ABSTRACT

Characterization of ZIIR Element of BZLF1 Promoter in the Context of the Whole Epstein - Barr Virus Genome

Latent infection of Epstein-Barr virus (EBV) is associated with many human cancers. Induction of lytic replication of EBV may lead to destruction of these tumor cells. The viral immediate-early gene, *BZLF1*, plays a key role in switching EBV infection from latent to lytic replication. Expression of the *BZLF1* gene is down-regulated by a potent silencer element, ZIIR, located in the *BZLF1* promoter (Zp). I introduced a 6-bp substitution mutation into the ZIIR element of Zp by mutagenesis of the genome of EBV strain B95.8, and studied the phenotype of this mutation in the context of the whole EBV genome. The human embryonic kidney cell line 293 was transfected with ZIIR mutant (ZIIRmt) EBV DNA, and independent clones of latently infected cells were grown out into cell lines. Strikingly, 293 cells latently infected with ZIIRmt spontaneously replicated EBV DNA and produced infectious virus, while cells latently infected with wild –type (WT) did not. Immunoblot analyses showed that ZIIRmt –infected 293 cells accumulated at least 30-fold more viral immediate-early proteins Zta and Rta and early protein BMRF1 than the WT-infected cells. Immunofluorescence staining confirmed the abundant presence of Zta, Rta, BMRF1 and viral late proteins gp125 and gp350 in ZIIRmt- infected cells, while these proteins were not detectable in WT-infected cells. Thus, the ZIIR element of Zp is crucial in the regulation of EBV's switch from latency to lytic replication.

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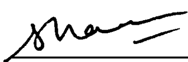
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COVER SHEET

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Characterization of ZIIR Element of BZLF1 Promoter in the Context of the Whole Epstein - Barr Virus Genome

By

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Under the direction of

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Senior Honors Thesis

May 15, 2007

ABSTRACT

Latent infection of Epstein-Barr virus (EBV) is associated with many human cancers. Induction of lytic replication of EBV may lead to destruction of these tumor cells. The viral immediate-early gene, *BZLF1*, plays a key role in switching EBV infection from latent to lytic replication. Expression of the *BZLF1* gene is down-regulated by a potent silencer element, ZIIR, located in the *BZLF1* promoter (Zp). I introduced a 6-bp substitution mutation into the ZIIR element of Zp by mutagenesis of the genome of EBV strain B95.8, and studied the phenotype of this mutation in the context of the whole EBV genome. The human embryonic kidney cell line 293 was transfected with ZIIR mutant (ZIIRmt) EBV DNA, and independent clones of latently infected cells were grown out into cell lines. Strikingly, 293 cells latently infected with ZIIRmt spontaneously replicated EBV DNA and produced infectious virus, while cells latently infected with wild –type (WT) did not. Immunoblot analyses showed that ZIIRmt –infected 293 cells accumulated at least 30-fold more viral immediate-early proteins Zta and Rta and early protein BMRF1 than the WT-infected cells. Immunofluorescence staining confirmed the abundant presence of Zta, Rta, BMRF1 and viral late proteins gp125 and gp350 in ZIIRmt- infected cells, while these proteins were not detectable in WT-infected cells. Thus, the ZIIR element of Zp is crucial in the regulation of EBV's switch from latency to lytic replication.

INTRODUCTION

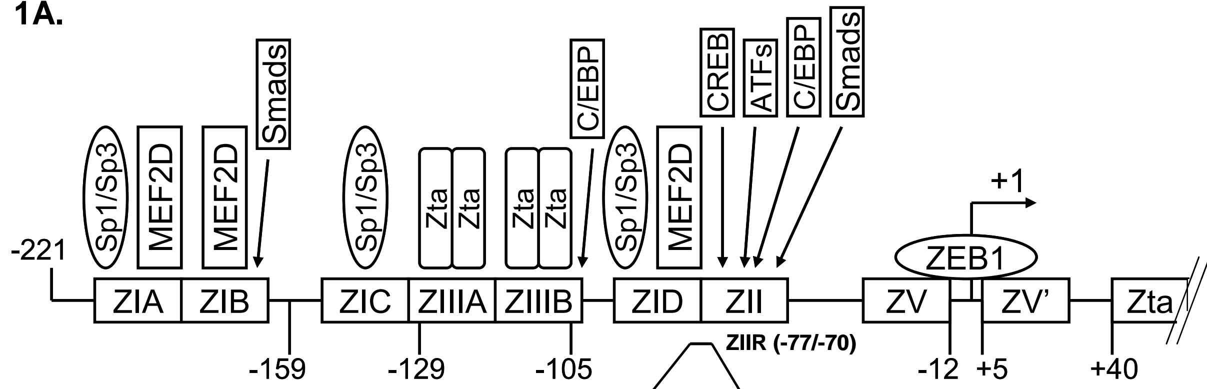
Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is estimated to infect 90% of the world's adult population (Niederman *et al*., 1970). The virus infects B lymphocytes, transforming them into permanent, latently infected lymphoblastoid cell lines (LCLs), that persists lifelong in the host (Golden *et al.,* 1973). During this phase, the virus expresses only a small subset of its genome, which is sufficient to immortalize B cells (Knutson & Sugden, 1989). Latent EBV infection is associated with many cancers, including Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma.

