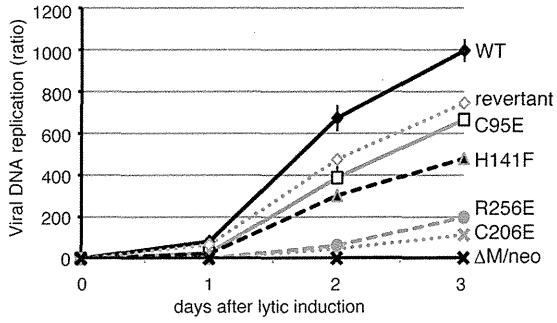

FIG. 3. Viral DNA synthesis in cells harboring BMRF1-mutated recombinant viruses after induction of lytic replication. HEK293 cells with the indicated EBV genomes were transfected with the BZLF1 expression plasmid and harvested at the indicated days. Levels of viral DNA synthesis were determined by quantitative real time-PCR assay as described in Materials and Methods and plotted as a ratio to each latency value (day 0) ± the standard error of the mean (SEM) from three independent experiments. WT, wild type.

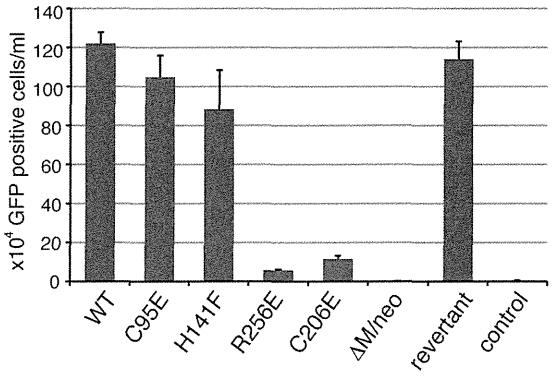

FIG. 4. Production of infectious progeny viruses. HEK293 cells carrying the indicated EBV genomes were transfected with both BZLF1 and BALF4 expression plasmids and harvested at 3 days post-transfection. EBV-negative Akata cells (2×10^6 cells) were infected with serially diluted (1-, 2-, or 10-fold) virus mixtures as described in Materials and Methods. The cells were analyzed by FACS analysis using FL1 and FL2 channels, and GFP-expressing cells were identified by shift of fluorescence intensity in the FL1 channel. Bars represent mean infectious viral particle units per ml ($\times 10^4$ GFP-positive cells/ml) ± the SEM from three independent experiments.

which is defective in DNA binding and polymerase processivity (38), was significantly restricted.

Production of infectious progeny viruses. To determine the yields of progeny viruses, HEK293 cells carrying each BAC DNA clone were transfected with the BZLF1 expression vector to induce lytic replication and harvested at 3 days after transfection as described in Materials and Methods. The viral titer with 1.0 ml of virus solution was calculated by counting GFP-positive cells (Fig. 4). The titer of the C95E (1.05×10^6/ml) or H141F (0.84×10^6/ml) virus was almost the same as that of wild-type (1.22×10^6/ml) or revertant (1.14×10^6/ml) virus. Corresponding to the levels of DNA synthesis, progeny virus production was highly restricted with the R256E (0.06×10^6/ml) and C206E (0.12×10^6/ml) substitutions.

Processing of viral DNA intermediates. To ascertain whether each mutated BMRF1 protein influences the maturation of viral replication products, we performed pulsed-field gel electrophoresis (PFGE), followed by Southern blot analysis with EBV W-repeat sequences as probes (Fig. 5). HEK293 cells carrying the wild-type genome were transfected with the BZLF1 expression vector to induce the lytic cycle of the virus in the absence or presence of 0.4 mg/ml of phosphonoacetic acid (PAA), a specific inhibitor of viral DNA polymerase, and then harvested on days 1, 2, and 3 (Fig. 5A). In the gels, three major bands of viral DNA were detected, as was expected in other herpesviruses. It has been previously reported that significant amounts of amplified viral DNA are unable to enter the gel and are detected at wells, because huge replicated DNA forms are complicated branched and nonlinear structures (18, 33, 46). We also observed similar accumulation of viral DNA (Fig. 5A). A fraction of the amplified viral DNA migrated to the same position as host chromosomal DNA, and the remainder was found at about 170 kb, which corresponds to the size of unit-length mature virion DNA (Fig. 5A). A band coinciding with circular DNA was not detectable (Fig. 5A).

We then tested cells with viruses carrying each mutation at 3 days after induction (Fig. 5B). In the C95E and H141F virus-infected cells, huge well DNA and mature unit-length virion DNA were produced, as in the cases of wild-type and revertant viruses. With the R256E and C206E mutant-infected cells, however, markedly reduced levels of the mature unit-length viral DNA were observed in proportion to the reduced yields of the progeny viruses (Fig. 4).

Nuclear retention and replication compartment formation. We next assessed the nuclear retention capacity of BMRF1 proteins in HEK293 cells with wild-type and recombinant viruses. Cells were harvested at 2 days postinduction and lysed in 0.5% Triton X-100 solution containing 50, 100, or 150 mM NaCl. After centrifugation, the supernatant (S) and the pellet (P) fractions were separated by centrifugation (referred to as cytoplasmic and nuclear fractions, respectively). Levels of the BMRF1 proteins in each fraction were detected by immunoblot assay (Fig. 6A). The intensity of the fluorescence was assessed with a Lumi Vision Analyzer 2.0 (AISIN), and nuclear retention was estimated by calculating the proportions of the BMRF1 proteins in the nuclear fractions [P/(S + P)]. The H141F and C206E mutant proteins behaved similarly to wild-type BMRF1, whereas the C95E protein showed slightly decreased association with the nuclear fraction. In contrast, the R256E mutant demonstrated very weak retention in the nucleus (Fig. 6A). We previously demonstrated that the *in vitro* DNA binding activities of C95E and R256E mutant BMRF1 proteins, as assessed by filter binding assay, were disabled moderately and completely, respectively (38). Therefore, it is likely that the nuclear retention simply reflects the DNA binding activity of BMRF1 mutants.

EBV lytic DNA replication takes place at discrete sites in nuclei, which are called replication compartments. Components of the viral DNA replication machinery, including BMRF1 and BALF2, are localized in these compartments (10). We thus examined whether BMRF1 mutants could gather to form the replication compartments in lytically infected cells by using immunofluorescence analysis (Fig. 6B). Cells were harvested at 24 h postinduction, fixed after treatment with or without 0.5% Triton X-100–mCSK buffer (150 mM NaCl), and then visualized with anti-BMRF1 mouse

