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Involvement of Jun Dimerization Protein 2 (JDP2) in the Maintenance of Epstein-Barr Virus Latency*[S]

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Reactivation of the Epstein-Barr virus from latency is dependent on expression of the BZLF1 viral immediate-early protein. The *BZLF1* promoter (Zp) normally exhibits only low basal activity but is activated in response to chemical inducers such as 12-*O*-tetradecanoylphorbol-13-acetate and calcium ionophore. We found that Jun dimerization protein 2 (JDP2) plays a significant role in suppressing Zp activity. Reporter, EMSA, and ChIP assays of a Zp mutant virus revealed JDP2 association with Zp at the ZII *cis*-element, a binding site for CREB/ATF/AP-1. Suppression of Zp activity by JDP2 correlated with HDAC3 association and reduced levels of histone acetylation. Although introduction of point mutations into the ZII element of the viral genome did not increase the level of BZLF1 production, silencing of endogenous *JDP2* gene expression by RNA interference increased the levels of viral early gene products and viral DNA replication. These results indicate that JDP2 plays a role as a repressor of Zp and that its replacement by CREB/ATF/AP-1 at ZII is crucial to triggering reactivation from latency to lytic replication.

The Epstein-Barr virus (EBV)[2] is a human γ-herpesvirus that predominantly establishes latent infection in B lymphocytes. Only a small percentage of infected cells switch from the latent stage into the lytic cycle to produce progeny viruses. Although the mechanism of EBV reactivation *in vivo* is not fully understood, it is known to be elicited by treatment of latently infected B cells with chemical or biological reagents, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), calcium ionophore, sodium butyrate, or anti-immunoglobulin, at least in cultured cells. Stimulation of the EBV lytic cascade by any of these leads to expression of two immediate-early genes, *BZLF1* and *BRLF1*. The BZLF1 protein is a transcriptional activator that shares structural similarities to basic leucine zipper (b-Zip) family transcriptional factors and acts as an oriLyt binding protein essential for lytic viral DNA replication. BZLF1 expression alone can trigger the entire reactivation cascade (1–3).

Expression of the *BZLF1* gene is tightly controlled at the transcriptional level. The *BZLF1* promoter (Zp) normally exhibits low basal activity and is activated in response to TPA or the other reagents described above. The minimal sequence of Zp necessary for activation by the inducers is 233 bp in length (4). The region harbors at least three types of *cis* regulatory elements, referred to as ZI, ZII, and ZIII. Four copies of the ZI element (ZIA–D) are distributed within the minimal Zp. The myocyte enhancer factor 2D binds to ZIA, ZIB, and ZID (5), whereas Sp1 or Sp3 can bind to ZIA, ZIC, and ZID (6). A single ZII element is located near TATA, sharing homology with binding sites for the cyclic AMP-response element-binding protein (CREB), activating transcription factor (ATF), and activator protein-1 (AP-1) family transcriptional factors such as JunB and JunD (7, 8). Two copies of the ZIII element (ZIII-A and -B) bind to the BZLF1 protein. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation of the promoter by TPA/ionophore or anti-surface immunoglobulin IgG (2). Then, the expressed BZLF1 protein further activates Zp by binding to ZIIIA and -B (9). BZLF1 also activates transcription of other viral immediate-early or early genes and enhances the lytic infection cycle of the virus.

The Jun dimerization protein 2 (JDP2) was initially identified as a binding partner of the AP-1 transcription factor, c-Jun (10). It appears ubiquitously expressed and is involved in a variety of biological phenomena, such as cell differentiation (11–14), apoptosis (15, 16), and tumorigenesis (17–22). It can dimerize, through its b-Zip motif, with itself or other b-Zip proteins, such as c-Jun, JunB, JunD, or ATF-2 (10, 11, 23), and function as a general repressor of, at least, AP-1, cAMP-response element, and TPA responsive element-dependent transcription (10, 23). It has been reported that JDP2 recruits histone deacetylase 3 (HDAC3) to the promoters of target genes and inhibits histone acetyltransferase activity, thereby suppressing transcriptional activity (14, 24). Depending on the context and cell type, however, it can alternatively act as a transcriptional activator (25, 26).

In the present study, we obtained evidence that JDP2 suppresses Zp mainly through interaction with the ZII *cis*-element.

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[2] The abbreviations used are: EBV, Epstein-Barr virus; Zp, *BZLF1* promoter; JDP2, Jun dimerization protein 2; CREB, cyclic AMP-responsive element-binding protein; AP-1, activator protein-1; ATF, activating transcription factor; XBP1(s), spliced form of X-box binding protein 1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; b-Zip, basic leucine zipper; CMV, cytomegalovirus; HDAC, histone deacetylase; KO, knock-out.

The lytic life cycle of the virus was found to be significantly enhanced by silencing of endogenous JDP2 expression. We also found that JDP2 supported recruitment of HDAC3 to Zp. These results indicate that JDP2 plays critical roles in regulation of the latent-lytic switch in EBV infection.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—HEK293T and EBV-BAC 293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. HEK293 cells with *BZLF1* knock-out (BZLF1KO) EBV were prepared as described previously (27). B95-8 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. TPA and A23187 were added to induce lytic replication of EBV. Anti-human IgG (Dako Cytomation, A0423) was used for viral lytic induction in Akata cells, which were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. Anti-JDP2 rabbit antiserum was a gift from Dr. A. Aronheim (The Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Israel). Anti-GAPDH, -HDAC3, and acetylated histone H3K9 antibodies were from Ambion, Abcam, and Active Motif, respectively. Both mouse and rabbit anti-FLAG antibodies were from Sigma. Rabbit anti-BZLF1, -BMRF1, -BALF2, and -BALF5 antibodies were as reported previously (28). Horseradish peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences.

Plasmid Construction—The expression vector for BZLF1 (pcDNABZLF1), b-Zip deletion form of BZLF1 (pcDNAdBZLF1), the reporter plasmid pZp-luc and its derivatives, pZpmZII-luc, pZpmZIII-luc, and pZpmZII+III-luc, were constructed as described previously (29). An expression vector for FLAG-tagged BZLF1 (pcDNAFlagBZLF1) was prepared by inserting the *BZLF1* cDNA sequence into the EcoRI/XbaI site of pcDNAFLAG (29). FLAG-tagged expression vectors for CREB (30) and c-Jun (31) were as reported previously. For pcDNAFlagXBP1(s), the cDNA sequence for XBP1(s) (32) was amplified using the following primers: 5′-CATGGACTACAAGGACGACGATGACAAGATGGTGGTGGTGGCAGCCGC-3′ and 5′-CTTAGACACTAATCAGCTGGG-3′. Underlined nucleotides indicate the FLAG epitope. The amplified DNA was phosphorylated by polynucleotide kinase and then inserted into the EcoRV site of the pcDNA3 vector. The pCMV-RL reporter plasmid was obtained commercially (Stratagene). pCMV-FlagJDP2 was made by inserting human *JDP2* cDNA into the NotI site of pCMV_S-FLAG (RIKEN, RDB 5956). A deletion mutant at the b-Zip domain of *JDP2* was generated by PCR using primers 5′-CGCCCCACCTGCATCGTCC-3′ and 5′-TCGCTCCTCTTCCTCATCTAG-3′.

