Epstein-Barr Virus Polymerase Processivity Factor Enhances BALF2 Promoter Transcription as a Coactivator for the BZLF1 Immediate-Early Protein

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The Epstein-Barr virus (EBV) BMRF1 protein is an essential replication protein acting at viral replication forks as a viral DNA polymerase processivity factor, whereas the BALF2 protein is a single-stranded DNA-binding protein that also acts at replication forks and is most abundantly expressed during viral productive replication. Here we document that the BMRF1 protein evidently enhances viral BZLF1 transcription factor-mediated transactivation of the BALF2 gene promoter. Mutagenesis and electrophoretic mobility shift assays demonstrated the BALF2 promoter to harbor two BZLF1 protein-binding sites (BZLF1-responsive elements). Direct binding of the BZLF1 protein to BZLF1-responsive elements and physical interaction between BZLF1 and BMRF1 proteins are prerequisite for the BMRF1 protein up-regulation of the BALF2 promoter. A monomeric mutant, C95E, which is defective in homodimerization, could still interact and enhance BZLF1-mediated transactivation. Furthermore although EBV protein kinase phosphorylates BMRF1 protein extensively, it turned out that phosphorylation of the protein by the kinase is inhibitory to the enhancement of the BZLF1-mediated transactivation of BALF2 promoter. Exogenous expression of BMRF1 protein augmented BALF2 expression in HEK293 cells harboring the EBV genome but lacking BMRF1 and BALF5 genes, demonstrating functions as a transcriptional regulator in the context of viral infection. Overall the BMRF1 protein is a multifunctional protein that cannot only act as a DNA polymerase processivity factor but also enhances BALF2 promoter transcription as a coactivator for the BMRF1 protein, regulating the expression level of viral single-stranded DNA-binding protein.

The Epstein-Barr virus (EBV), a B lymphotropic γ-herpesvirus causing infectious mononucleosis, is also associated with several human cancers, including Burkitt lymphoma and nasopharyngeal carcinoma (1). EBV possesses two alternative life styles: latent and lytic. In latently infected cells, only limited numbers of viral genes are expressed (2) with no production of virus particles. Lytic infection differs in that multiple rounds of replication are initiated within the oriLyt region of the EBV genome (3). One of the first detectable changes is expression of the BZLF1 gene product, which is also called Zta or ZEBRA. The BZLF1 protein transactivates various viral promoters together with the BRLF1 gene product (4), leading to an ordered cascade of viral gene expression: activation of early genes followed by viral genome replication and late gene expression. In the viral productive cycle, the EBV genome is amplified more than 100-fold utilizing viral replication machinery consisting of the BZLF1 protein, an oriLyt-binding protein; the BALF5 protein, a DNA polymerase (Pol); the BMRF1 protein, a Pol processivity factor; the BALF2 protein, a single-stranded DNA-binding protein; and the BBLF4, BSLF1, and BBLF2/3 proteins, which are predicted to be helicase, primase, and helicase-primase-associated proteins, respectively (5). It has been suggested that all except the BMRF1 protein work together at replication forks to synthesize leading and lagging strands of the concatemeric EBV genome (6).

The EBV BALF2 gene encodes a single-stranded DNA-binding protein that is indispensable for the lytic phase of EBV DNA replication (5, 7). BALF2 protein is abundantly expressed in cells undergoing lytic replication and preferentially binds to single-stranded DNA rather than to double-stranded DNA or single-stranded RNA, thereby enhancing the DNA synthesis catalyzed by the BALF5 Pol catalytic subunit and yielding replication products with longer length (8). Stoichiometrically large amounts of the BALF2 protein are required at viral replication forks during the lytic phase of EBV DNA replication. It has been reported that the gene promoter is activated transcriptionally by both BZLF1 and BRLF1 proteins (9). Whether an additional mechanism contributes to augmented expression of the BALF2 gene remains to be clarified.

The BMRF1 gene product, which is also called early antigen diffused, is essential for processive DNA synthesis by the BALF5 polymerase catalytic subunit and is the major early phosphoprotein for EBV lytic replication (10–19). The BMRF1 protein shows homogenous, not dotlike, distribution throughout the replication compartments, completely coincident with...
the newly synthesized viral genome (20). The immunostaining data together with the findings that almost all of abundantly expressed BMRF1 proteins bind to DNA (20) indicate that it not only acts at viral replication forks as a polymerase processive factor but also is widely distributed on newly synthesized EBV genomic DNA. Recently it has been shown to transcriptionally activate the EBV BRLF1 promoter, one of two divergent early promoters located within the lytic origin of viral DNA replication, oriLyt (21–23), as well as transactivating a cellular gastrin promoter (24). Interaction between BMRF1 protein and promoter-bound cellular transcriptional factors, such as ZBP-89 and/or SP1, appears to be essential for formation of initial transcriptional replication complexes to enhance transcription (24, 25).

In the present study, we found that the BMRF1 protein evidently enhances BZLF1-mediated transcriptional activation of the BALF2 promoter. At least two BZLF1-binding sites, termed BZLF1-responsive element (ZReS), were identified within the BALF2 promoter region, and BZLF1 binding to the BALF2 promoter proved necessary for the BMRF1 augmentation of BALF2 promoter activity. A monomeric BMRF1 mutant, C95E, also enhanced the BZLF1-mediated transcriptional activation of the BALF2 promoter to an extent similar to that of wild-type BMRF1. However, phosphorylation of the BMRF1 protein by the EBV protein kinase prevented the enhancement, indicating that hypophosphorylated forms are functional to the augmentation of the BALF2 promoter activity. When HEK293 cells harboring the EBV genome but lacking BMRF1 and BALF5 genes were treated with chemical compounds to induce BALF2 gene expression, exogenous BMRF1 significantly augmented its expression, demonstrating functions as a transcriptional regulator in the context of viral lytic replication. This is the first report that the BMRF1 protein acts as a transcriptional coactivator for the BZLF1 protein.

