The Epstein-Barr Virus (EBV) BZLF1 Immediate-Early Gene Product Differentially Affects Latent versus Productive EBV Promoters

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The Epstein-Barr virus (EBV) BZLF1 gene product is thought to mediate the disruption of latent EBV infection. We have examined the regulatory effects of BZLF1 by studying its transactivating effects on seven different EBV promoters. We find that whereas the BZLF1 gene product increases the activity of the two early promoters, BMLF1 and BMRF1, it decreases the activity of three latent promoters (the *Bam*HI-C and *Bam*HI-W Epstein-Barr nuclear antigen promoters and the latent membrane protein promoter). The BZLF1-induced changes in promoter-directed chloramphenicol acetyltransferase activity occur in EBV-negative as well as EBV-positive cell lines and are accompanied by a similar change in chloramphenicol acetyltransferase mRNA. Deletion analysis of the *Bam*HI Z fragment indicates that a portion of the amino-terminal half of the BZLF1 gene product (amino acids 24 to 86) is not essential for positive transactivating effects but is required for down-regulating effects. Thus, different domains of the same EBV immediate-early gene product can either increase the function of EBV promoters active in productive infection or decrease the function of key promoters active in latent infection.

Epstein-Barr virus (EBV) infection of lymphoid cells is largely latent, with only rare cells supporting productive infection. Although the cellular and viral factors that determine whether EBV infection is latent or productive remain largely unknown, an EBV immediate-early (IE) gene product, BZLF1, has recently been implicated by several laboratories to play an important role in regulating viral latency. The observation that superinfection with the P3HR1 strain of EBV disrupts latency has been attributed to the presence of small defective viral particles which contain the BZLF1 open reading frame (ORF) (9, 15). The BZLF1 gene product has been demonstrated to encode a transactivating function which stimulates the expression of at least one EBV early promoter (7, 16). The BZLF1 IE gene product alone, if placed in a plasmid under the control of a strong heterologous promoter, is sufficient to disrupt latency when transfected into EBV-infected lymphoid cells (32).

In this report, we have examined the transregulatory effect of the BZLF1 gene product on different classes of EBV promoters. Three EBV promoters active in latent infection and four promoters active only in productive infection have been linked to the heterologous chloramphenicol acetyltransferase (CAT) gene. We show that whereas the BZLF1 gene product increases the CAT activity of the constructs directed by the BMLF1 IE promoter and the BMRF1 early promoter, the CAT activity directed by three latent promoters is decreased. The BZLF1-induced increase in BMLF1 promoter-directed CAT activity is accompanied by a similar increase in CAT mRNA. We show that the BZLF1 transactivator can function in a variety of cell types (both EBV positive and negative). In addition, we have constructed deletion mutants of the *Bam*HI Z fragment and studied the ability of these mutants to transregulate the EBV-CAT constructs and disrupt latency in Raji cells.

MATERIALS AND METHODS

Cell lines. All lymphoid cell lines were maintained in RPMI 1640 medium with 10% fetal calf serum. The EBV-positive cell lines included Raji (a latently infected human Burkitt's lymphoma line) and B95-8 (a productively infected marmoset B-cell line). The EBV-negative cell lines included the B-cell line Loukes (a human Burkitt's lymphoma line), HeLa (a cervical epithelial line), and Jurkat (a human T-cell line).

DNA transfection. Plasmid DNA was purified through two sequential cesium chloride gradients. Transfection into lymphoid cell lines was accomplished by using the electroporation technique (33). Cells (10^7 per condition) were shocked at 1,500 V with the Zapper electroporation unit from the University of Wisconsin medical electronics shop. Transfection into HeLa cells was performed by using the calcium phosphate method (14).

EBV-CAT plasmids. Seven different EBV-CAT plasmids were constructed by inserting putative EBV promoters into the *Bgl*II site of the vector pCAT3M (25). The pCAT3M vector contains the bacterial gene encoding CAT and a simian virus 40 polyadenylation site but no eucaryotic promoter element. The amount of CAT activity produced by each plasmid is thus a reflection of the activity of the upstream EBV promoter. Using the primer extension method (28), we have confirmed that the CAT mRNA in the pC-CAT construct originates from the expected start site (6) (data not shown). Primer extension mapping with the pM-CAT construct is presented in Results. The promoter sequences and mRNA start sites in the other EBV constructs are presumed from the EBV sequence and RNA mapping studies of previous investigators (3, 5, 8, 20, 26, 30). Table 1 shows the EBV sequence (1) included in each construct.