The switch of EBV infection from latency to lytic replication is required for the propagation of EBV from host to host, and is dependent upon the activation of an estimated 100 or more viral genes (Kieff & Liebovitz, 1990). Activation of the lytic cascade depends initially on the expression of two viral immediate-early (IE) genes, *BZLF1* and *BRLF1*. The expression of the gene products of *BZLF1* and *BRLF1*, Zta and Rta respectively, leads to the activation of early viral genes and ultimately lytic viral replication. Zta has been shown to be a transcriptional activator (Countryman & Miller, 1985), and its expression alone can initiate the entire lytic cascade (Miller *et al.,* 1984). Thus regulation of Zta appears to be central to regulating the switch from latency to lytic replication.

Many regulatory elements on the *BZLF1* promoter (Zp) have been identified to be located within the nucleotide (nt.) -221 to +12 region relative to Zp transcription initiation site (Daibata et. al., 1994; Figure 1A). Several distinct types of response elements have been defined. The first are four AT-rich domains named ZIA-D, which can bind the transcription factors Sp1, Sp3 and the myocyte enhancer factor 2D (MEF2D). A second element, ZII, shares sequence similarity with the consensus CRE/AP-1 binding site and can bind to CREP, ATF1-like and AP1-like

Figure 1: ***BZLF1* promoter.** (A) Schematic indicating the *cis*-acting regulatory elements present in the nt. -221 through +20 region of Zp. Rectangles along Zp indicate approximate locations of known regulatory elements; their *trans*-acting factors are indicated above them. The transcription initiation site is indicated by a rightward arrow. (B) Nucleotide sequences of the nt. -86 to -61 region relative to the transcription initiation site of Zp. The ZIIR element is boxed, with the 6-bp substitution mutation in it studied here shown below the sequence. The mutation in ZIIR created a cleavage site for restriction endonuclease *Eco*RI.

1A.



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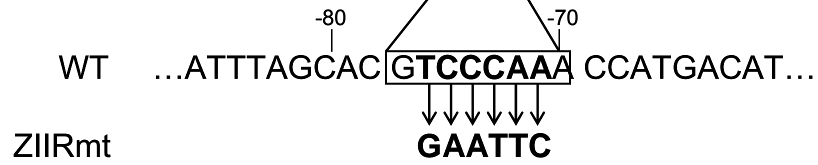

factors. A third element, ZIII, has two sites, ZIIIA and ZIIIB, which can bind Zta (Speck et. al, 1997; Israel & Kenney, 2005). Induction of the *BZLF1* gene has been suggested to occur in two steps: (i) initial activation by exogenous inducers mediated through the ZI and ZII domains, resulting in low-level transcription of the *BZLF1* gene; and (ii) auto-activation of *BZLF1* promoter through Zta binding to the ZIII domains. In addition, our laboratory identified ZV, a *cis*-acting element located at nt. -12 to -17 relative to the Zp transcription initiation site that has been shown to be a major contributor to Zp repression (Kraus et. al., 2001). Recently, a second element was identified, ZV', located at nt. +5 to +10 relative to the Zp transcription initiation site which, in combination with ZV, allows very high affinity binding of ZEB1, a cellular *trans*-acting factor that represses transcription from Zp (Yu, Wang, and Mertz, manuscript in preparation).

It has been reported that base substitution mutations in a negative, *cis* –acting element, located at nt. -70 to -77 relative to Zp transcription initiation site (Figure 1B), termed ZIIR, led to a 10- to 20-fold increase in both basal and induced Zp activity in B-lymphocytic cells using reporter plasmid assays (Liu & Speck, 1998). Previous studies conducted in our laboratory using reporter plasmid assays showed that a 6-base pair (bp) substitution mutation in the ZIIR element led to a 10-fold increase in Zp activity in B lymphocytic DG-75 cells; combination of the ZIIR mutation with a mutation in ZV element, led to an over 20-fold increase in Zp activity. Thus, the ZIIR element may play an important role in maintaining latency by repressing transcription from Zp. So far, the regulatory *trans*-acting factor(s) that binds to the ZIIR site to mediate repression has yet to be identified.

Here, I report the phenotype of the 6-bp substitution mutation in the ZIIR element of Zp in the context of the whole EBV genome. This mutation of ZIIR led to abundant expression of

viral IE, early (E) and late (L) proteins in latently infected 293 cells. The ZIIR mutant (ZIIRmt)-infected 293 cells also spontaneously replicated EBV DNA and produced infectious virus, while the wild type (WT)-infected ones did not. Thus, the ZIIR element of Zp plays a crucial role in maintaining latency in EBV-infected epithelial and B-lymphocytic cells.