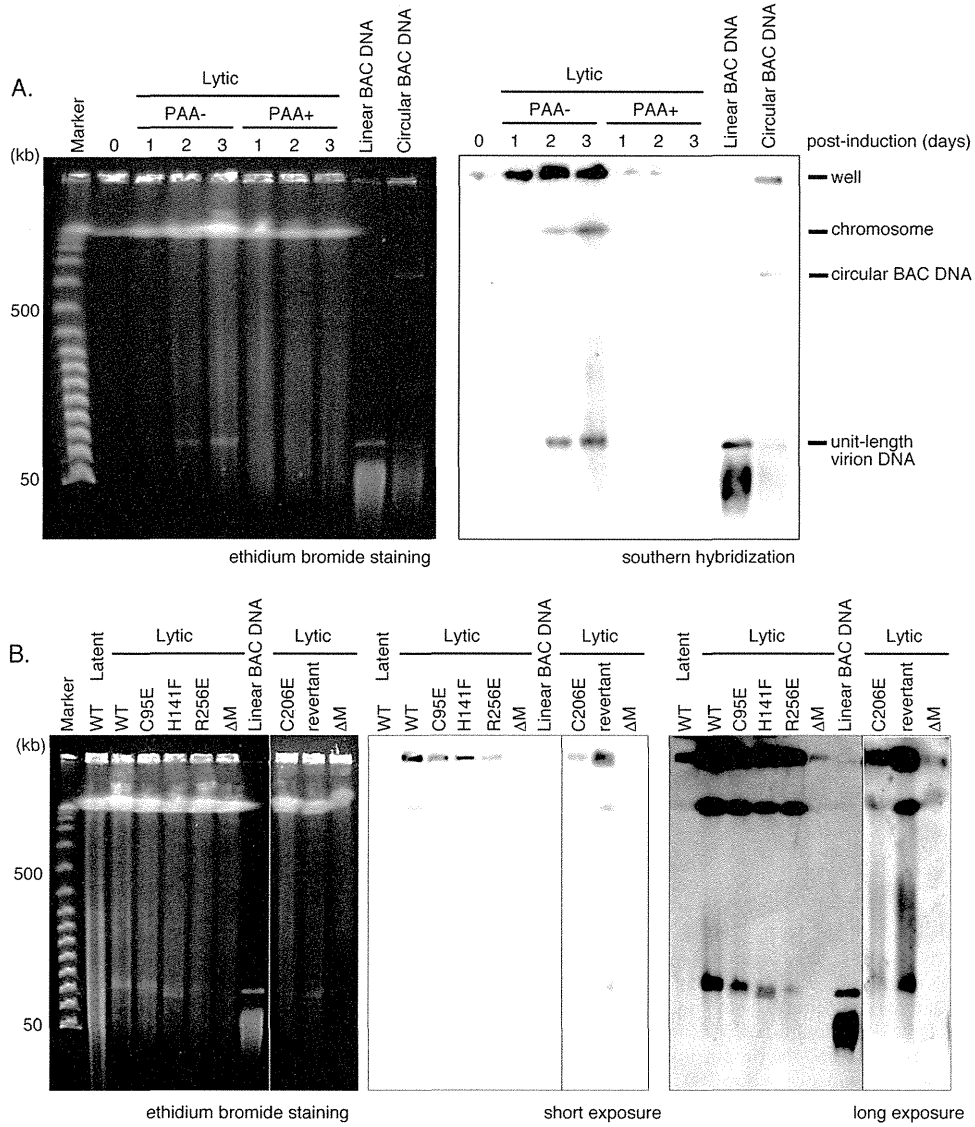

FIG. 5. Viral replication intermediates in lytic replication-induced cells with recombinant viruses. (A) HEK293 cells carrying the wild-type EBV genome were transfected with BZLF1 expression vector and incubated with (+) or without (−) 0.4 mg/ml of phosphonoacetic acid (PAA). The cells were harvested at 1, 2, and 3 days after transfection. (B) HEK293 cells carrying the indicated EBV genomes were transfected with the BZLF1 expression plasmid and harvested 3 days thereafter. Plugs (1.5×10^5 cells per plug) were prepared as described in Materials and Methods and subjected to PFEG in a 1.0% agarose gel. Lambda ladder PFG marker (marker), restriction enzyme, IppoI-digested EBV BAC DNA (linear), and undigested EBV BAC DNA (circular) were used as size markers. Southern blots of the gels were probed with digoxigenin (DIG)-labeled EBV W-repeat fragments (see Materials and Methods).

monoclonal antibody and anti-BALF2 rabbit polyclonal antibody (green). Without detergent treatment, all the wild-type BMRF1 and mutants were localized throughout nuclei (Fig. 6C). When cells were treated with detergent, two BMRF1 mutants, the C95E and H141F mutants, were retained in replication compartments, just like wild-type BMRF1. On the other hand, in a number of cells harboring the R256E mutation in the EBV genome, the mutant BMRF1 protein neither was retained nor colocalized with BALF2 proteins, which were distributed throughout nuclei as small dots by the treatment. The same staining pattern was observed in lytic replication-induced B95-8 cells in the presence of phosphonoacetic acid (10). Thus, formation of replication compartments was not apparent in the R256E-infected cells. In some cells harboring the C206E mutation, the staining pattern was the same as that of the cells harboring the R256E mutation (Fig. 6B, C206Ea). However, in others, replication compartments were formed although the BMRF1 was not stained as strongly as in cells harboring the wild-type EBV genome (Fig. 6B, C206Eb). With an increase in the sensitivity of the BMRF1, staining was visible as shown in Fig. 6B, C206Ec.

***trans*-Complementation of reduced viral replication in BMRF1-deficient virus-infected cells.** So far, we have documented the crucial roles of the Cys^{206} and Arg^{256} residues in the context of infection, using recombinant EBV carrying designated mutations. However, we still could not deny the pos-

