

FIG. 7. *trans*-Complementation of impaired viral replication in BMRF1-knockout BAC $\Delta$ M/neo-harboring cells. (A) *trans*-complementation of viral DNA synthesis in BMRF1. Increasing amounts of FLAG-tagged BMRF1 expression vector were transfected into BAC $\Delta$ M/neo cells ( $6 \times 10^5$  cells) with the BZLF1 expression vector and harvested 3 days thereafter. Levels of viral DNA synthesis were determined by quantitative real-time-PCR assay as described in Materials and Methods. The values from three independent experiments are plotted as ratios to values for latent cells. The solid line is the best-fit curve for the data. Cell extracts from each transfected cell preparation were electrophoresed on 10% polyacrylamide gels. Immunoblotting was carried out with anti-BALF2, -Flag, and -BZLF1 antibodies (bottom panel). (B) Viral DNA synthesis levels. A 400-ng aliquot of the indicated FLAG-tagged BMRF1 expression vector was cotransfected with the BZLF1 expression vector into BAC $\Delta$ M/neo cells ( $6 \times 10^5$  cells), which were harvested at various time points thereafter, followed by processing as described for panel A. (C) Production of infectious progeny viruses. A 400-ng aliquot of the indicated FLAG-tagged BMRF1 expression vector was cotransfected with 200 ng of BZLF1 and BALF4 expression vectors into HEK293 cells carrying BAC $\Delta$ M/neo ( $6 \times 10^5$  cells), and viruses were harvested 3 days thereafter. The viruses were then inoculated with EBV-negative Akata cells, followed by determination of infectivity by FACS analysis.

ultracentrifugation and blue native gel electrophoresis. However, some C-shaped head-to-head homodimers can associate through their tail-to-tail interfaces and form tetrameric ring structures. The molecular contact surfaces within this ring

form continuous  $\beta$ -sheets in "head-to-head" ( $\beta I_1$ - $\beta I_1'$ ) and "tail-to-tail" ( $\beta D_2$ - $\beta D_2'$ ) manners (where "prime" indicates the neighboring molecule) (see supplemental Fig. 2 in reference 38). The head-to-head contact surface area is larger (ap-

proximately 930 Å<sup>2</sup>) than the tail-to-tail contact area (340 Å<sup>2</sup>). In addition to these main-chain/main-chain interactions, disulfide bonds (Cys95-Cys95' and Cys206-Cys206') are present on both contact surfaces of the crystal ring structure (38, 39). Although a Cys95 mutation to a serine residue had no effect at all on the dimer formation (data not shown), replacement with a negatively charged glutamic acid, C95E, impaired dimer formation *in vitro*, with only very weak DNA binding under *in vitro* specific conditions (38), strongly suggesting that head-to-head homodimer formation is mediated mainly through  $\beta$ -sheets ( $\beta 1_1\text{-}\beta 1_1'$ ) rather than through C95 residues. Since the C95E mutation had only a marginal effect on viral replication *in vivo*, as shown in the present study, it would not be expected to affect dimer formation in the context of infection, unlike with the *in vitro* results. On the other hand, viral replication of the C206E mutant, whose mutation does not affect *in vitro* biochemical activity of the protein, was found to be severely restricted, suggesting that tail-to-tail interactions are significant for virus replication. Since the head-to-head contact area is larger than the tail-to-tail interface, the tail-tail interaction via the Cys<sup>206</sup> residue might be crucial for tetramer formation *in vivo*. Therefore, we speculate that tetrameric ring formation might be required for efficient lytic EBV replication, and this would explain why replacement of the Cys<sup>206</sup> residue at the tail-to-tail interface had a very distinct influence on viral replication. Another possibility is that a particular viral factor might help the head-to-head association even in the absence of the Cys<sup>95</sup> residue, while the tail-to-tail contact could not be restored by any factor. Because BALF5, the catalytic subunit of viral DNA polymerase, is estimated to associate with the contact area of the head-to-head dimer of BMRF1 (38), BALF5 might somehow enforce the dimer formation of BMRF1.

The Arg<sup>256</sup> residue is located inside the ring-shaped tetramer BMRF1 (Fig. 1). As the positive charge of the residue is crucial for binding to DNA, substitution with an acidic amino acid (R256E) causes complete loss of DNA binding activity *in vitro* (38). In fact, viral replication was also severely restricted by the mutation, suggesting an importance of DNA binding by the processivity factor for efficient function. The C95E mutant BMRF1, however, exhibited normal viral replication, although the mutation caused significant loss of DNA binding *in vitro* at lower concentrations (38). Even so, we speculate that DNA binding is a crucial quality of BMRF1 in order for it to act as a processivity factor, because the C95E mutant BMRF1 did bind with DNA as efficiently as wild-type BMRF1 at high concentration (38), corresponding with the fact that the BMRF1 protein is abundantly present at replication compartments. Supporting data for this speculation are provided by BMRF1 homologues of human cytomegalovirus (HCMV) and herpes simplex virus (HSV): the DNA binding ability of HCMV UL44 (3, 31) and HSV UL42 (45) is essential for their function as processivity factors.

We then asked why DNA binding is so important for the processivity function and examined the intracellular localization of BMRF1 (Fig. 6). Interestingly, retention of the R256E mutant BMRF1 with the nuclear fraction proved to be severely impaired, and the mutant virus failed to form replication compartments, suggesting that DNA binding ability is required for nuclear retention and proper localization to the compartments. Similarly, DNA binding ability of HCMV UL44 is required for

its nuclear retention (3). The C206E BMRF1, on the other hand, showed normal association with the nuclear fraction, but it also failed to accumulate at replication compartments. Our previous report indicated that efficient DNA replication is needed for appropriate accumulation of viral replication proteins to the compartments, since inhibition of viral DNA replication by the addition of PAA caused similar mislocalization of viral replication factors (10). Therefore, failure of BMRF1 to accumulate at replication compartments could be the reason for impaired replication of the mutants, but we could not explain with our current data why the mutant demonstrates such impaired accumulation. Another way to account for the importance of DNA binding is that BMRF1 might not only act at viral replication forks as a polymerase processivity factor but also widely attach on newly synthesized EBV genomic DNA to maintain the DNA's integrity or to protect it from degradation (10).

The BMRF1 phenotypes of the mutants *in vitro* and *in vivo* are summarized in Table 1. Since the previous results for BMRF1 processivity measured *in vitro* were not always found to agree with the data presented in this paper, research *in vivo* must be accorded great importance. To give a concrete example, the C206E mutant, which is defective in the disulfide bond of the tail-to-tail contact, exhibited as efficient processivity as wild-type BMRF1 *in vitro*, but the mutation severely affected virus replication. This is the first report that the correct conformation of tetramer BMRF1 protein is necessary for lytic replication. With crystal structural imaging, such information might be of great value in creating novel antiviral medicines.

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# Transcriptional Repression by Sumoylation of Epstein-Barr Virus BZLF1 Protein Correlates with Association of Histone Deacetylase<sup>\*S</sup>

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The transition from latent to lytic phases of the Epstein-Barr virus life cycle is triggered by expression of a viral transactivator, BZLF1, that then induces expression of the viral immediate-early and early genes. The BZLF1 protein is post-translationally modified by a small ubiquitin-related modifier-1 (SUMO-1). Here we found that BZLF1 is conjugated at lysine 12R not only by SUMO-1 but also by SUMO-2 and 3. The K12R mutant of BZLF1, which no longer becomes sumoylated, exhibits stronger transactivation than the wild-type BZLF1 in a reporter assay system as well as in the context of virus genome with nucleosomal structures. Furthermore, exogenous supply of a SUMO-specific protease, SENP, caused de-sumoylation of BZLF1 and enhanced BZLF1-mediated transactivation. Immunoprecipitation experiments proved that histone deacetylase 3 preferentially associated with the sumoylated form of BZLF1. Levels of the sumoylated BZLF1 increased as lytic replication progressed. Based on these observations, we conclude that sumoylation of BZLF1 regulates its transcriptional activity through histone modification during Epstein-Barr virus productive replication.

The Epstein-Barr virus (EBV)<sup>2</sup> is a human  $\gamma$ -herpesvirus that predominantly establishes latent infection in B lymphocytes and epithelial cells. Only a small percentage of infected cells switch from the latent stage into the lytic cycle to produce progeny viruses. This reactivation has been associated with the emergence of human cancers (1, 2), suggesting that the EBV switching mechanism is a key determinant of EBV pathogene-

sis. Although the details of EBV reactivation *in vivo* are not fully understood, it is known that viral lytic replication can be achieved by treatment of latently infected B cells with some chemical or biological reagents such as 12-*O*-tetradecanoylphorbol-13-acetate, calcium ionophore, sodium butyrate, or anti-immunoglobulin. Through different pathways these reagents lead to expression of two transcriptional regulators, BZLF1 (also known as Zta or ZEBRA) and BRLF1 (Rta), which are the products of the two immediate-early genes, *BZLF1* and *BRLF1*. BZLF1 is a transcriptional activator that shares structural similarities to the basic leucine zipper (b-Zip) family transcriptional factors and is involved in the activation of replication origin, *oriLyt*, used in the lytic cycle. BZLF1 expression alone can trigger the entire reactivation cascade, suggesting that this is a primary event (3–5).

In latently infected cells, EBV DNA is present as multicopy episomes assembled into nucleosomal structures that are similar to cellular chromatin (6). Changes in histone acetylation in the *BZLF1* promoter region results in activation of *BZLF1* gene expression leading to reactivation from latency, as treatment with histone deacetylase (HDAC) inhibitors can activate viral lytic gene expression (7–9). It is reported that, in latently infected cells, the silent state of the virus genome is maintained, at least in part, by MEF2-mediated recruitment of HDAC proteins to the *BZLF1* promoter region (10). Once BZLF1 protein is expressed, the viral transcriptional activator recruits cAMP-response element-binding protein (CREB)-binding protein (CBP), a histone acetyltransferase, to BZLF1-responsive sequences to activate viral early promoters (11–15) followed by a coordinated cascade of viral lytic steps such as viral DNA replication, late gene expression, and progeny virus production. Thus, interplay between histone deacetylation and acetylation is associated with repression or activation of transcription, regulating latency and the replicative cycle of EBV.

Reversible posttranslational modifications are widely used to dynamically regulate protein activity. Proteins can be modified by small chemical groups, sugars, lipids, and even by covalent attachment of other polypeptides. Conjugation of target proteins by the small ubiquitin-related modifier (SUMO) (16) is a polypeptide post-translational modification that takes place at the lysine residue(s) of the target protein. SUMO is an 11-kDa protein that is structurally

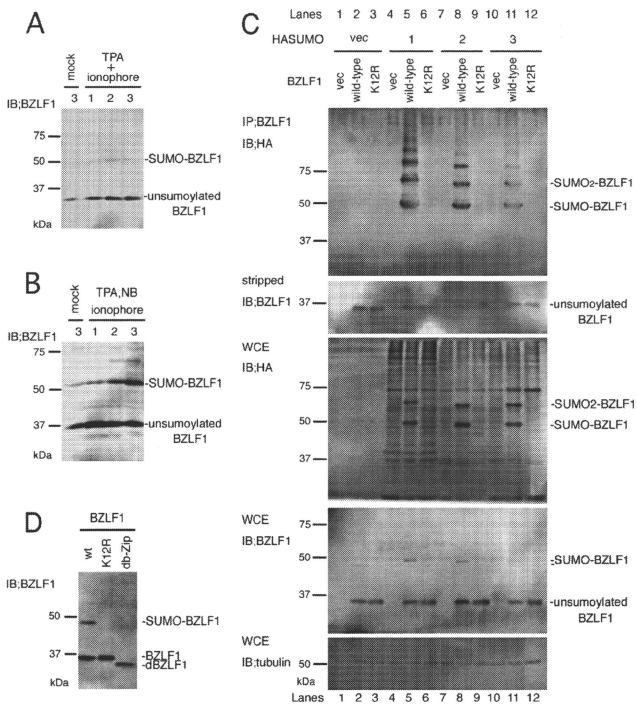
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<sup>2</sup>The abbreviations used are: EBV, Epstein-Barr virus; SUMO, small ubiquitin-related modifier; CBP, cAMP-response element-binding protein (CREB)-binding protein; HDAC, histone deacetylase; b-Zip, basic leucine zipper; TSA, trichostatin A; WCE, whole cell extracts; KSHV, Kaposi's sarcoma-associated herpesvirus; GFP, green fluorescent protein; HA, hemagglutinin; K-bZip, KSHV b-Zip.

