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Identification and Characterization of CCAAT Enhancer-binding Protein (C/EBP) as a Transcriptional Activator for Epstein-Barr Virus Oncogene Latent Membrane Protein 1^{*[S]}

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Background: Expression mechanism of EBV oncogene *LMP1* is not fully understood.

Results: C/EBP was newly isolated to enhance the *LMP1* promoter in our transient assay system.

Conclusion: C/EBP transactivate the *LMP1* promoter at physiological levels.

Significance: This is the first report that showed the significance of C/EBP on *LMP1* expression.

Epstein-Barr virus *LMP1*, a major oncoprotein expressed in latent infection, is critical for primary B cell transformation, functioning as a TNFR family member by aggregation in the plasma membrane resulting in constitutive activation of cellular signals, such as NF- κ B, MAPK, JAK/STAT, and AKT. Although transcription of *LMP1* in latent type III cells is generally under the control of the viral coactivator EBNA2, little is known about EBNA2-independent *LMP1* expression in type II latency. We thus screened a cDNA library for factors that can activate the *LMP1* promoter in an EBNA2-independent manner, using a reporter assay system. So far, we have screened >20,000 clones, and here identified C/EBP ϵ as a new transcriptional activator. Exogenous expression of C/EBP α , - β , or - ϵ efficiently augmented *LMP1* mRNA and protein levels in EBV-positive cell lines, whereas other members of the C/EBP family exhibited modest or little activity. It has been demonstrated that *LMP1* gene transcription depends on two promoter regions: proximal (*ED-L1*) and distal (*TR-L1*). Interestingly, although we first used the proximal promoter for screening, we found that C/EBP increased transcription from both promoters in latent EBV-positive cells. Mutagenesis in reporter assays and EMSA identified only one functional C/EBP binding site, through which activation of both proximal and distal promoters is mediated. Introduction of point mutations into the identified C/EBP site in EBV-BAC caused reduced *LMP1* transcription from both *LMP1* promoters in epithelial cells. In conclusion, C/EBP is a newly

identified transcriptional activator of the *LMP1* gene, independent of the EBNA2 coactivator.

The Epstein-Barr virus (EBV)³ is a human γ -herpesvirus that mainly infects and establishes latent infection in B lymphocytes, but it also can infect other types of cells, including NK, T, and epithelial cells. Infection of EBV has been implicated in a variety of malignancies, and the expression pattern of viral latent genes varies depending on the tissue of origin and the state of the tumors. Neoplasms such as Burkitt lymphoma or gastric carcinoma express only the *EBER* and *EBNA1* (type I latency), whereas some Hodgkin lymphomas, nasopharyngeal carcinomas (NPC), and NK/T lymphomas produce *EBER*, *EBNA1*, *LMP1* and *LMP2* genes (type II latency). In addition to the type II genes, *EBNA2*, *EBNA3*, and *EBNA-LP* are also expressed in immunosuppression-related lymphomas or lymphoblastoid cell lines (type III latency).

EBV latent infection integral membrane protein 1 (*LMP1*) is frequently expressed in latent EBV infections associated with B cell proliferation and NPC. It is uniformly expressed in latent type III EBV infection with human B lymphocyte proliferation *in vitro*, in resultant lymphoblastoid cell lines, in primary human infection *in vivo*, and in lymphoproliferative disorders in transplant recipients. *LMP1* is also expressed in latent type II EBV infection in Hodgkin disease B lymphocytes and NPC epithelial cells.

Because it functions as a constitutive TNFR family member by aggregation in the plasma membrane, resulting in constitutive activation of cellular signaling, through NF- κ B, MAPK, JAK/STAT, and AKT (1–4), *LMP1* is assumed to be the most major oncogene encoded by EBV.

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³ The abbreviations used are: EBV, Epstein-Barr virus; C/EBP, CCAAT enhancer-binding protein; *LMP1*, latent membrane protein 1; NPC, nasopharyngeal carcinomas; TR, terminal repeats; Fli1, Friend leukemia virus integration 1; CREB, cAMP-response element-binding protein.

Two promoters regulate *LMP1* gene transcription with mechanisms that differ between type II and type III infection. In latency III lymphocyte infection, *LMP1* transcription is turned on by EBNA2 and EBNA-LP from the *ED-L1* promoter (5–7). Although EBNA2 does not feature DNA binding activity, it enhances *LMP1* promoter activity by acting as a cofactor. It associates with cellular transcriptional factors, including RBP-J κ and PU.1, which are then recruited onto the *LMP1* promoter for transactivation. EBNA-LP also associates with the complex and further helps the activation process (8).

On the other hand, *LMP1* is expressed in an EBNA2-independent manner in type II latency, because neither EBNA2 nor EBNA-LP are available in such type II cells. It has been frequently reported that cytokines, such as IL-4, IL-6, IL-10, IL-13, and IL-21, activate the JAK/STAT pathway, thereby inducing *LMP1* gene expression through STAT (9–14). In certain latency II-infected cells including NPC cells, *LMP1* transcription originates from a STAT-regulated upstream promoter, termed *TR-L1*, located within the terminal repeats (TR), in addition to the proximal *ED-L1* promoter (10, 13, 15, 16). Involvement of transcriptional factors, such as ATF/CREB (17), SP1/3 (18), and IRF7 (19) has also been indicated. Despite the presence of these well targeted, focused reports, there is still a possibility of other yet unknown factor(s) that play(s) essential roles in EBNA2-independent *LMP1* expression, because exhaustive investigations have hitherto not been performed.

In the present study, we therefore adopted a comprehensive approach and screened a cDNA library for cellular factors that can activate *LMP1* transcription. We newly cloned the CCAAT enhancer-binding protein (C/EBP) family transcription factor that augments both proximal and distal promoter activation of *LMP1* by binding to a motif in the proximal promoter. A functional C/EBP binding site for the *LMP1* promoter was identified by reporter mutagenesis and EMSA. We also constructed a mutant EBV with a point mutation in the C/EBP binding site, and confirmed the importance of binding for *LMP1* expression in latent cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293T, HeLa-CR2/GFP-EBV, and 293EBV-BAC cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. C666-1, Akata(–), and AGS cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. To prepare HeLa-CR2/GFP-EBV cells, EBV-negative HeLa cells were stably transformed with CR2 (CD21), the receptor for the EBV expression vector, and infected with GFP-EBV (20). AGS-CR2 was prepared by retroviral transduction of the viral receptor CR2 (CD21) into AGS cells. Anti-FLAG, -C/EBP α , and -tubulin antibodies were purchased from Sigma, Cell Signaling, and Santa Cruz, respectively. The anti-LMP1 antibody has been described previously (21). Horseradish peroxidase-linked goat antibodies to mouse/rabbit IgG were from Amer-sham Biosciences.

Library and Plasmids—A SuperScript pre-made cDNA library (from human bone marrow) was purchased from Invitrogen and used for screening after exclusion of clones with junk inserts. Control reporter pCMV-RLuc was reported previously

(22). For pLMP1/ED-L1-FLuc, the *ED-L1* promoter sequence of *LMP1* was amplified from the B95-8 genome using ED-L1pFor and ED-L1pRev primers (supplemental Table S1). The amplified DNA was digested with XhoI and NcoI, and then inserted into the XhoI/NcoI sites of pGL4.10 (Promega). Likewise, luciferase reporter constructs containing various TR sequences were prepared using the following primers: for pLMP1/ED-L1+TR-L1-FLuc, ED-L1p+TR-L1pFor, and ED-L1p+TR-L1pRev, for pLMP1/TR-L1-FLuc, TR-L1pFor, and TR-L1pRev, for pLMP1/TR-L1+BS-FLuc, TR-L1p+BSFor, and TR-L1p+BSRev (supplemental Table S1). Truncated or point-mutated derivatives of the reporter, pLMP1/ED-L1-FLuc, were made by the inverse PCR method using primers shown in supplemental Table S1 (from 417For to 268mtRev). The C/EBP expression vectors were made by inserting cDNA fragments of the proteins into EcoRI/XhoI sites of pcDNA3-FLAG (23). RNA was obtained from Akata or HEK293T cells, and subjected to RT-PCR using the SuperScript III First-strand System (Invitrogen) and KOD DNA polymerase (TOYOBO). Primers used for the PCR are listed in supplemental Table S1.

Transfection, Luciferase Assay, and Immunoblotting—Transfections were carried out by lipofection using Lipofectamine 2000 reagent (Invitrogen) or by electroporation using a Microporator (Digital Bio). The total amounts of plasmid DNAs 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. Immunoblotting was carried out as described previously (23).

Short Hairpin RNA (shRNA) Vector—Knockdown of C/EBP was carried out by the retrovirus shRNA system (24).⁴ Target sequences for the shRNAs are shown in supplemental Table S2.

Electromobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP)—EMSA was carried out as described previously (25). FLAG-tagged C/EBP α and - ϵ 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 the Klenow fragment (TOYOBO) and [³²P]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 MgCl₂, 0.5 mg/ml of poly(dI-dC)) at room temperature for 30 min. The samples were then separated in a 4% nondenaturing polyacrylamide gel in 0.5 \times TBE buffer and radioactivity was visualized using the BAS2500 system (Fuji Film). The sequences of oligonucleotide probes are listed in supplemental Table S3. ChIP assays and real time PCR were carried out as described previously by using anti-C/EBP ϵ antibody (Santa Cruz Biotechnology) (22, 25). Primers used for the real time PCR are indicated in supplemental Table S4.