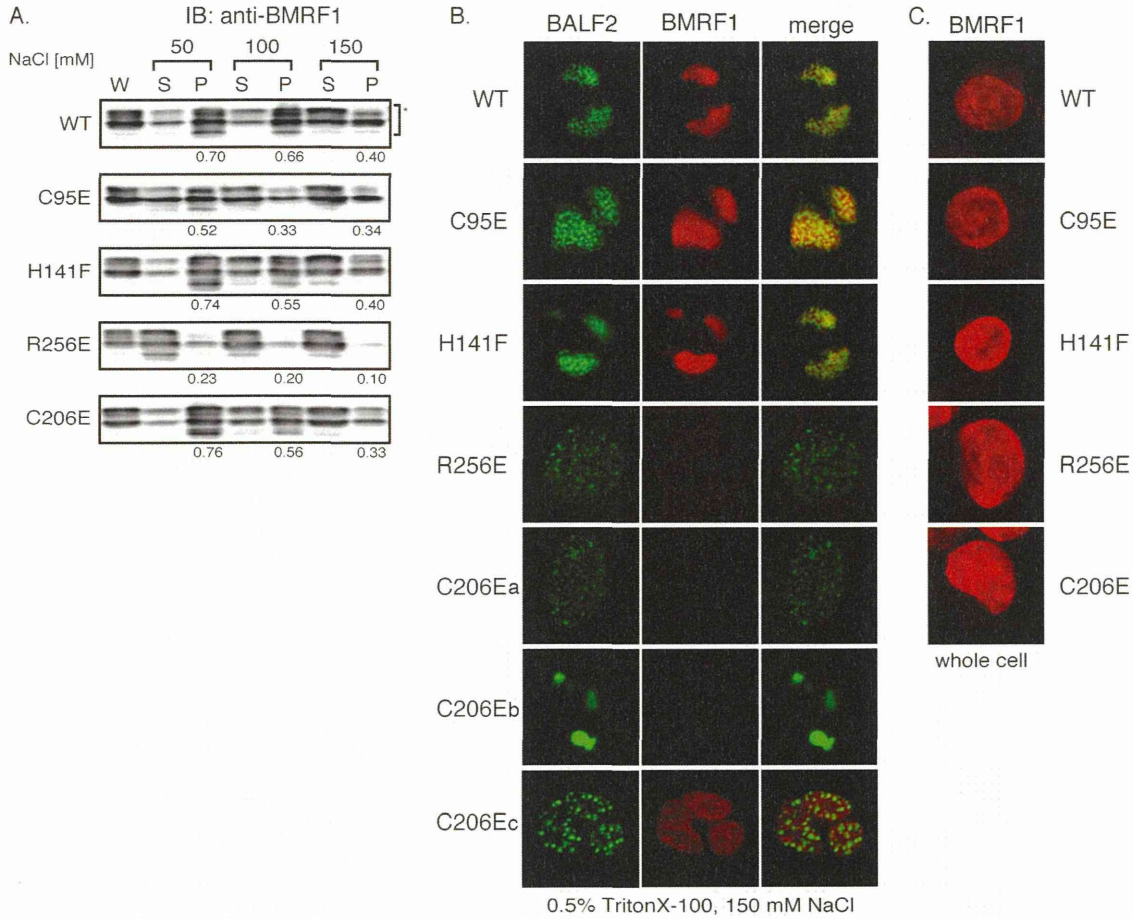

FIG. 6. Nuclear retention of BMRF1 proteins and formation of replication compartments. HEK293 cells carrying the indicated EBV genomes were transfected with the BZLF1 expression plasmid and harvested at 48 h posttransfection. (A) Nuclear retention of BMRF1 proteins. Lytic replication-induced cells were treated with lysis buffer containing one of several concentrations of NaCl (50, 100, or 150 mM). Whole-cell lysate (W), extractable supernatants (S), and extracted nuclear pellets (P) were prepared as described in Materials and Methods. Immunoblot analysis was performed using anti-BMRF1 antibodies. The intensity of the fluorescence was counted with a Lumi Vision Analyzer 2.0 (AISIN), and nuclear retention was estimated by calculating the proportion of the BMRF1 proteins in the nuclear fraction, P/(S + P). (B) Formation of viral replication compartments. Infected cells were extracted with 0.5% Triton X-100–mCSK buffer and fixed with methanol. The fixed cells were immunostained with anti-BMRF1 monoclonal and anti-BALF2 polyclonal antibodies, followed by treatment with Alexa Fluor 594-conjugated anti-mouse IgG secondary antibody (BMRF1, red) or with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (BALF2, green), respectively. The right panels are the merged images. (C) The lytic replication-induced cells were harvested 24 h postinduction and fixed with methanol. The fixed cells were immunostained with anti-BMRF1 monoclonal antibody, followed by treatment with Alexa fluor 594-conjugated anti-mouse IgG secondary antibody (BMRF1, red).

sibility that those mutant viruses might carry an additional unexpected mutation(s) in the genome. In order to preclude this possibility and further verify the significance of these residues, we tested whether ectopic expression of wild-type or mutant BMRF1 proteins could restore the attenuated viral replication in BMRF1 knockout virus-infected cells. HEK293 cells latently infected with BMRF1-deficient virus (BACΔM/neo) were transfected with panels of the FLAG-tagged BMRF1 expression plasmid, together with the BZLF1 expression plasmid. Viral DNA replication levels (Fig. 7A and B) and progeny virus production (Fig. 7C) were measured by real-time PCR and fluorescence-activated cell sorter (FACS) analysis, respectively. Overall, expression of the C95E and H141F mutants could complement the impaired replication of knockout BMRF1 just like wild-type BMRF1, but recovery by the R256E and C206E mutants was very limited. In addition, impaired processing of the viral genome DNA into unit lengths with the BMRF1 knockout virus was detected upon exogenous supply of the wild-type, C95E, or H141F BMRF1 (data not shown). These results further support our conclusion that the Cys^{206} and Arg^{256} residues of EBV BMRF1 are important for viral replication, especially in the context of infection.

DISCUSSION

The EBV BMRF1 gene product functions as the viral DNA polymerase processivity factor, playing an essential role in lytic replication (7, 16, 25, 26, 29, 50). We have recently solved the crystal structure of BMRF1, and, taking advantage of the data, several BMRF1 mutant proteins were prepared and characterized biochemically (38, 39). Most BMRF1 exists as a C-shaped head-to-head homodimer in solution, as judged by analytical

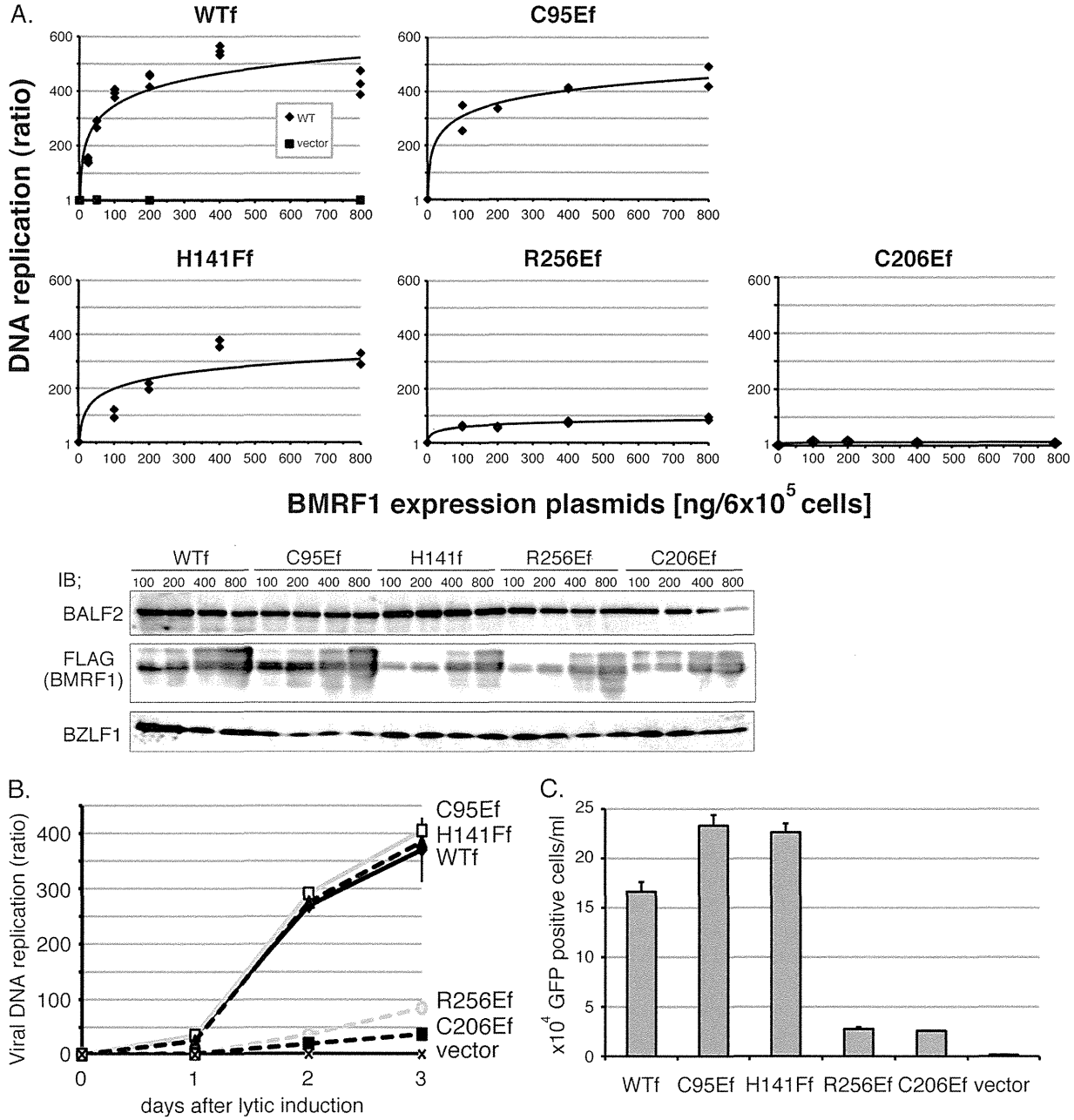

FIG. 7. *trans*-Complementation of impaired viral replication in BMRF1-knockout BACΔM/neo-harboring cells. (A) *trans*-complementation of viral DNA synthesis in BMRF1. Increasing amounts of FLAG-tagged BMRF1 expression vector were transfected into BACΔM/neo cells (6×10^5 cells) with the BZLF1 expression 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 BACΔM/neo cells (6×10^5 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 BACΔM/neo (6×10^5 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 β-sheets in "head-to-head" (βI_1-$\beta I_1'$) and "tail-to-tail" (βD_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^2) than the tail-to-tail contact area (340 Å^2). 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 β-sheets (βI_1-$\beta I_1'$) 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^{206} 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^{206} 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^{95} 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^{256} 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 processive 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*[S]

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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 12 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)[2] is a human γ-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 pathogenesis. 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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[2] 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, tricostatin A; WCE, whole cell extracts; KSHV, Kaposi's sarcoma-associated herpesvirus; GFP, green fluorescent protein; HA, hemagglutinin; K-bZIP, KSHV b-Zip.