Transfection, Luciferase Assay, and Immunoblotting—Plasmid DNA was transfected into HEK293T or EBV-BAC 293 cells using Lipofectamine 2000 reagent (Invitrogen). The total amounts of plasmid DNA were standardized by 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 activities were measured using the kit. Akata cells were electronically transfected using a Microporator (Digital Bio). Protein samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies as described previously (29).

Electromobility Shift Assay (EMSA)—FLAG-tagged JDP2 proteins were produced using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. The probe was prepared by 3′-end labeling using Klenow fragment (TOYOBO) and [^{32}P]dATP (Institute of Isotopes Co., Hungary). Unincorporated deoxynucleotide triphosphates were removed with Chromaspin-10 columns (Clontech). The *in vitro* translated protein and labeled DNA sequences were incubated in the EMSA binding buffer (20 mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 30 mM KCl, 3 mM $MgCl_2$, 0.5 mg/ml of poly(dI-dC)) at room temperature for 30 min. The samples were then separated in a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffer and the radioactivities were visualized by using BAS2500 system (Fuji Film). The sequences of oligonucleotide probes were as follows: ZII, 5′-CCCAAACCATGACATCACAGAG-3′ and 5′-CTCCTCTGTGATGTCATGGTTTGGG-3′; ZIIIA, 5′-AACTATGCATGAGCCACAGG-3′ and 5′-ATGCCTGTGGCTCATGCATAGTT-3′; ZIIIB, 5′-CACAGGCATTGCTAATGT-3′ and 5′-GAGGTACATTAGCAATGCCTGTG-3′.

siRNA, Exogenous Expression, and RT-PCR—Duplexes of a 21-nucleotide small interfering RNA (siRNA) specific to *JDP2* mRNA, including two nucleotides of deoxythymidine at the 3′ end, were synthesized and annealed (Gene Design, Inc). The sense and antisense sequences of the duplex were 5′-GUGAGCUAGAUGAGGAAGAdTdT-3′ and 5′-UCUUCCUCAUCUAGCUCACdTdT-3′ (si-JDP2.1) and 5′-GCACAUACUCACCGAAUGUdTdT-3′ and 5′-ACAUUCGGUGAGUAUGUGCdTdT-3′ (si-JDP2.2), respectively. The control siRNA sequences were 5′-GCAGAGCUGGUUUAGUGAAdTdT-3′ and 5′-UUCACUAAACCAGCUCUGCdTdT-3′. Akata or B95-8 cells (1×10^5) were transfected with 50 pmol of the duplex RNA or with 300 ng of expression plasmid per well of a 24-well plate, using a Microporator (Digital Bio). 24 or 48 h after transfection, TPA plus A23187, or anti-IgG was added to induce lytic replication and incubated for another 12 or 24 h. Cells were then harvested for quantification by real time RT-PCR using the One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa). PCR was performed in 10 μl of solution containing 0.2 μM primers, 0.2 μl of ROX Dye, and the sample RNA in 1× One-Step SYBR RT-PCR buffer. Intensity of ROX Dye was used to compensate the volume fluctuations among the tubes. PCR included 5 min at 42 °C, 10 s at 95 °C, and 40 cycles at 95 °C for 5 s followed by 40 s at 60 °C. Immediately after the RT-PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. An arbitrary RNA was set to 1.0 and a standard curve was constructed using serial dilutions of RNA from the RNA set to 1.0. The amount of mRNA was quantitated based on the standard curve. Real time PCR with RNA polII primers was also performed to serve as an internal control for input RNA. Primers used for the real time RT-PCR were as follows: for *JDP2* mRNA, 5′-CTGTGGAGGAGCTGAAATAC-3′ and 5′-ATCTAGCTCACTTTTCACGG-3′, for *BZLF1* mRNA, 5′-AACAGCCAGAATCGCTGGAG-3′ and 5′-GGCACATCTGCTTCAACAGG-3′, for *RNApolII* 5′-GCACCAC-

GTCCAATGACAT-3′ and 5′-GTGCGGCTGCTTCCATAA-3′. Viral DNA levels were assayed by dot-blot hybridization as described previously (28, 33).

Genetic Manipulation of EBV-BAC DNA and Cloning of HEK293 Cells with EBV-BAC—EBV-BAC DNA was provided by W. Hammerschmidt (34). Homologous recombination was carried out in *Escherichia coli* as described previously (28).

To prepare an mZII mutant of EBV-BAC, a transfer DNA fragment for the first recombination was generated by PCR using PpsL-neo (Gene Bridges) as the template, with the following primers: 5′-AGCCACAGGCATTGCTAATGTACCTCA-TAGACACACCTAAATTTAGCACGTCCCAAACCAGGC-CTGGTGATGATGGCGGGATC-3′ and 5′-GAGTTACCT-GTCTAACATCTCCCCTTTAAAGCCAAGGCACCAGCC-TCCTCTGTGATGTCATCAGAAGAACTCGTCAAGAAG-G-3′. After the recombination, kanamycin-resistant colonies were selected and checked to make intermediate DNA. The NeoSt+ cassette in the intermediate DNA was then replaced using the next transfer vector DNA, containing a mutation in the ZII *cis*-element of Zp. The transfer vector was made by PCR using pZpmZII-luc as the template with the following primers: 5′-ACCAGCTTATTTTAGACACTTC-3′ and 5′-GTTTGG-GTCCATCATCTTCAG-3′. Streptomycin-resistant colonies were cloned and checked to make the EBV-BAC mZII mutant. Likewise, a revertant of the mZII mutant (EBV-BAC mZII/R) was created by re-insertion of the NeoSt+ cassette and its rescue using a wild-type *BZLF1* promoter sequence.

Electroporation of *E. coli* was performed using Gene Pulser III (Bio-Rad) and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel). Recombination was confirmed with PCR products of the Zp region, electrophoresis of the BamHI-digested viral genome, and sequencing analysis.

EBV-BAC DNA was transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen), which were then cultured on 10-cm dishes with 100–150 μg/ml of hygromycin B for 10–15 days for cloning of GFP-positive cell colonies as described previously (28). Briefly, for each recombinant virus, we picked up more than 10 hygromycin-resistant, GFP-positive cell colonies to obtain at least 3 typical clones exhibiting minimal spontaneous expression of viral lytic proteins and significant induction of these upon BZLF1 transfection.

ChIP Assays—ChIP assays were performed essentially as described (Upstate Biotechnology, Inc.) with formaldehyde cross-linked chromatin from 1×10^6 cells for each reaction. Cells were lysed, and chromatin was sonicated to obtain DNA fragments with an average length of 300 bp. Following centrifugation, the chromatin was diluted 10-fold with ChIP dilution buffer, and precleared with protein A-agarose beads containing salmon sperm DNA (Upstate). Anti-FLAG antibody or normal rabbit IgG was added to the sample and incubation proceeded overnight with rotation. Immune complexes were then collected by addition of protein A-agarose beads and DNA was purified using a QIAquick PCR Purification Kit (Qiagen) after uncoupling of the cross-linking and proteinase K digestion. The recovered DNA was amplified by PCR using primers specific for Zp: 5′-TAGCCTCGAGGCCATGCATATTTCAACTGG-3′, 5′-GCCAAGCTTCAAGGTGCAATGTTTAGTGAG-3′, and for the EBNA1 open reading frame: 5′-GTCATCATCAT-CCGGGTCTC-3′ and 5′-TTCGGGTTGGAACCTCCTTG-3′. The PCR products were then analyzed by agarose gel electrophoresis and visualized with ethidium bromide. The samples were also subjected to real time PCR for quantification of DNA sequences using the same primers and SYBR Premix Ex TaqII (TaKaRa). Real time PCR was performed in 10 μl of solution containing 0.2 μM primers, 0.2 μl of ROX Dye, and the sample DNA in 1× One-Step SYBR RT-PCR buffer. Intensity of ROX Dye was used to compensate the volume fluctuations among the tubes. PCR included 10 s at 95 °C and 40 cycles at 95 °C for 5 s followed by 45 s at 60 °C. Immediately after the PCR, we carried out dissociation curve analysis and confirmed the specificity of each PCR product. An arbitrary DNA was set to 1.0 and a standard curve was constructed using serial dilutions of DNA from the DNA set to 1.0. The amount of DNA was quantitated based on the standard curve.

