Shutoff of BZLF1 Gene Expression Is Necessary for Immortalization of Primary B Cells by Epstein-Barr Virus

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The *BZLF1* gene controls the switch between latent and lytic infection by Epstein-Barr virus (EBV). We previously reported that both the ZV and ZIIR elements within the *BZLF1* promoter, Zp, are potent transcription silencers within the context of an intact EBV genome. We report here identification of another sequence element, ZV', which synergized with ZV in repressing Zp via binding ZEB1 or ZEB2. We then determined the phenotype of a variant of EBV strain B95.8 in which the ZV, ZV', and ZIIR elements were concurrently mutated. HEK293 cell lines infected with this triple mutant (tmt) virus spontaneously synthesized 6- to 10-fold more viral *BZLF1*, *BRLF1*, *BMRF1*, and *BLRF1* RNAs, 3- to 6-fold more viral Zta, Rta, and EAD proteins, 3- to 5-fold more viral DNA, and 7- to 9-fold more infectious virus than did 293 cell lines latently infected with either the ZV ZV' double mutant (dmt) or ZIIR mutant (mt) virus. While ZV ZV' ZIIR tmt EBV efficiently infected human primary blood B cells *in vitro*, it was highly defective in immortalizing them. Instead of the nearly complete silencing of *BZLF1* gene expression that occurs within 4 days after primary infection with wild-type EBV, the ZV ZV' ZIIR tmt-infected cells continued to synthesize *BZLF1* RNA, with 90% of them dying within 9 days postinfection. BL41 cells infected with this "superlytic" virus also exhibited increased synthesis of *BZLF1* and *BMRF1* RNAs. Thus, we conclude that the ZV, ZV', and ZIIR silencing elements act synergistically to repress transcription from Zp, thereby tightly controlling *BZLF1* gene expression, which is crucial for establishing and maintaining EBV latency.

Epstein-Barr virus (EBV) is a causative agent of infectious mononucleosis and is associated with several epithelial and B-cell cancers, including Burkitt's lymphoma (BL), Hodgkin's disease, nasopharyngeal carcinoma, and some gastric carcinomas (reviewed in reference 44). EBV can infect primary B lymphocytes, establishing a latent form of infection in which its genome is maintained as an episome replicating in synchrony with its host's cell DNA. EBV can also lytically infect normal human oropharyngeal epithelial cells, while establishing latency in most EBV-associated epithelial malignancies (reviewed in reference 29).

The switch from EBV latency to lytic replication in B cells is usually initiated via activating expression of the immediate-early (IE) *BZLF1* gene. The *BZLF1* gene encodes a sequence-specific DNA-binding protein, Zta (also called "Z," "Zebra," and "EB1"), a member of the bZIP family of leucine-zipper transactivators. The activities of Zta include directly participating in EBV replication via binding to the viral DNA origin of lytic replication, oriLyt, downregulating the latency-associated promoters Cp and Wp, and serving as a transcriptional transactivator of its own promoter, other IE and early (E) viral promoters, including the IE *BRLF1* promoter, Rp, the E *BMRF1* promoter, and several cellular promoters. The *BRLF1* gene encodes a second viral transactivator, Rta (also called "R"). Acting together, Zta and Rta play multiple roles in lytic replication of EBV (12). The *BMRF1* gene encodes a viral DNA polymerase processing factor called "early antigen diffuse" (EAD). It is a marker often indicative of onset of viral lytic replication (reviewed in references 21, 27, and 33).

The *cis*-acting elements of Zp sufficient for both basal and induced activities lie within the region from nucleotide (nt) -221 to +12 (mini-Zp) relative to Zp's transcription initiation site (Fig. 1). Three positive regulatory *cis*-acting elements, named ZI, ZII and ZIII, have been identified within mini-Zp (reviewed in reference 47). The ubiquitous transcription factors Sp1 and Sp3 can bind to the AT-rich ZIA, ZIC, and ZID elements, while the myo-

cyte enhancer factor 2D (MEF-2D) can bind to the ZIA, ZIB and ZID elements (6, 36, 37). ZII is a cyclic AMP (cAMP) response element-like motif that binds CREB, ATF family members, C/EBPs, and the AP-1 family of transcription factors (5, 14, 19, 48, 50, 52). The ZIII element contains multiple Zta-binding sites (15). In addition, we and others have identified multiple Smad-binding elements (SBEs) in Zp through which transforming growth factor β , a cellular cytokine, can activate *BZLF1* gene expression (20, 32).

Given that the *BZLF1* gene functions as the key switch between latent and lytic replication of EBV in B cells, Zp needs to be tightly repressed to maintain latency. This end is achieved by the presence within Zp of multiple negative regulatory elements. Three silencing elements have been identified to date within the mini-Zp region: ZIIR, HI ϵ , and ZV (30, 31, 35, 46, 53). In addition, a phosphorylated form of MEF-2D bound to ZIA, ZIB, and ZID can also repress Zp by recruiting histone deacetylases (HDACs) to maintain chromatin in a repressed state (7). Other silencing elements of Zp, ZIV and HI ϵ -HI δ , lie within the region from nt -551 to -222 of the promoter (41, 42, 46, 49). However, they have not been extensively characterized, and their impact on *BZLF1* gene expression and establishment and maintenance of EBV latency remains to be determined in the context of an intact EBV genome.

We previously reported that the ZV element, located at nt -17 to -12 relative to the transcription initiation site of Zp, contributes to silencing of *BZLF1* gene expression by being a binding site for the cellular zinc-finger, E-box binding proteins ZEB1 and

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FIG 1 Schematic diagram indicating the *cis*-acting regulatory elements present within the region from nt -237 through +40 of Zp relative to the transcription initiation site. Rectangles along Zp indicate approximate locations of known regulatory elements; their known *trans*-acting factors are indicated above them. The small rectangles below Zp indicate Smad-binding elements (SBEs) 1 through 5 (20, 32). The nucleotide sequences of the ZIIR, ZV, and ZV' elements are shown, with the base substitution mutations introduced into the mutant oligonucleotides and variant EBVs studied here indicated below the downward arrows.

