

Either ZEB1 or ZEB2/SIP1 Can Play a Central Role in Regulating the Epstein-Barr Virus Latent-Lytic Switch in a Cell-Type-Specific Manner[†]

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Received 23 December 2009/Accepted 29 March 2010

We previously reported that the cellular protein ZEB1 can repress expression of the Epstein-Barr virus (EBV) *BZLF1* gene in transient transfection assays by directly binding its promoter, Zp. We also reported that EBV containing a 2-bp substitution mutation in the ZEB-binding ZV element of Zp spontaneously reactivated out of latency into lytic replication at a higher frequency than did wild-type EBV. Here, using small interfering RNA (siRNA) and short hairpin RNA (shRNA) technologies, we definitively show that ZEB1 is, indeed, a key player in maintaining EBV latency in some epithelial and B-lymphocytic cell lines. However, in other EBV-positive epithelial and B-cell lines, another zinc finger E-box-binding protein, ZEB2/SIP1, is the key player. Both ZEB1 and ZEB2 can bind Zp via the ZV element. In EBV-positive cells containing only ZEB1, knockdown of ZEB1 led to viral reactivation out of latency, with synthesis of EBV immediate-early and early lytic gene products. However, in EBV-positive cells containing both ZEBs, ZEB2, not ZEB1, was the primary ZEB family member bound to Zp. Knockdown of ZEB2, but not ZEB1, led to EBV lytic reactivation. Thus, we conclude that either ZEB1 or ZEB2 can play a central role in the maintenance of EBV latency, doing so in a cell-type-dependent manner.

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects 90% of the world's population. Latent EBV infection is associated with several types of malignancies in epithelial and B-lymphocytic cells, including nasopharyngeal carcinoma (NPC) (15), posttransplant lymphoproliferative disease (PTLD) (56), Burkitt's lymphoma (BL) (19), Hodgkin's disease (2, 80), and some gastric cancers (71; reviewed in references 66 and 67). Reactivation into lytic replication is necessary for the viral progeny to pass from host to host. It occurs in infected individuals at a low level; periodic shedding of the virus into saliva allows for transmission (67). It remains unclear how reactivation occurs *in vivo*. The product of the *BZLF1* gene, known as BZLF1 (also called ZEBRA, Z, Zta, and EB1), is a key player in the switch from EBV latency into lytic replication in cells in culture (12, 13, 20, 72; reviewed in references 66 and 67). BZLF1 is a multifunctional DNA-binding protein belonging to the bZIP family of transcription factors (9). It can directly bind to the origin of lytic replication, *oriLyt*, within the viral genome and induce expression of other immediate-early, early, and late EBV lytic genes (35, 44, 66, 69, 70). BZLF1 can also interact with several cellular proteins, affecting the activities or localization of these proteins, further contributing to viral reactivation (53, 66).

Our laboratory previously identified a cellular protein, ZEB1 (also known as δ EF1, TCF8, AREB6, ZFHEP, NIL-2A, ZFH1A, and BZP), that binds via its two zinc fingers to two sequence elements, named ZV and ZV', surrounding the transcription initiation site of the *BZLF1* promoter, Zp (see Fig. 1A and B) (40, 41, 83). Exogenous expression of ZEB1 led to

transcriptional repression of Zp activity in transient transfection assays (41). Mutation of the ZV element in the context of a whole EBV genome led to increased expression of EBV lytic proteins in B-lymphocytic BJAB cells and spontaneous production of infectious virus in 293 cells (83). We also showed that a highly lytic form of EBV infection exists in EBV-positive, ZEB1-negative AGS cells; exogenous addition of ZEB1 leads to a reduction in the expression of EBV lytic proteins (25). On the other hand, EBV exists in a highly latent form of infection in EBV-positive HeLa cells that express ZEB1 at a high level (25). Taken together with our other data, these findings strongly suggested, but did not definitively prove, that ZEB1 binding to Zp via the ZV element likely plays a central role in maintenance of EBV latency.

However, two ZEB family members exist: ZEB1 and ZEB2 (also known as SIP1, SMADIP1, ZFH1B, and KIAA0569). The ZEB family members are structurally similar, containing (i) two zinc finger-binding domains, (ii) a central repression region including CtBP- and Smad-interacting domains, and (iii) N- and C-terminal p300-interacting domains (74) (see Fig. 1A). Both family members bind sequence specifically to target DNAs via E-box-binding sequences resembling 5'-CAC(C/G)(T/G)(G/T)-3'. Depending upon interactions with coactivators, corepressors, and histone deacetylases (HDACs), ZEB1 can either activate or repress transcription of its target genes (59, 60, 62, 63, 75, 76). Whether ZEB2 can function as an activator as well as a repressor remains unknown. Due to the high sequence and structural similarity in the zinc finger domain regions of ZEB2 and ZEB1, we hypothesized that ZEB2/SIP1 likely can also interact with Zp via binding its ZV and ZV' elements.

Based upon data from small interfering RNA (siRNA) and short hairpin RNA (shRNA) knockdown studies of several EBV-positive epithelial and B-lymphocytic cell lines, we now show that ZEB1 does, indeed, play a key role in transcriptional

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[†] Published ahead of print on 7 April 2010.

repression of the *BZLF1* gene, maintaining EBV in a latent state in some cell lines. However, in other EBV-positive cell lines, ZEB2, not ZEB1, is the key player. Thus, we conclude that both ZEB1 and ZEB2 contribute to maintenance of EBV latency, doing so in a cell-type-specific manner.

MATERIALS AND METHODS

Cells. All EBV-positive B-lymphocytic cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin and streptomycin per ml. MutuI and MutuIII cells are EBV-positive Burkitt's lymphoma (BL) cell lines in latency type I and III, respectively, derived from clonal isolates of the cell line Mutu (30). Cell line 721 is a type III latency lymphoblastoid cell line (LCL). Jijoye is a type III latency BL cell line, and GG68 is a type III latency BL cell line derived from a clonal isolate of the cell line P3HR1; they were obtained from Bill Sugden and originally described in references 36 and 78. Akata is an EBV-positive BL cell line in type I latency derived by reinfection of EBV-negative Akata BL cells with the EBV strain that had been lost during growth in culture (originating from the laboratory of Kenzo Takada [73]; obtained from Bill Sugden). BJAB^{B95.8} was derived by infection of the EBV-negative BL cell line BJAB with the B95.8 strain of EBV (83). The media for growing EBV-positive Akata and BJAB^{B95.8} cells also included 500 µg/ml G418 and 300 µg/ml hygromycin, respectively. Epithelial gastric carcinoma AGS^{B95.8} cells (a gift from Shannon Kenney) were maintained in F12 medium supplemented with 10% FBS and 100 U penicillin and streptomycin per ml as previously described (25). Epithelial nasopharyngeal carcinoma (NPC) HONE-1^{Akata} (a gift from Lawrence Young via Shannon Kenney; originally described in reference 28), CNE1^{Akata}, and CNE2^{Akata} (gifts from Diane Hayward, with permission from Kwok Wai Lo) were maintained in RPMI plus 10% FBS and 100 U penicillin/streptomycin, additionally supplemented with 500 µg/ml G418 as described in reference 49. Neuronal 293^{B95.8} and 293^{ZVmt} cells were obtained by infection of 293D cells with the bacmid p2089 or a variant of it containing a 2-bp substitution mutation in the ZV element of the *BZLF1* promoter, respectively, as previously described (83). The following EBV-negative cell lines were used. Breast carcinoma MCF-7 cells (ATCC) were maintained in Dulbecco modified Eagle medium (DMEM)/F12 supplemented with 10% FBS, 6 ng/ml insulin, 2 mM L-glutamine, 100 µM nonessential amino acids, and 100 U penicillin/streptomycin. HEK 293T and 293D cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. All cells were maintained at 37°C in a 5% CO₂ atmosphere.

Plasmids. The expression plasmid pcDNA4hismaxCZEB1 contains human ZEB1 cloned between the EcoRI and XbaI sites of pcDNA4hismaxC (Invitrogen); it was constructed by recombining the N-terminal 438-bp region of a ZEB1 cDNA generated by reverse transcriptase PCR (RT-PCR) amplification of polyadenylated RNA obtained from 293 cells with the remainder of a ZEB1 cDNA clone provided by Tom Genetta (27).

The expression plasmid pcDNA4hismaxCZEB2, encoding human ZEB2, was constructed as follows. A cDNA clone of human ZEB2 was provided by Takahiro Nagase (Kazusa DNA Research Institute, Japan) (54, 55). PCRs were used to generate two fragments approximately 1.8 kbp in length that included the entire ZEB2-coding region and an overlapping region containing a BspHI site. These fragments were digested with either NotI and BspHI or XbaI and BspHI. The fragments were ligated into pcDNA4hismaxC, and the presence of a complete ZEB2-coding region was confirmed by DNA sequencing.

The luciferase reporter plasmid pZpWT-luc contains the nucleotide (nt) -221 to +30 region of Zp relative to the transcription initiation site cloned into the pGL3-Basic vector (Promega). It was constructed by ligation of a PCR-amplified DNA fragment corresponding to the nt -221 to +30 region of Zp into the KpnI and HindIII sites of pGL3-Basic. The luciferase reporter construct pZpZVmt-luc was constructed by site-directed mutagenesis of pZpWT-luc; it contains base pair substitution mutations of T to C at nt -13 and of A to C at nt -12 within the ZV element of Zp. The expression plasmid pSG5-Z (provided by Diane Hayward via Shannon Kenney) encodes the EBV intermediate-early (IE) *BZLF1* protein (16).

Transfections. The expression plasmids pcDNA4hismaxCZEB1 and pcDNA4hismaxCZEB2 and their empty parental vector, pcDNA4hismax, were transiently transfected into CNE1^{Akata} (see Fig. 6) and 293^{B95.8} (see Fig. 8) cells using TransIT-LT1 reagent (Mirus). When working with CNE1^{Akata} cells, 20 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 3 mM sodium butyrate were added 24 h posttransfection to reactivate the virus. Twenty-four hours after addition of TPA plus butyrate, the cells were harvested, and the proteins were analyzed by SDS-PAGE followed by immunoblotting. When working with

293^{B95.8} cells, lytic reactivation was induced by transfection of ZEB2 (catalog no. AM16708, item no. 108634; Applied Biosystems) siRNAs 24 h later (see paragraph below for transfection of siRNAs) and then treated 24 h after siRNA transfection with 20 ng/ml TPA and 3 mM sodium butyrate, as indicated below (see Fig. 8). Forty-eight hours after the addition of TPA plus butyrate, the cells were harvested, and the proteins were analyzed by SDS-PAGE followed by immunoblotting.