RT-PCR—Total cell RNA was purified using TriPure Isolation Reagent (Roche Applied Science) and subjected to reverse

⁴ S. Tsuzuki, unpublished data.

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transcription and PCR using the SuperScript III First Strand Synthesis System (Invitrogen) and GoTaq Green Master Mix (Promega). Primers used for the RT-PCR are listed in supplemental Table S5. The PCR products were then subjected to agarose gel electrophoresis for detection.

Genetic Manipulation of EBV-BAC DNA and Cloning of HEK293 Cells with EBV-BAC—EBV-BAC DNA was provided by W. Hammerschmidt (26). Homologous recombination was carried out in *Escherichia coli* as described previously (27).

To prepare a C/EBP binding site 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 Neo/stFor and Neo/stRev primers (supplemental Table S6). After the recombination, kanamycin-resistant colonies were selected and checked to make intermediate DNA. The Neo/st cassette in the intermediate DNA was then replaced using the next transfer vector DNA, containing a mutation in the C/EBP binding site of the *LMP1* promoter. The transfer vector was made by PCR using pLMP1/−268mt-FLuc as the template with the primers listed in supplemental Table S6. Streptomycin-resistant colonies were cloned and checked to make EBV-BAC C/EBP BSmt.

Electroporation of *E. coli* was performed using a 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 promoter region, by electrophoresis of the BamHI-digested viral genome and sequencing analysis.

EBV-BAC DNA was transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen), followed by culture on 10-cm dishes with 100–150 μ g/ml of hygromycin B for 10–15 days for cloning of GFP-positive cell colonies as described previously (27). 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.

RESULTS

Screening of Cellular Factors Transactivating the *LMP1* Promoter—To exhaustively search for cellular factor(s) that enhance(s) *LMP1* transcription, we screened a human bone marrow cDNA expression library for the ability to enhance the promoter activity, using reporter assay systems. To this end, we cloned proximal *LMP1* promoter (*ED-L1*) into the promoterless firefly luciferase vector (pGL4.10) to make pLMP1/ED-L1-FLuc. As a control, pCMV-RLuc, featuring the *CMV IE* promoter upstream of the *Renilla* luciferase gene, was used to normalize for transfection efficiency. An example of our screen is shown under supplemental Fig. S1. To maximize the number of the cDNAs that could be assayed while assuring that any positive clone would not be missed, we generated cDNA pools with 10 cDNAs per pool. Each cDNA pool was transfected into HEK293T cells together with pLMP1/ED-L1-FLuc and pCMV-RLuc. A pool was considered positive when the pLMP1/ED-L1-FLuc reporter was activated 2-fold or more, as compared with the control pCMV-RLuc. Then, the positive pool was re-cloned and assayed again to single out the positive clone, followed by

sequencing. So far, we have screened more than 2,000 pools, which means 20,000 clones, and after pseudo positives were excluded, we identified at least 9 clones as possible positive regulators of the *LMP1* promoter. All of the hits cloned in the screen turned out to be transcription factors. Among them, we found that Ets domain family transcription factors were frequently isolated: two clones of the hits encode Friend leukemia virus integration 1 (*FLI1*), and four clones encode *PU.1*, also known as spleen forming virus proviral integration 1 (*SP11*). Exogenous expression of *FLI1* or *PU.1* elicited *LMP1* promoter activity about 40–50- or 3–5-fold, respectively, in the reporter assays. Likewise, one clone of SP3 was isolated that activated the promoter about 4–8-fold. Because Ets family transcription factor PU.1 (5, 6) and SP1/3 (18) have been reported to bind and activate the proximal *LMP1* promoter, we assume credibility of our screen system was proven. Last, we identified one new clone of C/EBP ϵ , which encodes a b-Zip type transcriptional factor, as a *LMP1* transcriptional activator. Although CREB/ATF, members of the b-Zip transcriptional factors, are reported to activate the *ED-L1* proximal *LMP1* promoter (17), we assumed C/EBP to act in a different mode from, because the DNA binding consensus sequence of C/EBP (28, 29) is quite distinct from CREB/ATF. Therefore, we decided to further analyze molecular mechanisms underlying the activation.

C/EBP α , β , and ϵ Efficiently Transactivate the *LMP1* Promoter—After newly identifying the transcriptional factor C/EBP ϵ as an activator, we tested if other members of the C/EBP family could also function as transcriptional activators, using a reporter assay system (Fig. 1A). C/EBP α or ϵ transactivated the promoter relatively efficiently, C/EBP β had a moderate effect, whereas others had little effects. Only one negative regulator of the family, C/EBP γ , which lacks an activation domain and therefore represses gene transcription by forming inactive heterodimers with other members (30), actually reduced the transcription as expected.

We then transfected the C/EBP expression vectors into an EBV-positive cell line, HeLa-CR2/GFP-EBV, to check the effects (Fig. 1B). This cell line was prepared by infecting HeLa cells stably expressing CR2 (CD21), the cell surface receptor of the virus, with EBV. We here used the cells for two reasons: first, the EBV in this cell line features type II latency, in which *LMP1* is produced in the EBNA2-independent manner, and second, transfection efficiency is very high and thus easy to handle when compared with other cells featuring type II EBV latency. When C/EBP α or ϵ were exogenously expressed, increased levels of the *LMP1* protein were readily detected by immunoblotting (Fig. 1B), whereas other members did not appreciably increase the *LMP1* levels. We also tested C666-1, a nasopharyngeal carcinoma cell line naturally infected with EBV (Fig. 1C). Expression of C/EBP α increased the *LMP1* mRNA level. In AGS-CR2/GFP-EBV-Bac cells, *LMP1* was increased by the exogenous supply of not only C/EBP α and ϵ , but also β (supplemental Fig. S2).

Identification of a C/EBP Binding Site in the *LMP1* Promoter—Because we confirmed the C/EBP potentiating effect of *LMP1* transcription, we then carried out truncation and mutagenesis analysis of the promoter region to identify any *cis*-element that might be responsible for the activation. We first prepared

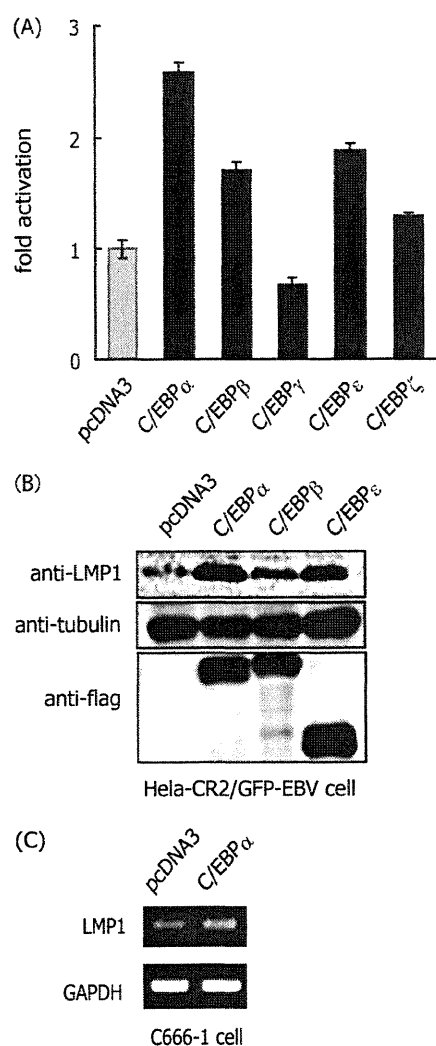


FIGURE 1. C/EBP efficiently transactivates the LMP1 promoter. A, C/EBP α and ϵ augmented LMP1 promoter function in reporter assays. HEK293T cells were transfected with 10 ng of reporter plasmid pLMP1/ED-L1-FLuc, 1 ng of control pCMV-RLuc, and 100 ng of indicated C/EBP family expression vector or the empty vector (pcDNA3). Luciferase assays were carried out after 1 day as described under "Experimental Procedures." The firefly luciferase activity was normalized to Renilla luciferase activity and shown as mean fold-activation of that with the control vector (pcDNA3) and S.D. B, potentiation of LMP1 levels by ectopic expression of C/EBP α and ϵ in HeLa cells latently infected with EBV. HeLa-CR2/GFP-EBV cells were transfected with empty vector (pcDNA3) or the indicated C/EBP family expression vector. After 60 h, cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -tubulin, and -FLAG antibodies. C, C/EBP α transactivated LMP1 levels in a nasopharyngeal carcinoma cell line. C666-1 cells were transfected with empty vector or C/EBP α expression vector. After 48 h, cell RNAs were collected and subjected to RT-PCR.

reporter vectors in which the promoter sequences were gradually deleted as shown in Fig. 2A. Although truncation of the sequence to -320 relative to the transcription start site (+1) did not impair the promoter response to C/EBP α , severing the sequence between nucleotides -320 and -229 markedly diminished the response (Fig. 2B), implying the presence of the responsible motif(s) between -320 and -229 . We thus searched this region for sequences conforming to the consensus C/EBP binding motif, RTTGCGCYAAY, where R indicates A or G, and Y indicates C or T (28), and found three such