ZEB2 in a cell-type-dependent manner. Cells latently infected with a variant of the B95.8 strain of EBV containing a 2-bp substitution mutation in the ZV element spontaneously reactivate into lytic replication with production of infectious virus (10, 11, 13, 30, 31, 54). Furthermore, addition of either of the cellular microRNAs (miRNAs) 200b or 429 can induce EBV lytic replication in EBV-positive cells by downregulating expression of ZEB1 and ZEB2 (11, 34). Here, we show that Zp contains a second ZEB-binding element, named ZV', located at nt +5 to +10; it synergizes with the ZV element to enable high-affinity binding of ZEB1 and ZEB2 via their two zinc-finger domains (Fig. 1).

The ZIIR silencing element, initially identified by Liu et al. (35), is located at nt -77 to -70 juxtaposed between the positive regulatory elements ZID and ZII (Fig. 1). We recently reported that variants of EBV strain B95.8 harboring base pair substitution mutations in the ZIIR element are somewhat defective in establishing proliferating B-cell colonies; the EBV-infected cells that do manage to grow out spontaneously reactivate into lytic replication, doing so at a very high frequency in the presence of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (53). The identity of the cellular factor(s) through which ZIIR silences *BZLF1* gene expression remains unknown.

Here, we report the phenotype of variants of EBV concurrently containing substitution mutations in the ZV, ZV', and ZIIR elements of Zp. We show that the ZV ZV' ZIIR triple mutant (tmt) EBV is much more defective in establishing latency than are EBVs singly mutated in these silencing elements. ZV ZV' ZIIR tmt EBV is highly defective in immortalizing human primary blood B cells *in vitro* because the cells die. HEK293 cells that manage to survive infection with ZV ZV' ZIIR tmt EBV spontaneously reactivate into lytic replication at a high frequency. BL41 cells infected with this "superlytic" EBV synthesize a much higher level of *BZLF1* RNA than do cells infected with either the ZV ZV' double mutant (dmt) or ZIIR single mutant (mt) EBV. Thus, we conclude that synergistic repression of Zp by multiple transcriptional silencers plays a crucial role in establishing and maintaining EBV latency.

MATERIALS AND METHODS

Cells and plasmids. The HEK293 and Burkitt's lymphoma (BL) cell lines Raji and DG-75 used here were previously described (54). BJAB and BL41 are EBV-negative BL cell lines obtained, respectively, from Bill Sugden and Elliott Kieff. These BL cell lines and human blood primary B cells were

maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The 293 cell lines infected by EBV were maintained in the same medium, additionally supplemented with hygromycin (100 µg/ml). The ZEB1 and ZEB2 expression plasmids were previously described (10).

The base pair substitution mutations in the ZV, ZV', and ZIIR elements were introduced into Zp by PCR-based, site-directed mutagenesis. The pZp-luc reporter plasmids (Fig. 2B) were constructed by cloning the region from nt -221 to +32 relative to the transcription initiation site of the wild-type (WT) or mutant Zp between the KpnI and HindIII sites of the pGL3-Basic luciferase vector (Promega). The bacmid p2089 (9) and *Escherichia coli* strains and plasmids used for mutagenesis of the EBV genome were described previously (54).

Transient-transfection and luciferase assays. HEK293 cells grown in 24-well plates were transfected with 0.2 µg DNA/well using TransIT LT1 reagent (Mirus) and harvested after incubation at 37°C for 48 h. For transient transfection of DG-75 and BJAB cells, 1×10^7 cells were resuspended in 500 µl of RPMI 1640 medium supplemented with 10% FBS, mixed with 4 µg of the indicated reporter plasmid, and transferred to a 4-mm cuvette (Bio-Rad). Cells were electroporated at 1.5 kV and 25 µF and 0.75 kV and 25 µF, respectively, using a Gene Pulser device (Bio-Rad). Cells were then grown in fresh medium and harvested after incubation at 37°C for 48 h. Preparation of whole-cell extracts and luciferase assays were performed according to the manufacturer's instructions (Promega). Luciferase activities were normalized to protein concentrations determined by a modified Bradford assay (Bio-Rad).

Isolation of ZV ZV' ZIIR tmt virus. Base pair substitution mutations were introduced into bacmid p2089 as previously described (54). The variants were identified by a PCR-based screen. The presence of the desired mutations was confirmed by DNA sequence analysis of the region from nt -600 to +500 of Zp relative to its transcription initiation site. The WT and mutant bacmids were also checked for presence of any major genome alterations by analysis of EcoRI, BamHI, SalI, and HindIII restriction fragment patterns. A WT revertant of the p2089-ZV ZV' ZIIR tmt, p2089-ZV ZV' ZIIR tmtRev, was likewise constructed by mutagenesis of the p2089-ZV ZV' ZIIR tmt.

The transfection of 293 cells with bacmids, isolation of WT- and mutant EBV-infected 293 cell lines, and preparation of virus stocks by induction of latently infected 293 cell lines were performed as previously described (54). The Zp region of EBV was amplified by nested PCR of virus stocks, and the mutations in ZV, ZV', and ZIIR (or lack of them) were confirmed by DNA sequence analysis.

IFS and infectious virus assays. Immunofluorescence staining (IFS) was performed as previously described (53, 54). Titers of infectious virus were quantified by a Raji assay using 3-fold serial dilutions of each sample

FIG 2 The ZV' element is a ZEB-binding site that contributes to repression of transcription from Zp. (A) Autoradiogram of competition EMSAs showing that both the ZV and ZV' elements are ZEB1-binding sites within Zp. Competition EMSAs were performed by incubation of 25 μ g of protein from WCEs prepared from ZEB1 expression plasmid-transfected 293 cells with radiolabeled double-stranded oligonucleotide (Oligo) probe corresponding to the region from nt -30 to +30 of Zp and various amounts (20, 40, and 80 times molar excess) of the indicated competitor oligonucleotides. The position of the ZEB1-DNA complexes is indicated on the left. (B) Schematic showing the structure of the Zp-luc reporter plasmid containing the region from nt -221 to +32 of Zp inserted into pGL3-Basic (Promega). (C) Luciferase assays showing that the ZV and ZV' elements synergistically repress transcription from Zp. 293, DG-75, and BJAB cells were transiently transfected with pZp-lucWT and the indicated mutant variants of it shown in Fig. 1. Forty-eight hours later, the cells were harvested, and luciferase activities were determined, with normalization to the concentration of protein present in each extract. The histogram shows means plus standard errors of the means from one representative set of experiments performed in triplicate on four occasions. (D) Immunoblot showing ZV and ZV' elements both contribute to repression of transcription from Zp in EBV-infected 293 cell lines. Shown here are the relative levels of viral Zta, Rta, and EAD proteins present in 293-WT, 293-ZV mt, 293-ZV ZV' dmt, and 293-ZV mtRev (a WT revertant of the 293-ZV mt). Samples were run in the same gel. The cellular protein β -actin present in the same samples served as a loading control.

on two or more occasions as previously described (1, 54). Relative titers of spontaneously produced infectious virus were determined in parallel for multiple independently isolated clones of 293 cells infected with the triple mutant versus the ZV ZV' dmt and ZIIR mt.