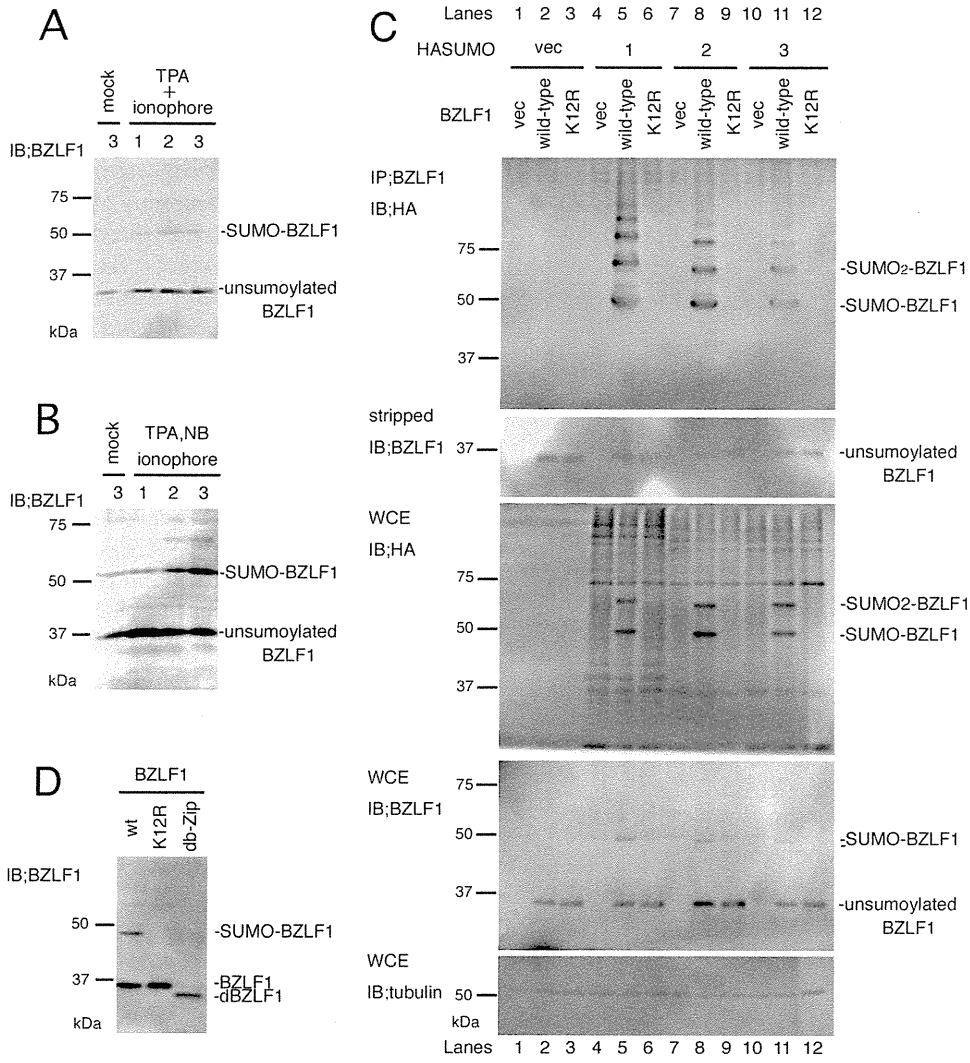

FIGURE 1. **BZLF1 is modified by SUMO.** *A* and *B*, sumoylation of BZLF1 in infected cells is shown. *A*, GTC-4 cells were treated with or without 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*, 20 ng/ml) and calcium ionophore A23187 (0.5 mM) for 1, 2, or 3 days. Cell lysates were subjected to immunoblotting (*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 pcDNABZLF1(wild type) or pcDNABZLF1(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 pcDNABZLF1(wild type), pcDNABZLF1(K12R), or pcDNABZLF1(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-HA 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

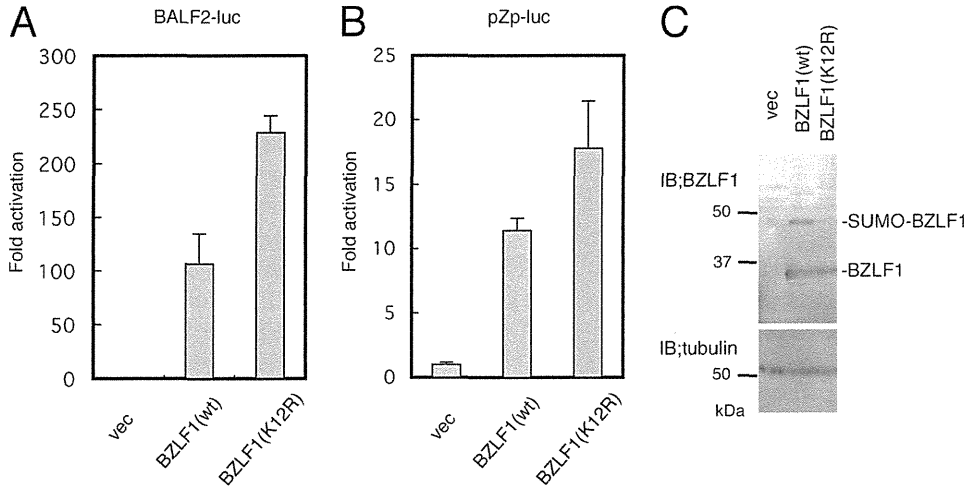

FIGURE 2. **K12R mutant BZLF1 activates promoters with BZLF1-responsive elements more efficiently than wild-type BZLF1.** *A*, HEK293T cells were transfected with 10 ng of BALF2-luc, 1 ng of pCMV-RL, and 10 ng of pcDNABZLF1(wild type) or pcDNABZLF1(K12R). Counts are shown as -fold activation of that 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 μl 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 μl 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-BZLF1antibodies, 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, AISIN 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′-AGGAGGCTGGTGCCTTGGCTTTAAAGGGGAGATGTTAGACAGGTAACTCACTAAACATTGGGCCTGGTGATGATGGCGGGATC-3′ and 5′-ATTTATTAATATTCCATTAGTAAACGAGGCGTGAAGCAGGCGTGGTTTCAATAACGGGAGTCAGAAGAACTCGTCAAGAAGG-3′. After the recombination, kanamycin-resistant colonies were selected and checked to make the intermediate, dBZLF1/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′-AGAGAGCCGACAGGAAGATATTTATTAATATTCCATTAGTAAACGAGGCGTGAAGCAGGCGTGGTTTCAATAACGGGAGTTACACGGCGATCTTGCCGC-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′-GCTCCTGAGAATGCTTATCAAGCTTATGCAGCACCTCAGCTGTTCCCAGTCTCCGACATAGGCCTGGTGATGATGGCGGGATC-3′ and 5′-CCGGCATTTTCTGGAAGCCACCCGATTCTTGTATCGCTTTATTTCTAGTTCAGAATCGCATCAGAAGAACTCGTCAAGAAGG-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′-TAGCCTCGAGGCCATGCATATTTCAACTGG-3′ and 5′-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3′; for BALF2 promoter, 5′-ACCAAGCTTGATGCCCAAGGTATCGCCCCG-3′ and 5′-CTGGCCCTCGCTAGCA-