Immunoprecipitation Assay—For immunoprecipitation to detect the association between JDP2 and HDAC3, cells were lysed in Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and protease inhibitor mixture). After sonication and centrifugation, lysates were precleared with protein G-Sepharose, then mixed with anti-FLAG antibody and protein G-Sepharose for 4 h. The resin was washed 5 times with the same buffer and then samples were subjected to SDS-PAGE followed by immunoblotting with anti-JDP2 or FLAG antibody.

Data Analysis and Statistics—Data are presented as mean ± S.D. Statistical analysis was carried out using Student's *t* test and values considered significantly different when $p < 0.05$.

RESULTS

JDP2 Represses Transcription from EBV Zp—Because JDP2 represses transcriptional activation of reporter constructs containing cAMP-response element or TPA-responsive elements (23), we hypothesized that JDP2 might regulate the *BZLF1* transcription and thereby affect EBV reactivation. To investigate this possibility, we first carried out reporter gene assays. Co-transfection of the JDP2 expression vector clearly repressed reporter gene expression from pZp-luc, in the presence or absence of BZLF1 (data not shown). In this reporter assay, however, we co-transfected pCMV-RL, a *Renilla* luciferase reporter driven by the CMV immediate early promoter at the same time, and this reporter strongly responded negatively, regardless of BZLF1 (data not shown). This result pointed to a possibility that ectopic overexpression of JDP2 suppresses the CMV immediate-early promoter, as well as *BZLF1* promoter and that this system is not suitable for analyses of specific effects of JDP2 on the Zp.

Therefore, we adopted siRNA technology, using synthetic oligonucleotides that form duplex RNA encoding partial nucleotides from *JDP2*. As shown in Fig. 1*A*, cotransfection with the BZLF1 expression vector (Z) caused increased Zp activity, due to its autoactivation ability. Treatment with an siRNA (si-JDP2.1) significantly elevated the transcriptional activity from the wild-type *BZLF1* promoter (pZp-luc(wt)), either in the presence or absence of BZLF1 (Fig. 1*A*). Another siRNA (si-JDP2.2) also resulted in increased Zp activity (Fig. 1*A*). Interest-

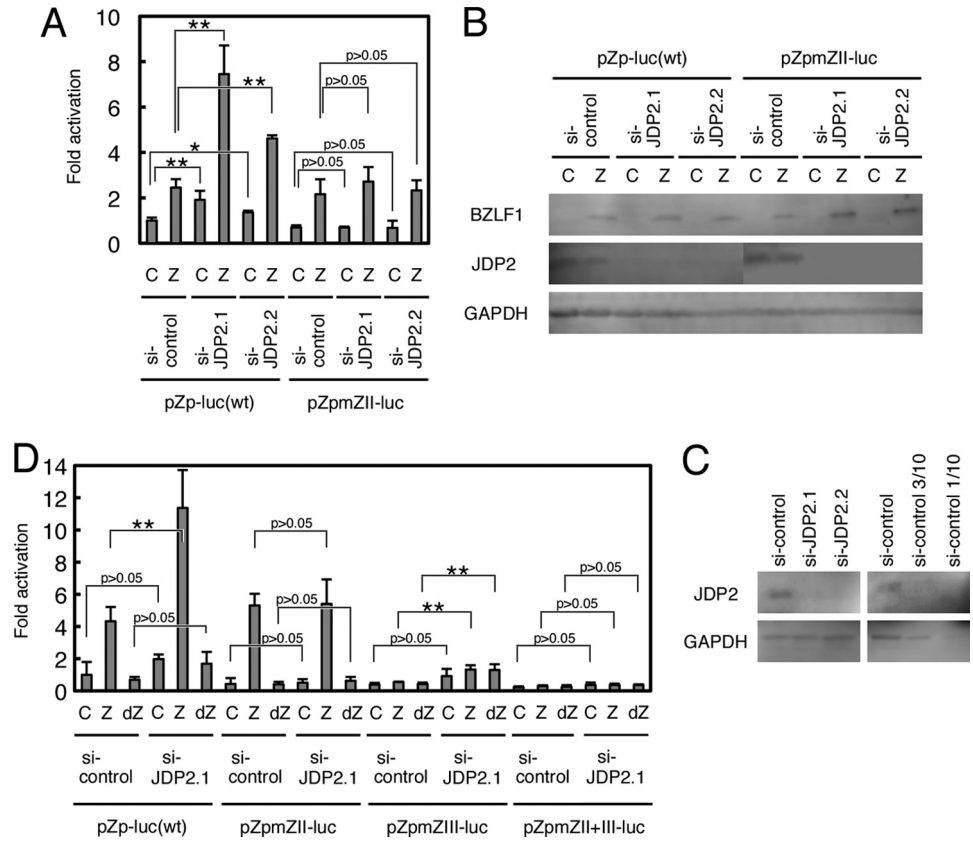

FIGURE 1. **Knockdown of endogenous JDP2 enhances transcription from Zp.** *A–C*, HEK293T cells were transfected with duplexes of a 21-nucleotide siRNA against JDP2 (*si-JDP2.1* or *si-JDP2.2*) or a control (*si-control*) siRNA, together with 10 ng of pZp-luc or its derivative, pZpmZII-luc, and 1 ng of pCMV-RL with 10 ng of empty vector (*C*) or pcDNABZLF1 (*Z*). Luciferase assays (*A*) were carried out 36 h after the transfection, as described under "Experimental Procedures." Each *bar* illustrates the mean ± S.D. of three independent transfections. The luciferase activities are shown as fold-activation of that for pZp-luc(wt), with the si-control and empty vector (*leftmost bar*). *B*, protein levels of BZLF1, JDP2, and GAPDH in the assay were examined by immunoblotting. *C*, JDP2 levels in samples transfected with si-JDP2.1 or si-JDP2.2 in the assay were compared with 100, 30, or 10% of the control. *D*, HEK293T cells were transfected with siRNA against JDP2 (si-JDP2.1) or a control (si-control) siRNA, together with 10 ng of pZp-luc or its derivatives and 1 ng of pCMV-RL with 10 ng of empty vector (*C*), pcDNABZLF1 (*Z*), or pcDNAdBZLF1 (*dZ*). pcDNAdBZLF1 (*dZ*) expresses BZLF1 protein lacking the b-Zip domain. Luciferase assays were carried out 36 h after the transfection, as described under "Experimental Procedures." * or ** indicates $p < 0.05$ or $p < 0.02$, respectively.