A 30 nM concentration of small interfering RNAs (siRNAs) to ZEB1 (catalog no. AM16708, item no. 109652), to ZEB2 (catalog no. AM16708, item no. 108634), to both ZEBs, and "scrambled" as a negative control (catalog no. AM4613; Ambion/Applied Biosystems) was transfected into the epithelial cells using Lipofectamine RNAiMAX (Invitrogen) according to the company's instructions. Alternatively, experiments were performed using another ZEB2 siRNA (catalog no. AM16708, item no. 5799; Applied Biosystems; referred to as ZEB2a [see Fig. 5A and 7C]), targeting a different region of the ZEB2 mRNA, using methods described above (see Fig. 5). The cells were subsequently incubated for at least 72 h to achieve knockdown of expression of the target genes. When working with 293^{B95.8} cells, we also added 20 ng/ml TPA and 3 mM sodium butyrate (see Fig. 7) to the medium 48 h prior to harvesting the cells.

Infection with lentiviruses expressing short hairpin RNAs (shRNAs). A set of five lentivirus shRNAiR expression plasmids targeting ZEB1 (target set NM_011546), ZEB2 (target set NM_014795), and the universal negative control, pLKO.1 (RHS4080), were purchased from Open Biosystems (ThermoScientific). To generate virus, mycoplasma-free 293T cells grown in 10-cm dishes were transfected using LT1 reagent with 10 µg total of a mixture containing shRNAiR ZEB1, ZEB2, ZEB1 plus ZEB2, or pLKO.1 as indicated, 3 µg DNA encoding HIV Gag/Pol (provided by Caroline Alexander) (46), and 0.5 µg of a plasmid encoding VSVG (provided by Bill Sugden) (42, 43). Alternatively, one clone of the ZEB2 shRNA set (clone ID number AAC71-H2; Open Biosystems; referred to as ZEB2a [see Fig. 5B and 7D]) was used to eliminate the possibility of nonspecific knockdown of ZEB1, using methods as described above for generating virus (see Fig. 5B and 7D). The medium was changed 24 h posttransfection to RPMI with 10% FBS, additionally supplemented with 50 mM HEPES, pH 7.3 (Invitrogen). Virus in the supernatant was collected after 24 h and passed through a 0.45-µm-pore-size filter. The indicated B-lymphocytic cells were infected daily with the indicated virus for 72 h. When working with BJAB^{B95.8} cells, 20 ng/ml TPA and 3 mM sodium butyrate were added 48 h prior to harvest.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (37, 38) with a radiolabeled double-stranded DNA corresponding to nt -30 to +20 relative to the transcription initiation site of the *BZLF1* gene serving as a probe (see Fig. 1C). Recombinant human ZEB1 and ZEB2, synthesized in a coupled transcription-translation rabbit reticulocyte lysate system (Promega), served as the protein sources. Immunoshift assays were performed as previously described (41) by incubation of the reaction mixture for 20 min with the indicated ZEB-specific antiserum or unprogrammed reticulocyte lysate as a control prior to addition of the radiolabeled probe. The ZEB2-specific antiserum, a gift from Michel Sanders, was a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acid residues 1197 through 1214 of human ZEB2; it was further purified by protein A agarose. The ZEB1-specific antiserum was a rabbit polyclonal antibody (sc-25388; Santa Cruz Biotechnologies).

EMSAs showing competition between ZEB1 and ZEB2 binding were performed as previously described (37, 38), with a radiolabeled double-stranded DNA corresponding to nt -30 to +20 relative to the transcription initiation site of the *BZLF1* gene serving as a probe. ZEB1 and ZEB2 reticulocyte lysates served as the protein sources and were synthesized as described above. Equal amounts of ZEB1 were loaded into each lane, along with increasing amounts of ZEB2, as well as unprogrammed reticulocyte lysate to ensure equal loading. Electrophoresis was performed at 4°C for 2 h at 220 V in a 4% native polyacrylamide gel. Gels were dried for 1 h at 80°C and imaged on a STORM 840 phosphorimager (GE Healthcare).

Competition EMSAs were performed as previously described (40, 41). The protein source was whole-cell extracts prepared from 293 cells transfected 48 h earlier with pcDNAhismaxCZEB1 or pcDNAhismaxCZEB2. The ZV mutant (ZVmt) double-stranded competitor DNA contained the sequence indicated in Fig. 1C. The whole-cell extracts containing ZEB1 or ZEB2 were incubated with various amounts of nonradioactive nt -30 to +20 wild-type (WT) or ZVmt competitor DNA for 20 min on ice, radiolabeled WT nt -30 to +20 probe DNA was added, incubation was continued for 15 min, and electrophoresis was performed for 2 h at 220 V in a native 4% polyacrylamide gel. Gels were imaged as indicated above.

Transient transfection assays. MCF-7 cells grown in 12-well dishes were cotransfected using LT1 reagent with (i) 0.5 µg of pZpWT-luc, pZpZVmt-luc, or

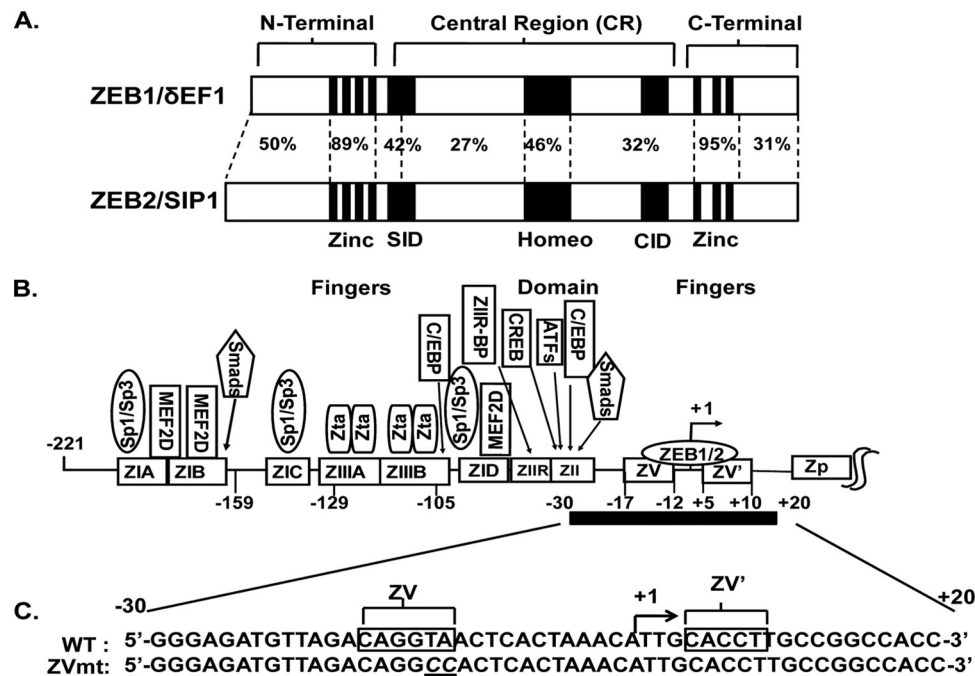


FIG. 1. Schematic diagrams showing structures of ZEB1 and ZEB2 proteins (A), promoter region of the *BZLF1* gene indicating its *cis*-acting elements and their *trans*-acting factors (B), and the sequences of the wild-type (WT) and ZV mutant (mt) DNA probes used in the EMSAs (C). The ZV and ZV' elements are indicated by boxes in panel C, with the 2-bp substitution mutation in the ZV element indicated by italicized, underlined letters. SID, Smad-interacting domain; CID, CtBP-interacting domain.

pGL3-Basic as a control, together with (ii) 0.05 µg of pcDNAhismax4ZEB1, pcDNAhismax4ZEB2, or pcDNA4hismaxC as a control. All samples were concurrently transfected with 0.05 pCMXLuc (Stratagene) as an internal control. Cells were harvested 48 h posttransfection into passive lysis buffer (Promega). Luciferase activity was assayed with a Monolight 3010 luminometer according to the manufacturer's protocol. Data were normalized both internally to renilla luciferase expression and externally to the controls transfected in parallel as indicated in the figure legends.

Immunoblot analysis. Cells were harvested in SUMO buffer (1) and lysed by sonication as previously described (1). To detect BZLF1 and BMRF1, the proteins were resolved by electrophoresis for 1 h at 120 V in SDS gels containing 12% polyacrylamide (ISC Bioexpress) and transferred to a nitrocellulose membrane by electrophoresis at 350 mA for 2 h at 4°C. After incubation in 5% milk in TBST (100 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) for 1 h, the membranes were incubated overnight at 4°C in 5% milk/TBST containing antibody specific to BZLF1 (1:250 sc-53904; Santa Cruz) or BMRF1 (1:250 VP-E608; Vector Laboratories). Afterward, the blots were washed, incubated for 1 h in 5% milk-TBST containing the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; ThermoScientific), washed again with TBST, incubated for 1 min in ECL (Roche), and exposed to X-ray film.

To detect ZEB1 and ZEB2, the proteins were resolved by electrophoresis for 1 h at 120 V in 3 to 8% gradient polyacrylamide-Tris-acetate gels (Invitrogen), and subsequently processed as described above, except the primary antibody was rabbit polyclonal anti-ZEB1 (1:500 sc-25388; Santa Cruz) or ZEB2 (1:2,000 dilution) and the secondary antibody was ECL horseradish peroxidase-conjugated goat anti-rabbit IgG (GE Healthcare).

Quantitative real-time PCR. For reverse transcription quantitative real-time-PCR (RT-qPCR) analysis, total RNA from approximately 10⁶ cells was isolated using an RNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. RNA concentration was quantified by Nanodrop Technologies, and cDNA was synthesized from 10 ng of total RNA using an iScript cDNA Synthesis kit (Bio-Rad) and reverse transcribed according to the manufacturer's instructions (Bio-Rad). BZLF1 RNA levels were determined by RT-qPCR performed on a MyIQ quantitative real-time PCR detection system (Bio-Rad). The PCR conditions were as follows: 95°C for 10 min, 95°C for 1 min, 60°C for 15 s (45 cycles). Data were normalized to Po rRNA levels present in the same samples using the $\Delta\Delta C_T$ method; they are shown relative to the negative control included in each experiment. Primer sets used were as follows: ZEB1, forward (FWD),

5'-AGCAGTGAAAGAGAAGGG-3', and reverse (REV), 5'-GGTCCTCTTCAGGTGCCT-3'; ZEB2, FWD, 5'-CAAGAGGCGCAAACAAGC-3', and REV, 5'-GGTTGGCAATACCGTCAT-3'; BZLF1, FWD, 5'-CTGCTCCTGA GAATGCTT-3', and REV, 5'-CGGCTTGTTGGTCTGTT-3'; Po, FWD, 5'-G ACAATGGCAGCATCTAC-3', and REV, 5'-GCAGACAGACACTGGTCA-3'. Pearson's correlation coefficient was calculated using the MSTATs program (<http://www.mcardle.wisc.edu/mstat>). The primer set used to assay for BZLF1 RNA can also detect bicistronic mRNA initiated from the promoter for the upstream *BRLF1* gene (50); however, this fact is unlikely to affect significantly our measurement of the relative amounts of BZLF1 RNA present in cells, especially since these two immediate-early genes autoregulate each other (64, 65, 84).