## HDAC Association with Sumoylated BZLF1



**FIGURE 1. BZLF1 is modified by SUMO.** A and B, sumoylation of BZLF1 in infected cells is shown. A, GTC-4 cells were treated with or without 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml) and calcium ionophore A23187 (0.5 mM) for 1, 2, or 3 days. Cells were subjected to immunoblotting (IB) with anti-BZLF1 antibodies. B, likewise, AGS-CR2/GFP-EBV cells were treated with or without 12-*O*-tetradecanoylphorbol-13-acetate (20 ng/ml), sodium butyrate (NB, 5 mM), and calcium ionophore A23187 (0.5 mM) for 1, 2, or 3 days. Cell lysates were subjected to immunoblotting with anti-BZLF1 antibodies. NB, sodium butyrate. C, BZLF1 protein is a substrate for SUMO-1, -2, and -3 modification. HEK293T cells were transfected with pcDNA3-BZLF1(wild type) or pcDNA3-BZLF1(K12R) and pCHASUMO-1, -2, or -3. Immunoprecipitation (IP) was carried out using anti-BZLF1 antibodies. After immunoblotting (IB) with anti-HA antibodies (top panel), the membrane was stripped and reprobed with anti-BZLF1 antibodies (second panel). As controls, WCE from the same samples were also stained with HA (third panel), BZLF1 (fourth panel), and tubulin (bottom panel) antibodies. D, the b-Zip domain of BZLF1 is crucial for its sumoylation. HEK293T cells were transfected with pcDNA3-BZLF1(wild type), pcDNA3-BZLF1(K12R), or pcDNA3-BZLF1(db-Zip). Cell proteins were harvested for immunoblotting using anti-BZLF1 antibodies.

related to ubiquitin, and its covalent modification of proteins regulates various important cellular functions, such as nuclear transport, activation/suppression of signal transduction, cell cycle progression, and protein degradation (17–19). Three SUMO homologs have been described in mammals. SUMO-1 and SUMO-2/3 appear to partly share their substrates. Thus, some substrates may be simultaneously modified by SUMO-1 and SUMO-2/3, whereas RanGAP1, for example, is predominantly modified by SUMO-1, and topoisomerase II is also predominantly modified by SUMO-2/3 (20, 21).

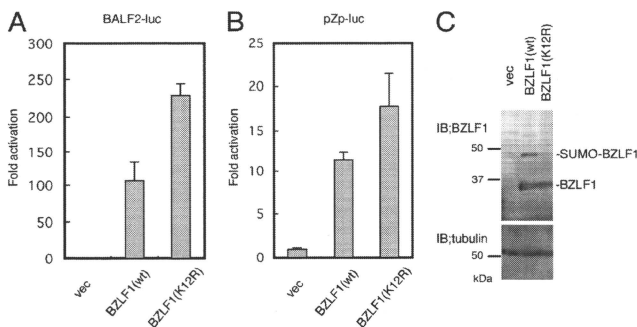
Adamson and Kenney first reported in 2001 (22) that BZLF1 protein is modified by SUMO-1, most likely at lysine

12, and that BZLF1 is responsible for disruption of promyelocytic leukemia bodies upon induction of EBV lytic replication. However, sumoylation of BZLF1 does not appear to be involved in promyelocytic leukemia body disruption (22). Subsequently, with a sumoylation-defective mutant of BZLF1 (Zm12/13), Adamson (23) demonstrated that the SUMO-1 modification decreases the transcriptional activity of BZLF1 without its degradation. The physiological significance of the SUMO modification of BZLF1, however, remains elusive.

In this study we show that BZLF1 is conjugated not only by SUMO-1 but also by SUMO-2 and 3 and that sumoylated BZLF1 is deconjugated by SENP reversibly. SUMO modification of BZLF1 negatively modulated its transcriptional activity. We further found that transcriptional repression by SUMO modification is correlated with association of repressor complexes, which at least include HDAC3 in the context of infection. These results indicate that the virus utilizes SUMO modification to provide favorable conditions for its optimal replication in the host cell.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Reagents**—GTC-4, AGS-CR2/GFP-EBV, HEK293T, EBV-Bac/Zp-luc, and BZLF1KO cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. GTC-4 is a cell line established from an EBV-positive gastric cancer (24). To prepare AGS-CR2/GFP-EBV cells, an EBV-negative cell line from gastric cancer, AGS, was stably transfected with CR2 (CD21), the receptor for EBV expression vector (25) and infected with GFP-EBV (26) followed by G418 selection. EBV-Bac/Zp-luc cells were prepared by transfection of Bac/Zp-luc DNA into HEK293 cells (27). Anti-EBV antibodies were purchased from Roche Applied Science, and anti-tubulin antibodies were from Cell Signaling. Anti-GFP, -CBP, and -HDAC3 antibodies were from MBL, Santa Cruz, and Abcam, respectively. Rabbit anti-BZLF1, -BMRF1, -BALF2, and -BALF5 antibodies were as reported previously (27). Horseradish peroxidase (HRP)-linked goat antibodies to mouse/rabbit IgG and TrueBlot ULTRA HRP anti-rabbit/mouse IgG were from



**FIGURE 2. K12R mutant BZLF1 activates promoters with BZLF1-responsive elements more efficiently than wild-type BZLF1.** A, HEK293T cells were transfected with 10 ng of BALF2-luc, 1 ng of pCMV-RL, and 10 ng of pcDNABZLF1 (wild type) or pcDNABZLF1(K12R). Counts are shown as -fold activation of that without the BZLF1 expression vector (vec). B, HEK293T cells were transfected with 10 ng of pZp-luc, 1 ng of pCMV-RL, and 10 ng of pcDNABZLF1 (wild type) or pcDNABZLF1(K12R). Counts were shown as -fold activation of that without the BZLF1 expression vector. C, the expression levels of BZLF1 and tubulin proteins in the assay were measured by immunoblotting (IB).

Amersham Biosciences and eBioscience, respectively. Tricostatin A (TSA) was purchased from Sigma.

**Plasmid Construction**—The expression vector for BZLF1 was constructed as detailed previously (28), and the K12R point mutation was introduced by PCR using appropriate primers. Vectors for HA-SUMO-1, -2, and -3 and SENPs were also described previously (29–32) as was construction of the pZp-luc and pBALF2-luc reporter plasmids (28, 33). pCMV-RLuc was from Promega. The pcDNA3-based expression vector for SUMO-2/BZLF1 fusion protein was made by PCR using appropriate primers.

**Immunoprecipitation and Immunoblotting**—To detect sumoylated BZLF1, we carried out immunoprecipitation under stringent conditions (34). In brief, cells were solubilized and sonicated in 100  $\mu$ l of SDS(+) lysis buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 1% SDS, and a protease inhibitor mixture). Lysates were boiled for 5 min to completely denature proteins and disrupt non-covalent interactions. Cell lysates were then diluted with 900  $\mu$ l of SDS(-) lysis buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and protease inhibitor mixture) and precleared with protein G-Sepharose (Amersham Biosciences). Supernatants were then incubated with anti-BZLF1 antibodies, and immunocomplexes were recovered by interaction with protein-G-Sepharose for 1 h, after which the resin was washed five times with SDS(-) lysis buffer. Samples were subjected to SDS-PAGE followed by immunoblotting with indicated antibodies. Immunoblotting was carried out as described previously (28). For measurement of protein levels, imaging, and densitometry software (Lumi Vision Analyzer version 2.1, AINIS SEIKI, Co.) were used. To detect associations with sumoylated BZLF1, we used the lysis buffer described previously (28) with the slight modification that 10 mM *N*-ethylmaleimide was added to preserve the sumoylation of cellular proteins (35).

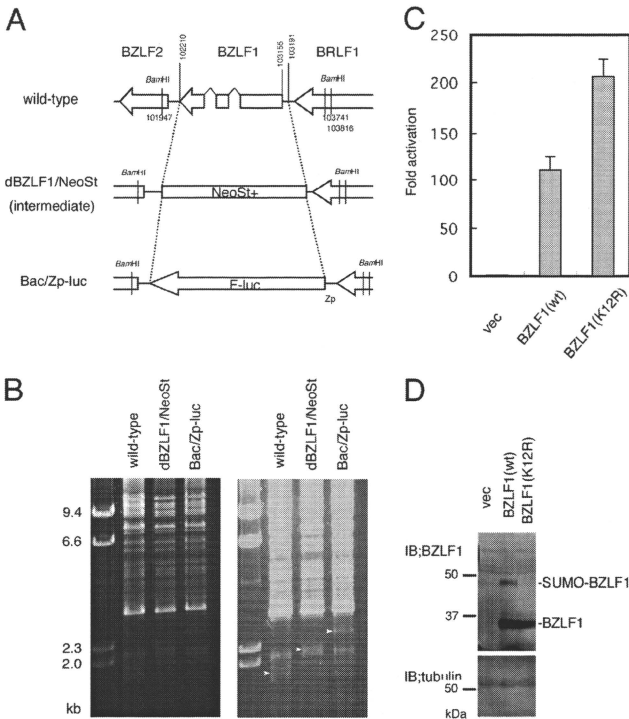
**Transfection and Luciferase Assays**—Plasmid DNA was transfected into HEK293T cells by lipofection or electroporation using Lipofectamine 2000 reagent (Invitrogen) or Microporator (Digital Bio). The total amounts of plasmid DNAs were standardized by the addition of an empty vector. Proteins were extracted from cells with the lysis buffer supplied in a Dual-Luciferase Reporter Assay System (Promega) kit, and luciferase activity was measured using the kit. The counts for firefly luciferase were normalized to those for renilla luciferase.

**Genetic Manipulation of EBV-Bac DNA**—EBV-Bac DNA was provided by Hammerschmidt and co-workers (36). Homologous recombination was carried out in *Escherichia coli* as described previously (27).

To prepare Bac/Zp-luc, transfer DNA fragment for the first recombination was prepared by PCR using PpsL-neo (Gene Bridges) as the template with the primers 5'-AGGAGCTGGTGCCTTGGCTTTAAAGGGGAGATGTTAGACAGGTAACACTCACTAAACATTGGGCCTGGTGATGATGGCGGATC-3' and 5'-ATTTAATTAATTCATTAGTAACGAGCGGTGAAGCAGCGCGGTTCACATAACGGGAGTCAAGAAGAACTCGTCAAGAAGG-3'. After the recombination, kanamycin-resistant colonies were selected and checked to make the intermediate,  $\delta$ BZLF1/NeoSt. The NeoSt+ cassette in the intermediate DNA was then replaced using the next transfer vector DNA which contained the firefly luciferase gene with Zp. The transfer vector was made by PCR using pZp-luc (28) as the template with the primers 5'-GCCATGCATATTTCAACTGG-3' and 5'-AGAGAGCCGACAGGAAGATATTTAATAATTCATTAGTAACGAGCGGTGAAGCAGCGCGGTTCACATAACGGGAGTTACAGCGCATCTTGGCGC-3'. Streptomycin-resistant colonies were cloned and checked to make Bac/Zp-luc. To construct the BZLF1KO virus, the following primers were used for PCR: 5'-GCTCCTGAGAATGCTTATCAAGCTTATGCGACACCTCAGCTGTTCCAGTCTCCGACATAGGCTGGTGATGATGGCGGATC-3' and 5'-CCGGCATTTCTGGAGCCACCCGATCTTGATCGGTTTATTCTAGTTTCAAGTATCGCATCAAGAAGAACTCGTCAAGAAGG-3'. Electroporation for *E. coli* was performed using Gene Pulser III (Bio-Rad), and purification of EBV-Bac DNA was achieved with NucleoBond Bac100 (Macherey-Nagel). Recombinant EBV-Bac viruses were confirmed in supplemental Fig. 1.