B-cell proliferation assay. Human primary blood B cells were purchased from AllCells (Emeryville, CA), infected with the indicated amount of virus in green Raji units (GRU)/cell, aliquoted at 1×10^4 cells per well in 96-well plates, incubated at 37°C for 6 weeks, and examined for the percentage of wells containing proliferating, GFP-positive B cells as previously described (53).

EBV infection of B cells. For analysis of viral RNA production, human primary blood B cells were infected with WT or mutant EBV at a multiplicity of infection (MOI) of 0.1 GRU/cell by incubation at 4°C for 2 h with slow rotation, spun down, washed, resuspended in fresh medium, and plated at 5×10^5 cells in 3 ml medium per well of 12-well plates. Whole-cell RNA was isolated after incubation of the infected cells at 37°C for 1, 2, 4, 6, and 8 days. EBV-negative BL41 cells were infected at an MOI of 0.1 GRU/cell, incubated at room temperature for 6 h with slow rotation, washed, resuspended in fresh medium, plated at 2×10^6 cells in 3 ml medium per well of 12-well plates, and harvested for whole-cell RNA after incubation at 37°C for 4 days.

RT-qPCR analysis of EBV RNA. Total RNA was isolated from infected primary B cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Contaminating genomic DNA was removed from the samples using RNase-free DNase (Ambion). Total RNA was isolated from infected 293 and BL41 cells using an RNeasy RNA isolation kit (Qiagen); the contaminating genomic DNA was removed from the samples using RNase-free DNase (Qiagen). When PCR analysis of these RNA samples was carried out using a primer set which detected EBV genomic DNA, no contaminating genomic DNA was detected after DNase treatment. The RNA concentration was quantified with a Nanodrop spectrophotometer, and cDNA was synthesized from 150 ng of RNA from primary B cells or 200 to 1200 ng of RNA from 293 and BL41 cells using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. For

real-time quantitative PCR (RT-qPCR), the cDNA, a pair of PCR primers, and the SYBR green PCR master mix (Applied Biosystem) were mixed, and the levels of the viral RNAs, green fluorescent protein (GFP) RNA, and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA were determined using a MyiQ thermal cycler (Bio-Rad). The RT-qPCR conditions were as follows: 50°C for 2 min and 96°C for 10 min, followed by 96°C for 15 s and 57 to 60°C (57°C for *BRLF1* and *BLLF1* primer sets; 58°C for GAPDH, *BZLF1*, and *BMRF1* primer sets; 60°C for GFP primer set) for 1 min for 40 cycles. Data obtained from RNA isolated from 293 and primary B cells were normalized to cellular GAPDH RNA. Data obtained from BL41 RNA were normalized to GFP RNA using the threshold cycle ($\Delta\Delta C_T$) method (38). cDNA samples obtained from RNA isolated from mock-infected cells were included as a negative control. The PCR primers used are shown in Table 1. The conditions for qPCR yielded over 95% PCR efficiency and an r^2 value greater than 0.99 for each primer set.

RT-qPCR analysis of EBV DNA. Total genomic DNA was isolated from 293 cell lines latently infected with WT and mutant variants of EBV using DNeasy blood and tissue kits (Qiagen). The DNA concentration was quantified with a Nanodrop spectrophotometer. Approximately 75 to 100 ng of DNA was used in each reaction. The levels of EBV DNA and cellular β_2 -microglobulin were determined by RT-qPCR as described above. The RT-qPCR conditions were as follows: 50°C for 2 min and 96°C for 10 min, followed by 96°C for 30 s and 62°C for 1 min for 40 cycles. Data obtained from EBV DNA were normalized to cellular β_2 -microglobulin using the $\Delta\Delta C_T$ method. The PCR primers used are shown in Table 1.

Semiquantitative PCR detection of EBV latent RNAs. Two microliters of cDNA sample was included in each PCR (20- μ l total reaction volume) performed with a REDTag ReadyMix PCR kit (Sigma). The PCR conditions were as follows: 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min for 30, 36, or 40 cycles as indicated in Fig. 10. The sequences of the primers used and their product sizes are shown in Table 1.

Cell viability. Cell viability was assayed by performing a trypan blue exclusion test. Briefly, at various times after EBV infection of primary blood B cells (a generous gift from Jenny Gumperz, University of Wisconsin-

TABLE 1 Sequences of PCR primers used in this study^a

Primer	Product Size (bp)	Direction	Sequence (5'→3')	Reference
BZLF1	91	Forward Reverse	AAATTTAAGAGATCCTCGTGTAACATC CGCCTCCTGTTGAAGCAGAT	45
BRLF1	96	Forward Reverse	ATGGAACATGCGTCGTTG AATGGCCACGCTCAACAT	—
BMRF1	102	Forward Reverse	CAACACCGCACTGGAGAG GCCTGCTTCACTTTCTTGG	18
BLLF1	110	Forward Reverse	ATCCAGTTGTATTCAAGGTAGG ACTCATTATCACACGAACGG	—
GAPDH	173	Forward Reverse	TTGCCATCAATGACCCCTTCA CGCCCCACTTGATTTTGA	—
EGFP	121	Forward Reverse	CTGGACGCGACGTAAAC CGGTGGTGCAGATGAACCT	16
BamHIW	83	Forward Reverse	GCAGCCGCCAGTCTCT ACAGACAGTGCACAGGAGCCT	45
β ₂ -Microglobulin	80	Forward Reverse	GGAATTGATTTGGGAGAGCATC CAGGTCCTGGCTCTACAATTTACTAA	22
BALF5	95	Forward Reverse	AGTCCTTCTGGCTAGTCTGTTGAC CTTTGGCGCGGATCCTC	22
EBNA1	220	Forward Reverse	TGCCTGAACCTGTGGTTGG GTACCTGGCCCCCTCGTCA	3
EBNA2	95	Forward Reverse	GCTTAGCCAGTAACCCAGCACT TGCTTAGAAGGTTGTTGGCATG	3
LMP1	132, 209	Forward Reverse	AATTTGCACGGACAGGCATT AAGGCCAAAAGCTGCCAGAT	3
LMP2A	284	Forward Reverse	CGGGATGACTCATCTCAACACATA CATGTTAGGCAAATTGCAAA	3