Chromatin immunoprecipitation (ChIP) assays. ChIP analysis was carried out as described previously (79). Briefly, protein-DNA complexes were cross-linked by treating the cells with 1.5% formaldehyde. Nuclei were isolated and resuspended in nuclei lysis buffer (79), and the chromatin was sheared by sonication to a fragment size of approximately 500 bp by three to five pulses of 15 s each on a Sonic Dismembrator model 100 (Fisher Scientific). The samples were cleared by centrifugation at 14,000 rpm for 10 min, and the supernatants were collected. Protein A Sepharose beads (GE Healthcare) were blocked by incubation with gentle rocking at 4°C overnight with 1 mg/ml of herring sperm DNA (Applied Biosystems) and 1 mg/ml of UltraPure bovine serum albumin (BSA) (Applied Biosystems). The sheared chromatin samples were incubated overnight at 4°C with 5 µl of (i) ZEB1 (sc-25388; Santa Cruz), (ii) ZEB2 (sc-48789; Santa Cruz), or (iii) IgG (sc-2027; Santa Cruz) as a negative control, followed by addition of the protein A Sepharose beads and incubation for 1 h at 4°C. After washing of the beads, the immunoprecipitates were eluted and the cross-links were reversed. The resulting DNAs were purified using QIAquick PCR purification kits (Qiagen) and amplified by PCR with a GeneMate Genius PCR machine using 200 nM each primer set and *Pfu* Turbo (Stratagene) according to the manufacturer's instructions. The amplification conditions were as follows: 95°C for 10 min, 95°C for 30 s, 60°C for 1 min (25 cycles). The primers were as follows: BZLF1, FWD, 5'-TTACCTGTCTAACATCTCCCTTT-3', and REV, 5'-TTGACACCAGCTTATTTTAGACAC-3'; negative control (5 kbp upstream of the EBV ZV element of Zp), FWD, 5'-AGAAGGAGACACATCTG-3', and REV, 5'-AACTTGGACGTTTGTGGG-3'; and IL-2, FWD, 5'-GTTTCATAC AGCAGGCGT-3', and REV, 5'-TTTCTCTTCTGATGACT-3'. PCR prod-

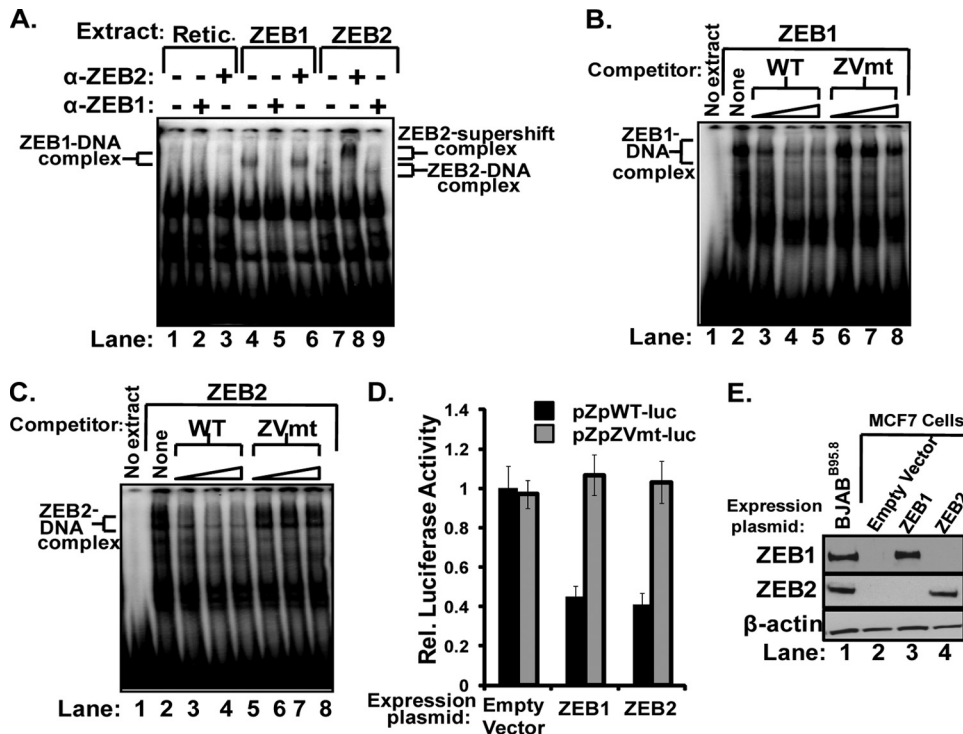


FIG. 2. Both ZEB1 and ZEB2 repress transcription of Zp via binding the ZV element. (A) EMSAs showing ZEB1 and ZEB2 binding to Zp. ZEB1 and ZEB2, synthesized in a cell-free system (TNT), were incubated with a radiolabeled double-stranded WT DNA corresponding to the nt -30 to +20 region of Zp (Fig. 1C) as a probe in the presence or absence of antibody specific to ZEB1 or ZEB2. Lanes 1 to 3, EMSAs performed in parallel with unprogrammed reticulocyte (Retic.) lysate as a negative control. (B and C) Competition EMSAs showing ZEB1 and ZEB2 bind via the ZV element to Zp. Extracts prepared from 293D cells transiently transfected with plasmids expressing ZEB1 (B) or ZEB2 (C) were incubated with the same radiolabeled DNA probe used in panel A together with a 0, 20, 40, or 80 M excess of nonradiolabeled, double-stranded DNA corresponding to the WT or ZVmt sequence shown in Fig. 1C. (D) MCF-7 cells were cotransfected as indicated with 0.5 μ g of the reporter plasmid pZpWT-luc or pZpZVmt-luc (i), 0.5 μ g of pCMXRluc as an internal control (ii), and 0.05 μ g of an expression plasmid encoding ZEB1, ZEB2, or their empty parental vector, pcDNA4hismaxC (iii). Forty-eight hours later, the cells were harvested, and relative (Rel.) luciferase activity was measured. Data were internally normalized to the value for renilla luciferase present in the same sample and externally normalized to the value obtained for the cells cotransfected with pZpWT-luc, pCMXRluc, and pcDNA4hismaxC. Shown here is a representative experiment indicating the means \pm standard errors of the means from assays performed in triplicate. (E) Immunoblot analysis of MCF-7 cells transfected as described for panel D with 0.05 μ g of a plasmid expressing ZEB1, ZEB2, or the empty vector pcDNA4hismaxC as a control. The cells were harvested 48 h after transfection, and 30 μ g of whole-cell protein was loaded per lane and analyzed as described in Materials and Methods for ZEB1 and ZEB2 protein, with β -actin serving as a loading control. Lane 1 was loaded with 30 μ g of whole-cell protein obtained from BJAB^{B95.8} cells as a control for endogenous levels of ZEB1 and ZEB2 protein present in latently infected cells.

ucts were analyzed by electrophoresis in a 1% agarose gel and visualized by UV light after staining with ethidium bromide.

Quantitative real-time PCR (qPCR) analysis was carried out as described above, except 1 μ l of input and 4 μ l of ChIP-assayed sample and 400 nM the above primer sets were used. qPCR was performed with the MyIQ quantitative real-time PCR detection system (Bio-Rad). Data were calculated as percent input by the $\Delta\Delta CT$ method.

RESULTS

ZEB2 binds the ZV element of Zp, repressing transcription.

We previously reported that ZEB1 can bind Zp via its ZV element, repressing *BZLF1* gene expression both in luciferase reporter assays (40, 41) and in cell lines latently infected with EBV (83). Based upon these findings, we concluded that ZEB1 plays a central role in regulating *BZLF1* gene expression in at least some cell types. However, a second cellular zinc finger E-box binding protein exists, ZEB2 (also frequently called SIP1 for Smad-interacting protein 1), with an arrangement of functional domains and DNA-binding specificity very similar to those of ZEB1 (Fig. 1A) (74). Thus, we hypothesized that

ZEB2 may also repress *BZLF1* gene expression via binding the ZV element of Zp when present.

To test this hypothesis, we first generated a human ZEB2 expression plasmid, pcDNA4hismaxZEB2, and antisera specific to ZEB2. Using these reagents, along with ones previously reported for our studies with ZEB1 (40, 41), we tested whether ZEB2 can repress transcription from Zp via binding its ZV element. First, we determined using an electrophoretic mobility shift assay with a radiolabeled DNA probe corresponding to the nt -30 to +20 region of Zp relative to the transcriptional initiation site of the *BZLF1* gene (Fig. 1C) that ZEB2 did, indeed, bind to Zp (Fig. 2A, lane 7). Inclusion of a ZEB2-specific antiserum in the reaction mixture led to loss of most of the DNA migrating in this region of the gel along with the appearance of a slower-mobility band indicative of a supershifted antibody-ZEB2-DNA complex (Fig. 2A, lane 8). This antiserum was specific for ZEB2; i.e., it did not affect the mobility of the ZEB1-DNA complex (Fig. 2A, lane 6). Thus, we conclude that ZEB2 can bind to the nt -30 to +20 region of Zp.

Next, we performed competition EMSAs to show that, as with ZEB1 (41), ZEB2 binding to Zp is mediated in large part through the ZV element located within nt -17 to -12 of Zp. As expected, nonradiolabeled wild-type (WT) double-stranded DNA competed well with the radiolabeled probe for binding ZEB1 (Fig. 2B, lane 2 versus lanes 3 to 5), while the ZV mutant DNA competed poorly (Fig. 2B, lane 2 versus lanes 6 to 8). The results obtained for binding of ZEB2 were similar; i.e., the WT DNA competed well (Fig. 2C, lane 2 versus lanes 3 to 5), while the ZVmt DNA competed poorly for binding ZEB2 (Fig. 2C, lane 2 versus lanes 6 to 8). The modest reduction in ZEB1 and ZEB2 binding observed at the highest concentration of ZVmt competitor DNA is due to the presence of a weaker, secondary ZEB-binding site in Zp, named the ZV' element, that synergizes with the ZV element to enable the ZEBs to bind concurrently via their two zinc fingers to these two elements in Zp (Fig. 1B; X. Yu, P. McCarthy, Z. Wang, H. J. Lim, T. Iempridee, D. Gorlen, R. Kraus, and J. E. Mertz, unpublished data). Thus, we conclude that ZEB2 primarily binds via the ZV element to Zp.