**Chromatin Immunoprecipitation Assay**—This assay was performed as described previously (28). The recovered DNA was amplified by PCR using the following primers: for BZLF1 promoter, 5'-TAGCCTCGAGGCCATGCATATTTCAAC-TGG-3' and 5'-GCCAAGCTTCAAGTTGCAATGTTTAGT-GAG-3'; for BALF2 promoter, 5'-ACCAAGCTTAGTGGCCCAAGGTATCGCCCG-3' and 5'-CTGGCCCTCGCTAGCA-

## HDAC Association with Sumoylated BZLF1



**FIGURE 3. The K12R mutant activates the BZLF1 promoter more efficiently than wild-type BZLF1 in the context of the viral genome.** *A*, shown is a schematic arrangement of the recombination of the EBV genome using tandemly arranged neomycin resistance and streptomycin sensitivity genes (*NeoSt<sup>r</sup>*). The region between nucleotide 102,210 and 103,191 of the B95-8 genome (V01555) was replaced with the *NeoSt<sup>r</sup>* cassette to make the intermediate, dBZLF1/*NeoSt*. The *NeoSt<sup>r</sup>* cassette was then replaced with a vector sequence with firefly luciferase (*F-luc*) flanked with the 5' and 3'-UTRs of the *BZLF1* gene to construct Bac/*Zp-luc*. The Bac DNA was introduced into HEK293 cells followed by hygromycin selection. Resultant cell clones were tested for lytic induction, and one of the typical clones was used as EBV-Bac/*Zp-luc* cells in the next panels. *B*, electrophoresis of the recombinant viruses is shown. EBV-Bac DNAs were digested with *Bam*HI and separated in an agarose gel. Brightness was enhanced in the right panel to clearly show *Bam*HI-*Z* fragment of the virus. White arrowheads indicate the size of *Bam*HI-*Z* fragment (wild type) or the sizes of the fragments plus inserts. *C*, EBV-Bac/*Zp-luc* cells were transfected with 1 ng of pCMV-RL and 30 ng of pCDNA3BZLF1(wild type) or pCDNABZLF1(K12R). Counts are shown as fold activation of that without the BZLF1 expression vector. *D*, the expression levels of BZLF1 and tubulin proteins in the assay were measured by immunoblotting (IB).

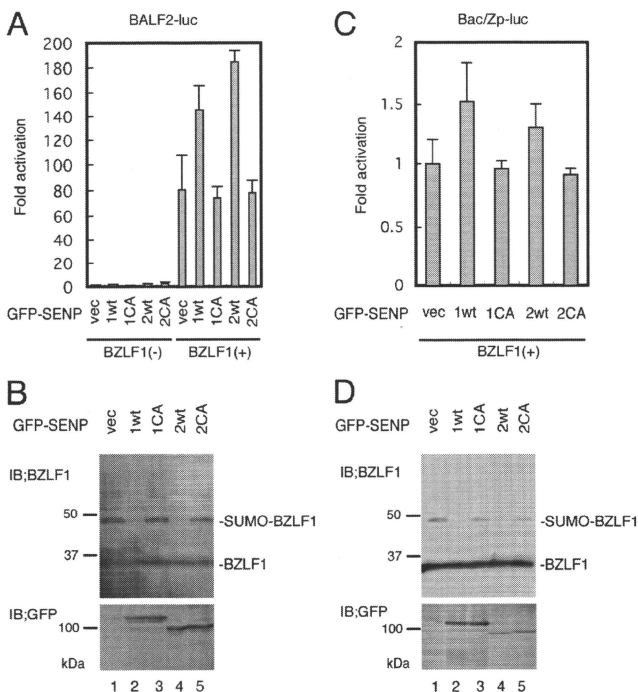
GACTCTGGTTTGCG-3'; for Ori-Lyt, 5'-CCGGCTCGCCT-TCTTTTATCCTC-3' and 5'-CCTGGTTCAACCCATGG-AGGGGAC-3'; for the EBNA-1 open reading frame, 5'-GTC-ATCATCATCCGGGTCTC-3' and 5'-TTCGGGTTGGAA-CCTCCTTG-3'.

### RESULTS

**BZLF1 Protein Is a Substrate for SUMO-1, -2, and -3 Modifications**—It was previously reported that the BZLF1 protein of EBV is modified by SUMO-1, most likely at lysine 12 (22). We first examined the conjugation in the lytic replication-

induced cells (Fig. 1, *A* and *B*). Densitometry analysis demonstrated in GTC-4 (Fig. 1*A*) cells that unsumoylated BZLF1 levels were 31% without induction (mock) and reached 91, 100, and 95% 1, 2, and 3 days after induction, respectively, when sumoylated BZLF1 levels were 1.8, 7.0, 13, and 9.8% in mock, 1-, 2-, and 3-day samples (value of the highest density (unsumoylated BZLF1 band, day 2 in Fig. 1*A*) was set 100%). The sumoylation was more conspicuous in AGS-CR2/GFP-EBV cells (Fig. 1*B*). A significant amount of sumoylated and unsumoylated BZLF1 was spontaneously produced in the cells even without lytic induction (22 and 54%, respectively), as AGS cells support significantly high levels of persistent lytic infection of the virus (37). Unsumoylated BZLF1 levels reached a maximum on day 1 (100%) and gradually declined on days 2 and 3 (75 and 64%, respectively), whereas levels of SUMO-conjugated BZLF1 rose 29, 36, and 41% on days 1, 2, and 3. In EBV-positive lymphocytes, however, the SUMO modification of BZLF1 was very low (data not shown), suggesting the physiological significance of the modification, especially in epithelial cells.

We then performed a detailed biochemical analysis of the modification. In humans, three paralogs of SUMO proteins, SUMO-1, -2, and -3, have been identified. Despite the similarities between the proteins, recent studies suggest that SUMO-1 and SUMO-2/3 conjugate to distinct substrates in some cases. Thus, we then tested if BZLF1 is also modified by SUMO-2 and SUMO-3. In Fig. 1*C*, HEK293T cells were transfected with wild-type or K12R mutant of BZLF1 expression vector together with each of HA-tagged SUMO-1, -2, and -3 expression vectors. An aliquot of whole cell extract was reserved for immunoblotting, whereas the remainder of the sample was subjected to immunoprecipitation using anti-BZLF1 antibody followed by immunoblotting. Unsumoylated BZLF1 proteins (wild type and K12R) with a molecular mass of 35 kDa were detectable in the whole cell extracts (WCE; Fig. 1*C*, fourth panel) and in the immunoprecipitated samples (*IP:BZLF1*) (second panel), proving comparable expression of the proteins and successful immunoprecipitation. When co-expressed with either of the SUMO proteins,



**FIGURE 4. SENP proteins deconjugate SUMO from BZLF1.** A and B, HEK293T cells were transfected with 10 ng of pcDNABZLF1(wild type) and 50 ng of GFP-tagged, wild type (wt), or inactive (CA) SENP expression vectors (vec) together with 10 ng of pZp-luc and 1 ng of pCMV-RL. Cell proteins were subjected to luciferase assay (A) and immunoblotting (B) using anti-BZLF1 (upper panel) and GFP (lower panel) antibodies (B). C and D, EBV-Bac/Zp-luc cells were transfected with 1 ng of pCMV-RL and 30 ng of pcDNABZLF1(wild type) or pcDNABZLF1(K12R). Counts are shown as -fold activation of that without the BZLF1 expression vector. Cell proteins were subjected to luciferase assay (C) and immunoblotting using anti-BZLF1 (upper panel) and GFP (lower panel) antibodies (D).

wild-type BZLF1 gave evidence of multiple sumoylation (top panel, lanes 5, 8, and 11), whereas the K12R mutant did not at all (lanes 6, 9, and 12). In addition, sumoylation of BZLF1 was so efficient that at least mono- and di-sumoylated forms of BZLF1 became visible even in the HA staining of WCE (third panel, lanes 5, 8, and 11). Together, BZLF1 is modified not only by SUMO-1 but also by SUMO-2 and -3 at lysine 12 residue. We also found that when the b-Zip domain of BZLF1 was removed, sumoylation of the protein diminished (Fig. 1D), just as reported in Kaposi's sarcoma-associated herpesvirus (KSHV) (38).

**K12R Mutant BZLF1 Activates BZLF1-responsive Promoters More Efficiently Than Wild-type BZLF1**—To assess the effects of BZLF1 sumoylation on its transcriptional activity, we analyzed whether a sumoylation-defective mutant of BZLF1 enhances BZLF1-responsive promoter activity (Fig. 2). We used the BALF2 promoter, which is responsive to BZLF1 (33). Although the expression of wild-type BZLF1 exhibited 106-fold

transactivation, expression of the K12R mutant exhibited 228-fold transactivation. As shown in Fig. 2C, expression levels of wild-type and K12R BZLF1 were comparable, and the K12R mutation disrupted SUMO conjugation. Thus, the mutation enhanced transcription from the BALF2 promoter by 2.2-fold. Likewise, BZLF1-mediated transcriptional activity of the BZLF1 promoter was also up-regulated by K12R mutation by 1.6-fold (Fig. 2B). These results clearly support the previous report that sumoylation of BZLF1 results in attenuated transactivation using a reporter assay system with the EBV BMRF1 promoter (23).

**Stimulation of BZLF1-responsive Promoter Activity by K12R Mutation in the Context of the Viral Genome**—To extend the above reporter gene experiment, we further examined whether SUMO conjugation of BZLF1 could influence its transcriptional activity on the BZLF1 promoter of the viral genome with nucleosomal structures. To this end we generated EBV-Bac DNA in which the entire open reading frame of the BZLF1 gene was replaced with the firefly luciferase gene (Fig. 3A, Bac/Zp-luc). As this Bac DNA contains the luciferase gene under the control of native BZLF1 gene promoter, reporter activity reflects transcription from the promoter in the EBV genome. The Bac DNA construction was confirmed by BamHI digestion followed by electrophoresis (Fig. 3B). Bac DNA was introduced into HEK293 cells followed by hygromycin selection to establish cell lines in which multiple copies of Bac/Zp-luc DNA were maintained as an episome. We confirmed that viral lytic gene expression of elements such as BMRF1 and BALF2 could be induced by the BZLF1 expression vector into cells (data not shown). As shown in Fig. 3C, expression of wild-type BZLF1 induced luciferase reporter activity 111-fold, whereas K12R mutant BZLF1 induced 206-fold activity. Expression levels of wild-type and K12R BZLF1 proteins were comparable, and the mutation disrupted SUMO conjugation (Fig. 4D). Thus, the sumoylation of BZLF1 at Lys-12 repressed BZLF1-dependent transcription by 47% in the context of the viral genome.

**SENP Proteins Deconjugate SUMO from BZLF1 and Enhance BZLF1-mediated Transcription**—We then focused on the desumoylation of BZLF1. As cleavage of isopeptide bonds



## HDAC Association with Sumoylated BZLF1

between SUMO and its targets is mediated by the SUMO-specific protease, SENP, we examined whether the protease could affect BZLF1 transcription through sumoylation (Fig. 4B). Two SENP proteins, 1 and 2, and their CA mutants, in which the conserved cysteine residue within the catalytic domain is changed to alanine, were used. SUMO-conjugated BZLF1 was detected in cells without SENP proteins (*lane 1*), and expression of SENP1 or SENP2 completely abrogated the modification (*lanes 2 and 4*). In contrast, catalytically inactive mutants of SENP proteins failed to deconjugate the modification (*lanes 3 and 5*). These results clearly confirmed the BZLF1 protein to be sumoylated as the SUMO-specific proteases cleaved the conjugation.