MATERIALS AND METHODS

Cells. 293 is a human embryonic kidney epithelial cell line. Raji is an EBV-positive, Burkitt's lymphoma cell line. The 293 cells harboring the maxi EBV plasmid (WT-infected 293 cells) were obtained from B. Sugden. All cell lines were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

Plasmids. A pSP65 plasmid with a 1,100-bp DNA fragment insertion containing the 6-bp substitution mutation in the ZIIR element near the center of the fragment was provided by Z. Wang. Plasmid pGS284/ZIIR^{-} was constructed by subcloning the same fragment into pGS284. The maxi-EBV plasmid, provided by W. Hammerschmidt, contains the complete genome of the B95.8 strain of EBV along with a mini F plasmid and expression cassettes for hygromycin phosphotransferase, green fluorescence protein (GFP), and chloramphenicol acetyl transferase (Delecluse et al., 1998). The ZIIRmt maxi-EBV plasmid was generated through a two-step homologous recombination in *E. coli* cells as described elsewhere (Fig. 2; Yu, Wang, and Mertz, manuscript in preparation). Multiple independently isolated clones harboring ZIIRmt maxi-EBV plasmid were then characterized extensively. The EBV DNA was extracted from each of these clones. First, the -600 to +500 region of the *BZLF1* gene relative to the transcription initiation site was PCR-amplified and sequenced; only the expected 6-bp substitution mutations were observed. Second the EBV DNAs were digested with restriction endonuclease *Eco*RI and *Bam*HI, and resolved in agarose gels. No differences were observed in any of the restriction fragment digestion patterns compared to the parental maxi-EBV plasmid (data not shown). Thus, the EBV genomes present in these ZIIRmt clones likely do not differ in any significant way from the parental maxi-EBV plasmid except for the 6-bp substitution mutation in the ZIIR element of Zp (Figure 1B).

Figure 2: **Mutagenesis of ZIIR element of Zp via homologous recombination.** *E. coli* strains harboring pGS284/ZIIRmt and maxi-EBV plasmid, respectively, were conjugated. Integration of pGS284/ZIIRmt into maxi-EBV and its subsequent excision via homologous recombination were sequentially selected using the markers present in these two plasmids. PCR screening indicated that approximately 7% of the maxi-EBV revertants had lost the WT ZIIR element and retained the ZIIRmt one. The sizes of the pGS284/ZIIRmt and maxi-EBV plasmid are not drawn to scale.

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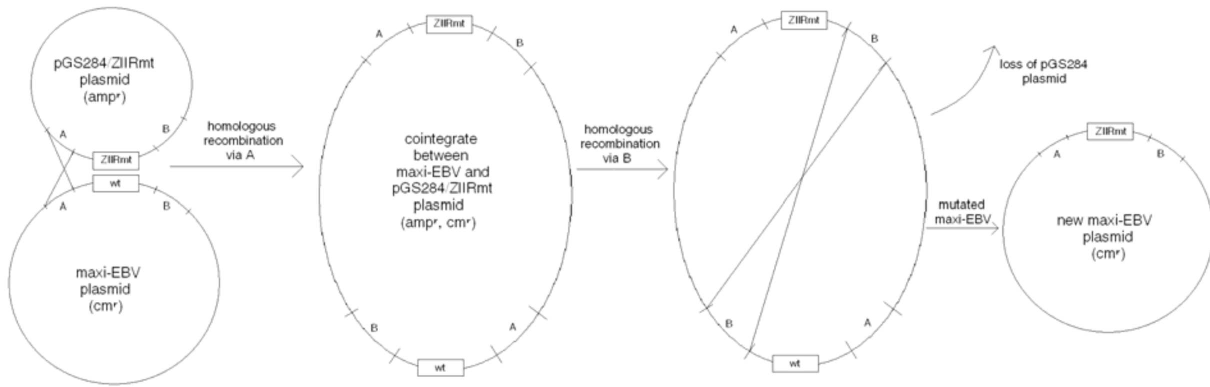

Isolation of latently infected 293 cell lines. The ZIIRmt maxi-EBV DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients, introduced into 293 cells with Lipofectamine 2000 (Invitrogen), and selected by incubation in the presence of 100 μg/ ml hygromycin as described by Neuhierl et al. (2002). GFP-positive 293 cell colonies were picked four-to-six weeks later and grown out. To make virus stocks, these latently infected cells were co-transfected with pCMV-BZLF1 (5 μg/100 mm dish) and p2670 (5 μg/100 mm dish) using Lipofectamine 2000. The culture medium was harvested 72 h later, passed through a 0.8 μm filter, and stored at 4°C.

Assay for infectious EBV. To assay for spontaneous reactivation, latently infected 293 cells were plated in 100-mm dishes and incubated for 3 days until ~80% confluent. The medium was harvested, passing through a 0.8 mm filter, and adjusted to 8 mls total volume. The virus titers were determined by a Raji cell assay and the absolute number of GFP^{+} cells was counted by ultraviolet microscopy using a hemocytometer (Altmann and Hammerschmidt, 2005). This assay underestimates the concentration of infectious virus by a factor of at least 10 (Altmann and Hammerschmidt, 2005). When virus titer was low, the virus particles were concentrated by centrifugation in an 80Ti Beckman rotor for 2 h at 17,500 rpm prior to infection of the Raji cells.