**EXPERIMENTAL PROCEDURES**

**Cells**—The B95-8 B lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO₂. HEK293 and HEK293T cells were grown and maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum. For induction of lytic replication in B95-8 cells, 12-O-tetradecanoylphorbol-13-acetate (TPA) at a final concentration of 100 ng/ml, 5 mM sodium azide, and 0.5 μg/ml calcium ionophore (A23187) were added to the culture medium, and then the cells were incubated for 48 h.

**Plasmids**—The BZLF1 protein expression vector (pBZLF1) was kindly provided by Dr. K. Kuzushima (Aichi Cancer Center Research Institute, Nagoya, Japan). The expression vectors for wild-type BGLF4 and for kinase-dead mutant K102I possessing a mutation at the catalytic lysine were constructed as described in our recent published report (26). Coding regions of the BRLF1 and BMRF1 genes were amplified by PCR using B95-8 genomic DNA as the template and inserted into EcoRI-HindIII sites of pcDNA3.1(+) (Invitrogen) to generate pBRLF1 and pBMRF1, respectively. A carboxyl-terminal FLAG-tagged BMRF1 expression vector (pWT-f) was obtained by subcloning a PCR-amplified insert using primers containing the FLAG tag sequence. BMRF1 deletion mutants were obtained by PCR using pWT-f as a template. The pBMRF1(C95E) point mutant was generated by site-directed mutagenesis replacing Cys-95 with glutamic acid. The quadruple mutant pBMRF1(S337A/T344V/S349A/T355V) (27) was generated by stepwise site-directed mutagenesis.

For construction of BZLF1-E2 fusion protein expression vector (pBZLF1:E2), a PCR-amplified fragment containing the E2 DNA binding region sequence (nt 652–1232) was inserted into the XbaI-Xhol site of a mutated pBZLF1 whose stop codon was eliminated by inserting an Xbal site by site-directed mutagenesis. Bovine papillomavirus type-1 E2 DNA used as a template for the PCR was kindly provided by Dr. T. Kiyono (National Cancer Center Research Institute, Tokyo, Japan). This allowed generation of the plasmid pBZLF1:E2 expressing a chimeric protein consisting of amino acids (aa) 1–245 of BZLF1 and aa 128–410 of E2. Carboxyl-terminal FLAG-tagged E2 DNA binding region (aa 128–410) (pE2-FLAG) was obtained by subcloning a PCR-amplified insert using primers containing the FLAG sequence.

The BALF2 promoter region (nt 358 to +12) (9) was subcloned into the pGL4.10 (Promega) reporter plasmid containing luciferase gene (luc2). The B95-8 genomic DNA was used as a template for the PCR. The PCR products were double digested with Nhel and HindIII and then subcloned into pGL4.10. The resultant plasmid was designated pBALF2P. A reporter vector with a truncated BALF2 promoter was generated by amplifying each length of the promoter and subcloning into the Nhel and HindIII sites of pGL4.10.

To construct reporter plasmids, pE2-BA2P1, in which the putative ZRE1 was replaced with the E2-binding sites (S’-ACCGTTTTCCGT-3’) (28), site-directed mutagenesis was carried out twice. Other mutant reporters, pE2-BA2P2 and pE2-BA2P3, were generated likewise.

Oligonucleotide primers and DNA templates used for PCR or site-directed mutagenesis to construct expression vectors or reporter genes are specified in supplemental Tables 1 and 2. The inserted DNA sequence of each vector was confirmed by DNA sequencing.

**Luciferase Reporter Assay**—All transfections were in triplicate on 24-well plates of HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In all experiments, 10 ng of the pCMV-Rluc plasmid was co-transfected as an internal control. The amount of transfected DNA was kept constant by adding empty vector. Luciferase activity was measured with a Dual-Luciferase assay kit (Promega) according to the manufacturer’s instructions using a luminometer (Berthold).

**Immunoblot Analysis**—Transfected HEK293 cells were suspended in Passive Lysis Buffer (Promega), incubated for 15 min at room temperature, and then vortexed for 10 s. The proteins were separated by 10% SDS-PAGE and transferred onto Immobilon transfer membranes (Millipore) followed by detection using Western Lightning (PerkinElmer Life Sciences). Images were processed with LumiVision PRO 400EX (Aisin/Taitec, Inc.).

**Antibodies**—An anti-BMRF1-specific mouse monoclonal antibody, R3, and an anti-glyceraldehyde-3-phosphate dehydrogenase were purchased from Chemicon International Inc.
An anti-BRLF1-specific monoclonal antibody and an anti-FLAG-specific mouse monoclonal antibody, M2, were purchased from Argen Inc. and Sigma, respectively. Affinity-purified anti-BALF2-, anti-BZLF1-, and anti-BMRF1-specific polyclonal antibodies were prepared as described previously (8, 20, 29). The anti-BGLF4 antibody was kindly provided by Dr. Kawaguchi (Tokyo University) (30).

**Electrophoretic Mobility Shift Assay (EMSA)**—EBV BZLF1 protein was purified from SF21 cells infected with recombinant baculovirus expressing BZLF1 protein (31). SF21 cells were infected with recombinant baculovirus. Cells were harvested 72 h postinfection and suspended in hypotonic buffer (HB; 40 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin) followed by homogenization using a Dounce homogenizer. Nuclei were then centrifuged at 10,000 × g for 20 min, resuspended in 0.1 M NaCl, HB containing 0.2% Triton X-100, and precipitated again. After one more washing with 0.1 M NaCl, HB containing 0.2% Triton X-100, precipitates were washed with 0.2 M NaCl, HB containing 0.2% Triton X-100 twice, and then BZLF1 protein was extracted with 0.6 M NaCl, HB containing 0.2% Triton X-100. Purity of the protein was greater than 90%.