^a EGFP, enhanced green fluorescent protein; —, designed by the authors.

sin—Madison), the cells were washed twice with phosphate-buffered saline (PBS), resuspended in PBS, mixed with an equal volume of 0.4% trypan blue, incubated for 10 min at room temperature, and visually counted by light microscopy with the aid of a hemacytometer for the percentage of blue-stained (i.e., nonviable) cells.

RESULTS

Identification of a secondary ZEB-binding site, ZV', in Zp. We previously reported the identification of the ZV silencing element that contributes to repression of *BZLF1* gene expression by binding the cellular factor ZEB1 or ZEB2. ZEB1 and ZEB2 both contain two zinc finger domains. While these proteins can bind DNA via either of their zinc finger domains, they bind with higher affinity if bound concurrently via both zinc finger domains to two properly spaced binding sites. ZEB-binding sites found in eukaryotic promoters are often bipartite, consisting of a high-affinity element with consensus sequence 5'-CACCTG-3' and a low-affinity element with consensus sequence 5'-CACCT-3' (43). This prompted us to search for an additional ZEB-binding element located near the ZV element, leading to identification of the sequence 5'-CACCTT-3' between nt +5 and +10 relative to the transcription initiation site of Zp, named here "ZV'" (Fig. 1).

To examine whether the ZV' element is a binding site for ZEB1, we performed competition electrophoretic mobility shift assays (EMSAs). Whole-cell extracts (WCEs) prepared from 293 cells transfected with a ZEB1 expression plasmid were incubated with a radiolabeled oligonucleotide corresponding to the region from nt -30 to +30 of WT Zp and with various amounts of WT or the indicated mutant oligonucleotide as competitor. While the ZV' mt oligonucleotide competed much more efficiently than did the ZV mt one for binding ZEB1 (Fig. 2A, lanes 9 to 11 versus lanes 12 to 14, respectively), it did not compete as well as did the WT oligonucleotide (Fig. 2A, lanes 3 to 5). Interestingly, the ZV ZV' dmt oligonucleotide was almost completely defective in competing for binding ZEB1 (Fig. 2A, lanes 6 to 8). Similar results were observed when WCE prepared from 293 cells overexpressing ZEB2 was used as the source of protein in the competition EMSAs (data not shown). Base pair substitution mutations in other nucleotides within the ZV' element showed even less of an effect on ZEB1 binding (data not shown). Therefore, we conclude that the ZV' element is also a ZEB-binding site, albeit a much weaker one than is the ZV element.

We performed transient-transfection assays with reporter

FIG 3 Luciferase assays showing the synergistic effect of mutations in the ZV, ZV', and ZIIR elements on transcription from Zp in both DG-75 (A) and BJAB (B) cells. The base pair substitution mutations in the ZV, ZV', and ZIIR elements are the ones indicated in Fig. 1. Luciferase activities were normalized to the concentration of protein present in each extract. Histograms show means plus standard errors of the means from a representative set of experiments performed in triplicate on four occasions. rel., relative.

plasmids to examine the effects of mutations in these ZEB-binding elements on transcription from Zp. In 293 cells, concurrent mutation of both the ZV and ZV' elements led to stronger derepression of Zp than did mutation of either element by itself, with the ZV' mt making a smaller contribution than the ZV mt to this derepression (Fig. 2C). This finding is fully consistent with the relative affinities of these two elements for binding by the ZEBs. Similar results were also obtained in the BL cell lines DG-75 and BJAB (Fig. 2C).

To examine whether the ZV' element contributes to repression of *BZLF1* gene expression in the context of an intact EBV genome, we constructed a mutant variant of EBV strain B95.8 containing the base pair substitution mutations indicated in Fig. 1 in both the ZV and ZV' elements within the bacmid p2089. HEK293 cells were transfected with this mutant bacmid, and clones of cells latently infected with it were selected based upon resistance to hygromycin. Immunoblot analysis showed that cells infected with the ZV ZV' double mutant virus contained approximately 2-fold more IE Zta and E EAD protein than did cells infected with the ZV mutant virus (Fig. 2D, lane 3 versus lane 2). Thus, we conclude that the ZV' element contributes to repression of Zp, albeit modestly, as a low-affinity ZEB-binding site; together with the ZV element, it forms a high-affinity ZEB-binding site through which ZEB1 or ZEB2 can strongly repress transcription from Zp in the context of an intact EBV genome.

Synergistic effects of ZV, ZV', and ZIIR elements on transcription. We reported previously that both the ZV and ZIIR elements function as potent silencers of Zp in the context of an intact EBV genome (53, 54). To begin to understand how the ZV, ZV', and ZIIR elements collectively function to regulate transcription from Zp, we first assessed the effects of mutations in these elements individually versus concurrently using a transient-transfection assay. DG-75 and BJAB cells were electroporated with pZp-luc reporter plasmids containing either the wild-type or combinations of the mutant variants of Zp indicated in Fig. 1. In both cell lines, the reporter plasmid mutated in all three elements consistently exhibited a level of derepression that was more than the sum of the derepression relative to the wild type observed when only the ZEB-binding elements or ZIIR element were mu-

FIG 4 Immunoblot showing that 293-ZV ZV' ZIIR tmt cell lines contain more viral IE and E proteins than do either 293-ZV ZV' dmt or 293-ZIIR mt cell lines. The 293-ZV ZV' ZIIR tmt lines 1 through 3 were established by transfection of 293 cells with three completely independent isolates of the p2089-ZV ZV' ZIIR tmt bacmid. Cellular α-tubulin served as a loading control.

tated (Fig. 3). Thus, we conclude that ZV, ZV', and ZIIR act synergistically to repress transcription from Zp in BL cell lines.