Immunoblot analyses and immunofluorescence staining. To detect Zta, Rta and BMRF1 proteins by immunoblot, 293 cells in 100-mm dishes were lysed in 75µl of SUMO lysis buffer (Adamson and Kenney, 2001) per 100-mm dish. Proteins in whole-cell extract were separated by SDS-12% PAGE, transferred to a nitrocellulose membrane, and detected by incubation with monoclonal anti-Zta (Argene, 1:200 dilution), anti-Rta (Argene, 1:200 dilution), anti-BMRF1 (Capricorn, 1:150 dilution), and anti-β-actin (Sigma, 1:5000 dilution) antibody, followed by incubation with goat anti-mouse IgG, horseradish-conjugated second antibody (Pierce, 1:5000

dilution). The bound second antibody was visualized using a chemiluminescence kit (Roche). For immunofluorescence staining, the cells were fixed with methanol:acetone (1:1) for 10 min at room temperature, pre-incubated at room temperature with phosphate-buffered saline (PBS) containing 10% FBS, incubated with monoclonal anti-Zta (1:50 dilution), anti-Rta (1:50 dilution), anti-BMRF1 (1:50 dilution), anti-gp125 (Chemicon, 1:40 dilution) or anti-gp350 (Chemicon, 1:40 dilution) antibody, washed with PBS, incubated with a Texas Red-conjugated, anti-mouse IgG second antibody (Jackson Laboratories, 1:100), washed with PBS, embedded in mounting medium (Vector Laboratories, Inc.), and examined with a fluorescence microscope (Zeiss).

EBV termini assay. The Southern blot assay to detect the presence of linear and circular episomal EBV genome was described by Raab-Traub and Flynn (1986). Briefly, the total DNA isolated from three 293 cell lines latently infected with ZIIRmt EBV and one 293 cell line latently infected with WT EBV using a DNeasy kit (Qiagen) was digested with restriction endonuclease *Bam*H1, separated by electrophoresis in a 0.8% agarose gel, transferred to a Hybond-N membrane (GE Healthcare), and probed with an EBV probe specific to the ends of the EBV genome.

RESULTS

Isolation of cell lines latently infected with ZIIRmt EBV. Three completely independent isolates of ZIIRmt maxi-EBV plasmid were transfected in parallel into 293 cells, and the cells were grown thereafter in the presence of hygromycin. Numerous hygromycin-resistant, GFP-positive colonies were visible within 3-4 weeks after transfection with ZIIRmt maxi-EBV DNA. Independent clones of latently infected cells were grown out into cell lines. Virus stocks of WT and ZIIRmt EBV were generated by co-transfection of the cell lines with plasmids expressing the EBV-encoded proteins Zta and glycoprotein 110, respectively, to induce lytic replication and production of infectious virus. The titers of these virus stocks were determined by a B-lymphocytic Raji cell assay (Altmann and Hammerschmidt, 2005). Multiple independent 293 cell lines which consistently yielded infectious virus with titers of 10^4 green Raji units (GRUs) per ml of medium (Figure 3, Table 1) following induction were chosen for further analysis.

To confirm the presence of the ZIIR mutations in all these cell lines, the viral DNA was extracted from these cells, PCR-amplified and sequenced; only the expected 6-bp substitution mutation was observed. Southern blot analysis also showed WT and ZIIRmt EBV DNAs were present as episomes in 293 cells (see below). Three independently isolated cell lines were chosen for further study.

Spontaneous reactivation of ZIIRmt EBV. 293 cells latently infected with the B95.8 strain of EBV rarely, if ever, spontaneously produce infectious virus because expression of the *BZLF1* and *BRLF1* genes is strongly repressed (Altmann & Hammerschmidt, pers. comm.). To test whether introduction of ZIIR mutation into Zp disrupted the latency of EBV in 293 cells, I assayed the cell lines for production of infectious virus. Strikingly, all three ZIIRmt-infected cell

Figure 3: **Production of infectious virus from ZIIRmt-infected 293 cells.** Green Raji cell assay showing production of infectious virus from ZIIRmt-infected cell line 1, but not the WT-infected cell line. The EBV virus released into the medium was concentrated by centrifugation and used to infect Raji cells. Fields of Raji cells were examined with visible light (visible) for total cell number versus ultraviolet (UV) light for EBV-infected, GFP-positive cells.