The DNA sequences of each oligonucleotide probe were as follows: B2ZRE1 (containing from nt −137 to −114), 5′-CAG-TTGAGACGTCAAGTTCCCTCCCAGCTG-3′ (sense) and 5′-ACGTACGTACGAGGGGAACTGACGTCTCA-3′ (antisense); B2ZRE2 (containing from nt −80 to −57), 5′-CAGTCTTTGG-TAAATGAAATACAGTGGG-3′ (sense) and 5′-ACGTCCACACTGTTATTCTATTTAAG-3′ (antisense); ZRE2 (containing two concatenated E2 binding sequences), 5′-CAGTACCTTTGGTACCTTGGTT-3′ (sense) and 5′-ACGTACCCGAAAAACCGTACAGGAA-3′ (antisense). Equal molar ratios of each pair of sense and antisense oligonucleotides were mixed, denatured at 95 °C, and annealed by cooling gradually to room temperature. For labeled probes were prepared by 3′-end labeling using the Klenow fragment of *E. coli* DNA polymerase I and 32P[dCTP (Amersham Biosciences). Unincorporated deoxynucleotide triphosphates were removed with Chromaspin-10 columns (Clontech). Binding reactions were performed at room temperature with 50 ng of purified BZLF1 protein in a buffer consisting of 20 mM HEPES, KCl, 0.1 mM EDTA, 10% (v/v) glycerol, 2 mM MgCl2, 1 mM dithiothreitol, 0.25 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml poly(dI-dC)-poly(dI-dC). After adding 1 μg of the labeled probe and 20 ng (×20) or 100 ng (×100) of the cold probe as a competitor, the mixture was incubated for 30 min. The sample was then loaded onto a 5% polyacrylamide gel and run in 0.5× Tris borate-EDTA buffer. Gels were dried and used to expose Fuji imaging plates for analysis by a Fuji Image Analyzer BAS 2500.

**Blue Native Polyacrylamide Gel Electrophoresis**—Cell lysates were prepared using a NativePAGE Sample Prep kit (Invitrogen) according to the manufacturer’s instructions. A precast 4–16% NuPAGE Bis-Tris gel (Invitrogen) was used to resolve the protein. NativeMark standard (Invitrogen) was used as a molecular weight standard.

**Pulldown Assay**—Each of the expression plasmids for carboxyl-terminal FLAG-tagged BMRF1 wild type and mutants or BZLF1 protein were individually transfected into HEK293T cells. After 48 h post-transfection cells were lysed in 500 μl of a lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2 mM Na3VO4, and protease inhibitor mixture (Roche Applied Science) and then sonicated followed by centrifugation at 18,000 × g for 15 min at 4 °C. The protein extract containing each BMRF1 protein (100 μg) and the BZLF1 cell extract (100 μg) were mixed and then applied for immunoprecipitation using 40 μl of anti-FLAG M2 affinity gel (Sigma) with gentle rocking overnight at 4 °C. Immunocomplexes were collected by centrifugation and washed five times with wash buffer (20 mM HEPES-KOH (pH 7.6), 75 mM KCl, 25 mM MgCl2, 0.15% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol). The immunoprecipitates were then subjected to SDS-PAGE followed by immunoblot analysis with the anti-BZLF1-specific antibody.

**Immunoprecipitation (IP)**—B95-8 cells were treated with TPA, A23187, and sodium butyrate to induce lytic replication; harvested 48 h post-treatment; and lysed in IP buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.2% Nonidet P-40, 10 mM NaF, 2 mM Na3VO4, protease inhibitor mixture) containing 500 mM NaCl. After incubation for 10 min on ice, cells were sonicated followed by centrifugation at 18,000 × g for 20 min at 4 °C. The supernatants were diluted 5-fold with IP buffer, pre-cleaned with Dynabeads protein A (Dynal), and mixed with anti-BZLF1-specific rabbit IgG or normal rabbit IgG for 2 hours at 4 °C. The supernatants were added 5-fold with IP buffer, pre-cleaned with Dynabeads protein A (Dynal), and mixed with anti-BZLF1-specific antibody. The supernatants were further incubated for 1 h at 4 °C. The beads were then washed five times with IP buffer containing 100 mM NaCl. The immunoprecipitates were then subjected to SDS-PAGE followed by immunoblot analysis with the anti-BMRF1-specific monoclonal antibody.

**Construction of Deletion Mutant EBV BAC DNA**—For DNA of human EBV B95-8 (B95-8/F-BAC) was provided by Hammerschmidt and co-workers (32). Homologous recombination was carried out in *E. coli* as described previously (33, 34). To generate a deletion mutant BAC of BMRF1 gene, linear target constructs for recombination were prepared by PCR. To construct EBV BACΔM/ne containing a kanamycin resistance gene and streptomycin sensitivity gene, RpsLneo (purchased from Gene Bridges, Dresden, Germany) was used as a PCR template. A pair of oligonucleotides was designed to carry oligonucleotide homologous to the target region of B95-8/F-BAC at their 3′-ends. The following oligonucleotide pair was used: BM1neoF, 5′-GTTAATTCCTTCTGTTGAATACTTAGGAGGCCCCCGGGTGGGGCCGTGCGTGTGGCACAAGGCGGcgcctttgatgtaggcccagc-3′; and BM1neoR, 5′-TGTTGGAGTTCCTGTGCCCCAGTTGTGCACCGGGGGAGCTTTATCTCTTGCGTcagactgcagactgcagactgcagaggg-3′. Upcasecar and lowercase letters indicate regions homologous to BMRF1 gene of B95-8/F-BAC and RpsLneo plasmid, respect-
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The PCR products were digested with DpnI to remove residual template DNA. Approximately 200 ng of the linear PCR product was electroporated into recombinase-induced E. coli DH10B electrocompetent cells (harboring pREDE5 and EBV BAC), which were prepared as described previously (3). Electroporation was performed using a Bio-Rad Gene Pulser II electroporation system (0.1-cm cuvette, 1.6 kV, 25 microfarads, 200 ohms, 50 μl of cells). Colonies of recombinant BAC DNA were identified by plating cells on an LB plate containing chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml).