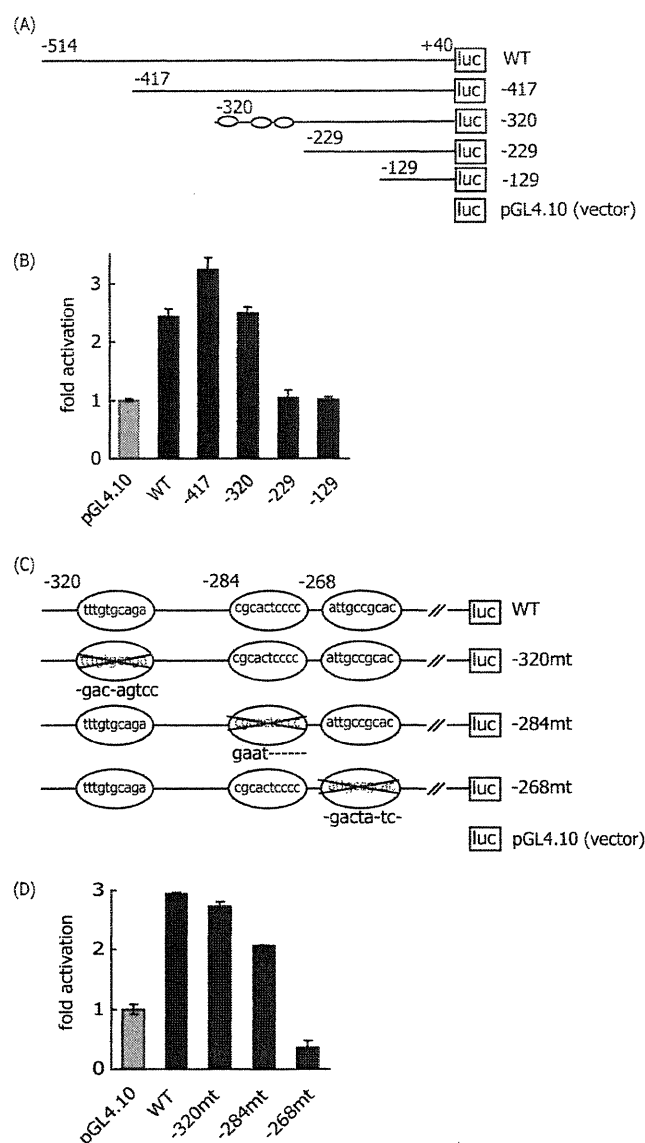


FIGURE 2. Identification of the sequence motif responsible for activation of the LMP1 ED-L1 promoter by C/EBP α . A, schematic representation of reporter constructs with truncated LMP1 ED-L1 promoter sequences. Possible C/EBP binding sites between -320 and -229 are ringed. B, the C/EBP α expression plasmid or its empty vector were cotransfected into HEK293T cells with the truncated reporter plasmid in A and pCMV-RLuc. Luciferase assays were carried out after 1 day as described under "Experimental Procedures." The firefly luciferase activity was normalized to Renilla luciferase activity. Bars indicate averages of the fold-activation on transfection of C/EBP α , compared with those with the empty vector, and S.D., for each reporter. C, schematic representation of the mutated derivatives of pLMP1/ED-L1-FLuc. Possible C/EBP binding sites between -320 and -229 are ringed. The putative C/EBP binding motifs were replaced with the sequences below. D, C/EBP α expression plasmid or its empty vector were cotransfected into HEK293T cells with the mutated reporter plasmid in C and pCMV-RLuc. Luciferase assays were carried out after 1 day as described under "Experimental Procedures." The firefly luciferase activity was normalized to Renilla luciferase activity. Bars indicate averages of the fold-activation by transfection of C/EBP α , compared with those with empty vector, and S.D., for each reporter. The numbers indicate nucleotide positions relative to the transcription start site (+1).

possible motifs as shown in Fig. 2C. We named the possible binding motifs as -320 , -284 , and -268 , according to their positions, and introduced point mutations into each as shown in Fig. 2C to determine which might be functional. Luciferase

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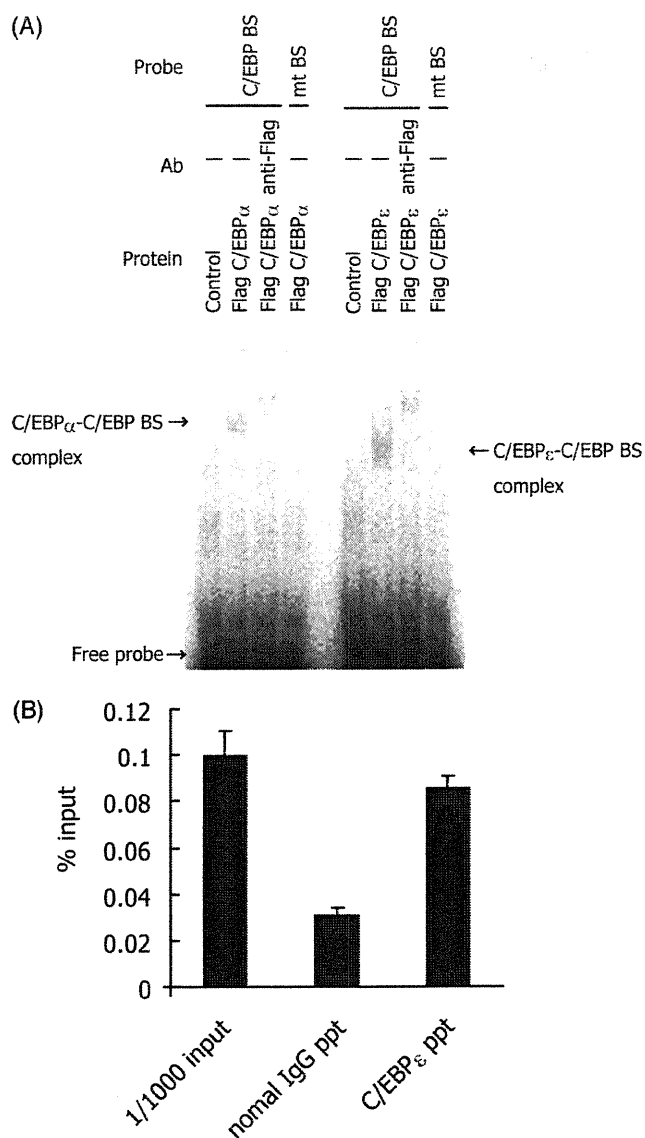


FIGURE 3. Binding of C/EBP α and ϵ to the binding site in the LMP1 ED-L1 promoter. A, EMSA was carried out as described under "Experimental Procedures." FLAG-tagged C/EBP α (left panel) or ϵ (right panel) were produced *in vitro* and incubated with 32 P-labeled wild-type (C/EBP BS) or point-mutated (mtBS) probe. Supershift analysis was performed using mouse anti-FLAG monoclonal antibodies. The samples were then separated in a 4% polyacrylamide gel and analyzed with Fuji Image Analyzer BAS2500. B, binding of endogenous C/EBP ϵ to LMP1 promoter. AGS-CR2/GFP-EBV-Bac cells, latently infected with EBV, were subjected to ChIP assays using anti-C/EBP ϵ antibody (Santa Cruz), followed by real time PCR analysis for quantification.

assays revealed that C/EBP α -mediated transactivation was severely attenuated when the putative motif at -268 was mutated, whereas replacement of the other two possible motifs did not cause any defect (Fig. 2D). These results suggest that the ATTGCCGCAC motif at the -268 of ED-L1 promoter is the *cis*-element responsible for the response to C/EBP.

We then used EMSA to examine whether the C/EBP protein could actually bind to the ATTGCCGCAC motif at -268 in the ED-L1 promoter (Fig. 3A). Addition of FLAG-tagged C/EBP α or ϵ produced a specific band for the C/EBP-nucleotide complex when the wild-type C/EBP binding site at the -268 of

ED-L1 (C/EBP BS) sequence was used, whereas this failed to be produced with mtC/EBP BS, the mutated sequence. Supershift analysis with anti-FLAG antibody demonstrated that the band actually contained FLAG-tagged C/EBP protein. Therefore, C/EBP binds to the ATTGCCGCAC motif in question.

We also tried to detect binding of endogenous C/EBP to the LMP1 promoter. In AGS-CR2/GFP-EBV-Bac cells, C/EBP ϵ was detected on the promoter sequence (Fig. 3B), although C/EBP α was undetectable (not shown). Because the amount of C/EBP α is very low in the cell line, we speculate that the ChIP result simply reflects the expression level of the family member.