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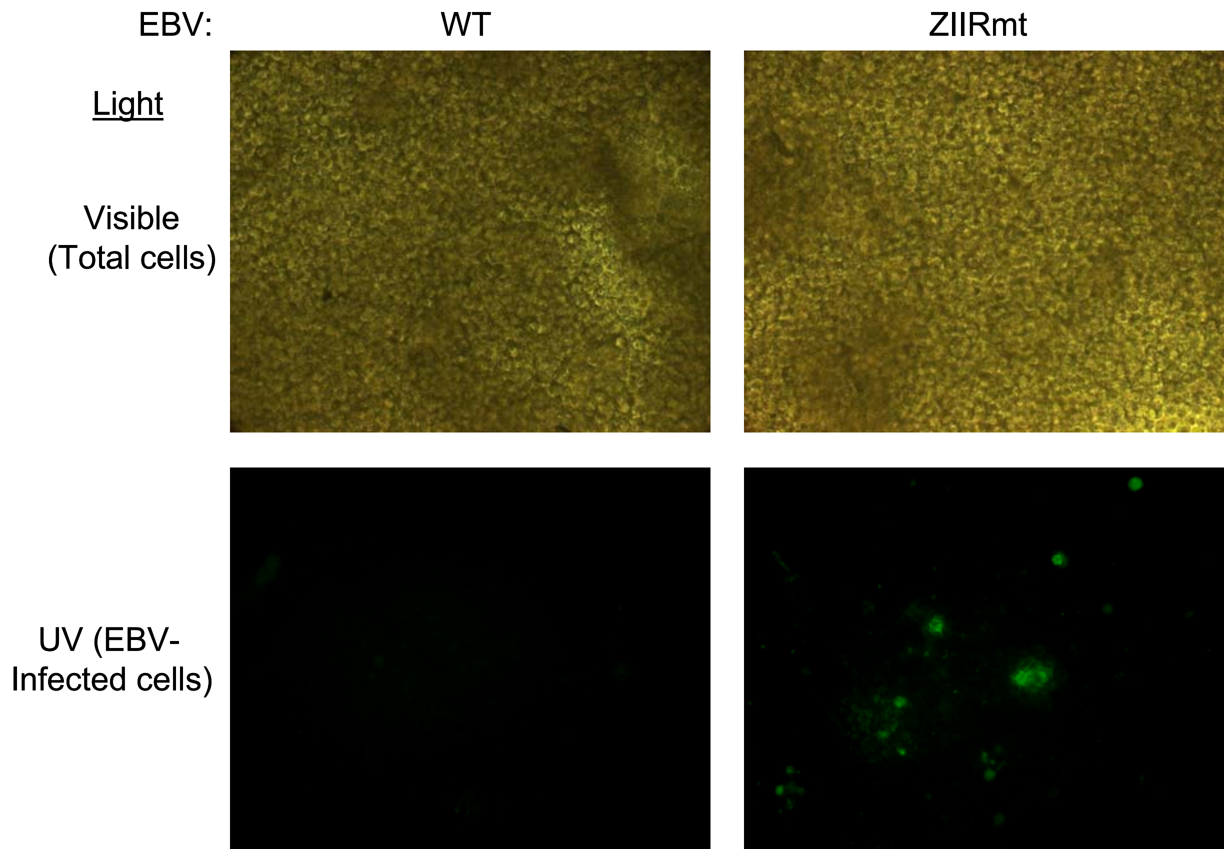

Table 1: **Spontaneous and induced reactivation of wild-type and ZIIRmt EBV in 293 cells (GRU/mL) [a].**

Treatment	Wild-type cell line	ZIIR mutant cell line		
		1	2	3
None	<0.1	$5x10^3$	$7x10^3$	$5x10^3$
Zta	$1x10^5$	$4x10^4$	$6x10^4$	$5x10^4$

a. Virus titer in the culture medium was determined by a Raji cell assay. Except for cells over-expressing Zta, media were concentrated prior to the assay.

lines spontaneously produced at least 10^3 green Raji units (GRUs) per ml of medium (Figure 3, Table 1). On the other hand, infectious virus was never detected in any of the WT-infected cell lines unless the cells were transfected with expression plasmids encoding Zta and gp110 (Figure 3; Table 1).

To confirm that the results observed with the Raji cell assay were truly due to replication of EBV DNA in 293 cells, I also performed a Southern blot assay to determine the termini of the EBV genomes present in these cell lines. When EBV exists solely in a latent state as a circular episomal plasmid, the ends of its genome are fused together; cleavage of the DNA with restriction endonuclease *Bam*H1 generates large DNA fragments, somewhat heterogenous in length due to variability in the exact number of copies of a tandemly repeated sequence present in this region of the EBV genome (Rabb-Traub and Flynn, 1986). However, in lytically infected cells, cleavage of linear EBV genome with *Bam*H1 generates smaller DNA fragments, also heterogeneous in length. As expected, the EBV DNA termini isolated from the ZIIRmt-infected 293 cell lines had sizes consistent with the presence of both circular and linear EBV genomes (Fig. 4, lanes 2-4). No smaller DNA terminal fragments were detected with the DNA isolated from the WT-infected 293 lines (Fig.4, lane 1). Therefore, I conclude that presence of the 6-bp mutation in the ZIIR element of Zp led, not only to derepression of the *BZLF1* promoter, but also to the entire subsequent series of events in EBV's lytic replication cycle necessary for production of infectious virus.

Immunoblot and immunofluorescence staining confirmed the expression of IE, E and L viral proteins in 293 cells infected with ZIIRmt EBV. Immunoblot analyses showed that the ZIIRmt EBV-infected 293 cells contained at least 30-fold more Zta, Rta, and BMRF1 than did the WT-infected ones (Fig. 5, lane 1 vs. lanes 2-4). BMRF1, an EBV early gene product, is only

Figure 4: **EBV termini assay for presence of linear and circular episomal EBV genomes.** Total cellular DNA was isolated from WT-infected and ZIIRmt-infected 293 cell lines 1-3, cleaved with restriction endonuclease *Bam*H1, separated by electrophoresis in a 0.8% agarose gel, transferred to a Hybond-N membrane (GE Healthcare), and probed with an EBV probe specific to the ends of the EBV genome. The size markers indicated on the right consisted of a DNA ladder (New England Biolabs) run in the same gel. The positions of circular EBV genomes with fused termini and linear EBV genomes are indicated on the left.