To construct EBV BACΔM by removing the marker gene from EBV BACΔM/neo, double-stranded DNAs for recombin- nation were prepared by two-step PCR. Two oligonucleotides (BMOVf and BMOVr) were designed to carry oligonucleotide homologous to 5’-ends and 3’-ends of the outside of the region deleting BMRF1 gene. Another pair of primers was designed upstream and downstream of the region deleting BMRF1 gene. The following primers were used: BMOVf, 5’-GCTGCTTTGG-Gagccagactaataagcc-3’; BMOVr, 5’-ATCTTCTGGCTC- cacaagcagcaagaag-3’. BMupF1, 5’-GAGGGGGTTATACCTT-GGTGGAAGTG-3’; and BMRF1R, 5’-TTAAATGAGG- GGTTAAAGGCCTG-3’. First PCRs were performed using pairs of primers (BMupF1 and BMRF1R or BMRF1F and BMRF1R) and B95-8/F-BAC as a template. A second PCR was performed using a pair of primers (BMupF1 and BMRF1R) and the first PCR products as a template. Approximately 200 ng of the resultant PCR product was electroporated into recombinase-induced E. coli HS996 electrocompetent cells (harboring pREDE5 and EBV BACΔM/neo). Colonies of recombinant BAC DNA were identified by plating cells on an LB plate containing chloramphenicol and streptomycin.

To generate a double deletion mutant BAC of BMRF1 and BALF5 genes, EBV BACΔMΔPol, double-stranded DNAs for recombination were prepared by PCR as described above. The following primers were used: BA5neoF, 5’-AGGGGCGA-GGCGAGGAGACACCCACGCAGCGCCCTCGAGC-TTCCATTGCTACTAGAATAgccttgtgtgtatgctggagac-3’; and BA5neoR, 5’-GGTCCCGGTGTTGTTGGCGGCAGGGCGAGGCATCGGAAACGGCGGTCTCCTGCTCTGAAAtcaga-gagaactgtgactgag-3’. Approximately 200 ng of the linear PCR product was electroporated into recombinase-induced HS996 electrocompetent cells (harboring pREDE5 and EBV BACΔM). Colonies of recombinant BAC DNA were identified by plating cells on an LB plate containing chloramphenicol and kanamycin. Obtained BAC DNAs were analyzed by 0.6% agarose gel electrophoresis after digestion with BamHI restriction enzyme, and sequences near the deletion regions were confirmed by DNA sequencing.

Transfection of BAC DNAs into HEK293 Cells—HEK293 cells (5 × 10⁵) were transfected with 1–3 μg of BAC DNA using Lipofectamine 2000 (Invitrogen). The next day, transfected cells were reseeded on a culture plate in medium containing 150 μg/ml hygromycin. After 2 weeks, hygromycin-resistant clones were screened for the presence of BAC DNA.

RESULTS

BZLF1-mediated Transactivation of the BALF2 Promoter Is Enhanced by BMRF1 Protein Expression—When EBV lytic replication was induced in B95-8 cells with chemical compounds, viral early proteins, such as BALF2 or BMRF1, appeared at 12 h postinduction and reached plateaus at around 24 h (Fig. 1). The BALF2 protein was so abundantly expressed that its protein band (130 kDa) was visible when the cell lysates were analyzed by gel electrophoresis and Coomassie Blue staining (data not shown). The BALF2 protein binds to single-stranded DNA and facilitates movement of the EBV DNA polymerase stochiometrically at viral replication forks (5, 7, 8). The exceptional high level expression of the BALF2 protein prompted us to clarify the molecular mechanism of regulation of the BALF2 gene promoter.

To clarify molecular mechanisms governing the BALF2 gene expression, transient reporter assays were used. We constructed a reporter plasmid, pBALF2P, by inserting the promoter sequence between −358 and +12 of the BALF2 gene into the pGL4.10 luciferase reporter vector (see Fig. 3A). Transient expression of either the BZLF1 protein alone (Fig. 2A, lane 2) or the BRLF1 protein alone (Fig. 2B, lane 5) transactivated the BALF2 promoter as reported previously (9, 35).

When increasing amounts of BMRF1 were co-expressed with BZLF1, further enhancement of the BZLF1-mediated transactivation of the BALF2 promoter was observed in a dose-dependent manner (Fig. 2A), whereas expression of the BMRF1 protein alone did not exhibit any transactivation (Fig. 2A, lane 6). Furthermore co-expression of the BMRF1 protein exhibited a minimal effect on BRLF1-mediated transactivation of the BALF2 gene promoter (Fig. 2B, lane 6). Thus, the BMRF1 protein, known to be a polymerase accessory factor, appears to work as a transcriptional cofactor and specifically enhance BZLF1-mediated transactivation of the BALF2 promoter.
It has been reported that the BALF2 promoter is synergistically activated by simultaneous expression of BZLF1 and BRLF1 (9). We then examined how BMRF1 expression affects the synergistic transactivation. Co-expression of BMRF1 further enhanced the BALF2 gene transactivation mediated by simultaneous expression of BZLF1 and BRLF1 (Fig. 2B, lanes 7 and 8). Overall these results allow the hypothesis that BMRF1 specifically interacts with BZLF1 protein and that, together with BRLF1 protein, they strongly transactivate the BALF2 promoter, leading to abundant expression during the lytic phase in infected cells.

At Least Two Putative BZLF1-responsive Elements Are Present in the BALF2 Promoter—To identify the regions of the BALF2 promoter responsible for up-regulation by BZLF1 and BMRF1, we constructed reporter constructs featuring a series of deletions introduced into the BALF2 promoter region (Fig. 3A). When HEK293 cells were transfected with the reporter constructs and the BZLF1 expression vector (Fig. 2B, white bars), we observed evident attenuation of the luciferase activity by deleting nucleotides −358 to −113 (pBALF2P-113). The reporter (pBALF2P-113) also exhibited minimal transactivation when the BMRF1 and the BZLF1 proteins were co-expressed (Fig. 3B; only 1.6-fold activation), whereas wild type, −179, and −133 mutants exhibited 5.1-, 4.0-, and 4.7-fold transactivation, respectively. These results indicate that the region between nucleotides −133 and −114 contains a putative ZRE. It should be noted that deletion of sequences from −358 to −179 increased the basal luciferase activity (Fig. 3B, compare pBALF2P-179 with pBALF2P). The increase may imply the presence of repressing cis element(s) within the region.