Because the desumoylation by the proteases was confirmed, we examined whether an exogenous supply of SENPs might affect the BZLF1-mediated transcription of the *BALF2* promoter in a reporter gene assay (Fig. 4A). Without SENPs, the addition of the BZLF1 expression vector activated the *BALF2* promoter by 81-fold. In the presence of wild-type SENP1 or -2, it reached 146- or 185-fold activation, respectively. In contrast, when catalytically inactive mutants of SENP1 or -2 were transfected, the transactivation became 74- or 78-fold, respectively, which was essentially the same as the level of transactivation obtained with BZLF1 transfection itself.

We then tested if SUMO deconjugation of BZLF1 by SENP could influence its transcriptional activity on the *BZLF1* promoter of the viral genome using Bac/Zp-luc (Fig. 4, C and D). As expected, desumoylation of BZLF1 by either SENP1 or SENP2 resulted in increased transcription from the viral genome. These results support our data that sumoylation of BZLF1 results in repression of its transcriptional activity and also provide evidence that BZLF1 sumoylation is a reversible process.

**Involvement of Histone Deacetylation in Transcriptional Repression by BZLF1 Sumoylation**—It has been reported that BZLF1 activates viral gene expression by interacting with the CBP possessing histone transacetylase activity (11–15). Immunoprecipitation assays indicated that BZLF1 did associate with CBP, but the interaction between sumoylated BZLF1 and CBP was somehow attenuated (data not shown), suggesting that conjugation of SUMO molecule to BZLF1 protein might reduce its association with the CBP transcriptional co-activator.

Next, we also examined whether BZLF1 interacts with histone deacetylase (Fig. 5). Surprisingly, sumoylated BZLF1 protein, but not the unmodified form, was pulled down with HDAC3 (Fig. 5, *lane 2*). A similar result was obtained when anti-HDAC7 antibodies were used (not shown).

From these observations we hypothesized that the transcriptional inhibition by BZLF1 sumoylation is mediated mainly by HDAC recruitment. To test this possibility, we analyzed the effect of TSA, a potent inhibitor of HDACs, on BZLF1-mediated transcription from the *BALF2* and *BZLF1* promoters in reporter gene assays. HEK293T cells were transfected, incubated in the presence or absence of TSA, harvested, and subjected to luciferase assays (Fig. 6, A and B) and immunoblotting (Fig. 6C). TSA treatment reversed the inhibitory action of BZLF1 sumoylation. We further exam-

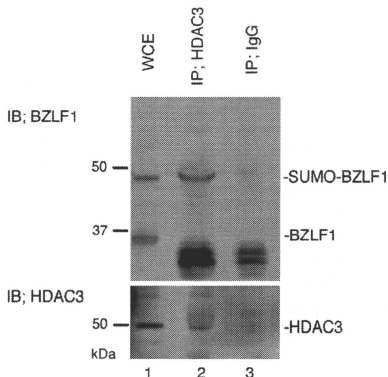


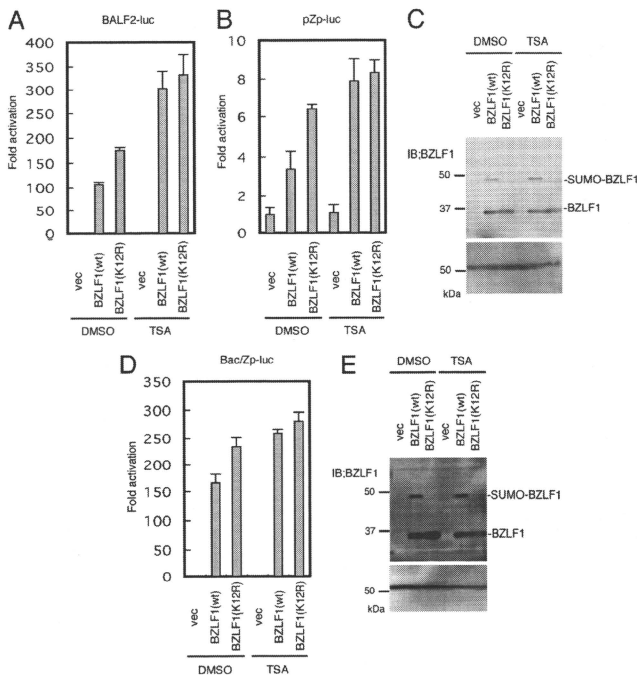
FIGURE 5. **SUMO modification of BZLF1 mediates HDAC3.** Proteins from HEK293T cells transfected with wild-type pcDNA3BZLF1 were subjected to immunoprecipitation (IP) using normal IgG or anti-HDAC3 followed by immunoblotting (IB) with anti-BZLF1 (upper panel) and HDAC3 (lower panel) antibodies. WCE (1/50) from the same lysate was also blotted.

ined the effect of TSA in the context of the viral genome (Fig. 6, D and E). As expected, TSA treatment abrogated the inhibitory action of BZLF1 sumoylation. Collectively, our results strongly suggested that BZLF1 represses BZLF1-responsive promoters by recruiting histone deacetylase complexes through sumoylation.

**HDAC Is Recruited to BZLF1-responsive Promoters through Association with BZLF1**—Next, we determined whether HDAC is actually recruited to viral promoters bound by sumoylated BZLF1. However, the experiment was hampered by low levels of SUMO conjugation. Thus, an expression vector for SUMO-2 and BZLF1 fusion protein was constructed to mimic sumoylated BZLF1, as it has been reported that SUMO fusion to target proteins produces similar effects (39–41). We speculated that HDAC could be recruited onto BZLF1-responsive promoters to repress transcription in the presence of the SUMO-2/BZLF1 fusion protein. We first examined the expression of the fusion protein (Fig. 7A).

We then tested the effect of the fusion protein on the *BZLF1* promoter of the viral genome using Bac/Zp-luc (Fig. 7B). As expected, SUMO-2/BZLF1 fusion protein failed to transactivate the *BZLF1* promoter, whereas wild-type BZLF1 protein clearly did transactivate the promoter by 66-fold.

Results of immunoprecipitation assay using anti-HDAC3 antibody confirmed the SUMO-2/BZLF1 fusion protein to be associated with HDAC3, just like sumoylated BZLF1 (Fig. 7C). Because the SUMO-2/BZLF1 fusion protein acted as expected, we further confirmed the interactions. When anti-BZLF1 antibody was used for precipitation (Fig. 7D), HDAC3 co-precipitated more efficiently with SUMO-2/BZLF1 fusion protein than BZLF1 protein. On the other hand, CBP preferentially associated with BZLF1, but the association was weak with SUMO-2/BZLF1. We here used BZLF1 K12R and SUMO-2/BZLF1 K12R mutants to elimi-



**FIGURE 6. An HDAC inhibitor reverses the effect of BZLF1 sumoylation.** *A* and *B*, HEK293T cells were transfected with 10 ng of BALF2-luc (*A*) or pZp-luc (*B*), 1 ng of pCMV-RL, and 10 ng of pcDNABZLF1 (wild type) or pcDNABZLF1 (K12R). After 4 h, cells were then exposed to TSA (100  $\mu$ M) for 20 h. Counts are shown as -fold activation of that without the BZLF1 expression vector (vec). *C*, The expression levels of BZLF1 and tubulin proteins in the assay were measured by immunoblotting (IB). *D*, EBV-Bac/Zp-luc cells were transfected with 1 ng of pCMV-RL and 30 ng of pcDNABZLF1 (wild type) or pcDNABZLF1 (K12R). After 4 h cells were then exposed to TSA (100  $\mu$ M) for 20 h. Counts are shown as -fold activation of that without the BZLF1 expression vector. *E*, The expression levels of BZLF1 and tubulin proteins in the assay were measured by immunoblotting.

nate the SUMO conjugation at the residue, which might provoke confusion.

To examine if HDAC is actually recruited to BZLF1-responsive elements in viral promoters, we performed chromatin immunoprecipitation assays in the presence of the SUMO-2/BZLF1 fusion protein (Fig. 7E). With either wild-type or SUMO-2-fused BZLF1, BZLF1 and BALF2 promoter sequences and *oriLyt*, all of which contain BZLF1-responsive elements, co-precipitated with anti-BZLF1 antibodies (Fig. 7E, lanes 3 and 7), demonstrating that BZLF1 binds to BZLF1-responsive elements even when N-terminal-fused with SUMO-2. Immunoprecipitation with anti-HDAC3 antibodies (Fig. 7E, lanes 4 and 8) revealed the SUMO-2/BZLF1 fusion protein to exhibit more efficient recruitment of the deacetylase to the BZLF1 and BALF2 promoters and *oriLyt* than wild-type BZLF1 protein. This finding is consistent with our speculation that BZLF1 represses BZLF1-responsive promoters by recruiting HDAC complexes through sumoylation.

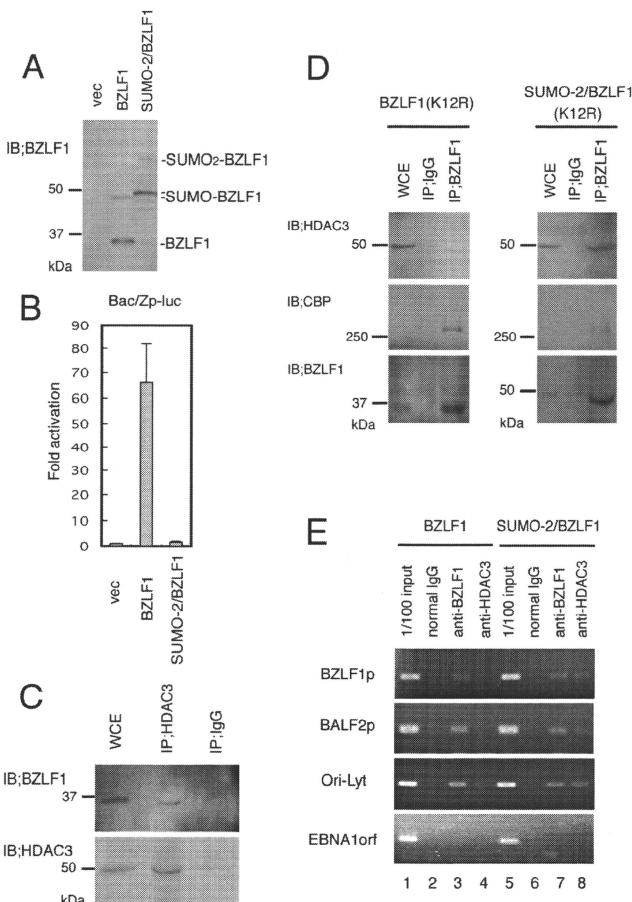
**Activation of Viral Early Genes in BZLF1KO Virus-infected Cells**—In Fig. 3 we assayed the effect of BZLF1 sumoylation on the BZLF1 promoter. Because viral early genes such as BALF2 and BMRF1 are also responsive to BZLF1, we made the BZLF1KO virus (Fig. 8, A and B) and examined how the sumoylation affected on its early gene expressions (Fig. 8, C–E). As shown in Fig. 8C, K12R mutant of BZLF1 induced BALF2 and BMRF1 proteins more efficiently than wild-type BZLF1. On the other hand, with or without K12R mutation, SUMO-2/BZLF1 fusion protein did not enhance the early genes (Fig. 8C). Co-expression of wild-type SENP1 correlated with the disappearance of sumoylated BZLF1 and increased early gene productions (Fig. 8D). Finally, a HDAC inhibitor TSA augmented amounts of early gene products without affecting the BZLF1 sumoylation levels (Fig. 8E). These results support the idea that sumoylated BZLF1 represses transcription from BZLF1-responsive promoters and that histone acetylation levels play a significant role in that process.

## DISCUSSION

In this report we document evidence that SUMO conjugation of BZLF1 negatively affects its transcriptional activity. In contrast to ubiquitination, which most frequently is regarded as an earmark for proteasome-dependent degradation, sumoylation has a wide range of substrate-specific functions. With transcription factors, SUMO modification is mostly related to repression. We also suggest that negative regulation is brought by epigenetic alterations of virus gene promoters. It has been previously demonstrated that the BZLF1 protein interacts physically with CBP through its N-terminal activation domain to enhance viral early gene transcription (11–14). As BZLF1 sumoylation takes place at the N terminus, it is tempting to speculate that structural alteration caused by the conjugation weakens the association, thereby inhibiting the transcriptional activity.