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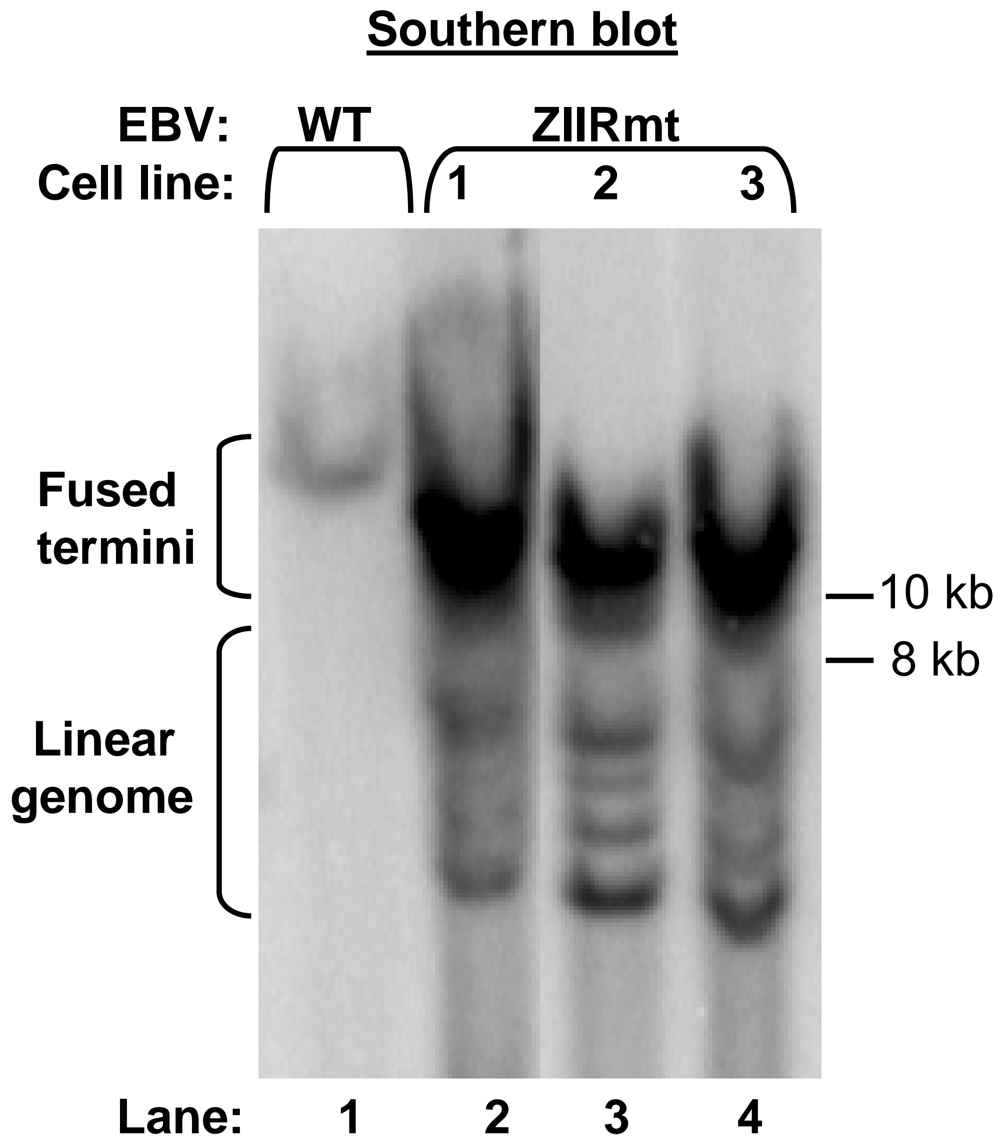

Figure 5: **Mutation of Zp's ZIIR element leads to increased synthesis of the viral Rta, Zta, and BMRF1 proteins.** Immunoblot analyses were performed with lysates of the indicated cell lines and antisera to Zta, Rta, BMRF1. The β-actin served as a loading control.