We then constructed a reporter construct (pE2-BA2P1) in which the putative ZRE (ZRE1) located between nucleotides −133 and −114 was replaced with the BPV E2 binding sequence (5′-accggttcgccg-3′) (Fig. 4A) to obtain supporting evidence.

FIGURE 2. BMRF1 enhances BZLF1-dependent transcriptional activation from the BALF2 promoter. A, luciferase assays using the BALF2 promoter-reporter plasmid pBALF2P were performed. Increasing amounts of pBMRF1 (0, 100, 200, and 400 ng) were co-transfected into HEK293 cells with 10 ng of pBZLF1 and 100 ng of pBALF2P for the luciferase assay. B, BMRF1 enhances BALF2-dependent transcription from the BALF2 promoter but not BRLF1-dependent transcription. pBMRF1 (400 ng) was co-transfected with pBZLF1 (10 ng) or, pBRF1 (15 ng), or both as indicated, and cells were then subjected to luciferase assay at 48 h post-transfection. In all experiments, 10 ng of the pcMV-Rluc plasmid was co-transfected as an internal control. Bars represent mean -fold activation of the luciferase activity obtained by transfecting the empty vector with pBALF2P ± S.E. from three independent experiments. The -fold activations by transfection of pBMRF plasmid (compared with that without pBMRF1) are given in the graphs. Expression levels of BZLF1, BMRF1, and BRLF1 proteins are shown by immunoblotting (IB) with specific antibodies. Lysate from B95-8 cells treated with TPA, A23187, and sodium butyrate for 48 h was included as a positive control (Lytic B95-8).

FIGURE 3. Deletion analysis of the BALF2 promoter. A, schematic representation of constructs with a truncated BALF2 promoter. A map of the BALF2 promoter region (−358 to +12 relative to the transcription start site (+1)) is depicted in the upper part of A (9). TATA box (boxed TATA), putative AP-1/ZRE (white circle), and AP-1 (gray circle) sites are shown according to the report of Hung and Liu (9). Another putative ZRE, speculated from the luciferase assay (B), is shown as a white box. B, pBMRF1 (200 ng) was co-transfected with pBZLF1 (10 ng) and 100 ng of each reporter plasmid into HEK293 cells. In all experiments, 10 ng of the pcMV-Rluc plasmid was co-transfected as an internal control. Cells were then subjected to luciferase assay at 48 h post-transfection. Bars represent mean -fold activation of the activity obtained by transfecting the empty vector with pBALF2P ± S.E. from three independent experiments. The -fold activations by transfection of pBMRF plasmid (compared with that without pBMRF1) are given in the graphs.
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A.

Plasmid BALF2 promoter

-358
-140
-133
-114
-107
-74
-70
-63
-55
+1
+mRNA

pBALF2P(WT) CGCTGAGCTACATCCCCGGACGTGGG.../GTAATGATTTACAGTTGGG... pE2-BA2P1 CCGaccttttctttaccctttccgtTGGG.../GTAATGATTTACAGTTGGG...

pE2-BA2P2 CGCTGAGCTACATCCCCGGACGTGGG.../GTAaccccttttccgtTGGG...
pE2-BA2P3 CCGaccttttctttaccctttccgtTGGG.../GTAaccccttttccgtTGGG...

BPV E2 protein binding sequence

B.

FIGURE 4. Mutational analysis of the two putative BZLF1 protein-binding sites (ZREs) in the BALF2 promoter. A, schematic representation of the mutated constructs of pBALF2P. The sequences from nt -140 to -107 and from nt -74 to -55 relative to the transcription start site (+1) are shown. The ringed sequence (nt -70 to -63) is the putative ZRE reported by Hung and Liu (9), and the other putative ZRE from our result (Fig. 3) is boxed (nt -133 to -114). For mutagenesis, sequences were replaced with the BPV E2 binding sequence (5'-acgttttcggt-3'). B, HEK293 cells were transfected with 200 ng of pBMRF1 together with 10 ng of pBZLF1 and 100 ng of each reporter plasmid. Luciferase assays were carried out at 48 h after transfection. In all experiments, 10 ng of the pCMV-Rluc plasmid was co-transfected as an internal control. Bars represent mean ± S.E from three independent experiments. The fold activations by transfection of pBMRF plasmid (compared with that without pBMRF1) are given in the graphs. BPV, bovine papillomavirus; WT, wild type.

evidence that the BZLF1 protein recognizes and binds to the region. Such replacement should affect enhancement of BZLF1-mediated transactivation of the BALF2 promoter by the BMRF1 protein as the BZLF1 protein cannot bind to the E2 binding motif (36). In addition, another putative ZRE (ZRE2), located between nucleotides -70 and -63, has been reported by Hung and Liu (9) on the basis of computer analysis. Thus, we constructed a reporter construct (Fig. 4A, pE2-BA2P2) similarly by replacing ZRE2 with the E2 binding motif. A third mutant was constructed in which both the ZRE1 and the ZRE2 were mutated (Fig. 4A, pE2-BA2P3).

 Luciferase assays revealed that BZLF1-mediated transactivation was severely attenuated when any of the three mutated reporter constructs were subjected to assays (Fig. 4B). Importantly replacing both ZREs completely abolished the BZLF1-mediated transactivation, indicating both to be truly functional BZLF1-binding sites. Interestingly enhancement of the BZLF1-mediated transactivation by the BMRF1 protein was also impaired with all mutants. We conclude that BMRF1 recognizes BZLF1 protein bound to ZREs.