ingly, silencing of endogenous *JDP2* did not affect the CMV promoter as monitored by the reporter assay (data not shown). On the other hand, when the ZII motif in the Zp was mutated (pZpmZII-luc), a significant response to JDP2 knockdown was not observed (Fig. 1*A*). Knockdown of the JDP2 protein was checked by immunoblotting (Fig. 1*B*). Real time PCR analysis indicated that si-JDP2.1 and si-JDP2.2 reduced the *JDP2* mRNA levels down to 23 and 43% of the control (data not shown). We also examined that levels of the BZLF1 protein were relatively constant (Fig. 1*B*). To quantify the levels of JDP2 knockdown, protein samples transfected with si-JDP2.1 or si-JDP2.2 were compared with those from control cells after serial dilution (Fig. 1*C*). Either of the siRNAs reduced the JDP2 protein levels to ~30% or less in HEK293T cells. These results suggest that JDP2 inhibit Zp through the ZII *cis*-element, which is known to be bound by cellular transcription factors such as CREB/ATF/AP-1.

The site-directed mutation of the ZIII element, which prevents BZLF1 binding, failed to respond to ectopic expression of the BZLF1 protein (Fig. 1*D*) as expected, but its promoter activity was increased by si-JDP2 treatment (Fig. 1*D*, pZpmZIII-luc). Although *p* values indicate that JDP2 knockdown did not always cause statistically significant augmentation of wild-type or mZIII promoters, these results imply that JDP2 decreases Zp activity and that the suppressive effect of JDP2 is mediated through the ZII motif, but not through the ZIII of *BZLF1* promoter.

JDP2 Binds to ZII Motif in Vitro—Reporter assays suggested JDP2 association with the ZII element, but not with the ZIII elements. To confirm the binding of JDP2 to the ZII *cis*-element, EMSA was carried out in Fig. 2. Addition of FLAG-tagged JDP2 produced a specific band of JDP2-ZII complex (Fig. 2, *lane 2*). Excess amounts of unlabeled ZII competitor clearly reduced the intensities of the shifted band (*lane 3*), whereas the cold ZIIIA or ZIIIB did not (*lanes 4* or *5*, respectively). When JDP2 lacking b-Zip domain (FlagdJDP2, *lane 8*) was used instead of wild-type FlagJDP2 (*lane 7*), no obvious shift was detected (Fig. 2, *right*). Supershift analysis with anti-FLAG antibody demonstrated that the band actually contained FLAG-tagged JDP2 protein (*lane 9*). Therefore, JDP2 binds to ZII, but not to the ZIIIA or ZIIIB *cis*-elements of Zp.

Association of JDP2 with the BZLF1 Promoter in Vivo—To further verify the reporter assays and EMSA results, we performed ChIP analysis using cells containing wild-type or *BZLF1* knock-out (BZLF1KO) EBV-BAC (27). We used the BZLF1KO mutant strain, to exclude the possibility that BZLF1 is involved

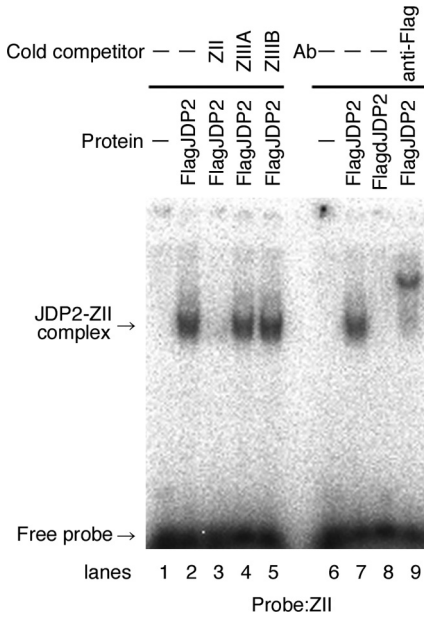

FIGURE 2. **JDP2 binds to ZII motif in the *BZLF1* promoter *in vitro*.** EMSA was carried out as described under "Experimental Procedures." FLAG-tagged wild-type JDP2 protein (*FlagJDP2*) or JDP2 protein lacking b-Zip domain (*FlagdJDP2*, *lane 8*) was produced *in vitro* and incubated with ^{32}P-labeled probe and excessive amounts of cold competitors (ZII (*lane 3*), ZIIIA (*lane 4*), and ZIIIB (*lane 5*)). Supershift analysis was performed using mouse anti-FLAG monoclonal antibody (*lane 9*). The samples were then separated in a 4% polyacrylamide gel and analyzed with Fuji Image Analyzer BAS2500.

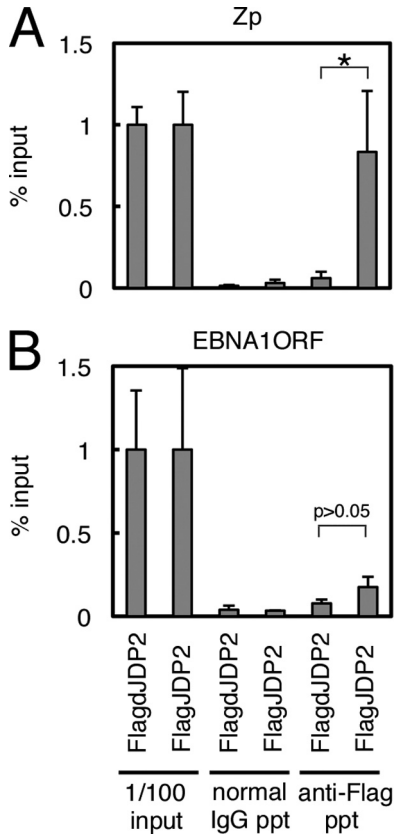

FIGURE 3. **Association of JDP2 with the *BZLF1* promoter.** HEK293 cells with *BZLF1* knock-out (BZLF1KO) EBV-BAC DNA were transfected with the FlagJDP2 expression vector (*FlagJDP2*), or the FlagJDP2 expression vector with a deletion in the b-Zip domain (*FlagdJDP2*). After 24 h, ChIP experiments were carried out using cross-linked DNA-protein complexes from the cells, precipitated using normal IgG or anti-FLAG antibody, followed by DNA extraction and PCR to detect the Zp (*A*). A fragment for the EBNA1 open reading frame was also detected as a negative control (*B*). * indicates $p < 0.05$.

in the association between JDP2 and the *BZLF1* promoter. The *BZLF1* promoter region was obviously co-precipitated with FLAG-tagged JDP2 (supplemental Fig. S1*A*), either in wild-type or BZLF1KO. A primer set for the EBNA1 coding region was included as a negative control to prove that the signal for the Zp was specific. This experiment of BZLF1KO was repeated and quantified in Fig. 3. With wild-type JDP2, the Zp was significantly concentrated when compared to the level of dJDP2 (*Fig. 3A*). Thus, the association proved to be intact, indicating that BZLF1 is not needed for JDP2 binding to the *BZLF1* promoter sequence.