Phenotype of ZV ZV' ZIIR tmt EBV in 293 cells. To test whether this synergy between the ZV, ZV', and ZIIR elements also occurs in the context of an intact EBV genome, we introduced the same base pair substitution mutations into the Zp region of p2089, a bacmid containing the complete B95.8 strain of EBV (9). Three completely independent ZV ZV' ZIIR tmt bacmids were isolated in *E. coli*. The region from nt -600 to +500 of Zp relative to its transcription initiation site was PCR amplified and sequenced to confirm the presence of only the expected substitution mutations within the ZV, ZV', and ZIIR elements. The three tmt bacmids were also separately digested with EcoRI, BamHI, SalI, and HindIII restriction endonuclease; no differences in any of the restriction fragment patterns were observed compared to the parental p2089 DNA, except for introduction of a new EcoRI site due to the ZIIR mutation.

To further rule out the possibility that extraneous mutations may have been arisen outside the region sequenced that significantly affected the phenotype of the mutant EBVs, we transfected each of these three tmt bacmids individually into 293 cells and hygromycin selected clones of cells latently infected with each bacmid. Again, the bacmids in these cell lines were thoroughly checked as described above for the presence of the described base pair substitution mutations and the patterns generated by restriction enzyme digestion. In addition, they were tested for the ability to produce infectious virus upon transfection with a Zta expression plasmid; high-titer virus stocks (i.e., 10^4 to 10^5 GRU/ml) could be obtained from 293 cells infected with each of these tmt bacmids. Immunoblot analysis indicated that these 293-tmt-infected cell lines were similar with respect to expression of viral lytic genes, accumulating 3- to 6-fold more Zta and EAD protein than did either ZV ZV' dmt- or ZIIR mt-infected cell lines grown in culture for a similar amount of time (Fig. 4, lanes 4 to 6 versus lanes 2 and 3, respectively). Given the phenotypes of the 293-tmt-infected cell lines were similar, our subsequent work was primarily performed with the first of these bacmids, referred to hereafter as the "ZV ZV' ZIIR tmt."

To demonstrate that the phenotype of this bacmid was truly the result of the mutations we had introduced into Zp, we also constructed a wild-type revertant of it, ZV ZV' ZIIR tmtRev. The

FIG 5 Phenotype of ZV ZV' ZIIR tmt virus in 293 cells. (A) RT-qPCR analysis of the indicated viral IE, E, and L RNAs isolated from clones of 293 cells infected with the indicated mutant variants of EBV. Histogram shows means plus standard errors of the means from one representative set of experiments performed in triplicate on three occasions. (B) Representative photomicrographs showing IFS of 293 cells latently infected with WT and the indicated mutant EBVs. Fields of cells were photographed with different filters to show the total cells (GFP) versus the subset of them expressing gp350 protein. The percentages of gp350-positive cells (% Pos.) are indicated at the bottom of each field; these values were obtained by scoring 5 fields of cells. (C) Quantitative PCR analysis of viral DNA isolated from individual clones of 293 cells infected with WT EBV and the indicated mutant variants of EBV. The histogram shows means plus standard errors of the means from one representative set of experiments performed in triplicate on three occasions. (D) Green Raji unit assay showing relative (rel.) levels of production of infectious virus from 293 cells latently infected with WT and mutant EBVs. Fields of Raji cells were examined with visible light for total cell number versus fluorescence for EBV-infected, GFP-positive cells.

phenotype of this revertant was similar to that of the original parental WT bacmid, p2089. For example, as observed with 293-WT cell lines (e.g., Fig. 4, lane 1), neither IE Zta nor E EAD protein could be detected by immunoblot analysis in WCEs made from 293-ZV ZV' ZIIR tmtRev cell lines (data not shown). Likewise, the WT and ZV ZV' ZIIR tmtRev viruses synthesized similar amounts of viral IE *BZLF1* and E *BMRF1* RNAs at 4 days after infection of BL41 cells (see Fig. 8 below).

Three independent clones of cells infected with this triple mutant bacmid were analyzed for viral lytic gene expression by RT-qPCR. Again, their phenotypes were similar, with the levels of the viral IE *BZLF1* and *BRLF1*, E *BMRF1*, and L *BLLF1* RNAs being 6- to 10-fold higher than the levels observed in the cells infected with either ZV ZV' dmt or ZIIR mt (Fig. 5A). Immunoblot analysis of the viral Zta, Rta, and EAD protein levels present in these cell lines correlated well with the RT-qPCR results (data not shown). IFS assays indicated that 3- to 5-fold more of the 293 cells infected with the triple mutant were positive for Zta, Rta, EAD, and gp350 proteins than were the 293 cells infected with the ZIIR mt EBV (Fig. 5B). The viral glycoprotein gp350 is synthesized from *BLLF1* RNA following viral DNA replication. Consistent with this finding, we also observed 3- to 5-fold higher levels of replicated EBV DNA (Fig. 5C) and 7- to 9-fold greater spontaneous production of infectious virus (Fig. 5D) in the 293 cell lines infected with the triple mutant than in the ones infected with ZIIR mt EBV. Thus, we conclude that the ZV, ZV', and ZIIR elements synergistically act to strongly silence *BZLF1* gene expression in the context of an intact EBV genome; abrogation of these functions can lead to high-level spontaneous reactivation into lytic replication, with the

production of infectious virus in 293 cells in which WT EBV is usually highly latent.

Phenotype of ZV ZV' ZIIR tmt EBV in primary and BL B cells. To examine the phenotype of the ZV ZV' ZIIR tmt EBV in a physiologically relevant cell type, we infected human primary blood B cells in parallel with titer-determined virus stocks of WT, ZVmt, ZV ZV' dmt, ZIIR mt, and ZV ZV' ZIIR tmt EBV at a multiplicity of infection (MOI) of 25 green Raji units per 10^4 cells and aliquoted the infected cells at 10^4 cells per well in 96-well plates. After incubation for 6 weeks at 37°C, we scored the wells for presence of GFP-positive, proliferating B-cell colonies. While mutations in ZV, ZV ZV' mt, and ZIIR reduced the efficiency of induction of proliferating B-cell colonies by approximately 2-, 5-, and 10-fold relative to WT EBV, respectively, no proliferating colonies were observed in B cells infected with the ZV ZV' ZIIR tmt EBV (Fig. 6). We repeatedly attempted, without success, to isolate B-cell colonies infected with ZV ZV' ZIIR tmt EBV, including using virus stocks made from each of three independent 293-ZV ZV' ZIIR tmt cell lines and B cells obtained from 3 different donors.