**BZLF1 Protein Directly Binds to Two ZREs within the BALF2 Promoter—**We then used EMSA to examine whether the purified BZLF1 protein can directly bind to the ZREs within the BALF2 promoter. Four 32P-labeled probes were used for the assay (Fig. 5A): B2ZRE1 and B2ZRE2 derived from the BALF2 promoter sequence (nt -137 to -114 and nt -80 to -57, respectively), 2×E2 consisting of two copies of E2 binding motifs as a negative control, and one with ZRE5 (derived from EBV oriLyt) as a positive control. Purified BZLF1 protein was obtained from Sf21 cells infected with recombinant baculovirus AcBZLF1 (see “Experimental Procedures”). EMSA demonstrated the purified BZLF1 protein to bind to both B2ZRE1 and B2ZRE2 as expected but not to any appreciable extent to 2×E2 (Fig. 5B).

We then carried out competition experiments. As demonstrated in Fig. 5C, addition of excess amounts of unlabeled probes (B2ZRE1 in the left panel and B2ZRE2 in the right panel) clearly reduced the intensities of the shifted bands, whereas the unlabeled 2×E2 probe did not. The results, together with the data shown in Fig. 4, support the idea that the BZLF1 protein directly binds to the two ZREs in the BALF2 promoter. Furthermore using labeled B2ZRE2 as a probe, we performed competition assays with unlabeled B2ZRE1 or B2ZRE2 as a competitor, respectively. The competitor B2ZRE2 competed more efficiently than B2ZRE1, indicating higher binding affinity of BZLF1 protein to B2ZRE2 than to B2ZRE1 (Fig. 5C, right panel).

**BZLF1 Protein Binding to the Promoter Is Necessary and Sufficient for Transcriptional Enhancement—**The BZLF1 protein bound to two ZREs in the BALF2 promoter (Fig. 6), and this was necessary for transcriptional enhancement by the BMRF1 protein (Fig. 4). To demonstrate that the BZLF1 binding is not only necessary but also sufficient for enhancement, we prepared a vector for expression of a BZLF1-E2 fusion protein (28, 36) expected to bind to the E2 binding sequence in the pE2-BA2P3 vector. FLAG-tagged E2 served as a negative control. The expressions of these fusion proteins were confirmed by immunoblot assay using anti-BZLF1 (left panel) and anti-FLAG (right panel) antibodies as shown in Fig. 6A. The BZLF1 protein itself and the FLAG-E2 fusion protein barely transactivated the pE2-BA2P3 reporter (Fig. 6B) as we already described above. In contrast, strong transactivation was noted with the BZLF1-E2 fusion protein. Thus, replacing the ZREs with E2-binding sites within the BALF2 promoter resulted in the loss of BZLF1-me-
mediated transactivation, but this could be almost completely restored using the BZLF1-E2 fusion protein as a trans-acting factor, compatible with the idea that direct binding of the BZLF1 protein to ZREs is necessary and sufficient for BZLF1-mediated BALF2 promoter transactivation.

Interaction with BZLF1 Protein Is Prerequisite for BMRF1 Enhancement of BZLF1-mediated Transactivation of the BALF2 Promoter—As shown in Fig. 7F, the BMRF1 protein interacts with the BZLF1 protein under physiological condition, corresponding with the previous report (21). The physical interaction requires the amino-terminal region of the former and the basic leucine zipper domain (aa 175–220) of the latter.

The region spanning aa 379–388 of the BMRF1 protein is considered to be a transactivation domain because deletion mutants BMRF1/H9004379–383 and BMRF1/H9004385/387/388 did not transactivate either the BHLF1 promoter (23) or the gastrin promoter (24). As shown in Fig. 7D, lane 6, the mutant BMRF1/H9004379–383 did not enhance BZLF1-mediated transactivation of the BALF2 promoter (Fig. 7E, lane 5). The BMRF1 protein directly transactivates the BHLF1 and gastrin promoters, whereas BZLF1 protein is prerequisite for the enhancement of the BALF2 promoter by the BMRF1 protein. Thus, the region between aa 379 and 383 would be a common domain involved in transactivation activity for gene expression. Another deletion mutant, BMRF1 Δ316–378, activated the BALF2 promoter as well as the wild-type BMRF1 did (Fig. 7D, lane 5), compatible with the results obtained using the BHLF1 (23) or the gastrin promoters (24).

BMRF1 Monomers Can Interact with BZLF1 Protein and Enhance BZLF1-mediated Transactivation of the BALF2 Promoter—It is known that the human cytomegalovirus Pol accessory protein UL44 (38) and PF-8 of Kaposi sarcoma-associated herpesvirus (39) form homodimers in solution, whereas the herpes simplex virus homologue UL42 exists as a monomer (40–42). To determine whether the EBV BMRF1 protein forms (21, 37). It is reported that the amino-terminal 45 amino acids in the BMRF1 protein are not required for DNA binding activity or polymerase processivity function (15). To test whether the interaction between the BMRF1 and BZLF1 gene products is important for enhancement of the BZLF1-mediated transcriptional activation by the BMRF1 protein, we prepared amino-terminally truncated mutants, BMRF1/H90041–10 and BMRF1/H90041–30. The wild-type and the mutant BMRF1 proteins are fused to FLAG tag at their carboxyl-terminal ends (Fig. 7A). A pulldown assay with anti-FLAG M2 resin revealed that both mutants hardly interacted with the BZLF1 protein (Fig. 7E, lanes 2 and 3). In agreement, the amino-terminally truncated mutants did not enhance BZLF1-mediated transactivation of the BALF2 promoter (Fig. 7D, lanes 3 and 4).

The region spanning aa 379–388 of the BMRF1 protein is considered to be a transactivation domain because deletion mutants BMRF1 Δ379–383 and Δ385/387/388 did not transactivate either the BHLF1 promoter (23) or the gastrin promoter (24). As shown in Fig. 7D, lane 6, the mutant BMRF1 Δ379–383 did not enhance BZLF1-mediated transactivation of the BALF2 promoter but exhibited retained binding to BZLF1 (Fig. 7E, lane 5). The BMRF1 protein directly transactivates the BHLF1 and gastrin promoters, whereas BZLF1 protein is prerequisite for the enhancement of the BALF2 promoter by the BMRF1 protein. Thus, the region between aa 379 and 383 would be a common domain involved in transactivation activity for gene expression. Another deletion mutant, BMRF1 Δ316–378, activated the BALF2 promoter as well as the wild-type BMRF1 did (Fig. 7D, lane 5), compatible with the results obtained using the BHLF1 (23) or the gastrin promoters (24).
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To further confirm the interaction between the BZLF1 protein and the mutant BMRF1(C95E) protein, we performed pull-down assays (Fig. 7E, lane 6). As expected, the mutant BMRF1(C95E) protein, like the wild-type protein, retained the ability to interact with BZLF1 protein. Taken together, these results indicate that the monomer BMRF1 protein can interact with the BZLF1 protein and can enhance BZLF1-mediated transactivation of the BALF2 promoter.