Because the above documented results indicated JDP2 acts to suppress the *BZLF1* promoter by binding to the ZII *cis*-element, we generated recombinant EBV with a point mutation in the ZII element (Fig. 4*A*). To this end, we first replaced the ZII element with a marker cassette (NeoSt+), and then exchanged this with the mutated ZII sequence, to prepare EBV-BAC mZII. The mutated ZII sequence of the EBV was again swapped with a NeoSt+ cassette, followed by replacement with the wild-type sequence, to generate a revertant strain, EBV-BAC mZII/R. Sequencing analysis confirmed that the EBV-BAC mZII DNA had the same mutation as the pZpmZII-luc vector (Fig. 1), and that the EBV-BAC mZII/R had the same sequence as the wild-type virus, as intended. Integrity of the BAC DNA was checked by BamHI digestion followed by electrophoresis to confirm that the recombinant viruses did not carry obvious deletions or insertions (Fig. 4*B*). The BamHI-Z fragment was too short to be clearly detected.

Recombinant EBV-BAC DNA was introduced into HEK293 cells, followed by hygromycin selection, to establish cell lines in which multiple copies were maintained as an episome. More than 10 cell colonies from each recombinant virus were obtained and viral protein expression levels were examined. Fig. 4*C* shows immunoblotting data for typical cells. When the BZLF1 expression vector was transfected, the cells all responded and expressed viral genes, as expected. In the absence of BZLF1, however, levels of the viral genes in EBV-BAC mZII cells were noticeably lower than those in wild-type or revertant cells. Thus, introduction of point mutations into the ZII element of the viral genome did not increase BZLF1 production. We speculate that the suppressive JDP2 does bind to the ZII element in the latent phase but that its negative effect is counteracted by powerful stimulatory effects of other transcriptional factors, such as CREB/ATF/AP-1 family proteins. We here first demonstrated the significance of the ZII element in the context of the viral genome.

Although effects of JDP2 on *BZLF1* promoter activation were not observed in recombinant viruses, the ZII-mutant and its revertant viruses could still be used for binding assays. Supplemental Fig. S1*B* shows ChIP results. JDP2 was recruited to the *BZLF1* promoter in cells with the wild-type and revertant viruses, but this was impaired in cells with the mZII virus. Additionally, JDP2 with deletion in the b-Zip domain of the protein failed to target the *BZLF1* promoter (supplemental Fig. S1*B*, *right panels*). Taken together, the results indicate that JDP2 is

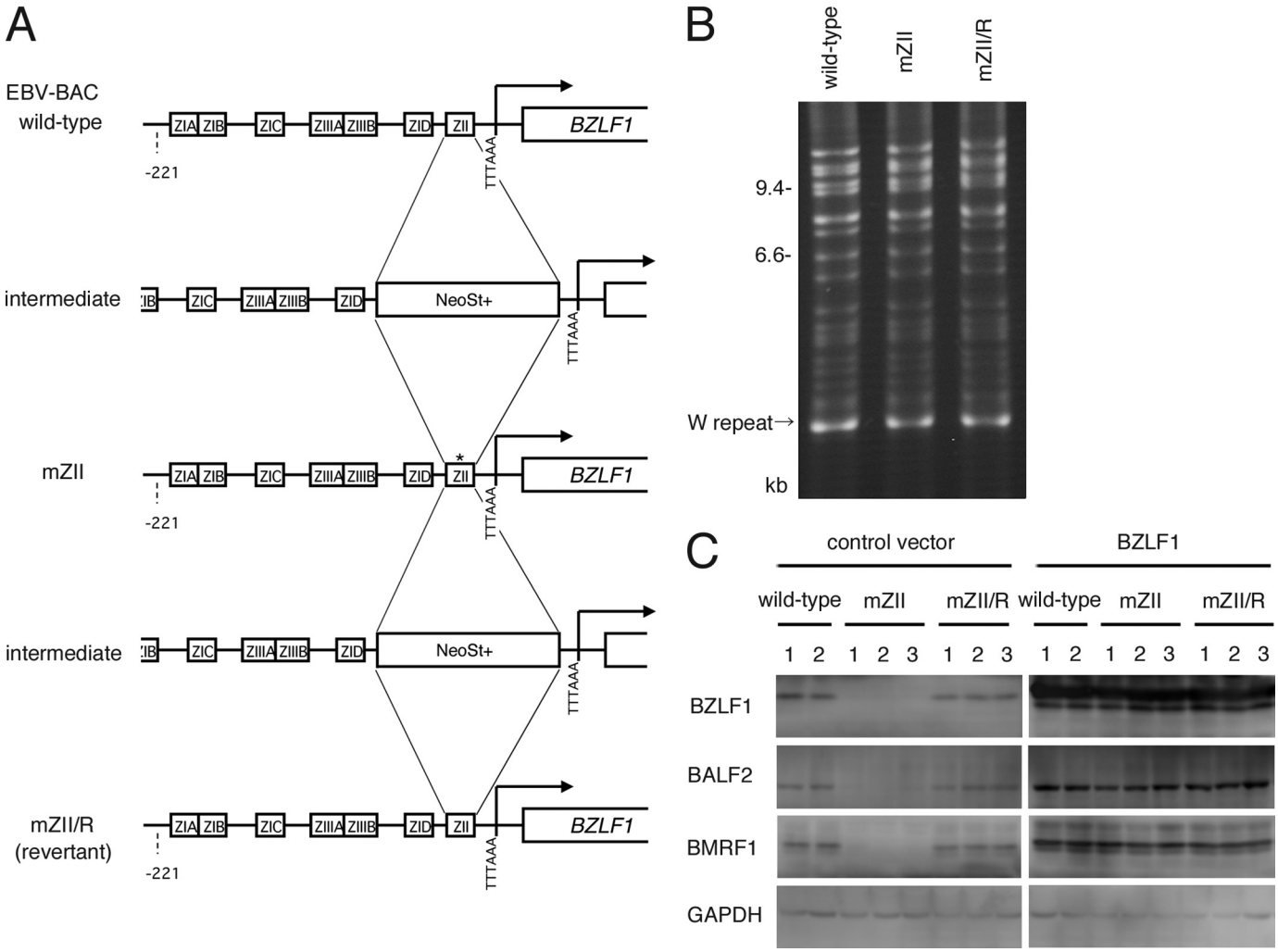

FIGURE 4. **Construction of recombinant EBV featuring point mutation in the ZII *cis*-element of the *BZLF1* promoter.** *A*, schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin resistance and streptomycin sensitivity genes (*NeoSt+*). The ZII element of the B95-8 genome was first replaced with the NeoSt+ cassette, which was then replaced with a point-mutated ZII sequence (*asterisk*) to construct EBV-BAC mZII. The mutated ZII sequence was replaced again with the NeoSt+ marker cassette and swapped with the wild-type ZII sequence to prepare the revertant clone, EBV-BAC mZII/R. *B*, electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel. *C*, expression of viral proteins from the recombinant viruses. The recombinant EBV-BAC DNAs were introduced into HEK293 cells, followed by hygromycin selection. Resultant cell clones were tested for protein viral expression. The indicated clones of cells were transfected with the BZLF1 expression vector (BZLF1) or its empty control vector (control vector). After 48 h, cell proteins were harvested, and immunoblotting was performed using anti-BZLF1, -BALF2, -BMRF1, and -GAPDH antibodies.

recruited to the *BZLF1* promoter and binds to the ZII element through its b-Zip domain.