Construction of the BZLF1 plasmid pEBV-ZIE. Since the promoter of the BZLF1 gene is quite weak (23), we inserted the EBV *Bam*HI Z fragment into the *Bam*HI site of the vector pH1013 (a gift from Michelle Davis) (22) and named

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TABLE 1. Construction of EBV-CAT plasmids

Plasmid	Promoter	EBV <i>Bam</i> HI fragment	Sequences of promoter insert ^a	Gene category
pW-CAT	EBNA (18, 19, 30)	W	44,183–45,084	Latent
pC-CAT	EBNA (6)	C	10,312–11,336	Latent
pLMP-CAT	Latent membrane protein (8, 12, 34)	N	169,924–169,476	Latent
pZ-CAT	BZLF1 (5)	Z	103,625–103,181	IE
pM-CAT	BMLF1 (26, 31)	M	84,770–84,333	IE
pEA-CAT	Early antigen diffuse (BMRF1) (8, 31, 35)	M	79,538–79,870	Early
pLATE-CAT	gp350/220 (BLLF1) (3)	L	92,703–92,160	Late

^a The EBV promoter sequences shown were inserted into the *Bgl*II site of the vector, pCAT3M (25), upstream of the CAT gene. The EBV inserts (1) were blunt ended with either the Klenow enzyme or T4 polymerase, ligated into the *Hinc*II site of the PUC-7 vector, and then excised by *Bam*HI digestion for ligation in pCAT3M.

the resulting construct pEBV-ZIE (Fig. 1). In the pEBV-ZIE plasmid, the BZLF1 gene is driven by the strong human cytomegalovirus (CMV) IE promoter. To define the *Bam*HI-Z sequences required for transregulatory effects, we also made a series of plasmids that remove different portions of *Bam*HI-Z from the pEBV-ZIE construct. These deletion mutants are shown in Fig. 2.

In vitro transcription and translation of BZLF1 deletion mutants. Since the pHD1013 vector was originally derived from the pGem2 plasmid (Promega) and contains the Sp6 promoter, the Sp6 polymerase enzyme (Promega) was used to generate sense RNA from plasmids pEBV-ZIE (Δ HindIII-HindIII) and pEBV-ZIE (Δ Sma-Pst) suitable for in vitro translation. The RNA was translated in rabbit reticulocyte lysate (Promega) with 60 μ Ci of [³⁵S]methionine (1,134 Ci/mmol) in a 50- μ l volume containing 4 μ g of RNA at 30°C for 1 h. Samples were then taken and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

CAT assays. Each EBV-CAT plasmid (5 μ g) was cotransfected with either the pHD1013 vector or 5 μ g of the pEBV-ZIE plasmid or its derivative with 10⁷ lymphoid cells per condition. Forty-eight hours after transfection, an extract of the cells was prepared (200 μ l per condition), and 100 μ l of the extract was incubated for 1 h at 37°C with [¹⁴C]-labeled chloramphenicol as previously described (13). The percent acetylation of chloramphenicol was quantitated

by thin-layer chromatography followed by autoradiography and scintillation counting. All CAT assays were repeated with smaller amounts of extract if the results were not within the linear range (less than 70% chloramphenicol acetylation).

RNA analysis. Total cellular RNA was prepared 2 days after transfection by a modification of the Favaloro method (11, 24). For S1 nuclease quantitation of pM-CAT mRNA, a complementary RNA probe to pLATE-CAT (Table 1) mRNA was made by inserting a fragment from pLATE-CAT containing the EBV promoter plus 250 base pairs (bp) of the CAT gene into the pGem-2 vector (Promega) next to the Sp6 promoter. Total cellular RNA (35 μ g) was hybridized overnight to the [³²P]-labeled single-stranded RNA probe (6.5 $\times$ 10⁴ cpm). The hybrid probe was then digested with S1 nuclease (4), and the S1-protected fragment was electrophoresed on a 6% acrylamide gel with 7 M urea. We used the pLATE-CAT probe for S1 analysis of pM-CAT mRNA in Raji cells since we found that BZLF1 transfection of Raji cells results in the production of messages from the endogenous EBV genome which hybridize to the pM-CAT probe and thereby confuse the interpretation of total CAT mRNA (data not shown). In contrast, the pLATE-CAT probe allows for quantitative analysis of total CAT mRNA in Raji cells in the presence and absence of the BZLF1 transactivator but gives no information regarding the 5' start site.