Phosphorylation of the BMRF1 Protein by the EBV Protein Kinase Inhibits the Enhancement of BZLF1-mediated Transactivation of the BALF2 Promoter—The BMRF1 protein has been found to be phosphorylated mainly by the EBV protein kinase, BGLF4 protein, during lytic replication, and the BGLF4 protein co-localizes with BMRF1 protein in the viral replication compartments (43–45). When B95-8 cells were treated with chemical compounds to induce lytic replication, the hyperphosphorylated BMRF1 proteins mainly appeared in early stages of the lytic infection, whereas the dephosphorylated forms accumulated in late stages (Fig. 1) (46). Knockdown of BGLF4 expression by RNA interference resulted in an absence of hyperphosphorylated isoforms, confirming that the phosphorylation of BMRF1 protein is mainly mediated by BGLF4 kinase (47). So we examined the effect of EBV protein kinase on the enhancement of BZLF1-mediated transactivation of the BALF2 promoter by BMRF1 protein. As shown in Fig. 8A, expression of the BGLF4 kinase, but not kinase-dead BGLF4, inhibited enhancement of the BZLF1-dependent transcription from the BALF2 promoter by the BMRF1 protein in a dose-dependent manner. There is a proline-rich hinge region between amino acids 316 and 378 of the BMRF1 protein where Ser-337, Thr-344, Ser-349, and Thr-355 are reported to be the major BGLF4 target sites for phosphorylation (27). Although the quadruple mutant (S337A/T344V/S349A/T355V) enhanced the BZLF1-mediated transcription (Fig. 8B, lane 5) as well as the BMRF1 Δ316–378 deletion mutant (Fig. 7D, lane 5), the viral kinase still inhibited the enhancement by the quadruple mutant (Fig. 8B, lane 6). Unlike the claim by Yang et al. (27) that BGLF4-induced hyperphosphorylation was abolished in the BMRF1(S337A/T344V/S349A/T355V) mutant, co-expression of BGLF4 slightly shifted the band up (Fig. 8B, IB panel, compare lane 5 with 6),

A. Transfection/293T

B. Fold Activity

FIGURE 6. Rescue of enhancement of BZLF1-dependent transcriptional activation by the BZLF1-E2 fusion protein with the reporter gene in which both ZRESs were replaced with the E2 binding motif. A, expression of fusion proteins. HEK293T cells were transfected with plasmids expressing wild-type BZLF1 (BZLF1 WT; lane 3), BZLF1-E2 fusion protein (BZLF1:E2; lane 4), FLAG-tagged E2 DNA binding region (E2-flag; lane 5), or empty vector (Vector; lane 6). Immunoblot analysis was carried out using anti-BZLF1 protein antibodies (left panel) or anti-FLAG antibodies (right panel). Lyase from B95-8 cells treated with TPA, A23187, and sodium butyrate (B95-8 Lytic; lane 2) was included as a control. Black, black, and gray arrowheads indicate the BZLF1-E2 fusion protein, wild-type BZLF1 protein, and FLAG-tagged E2 DNA binding region protein, respectively. B, luciferase assays were performed with a variety of combinations of expression vectors and the reporter gene as indicated. Four hundred nanograms of pBMRF1 (black bars) or vector (white bars) were co-transfected with pBZLF1 wild type (10 ng), pBZLF1:E2 (10 ng) or pE2-FLAG (10 ng) and 100 ng of each reporter plasmid, pBALF2 or pE2-BA2P3 (Fig. 4A) in HEK293 cells. In all experiments, 10 ng of the pCMV-Rluc plasmid was co-transfected as an internal control. Luciferase assays were carried out at 48 h post-transfection. Bars represent mean -fold activation of the activity obtained by transfecting the empty vector with pBALF2P or pE2-BA2P3 ± S.E. from three independent experiments. The -fold activations by transfection of pBMRF plasmid (compared with that without pBMRF1) are given in the graphs. pE2-FLAG was used as a negative control.

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suggesting that other BGLF4-targeted phosphorylation site(s) in the BMRF1 protein would diminish its ability to enhance the BZLF1-mediated transactivation of the BALF2 promoter.

Because EBV protein kinase is known to phosphorylate BZLF1 protein as well as BMRF1, we could not deny the possibility that the inhibition of BZLF1-mediated transactivation of the BALF2 promoter by BMRF1 protein might be due to BZLF1 phosphorylation by EBV protein kinase. Therefore, we checked the effect of the protein kinase on BZLF1-dependent transcription through phosphorylation of the BZLF1 protein, strongly suggesting that the inhibition is most likely due to the phosphorylation of BMRF1 but not BZLF1. Considering these results, hypophosphorylated forms of the BMRF1 protein increase with progression during lytic replication (Fig. 1) (46, 48).
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A.