By means of simple immunoblotting experiments, we could show that the level of the sumoylated form of BZLF1 is much less than with its un-sumoylated counterpart. Nevertheless, the K12R mutant of BZLF1, which cannot be sumoylated, enhanced more efficiently than the wild-type BZLF1. Therefore, we speculate that there must be much more active and powerful repres-

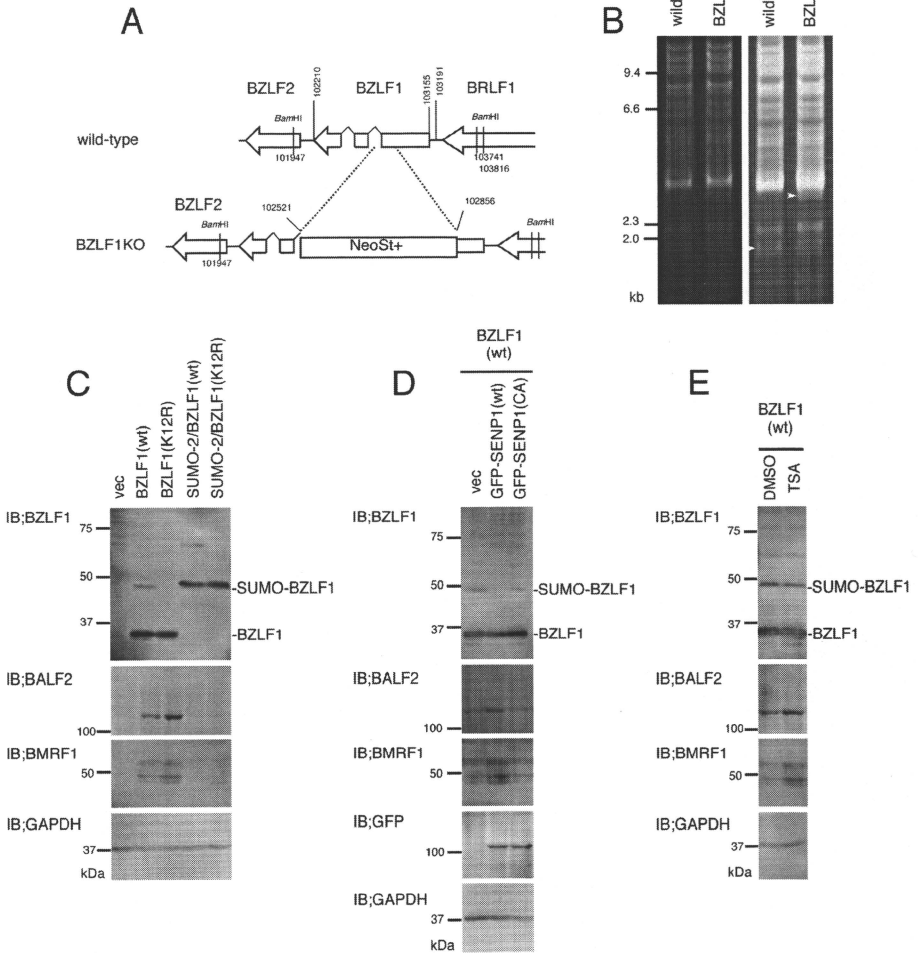
## HDAC Association with Sumoylated BZLF1



**FIGURE 7. HDAC is recruited to BZLF1-responsive promoters through association with SUMO-BZLF1.** *A*, pcDNASUMO-2/BZLF1, the expression vector for SUMO-2/BZLF1 fusion protein, was constructed as described under "Experimental Procedures," and its expression in HEK293T cells was confirmed by immunoblotting (IB). *B*, EBV-Bac/Zp-luc cells were transfected with 1 ng of pCMV-RL and 30 ng of pcDNABZLF1 (wild type) or pcDNASUMO-2/BZLF1. Counts are shown as -fold activation of that without the BZLF1 expression vector (*vec*). *C*, SUMO fusion with BZLF1 mediates HDAC3. Proteins from HEK293T cells transfected with pcDNASUMO-2/BZLF1 were subjected to immunoprecipitation (IP) using normal IgG or anti-HDAC3 followed by immunoblotting with anti-BZLF1 (*upper panel*) and HDAC3 (*lower panel*) antibodies. WCE (1/50) from the same lysate was also blotted. *D*, proteins from HEK293T cells transfected with pcDNABZLF1(K12R) or pcDNASUMO-2/BZLF1(K12R) were subjected to immunoprecipitation using normal IgG or anti-BZLF1 followed by immunoblotting with anti-HDAC3 (*top panel*), CBP (*middle panel*), and BZLF1 (*lower panel*) antibodies. WCE (1/50) from the same lysate was also blotted. *E*, chromatin immunoprecipitation experiments were carried out using cross-linked DNA-protein complexes from EBV-Bac/Zp-luc cells transfected with pcDNABZLF1 or pcDNASUMO-2/BZLF1. DNA-protein complexes were precipitated using normal IgG, anti-BZLF1, anti-CBP, or anti-HDAC3 followed by DNA extraction and PCR reactions to detect the *BZLF1* and *BALF2* promoters or *Ori-Lyt*, which contains binding sites for BZLF1. A fragment for the EBNA-1 open reading frame was also detected as a negative control.

tion mechanism rather than just inhibition of the interaction with CBP. Recently, it has been reported that sumoylation of cellular transcription factors promotes interaction with repressor complexes including HDAC (42–48). We, therefore, tested the interaction between SUMO-modified BZLF1 and HDAC and showed that at least HDAC3 associates with the sumoylated form of BZLF1 but not clearly with the unsumoylated form, suggesting that SUMO-mediated acetylation levels of chromatin affect the BZLF1-dependent transcription.

Sumoylation is often regulated by phosphorylation at a neighboring residue of the target protein. It is very well established that a phosphorylation-dependent sumoylation motif ( $\Phi$ KX(D/E)XX(S/T)P, where  $\Phi$  is a hydrophobic amino acid residue, X represents any residue, and D or E is an acidic residue) is present in some transcription factors, including heat-shock factor 1, MEF2, and estrogen-related receptors. Phosphorylation at the phosphorylation-dependent sumoylation motif, which contains a SUMO consensus motif ( $\Phi$ KX(D/E)) and an adjacent proline-directed phosphoacceptor, up-regulates SUMO conjugation of those transcription factors (49–51). Although phosphorylation-dependent sumoylation motif is not a feature of BZLF1, we found a remarkable similarity except in the Thr-14 residue in BZLF1 when the surrounding sequence of BZLF1 Lys-12 ( $^{11}$ VKFTP $^{15}$ ) was compared with the consensus motif for SUMO modification ( $\Phi$ KX(D/E)). It could be envisaged that the non-functional motif,  $\Phi$ KX(S/T), might be converted to a functional substrate for sumoylation after phosphorylation at Ser or Thr residues (17). In fact, BZLF1 Thr-14 is strongly phosphorylated (52). We, thus, examined if a single point mutation at Thr-14 affected the BZLF1 sumoylation. However, the levels of sumoylation were neither increased nor decreased by the T14D



**FIGURE 8. Viral early gene expression was decreased by BZLF1 sumoylation.** *A*, shown is a schematic arrangement of the recombination of the EBV genome using the neomycin resistance and streptomycin sensitivity genes (NeoSt<sup>+</sup>). The region between nucleotide 102,521 and 102,856 of the B95-8 genome (V01555) was replaced with the NeoSt<sup>+</sup> cassette to make the BZLF1KO virus. The Bac DNA was introduced into HEK293 cells followed by hygromycin selection. Resultant cell clones were tested for lytic induction, and one of the typical clones was used as BZLF1KO cells in the next panels. *B*, electrophoresis of the recombinant viruses is shown. EBV-Bac DNAs were digested with BamHI and separated in an agarose gel. Brightness was enhanced in the right panel to clearly show BamHI-Z fragment of the virus. White arrowheads indicate the size of BamHI-Z fragment (wild type) or the size of the fragment plus the cassette. *C*, effect of BZLF1 mutants on early gene expressions of BZLF1KO virus. HEK293 cells with the BZLF1KO virus were transfected with indicated BZLF1 mutants. After 15 h, cell lysates were subjected to immunoblotting (IB) using anti-BZLF1, BALF2, BMRF1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. *D*, the effect of SENP on early gene expressions of BZLF1KO virus is shown. HEK293 cells with the BZLF1KO virus were transfected with BZLF1(wt) together with GFP-SENP expression vectors. After 15 h, cell lysates were subjected to immunoblotting using anti-BZLF1, BALF2, BMRF1, and glyceraldehyde-3-phosphate dehydrogenase antibodies. *E*, shown is the effect of an HDAC inhibitor on early gene expressions of BZLF1KO virus. HEK293 cells with the BZLF1KO virus were transfected with BZLF1(wt) expression vectors. After 4 h, TSA was added to the medium followed by incubation for 11 h. Cell lysates were subjected to immunoblotting using anti-BZLF1, BALF2, BMRF1, and glyceraldehyde-3-phosphate dehydrogenase antibodies.

## HDAC Association with Sumoylated BZLF1

or T14A mutation of BZLF1 (data not shown), indicating that phosphorylation at Thr-14 has little or no effect on the conjugation.

The information about KSHV K-bZIP sumoylation provided by Izumiya *et al.* (38) may be of great value in determining the nature of sumoylation of the EBV homologue, BZLF1. K-bZIP is thought to be a structural homolog of EBV BZLF1, as they share the b-Zip domain located near the C terminus and a presumptive regulatory region in the N terminus, although the homology of the sequences is not very high (about 37%). Interestingly, although the EBV BZLF1 gene product functions as a transcriptional activator, K-bZIP has strong repression activity. Izumiya *et al.* (38) have indicated that transcriptional repression by K-bZIP is caused by SUMO. As they showed a physical interaction between K-bZIP and the SUMO E2 ligase, Ubc9, we also examined the possibility of such interaction with EBV BZLF1. Co-immunoprecipitation assays in fact did reveal a weak association (data not shown) suggesting that the affinity with the ligase may at least in part define the opposite actions of the viral b-Zip protein.

EBV BZLF1 sumoylation takes place at the N terminus, and the residue for K-bZIP is in the C terminus region. Despite the difference in residues for the SUMO conjugation, we found another similarity. Just like truncation of the b-Zip domain from KSHV K-bZIP abrogates SUMO modification of the protein (38), SUMO conjugation to EBV BZLF1 did not take place when the EBV BZLF1 b-Zip motif was removed (Fig. 2). This result suggests that DNA binding of the proteins may play significant roles in SUMO conjugation. Alternatively, the SUMO E2 ligase, Ubc9, may not be able to associate with the b-Zip null mutant of BZLF1, just as is the case with K-bZIP (38) and some other b-Zip proteins (53, 54). The SUMO-specific protease SENP might be involved in this process, as it deconjugates SUMO molecules from BZLF1 (Fig. 5).

KSHV K-bZIP sumoylation is known to be decreased by phosphorylation at Thr-111 of K-bZIP by the virus PK (55). Because the BGLF4 PK of EBV, the structural and functional homolog of KSHV vPK, interacts with and phosphorylates BZLF1 (56, 57), its effects on BZLF1 sumoylation were examined (data not shown). EBV BZLF1 sumoylation was also decreased by the virus BGLF4 PK, but unlike K-bZIP, phosphorylation of EBV BZLF1 at Ser-209 by BGLF4 (57) did not influence the SUMO modification (data not shown). Those results were clearly repeated by Hagemeyer *et al.* in their very recent article (58). We assume that BGLF4 PK may suppress BZLF1 sumoylation through phosphorylation of another factor. One possibility is that PK might structurally inhibit SUMO conjugation of BZLF1 through their interaction because K102I mutation of BGLF4 PK disables its association with BZLF1 (57).