To determine the actual 5' start site(s) in the pM-CAT plasmid, we used the primer extension method. A 20-bp oligonucleotide sequence homologous to the CAT gene in the region 15 to 34 bp downstream of the ATG codon was synthesized and labeled with [³²P]ATP. Labeled oligonucleotide (10⁵ cpm) was hybridized overnight with 50 μ g of RNA at 30°C in a solution containing 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, and 1 mM EDTA. Primer extension was then performed at 42°C for 90 min with avian myeloblastosis virus reverse transcriptase as previously described (28). After RNase digestion, the primer extension products were electrophoresed on a 6% acrylamide-urea gel.

Assay of EBV productive antigen synthesis. Raji cells were transfected with 10 μ g of the pEBV-ZIE plasmid (or a pEBV-ZIE deletion mutant), and then cells were harvested by centrifugation at 4 or 6 days after transfection and washed three times with cold phosphate-buffered saline solution. Cell smears were prepared on standard painted microscope slides. After drying, the smears were fixed by cold (−20°C) acetone for 5 min. Assay of EBV productive antigens was carried out by the indirect immunofluorescence method with pooled sera from patients with nasopharyngeal carcinoma and infectious mononucleosis.

FIG. 1. Construction of pEBV-ZIE. The EBV *Bam*HI Z fragment, which contains the intact BZLF1 ORF plus portions of two other ORFs (BZLF2 and BRLF1), was inserted into the *Bam*HI site of vector pHD1013 (22). The resultant plasmid, pEBV-ZIE, had the BZLF1 gene driven by the strong human CMV IE promoter.

TABLE 2. Effect of the BZLF1 transactivator in different cell types

Promoter	% CAT acetylation without BZLF1 (fold stimulation with BZLF1)			
	EBV-positive cells		EBV-negative cells	
	Raji	B95-8	Jurkat	HeLa
Latent				
pW-CAT	2.2 (0.2)	0.3 (1.0)	16.3 (0.1)	
pC-CAT	14.1 (0.2)	1.6 (1.6)	11.5 (0.2)	
pLMP-CAT	1.6 (0.5)	1.6 (0.9)	3.4 (0.3)	
Productive				
pZ-CAT	0.2 (1.1)	0.4 (1.8)		
pM-CAT	0.3 (46.6)	1.8 (5.8)	1.4 (1.9)	0.2 (3.0)
pEA-CAT	0.4 (8.8)	2.1 (3.3)	0.7 (6.8)	0.2 (30.0)
pLATE-CAT	0.3 (1.6)	0.4 (1.4)		

RESULTS

The BZLF1 IE gene product differentially affects latent versus productive EBV promoters. To examine the effect of the BZLF1 IE gene product on different classes of EBV promoters, 5 µg of each EBV-CAT plasmid was cotransfected with either 5 µg of the pHD1013 vector or 5 µg of the pEBV-ZIE vector. Table 2 shows the results of these experiments. In the latently infected B-cell line, Raji, the activity of the two CAT vectors containing the BMLF1 (pM-CAT) and BMRF1 (pEA-CAT) productive promoters, which are normally active only in productive infection, was stimulated by cotransfection with the pEBV-ZIE plasmid. On the other hand, the activity of the three promoters which are actively expressed during latent EBV infection was consistently down regulated by cotransfection with pEBV-ZIE. This decrease in latent promoter activity could not be accounted for by a promoter competition effect, since the pHD1013 control vector also contains the human CMV IE promoter. Furthermore, this down-regulating effect on latent promoters was observed with multiple plasmid preparations of pEBV-ZIE. The activity of two other CAT constructs, pRSV-CAT and pHIV-CAT, which contain the Rous sarcoma virus (22) and human immunodeficiency virus promoters (13), was not affected by cotransfection with pEBV-ZIE (data not shown).

The ability of BZLF1 to function as a transregulator was studied in several different cell lines. The BZLF1 gene product increased the activity of the pM-CAT and pEA-CAT constructs in both latently (Raji) and productively (B95-8) infected B-cell lines, as well as in the epithelial line, HeLa, and the T-cell line, Jurkat. The latent promoters were down regulated by BZLF1 in Raji and Jurkat cells but not in B95-8 cells (productively infected B cells). In HeLa cells, the constitutive activity of these promoters is too low to observe any negative effect. The BZLF1 IE promoter (pZ-CAT) and the gp350/220 late promoter (pLATE-CAT) were not significantly affected by cotransfection with the BZLF1 gene product in any cell type.