Because JDP2 and CREB, ATF, and AP-1 all bind to the ZII *cis*-element of the *BZLF1* promoter, we examined whether JDP2 could competitively block the binding of these b-Zip proteins (supplemental Fig. S2). HEK293 cells harboring wild-type EBV-BAC were transfected with siRNA against JDP2 or its control siRNA, together with or without the expression vector for FLAG-tagged -BZLF1, -CREB, and -c-Jun. We used the viral b-Zip protein BZLF1 as a control, which associates with the ZIII element of the *BZLF1* promoter (9). As expected, FLAG-BZLF1 bound to Zp, and cotransfection of the JDP2 siRNA did not alter the binding efficiency to the promoter (supplemental Fig. S2, *left panels*). On the other hand, binding was lower with control siRNA, and si-JDP2 treatment significantly increased the association of FLAG-tagged CREB and c-Jun with the promoter, compared with the si-control results (supplemental Fig. S2, *left panels*). Because a spliced form of X-box binding protein 1 (XBP1(s)) also reportedly targets the ZII element of Zp (35), we also tested for association. We could observe XBP1(s) binding to the Zp to be increased by si-JDP2 treatment, although this was not obvious in control-si experiments.

The competitive nature of JDP2 with CREB was reconfirmed and quantified in Fig. 5. When JDP2 was knocked down, FLAG-tagged CREB association with the Zp was significantly higher than that with the control siRNA (Fig. 5*A*). Immunoblotting data indicated the expression level of FLAG-CREB was not affected by si-JDP2 (Fig. 5*C*). These data indicate that JDP2 inhibits transcriptional activity of Zp by competitively occupying the ZII binding site.

Role of JDP2 in EBV Reactivation from Latency—To examine the role of JDP2 in EBV reactivation from latency under physiological conditions, B95-8 cells, latently infected with

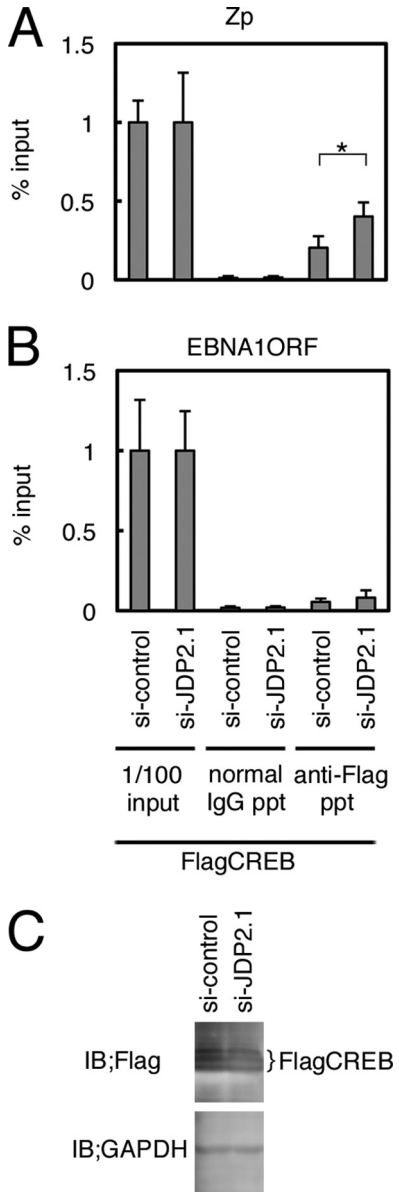

FIGURE 5. **Competitive binding of JDP2 with other b-Zip transcription factors.** HEK293 cells with wild-type EBV-BAC DNA were transfected with the siRNA against JDP2 (*si-JDP2.1*) or the control (*si-control*), together with FLAG-tagged expression vectors for CREB. After 36 h, ChIP experiments were carried out using cross-linked DNA-protein complexes from the cells, precipitated using normal IgG or anti-FLAG antibody, followed by DNA extraction and PCR to detect the Zp (*A*). A fragment for the EBNA1 open reading frame was also detected as a negative control (*B*). Levels of FLAG-tagged CREB protein were examined by immunoblotting (*IB*) (*C*). * indicates $p < 0.05$.

EBV, were treated with the siRNAs against JDP2. As shown in Fig. 6*B*, 73 or 49% suppression of the levels of *JDP2* mRNA resulted by si-JDP2.1 or si-JDP2. Although the knockdown of *JDP2* mRNA might not be perfect, it did reduce JDP2 protein levels (supplemental Fig. S3). JDP2 knockdown, in the absence of chemical lytic induction, augmented *BZLF1* levels 3.6- or 3.4-fold by si-JDP2.1 or si-JDP2.2, respectively (Fig. 6*A*), and the augmentation by si-JDP2.2, especially, was statistically significant. Treatment of TPA and A23187, a calcium ionophore, caused overall induction of the *BZLF1* gene, and si-JDP2.1 significantly induced *BZLF1* mRNA levels (Fig. 6*A*). A similar increase was also observed by si-JDP2.2 (Fig. 6*A*).

We then examined whether silencing of JDP2 influence viral lytic replication of another cell line (supplemental Fig. S4). Akata cells featuring latent infection were treated with the siRNA against JDP2. The levels of BZLF1 protein and early proteins, such as BMRF1, BALF5, and BALF2, were also enhanced, especially when treatment was combined with exposure to anti-IgG (supplemental Fig. S4).

To further confirm the role of JDP2, the effect of exogenous overexpression of JDP2 was monitored in B95-8 (Fig. 6, *C* and *D*) or HEK293 (Fig. 6, *E* and *F*) cells latently infected with EBV. Electroporation of FLAG-tagged JDP2 expression vector (FlagJDP2) or its deletion mutant (FlagdJDP2) elicited *JDP2* mRNA levels up to approximately 1,000-fold or more (Fig. 6, *D* and *F*). Expression of the dJDP2 mutant served as a negative control, as it lacks its b-Zip domain, and thus is not functional. In B95-8 cells, *BZLF1* mRNA levels were reduced significantly to about 50% by JDP2 overexpression with TPA and ionophore (Fig. 6*C*). Importantly, *BZLF1* expression patterns in HEK293 EBV/BAC cells with wild-type or the revertant strain (mZII/R) responded in similar ways (Fig. 6*E*) to JDP2 overexpression, whereas the *BZLF1* mRNA levels were not significantly affected by the overexpression in cells with the mZII mutant virus (Fig. 6*E*). These experiments strongly suggest that JDP2 suppresses *BZLF1* expression through the ZII motif in the promoter upon reactivation from latency.

JDP2 Mediates HDAC3 Recruitment to the BZLF1 Promoter—It has been reported that JDP2 recruits HDAC3 to the promoter of target genes and inhibits histone acetyltransferase activity, thereby suppressing transcriptional activity (14, 24). As exhibited previously, exogenously expressed FLAG-tagged JDP2 was able to associate with Zp (Fig. 7*A*, *left*), whereas its b-Zip deletion mutant lost the ability (Fig. 7*A*, *right*). Association of JDP2 with the *BZLF1* promoter correlated with increased HDAC3 recruitment to the promoter (Fig. 7*A*). This HDAC3 recruitment by JDP2 correlated to reduced levels of histone H3 acetylation (Fig. 7*B*). To extend these results, endogenous JDP2 was knocked down by siRNA (Fig. 7*C*). Recruitment of endogenous HDAC3 to Zp was weak although appreciable in the si-control case, whereas treatment with si-JDP2 decreased the HDAC3 interaction with Zp (Fig. 7*C*). The reduction in HDAC3 recruitment by si-JDP2 caused increments in histone H3 acetylation levels (Fig. 7*D*). Furthermore, JDP2 association with HDAC3 was confirmed by immunoprecipitation (Fig. 7*E*). These results suggest that HDAC3 recruitment to Zp, which coincides with lower levels of acetylated histone H3, is efficiently mediated by JDP2 protein.