When viral lytic replication was induced in GTC-4 or AGS cells latently infected with EBV, BZLF1 sumoylation levels were relatively low at 24 h and then increased at 48 and 72 h (Fig. 1, A and B). This indicates that transcriptional activity of BZLF1 is relatively intact at 24 h or earlier and then suppressed by SUMO conjugation thereafter. The virus may be taking advantage of the cellular SUMO system to improve its replication efficiency because transcriptional activity of BZLF1 is necessary for enhancement of viral early gene expressions at earlier times

after lytic induction and is not needed any longer after viral gene production is complete. We also tested if EBV-positive lymphocytes, including Akata, B95-8, and Raji, provide BZLF1 sumoylation. However, sumoylation levels of endogenous BZLF1 in those cell lines were very low, suggesting a significant role of the modification in epithelial cells.

To summarize, EBV reactivation from latency is dependent on the availability and association with other transcriptional co-factors and post-translational modification of BZLF1. The overall regulation appears very complicated, but with sumoylation, EBV may exploit the modification to fine-tune the levels of viral lytic replication. Further elucidation is needed for comprehensive understanding of the molecular mechanism that governs EBV reactivation from latency.

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# Transient increases in p53-responsible gene expression at early stages of Epstein-Barr virus productive replication

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**Key words:** p53, Epstein-Barr virus, transcription, BZLF1 protein

Expression of Epstein-Barr Virus BZLF1, a key protein initiating the switch from latent to lytic infection, is known to cause cell growth arrest by accumulating p53 and p21<sup>WAF1/CIP1</sup> in epithelial cells, but its molecular mechanism remains elusive. We found here that the BZLF1 protein stimulates p53 binding to its recognition sequence. The BZLF1 accelerated the rate of p53-DNA complex formation through the interaction with p53 protein and also enhanced p53-specific transcription *in vitro*. Furthermore, p53 protein was found to bind to its target promoter regions specifically in the early stages of lytic replication. Overexpression of p53 at the early stages of lytic replication enhanced viral genome replication, supporting the idea that p53 plays an important role in the initiation steps of EBV replication. Taking the independent role of BZLF1 on p53 degradation into consideration, we propose that the BZLF1 protein regulates p53 and its target gene products in two distinctive manners; transient induction of p53 at the early stages for the initiation of viral productive replication and p53 degradation at the later stages for S-phase like environment preferable for viral replication.

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## Introduction

The tumor suppressor p53 plays an important role in maintaining genomic integrity. Exposure of genotoxic stress to a normal cell causes phosphorylation of p53, resulting in stabilization of p53 and an increase in p53 protein level. The increase in p53 protein results in an increase in p53-dependent transcription of its target genes including p53 itself, which subsequently leads to cell cycle arrest or apoptosis.<sup>1,2</sup> Wild-type p53 binds to specific genomic DNA sites with a consensus recognition sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'. It is predicted that the expression of about 200–300 genes might be controlled by p53 transactivation. The ability of p53 to induce a G<sub>1</sub> arrest is mainly dependent on its ability to drive an increasing expression of the p21<sup>WAF1/CIP1</sup> protein, a cyclin-dependent kinase (CDK) inhibitor.<sup>3,4</sup>

Epstein-Barr virus (EBV), a human gamma-herpesvirus, is associated with several B-cell and epithelial-cell malignancies, and it chooses two alternative infection states; latent and lytic.<sup>5</sup> Infection is primarily latent, but EBV periodically reactivates and replicates in a lytic manner in a subset of cells, being essential for viral propagation and transmission. The reactivation of EBV from the latent phase to the productive lytic cycle not only produces

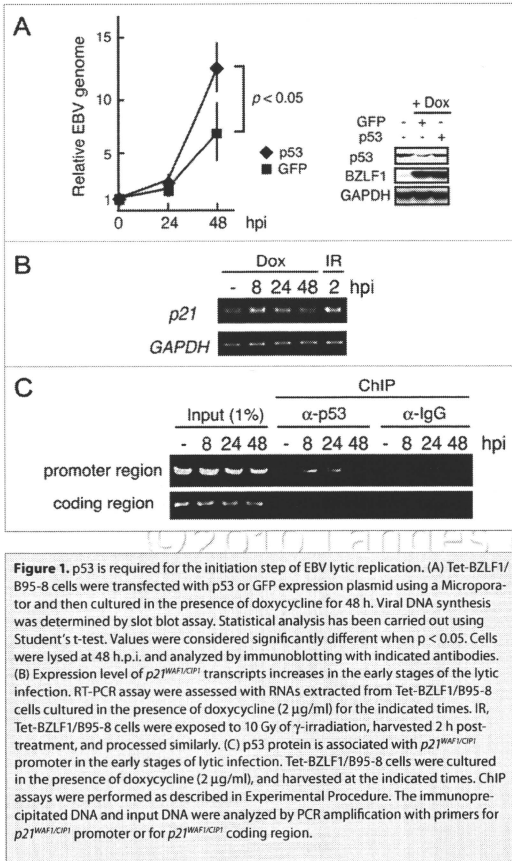
infectious viral progeny but also contributes to disease progression. Elevation of antibodies against EBV lytic gene products in the sera of patients with nasopharyngeal carcinoma is suggestive of a correlation between viral reactivation and human cancer.<sup>6,7</sup> In addition, the requirement of lytic gene expression for outgrowth of lymphoblastoid cell lines in a SCID mouse model suggests the importance of reactivation for EBV pathogenesis.<sup>8</sup> In latently-infected cells, p53 exists as a hypo-phosphorylated form and its expression level is regulated through Mdm2 ubiquitin ligase. The hypo-phosphorylated p53 exhibits weak binding to its recognition sequences. After induction of lytic replication, ATM-dependent DNA damage signal transduction pathway is activated,<sup>9</sup> resulting in phosphorylation of p53 at various sites including S15 after 24 h post-induction.<sup>10</sup> Although the phosphorylation of p53 prevents interaction with MDM2, the BZLF1 protein directly functions as an adaptor component of the ECS (Elongin B/C-Cul2/5- $\zeta$ SOCS-box protein) ubiquitin ligase complex targeting phosphorylated p53 for degradation, followed by sustaining S-phase like cellular environment with high CDK activities especially at middle and late stages of EBV lytic replication.<sup>10</sup>

The switch from latent to lytic infection is triggered by the BZLF1 immediate-early protein,<sup>11</sup> a b-Zip transcriptional factor which binds to AP-1-like sequences present in promoters of

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**Figure 1.** p53 is required for the initiation step of EBV lytic replication. (A) Tet-BZLF1/B95-8 cells were transfected with p53 or GFP expression plasmid using a Microportorator and then cultured in the presence of doxycycline for 48 h. Viral DNA synthesis was determined by slot blot assay. Statistical analysis has been carried out using Student's t-test. Values were considered significantly different when  $p < 0.05$ . Cells were lysed at 48 h.p.i. and analyzed by immunoblotting with indicated antibodies. (B) Expression level of  $p21^{WAF1/CIP1}$  transcripts increases in the early stages of the lytic infection. RT-PCR assay were assessed with RNAs extracted from Tet-BZLF1/B95-8 cells cultured in the presence of doxycycline (2  $\mu$ g/ml) for the indicated times. IR, Tet-BZLF1/B95-8 cells were exposed to 10 Gy of  $\gamma$ -irradiation, harvested 2 h post-treatment, and processed similarly. (C) p53 protein is associated with  $p21^{WAF1/CIP1}$  promoter in the early stages of lytic infection. Tet-BZLF1/B95-8 cells were cultured in the presence of doxycycline (2  $\mu$ g/ml), and harvested at the indicated times. ChIP assays were performed as described in Experimental Procedure. The immunoprecipitated DNA and input DNA were analyzed by PCR amplification with primers for  $p21^{WAF1/CIP1}$  promoter or for  $p21^{WAF1/CIP1}$  coding region.

early lytic genes.<sup>12,13</sup> The protein is a multi-functional protein and also serves as an oriLyf binding protein involved in the initiation step of viral lytic replication.<sup>14</sup> Furthermore, the expression of the BZLF1 protein alone arrests cells in  $G_0/G_1$  phase of the cell cycle in several epithelial cell lines.<sup>15,16</sup> In these cell lines, several lines of evidences suggest that the increased expression levels of p53 and  $p21^{WAF1/CIP1}$  elicited by the BZLF1 protein causes the cell cycle arrest.<sup>15,17</sup> Expression of BZLF1 protein alone does not induce p53 phosphorylation,<sup>9</sup> but increases the levels of p53 and its target gene product,  $p21^{WAF1/CIP1}$ .<sup>15</sup> However, the molecular mechanism by which the BZLF1 protein stimulates expression of p53 and  $p21^{WAF1/CIP1}$  remains unknown.

In this study, we found that the BZLF1 protein by itself stimulates the binding of p53 and TATA box-binding protein (TBP)

to their recognition sequences and enhances p53-specific transcription in vitro. Consistent with the recent report that p53 is involved in the regulation of initiation step of EBV replication,<sup>18</sup> overexpression of p53 at the early stages of lytic replication stimulated viral genome replication. Thus, we propose that the BZLF1 protein regulates p53 and its target gene products in two distinctive manners: transient induction of p53 at the early stages of the virus production and p53 degradation at the later stages of EBV lytic replication.

## Results

**Overexpression of p53 at the early stages of EBV lytic infection stimulates viral genome replication.** Expression of p53 at the middle and late stages of EBV lytic infection interferes with viral replication.<sup>10</sup> It has been, however, recently reported that p53 is involved in the augmentation of EBV lytic cycle initiation.<sup>18</sup> In order to examine the effect of p53 expression at early stages of the lytic replication on viral DNA synthesis, p53 expression vector was introduced into Tet-BZLF1/B95-8 cells before induction of the lytic replication, and viral DNA synthesis was determined at indicated times. As shown in **Figure 1A**, p53 expression at the early stages enhanced viral genome synthesis (**Fig. 1A**), corresponding well with the report by Chang et al.<sup>18</sup> These results suggest that p53 plays an important role specifically in the early stages of EBV lytic replication. p53 might be required as a transcriptional factor so that its target gene products contribute to the efficient replication of EBV at least at the early stages.

So, we next investigated whether transcription of the p53-target gene is enhanced after induction of the lytic replication. RT-PCR analyses were performed to quantify the mRNA-levels of  $p21^{WAF1/CIP1}$  that is one of the major targets of p53 in lytic replication induced Tet BZLF1/B95-8 cells (**Fig. 1B**). Levels of  $p21^{WAF1/CIP1}$  transcript increased transiently, reaching plateau at 8–24 h post-induction (h.p.i.), in the early stages in infection, but thereafter declined with progression of lytic replication, although the level of GAPDH transcripts as a loading control remained constant. It should be noted that the decrease of p21 expression in the middle and late stages of lytic replication is caused by the ubiquitin-proteasome-mediated degradation of p53.<sup>10,19</sup> Furthermore, the dynamics of p53 binding to  $p21^{WAF1/CIP1}$  promoter during EBV lytic infection was monitored using by ChIP assay. As shown in **Figure 1C**, p53 protein was found to bind to the p21 promoter region at 8–24 h.p.i. but to be hardly detected at 48 h.p.i., consistent with the expression pattern of p21-gene transcripts during the lytic infection (**Fig. 1B**). This result indicates that the increased levels of  $p21^{WAF1/CIP1}$  transcripts at the early stages of lytic replication could be due to the increase of p53-binding to the p21 promoter.