Next, we tested whether ZEB2 represses transcription initiated from Zp in luciferase reporter assays when bound to Zp via the ZV element. MCF-7 cells, lacking both ZEB1 and ZEB2 (3, 18), were cotransfected with (i) the WT and ZV mutant reporter plasmids pZpWT-luc and pZpZVmt-luc, respectively, and (ii) expression plasmids encoding ZEB1, ZEB2, or their empty parental vector, pcDNA4hismaxC, as a control. As expected, exogenous expression of either ZEB1 or ZEB2 led to an approximately 60% decrease in transcription driven from the wild-type promoter (Fig. 2D). On the other hand, exogenous expression of neither ZEB1 nor ZEB2 led to any significant effect on transcription driven from the ZV mutant promoter (Fig. 2D). The levels of ZEB1 and ZEB2 achieved in these transfected cells were similar to the endogenous levels of these proteins present in BJAB^{B95.8}, an EBV-positive B-lymphocytic cell line which harbors EBV in a latent form of infection (Fig. 2E). Thus, we conclude that ZEB2, like ZEB1, can repress transcription of the *BZLF1* gene via binding the ZV element of Zp when it is present at a physiological level in cells.

ZEB1 and ZEB2 protein and RNA levels vary among EBV-positive cell lines. Given the above findings, we next asked whether ZEB1 or ZEB2 is the primary ZV element-binding protein contributing to silencing *BZLF1* gene expression in cells latently infected with EBV. As a first step toward answering this question, we determined by immunoblot and reverse transcription-qPCR analyses the relative levels of ZEB1 and ZEB2 protein and RNA, respectively, present in multiple EBV-positive B-lymphocytic and epithelial cell lines (Fig. 3A to C). ZEB1 protein was observed in all B-lymphocytic cell lines tested, in most cases at a high level (Fig. 3A). ZEB1 was also present, albeit at a lower level, in all but one of the epithelial cell lines examined (Fig. 3B). In contrast, ZEB2 protein and RNA were present at significant levels in the neuronal HEK293 cells, only three of the seven B-lymphocytic cell lines, and none of the epithelial cell lines examined (Fig. 3A to C). These findings are consistent with previous findings of others regarding ZEB1 and ZEB2 protein and RNA levels in a variety of cell lines and tissue types (27, 68). The failure to find a perfect correlation between ZEB1 protein and RNA

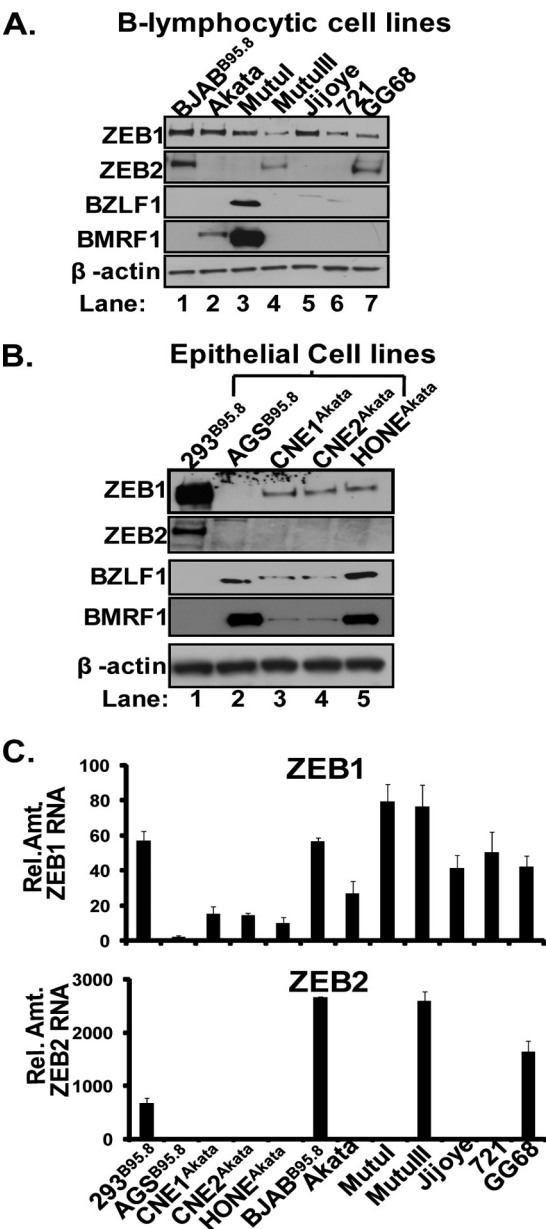


FIG. 3. ZEB1 and ZEB2 levels vary in different EBV-positive cell lines. (A and B) Immunoblots showing levels of endogenous cellular ZEB1 and ZEB2 and lytic EBV-encoded BZLF1 and BMRF1 proteins present in a variety of EBV-positive B-lymphocytic (A) and epithelial (B) cell lines. Whole-cell extracts were prepared from the indicated cell lines. Proteins (30 μ g per sample) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antisera as described in Materials and Methods. β -Actin served as a loading control. (C) RT-qPCR analysis of relative levels of ZEB1 and ZEB2 RNA present in a variety of EBV-positive cell lines. Samples were processed as described in Materials and Methods. Data were normalized to values for ribosomal Po RNA present in the same samples.

levels is due to the fact that expression of the ZEBs is regulated at the translational level by the 200 family of cellular micro-RNAs (6, 7, 10, 31, 32, 39, 57; A. Ellis, I. Xu, and J. E. Mertz, submitted for publication). Thus, for the purpose of this study, it is the ZEB protein, not RNA levels, that matters most, since

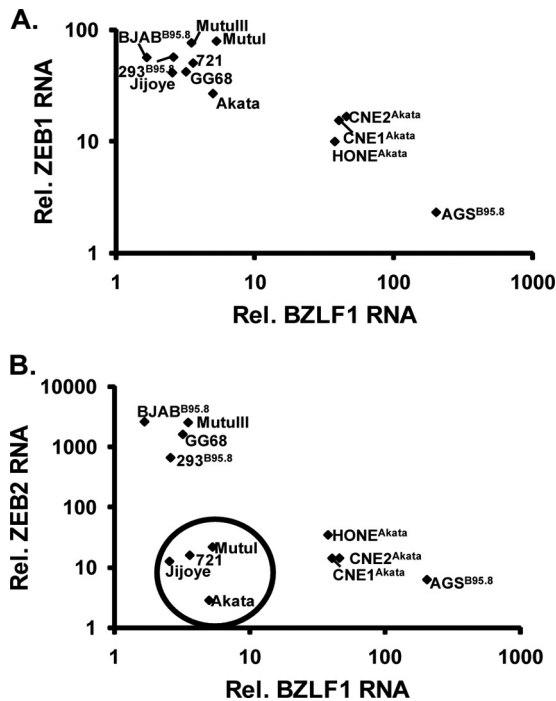


FIG. 4. ZEB1 and ZEB2 expression correlate with EBV latency. (A and B) Scatter plots showing correlation between level of BZLF1 RNA and level of ZEB1 (A) or ZEB2 (B) RNA in EBV-positive cell lines. Relative BZLF1, ZEB1, and ZEB2 RNA levels were determined by RT-qPCR as described in Materials and Methods. Data were normalized to values for ribosomal Po RNA present in the same samples.

it is the proteins that contribute to regulation of *BZLF1* gene expression.

Extracts of these same cell lines were examined likewise for their levels of the EBV-encoded immediate-early (IE) *BZLF1* and early (E) *BMRF1* lytic-gene products (Fig. 3A and B; Fig. 4), expression that is indicative of viral reactivation out of latency into lytic replication. Importantly, we found a strong negative Pearson correlation ($r = -0.70$; P value < 0.05) between ZEB1 and BZLF1 RNA levels (Fig. 4A). Cell lines with high levels of ZEB1 contained little or no BZLF1 RNA. Cell lines with moderate levels of ZEB1 contained moderate levels of BZLF1, and the cell line with no ZEB1 contained a high level of BZLF1 RNA. Likewise, little or no BZLF1 RNA or protein was detected in the EBV-positive cell lines containing both ZEBs (Fig. 3 and 4). There were a few exceptions to this strong negative correlation between BZLF1 and ZEB1 at the protein level. For example, the MutuI cell line expressed moderately high levels of both ZEB1 and BZLF1, suggesting that these cells might also contain high levels of activators of Zp such as c-Jun, ATF1/2, or C/EBP α that might overcome the repression by ZEB1 (25, 26, 77, 82). While some of the ZEB2-negative cell lines expressed BZLF1 at moderate to high levels as expected (Fig. 4), others, i.e., the ZEB1-positive ones within the circle in Fig. 4B, contained little to no BZLF1. Based upon these findings, we hypothesized that both ZEB1 and ZEB2 may play important roles in regulating *BZLF1* gene expression and maintaining EBV latency in a cell-type-specific manner, with ZEB1 being sufficient to silence *BZLF1* gene expression

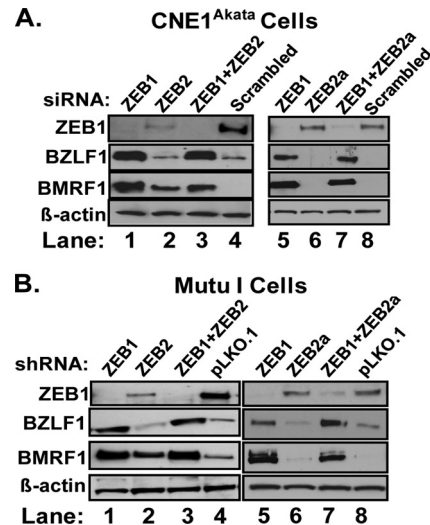


FIG. 5. Knockdown of ZEB1 leads to EBV lytic reactivation in ZEB2-negative cell lines. (A) Immunoblot showing effects of siRNAs to ZEB1 and ZEB2 on maintenance of EBV latency in epithelial NPC CNE1^{Akata} cells. CNE1 cells latently infected with the Akata strain of EBV were transfected with 30 nM the indicated siRNAs. Experiments were also performed using an alternative ZEB2 siRNA (indicated as ZEB2a) as indicated in Materials and Methods. Seventy-two hours later, whole-cell extracts were prepared, and protein levels were determined by immunoblot analysis. β -Actin served as a loading control. (B) Immunoblot showing effects of shRNAs to ZEB1 and ZEB2 on maintenance of EBV latency in EBV-positive BL MutuI cells. MutuI cells were infected with lentiviruses expressing the indicated shRNAs as described in Materials and Methods. Experiments were performed using an alternative ZEB2 shRNA (indicated as ZEB2a) as well. The cells were incubated, harvested, and analyzed as for panel A.

in some ZEB2-negative cell lines. The experiments presented below prove the validity of this hypothesis.