When we raised the MOI to 100 GRU per 10^4 cells, we detected proliferating B-cell colonies in 0, 1, and 4 wells of three 96-well plates of cells infected with the triple mutant on three different days. We were able to grow out lymphoblastoid cell lines (LCLs) from only 2 of these 5 wells. The EBV genome present in these 2 LCLs contained the expected substitution mutations in the ZV, ZV', and ZIIR elements based upon PCR amplification and DNA sequencing. However, we could not detect by immunoblot analysis any Zta or EAD protein in these cells (data not shown). Given

FIG 6 ZV ZV' ZIIR tmt EBV fails to induce proliferation of human primary blood B cells. Human primary blood B cells were infected in parallel with the indicated viruses, incubated for 6 weeks at 37°C, and scored for the presence of GFP-positive, proliferating B-cell colonies. Shown here are efficiencies of induction of proliferation by the indicated mutant viruses relative to WT virus, with means and standard errors of the means of data obtained from three sets of infections performed in parallel on different days starting with B cells obtained from three different donors.

that LCLs infected with ZIIR mt EBV synthesize high levels of Zta and EAD protein (53) and ZV ZV' ZIIR tmt EBV expresses lytic genes at even a higher level than does ZIIR mt EBV, we conclude that these rare tmt-infected LCLs probably escaped death due to the presence of either extraneous mutations or epigenetic modifications in the EBV genome that blocked synthesis of Zta. Thus, we conclude that the ZV ZV' ZIIR tmt EBV is highly defective in inducing proliferating colonies of human primary blood B cells.

The severe defect in the ability of ZV ZV' ZIIR tmt EBV to immortalize primary B cells is likely due to its failure to turn off *BZLF1* gene expression following infection. To directly test this possibility, we performed a time course experiment. Primary blood B cells were infected in parallel with WT and the various Zp mutant EBVs. Cellular RNA was extracted at various times postinfection, and the relative levels of IE *BZLF1* RNA were quantified by RT-qPCR. In agreement with Wen et al. (51), we readily observed *BZLF1* RNA 1 day after infection with WT EBV, but its amount rapidly decreased thereafter (Fig. 7). On the other hand, the level of *BZLF1* RNA present in the tmt-infected cells declined only gradually with time, possibly plateauing within a week at a level approximately one-third of the level observed at day 1. The levels of *BZLF1* RNA present in the cells infected with ZV ZV' dmt and ZIIR mt EBV were between these two extremes.

We also examined the phenotype of these mutants following infection of the EBV-negative cell line BL41. Again, the level of *BZLF1* RNA observed 4 days after infection was much greater in the tmt-infected BL41 cells than it was in the WT-infected BL41 cells and was significantly greater than the sum of the levels observed in the cells infected with ZV ZV' dmt and ZIIR mt EBV (Fig. 8A). The triple-mutant-infected BL41 cells also contained at 4 days after infection more lytic gene E *BMRF1* RNA than did the BL41 cells infected with WT EBV or the other mutant EBVs, albeit only a few fold more (Fig. 8B) rather than the 100-fold more seen for *BZLF1* RNA.

Thus, we conclude that EBV mutated in both of the ZV ZV' and ZIIR elements dramatically overexpresses its *BZLF1* gene throughout the first week following primary infection of B cells.

FIG 7 ZV ZV' ZIIR tmt EBV fails to shut off synthesis of *BZLF1* RNA following infection of primary blood B cells. Human primary blood B cells were infected in parallel with 0.1 GRU/cell of each of the indicated viruses. After incubation at 37°C for the times indicated, the cells were harvested. Whole-cell RNA was purified, and the level of *BZLF1* RNA was determined relative to the level of GAPDH RNA present in the same sample by RT-qPCR. Data are plotted relative to the level obtained for each virus on day 1.

Triple-mutant-infected primary blood B cells die. Why is the triple mutant EBV highly defective in inducing proliferating B-cell colonies? To answer this question, we assayed tmt- and WT-infected primary blood B cells for cell viability by trypan blue staining. While both the WT and the triple mutant similarly induced proliferation of the B cells within 2 days postinfection based upon the percentage of enlarged cells observed by light microscopy, approximately half of the tmt-infected cells went on to die by day 7 rather than continuing to proliferate. By day 9 postinfection, 90% of the tmt-infected cells were dead, whereas only 9% of the WT-infected cells were dead, as indicated by retention of trypan blue (Fig. 9A and B). Therefore, we conclude that infection of primary blood B cells with this triple mutant EBV fails to result in production of proliferating B-cell colonies because continued expression of the *BZLF1* gene leads, instead, to cell death.

Some EBV genes expressed during latency, including *EBNA1*, *EBNA2*, *LMP1*, and *LMP2A*, are essential for B-cell transformation and immortalization (2, 8, 40, 55). Overexpression of Zta may directly or indirectly affect expression of these genes (28). Thus, to determine whether failure of the triple mutant to immortalize B cells might be due to altered expression of some of these latency genes, we measured by semiquantitative PCR the accumulation of their transcripts in primary blood B cells 1 through 8 days postinfection with WT EBV versus the triple mutant EBV. We also measured likewise the same RNA samples for accumulation of *BZLF1* and *BALF5* RNAs. *BALF5* is an early lytic gene that encodes the viral DNA polymerase. While differences in *BZLF1* RNA levels were observed as expected, no significant differences were noted between the WT- and triple-mutant-infected cells with regard to either the kinetics of appearance or the abundance of these latency RNAs (Fig. 10). Neither were significant differences observed in accumulation of *BALF5* RNA, as one might expect if overexpression of Zta had led to significantly increased expression of EBV early lytic genes leading to lytic viral genome replication. Thus, we conclude that the cell death caused by infection of primary B cells with the ZV ZV' ZIIR tmt EBV was probably not caused by altered expression of EBV latency genes or lytic viral genome amplification.