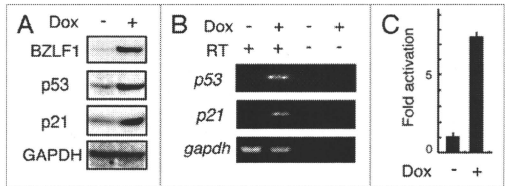
**Expression of BZLF1 protein induces p53 and  $p21^{WAF1/CIP1}$  gene expressions.** On the other hands, Cayrol and Flemington



previously showed that BZLF1 protein blocks cell cycle progression in  $G_1$  phase and induces the accumulation of p53 in HeLa cells.<sup>15</sup> We tried to confirm the effect of BZLF1 protein expression in the epithelial cells. As shown in Figure 2A, the treatment of doxycycline (Dox) induced the accumulation of p21<sup>WAF1/CIP1</sup> and p53 in Tet-BZLF1/HeLa cells, in which the BZLF1 protein is conditionally expressed under the control of tetracycline-regulated promoter.<sup>9</sup> Similar results were obtained when the BZLF1 protein was overexpressed in HeLa cells in a transient transfection experiment (data not shown). Since p21<sup>WAF1/CIP1</sup> is one of the major targets of p53 transcription factor,<sup>3,20</sup> it is likely that the  $G_1$  arrest induced by BZLF1 protein, as reported by Cayrol and Flemington,<sup>15</sup> is due to the induction of p53. RT-PCR analysis revealed apparent increases of both p53 and p21<sup>WAF1/CIP1</sup> mRNA levels in Dox-treated cells (Fig. 2B), indicating that the expressions of both p53 and p21<sup>WAF1/CIP1</sup> are transcriptionally-upregulated. Although Mauser et al. reported that BZLF1 expression using adenovirus vector in HeLa induced primarily a  $G_2/M$  block,<sup>21</sup> we observed that induction of BZLF1 protein in HeLa resulted in not  $G_2/M$  arrest but  $G_1$  arrest, as well as demonstrated by Cayrol and Flemington.<sup>15</sup>

To validate this observation, we performed a reporter assay. A PG13-Luc reporter plasmid, which harbors 13 copies of p53-binding sites, was introduced to Tet-BZLF1/HeLa cells to monitor the levels of p53-mediated transactivation. Although HeLa cells express HPV E6 protein that is a potential inhibitor of p53, we made choice of HeLa cells, since it is difficult to observe transient transactivation of p53 in other cell lines where the BZLF1 protein-dependent inhibition of p53 is dominant.<sup>19</sup> As can be seen in Figure 2C, the induction of BZLF1 protein by treatment with Dox resulted in the enhancement of p53-specific transcription by 7.5-fold in the cells, despite repression of p53 functions by E6. Thus, the BZLF1 protein can stimulate the p53-mediated transactivation.

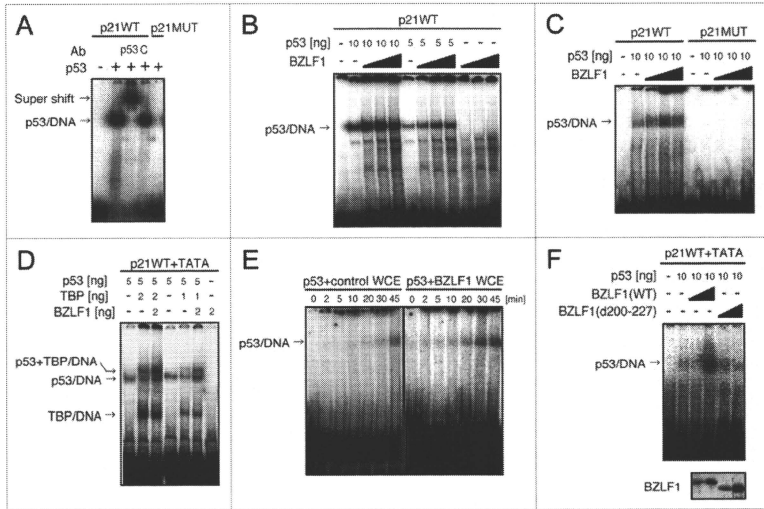
**The BZLF1 protein stimulates p53-binding to DNA containing its binding sites.** To examine how BZLF1 protein facilitates p21<sup>WAF1/CIP1</sup> transcription, we next performed the gel electrophoretic mobility shift assays (EMSA). Purified HA-tagged p53 protein, obtained from baculovirus-infected insect cells, was subjected to EMSA by using a <sup>32</sup>P-labeled p53 recognition sequence of the p21<sup>WAF1/CIP1</sup> gene promoter as a probe. A major shifted band was observed when p53 recognition sequence was used as a probe, while no shifted band was visible when a mutated sequence was used instead. The band super-shifted by the addition of anti-p53 IgG antibody, demonstrating that the band actually represents p53-DNA complex (Fig. 3A). When a constant amount of p53 and increasing amounts of BZLF1 protein were included in the DNA binding reaction, the band intensities of p53-DNA complex significantly increased accordingly to the increasing amounts of BZLF1 protein. Interestingly, the mobility of the shifted bands remained unaltered (Fig. 3B), suggesting that BZLF1 protein was not part of the p53-DNA complex. In fact, we confirmed that no super-shifted band was observed when anti-BZLF1



**Figure 2.** The BZLF1 protein induces the expression of p53 and p21 through the transactivation of p53. (A) Expression profiles of p53, and p21<sup>WAF1/CIP1</sup> after induction of BZLF1 protein expression. Tet-BZLF1/HeLa cells were cultured in the presence of 2  $\mu$ g/ml of doxycycline and harvested 48 h post-induction. Cell lysates were prepared, and applied for immunoblot analysis with specific antibodies as indicated. (B) RT-PCR assays were carried out with RNAs extracted from Tet-BZLF1/HeLa treated with or without doxycycline for 48 h. RT, reverse transcriptase. (C) Enhancement of p53-mediated transactivation by the BZLF1 protein. Tet-BZLF1/HeLa cells were transfected with PG13-Luc reporter plasmid. The cells were treated with Dox for 48 h and then luciferase activity was measured. Values are averages from three independent transfections ( $\pm$ S.E.) after normalization by total protein content.

antibody was added to the binding reaction (data not shown). Similar results were also reported with other proteins that stimulate the p53 DNA binding such as 421 antibody, Ref-1,<sup>22</sup> and c-Abl.<sup>23</sup> Moreover, no band-shift was observed when a mutated p53-binding site was used as a probe (Fig. 3C). Since p53 cooperates with TBP in binding to its responsible promoter,<sup>24</sup> we then investigated whether BZLF1 protein affects p53-TBP-DNA complex formation. A <sup>32</sup>P-labeled DNA fragment containing both a p53 recognition sequence and a TATA box was used as an EMSA probe. Incubation of both p53 and TBP with the probe generated three retarded protein-DNA complex bands; TBP-DNA complex, p53-DNA complex, and p53-TBP-DNA complex (Fig. 3D). While the BZLF1 protein did not affect TBP-DNA complex formation, p53-DNA complex and p53-TBP-DNA complex formation were markedly enhanced (Fig. 3D). The result implies that the BZLF1 protein specifically mediates the enhancement of p53-TBP-DNA complex formation through the interaction with p53 protein.

As is evident from EMSAs, although BZLF1 enhanced p53-DNA binding, the resultant p53-DNA complex did not contain the BZLF1 protein. Thus, the enhancement of p53-DNA binding by BZLF1 protein could be due to either the increased rate of p53-DNA complex formation or the decreased rate of p53-DNA dissociation. To determine whether the BZLF1 protein affects the rate of p53-DNA complex formation, p53 was incubated with the p21<sup>WT</sup> probe in the presence of whole cell extract containing BZLF1 protein. Whole cell extract containing human MCM7 was used in a control reaction. At different time points after mixing, aliquots of the reaction mixture were loaded on PAGE (Fig. 3E). The shifted bands of p53-DNA complex reached the maximal levels 30 min after incubation when the BZLF1 protein was included in the binding reaction, while it took more than 45 min to reach the maximum in case of control reactions. This result indicates that the BZLF1 protein increases the rate of p53-DNA complex formation. On the other hand, the BZLF1 protein did



**Figure 3.** The BZLF1 protein enhances binding of p53 to DNA at an association step. (A) p53 binds to its consensus sequence in the p21<sup>WT/CF1</sup> promoter. Super shift assays were performed with the p21WT probe containing a p53-binding site and the p21MUT probe containing a mutation in the p53 consensus sequence as described in the Experimental Procedures. Ten ng of HA-p53 recombinant protein, and 1  $\mu$ g of control mouse IgG (C) or p53 monoclonal (p53) antibodies were used. (B) DNA-binding activity of p53 is stimulated by BZLF1 protein. EMSA was performed with HA-p53 protein (10 or 5 ng). Increasing amounts of whole cell extract (BZLF1 WCE) from Sf21 cells infected with AcBZLF1 and <sup>32</sup>P-labeled p21WT as a probe. The bands below the p53/DNA complex are non-specific. (C) BZLF1 protein enhances p53 DNA-binding in a consensus sequence-specific manner. EMSA was performed with HA-p53 protein (10 ng), increasing amounts of whole cell extract (BZLF1 WCE) from Sf21 cells infected with AcBZLF1 and <sup>32</sup>P-labeled p21WT or p21MUT probes. (D) Stimulation of the recruitment of TBP to p53/DNA complex by BZLF1 protein. EMSA was performed with recombinant HA-p53 (5 ng), TBP (1 or 2 ng) and BZLF1 protein (2 ng), with <sup>32</sup>P-labeled p21WT-TATA as a probe. Arrows indicate p53 + TBP/DNA, p53/DNA and TBP/DNA complexes formed (top to bottom). (E) BZLF1 protein enhances the association of p53 with its binding site in the promoter. EMSA was performed with <sup>32</sup>P-labeled p21WT as a probe for determination of association rates of p53/DNA complexes. DNA-binding reactions were performed in the presence of WCE containing BZLF1 protein for different times. As a control, the same amount of WCE from Sf21 cells infected with AcMCM7 was used. (F) Interaction between p53 and the BZLF1 protein is required for the enhancement of p53-DNA binding. EMSA was performed with <sup>32</sup>P-labeled p21WT-TATA as a probe using purified recombinant HA-p53 (10 ng) and either wild-type BZLF1 protein or d200-227 lacking the association with p53. Purified BZLF1 proteins were subjected to SDS-PAGE followed by Coomassie blue staining.

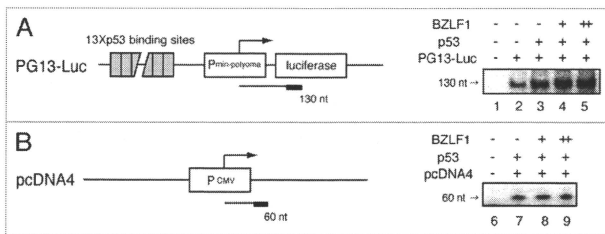
not affect the dissociation rates of p53-DNA complex when the p53-DNA complexes were challenged with 20-fold molar excess of unlabeled p21WT probe as a competitor (data not shown).

To further test whether the association of BZLF1 protein to p53 is required for the enhancement of the p53 DNA-binding activity, we performed EMSA using a BZLF1 mutant, d200-227 mutant which cannot bind to p53.<sup>25</sup> As shown in Figure 3F, the BZLF1 d200-227 mutant did not enhance the p53 DNA-binding activity at all. This finding indicates that the physical interaction between p53 and BZLF1 protein is required for the BZLF1-mediated enhancement of p53 DNA-binding activity. These results suggest that BZLF1 interacts with p53, thereby enhancing the p53 binding to its recognition element.

**BZLF1 protein enhances p53-mediated transcription in vitro.** According to these observations, it is most likely that BZLF1 protein stimulates p53-dependent transcription in vitro. Therefore, we carried out in vitro transcription assay using HeLa

nuclear extracts. The linearized PG13-Luc reporter plasmid, containing 13 copies of p53 DNA binding sites upstream of a basal promoter linked to a luciferase reporter gene,<sup>3</sup> was used as a template DNA, and the linearized DNA of pcDNA4 was used as an internal control (Fig. 4). The amounts of the transcripts were measured by primer extension analysis with <sup>32</sup>P-labeled primer. The expected sizes of the primer extension products are 130 and 60 nucleotide lengths, respectively. As shown in Figure 4, the addition of p53 protein specifically activated transcription from the template with p53 binding sites (PG13-Luc; lane 3), and the addition of BZLF1 protein further enhanced the transcription (PG13-Luc; lane 4 and 5). In contrast, the levels of pcDNA4-transcription were unaffected (pcDNA4; lane 6-9). These data clearly demonstrate that the BZLF1 protein stimulates p53-dependent transcription in vitro.