Previous reports demonstrated that a distal promoter, termed TR-L1, located within the TR of the viral genome, is also activated in addition to the proximal ED-L1 promoter in certain cell types with EBNA2-independent LMP1 expression (10, 13, 15, 16), we next examined, by RT-PCR, if C/EBP might affect the TR-L1 promoter, too. An antisense primer was designed to jump the first intron of the LMP1 gene (Fig. 4A, primer 3), so that the possibility of genomic contamination could be ignored, and one sense primer was set within the first exon (Fig. 4A, primer 1) and another sense set well upstream of the transcription start site (+1) of the ED-L1 promoter (Fig. 4A, primer 2). The result of the RT-PCR (Fig. 4B) indicated that C/EBP α markedly enhanced transcription from the TR-L1 promoter. It is not clear, from this result, whether the ED-L1 promoter is also activated or not.

Although we already identified the *cis*-element responsible for activation of the proximal ED-L1 promoter (Fig. 2), we then searched to find the *cis*-element that is crucial for the activation of the distal promoter, because the TR-L1 promoter of the LMP1 gene was markedly activated by C/EBP α (Fig. 4). We first prepared a firefly luciferase reporter construct by inserting the TR-L1 promoter (nucleotide -1115 to -544 , Fig. 5A, TR). Curiously, this reporter did not respond to exogenous expression of C/EBP α (Fig. 5B, TR), suggesting that a functional *cis*-element responsible for activation of the TR-L1 promoter does not exist in the sequence between nucleotides -1115 and -544 . Therefore, speculating that the C/EBP binding site located within the ED-L1 promoter might act to influence the TR-L1 promoter activity from downstream, the promoter sequence in the reporter construct was extended to -147 , to cover the C/EBP motif (Fig. 5A, TR+BS). Although this reporter contains part of the ED-L1 promoter, transcription from ED-L1 should not initiate because it does not contain the transcription start site (+1) of the ED-L1 promoter. As shown in Fig. 5B (TR+BS), the vector did respond to C/EBP α , and introduction of a point mutation at the C/EBP BS depressed the response (Fig. 5C, TR+BSmt). In addition, a reporter containing the TR-L1 and complete ED-L1 promoters (Fig. 5A, TR+ED) acted in a similar manner (Fig. 5D, TR+ED and TR+EDmt). These results suggest that activation of both the TR-L1 and ED-L1 promoters by C/EBP is mediated through the single C/EBP binding site in the ED-L1 promoter.

Mutation in the C/EBP Binding Site Attenuated Activity of Both LMP1 Promoters in the Context of the Viral Genome—Experiments so far have indicated there is one functional C/EBP binding site in the ED-L1 promoter through which activation of both ED-L1 and TR-L1 promoters is mediated. To further

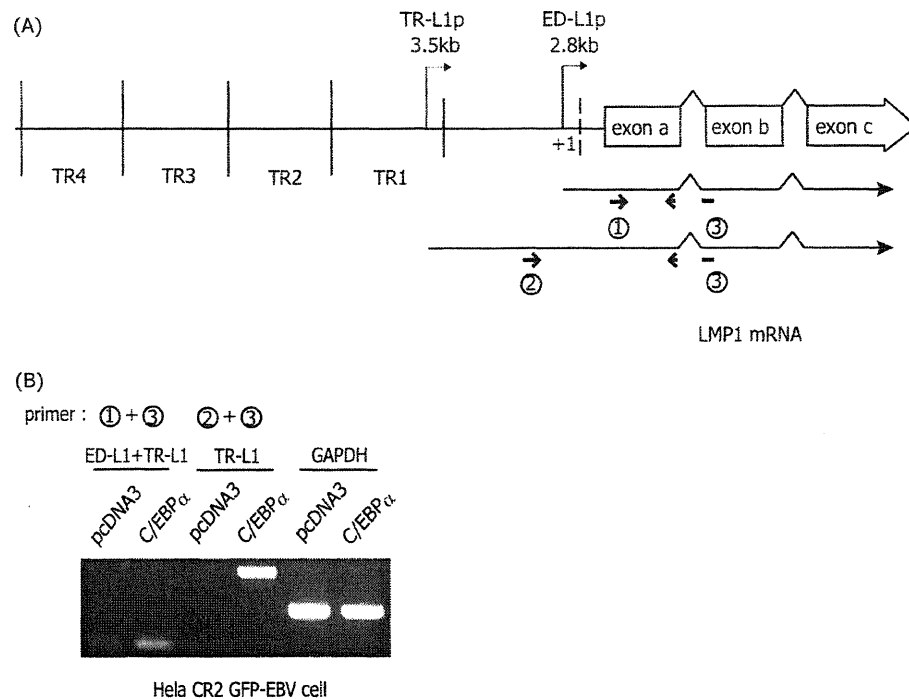


FIGURE 4. Activation of the *LMP1* TR-L1 promoter by C/EBPα in cells latently infected with EBV. *A*, schematic representation of the regulatory sequence of the *LMP1* gene. The 2.8- and 3.5-kb *LMP1* mRNAs and the primers used for RT-PCR in *B* are depicted. *B*, HeLa-CR2/GFP-EBV cells were transfected with empty vector (pcDNA3) or the indicated C/EBPα expression vector. After 60 h, cell RNAs were harvested and subjected to RT-PCR using the primers indicated above.

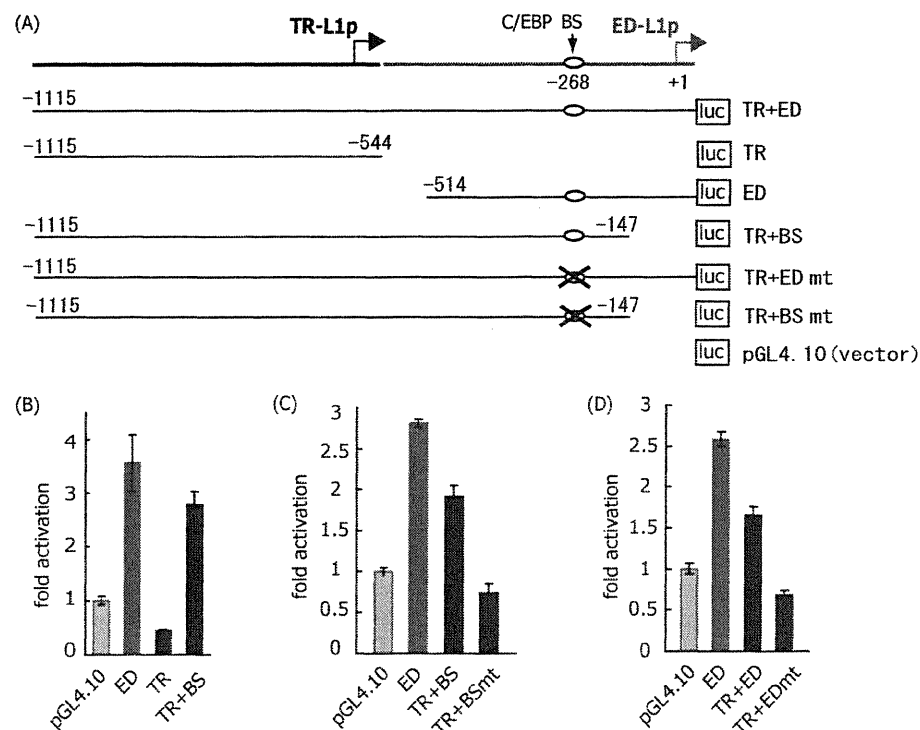


FIGURE 5. Identification of the sequence motif responsible for activation of the *LMP1* TR-L1 promoter by C/EBPα. *A*, schematic representation of reporter constructs with truncated and/or mutated *LMP1* promoter sequences. Identified C/EBP binding sites in the *ED-L1* promoter are ringed. *B*, the C/EBPα expression plasmid or its empty vector were cotransfected into HEK293T cells with the mutated reporter plasmid in *A* and pCMV-RLuc. Luciferase assays were carried out after 1 day as described under "Experimental Procedures." Firefly luciferase activity was normalized to *Renilla* luciferase activity. Bars indicate averages of fold-activation by transfection of C/EBPα, compared with those with the empty vector, and S.D., for each reporter. The numbers in the figure indicate nucleotide positions relative to the transcription start site (+1).