![Graph A]

**FIGURE 8. Effects of BMRF1 phosphorylation by BGLF4 protein kinase on BZLF1-dependent transactivation of BALF2 promoter.** A, increasing amounts of pBGLF4 (0, 50, 100, 200, 300, and 400 ng) were co-transfected into HEK293 cells with 10 ng of pBZLF1, 200 ng of pBMRF1, and 100 ng of pBALF2P. Bars represent mean -fold activation of the luciferase activity (×1) obtained by transfecting the empty vector with pBALF2P (lane 1) ± S.E. from three independent experiments. B, HEK293 cells were transfected with 400 ng of wild-type pBGLF4 or kinase-dead pBGLF4 mutant (KD) expression vector together with 200 ng of wild-type BMRF1 (WT) expression plasmid (lanes 3 and 4). The BMRF1 quadruple mutant S337A/T344V/S349A/T355V was co-transfected without or with pBGLF4 (lanes 5 and 6). Bars represent mean -fold activations of the activity (×1) after transfection of the empty vector with pBZLF1 and pBALF2P (lane 1) ± S.E. from three independent experiments. Expression levels of the BZLF1 and BMRF1 proteins are shown by immunoblotting (IB) with specific antibodies. Lysate from B95-8 cells treated with TPA, A23187, and sodium n-butyrate for 48 h was included as a positive control (lane 8; Lytic B95-8). The asterisk indicates phosphorylated isoforms of the BMRF1 protein. In all experiments, luciferase assays were carried out at 48 h after transfection. Ten nanograms of the pCMV-Rluc plasmid was co-transfected as an internal control. C, increasing amounts of BGLF4 expression plasmid (pBGLF4) (0, 200, and 400 ng) were co-transfected into HEK293 cells with 10 ng of pBZLF1 and 100 ng of pBALF2P for luciferase assays. Bars represent mean -fold activation of the luciferase activity (×1) obtained by transfecting the empty vector with pBALF2P.

BACΔMΔPol) was constructed, and 293 cell lines latently infected with the recombinant DNA (293/EBV BACΔMΔPol) were screened in the presence of hygromycin (see “Experimental Procedures” and Fig. 9A). The BALF5 gene encoding the viral DNA polymerase was deleted from the genome to avoid the amplification of the viral genome and to analyze BALF2 gene expression only. The 293/EBV BACΔMΔPol cells were transfected with the BMRF1 expression plasmid (pBMRF1) or empty vector and incubated for 24 h followed by treatment with chemical compounds to induce lytic gene expressions. As shown in Fig. 9B, without the BMRF1 expression the BALF2 protein was expressed slightly, whereas the expression was obviously augmented in the cells expressing BMRF1 protein. Under the condition, expression of the BZLF1 immediate-early protein was marginal. Thus, it is likely that the BMRF1 protein enhances the BALF2 gene expression in the context of viral lytic replication.

**DISCUSSION**

The EBV BMRF1 gene product, also termed the early antigen diffuse protein, is the viral DNA polymerase accessory protein and thus plays an essential role in lytic replication (10, 14–16, 18, 49). The BMRF1 and BZLF1 proteins co-localize within intranuclear viral replication compartments (50). The possibility has been pointed out that the direct interaction between BMRF1 and BZLF1 proteins modulates viral replication (21, 25). In the present study, because deletion mutants BMRF1 Δ1–10 and Δ1–30 lacking the ability to bind to the BZLF1 protein did not enhance BZLF1-mediated transactivation of the BALF2 promoter (Fig. 7), functional interaction between BZLF1 and BMRF1 is critical for regulation of viral gene transcription.

We showed that the EBV protein kinase inhibited enhancement of the BZLF1-dependent BALF2 promoter transcription through phosphorylation of the BMRF1 protein. Although functional regulation of the BMRF1 protein through phosphorylation is not well understood, Yang et al. (27) have recently reported that BMRF1 phosphorylation by the BGLF4 kinase down-regulates BMRF1-dependent transcriptional activation of the BHLF1 gene. Thus, hypophosphorylated forms of the BMRF1 protein might be involved in the activation, corresponding to an increase in levels of hypophosphorylated forms of the BMRF1 protein with progression during lytic replication (46, 48). These results suggest that dephosphorylation of the BMRF1 protein would be meaningful in the lytic replication.

Unlike the case of the BALF2 promoter, expression of the BMRF1 protein alone can activate the BHLF1 (21, 22) and the gastrin promoters (24) without expression of BZLF1. It was...
Therefore, it is possible that the BMRF1 protein functions as a transcriptional cofactor in the regulation of the BZLF1-mediated transactivation of the BALF2 promoter (Fig. 7). In addition, it also activated the BHLF1 promoter (data not shown). Taken together, these results lead us to the hypothesis that monomer BMRF1 protein interacts with viral and cellular transcription factors and regulates transcription as a cofactor.

To determine the contribution of the BMRF1 gene product to the expression of BALF2 protein during lytic EBV infection, HEK293 cells harboring the EBV BACΔMPol were treated with chemical compounds to induce BALF2 gene expression (Fig. 9). In this system the expression level of the BZLF1 protein induced by chemical compounds was too low to be confirmed by immunoblot analysis. However, the increase in the level of the BALF2 protein was evident when the BMRF1 protein was expressed in trans. It should be noted that the expression of the BMRF1 protein also resulted in an increase in the level of the BZLF1 protein. It should be noted that the expression of the BMRF1 protein also resulted in an increase in the level of the BZLF1 protein.

Regarding the structures of polymerase processivity factors, various oligomerization states, including monomers, have been reported so far. The UL42 of herpes simplex virus works as a monomer (40–42), UL44 of human cytomegalovirus works as a dimer (38), and PF-8 of Kaposi sarcoma-associated herpesvirus works as a dimer (39). We have recently succeeded in resolving the crystal structure of the carboxyl-terminally truncated BMRF1 protein (aa 1–314). Intriguingly, it appears that the BMRF1 protein may form a ring-shaped tetramer. In contrast, electrophoresis and sedimentation assay data indicate that the main component of BMRF1 in solution is a dimer (data not shown and Fig. 7C).

When several mutations were introduced to the amino acids constituting the dimer interface, the C95E mutation disrupted dimer formation. The monomeric mutant C95E retained the ability to bind to the BZLF1 protein and to enhance the BZLF1-mediated transactivation of the BALF2 promoter (Fig. 7). In addition, it also activated the BHLF1 promoter (data not shown). Taken together, these results lead us to the hypothesis that monomer BMRF1 protein interacts with viral and cellular transcription factors and regulates transcription as a cofactor.

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