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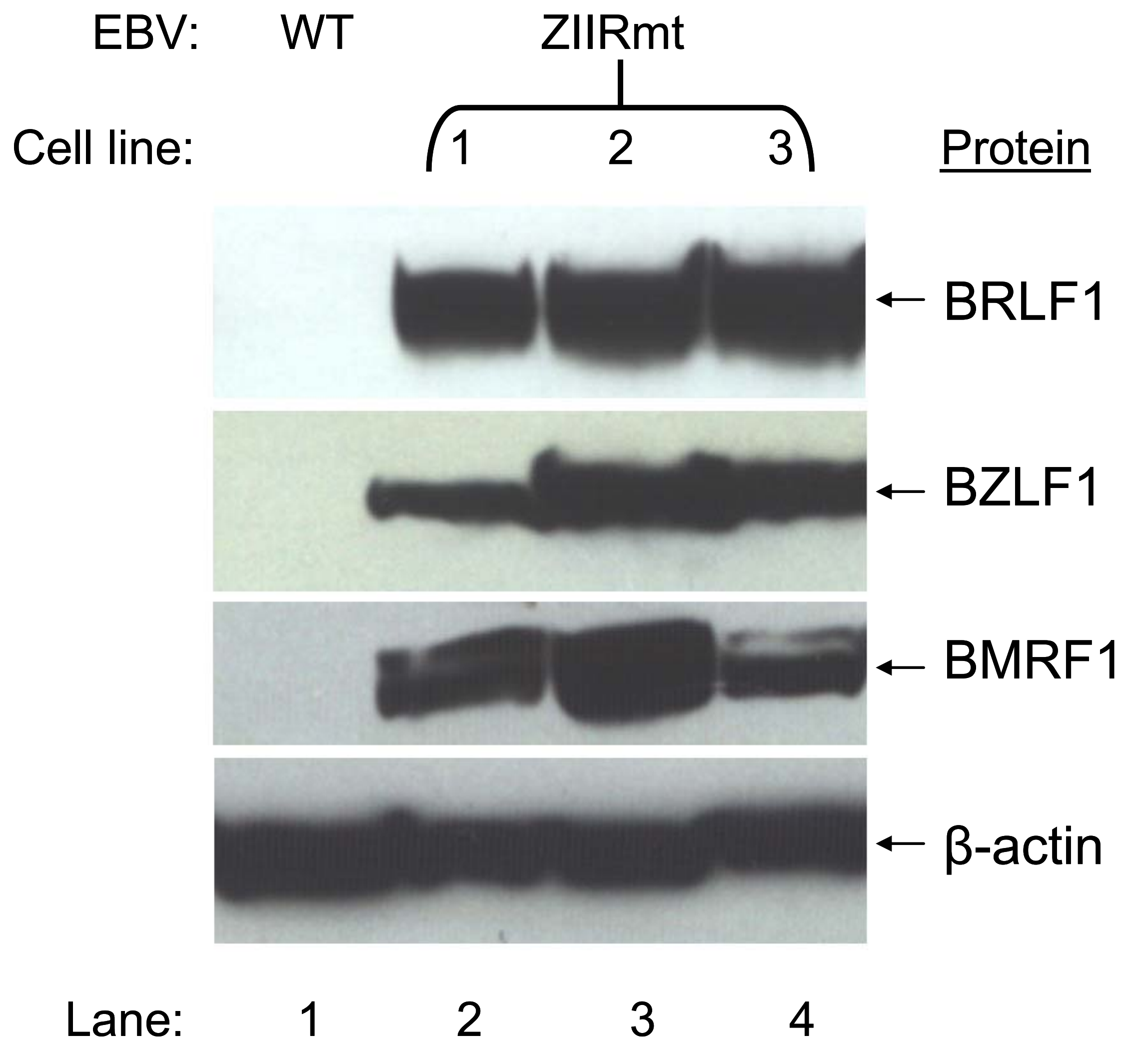

synthesized after Zta and Rta are present at sufficient levels to induce the later stages of EBV's lytic replication cycle. Immunofluorescence staining confirmed the abundant presence of Zta, Rta, and BMRF1 in approximately 2-10% of the ZIIRmt-infected cells adhering to the dishes in all three of these cell lines (Fig. 6, Table 2). It also showed the presence the viral glycoproteins, gp125 and gp350, in approximately 0.5-2% of the ZIIRmt-infected 293 cells (Fig. 6, Table 2). In contrast, I failed to detect any Zta-, Rta-, BMRF1-, gp125- or gp350-positive cells in the WT-infected 293 cell line (Fig. 6, Table 2). The percentage of positively stained cells listed in Table 2 is a lower-bound estimate of the percentage of spontaneously reactivated cells since lytic infected cells likely died and detached from the dish prior to or during washings, fixation, and staining of the cells. Thus, the 6-bp substitution mutation led to greatly increased expression of IE, E, and L genes of EBV in 293 cells.

Figure 6: **Indirect immunofluorescence staining (IFS) of WT- infected 293 cell line and ZIIRmt-infected 293 cell line 1 for presence of Zta, Rta, BMRF1, gp125, and gp350 protein.** The primary antibodies used are indicated on the left of each row of images. Fields of cells were photographed with different filters to show the total EBV-positive ones (GFP) versus the subset of them containing the indicated EBV-encoded protein (IFS).

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6.