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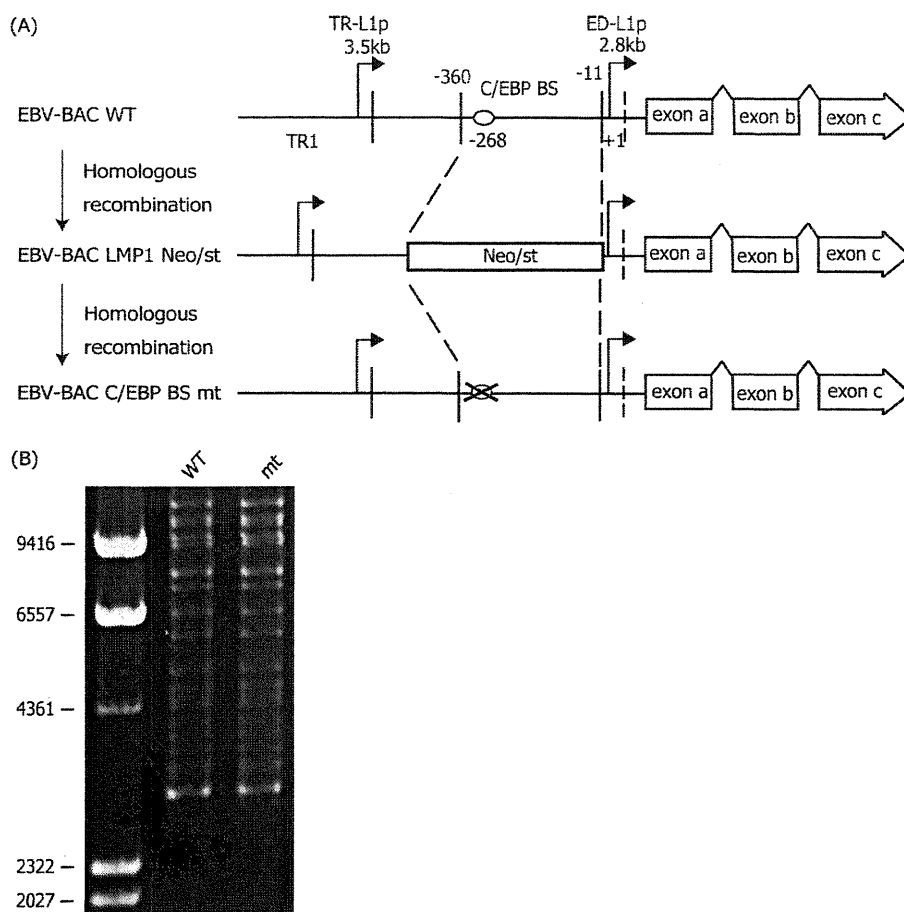


FIGURE 6. Construction of a recombinant EBV featuring point mutation in the C/EBP binding site of the LMP1 promoter. A, schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (*Neo/st*). The sequences around the C/EBP binding site (C/EBP BS, ringed) of the B95-8 LMP1 promoter (–360 to –11) were first replaced with the *Neo/st* cassette, which was then replaced with a point mutated C/EBP BS sequence (ringed X) to construct EBV-BAC C/EBP BS mt. B, electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel.

extend and verify the findings, recombinant EBV with a point mutation at the identified C/EBP binding site was prepared. As shown in Fig. 6A, part of the LMP1 *ED-L1* promoter sequence (–360 to –11), containing the C/EBP binding site (C/EBP BS, ringed in Fig. 6A), was first replaced with the marker cassette (*Neo/st*), and then this was exchanged with the mutated C/EBP binding site (C/EBP BSmt) sequence, to prepare EBV-BAC C/EBP BSmt. Sequencing analysis confirmed that the EBV-BAC C/EBP BSmt DNA had the same mutation as the pLMP1/–268mt-FLuc vector (Fig. 2C), 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. 6B). Recombinant EBV-BAC DNA was introduced into a virus-producing cell line, HEK293, 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 in the presence and absence of BZLF1 inductions were examined. The recombinant virus was then infected into AGS-CR2, expressing the cellular receptor for EBV, CR2 (CD21).

Protein levels were examined in the AGS cells, latently infected with wild-type or mutated EBV (Fig. 7A). Produc-

tion of the LMP1 protein in the AGS cells with virus carrying the point mutation at the C/EBP binding site (Fig. 7A, *mt*) was obviously lower than in the wild-type. The AGS cells expressed little or no EBNA2, in contrast to the lymphoblastoid cell line (Fig. 7A), indicating that the virus established type II latency in the cells (31). Promoter usage patterns were then checked by RT-PCR using the specific primers used for Fig. 4. Transcription from the *TR-L1* promoter was remarkably restricted with the mutant (Fig. 7B), although the effect of the mutation on the *ED-L1* promoter was not distinguishable from the data. We also checked that EBNA1 levels were comparable (Fig. 7, A and B).

Next, the effects of C/EBP exogenous expression were analyzed in cells carrying recombinant viruses. In AGS cells latently infected with wild-type EBV, intrinsic LMP1 protein was present and ectopic supply of C/EBP α caused a prominent increase in LMP1 protein levels (Fig. 7C). On the other hand, in cells with mutant EBV, the intrinsic LMP1 protein level was low and C/EBP α expression did not induce an increase (Fig. 7C). RT-PCR analysis clearly showed that transcriptional activation of the LMP1 gene by C/EBP α in wild-type, at least for the *TR-L1* promoter, was diminished in the mutant (Fig. 7D), indicating significance for the motif.

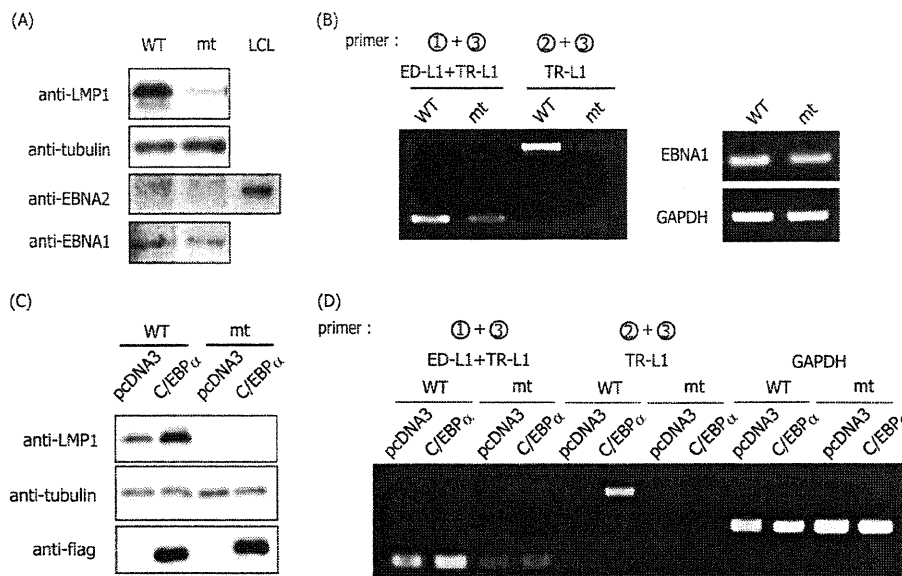


FIGURE 7. Decrease in LMP1 protein and mRNA levels with point mutation of the C/EBP binding site of the EBV-BAC LMP1 promoter. A, LMP1 protein levels in AGS cells latently infected with wild-type or point-mutated EBV-BAC. Immunoblotting was performed using anti-LMP1, -tubulin, and -EBNA2 antibodies. Proteins from the lymphoblastoid cell line were also included as a positive control for EBNA2. B, both *TR-L1* and *ED-L1* promoters were attenuated by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. RNA was collected from AGS cells latently infected with wild-type or point-mutated EBV-BAC, and subjected to RT-PCR using the primers shown in Fig. 5A. *EBNA1* and *GAPDH* levels were also checked. C, response to ectopic expression of C/EBPα was diminished by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. AGS cells latently infected with wild-type or point-mutated EBV-BAC were transfected with the C/EBPα expression vector or its empty vector (*pcDNA3*). After 60 h, cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -tubulin, and -FLAG antibodies. D, responses of both *TR-L1* and *ED-L1* promoters to ectopic expression of C/EBPα were diminished by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. AGS cells latently infected with wild-type or point-mutated EBV-BAC were transfected with C/EBPα expression vector or its empty vector (*pcDNA3*). After 60 h, RNA was collected and subjected to RT-PCR using the primers shown in Fig. 4A.

Knockdown of C/EBP Reduced LMP1 Levels—Last, we tested the effect of endogenous C/EBP proteins on LMP1 expression levels. To this end, α or ε members of the C/EBP family were ablated by shRNA technology. In HeLa-CR2/GFP-EBV cells, knockdown of either C/EBPα or -ε significantly restricted the amount of LMP1 (Fig. 8A and supplemental Fig. S3). In AGS-CR2/GFP-EBV-Bac cells, we tested knockdown of C/EBPε. Because levels of endogenous C/EBPα in the cells were very low, knockdown of C/EBPα was not done. Treatment of shC/EBPε also caused reduction of LMP1 protein in AGS cells (Fig. 8B). These results indicate that C/EBP proteins are involved in LMP1 production, and suggest that the effect is dependent on cell types.

DISCUSSION

The results documented here show clear involvement of C/EBP proteins in up-regulation of the *LMP1* gene. Initially, the C/EBPε protein was identified by our screening to increase the proximal *LMP1* (*ED-L1*) promoter activity. We are confident in the screening system, because factors like SP3- and Ets-type transcription factors, both of which have been implicated in the transcriptional regulation of *LMP1*, were isolated in the screen. Regarding Ets transcription factors, PU.1 has been reported to recruit the viral transcriptional activator EBNA2 and thereby enhance *LMP1* *ED-L1* promoter activity (5, 6), but it is understandable that PU.1 up-regulated transcription even without EBNA2 in the screening experiment, because PU.1 can functionally interact with basic transcriptional regulators, like CBP, TFIID, or TBP, or other transcription factors, like GATA or RUNX (32, 33). Another Ets family transcription factor FLI1

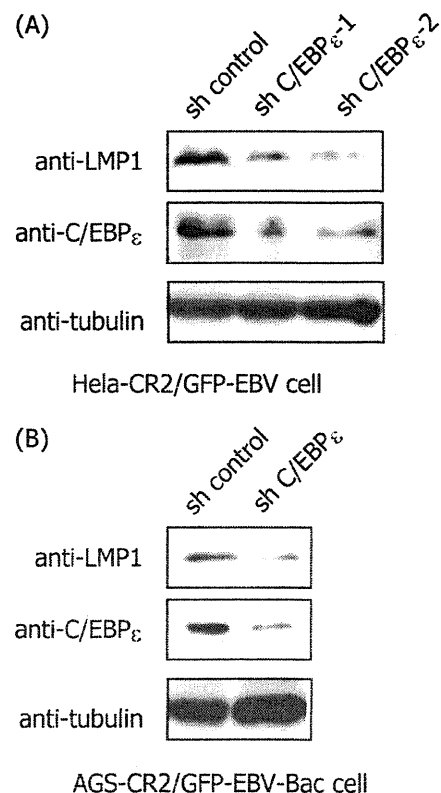


FIGURE 8. Knockdown of C/EBPε decreased levels of LMP1. HeLa-CR2/GFP-EBV (A) and AGS-CR2/GFP-EBV-Bac (B) cells were treated with control shRNA or shRNA for the C/EBPε. Cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -C/EBPε, and -tubulin antibodies.