DISCUSSION

In this report, we document evidence that JDP2 is able to suppress transcription from Zp, thereby inhibiting the entire life cycle in lytic infection. Our results indicate that JDP2 binds to the ZII *cis*-element of the *BZLF1* promoter, which has been reported to interact with b-Zip type transcriptional factors, such as CREB, ATF, and AP-1. Despite the fact that JDP2 represses Zp via the ZII *cis*-element, introduction of a

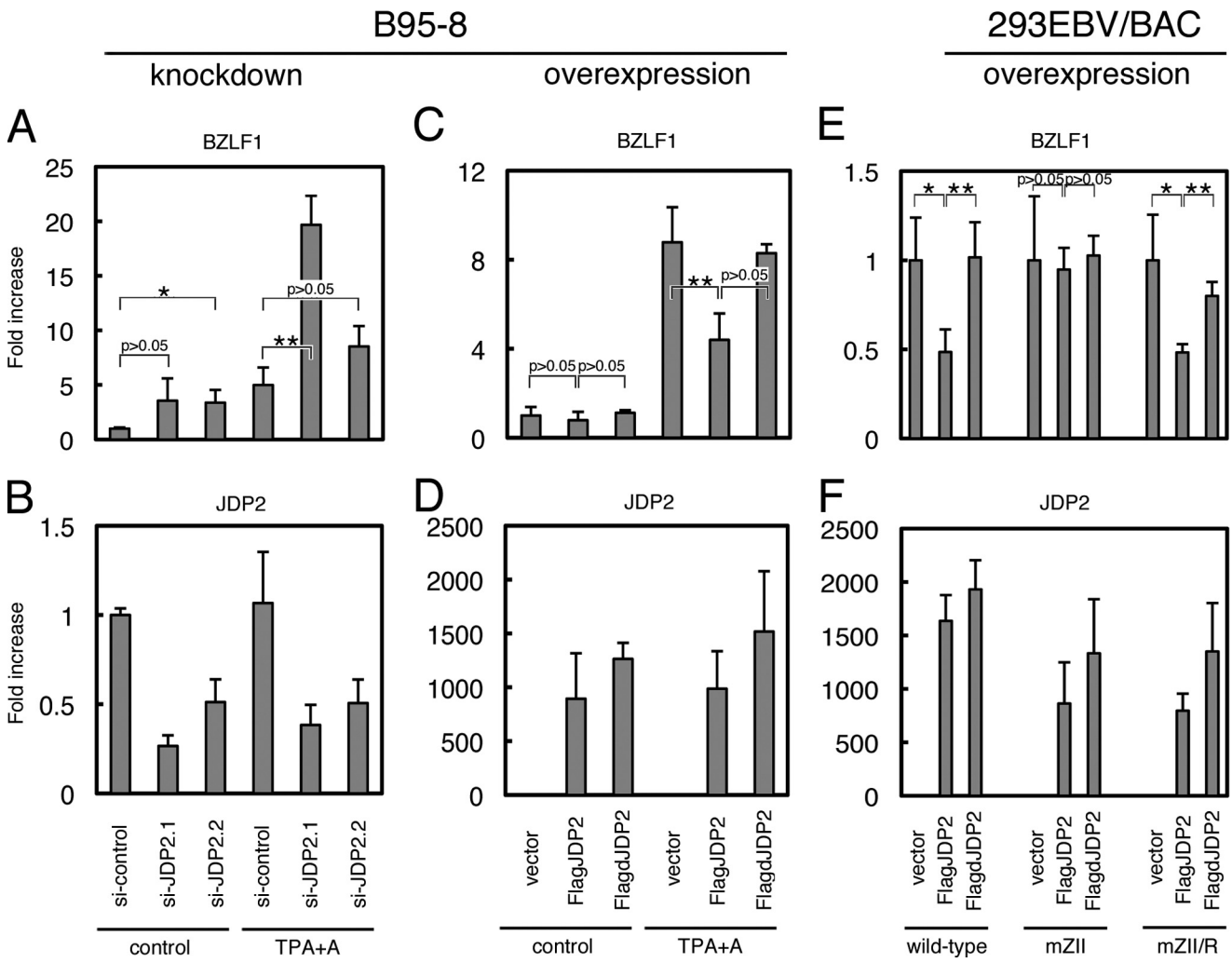

FIGURE 6. **JDP2 inhibits *BZLF1* expression.** *A* and *B*, knockdown of JDP2 enhances *BZLF1* expression. B95-8 cells transfected with siRNA against JDP2 (*si-JDP2.1* or *si-JDP2.2*) or a control (*si-control*) siRNA were cultured for 48 h, and then treated with TPA (20 ng/ml) and A23187 (1 mM) for an additional 12 h. Levels of *BZLF1* (*A*) and *JDP2* (*B*) were checked by real time RT-PCR. *C* and *D*, exogenous overexpression of JDP2 decreases *BZLF1* expression. B95-8 cells transfected with pCMV-FlagJDP2, pCMV-FlagdJDP2, which lacks b-Zip motif of JDP2, or the empty vector were cultured for 24 h, and then treated with TPA (20 ng/ml) and A23187 (1 mM) for an additional 12 h. Levels of *BZLF1* (*C*) and *JDP2* (*D*) were checked by real time RT-PCR. *E* and *F*, exogenous overexpression of JDP2 decreases *BZLF1* expression in HEK293 cells with recombinant EBV/BAC. Cells were transfected with pCMV-FlagJDP2, pCMV-FlagdJDP2, which lacks b-Zip motif of JDP2, or the empty vector. After 36 h, levels of *BZLF1* (*E*) and *JDP2* (*F*) mRNAs were quantified by real time RT-PCR. *BZLF1* and *JDP2* mRNA levels are normalized by *RNA polII* mRNA levels and shown as fold-activation of that for control (*leftmost bar*). * or ** indicates $p < 0.05$ or $p < 0.02$, respectively.

point mutation into the element did not activate BZLF1 expression. Instead, the mutation even reduced the basal expression of BZLF1 (Fig. 4*C*). We speculate that effects of the potential activators such as CREB, ATF, and AP-1 were more conspicuous than that of the negative regulator, JDP2 in the ZII element. Many reports have indicated a significance of the ZII domain for BZLF1 expression. For example, BZLF1 expression is highly leaky in AGS cells infected with EBV, at least partly due to abundant c-Jun and its binding to the ZII element (36). We are confident that JDP2 acts negatively on EBV reactivation, because knockdown of the gene substantially enhanced expression of viral immediate-early, early genes, and viral DNA replication (Fig. 6 and supplemental Fig. S4).