**Transactivation of p53-target genes correlates with occupancy of p53 at its promoter.** To validate the effect of BZLF1



**Figure 4.** Cooperation between p53 and the BZLF1 protein activates p53-specific transcription in vitro. In vitro transcription templates used are indicated (left). PG13-Luc contains thirteen copies of p53 binding sites upstream of the polyoma virus minimum promoter and the luciferase gene. This template is responsive to activation of p53. pcDNA4 containing CMV IE promoter was used as a control for the reactions. These templates were linearized by the restriction enzyme digestion. In vitro transcription reaction mixtures contained HeLa nuclear extract, linearized template DNA, p53 protein (15 ng) and purified BZLF1 protein (4 or 10 ng). In vitro transcription and primer extension were performed as described in the Experimental Procedures, the primer extension products of RNAs transcribed from PG13-Luc (A) and pcDNA4 (B) are 130 nucleotides (130 nt) and 60 nucleotides (60 nt) in length, respectively.

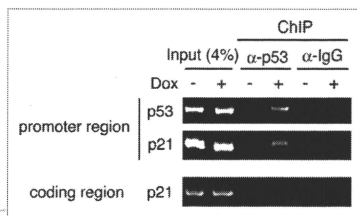
protein on the p53 DNA-binding activity in vivo, we utilized Tet-BZLF1/HeLa cells and examined the effect of BZLF1 expression on the dynamics of p53 binding to its target promoters ( $p21^{WAF1/CIP1}$  and p53 promoter)<sup>26</sup> by ChIP assay. As shown in Figure 5, following the induction of the BZLF1 protein, p53 protein was found to bind to both the  $p21^{WAF1/CIP1}$  and p53 promoter regions. This observation corresponds well with the expression pattern of  $p21^{WAF1/CIP1}$  and p53-gene transcripts following the induction of BZLF1 protein (Fig. 2B). These findings imply that BZLF1 protein induces the transactivation of p53 by stimulating the p53 protein binding to its recognition sequences in vivo. Induction of p53 should form a positive feedback loop regaling the p53 expression and should lead to amplification of p53-downstream signaling.<sup>26-28</sup>

## Discussion

Previous studies demonstrated that the EBV immediate-early protein BZLF1, which was either conditionally-expressed<sup>15,16</sup> or was overexpressed by a recombinant adenovirus<sup>21,29</sup> could induce G<sub>1</sub> arrest in some cell lines. The BZLF1 protein causes the accumulation of both mRNA and protein of CDK inhibitor p21<sup>WAF1/CIP1</sup>,<sup>15</sup> a well-known p53-target gene product. Thus, it appears that the BZLF1 protein affects p21 expression through transcriptional regulation.

In this study, we investigated the mechanism by which the BZLF1 protein enhances p53-dependent transactivation. Our data demonstrate the BZLF1 enhances the p53-DNA binding by accelerating the p53-DNA complex formation. Enhanced p53 binding to its genomic recognition sequences enables the efficient expression of p53-target genes, including p21<sup>WAF1/CIP1</sup> and p53 itself, which, in turn, causes cell cycle arrest in epithelial cells. Our study has clarified the pathway that links between the BZLF1 expression and the induction of p53-target genes.

However, we cannot rule out another possibility that the BZLF1 protein cooperates with bZIP transcription factor C/EBP $\alpha$  to promote cell cycle arrest. A group of Hayward found



**Figure 5.** BZLF1 protein induces the recruitment of p53 to its target promoters. ChIP assays were performed with Tet-BZLF1/HeLa cells. The p53 and p21<sup>WAF1/CIP1</sup> promoter and p21<sup>WAF1/CIP1</sup> coding region were detected by PCR amplification. IgG was used for a negative control.

that BZLF1 protein protects C/EBP $\alpha$  from proteasome-dependent degradation.<sup>30</sup> Since C/EBP $\alpha$  auto-regulates its own promoter, this rapidly results in enhanced C/EBP $\alpha$  expression. In addition, C/EBP $\alpha$  transactivates p21<sup>WAF1/CIP1</sup>. Thus, BZLF1 protein causes G<sub>1</sub> arrest through the interaction with C/EBP $\alpha$ . Moreover, the activation domain of BZLF1 protein also contributes to transactivate p27<sup>KIP1</sup>.<sup>31</sup> These results including this study suggest involvement of more than one mechanism behind the BZLF1 protein-mediated cell cycle arrest.

During the course of lytic replication, we propose that BZLF1 protein plays two distinctive roles in the regulation of p53-mediated transactivation depending on the phosphorylation states of p53. In the early phases of lytic replication, p53 is mainly in the hypo-phosphorylated form.<sup>9</sup> In general, the hypo-phosphorylated form of p53 exhibits weak DNA binding to its recognition sequences and is transcriptionally-inactive. The BZLF1 protein helps the hypo-phosphorylated p53 to bind to its recognition sequences, leading to the enhancement of p53 dependent transcription. This results in the transient increase in levels of p21<sup>WAF1/CIP1</sup>, which causes the cell cycle arrest at the early stages of the lytic replication. By contrast, at middle and late stages of lytic replication, the phosphorylation state of p53 dramatically

changes. When viral genome DNAs are synthesized after 24 h.p.i., the host cell DNA damage-sensing machinery recognizes the viral DNA as an abnormal DNA structures, activating ATM dependent DNA damage signaling. ATM, in turn, phosphorylates p53 at various sites including S15,<sup>9</sup> which liberates p53 from MDM2-mediated degradation. Instead, another pathway to degrade p53 protein becomes active. We have recently found that induction of EBV lytic program leads to the degradation of p53 via an ubiquitin-proteasome pathway, which is independent of MDM2.<sup>19</sup> The BZLF1 protein directly functions as an adaptor component of the ECS ubiquitin ligase complex targeting phosphorylated p53 for degradation.<sup>10</sup> As a result, the p53 target gene products are maintained at very low levels, and host cells are maintained in S-phase like condition,<sup>9,19,32</sup> which appears to be important for the progression of lytic replication. Supporting the idea, compensating the expression of p53 at middle to late stages of the lytic infection inhibits viral DNA replication and virus production during lytic infection.<sup>10</sup> This is in contrast to our observation that p53 expression enhanced viral genome replication at the early stages of infection. Overall, BZLF1 enables hypo-phosphorylated p53 to transactivate its target genes in the initial phase of lytic replication, while the activated p53 is degraded to maintain S-phase like environment for the efficient propagation of lytic replication at the later stages.

It is raised why p53 is required for viral proliferation at the early stages of the lytic infection. Several studies regarding p53 and human cytomegalovirus (HCMV), a member of beta-herpesvirus family, have demonstrated the elevated levels of p53 protein upon viral infection, whereas the cellular downstream targets of p53 are not activated.<sup>33,34</sup> It was reported that cells infected with HCMV in the absence of p53 produced fewer infectious viral particles, and that the loss of p53 caused the delays of viral protein production and its trafficking.<sup>35</sup> The observed delayed formation of replication centers could result in the delay of viral encapsidation.<sup>33</sup> Interestingly, HCMV genome has 21 potential p53 responsible sites.<sup>35</sup> HCMV genome-bound p53 molecules were most influential on HCMV gene expression at immediate-early and early stages of postinfection, implying a mechanism for the reduced and delayed production of virions in p53<sup>-/-</sup> cells. Similarly, potential p53 recognition sequences were also present on EBV genome (Murata T, et al. unpublished results). We showed previously that p53 is associated with EBV replication compartments.<sup>9</sup> Thus, it is tempting to speculate that p53 is recruited to EBV replication compartment through its direct binding to the recognition sequences within the EBV genome. Overall, the BZLF1-mediated enhancement of p53-DNA binding may contribute to the stimulated expression of viral genes that are transactivated by p53 at early stages of EBV lytic infection.

## Experimental Procedures

**Cloning of Tet-BZLF1/HeLa cells.** To generate Tet-BZLF1/HeLa cells, HeLa Tet-on cells (Clontech) was transfected with pCMSCV-RevTRE(hyg)-BZLF1.<sup>32</sup> Stable clones were obtained by double selection in the presence of G418 and hygromycin B. Clones resistant to G418 and hygromycin B were isolated by

limiting dilution and checked for expression of the BZLF1 with doxycycline by immunoblotting analysis.

**Cells and reagents.** Tet-BZLF1/HeLa cells were cultured in D-MEM containing 10% tetracycline free fetal calf serum in the presence of G418 and hygromycin B. Tet-BZLF1/B95-8 cells were maintained in RPMI medium containing 10% tetracycline free fetal calf serum in the presence of puromycin and hygromycin B.<sup>32</sup> To induce BZLF1 protein expression, the tetracycline derivative doxycycline (Dox) was added to the culture medium at a final concentration of 1 µg/ml. The anti-p53 (FL-393) and anti-p21<sup>WAF1/CIP1</sup> (C19G) antibodies were purchased from Santa Cruz Biotechnology. Affinity-purified anti-BZLF1 protein antibody was prepared as described previously.<sup>36</sup>

**Preparation of whole cell extracts.** Whole cell extracts (WCE) of Sf21 cells infected with the recombinant baculovirus expressing BZLF1 protein, AcBZLF1, were prepared as follows. Sf21 cells were infected with AcBZLF1 at a M.O.I. of 10, harvested 72 h postinfection, and suspended in insect cell lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1 mM DTT) containing a protease inhibitor cocktail. After 60 min incubation on ice, cell lysates were centrifuged and the supernatant was collected. As a control, whole cell extracts were also prepared similarly from Sf21 cells infected with the recombinant baculovirus expressing human MCM7, AcMCM7.

**Purification of proteins.** Purified TBP protein was purchased from Wako. HA-tagged p53 was expressed in Sf21 cells infected with recombinant baculovirus<sup>22</sup> that were kindly provided by Dr. C. Prives (Columbia University). HA-p53 protein was purified using monoclonal anti-HA agarose conjugate (Sigma) and elution was performed using HA peptide (Sigma). Purification of EBV BZLF1 protein was described previously.<sup>10</sup>

**Electrophoretic mobility shift assay (EMSA).** Gel mobility shift assays were carried out as described previously,<sup>22</sup> with some modifications. The p21WT and p21MUT probes were oligonucleotides containing the wild-type p53 consensus site in the p21<sup>WAF1/CIP1</sup> gene promoter<sup>37</sup> and mutant sequences,<sup>38</sup> respectively. The p21WT-TATA probe was a 130-bp DNA fragment generated by digestion with KpnI and XbaI of pBluescript II KS (Stratagene) including the p21WT probe sequence at the EcoRI site and the TATA box sequence (5'-CCT GTT ATA AAA GCA GTG-3') between PstI and BamHI sites. The probes were <sup>32</sup>P-labeled by the Klenow fragment of *E. coli* DNA polymerase I. Reaction mixtures (20 µl) contained 20 mM HEPES, pH 7.9, 25 mM KCl, 0.1 mM EDTA, 2 mM MgCl<sub>2</sub>, 0.5 mM DTT, 10% (v/v) glycerol, 2 mM spermidine, 0.025% NP40, 0.5 µg of double-stranded poly(dG-dC), 2 µg of BSA, 0.15 pmol of <sup>32</sup>P-labeled probe DNA, and protein samples and reactions were carried out at room temperature for 40 min. The amount of WCE was kept constant by adding control MCM7 WCE. For antibody supershift assays, 1 µg of antibody was added directly to the reaction mixtures, followed by incubation for 15 min. DNA-protein complexes were analyzed on native 4% polyacrylamide gels containing 0.5x Tris-borate-EDTA (TBE) buffer and 0.05% NP40 and were quantified by BAS 2500 Imaging analyzer (Fuji Photo Film). When experiments for association rate of p53-DNA were performed, incubation was for the indicated