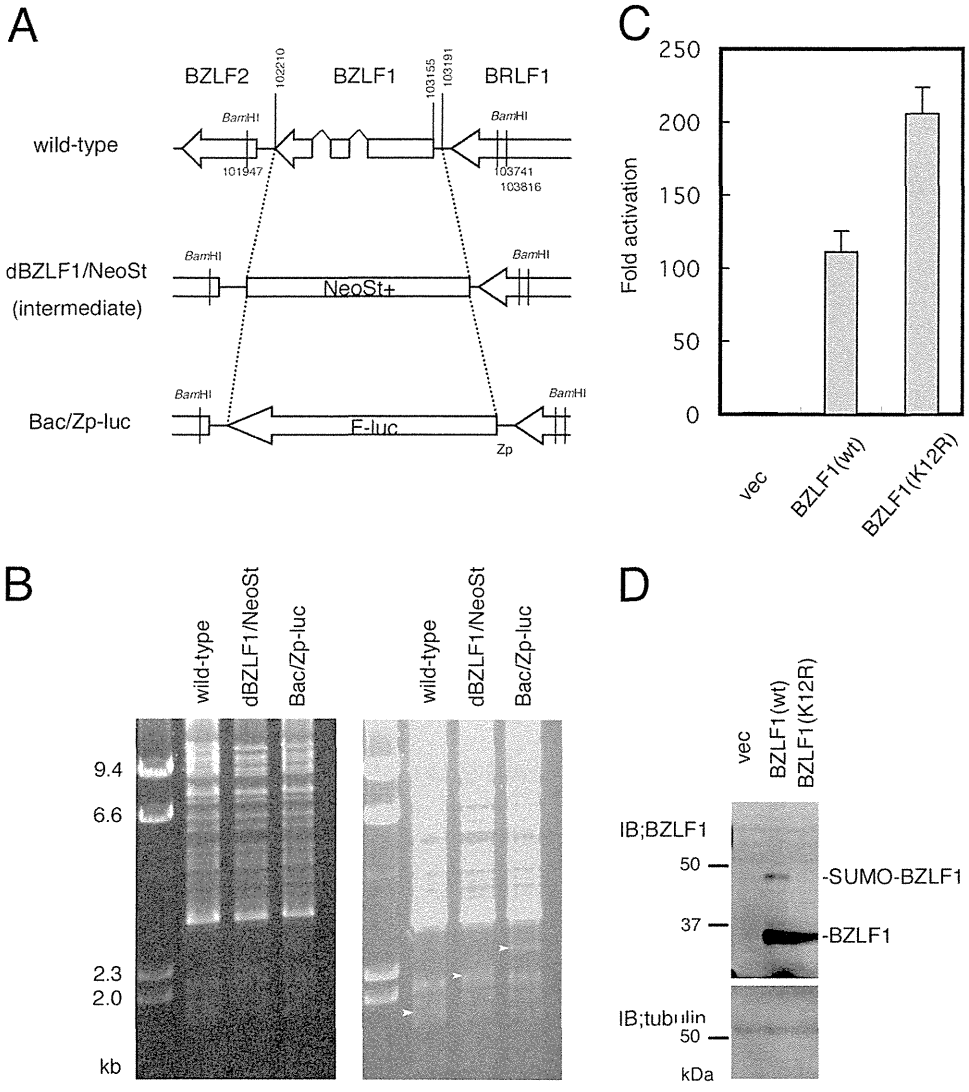

FIGURE 3. **The K12R mutant activates the *BZLF1* promoter more efficiently than wild-type BZLF1 in the context of the viral genome.** *A*, shown is a schematic arrangement of the recombination of the EBV genome using tandemly arranged neomycin resistance and streptomycin sensitivity genes (*NeoSt*$^{+}$). The region between nucleotide 102,210 and 103,191 of the B95-8 genome (V01555) was replaced with the NeoSt+ cassette to make the intermediate, dBZLF1/NeoSt. The NeoSt+ 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 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 sizes of the fragments plus inserts. *C*, 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. *D*, the expression levels of BZLF1 and tubulin proteins in the assay were measured by immunoblotting (*IB*).

GACTCTGGTTTGCG-3′; for Ori-Lyt, 5′-CCGGCTCGCCTTCTTTTATCCTC-3′ and 5′-CCTGGTTCAACCCTATGGAGGGGAC-3′; for the EBNA-1 open reading frame, 5′-GTCATCATCATCCGGGTCTC-3′ and 5′-TTCGGGTTGGAACCTCCTTG-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,