ZEB1 plays a central role in maintenance of EBV latency in ZEB2-negative cell lines. Although ZEB2 can repress *BZLF1* gene expression via binding the same sites in Zp as ZEB1 (Fig. 2), ZEB2 is not present in many EBV-positive cell lines (Fig. 3). Therefore, we initially hypothesized that ZEB1 may be the major repressor of *BZLF1* gene expression because it is more ubiquitously expressed in cells latently infected with EBV. To test the validity of this hypothesis, we determined whether knocking down the level of ZEB1 protein in EBV-positive B-lymphocytic and epithelial cell lines was sufficient to lead to lytic reactivation of the virus. Thus, we examined two of our EBV-positive, ZEB2-negative cell lines for this predicted property: the epithelial NPC cell line CNE1^{Akata} and the B-lymphocytic BL cell line MutuI. MutuI cells, derived from an EBV-positive Burkitt's lymphoma strain, contain the endogenous EBV strain from the patient still in type I latency (30). CNE1^{Akata} cells contain the Akata strain of EBV in the type II latency that is typically observed in EBV-positive NPCs; this human laboratory strain of EBV had been introduced after the endogenous EBV had been lost during passage in culture (49).

In the CNE1^{Akata} cell line, transfection with the ZEB1 siRNAs led to a reduction in the ZEB1 protein level (Fig. 5A, lane 1 versus lane 4). Concomitantly, the levels of the lytic IE BZLF1 and E BMRF1 proteins increased compared to those in cells transfected in parallel with a scrambled control siRNA

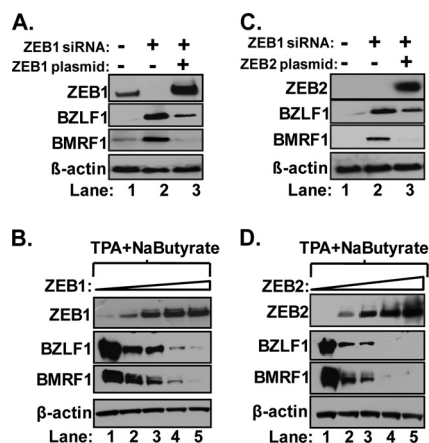


FIG. 6. Immunoblots showing that overexpression of either ZEB1 (A and B) or ZEB2 (C and D) can inhibit induction of EBV lytic reactivation. (A and C) ZEB2-negative CNE1^{Akata} cells were transfected with 1.0 μg of ZEB1 (A) or ZEB2 (C) expression plasmid or their empty parental plasmid, pcDNAhismaxC (-), as a control. Twenty-four hours later, the cells were transfected with 30 nM either ZEB1 siRNAs (+) or scrambled siRNA (-) as a control. Whole-cell extracts were prepared 72 h later and assayed for the indicated proteins by immunoblot analysis. (B and D) CNE1^{Akata} cells were cotransfected with 0, 0.1, 0.25, 0.5, or 1.0 μg of ZEB1 (B) or ZEB2 (D) expression plasmid along with pcDNAhismaxC DNA to apply 1.0 μg total DNA per well in a 6-well plate. Twenty-four hours later, TPA (20 ng/ml) and sodium butyrate (3 mM) were added to the medium and incubation was continued for an additional 24 h prior to harvesting for whole-cell protein and assaying for the indicated proteins by immunoblot analysis. β-Actin served as a loading control.

(Fig. 5A, lane 1 versus lane 4). The siRNAs to ZEB2 also somewhat knocked down the ZEB1 protein level, leading to some turn-on of lytic early gene expression as well (Fig. 5A, lane 2 versus lane 4). Using different siRNAs to ZEB2, the nonspecific knockdown of ZEB1 was eliminated, and the levels of the lytic IE BZLF1 and E BMRF1 proteins increased only in the presence of siRNAs to ZEB1 (Fig. 5A, lanes 5 and 7).

To examine the effect of knocking down the level of ZEB1 or ZEB2 in the B-lymphocytic Burkitt's lymphoma cell line MutuI, we infected the cells with lentiviruses expressing shRNAs to ZEB1, ZEB2, or pLKO.1, a scrambled sequence that is not known to knock down expression of any cellular mRNA, as a control. We achieved good knockdown of ZEB1 (Fig. 5B, lane 1 versus lane 4). Once again, knockdown of ZEB1 protein led to significant induction of synthesis of the lytic IE BZLF1 and E BMRF1 proteins above the level observed with the control shRNA (Fig. 5B, lane 1 versus lane 4). Similar results were seen using a single shRNA to ZEB2 as well (Fig. 5B, lanes 5 to 8). Taken together with our previous findings (41, 83) and those presented here (Fig. 2 and 3), these data provide definitive evidence that ZEB1 does, indeed, play a central role in maintaining EBV latency in ZEB2-negative cells.

To exclude the possibility that knocking down ZEB1 in ZEB2-negative cells induced EBV lytic replication via indirect, nonspecific effects of the ZEB1 siRNAs, we also tested whether overexpression of ZEB1 could inhibit lytic reactivation. CNE1^{Akata} cells were transfected with a ZEB1 expression plasmid or its empty parental plasmid as a control 24 h prior to transfection with the siRNAs to ZEB1. Under these condi-

tions, the ZEB1 protein was highly expressed despite the presence of the siRNAs to ZEB1 (Fig. 6A, lane 3 versus lane 2). Whereas presence of the ZEB1 siRNAs led to an induction of IE and E lytic EBV gene expression as expected (Fig. 6A, lane 2), concurrent overexpression of ZEB1 resulted in minimal induction of BZLF1 expression and no induction of early BMRF1 protein synthesis above the level observed with the control cells processed in parallel (Fig. 6A, lane 3 versus lane 1). Thus, we conclude that the EBV lytic reactivation induced by treating the cells with siRNAs to ZEB1 was, indeed, directly due to knocking down the level of ZEB1.

EBV lytic reactivation can also frequently be induced by treating EBV-positive cells with a variety of chemicals, including phorbol esters such as TPA and histone deacetylase (HDAC) inhibitors such as sodium butyrate (45, 85; reviewed in references 66 and 67) (Fig. 6B, lane 1). Thus, we asked whether overexpression of ZEB1 could also inhibit lytic reactivation by these chemical inducers. The CNE1^{Akata} cells were transfected with increasing amounts of the ZEB1 expression plasmid 24 h prior to addition of TPA plus butyrate and incubated for an additional 24 h before harvesting for protein. Strikingly, a strong negative correlation was observed between the level of ZEB1 protein in the cells and the levels of the EBV IE and E proteins, with the highest level of ZEB1 almost totally inhibiting EBV reactivation by these strong inducers (Fig. 6B). Thus, we conclude that ZEB1 can be a major player in maintaining EBV latency even when other factors are attempting to induce reactivation.

ZEB2 can also regulate maintenance of EBV latency. Given our finding that ZEB2 can functionally substitute for ZEB1 in regulating transcription from Zp via binding the ZV element (Fig. 2), we next asked whether it can also function as an alternative central player in maintaining EBV latency. To answer this question, we repeated the above experiments using a ZEB2 expression plasmid. The results were very similar; i.e., ZEB2 expression led to inhibition of IE and E lytic EBV protein synthesis induced by either the ZEB1 siRNAs (Fig. 6C) or TPA plus sodium butyrate (Fig. 6D), with a strong negative correlation existing between ZEB2 expression level and degree of reactivation (Fig. 6D). Thus, we conclude that ZEB2 can functionally substitute for ZEB1 in maintaining EBV in a latent form of infection.

ZEB2 is the major ZEB protein regulating EBV latency in ZEB2-positive cells. Given our findings with the ZEB2-negative cell lines (Fig. 6), we hypothesized that either ZEB1 or ZEB2 might play a central role in regulating the latent-lytic switch of EBV in a cell-type-specific manner. To test the validity of this hypothesis, we performed experiments as described above but using cell lines that contained both ZEB proteins. One readily transfectable, well-characterized EBV-positive cell line containing high levels of both ZEB1 and ZEB2 is 293^{B95.8} (Fig. 3B, lane 1). Therefore, we began by testing whether transfection of either ZEB1 or ZEB2 siRNAs into EBV-positive 293^{B95.8} cells could induce lytic reactivation of EBV. Unexpectedly, even with fairly good knockdown of both ZEB1 and ZEB2 in these cells, no BZLF1 or BMRF1 protein was detected; i.e., no EBV lytic reactivation occurred (Fig. 7A, lanes 2 to 5).

EBV exists in 293^{B95.8} cells in a very tightly latent state; i.e., reactivation with virus production has been observed only fol-

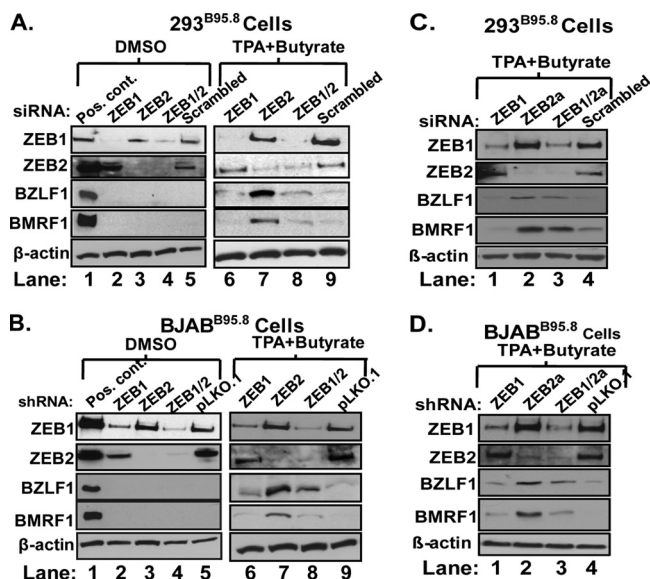


FIG. 7. ZEB2 is the major ZEB protein maintaining EBV latency in ZEB1⁺, ZEB2⁺ cells. (A) Immunoblots showing extent of lytic replication after treatment of 293^{B95.8} cells with various combinations of inducers. 293^{B95.8} cells were transfected with 30 nM the indicated siRNAs. Twenty-four hours later, we added to the medium dimethyl sulfoxide (DMSO) (lanes 2 to 5), the solvent for the chemicals, to 0.1% or TPA and sodium butyrate (lanes 6 to 9) to final concentrations of 20 ng/ml and 3 mM, respectively, and continued incubating the cells for an additional 48 h. Whole-cell extracts were prepared and the indicated proteins were assayed by immunoblot analysis. β -Actin served as the loading control. The positive-control (Pos. cont.) lane contained protein from cells reactivated by transfection with a BZLF1 expression plasmid. (B) Immunoblots showing extent of lytic replication after treatment of BJAB^{B95.8} cells with various combinations of inducers. B-lymphocytic BJAB^{B95.8} cells were infected with lentiviruses expressing the indicated shRNAs. Twenty-four hours later, DMSO (lanes 2 to 5) or TPA plus butyrate (lanes 6 to 9) was added to the medium, and the cells were subsequently treated as described for panel A. (C and D) Analysis of alternative ZEB2 siRNAs and shRNAs in EBV-positive cell lines. (C) 293^{B95.8} cells were transfected with 30 nM siRNAs to ZEB1, to an alternative ZEB2 (indicated as ZEB2a), or to both ZEBs or with negative-control scrambled siRNA. Experiments were performed identically to those described for panel A. (D) BJAB^{B95.8} cells were infected with lentiviruses expressing shRNAs to ZEB1, an alternative ZEB2 (indicated as ZEB2a), both ZEBs, or negative-control pLKO.1, and experiments were performed identically to those described for panel B.