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was also identified in our screening. Interestingly, whereas FLI1 markedly elicited promoter activity (about 40–50-fold) in the reporter assays, exogenous expression of FLI1 did not cause an increase in the levels of LMP1 protein in EBV-positive cells (data not shown). Likewise, PU.1 also did not significantly augment the LMP1 protein levels (data not shown). On the other hand, whereas the increment of the reporter activity by C/EBP ϵ was not very high (only 2–4-fold), exogenous expression of the gene clearly increased the mRNA and protein levels of LMP1 in EBV-positive cells (Figs. 1, B and C, 4, and supplemental Fig. S2). Therefore, we must conclude that transient reporter assays do not always reflect the actual promoter activity in the context of viral genome. The reason why overexpression of the Ets family transcription factors fail to increase the LMP1 protein levels in the context of infection is not clear. We speculate either that the promoter might already be occupied with certain Ets family proteins, or that the ability of the FLI1 or PU.1 to enhance the promoter activity might not be sufficiently strong as to counter epigenetic suppression of the gene but high enough for reporter assays.

Subsequent analyses demonstrated that C/EBP enhanced the distal *TR-L1* promoter of *LMP1*, and that the activation was mediated through one C/EBP binding motif in the proximal *ED-L1* promoter (supplemental Fig. S4). Therefore, the distal *TR-L1* promoter is activated by C/EBP binding downstream of the transcription start site of the *TR-L1* promoter. Because activation of a particular promoter by transcription factor binding downstream of the transcription start site has been demonstrated previously for various promoters (34, 35), we assume the activation of the *TR-L1* promoter by downstream C/EBP binding is reasonable when considered in light of our clear results of reporter assays and the point-mutated virus also. Although we obtained a substantial amount of evidence that C/EBP enhanced the distal *TR-L1* promoter of *LMP1*, activation of the proximal *ED-L1* promoter was checked only by the reporter assays. We were not able to confirm this, because PCR primers that detect only the *ED-L1* promoter could not be designed.

Previous studies have repeatedly demonstrated that cytokines such as IL-4, IL-6, IL-10, and IL-21 mediate *LMP1* gene expression in the absence of the viral coactivator EBNA2, namely in type II latent cells such as B cells (10–12, 36), NK/T lymphomas (11), epithelial HeLa cells, and the NPC-derived cell line CNE2 (14). More amazingly, expression patterns of EBV proteins even in type I or III cells could be modulated by cytokines to resemble those in type II latency (10–12, 36). These reports all showed engagement of the JAK/STAT signaling pathway in the process of induction of *LMP1* expression. We suggest new information on the involvement of the C/EBP family, which has been implicated in various physiological phenomena, such as differentiation, inflammation, and cell growth. In terms of inflammation, C/EBP proteins have been implicated in induction of a number of cytokines (37, 38). For example, C/EBP plays a role in transcriptional induction of certain genes by IL-6 (39). IL-10 activates expression of C/EBP and thereby activates transcription of IL-6 in epithelial cells (40). Therefore, it is strongly suggested that cytokines activate the *LMP1* promoter through C/EBP, besides JAK/STAT signaling. In addition to cytokine-induced expression of *LMP1*, it seems likely

that C/EBP contributes to produce *LMP1* even in the absence of cytokines, because expression of *LMP1* in the AGS cell line with recombinant EBV mutated at the C/EBP binding site was notably more subdued than with the wild-type virus (Fig. 7, A and B).

To summarize, we could successfully identify as a new factor C/EBP as the transcriptional activator of the major oncogene of EBV, *LMP1*, and made an initial characterization of the molecular mechanisms of how *LMP1* expression is reinforced by the transcription factor in an EBNA2-independent manner. Because *LMP1* is the major oncogene of EBV, suppression of *LMP1* gene expression by inhibiting the C/EBP family may provide potential targets of therapeutic drugs for EBV-positive cancers, especially for type II cancers, such as NK/T lymphomas, NPC, and Hodgkin lymphomas. Search for small molecules that inhibit *LMP1* expression is already under way.

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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 (ZIIIA 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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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S4.

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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.

JDP2 Suppresses EBV Reactivation

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 (pcDNAΔBZLF1), 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'-CATGGACTACAA-GGACGACGATGACAAGATGGTGGTGGTGGCAGCCG-C-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'-CGCCCCACCTGCATC-GTCC-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 [³²P]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 MgCl₂, 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'-CCCAAACCATGACATCAC-AGAG-3' and 5'-CTCCTCTGTGATGTCATGGTTTGGG-3'; ZIIIA, 5'-AACTATGCATGAGCCACAGG-3' and 5'-ATGCCTGTGGCTCATGCATAGTT-3'; ZIIIB, 5'-CACAG-GCATTGCTAATGT-3' and 5'-GAGGTACATTAGCAA-TGCCTGTG-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'-GUGAG-CUAGAUGAGGAAGAdTdT-3' and 5'-UCUCCUCAUCU-AGCUCACdTdT-3' (si-JDP2.1) and 5'-GCACAUACUCAC-CGAAUGUdTdT-3' and 5'-ACAUUCGGUGAGUAUG-UGCdTdT-3' (si-JDP2.2), respectively. The control siRNA sequences were 5'-GCAGAGCUGGUUAGUGAAdTdT-3' and 5'-UUCACUAAACCAGCUCUGCdTdT-3'. Akata or B95-8 cells (1 × 10⁵) 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'-GGC-ACATCTGCTTCAACAGG-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'-AGCCACAGGCATTGCTAATGTACCTCATAGACACACCTAAATTTAGCACGTCCCAACCAGGCCTGGTGATGATGGCGGGATC-3' and 5'-GAGTTACCTGTCTAACATCTCCCCTTTAAAGCCAAGGCACCAGCCTCCTCTGTGATGTCATCAGAAGAACTCGTCAAGAAGG-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'-GTTTGGGTCCATCATCTTCAG-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 pre-cleared 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. 1A, 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. 1A). Another siRNA (si-JDP2.2) also resulted in increased Zp activity (Fig. 1A). Interest-

JDP2 Suppresses EBV Reactivation

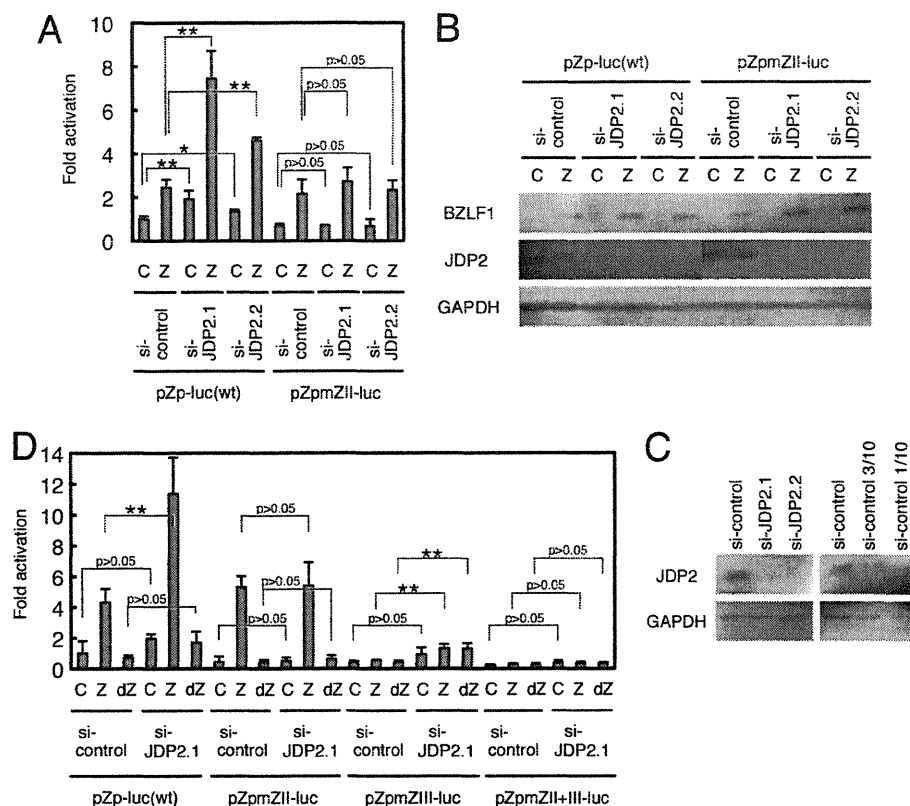


FIGURE 1. Knockdown of endogenous JDP2 enhances transcription from Zp. A–C, HEK293T cells were transfected with duplexes of a 21-nucleotide siRNA against JDP2 (si-JDP2.1 or si-JDP2.2) or a control (si-control) siRNA, together with 10 ng of pZp-luc or its derivative, pZpmZII-luc, and 1 ng of pCMV-RL with 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 \pm 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 pcDNABZLF1 (dZ). pcDNABZLF1 (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. 1A). Knockdown of the JDP2 protein was checked by immunoblotting (Fig. 1B). 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. 1B). 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. 1C). Either of the siRNAs reduced the JDP2 protein levels to $\sim 30\%$ 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. 1D) as expected, but its promoter activity was increased by si-JDP2 treatment (Fig. 1D, 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 (FlagJDP2, 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