FIG 8 Synergistic derepression of *BZLF1* and *BMRF1* gene expression by mutations in the ZV, ZV', and ZIIR elements of Zp. BL41 cells were infected with the indicated viruses. After incubation for 4 days at 37°C, whole-cell RNA was prepared, and relative amounts of *BZLF1* (A) and *BMRF1* (B) RNA were determined by RT-qPCR. They were internally normalized to GFP RNA present in the same RNA preparation and externally normalized to the amount of the EBV RNA presented in the WT-infected cells. Data are means plus standard errors from one representative experiment performed in triplicate. The inset in panel A shows the relative (rel.) amount of *BZLF1* RNA in WT-, ZV ZV' ZIIR tmtRev-, ZV mt1-, and ZV ZV' dmt1-infected cells drawn to a different scale.

DISCUSSION

During infection of primary blood B cells by EBV, the *BZLF1* gene is initially expressed; however, this expression is strongly silenced within a few days as the virus transitions into a latent form of infection (51; reviewed in reference 23). The question addressed here was what happens if silencing of *BZLF1* gene expression cannot occur. We largely inhibited this silencing by constructing a mutant variant of EBV in which the ZEB-binding ZV, ZV', and ZIIR elements in Zp are inactivated.

First, we identified a secondary ZEB-binding site, ZV', in Zp. Together with the ZV element, ZV' provides two sequences to which the two zinc-finger domains of ZEB1 or ZEB2 can bind simultaneously, thus allowing for tight transcriptional repression of Zp (Fig. 2). Next, we examined the functional cooperation of the ZV, ZV', and ZIIR elements in repressing Zp. Reporter assays showed that mutations in the ZV, ZV', and ZIIR elements led to synergistic derepression of Zp (Fig. 3). The same base pair substitution mutations in the context of an intact EBV genome in 293 cells also resulted in synergistic derepression of Zp, leading to increased synthesis of IE, E, and L viral RNAs and proteins and spontaneous production of infectious virus (Fig. 4 and 5) (data not shown). Strikingly, the ZV ZV' ZIIR tmt EBV was highly de-

fective in immortalization of human primary blood B cells (Fig. 6). RT-qPCR analysis of primary and BL B-cells infected with ZV ZV' ZIIR tmt EBV showed continued synthesis of *BZLF1* RNA and increased synthesis of *BMRF1* RNA (Fig. 7 and 8). A cell viability test indicated that primary blood B cells died within 9 days after infection with the triple mutant EBV, but not after infection with WT EBV (Fig. 9). This loss of viability was not due to altered expression of latent genes essential for transformation and immortalization of B cells or early lytic genes required for lytic viral genome amplification (Fig. 10). In all cell types examined, the ZV, ZV', and ZIIR elements acted synergistically to repress transcription from Zp (Fig. 2 to 8). Thus, we conclude that the synergistic repression of *BZLF1* gene expression via the ZV, ZV', and ZIIR elements plays a central role in the establishment and maintenance of EBV latency.

ZV ZV' ZIIR tmt EBV is highly defective in immortalizing primary B cells *in vitro* but not *in vivo*. Infection of human primary blood B cells from three different donors with stocks of the ZV ZV' ZIIR tmt virus obtained from three independently isolated 293-ZV ZV' ZIIR tmt cell lines rarely resulted in any clones of proliferating cells containing this mutant virus, while the same B cells infected in parallel with WT, ZV mt, ZV ZV' dmt, and

FIG 9 The triple mutant EBV kills primary B cells. (A) Representative photomicrograms showing viability of human primary blood B cells 9 days after infection with WT and triple mutant EBV. The cells were photographed with visible light following staining with trypan blue. The solid arrows indicate examples of EBV-infected proliferating cells which excluded trypan blue; the dotted arrows indicate examples of EBV-infected cells stained with the dye. The small dots in the background are uninfected primary B cells. (B) Viability of human primary blood B cells following infection with WT and triple mutant EBV. P.I., postinfection. The percentages of EBV-positive cells that were viable were determined by a trypan blue exclusion assay; they represent the means plus standard errors from 10 microscope fields of stained cells.

FIG 10 Increased accumulation of BZLF1 RNA in human primary blood B cells infected with the ZV ZV' ZIIR tmt mutant EBV does not significantly affect accumulation of viral latency gene EBNA1, EBNA2, LMP1, and LMP2A RNAs or early lytic BALF5 RNA. Shown here are 30-, 36-, and 40-cycle PCR amplifications of cDNAs derived from viral RNA harvested at the various indicated time points postinfection with the triple mutant and parental WT EBV, the same cDNA samples analyzed by RT-qPCR in Fig. 7. The top row shows the PCR amplifications with BZLF1 primers of the viral RNA samples prior to reverse transcription. The GAPDH panels served as an internal control. The left-pointing arrows indicate the locations of the PCR products.

ZIIR mt virus readily led to cell proliferation and establishment of LCLs (Fig. 6). This finding was not due to ZV ZV' ZIIR tmt EBV being defective relative to WT EBV in its ability to infect B cells since the kinetics of appearance and levels of latency EBV EBNA1, EBNA2, LMP1, and LMP2A RNAs were similar in WT- and the triple-mutant-infected B cells (Fig. 10).

In the accompanying article, Ma et al. (39) report that upregulation of Zta expression is also observed after infection of whole animals with the same ZV ZV' ZIIR tmt EBV in a mouse model with a humanized immune system that includes T as well as B cells. Surprisingly, they found that the triple mutant EBV induced lymphomas in these animals at a similar frequency to the parental wild-type EBV even though the mutant-infected tumor cells were up to 30% Zta positive versus less than 1% Zta positive in the WT EBV-infected lymphomas. Furthermore, the enhanced synthesis of Zta in the mutant-infected lymphomas only led to an abortively lytic form of EBV infection, with little or no expression of EBV early and late lytic genes. Thus, this superlytic EBV mutant immortalizes, rather than kills, infected B cells in these humanized mice. Zta facilitates proliferation of primary B cells infected with EBV in part through induction of interleukin-13 (IL-13) in the presence of T cells (26). Thus, part of the difference between our *in vitro* and *in vivo* results observed with this superlytic mutant EBV may relate to roles played by T cells in the establishment and maintenance of EBV latency. However, when T cells were inactivated in this humanized mouse model, the mutant-infected tumor cells still multiplied and divided just fine and failed to express EBV late lytic genes (39). Thus, factors in addition to T cells must be contributing to the differences observed in the phenotype of this triple mutant *in vivo* versus *in vitro* with purified B cells.