lowing transfection with BZLF1 expression plasmids (14; reviewed in reference 66). For example, while treatment with TPA plus butyrate readily induces EBV into lytic replication in many cell lines, it fails to reactivate the virus in 293^{B95.8} cells (e.g., see reference 83). Given neither TPA plus butyrate nor knockdown of ZEB1 plus ZEB2 is sufficient to induce reactivation in 293^{B95.8} cells, we next tested whether treating the cells with the combination of siRNAs to ZEB1 or ZEB2 together with TPA plus butyrate might do so, since these agents likely affect different regulators of *BZLF1* gene expression (5, 26, 29; reviewed in reference 66). Remarkably, the combination of the ZEB2 siRNAs together with TPA plus butyrate led to a significant increase in BZLF1 and BMRF1 protein levels compared to that of the control scrambled siRNA together with TPA plus butyrate (Fig. 7A, lane 7 versus lane 9). Similar results were seen using other siRNAs to ZEB2 as well (Fig.

7C). Unexpectedly, the combination of the ZEB1 siRNAs together with TPA plus butyrate did not significantly induce EBV lytic protein synthesis (Fig. 7A, lane 6). Thus, we conclude that ZEB2 is the main ZEB family member repressing transcription from Zp in 293^{B95.8} cells despite the abundant presence of ZEB1.

We likewise examined the ZEB1-positive, ZEB2-positive, EBV-positive B-lymphocytic BJAB^{B95.8} cell line to determine whether this conclusion applied to B cells as well. Again, knockdown of ZEB1, ZEB2, or both ZEBs with shRNAs to ZEB1 and ZEB2 resulted in no detectable viral reactivation (Fig. 7B, lanes 2 to 5). Again, lytic protein expression significantly increased above the pLKO.1 shRNA control when the BJAB^{B95.8} cells were treated with the combination of ZEB2 shRNAs and TPA plus butyrate (Fig. 7B, lane 7 versus lane 9; Fig. 7D, lane 2 versus lane 4). And also again, knockdown of ZEB1 together with treating the cells with the chemical inducers led to little reactivation (Fig. 7B and 7D, lane 6 and lane 1, respectively). Thus, we conclude that ZEB2 is the primary ZEB protein regulating maintenance of EBV latency through ZV when both ZEBs are present.

To rule out the possibility that these findings resulted from the ZEB siRNAs inadvertently affecting expression of some cellular genes in addition to the ZEBs, we also tested whether concurrent overexpression of ZEB1 or ZEB2 could inhibit siRNA-induced lytic reactivation. 293^{B95.8} cells were transfected with plasmids expressing ZEB1, ZEB2, or their empty parental plasmid as a control 24 h prior to transfection with the siRNAs to ZEB1, to ZEB2, or to both ZEBs or the scrambled control siRNA and incubation with the chemical inducers TPA plus butyrate as described above. Overexpression of ZEB1 failed to inhibit induction of lytic protein expression (Fig. 8A, lanes 2, 4, and 6 versus lanes 1, 3, and 5, respectively). We examined likewise the role of ZEB2 in affecting lytic reactivation. Consistent with the above findings, overexpression of ZEB2 led to a significant inhibition of induction of EBV lytic IE and E gene expression (Fig. 8B, lanes 2 and 4 versus lanes 1 and 3). We also examined the effects of the addition of increasing amounts of either ZEB1 or ZEB2 on lytic reactivation in the 293^{B95.8} cells. The cells were transfected with various amounts of either ZEB1 or ZEB2 expression plasmids or their empty parental plasmid as a control 24 h prior to their transfection with siRNAs to ZEB2 and incubation with chemical inducers TPA and sodium butyrate. Once again, large amounts of ZEB2 (Fig. 8D), but not ZEB1 (Fig. 8C), led to downregulation of lytic gene expression. Overall, we conclude that ZEB2, but not ZEB1, is the major regulator in ZEB1⁺, ZEB2⁺ cell lines.

ZEB2 can outcompete ZEB1 for binding to Zp. Given ZEB1 did not repress lytic reactivation in ZEB1⁺, ZEB2⁺ cells (Fig. 8), we next wanted to identify the mechanism for this unexpected finding. Postigo and colleagues have reported that ZEB2 has more cellular targets than ZEB1 and hypothesized that ZEB2 may bind better to its target sites than ZEB1 (60, 61). Therefore, we hypothesized that ZEB2 may outcompete ZEB1 for binding to Zp when present.

To test this hypothesis, we performed EMSAs in which various amounts of ZEB2 along with a constant amount of ZEB1 were incubated with the radiolabeled Zp probe DNA. As expected, addition of increasing amounts of ZEB2 led to corre-

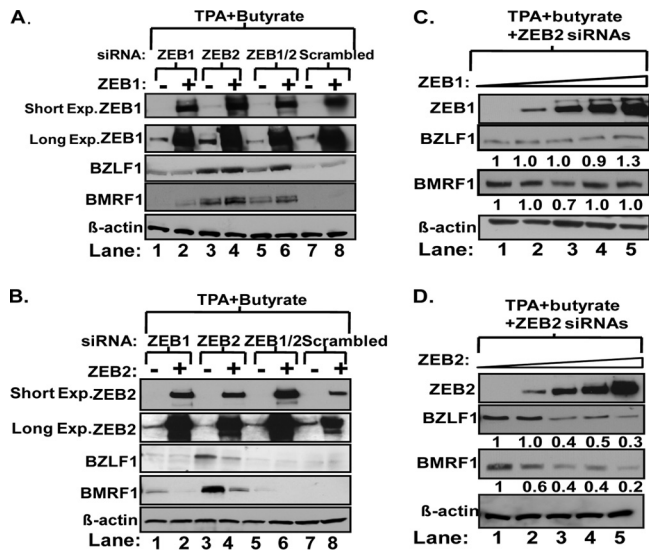


FIG. 8. Confirmation that ZEB2, not ZEB1, is the major ZEB protein inhibiting EBV lytic reactivation in ZEB1⁺, ZEB2⁺ cells. (A and B) Immunoblots showing extent of inhibition of lytic reactivation by overexpression of ZEB1 (A) and ZEB2 (B) in 293^{B95.8} cells subsequently induced by a combination of the indicated siRNAs and TPA plus butyrate. 293^{B95.8} cells were transfected with 1.0 μ g of ZEB1 (A), ZEB2 (B), or their empty parental expression plasmid (-). Twenty-four hours later, the cells were transfected with 30 nM the indicated siRNAs, incubated in the presence of TPA plus butyrate, and processed as described in the legend to Fig. 7. Exp., exposure. (C and D) Immunoblots showing extent of inhibition of lytic reactivation by overexpressed ZEB1 (C) versus ZEB2 (D) in 293^{B95.8} cells induced by BZLF1 protein. 293^{B95.8} cells were transfected with 0, 0.1, 0.25, 0.5, or 1.0 μ g of an expression plasmid encoding ZEB1 (C) or ZEB2 (D), along with 1.0, 0.9 0.75, 0.5, or 0 μ g of pcDNAhismaxC parental vector to allow for equal amounts of transfected DNA. Twenty-four hours later, cells were transfected with 30 nM siRNAs to ZEB2, incubated in the presence of TPA plus butyrate, and processed as described in the legend to Fig. 7. Fold changes in lytic protein expression were determined by densitometry, with internal normalization to β -actin and external normalization to samples containing no ZEBs (lane 1).

spondingly decreasing amounts of ZEB1/DNA complex (Fig. 9, lane 1 versus lanes 2 to 6). Concomitantly, it also led to correspondingly increasing amounts of ZEB2/DNA complexes migrating at two positions in the gel: the one expected for full-length ZEB2 and a faster-migrating one likely due to a carboxy-terminal truncated variant of ZEB2 resulting from premature termination of translation occurring during synthesis of the protein in the cell-free system. Consistent with these identifications, incubation with an antibody specific to ZEB2 eliminated these complexes and led to the appearance of a slower-migrating one (Fig. 9, lane 7). Thus, we conclude that ZEB2 does, indeed, compete with ZEB1 for binding Zp *in vitro*.

We next used chromatin immunoprecipitation assays to determine whether ZEB2 could also compete with ZEB1 for binding to Zp *in vivo*. Our laboratory previously reported that binding of ZEB1 to Zp was detectable in the ZEB1⁺, ZEB2⁺ 293^{B95.8} cell line in a ChIP assay; however, the immunoprecipitated DNA was PCR amplified using a highly sensitive method involving radiolabeled nucleotides (83). Undetermined at that time was whether ZEB2 is also bound to Zp in

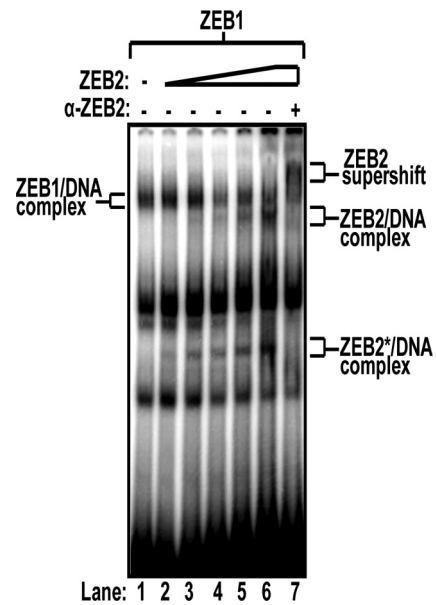


FIG. 9. ZEB1 and ZEB2 compete for binding to Zp *in vitro*. EMSAs in which ZEB1 and ZEB2, synthesized in a cell-free system (TNT), were incubated with a radiolabeled double-stranded WT DNA corresponding to the nt -30 to +20 region of Zp. Each lane contained the same amount of ZEB1 protein. Lanes 2 to 7 also contained ZEB2 protein such that the ratios of ZEB1:ZEB2 for lanes 2 to 6 were 4:1, 2:1, 1:1, 1:2, and 1:4, respectively, with unprogrammed reticulocyte lysate added as well to ensure that each reaction mixture contained the same total amount of protein lysate. Lane 7, the reaction mixture was identical to the one in lane 6 except for the inclusion as well of antibody specific to ZEB2. ZEB, truncated version of ZEB2.