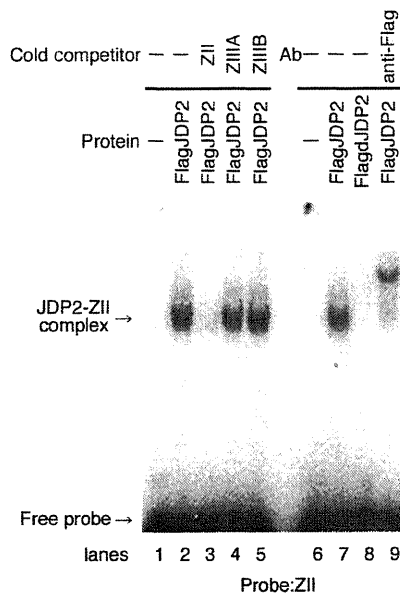


FIGURE 2. JDP2 binds to ZII motif in the *BZLF1* promoter *in vitro*. EMSA was carried out as described under "Experimental Procedures." FLAG-tagged wild-type JDP2 protein (FlagJDP2) or JDP2 protein lacking b-Zip domain (FlagdJDP2, lane 8) was produced *in vitro* and incubated with 32 P-labeled probe and excessive amounts of cold competitors (ZII (lane 3), ZIIA (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.

in the association between JDP2 and the *BZLF1* promoter. The *BZLF1* promoter region was obviously co-precipitated with FLAG-tagged JDP2 (supplemental Fig. S1A), 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. 4A). 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. 4B). 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

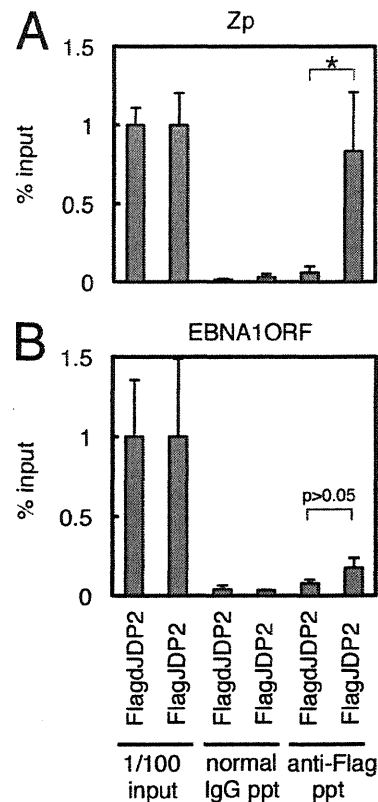


FIGURE 3. Association of JDP2 with the *BZLF1* promoter. HEK293 cells with *BZLF1* knock-out (BZLF1KO) EBV-BAC DNA were transfected with the FlagJDP2 expression vector (FlagJDP2), or the FlagJDP2 expression vector with a deletion in the b-Zip domain (FlagdJDP2). After 24 h, ChIP experiments were carried 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 $p < 0.05$.

than 10 cell colonies from each recombinant virus were obtained and viral protein expression levels were examined. Fig. 4C 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. S1B 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. S1B, right panels). Taken together, the results indicate that JDP2 is

JDP2 Suppresses EBV Reactivation

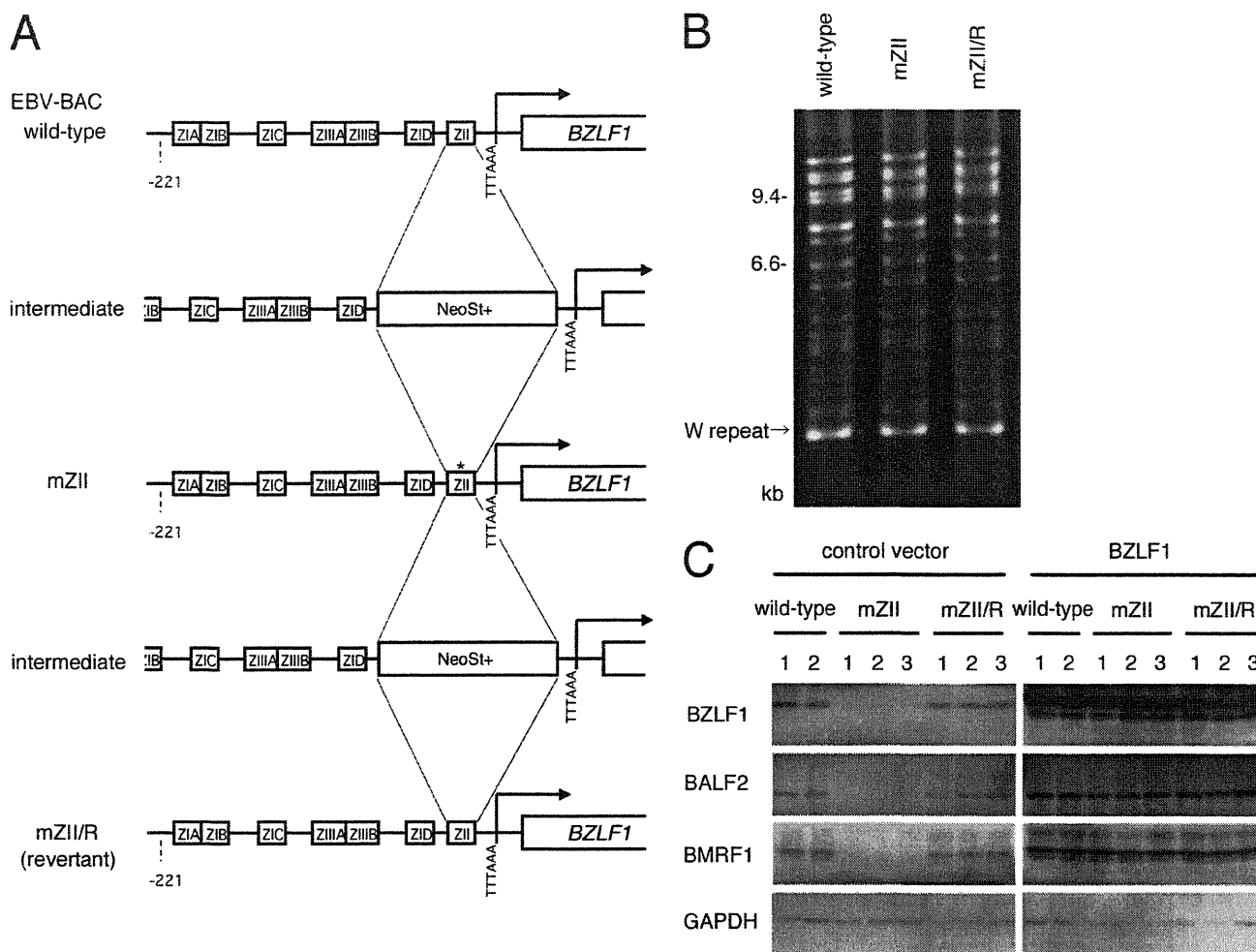


FIGURE 4. Construction of recombinant EBV featuring point mutation in the ZII cis-element of the *BZLF1* promoter. **A**, schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin resistance and streptomycin sensitivity genes (*NeoSt+*). The ZII element of the 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. 5A). Immunoblotting data indicated the expression level of FLAG-CREB was not affected by si-JDP2 (Fig. 5C). 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

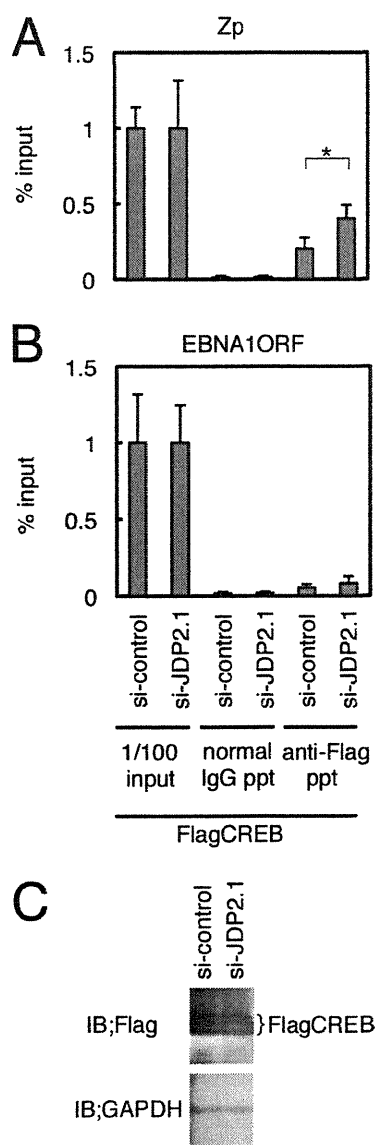


FIGURE 5. Competitive binding of JDP2 with other b-Zip transcription factors. HEK293 cells with wild-type EBV-BAC DNA were transfected with the siRNA against JDP2 (si-JDP2.1) or the control (si-control), together with FLAG-tagged expression vectors for CREB. After 36 h, ChIP experiments were carried 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. 6B, 73 or 49% suppression of the levels of *JDP2* mRNA resulted by si-JDP2.1 or si-JDP2.2. 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. 6A), 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 lev-

els (Fig. 6A). A similar increase was also observed by si-JDP2.2 (Fig. 6A).