To examine the underlying reason for the failure of ZV ZV' ZIIR tmt EBV to immortalize or stably infect purified B cells *in vitro*, we analyzed the BZLF1 RNA levels as a function of time after infection. In WT-infected B cells, the BZLF1 gene is transiently expressed and then shut off as the cells proceed into a latent form of EBV infection (23, 51; Fig. 7). On the other hand, in the triple-mutant-infected cells, BZLF1 RNA synthesis remained on (Fig. 7). B cells infected with the ZV ZV' dmt and ZIIR mt EBVs exhibited an intermediate phenotype, with respect to both inducing proliferating B-cell colonies (Fig. 6) and shutting down BZLF1 gene expression (Fig. 7). Along with continued synthesis of BZLF1 RNA, enhanced synthesis of E BMRF1 RNA was also observed in tmt-infected BL41 cells (Fig. 8). This low-level activation of BMRF1 gene expression by Zta was likely due to the fact that EBV virion DNA lacks CpG methylation; this methylation is only gradually acquired during the first month or so following initial infection (24). Zta activates a fully CpG-methylated BMRF1 promoter 4- to 6-fold more efficiently in 293 and Hone cells and 30- to 80-fold more efficiently in Akata and Daudi cells than it does an unmethylated one (4, 24; C. Wille and S. Kenney, personal communication).

The kinetics we observed for BZLF1 RNA synthesis in WT EBV-infected primary B cells is largely consistent with previous reports by Wen et al. (51) and Halder et al. (17). Wen et al. found that BZLF1 RNA was detectable by RT-PCR at 3 h, peaked at 12 h, and started to decline at 24 h postinfection of primary blood B cells with WT EBV. They also reported similar kinetics of BZLF1 RNA synthesis in EBV-negative BL cell lines Akata and Daudi. Likewise, Halder et al. reported that BZLF1 RNA was detectable at 6 h and peaked at 24 h postinfection. On the other hand, Kalla et al. (25) reported that BZLF1 RNA and Zta protein were detectable as early as day 1, peaked on day 4, and diminished after day 18 postinfection. Possible reasons for this discrepancy in the time course include the use by Kalla et al. of virus stocks prepared from B95.8 cells rather than 293 cells and primary B cells isolated from human adenoids rather than blood.

It has been hypothesized that transient synthesis of BZLF1 RNA early after infection is essential for establishment of EBV latency. Zta protein may play a key role in reversing the growth arrest of quiescent B cells by regulating certain cellular genes (17, 25, 51). However, the impact of continued synthesis of BZLF1 RNA and Zta protein after infection on B cell transformation and immortalization was not known. We showed here that primary human blood B cells infected with the triple mutant EBV largely died within 9 days (Fig. 9). Analysis of expression of viral latency genes following infection with the triple mutant EBV revealed that cell death is not due to reduction in their expression (Fig. 10). The exact reason for cell death remains to be determined. One likely possibility is that continued BZLF1 gene expression induces downregulation of surface CD74 in the tmt-infected B cells (56), yet without adequately turning on synthesis of the viral Bcl-2 homolog, BHRF1 (data not shown), thereby leading to cell death. Regardless of the exact mechanism, the result was that very few proliferating B-cell colonies were observed 6 weeks after infection with ZV ZV' ZIIR tmt EBV, and even fewer LCLs could be established. Both of the two tmt-infected LCLs that we managed to obtain from repeated attempts with B cells from three different donors no longer expressed the BZLF1 gene at a detectable level.

Functional coordination of multiple silencing elements in Zp. The BZLF1 promoter of EBV is a composite one with multiple

positive and negative regulatory elements. We previously reported that the negative regulatory elements ZV and ZIIR are both potent silencers of Zp that play key roles in EBV latency. Here we reported that concurrent mutation of both elements led to even greater derepression of Zp, with the ZV ZV' ZIIR tmt EBV being almost completely defective in establishing latency in B cells *in vitro*. Therefore, the turnoff of Zp during establishment of EBV latency is not likely due to lack of transactivators; rather, it is due to the presence of negative regulators of Zp in B cells. Furthermore, we found that the ZV, ZV', and ZIIR elements are not functionally redundant. Rather, they act synergistically to strongly silence *BZLF1* gene expression. Moreover, they probably repress Zp through different mechanisms. The cellular ZEB1 and ZEB2 proteins bind to the ZV and ZV' elements which span the transcription initiation site of Zp, blocking or interfering with the formation of transcription initiation complexes on Zp (10, 30, 31, 54). On the other hand, the ZIIR element likely represses Zp through an as-yet-unknown cellular factor(s) blocking the cross talk between the transcriptional activators bound to the adjacent ZI and ZII elements (35, 53).

This functional differentiation between these two silencers helps to provide a fail-safe system for repression of Zp and the maintaining of EBV latency. The fact that EBV has preserved multiple silencing elements in Zp during the course of evolution of its genome and natural selection of the virus has also made it possible for EBV to establish latency in a variety of host cell types: epithelial cells and lymphocytes and normal cells and tumor cells. The silencing elements repress transcription from Zp via the binding of a variety of cellular repressor proteins: ZEB1 and ZEB2 to the ZV and ZV' elements; the unidentified ZIIR-BP to the ZIIR element (53); and phosphorylated MEF-2D to the ZIA, ZIB, and ZID elements (7). The levels of these cellular repressor proteins vary greatly among different cell types (e.g., see reference 7) and change with the physiological state of the cell. This may explain our finding that the potency of a silencing element of Zp is cell type dependent: the ZIIR element represses Zp more efficiently than does the ZV or ZV' element in B cells (53), yet these two elements repress Zp with similar efficiency in 293 cells (Fig. 4). Therefore, the presence of multiple silencers may be essential for tight silencing of *BZLF1* gene expression during latency in a variety of cell types and growth conditions.

The collaboration between the ZV, ZV', and ZIIR elements in repressing Zp also suggests that lytic-induction therapy for treating EBV-associated cancers (reviewed in reference 27) might be more successful if the drugs target these cellular repressors of *BZLF1* gene expression along with inhibiting DNA synthesis (e.g., acyclovir or ganciclovir) and histone deacetylation (e.g., trichostatin A or valproic acid) to maximize the efficiency of EBV reactivation and killing of the tumor cells.

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