ZEB2-positive cells latently infected with EBV. BJAB^{B95.8} and 293^{B95.8} cells contain high levels of both ZEB proteins (lane 1 of Fig. 3A and B). Using a standard ChIP assay, we found that our ZEB2-specific antiserum, but not our ZEB1-specific antiserum, immunoprecipitated the ZV element-containing region of the BZLF1 promoter of the endogenous EBV genomes present in these cell lines at a level significantly above the level observed with the IgG control antiserum (Fig. 10A and B). As a positive control, we concurrently analyzed these same ChIP-assayed samples for ZEB1 and ZEB2 binding to the cellular interleukin 2 (IL-2) promoter, another DNA known to contain a ZEB-binding element (81); the two antisera immunoprecipitated the IL-2 promoter DNA to similar levels (Fig. 10A and B). As a negative control, we also concurrently analyzed these ChIP-assayed samples for an EBV sequence situated 5 kbp upstream of the ZV element of Zp; as expected, this sequence was not detected in the ChIP-assayed DNAs (Fig. 10A and B). We confirmed these findings by quantifying by real-time PCR the relative amounts of Zp DNA present in the ChIP-assayed samples. Approximately 3-fold more Zp DNA was immunoprecipitated from the chromatin obtained from the 293^{B95.8} and BJAB^{B95.8} cells with the ZEB2-specific antiserum than with the IgG serum (Fig. 10E and F, respectively). On the other hand, the amount of Zp DNA immunoprecipitated by the ZEB1 antiserum was not significantly different from the amount pulled down with the negative-control IgG antiserum.

We likewise performed ChIP analyses on chromatin obtained from ZEB1⁺, ZEB2⁻ CNE1^{Akata} and MutuI cell lines.

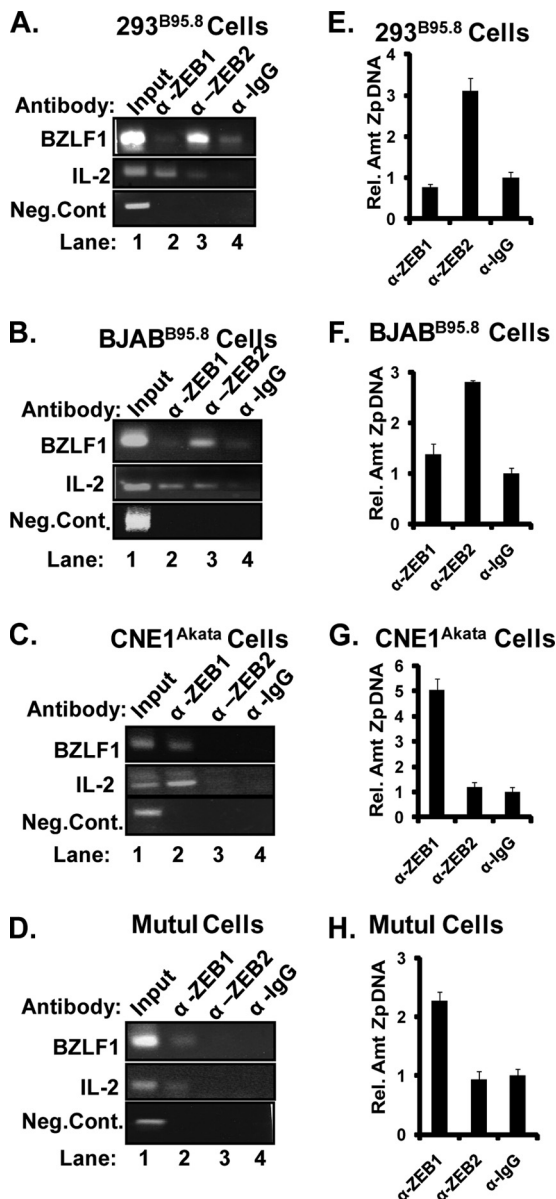


FIG. 10. ZEB1 and ZEB2 compete for binding to Zp *in vivo*. (A to D) ChIP assays indicating relative (Rel.) binding of ZEB1 and ZEB2 to Zp in ZEB2-positive (A and B) versus ZEB2-negative (C and D) cell lines. Shown here are UV light photographs of ethidium bromide-stained agarose gels containing the PCR products obtained from ChIP assays performed as described in Materials and Methods with the indicated antisera on chromatin obtained from EBV-positive 293^{B95.8} cells (A), BJAB^{B95.8} cells (B), CNE1^{Akata} cells (C), and MutuI cells (D). Binding of ZEB1 and ZEB2 was determined for the ZV region of Zp within the EBV genomes, a ZEB-binding region of the interleukin-2 (IL-2) promoter as a positive control, and a sequence within these EBV genomes located 5 kbp upstream of Zp as a negative control (Neg. Cont.). Antibodies to IgG were used as a negative control. (E to H) ChIP-assayed samples from panels A to D above were also analyzed by qPCR for binding of ZEB2 to Zp in 293^{B95.8} cells (E), BJAB^{B95.8} cells (F), CNE1^{Akata} cells (G), and MutuI cells (H), respectively. Data were normalized first to the percentage of input DNA and then to the anti-IgG control.

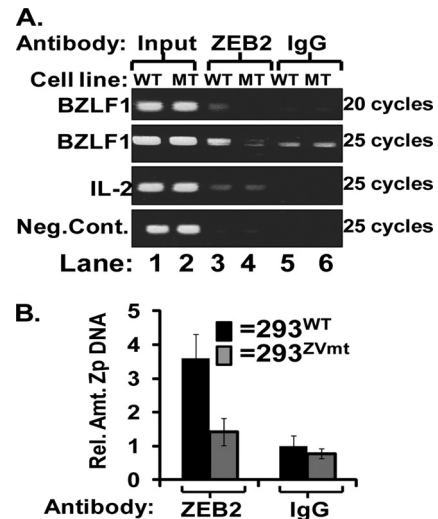


FIG. 11. ZEB2 binding to Zp *in vivo* is dependent upon presence of the ZV element. (A) ChIP assays showing relative binding of ZEB2 to Zp in 293^{B95.8} (WT) versus 293^{ZVmt} (MT) cells. Shown here are UV light photographs of ethidium bromide-stained agarose gels containing the PCR products obtained from chromatin immunoprecipitation assays performed as described in Materials and Methods with the indicated antisera and chromatin from EBV-positive 293^{B95.8} cells (lanes 1, 3, and 5) or 293^{ZVmt} cells (lanes 2, 4, and 6). Binding of ZEB2 was determined for the ZV region of Zp, the IL-2 promoter as a positive control, and a region 5 kbp upstream of Zp as a negative control (Neg. Cont.). (B) ChIP-assayed samples from panel A above were also analyzed by qPCR for binding of ZEB2 to the Zp region of the EBV genomes present in 293^{B95.8} cells versus 293^{ZVmt} cells. Data were normalized to values for both the input DNA and the anti-IgG control with WT EBV.

As expected, the antiserum specific to ZEB1, but not the antiserum specific to ZEB2, immunoprecipitated Zp and IL-2 DNA (Fig. 10C and D). The BZLF1 promoter DNA was enriched 5-fold and 2-fold by the ZEB1 antiserum relative to the negative-control IgG antiserum in the CNE1^{Akata} and MutuI cells, respectively (Fig. 10G and H). Thus, we conclude that ZEB2 outcompetes ZEB1 for binding to Zp *in vivo* when present in the cells at a high level.

ZEB2 binding to Zp *in vivo* is dependent upon the ZV element. We previously reported that 293 cells latently infected with EBV harboring a 2-bp substitution mutation in the ZV element of Zp (293^{ZVmt}) spontaneously reactivate into lytic replication at a significantly higher frequency than do 293^{B95.8} cells harboring WT virus (83). Given our finding that ZEB2 is the major repressor bound to the ZV region of Zp in 293^{B95.8} cells (Fig. 10A), we next asked whether this binding was reduced in this 293^{ZVmt} cell line. ChIP assays revealed, as expected, that ZEB2 bound to the ZV element region of Zp at a significantly higher level in the 293^{B95.8} cells than in the 293^{ZVmt} cells (Fig. 11A). Quantitative real-time PCR analysis of these ChIP-assayed DNAs indicated that the antiserum specific to ZEB2 immunoprecipitated wild-type Zp DNA at a 3-fold higher level than it did ZVmt Zp DNA (Fig. 11B). Therefore, we conclude that ZEB2 repression of BZLF1 gene transcription is dependent upon its ability to bind to the ZV element of Zp in the context of a whole EBV genome (Fig. 11) as well as in transient transfection assays (Fig. 2D).

DISCUSSION

In this study, we further investigated the roles of the ZEBs as transcriptional repressors of *BZLF1* gene expression and, thus, as regulators of the latent-to-lytic switch of the EBV life cycle. Previously, our laboratory reported the importance of ZEB1 as a direct repressor of *BZLF1* gene expression (25, 41, 83). Here, we demonstrated that ZEB2 can also bind to Zp via the ZV element, repressing transcription initiated from Zp (Fig. 2). Analysis of ZEB1 and ZEB2 levels in a comprehensive panel of EBV-positive epithelial and B-cell lines revealed that ZEB1 is present at moderate-to-high levels in most of the cell lines, while ZEB2 is present at detectable levels in only a small subset of them (Fig. 3A to C). Furthermore, a negative correlation was found to exist between expression of EBV lytic genes and the ZEBs (Fig. 4), a finding consistent with data from our laboratory (41, 83; reviewed in reference 58) that the ZEBs usually function as repressors of gene expression. We showed that ZEB1 does, indeed, play a central role in maintenance of EBV latency in ZEB1⁺, ZEB2⁻ cells, with knockdown of ZEB1 leading to lytic reactivation (Fig. 5). Furthermore, overexpression of ZEB1 leads to inhibition of viral reactivation by chemical inducers (Fig. 6). On the other hand, ZEB2, not ZEB1, was found to be the major inhibitor of EBV lytic reactivation in ZEB1-positive, ZEB2-positive cells (Fig. 7 and 8). We also show via EMSAs as well as ChIP assays the competitive binding between ZEB1 and ZEB2 to the ZV element-containing region of Zp within ZEB1⁺, ZEB2⁺ cells latently infected with EBV (Fig. 9 and 10). Taken together, these findings definitively show for the first time that both ZEB1 and ZEB2 can contribute to regulation of the switch between latency and lytic replication of EBV via binding the ZV element of Zp; while ZEB1 is the main repressor in many cell lines, ZEB2 is the main repressor in others.