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. 6C). Importantly, *BZLF1* expression patterns in HEK293 EBV/BAC cells with wild-type or the revertant strain (mZII/R) responded in similar ways (Fig. 6E) to JDP2 overexpression, whereas the *BZLF1* mRNA levels were not significantly affected by the overexpression in cells with the mZII mutant virus (Fig. 6E). 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. 7A, left), whereas its b-Zip deletion mutant lost the ability (Fig. 7A, right). Association of JDP2 with the *BZLF1* promoter correlated with increased HDAC3 recruitment to the promoter (Fig. 7A). This HDAC3 recruitment by JDP2 correlated to reduced levels of histone H3 acetylation (Fig. 7B). To extend these results, endogenous JDP2 was knocked down by siRNA (Fig. 7C). 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. 7C). The reduction in HDAC3 recruitment by si-JDP2 caused increments in histone H3 acetylation levels (Fig. 7D). Furthermore, JDP2 association with HDAC3 was confirmed by immunoprecipitation (Fig. 7E). 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

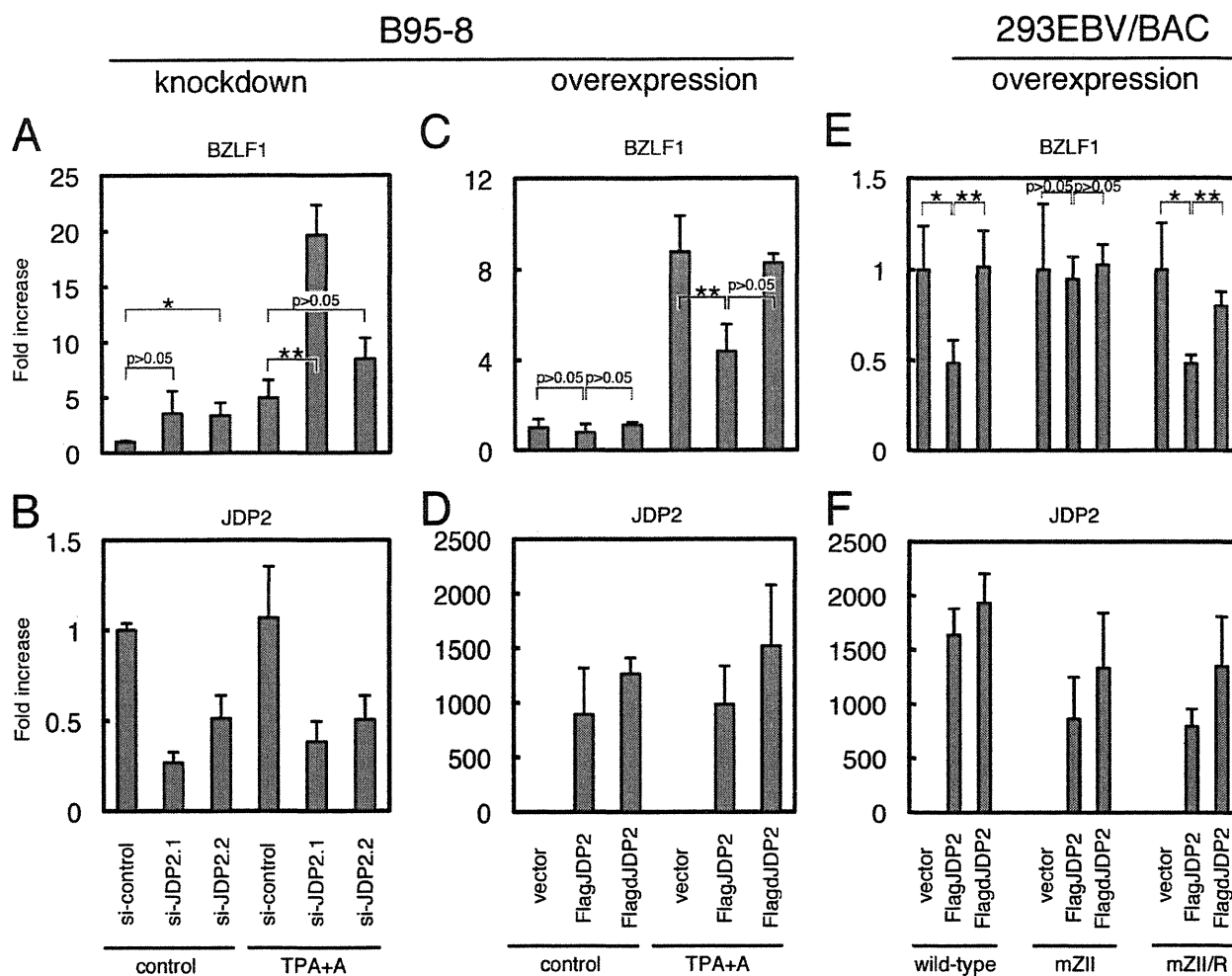


FIGURE 6. **JDP2 inhibits BZLF1 expression.** A and B, knockdown of JDP2 enhances BZLF1 expression. B95-8 cells transfected with siRNA against JDP2 (si-JDP2.1 or si-JDP2.2) or a control (si-control) siRNA were cultured for 48 h, and then treated with TPA (20 ng/ml) and A23187 (1 mM) for an additional 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. 4C). 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).

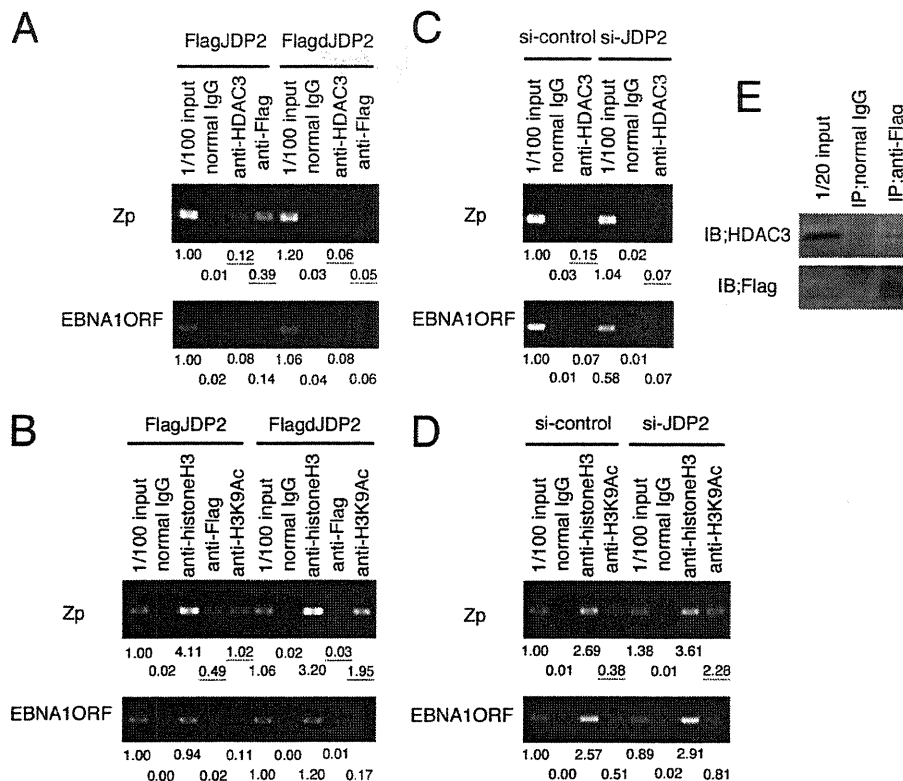


FIGURE 7. JDP2 mediates HDAC3 recruitment to the BZLF1 promoter. A and B, HEK293 cells with wild-type EBV-BAC DNA were transfected with the FLAG-JDP2 expression vector (*FlagJDP2*) or the expression vector with a deletion in the b-Zip domain (*FlagΔJDP2*). After 24 h, ChIP experiments were carried out 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.