In contrast to a report by other investigators (7), these data show that the BZLF1 transactivator clearly functions in EBV-negative as well as EBV-positive cell types. However, we did find that the BZLF1-induced increase in pM-CAT activity was consistently much greater in EBV-positive than in EBV-negative cell lines. This much greater effect on pM-CAT expression in EBV-positive cells suggests that another EBV gene product(s) may contribute to transactivation of the BMLF1 promoter by BZLF1. The BMRF1 promoter, on the other hand, is stimulated by BZLF1 at least as well in EBV-negative cells as in EBV-positive cells. The different effects of BZLF1 in the latently versus productively infected B-cell lines may be due to the constitutive expression of BZLF1 in the productive cell line, in addition to the expression of other EBV-encoded transactivating proteins such as the recently described *Bam*HI-R transactivator, BRLF1 (16).

The BZLF1 transactivator increases the level of mRNA from susceptible promoters. We next determined whether the positive stimulatory effect of the BZLF1 transactivator on the CAT activity of the pM-CAT construct is accompanied by a similar increase in CAT mRNA. We recently found that the EBV BMLF1 transactivator may function at least partly at a posttranscriptional level since it increases CAT activity but has little effect on CAT mRNA (22) in cotransfection experiments. In contrast, S1 nuclease analysis of the BZLF1 effect on CAT mRNA levels indicates that this transactivator significantly increases the steady-state level of CAT mRNA from susceptible promoters.

In the experiment shown in Fig. 3, the pM-CAT construct (5 µg) was cotransfected into Raji cells with either 5 µg of

FIG. 2. Construction of pEBV-ZIE deletion mutants. To construct each deletion mutant, pEBV-ZIE was cut with the restriction enzymes shown, blunt ended with T4 polymerase (except for the *Hind*III-*Hind*III mutant which was not blunt ended), and then religated. The hatched area shows the deleted portion of *Bam*HI-Z. EBV-ZIE (Δ MstII-*Bam*HI) contains the intact 200-amino-acid BZLF1 ORF but deletes a portion of the BRLF1 ORF. pEBV-ZIE (Δ HindIII-*Hind*III) has an in-frame deletion which removes amino acids 24 to 86 from BZLF1. pEBV-ZIE (Δ Sma-*Pst*) removes amino acids 132 to 200 from BZLF1. The above description of the pEBV-ZIE deletions assumes no splicing of the BZLF1 mRNA. However, a potential splice in the BZLF1 message creates a 245-amino-acid ORF which has the same 167 amino-terminal amino acids but an altered carboxy-terminal end (5, 21).

FIG. 3. Effect of the BZLF1 transactivator on steady-state CAT mRNA with S1 nuclease analysis. The pM-CAT (5 μ g) plasmid was cotransfected into Raji cells with either 5 μ g of the pH1013 vector, pEBV-MIE (22), which encodes the EBV BMLF1 transactivator, or pEBV-ZIE, which encodes the BZLF1 transactivator. At the level of CAT activity, cotransfection with pEBV-MIE and pEBV-ZIE increased pM-CAT activity 25-fold and 20-fold, respectively. After hybridization with an Sp6 polymerase-generated antisense pLATE-CAT probe, the expected size of the S1 nuclease-protected RNA fragment is 270 bp (the length of homology between the pLATE-CAT probe and the pM-CAT plasmid). Whereas the pEBV-MIE transactivator has little effect on pM-CAT mRNA levels, the pEBV-ZIE transactivator clearly increases steady-state CAT mRNA.

pH1013, 5 μ g of pEBV-MIE (22) (which has the BMLF1 transactivator under the control of the CMV IE promoter), or 5 μ g of pEBV-ZIE (BZLF1 transactivator controlled by the CMV IE promoter). In this experiment, cotransfection with pEBV-MIE increased the CAT activity of pM-CAT 25-fold, and cotransfection with the pEBV-ZIE plasmid increased CAT activity 20-fold. However, at the level of steady-state mRNA, the EBV-MIE transactivator had no effect, whereas EBV-ZIE increased mRNA 16-fold (as measured by laser densitometry). The effect of the BZLF1 transactivator on mRNA levels was analyzed by S1 nuclease analysis in three separate experiments, and in each case the increase in CAT mRNA produced was comparable to the increase in CAT activity.