In Fig. 6, a huge amount (1,000-fold or even more of endogenous level of *JDP2*) of forced expression of JDP2 caused only 50% reduction in *BZLF1* mRNA levels. One might argue that this reduction must have been more extensive. We speculate that the ZII motif of the promoter might already be occupied by endogenous b-Zip transcription factors, including JDP2, and thus exogenously supplied JDP2 could not replace effectively. Otherwise, JDP2 might be just one of several proteins that serve to restrict the BZLF1 expression, and the restriction by JDP2 could easily be achieved by the presence of a relatively small amount of the protein.

Because JDP2 and viral BZLF1 are both b-Zip-type transcriptional factors, we tested if they could interact with each other. Immunoprecipitation assays clearly demonstrated that JDP2 and BZLF1 associated through their b-Zip elements (data not shown), and we thus examined if the factors could act cooperatively on the *BZLF1* promoter (Fig. 1). However, we observed no evidence of such cooperation, and the ZIII *cis*-element, the binding motif for BZLF1, did not appear to be involved in the suppression by JDP2 (Fig. 1).

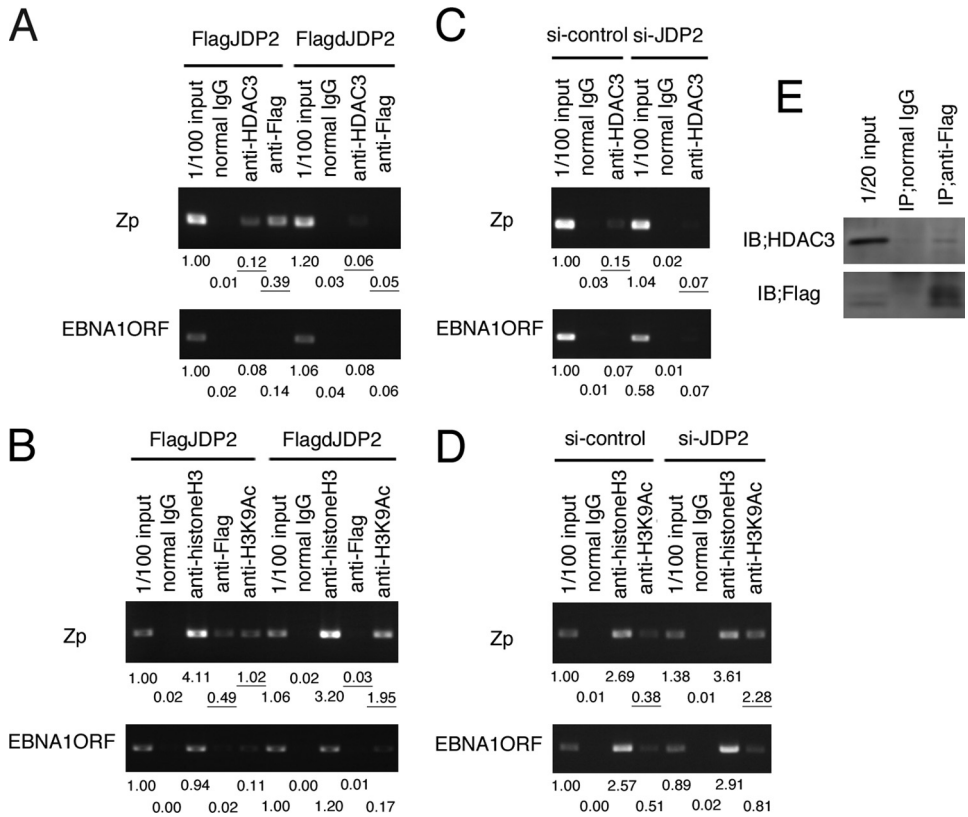

FIGURE 7. **JDP2 mediates HDAC3 recruitment to the *BZLF1* promoter.** *A* and *B,* HEK293 cells with wild-type EBV-BAC DNA were transfected with the FLAG-JDP2 expression vector (*FlagJDP2*) or the expression vector with a deletion in the b-Zip domain (*FlagdJDP2*). After 24 h, ChIP experiments were carried out using cross-linked DNA-protein complexes from the cells, precipitated using normal IgG, anti-HDAC3, anti-FLAG anti-histone H3, or anti-acetylated histone H3K9 antibody, followed by DNA extraction and PCR to detect the Zp. A fragment for the EBNA1 open reading frame was also detected as a control. *C* and *D*, HEK293 cells with wild-type EBV-BAC DNA were transfected with the siRNA against JDP2 (*si-JDP2.1*) or the control (*si-control*). After 48 h, ChIP experiments were carried out using normal IgG, anti-HDAC3 anti-histone H3, or anti-acetylated histone H3K9 antibody, followed by DNA extraction and PCR to detect the Zp and a fragment for the EBNA1 open reading frame as a control. The samples in *A–C* were also subjected to real time PCR analysis for quantification, and the relative values indicated *below* the bands. *E*, JDP2 associates with HDAC3. Proteins from HEK293T cells transfected with FLAG-tagged JDP2 expression vector were subjected to immunoprecipitation using normal IgG or mouse anti-FLAG monoclonal antibody followed by immunoblotting with rabbit anti-HDAC3 (*upper panel*) and rabbit anti-FLAG antibodies (*lower panel*).

Besides JDP2, several factors have been reported to suppress the *BZLF1* promoter. SMUBP-2, a transcription factor first identified through its interaction with the immunoglobulin Smu region, represses Zp activation by TPA (37). ZEB proteins restrict BZLF1 expression by binding to the ZV element of the promoter (38–40). E-box binding proteins, E2-2 (41), and Yin Yang1 (YY-1) (42) have also been implicated. Suppression by JDP2 is distinct from that of these factors, because it relies on binding to the ZII element. Identification and detailed analysis of negative regulators of *BZLF1* transcription should help to understand EBV reactivation mechanisms and provide a whole picture of the virus life cycle.

We found that the CMV promoter responded to JDP2 knockdown differently from the *BZLF1* promoter, although both were similarly suppressed by JDP2 overexpression (data not shown). We speculate that the affinity between the repressor and those promoters might vary. Aronheim's group (26) recently demonstrated that JDP2 activates transcription from TPA responsive element-dependent promoters through association with CHOP10, suggesting that JDP2 can be a transcriptional activator, as well, depending on the context. One possible hypothesis is that the CMV promoter might be regulated both positively and negatively by JDP2, and JDP2 knockdown act in both ways, whereas exogenous overexpression causes emphatic effects on the negative side. The reasons remain to be determined, but it is clear that JDP2 siRNA served, in this report, as the best tool to elucidate its biological roles under physiological conditions.

Knockdown of JDP2 by siRNA in EBV-positive Akata cells increased the levels of early gene expression and viral replication, when the viral lytic program was elicited by anti-IgG treatment (supplemental Fig. S4). Likewise, viral replication was augmented by such silencing in other cell lines, such as B95-8 and GTC-4 cells (data not shown), when treated with TPA and calcium ionophore. Interestingly, in EBV-positive HEK293 cells, knockdown of JDP2 still intensified viral lytic replication even when BZLF1 protein was exogenously supplied (data not shown). This result suggests that JDP2 can act at multiple steps in the lytic life cycle of the virus, and suppression at the *BZLF1* promoter is not the only inhibitory point.

In conclusion, we here have provided evidence that JDP2 is involved in regulation of the latent-lytic switch in EBV infection. Elucidation of associated factors may contribute to development of anti-EBV agents in the future.

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