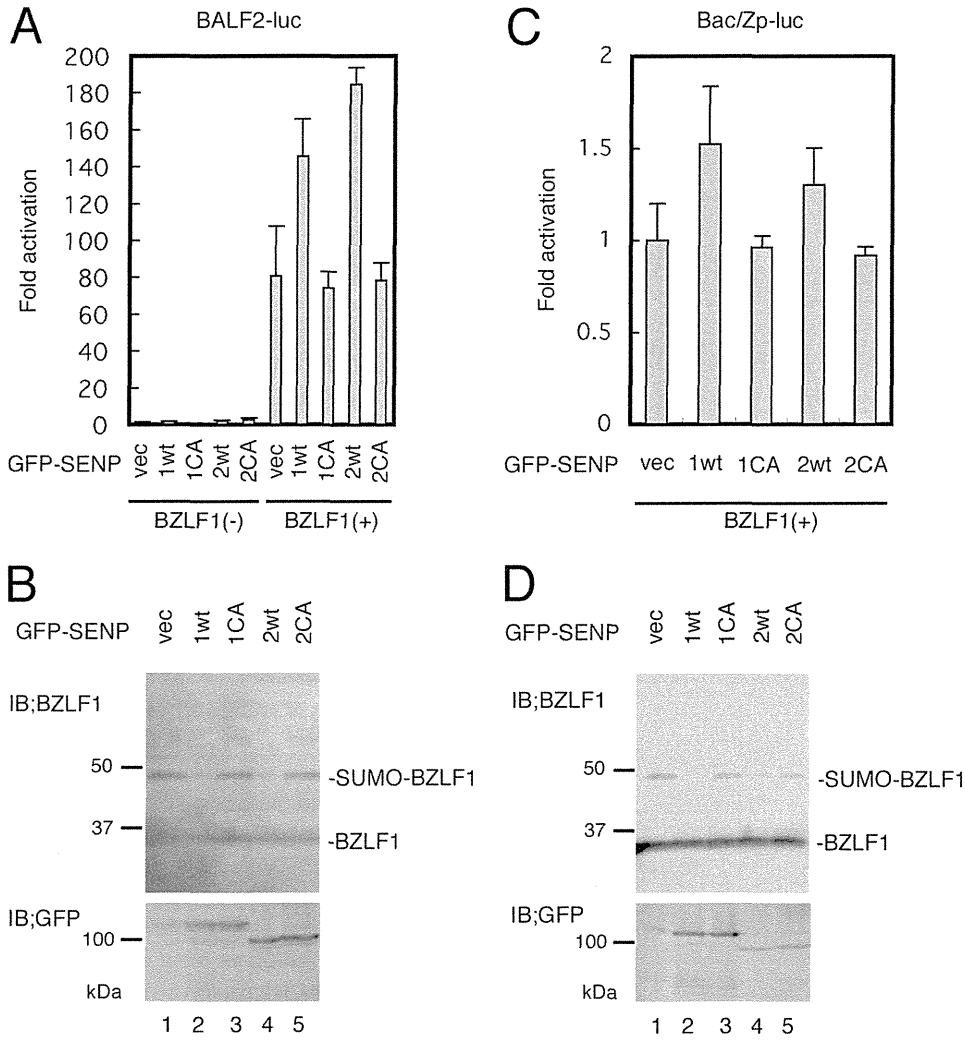

FIGURE 4. **SENP proteins deconjugate SUMO from BZLF1.** *A* and *B*, HEK293T cells were transfected with 10 ng of pcDNABZLF1(wild type) and 50 ng of GFP-tagged, wild type (*wt*), or inactive (*CA*) SENP expression vectors (*vec*) together with 10 ng of pZp-luc and 1 ng of pCMV-RL. Cell proteins were subjected to luciferase assay (*A*) and immunoblotting (*IB*) 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. 1*D*), 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. 2*C*, 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. 2*B*). 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. 3*A*, *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. 3*B*). 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. 3*C*, 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. 4*D*). 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

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. 4*B*). 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. 4*A*). 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. 6*C*). TSA treatment reversed the inhibitory action of BZLF1 sumoylation. We further examined 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.

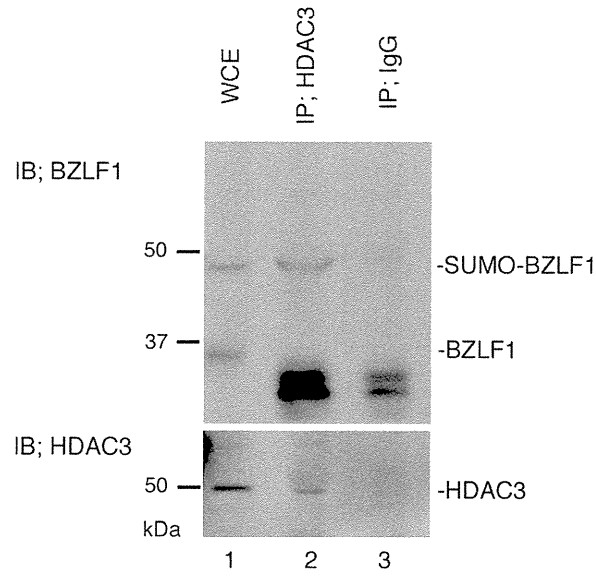

FIGURE 5. **SUMO modification of BZLF1 mediates HDAC3.** Proteins from HEK293T cells transfected with wild-type pcDNABZLF1 were subjected to immunoprecipitation (*IP*) using normal IgG or anti-HDAC3 followed by immunoblotting (*IB*) with anti-BZLF1 (*upper panel*) and HDAC3 (*lower panel*) antibodies. WCE (1/50) from the same lysate was also blotted.

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. 7*A*).

We then tested the effect of the fusion protein on the *BZLF1* promoter of the viral genome using Bac/Zp-luc (Fig. 7*B*). 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. 7*C*). Because the SUMO-2/BZLF1 fusion protein acted as expected, we further confirmed the interactions. When anti-BZLF1 antibody was used for precipitation (Fig. 7*D*), 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/BLZF1. We here used BZLF1 K12R and SUMO-2/BZLF1 K12R mutants to elimi-

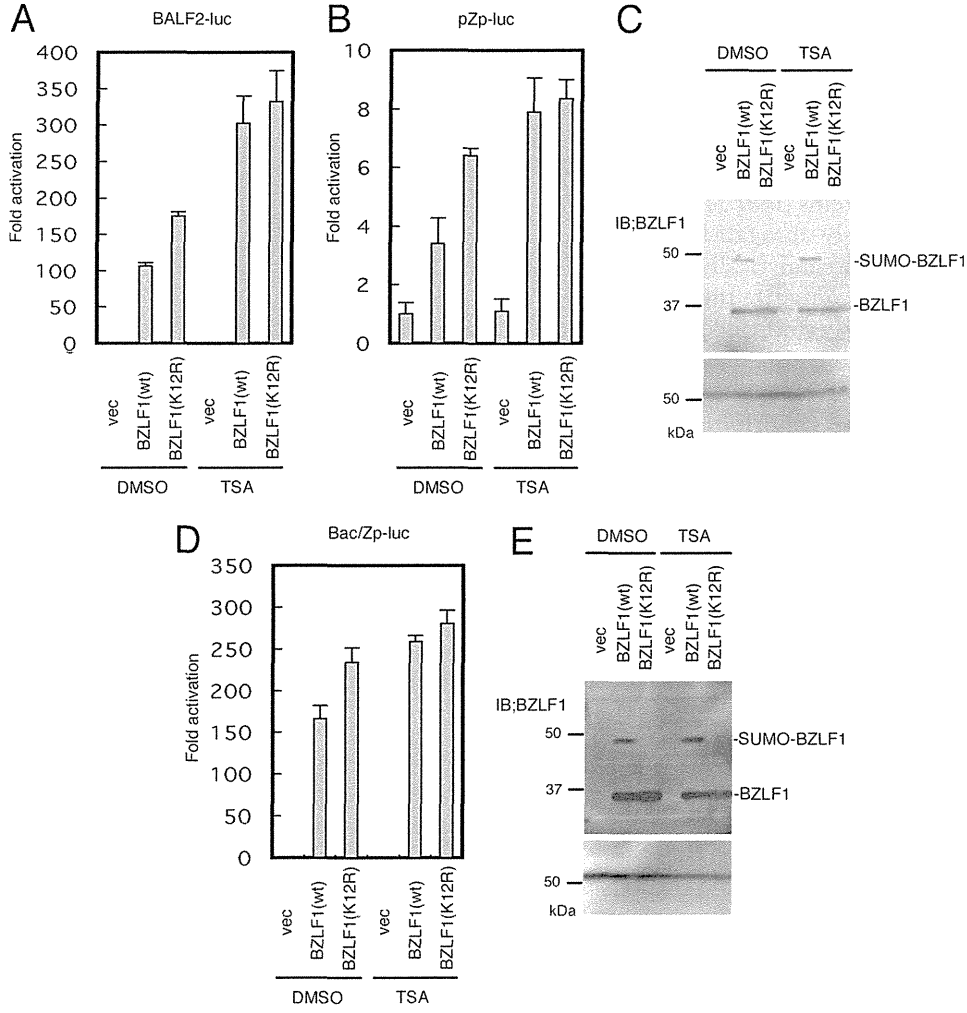

FIGURE 6. **An HDAC inhibitor reverses the effect of BZLF1 sumoylation.** *A* and *B*, HEK293T cells were transfected with 10 ng of BALF2-luc (*A*) or pZp-luc (*B*), 1 ng of pCMV-RL, and 10 ng of pcDNABZLF1(wild type) or pcDNABZLF1(K12R). After 4 h, cells were then exposed to TSA (100 μM) for 20 h. Counts 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 μM) 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.

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. 8*C*, 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. 8*C*). Co-expression of wild-type SENP1 correlated with the disappearance of sumoylated BZLF1 and increased early gene productions (Fig. 8*D*). Finally, a HDAC inhibitor TSA augmented amounts of early gene products without affecting the BZLF1 sumoylation levels (Fig. 8*E*). 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-

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. 7*E*). 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. 7*E*, *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. 7*E*, *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.