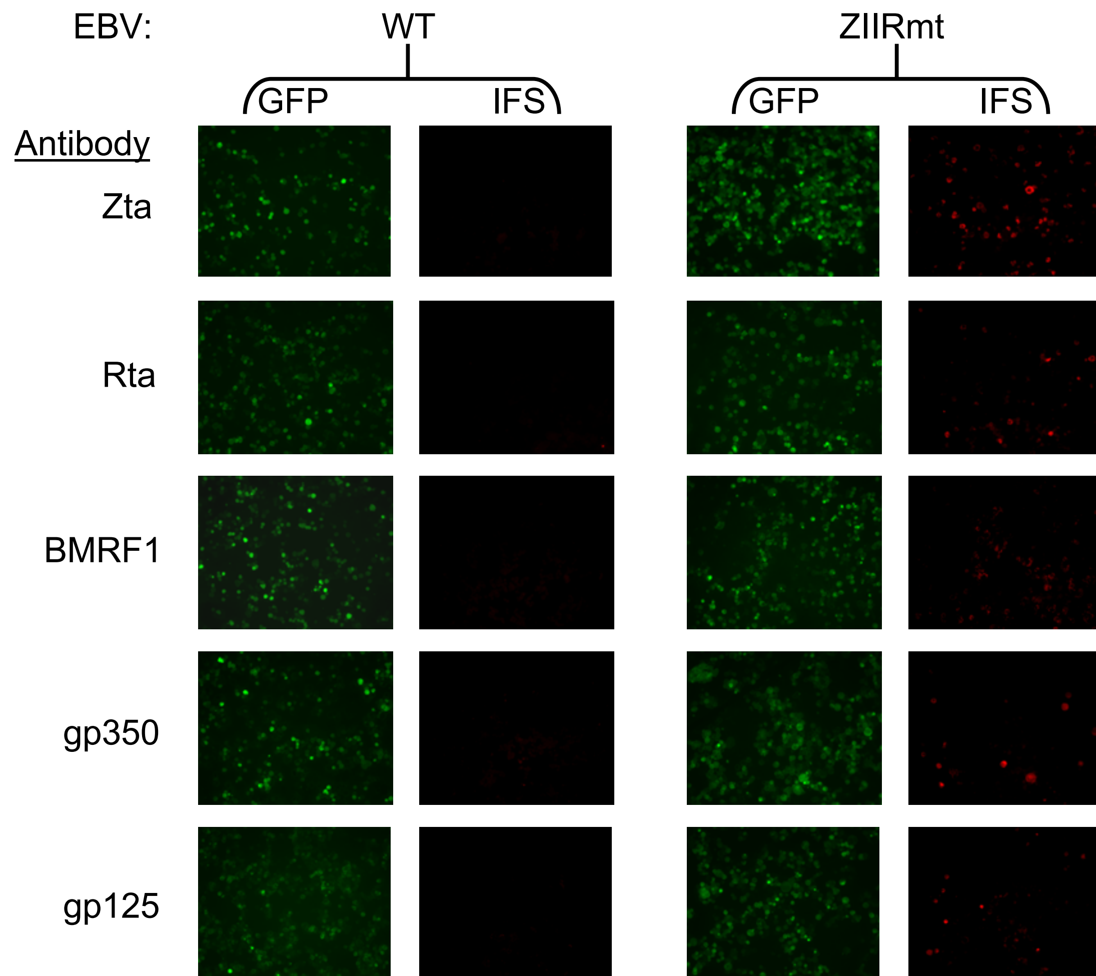

Table 2: **Percentage of 293 cells expressing EBV proteins following IFS of WT- and ZIIRmt-infected cells[a].**

Antibody	Wild-type cell line	ZIIR mutant cell line		
		1	2	3
Zta	<0.1	9	2	4
Rta	<0.1	10	6	4
BMRF1	<0.1	3	2	3
gp125	<0.1	1	1	-
gp350	<0.1	0.5	2	1

a. Approximately 1,000- 3,000 cells on each slide were counted, and the percentages of cells positively stained by the indicated antibodies was calculated.

DISCUSSION

I examined here the effect of a 6-bp substitution mutation in the ZIIR element of EBV's *BZLF1* gene on maintenance of latency and efficiency of reactivation of EBV in 293 cells. To the best of our knowledge, this is one of the first reports analyzing the role of a *cis*-acting element of Zp within the context of a whole EBV genome. I showed that a 6-bp substitution mutation in the ZIIR element can have dramatic effects on EBV's life cycle. While this particular ZIIRmt established latency in 293 cells, it was quite defective in maintaining this latency. Comparing to WT-infected cells, these cells grew more slowly, and many more cells detached from the plates during the course of short incubation, suggesting frequent lytic reactivation of the EBV genome. All of the ZIIRmt-infected 293 cell lines spontaneously synthesized Zta protein at levels sufficient to reactivate the virus into its lytic cycle, with synthesis of Rta, followed by early proteins including BMRF1, late proteins including gp125 and gp350, viral DNA replication and production of infectious virus. This spontaneous reactivation was observed with all three of the ZIIRmt-infected cell lines, but not with of the WT-infected one. All three lines produced at least 10^3 GRU/mL, approximately one order of magnitude higher than ZV mutant-infected 293 cells (Yu, Wang and Mertz, manuscript in preparation). Thus, I conclude that the ZIIR element is a key component of Zp, playing a crucial role in regulating the switch in EBV's life cycle between latency and lytic replication leading to production of infectious virus in both epithelial cells and B-lymphocytes.

My finding that ZIIRmt EBV can establish a latent infection in 293 cells with lytic expression of viral proteins and replication of viral genome suggests that, although ZIIR element is a major, physiologically important silencer of Zp, other negative regulatory elements (e.g., ZV/ZV') may also contribute to the tight repression of Zp. For full-blown activation of EBV

from latency, positive activation of Zp may also be required for Zta expression to efficiently reach the threshold level needed to trigger reactivation. Regardless, the ZIIR element of Zp plays a crucial role in regulating EBV's switch between latency and lytic replication and, ultimately, in dictating the pathological consequences of infection of the host.

Given the fact that a mere 6-bp substitution mutation in ZIIR of Zp led to dramatic increases in expression of EBV genes and production of infectious virus, this mutation in combination with another mutation (e.g., ZV/ZV'), may provide the basis for development of a constitutively lytic strain of EBV that could serve as a vaccine for immunization against EBV infection. It will also be interesting to identify and characterize the regulatory protein(s) which binds and regulates the function of ZIIR. Since ZIIR is a very potent silencer, therapeutic intervention targeted against its regulatory protein(s) may have the potential for the development of treatment of some EBV-associated diseases.

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