We previously showed that ZEB1 can bind Zp via the ZV element, repressing transcription initiated from Zp in luciferase reporter assays (41). We also showed that mutation of the ZV element of Zp in the context of a whole EBV genome led to an increased rate of spontaneous reactivation of the virus out of latency into lytic replication (83). However, we did not have definitive evidence that ZEB1 was exclusively responsible for this silencing of *BZLF1* gene expression via the ZV element of Zp during latency. Here, we show that knockdown of ZEB1 is sufficient in some cell lines to induce EBV lytic gene expression (Fig. 5), and overexpression of ZEB1 is sufficient to inhibit lytic reactivation by the strong chemical inducers TPA and butyrate (Fig. 6). Taken together, these findings provide conclusive evidence that ZEB1 is, indeed, a key player in maintenance of EBV latency in some physiologically relevant cell types.

However, we now reveal that ZEB2 can also play an important role in maintenance of EBV latency. ZEB1 and ZEB2 are functionally similar, with both binding to E-box consensus sequences as repressors of gene expression (58, 61, 74, 81). Importantly, ZEB2 has been shown by others to repress a larger number of target genes than ZEB1 (61) and been hypothesized to be a more potent repressor than ZEB1 on some promoters (60, 61). Consistent with these reports, we show here that ZEB2 is the major repressor of *BZLF1* gene expression in ZEB1⁺, ZEB2⁺, EBV-positive cell lines: (i) loss of ZEB2, not

ZEB1, leads to EBV lytic reactivation (Fig. 7); (ii) overexpression of ZEB2, but not ZEB1, inhibits reactivation by chemical inducers (Fig. 8); and (iii) ZEB2, rather than ZEB1, is bound to the ZV element-containing region of Zp in ZEB2⁺ cells (Fig. 9 and 10). Thus, when present, ZEB2 is a key repressor of *BZLF1* gene expression and, thereby, of EBV latency. Likely, the competition seen between ZEB1 and ZEB2 occurs in a cell-line- and promoter-dependent context. ZEB1 and ZEB2, while quite similar in several of their domains, do have unique domains lending to conformational differences and differences in interactions with corepressors (58, 61, 62, 76). ZEB2 may preferentially bind to Zp based on its conformational structure and interaction with corepressors, whereas with other cellular targets that ZEB1 and ZEB2 share, such as E-cadherin (11, 58), ZEB1 may be the dominant repressor bound. This hypothesis has yet to be tested.

Undoubtedly, factors in addition to ZEB1 and ZEB2 contribute to the stringency of EBV latency. For example, treatment of neuronal 293^{B95.8} or B-lymphocytic BJAB^{B95.8} cells with the chemical inducers TPA and sodium butyrate is insufficient to induce lytic replication (Fig. 7) (14). We achieved reactivation in these cell lines only by using a combination of siRNAs to ZEB2 together with these chemical inducers (Fig. 7). Additionally, we identified exceptions to the correlations found between ZEB1 and *BZLF1* expression; for example, the MutuI cell line expressed high levels of both ZEB1 and *BZLF1*, which could be attributed to high expression levels of activators of Zp, such as c-Jun, C/EBP α , or ATF1/2 (Fig. 3A) (25, 26, 77, 82). These findings indicate that more than one change in the status of transcription factors is necessary in these particular cell lines to achieve reactivation. This conclusion is not surprising given the fact that transcription from Zp is known to be regulated by multiple factors, activators as well as repressors (Fig. 1B) (4, 33, 37, 47, 48, 77, 82). The phorbol ester TPA is known to affect Sp1, Sp3, c-Jun, and ATF1, transcription factors that function in activation of *BZLF1* gene expression via binding to the ZI and ZII domains of Zp (25, 48, 77). HDAC inhibitors such as sodium butyrate affect the post-translational modifications on numerous transcription factors, including MEF2D, switching this ZI domain-binding factor from a repressor to activator of Zp (33, 37). An as-yet-unidentified factor that binds the ZIIR repressor element of Zp (47) also appears to play an important role in maintenance of EBV latency (Yu et al., unpublished). Thus, efficient EBV reactivation through turning on expression of the *BZLF1* gene likely usually requires a combination of events leading to loss of some repressors, switch of some repressors to activators, and gain of some activators of Zp. Therefore, the most surprising finding here was that knockdown of ZEB1 by itself is sufficient to induce EBV lytic replication in some types of latently infected cells (Fig. 5).

We propose here a working model consistent with our findings for regulation of *BZLF1* gene expression by ZEB1 and ZEB2 (Fig. 12). In cells such as CNE1^{Akata} that express ZEB1 but not ZEB2, ZEB1 functions during EBV latency as the master repressor, silencing *BZLF1* gene expression by binding directly over the transcription initiation site via the ZV and ZV' elements of Zp; in this case, loss of ZEB1 is sufficient to lead to reactivation into lytic replication (Fig. 12A). However, in cells such as 293^{B95.8} which express both ZEBs, ZEB2 func-

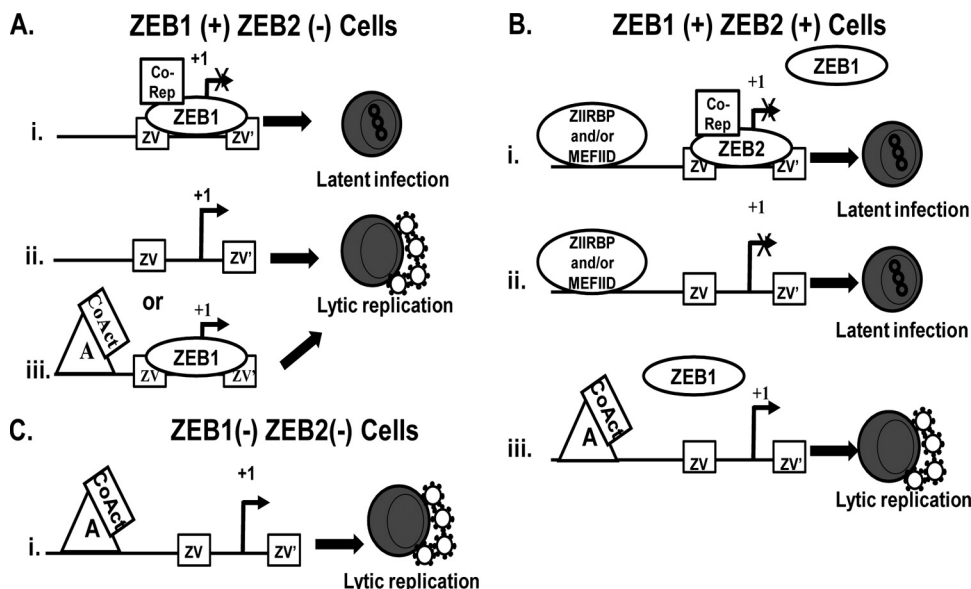


FIG. 12. Working model for how ZEB1 and ZEB2 contribute to regulating *BZLF1* gene expression. See Discussion for details. A, activator; CoRep, corepressor; CoAct, coactivator.

tions as one of two or more major repressors of Zp. In this case, loss of ZEB2 by itself is not sufficient to lead to lytic replication due to the presence of other repressors such as MEF2D (33, 37) and the factor that binds ZIIR (47); rather, induction requires a combination of loss of two or more repressors together with the presence of some coactivators (Fig. 12B). Finally, in cells such as AGS^{B95.8} that express neither ZEB, persistently lytic infection occurs (25) because inadequate silencing of *BZLF1* gene expression enables chronic synthesis of BZLF1 protein to occur, leading to expression of other EBV lytic immediate-early, early, and late genes (Fig. 12C).

Given our conclusion that both ZEB1 and ZEB2 are key players in regulating the latent-lytic switch of EBV, factors that control ZEB1 or ZEB2 levels or their activities may prove useful as agents for lytic induction therapies for treating patients with EBV-associated cancers (21–24, 51, 52). One such potential factor is specific cellular microRNAs that have recently been identified as key regulators of ZEB1 and ZEB2 expression levels in cells (6, 10, 31, 32, 39). Consistent with this idea, we have recently found that transfection of certain members of the 200 family of microRNAs into cells latently infected with EBV can lead to lytic replication of the virus (Ellis et al., submitted). Perhaps, presence of specific cellular microRNAs may account for the differential ZEB1 and ZEB2 protein levels observed in our panel of EBV-positive cell lines (Fig. 3). This hypothesis has yet to be tested.

Several latently expressed, EBV-encoded proteins, small RNAs, and microRNAs have been associated with uncontrolled cell growth, anti-apoptotic functions, and other hallmarks of cancer (8, 17, 34, 59; reviewed in reference 66). Turning on *BZLF1* gene expression is the key to switching between EBV latent infection and lytic replication (12, 13, 20, 72; reviewed in reference 66). Lytic induction therapy may provide a new paradigm for treating patients with EBV-associated malignancies (22–24, 38, 51, 52). Thus, identification of

factors that regulate transcription from Zp and, thereby, the EBV latent-to-lytic switch could lead to the discovery of novel candidate targets for the development of new drugs for such therapeutic approaches. The findings presented here indicate that the ZEBs are excellent targets of this type.

ACKNOWLEDGMENTS

We thank Diane Hayward, Shannon Kenney, Kwok Wai Lo, Bill Sugden, Kenzo Takada, and Lawrence Young for cell lines, Michel Saunders for the ZEB2-specific antiserum, Caroline Alexander, Tom Genetta, Diane Hayward, Shannon Kenney, Takahiro Nagase, and Bill Sugden for cDNA and plasmid constructs, and Shreyasi Das, Shannon Kenney, Richard Kraus, Bill Sugden, and Elizabeth Vu for helpful comments on the manuscript.

This work was funded by grants from the Pardee Foundation and U.S. Health and Human Services NIH grants T32 CA-09135, RO1 AI107034, PO1 CA22443, and P30 CA14520. Z.W. was additionally funded by a National Science Scholarship administered by the Agency for Science, Technology, and Research, Singapore.

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