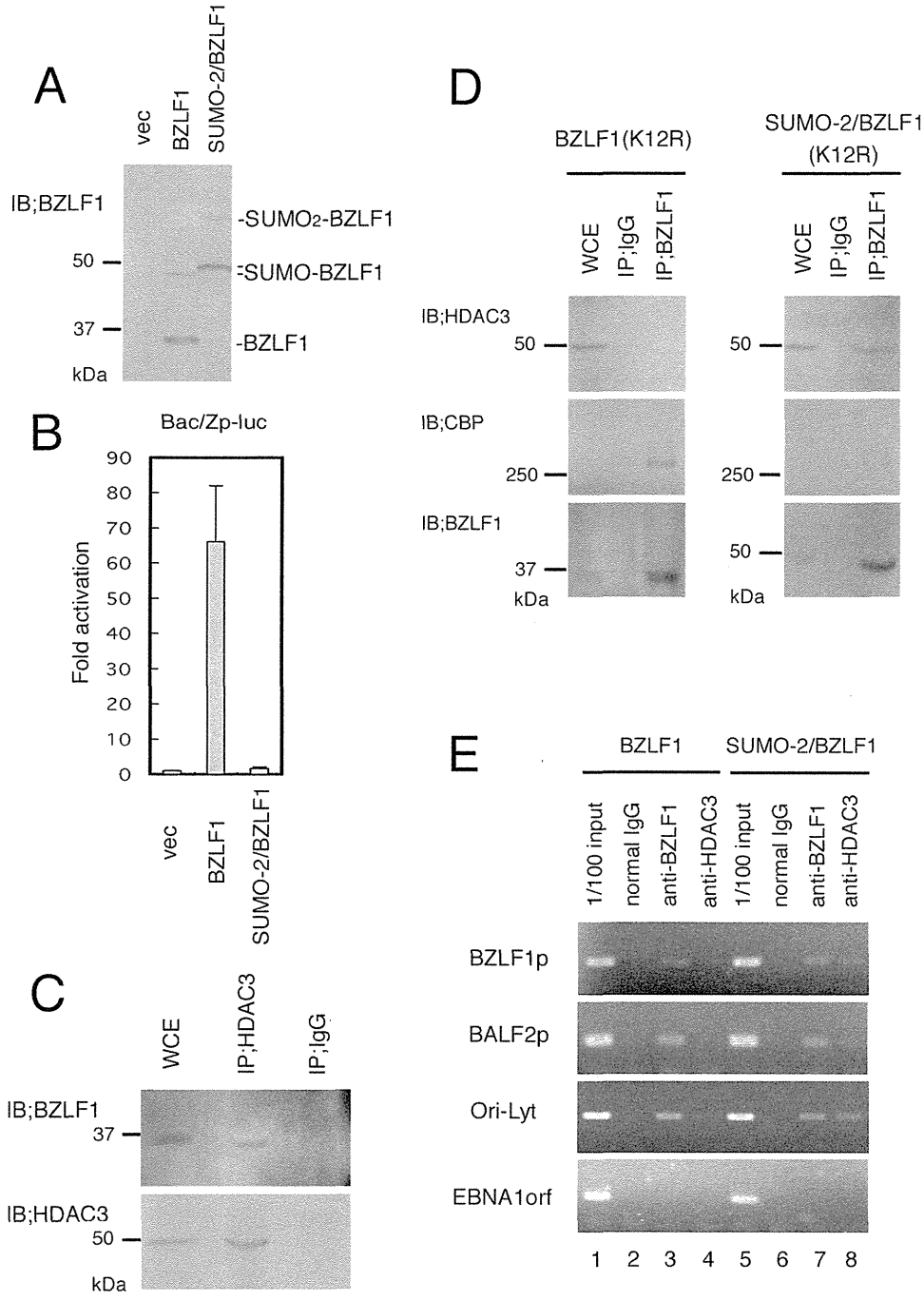

FIGURE 7. **HDAC is recruited to BZLF1-responsive promoters through association with SUMO-BZLF1.** *A*, pcDNASUMO-2/BZLF1, the expression vector for SUMO-2/BZLF1 fusion protein, was constructed as described under "Experimental Procedures," and its expression in HEK293T cells was confirmed by immunoblotting (*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.

sion 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 (ΦKX(D/E)*XX*(S/T)P, where Φ 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 (ΦK*X*(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 (ΦK*X*(D/E)). It could be envisaged that the non-functional motif, ΦK*X*(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

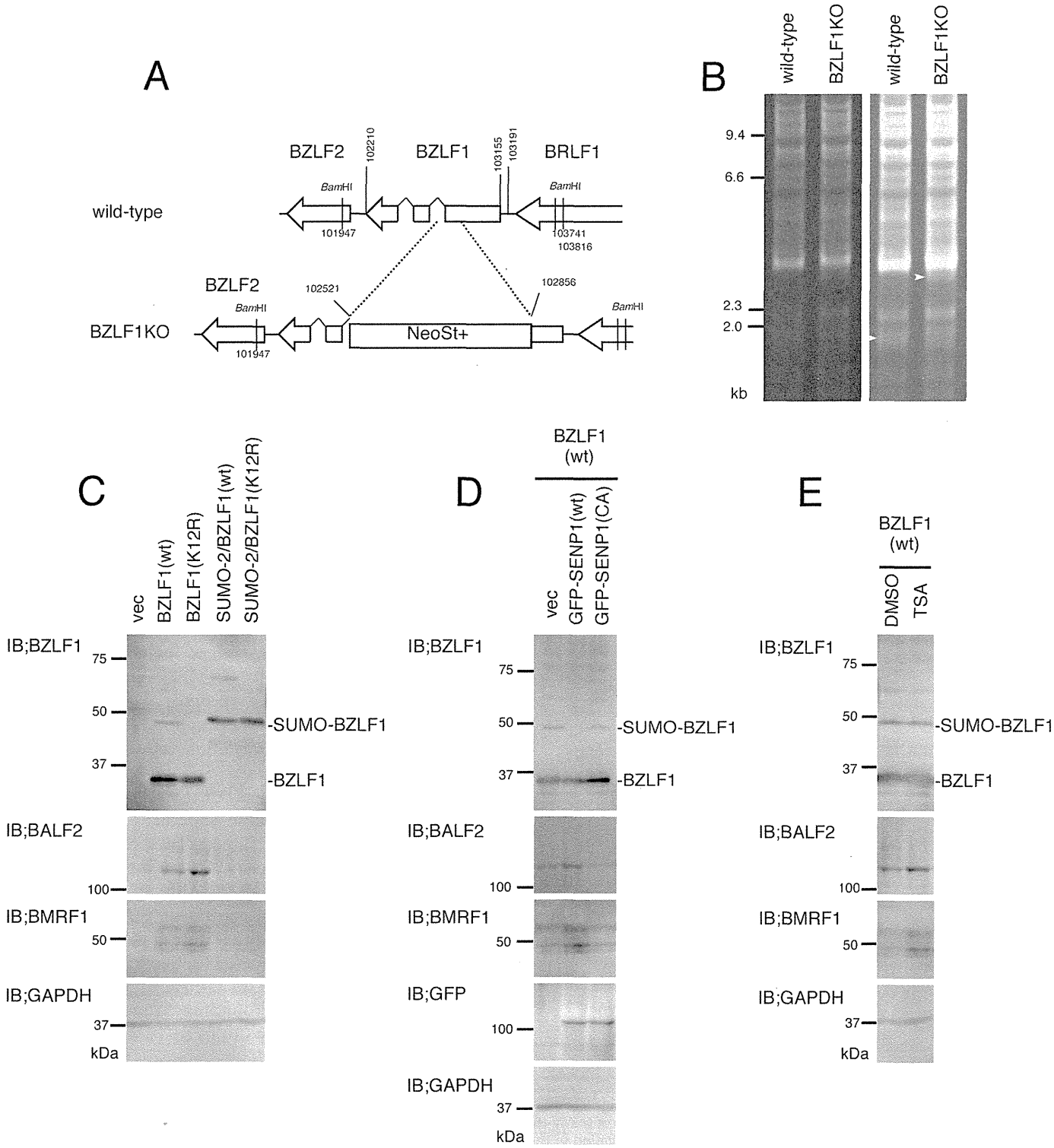

FIGURE 8. **Viral early gene expression was decreased by BZLF1 sumoylation.** *A*, shown is a schematic arrangement of the recombination of the EBV genome using the neomycin resistance and streptomycin sensitivity genes (*NeoSt*$^{+}$). The region between nucleotide 102,521 and 102,856 of the B95-8 genome (V01555) was replaced with the NeoSt+ 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.

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 Hagemeier *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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REPORT

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

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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 $p21^{WAF1/CIP1}$ 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.

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.[1,2] 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_1 arrest is mainly dependent on its ability to drive an increasing expression of the $p21^{WAF1/CIP1}$ protein, a cyclin-dependent kinase (CDK) inhibitor.[3,4]

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.[5] 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.[6,7] 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.[8] 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,[9] resulting in phosphorylation of p53 at various sites including S15 after 24 h post-induction.[10] 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-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.[10]

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

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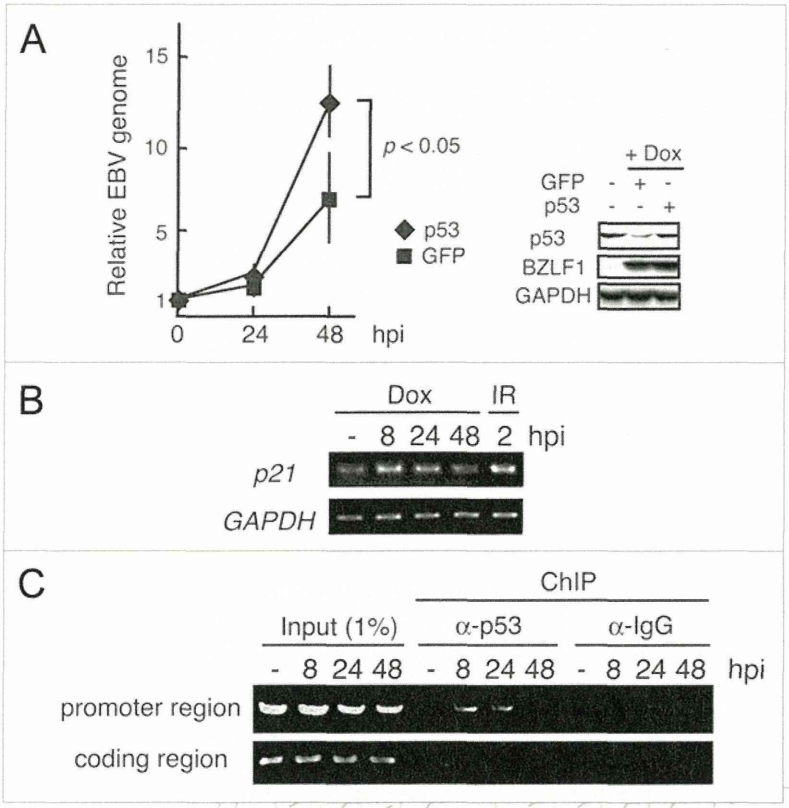

Figure 1. p53 is required for the initiation step of EBV lytic replication. (A) Tet-BZLF1/B95-8 cells were transfected with p53 or GFP expression plasmid using a Microporator and then cultured in the presence of doxycycline for 48 h. Viral DNA synthesis was determined by slot blot assay. Statistical 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 μg/ml) for the indicated times. IR, Tet-BZLF1/B95-8 cells were exposed to 10 Gy of γ-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 μg/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.[12,13] The protein is a multi-functional protein and also serves as an oriLyt binding protein involved in the initiation step of viral lytic replication.[14] 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.[15,16] 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.[15,17] Expression of BZLF1 protein alone does not induce p53 phosphorylation,[9] but increases the levels of p53 and its target gene product, p21$^{WAF1/CIP1}$.[15] 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 BZLF 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,[18] 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.[10] It has been, however, recently reported that p53 is involved in the augmentation of EBV lytic cycle initiation.[18] 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.[18] 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*$^{WAF-1/CIP-1}$ that is one of the major targets of p53 in lytic replication induced Tet BZLF1/B95-8 cells (**Fig. 1B**). Levels of *p21*$^{WAF-1/CIP-1}$ 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 system-mediated dergadation of p53.[10,19] Furthermore, the dynamics of p53 binding to *p21*$^{WAF-1/CIP-1}$ 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*$^{WAF-1/CIP-1}$ 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.[15] We tried to confirm the effect of BZLF1 protein expression in the epithelial cells. As shown in **Figure 2A**, the treatment of doxycyclin (Dox) induced the accumulation of $p21^{WAF1/CIP1}$ and p53 in Tet-BZLF1/HeLa cells, in which the BZLF1 protein is conditionally expressed under the control of tetracycline-regulated promoter.[9] Similar results were obtained when the BZLF1 protein was overexpressed in HeLa cells in a transient transfection experiment (data not shown). Since $p21^{WAF1/CIP1}$ is one of the major targets of p53 transcription factor,[3,20] it is likely that the G_1 arrest induced by BZLF1 protein, as reported by Cayrol and Flemington,[15] is due to the induction of p53. RT-PCR analysis revealed apparent increases of both p53 and $p21^{WAF1/CIP1}$ mRNA levels in Dox-treated cells (**Fig. 2B**), indicating that the expressions of both p53 and $p21^{WAF1/CIP1}$ are trasncriptionally-upregulated. Although Mauser et al. reported that BZLF1 expression using adenovirus vector in HeLa induced primarily a G_2/M block,[21] 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.[15]

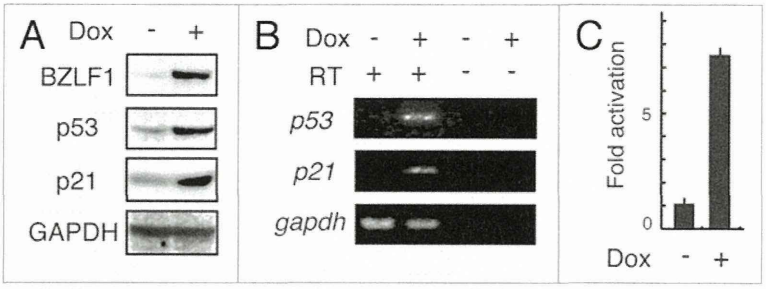

Figure 2. The BZLF1 protein induces the expression of p53 and p21 through the transactivation of p53. (A) Expression profiles of p53, and $p21^{WAF1/CIP1}$ after induction of BZLF1 protein expression. Tet-BZLF1/HeLa cells were cultured in the presence of 2 μg/ml of doxycycline and harvested 48 h post-induction. 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 (±S.E.) after normalization by total protein content.

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.[19] 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 *$p21^{WAF1/CIP1}$* transcription, we next performed the gel electrophoretic mobility shift assays (EMSAs). Purified HA-tagged p53 protein, obtained from baculovirus-infected insect cells, was subjected to EMSA by using a ^{32}P-labeled p53 recognition sequence of the *$p21^{WAF1/CIP1}$* 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 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,[22] and c-Abl.[23] 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,[24] we then investigated whether BZLF1 protein affects p53-TBP-DNA complex formation. A ^{32}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 p21WT 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