We also used the primer extension method to determine whether the presence of the BZLF1 transactivator can alter the 5' start site in the BMLF1 promoter region. These results are shown in Fig. 4. Although the BMLF1 mRNA start site has not been precisely mapped, a potential TATA box element has been noted upstream of the ATG codon (1). Our

FIG. 4. Primer extension analysis of pM-CAT RNA. The pM-CAT plasmid was cotransfected into Raji cells with either 5 μ g of the pH1013 vector (lane B) or 5 μ g of the BZLF1 transactivating plasmid, pEBV-ZIE (lane C). RNA (50 μ g) from untransfected Raji cells (lane A) or the pM-CAT transfected cells was hybridized overnight with a 32 P-labeled oligonucleotide primer homologous to CAT gene sequences 15 to 34 bp downstream of the ATG codon. Primer extension was then performed with reverse transcriptase at 42°C for 90 min (28). The expected size of the primer extension product if the BMLF1 promoter TATA box is used is approximately 100 bp. A band of the expected size is detected (lower arrow) in addition to a larger band mapping to the region approximately 20 bp upstream of the TATA box (upper arrow). The BZLF1 transactivator does not affect use of the mRNA start sites.

pM-CAT construct contains this TATA box element plus an additional 25 bp of downstream sequences. Preliminary primer extension analysis has revealed the expected start site downstream of the TATA box (the smaller band in Fig. 4), plus an additional unexpected band that maps to a region approximately 20 bp upstream of the TATA box (assuming no splicing occurs). This upstream start site was seen in two separate experiments and appeared to be a major start site in our plasmid. However, since our plasmid may not include the endogenous cap site downstream of the TATA box, this may favor use of the upstream start site. In any event, the BZLF1 transactivator was not found to promote the use of any additional mRNA start site.

Analysis of BamHI-Z sequences required for transactivation. Because the pEBV-ZIE construct contains the entire BamHI Z fragment, including not only the intact BZLF1 ORF but also two other partial ORFs, we made several deletion mutants of the pEBV-ZIE plasmid to ascertain whether it was in fact the BZLF1 gene product that was responsible for the observed transactivating effects (Fig. 2). To assure that the pEBV-ZIE deletion plasmids which alter the BZLF1 ORF can make a stable protein product of the predicted size, we used Sp6 polymerase to synthesize RNA

FIG. 5. In vitro translation of pEBV-ZIE deletion mutants. The pEBV-ZIE plasmid (lane C), the pEBV-ZIE (Δ HindIII-HindIII) mutant (lane B), and the pEBV-ZIE (Δ Sma-Pst) mutant (lane A) were transcribed in vitro with Sp6 polymerase and then translated in a rabbit reticulocyte lysate system in the presence of [35 S]methionine. The pEBV-ZIE plasmid, as expected, produces a protein of approximately 34 kilodaltons. The two deletion plasmids produce smaller protein products of the expected size. Numbers on the right give molecular mass in kilodaltons.

in vitro followed by in vitro translation of the RNA. The resultant protein products were labeled with [35 S]methionine and run on a sodium dodecyl sulfate-polyacrylamide gel (Fig. 5). With this technique, the parent pEBV-ZIE construct was found to make the expected protein product of approximately 34 kilodaltons (21). The pEBV-ZIE (Δ HindIII-HindIII) plasmid, which removes amino acids 24 to 86, was shown to produce a protein product of approximately 24 kilodaltons, and pEBV-ZIE (Δ Sma-Pst), which has amino acids 120 to 200 removed, produces a protein product of approximately 23 kilodaltons.

We next examined the ability of these pEBV-ZIE deletion constructs to transactivate the different EBV promoters (Table 3). The plasmid pEBV-ZIE (Δ Mst-BamHI), which removes most of the BRLF1 ORF but leaves the BZLF1 ORF intact, produces better transactivation of the pM-CAT construct than the parent plasmid, pEBV-ZIE, and also appears to function more efficiently as a down regulator of pC-CAT activity. The plasmid pEBV-ZIE (Δ HindIII-HindIII) contains an in-frame deletion which removes a large portion of the amino-terminal half of the 200-amino-acid BZLF1 ORF (amino acids 24 to 86 removed). In spite of this deletion, this plasmid still functions as a positive transactivator of pM-CAT, although less effectively than the parent, pEBV-ZIE (Table 3). On the other hand, the pC-CAT plasmid is consistently not down regulated by pEBV-ZIE (Δ HindIII-HindIII). The plasmid pEBV-ZIE (Δ Sma-Pst), which has the carboxy-terminal half (amino acids 120 to 200) of the BZLF1 ORF deleted, no longer functions as either a positive or negative transregulator. Thus, amino acids 24 to 86 in BZLF1 are not essential for the positive transactivating effects on the BMLF1 promoter, but may be required for negative regulation. The carboxy-terminal half of BZLF1 appears to be essential for both positive and negative regulation.

Disruption of viral latency by pEBV-ZIE deletion plasmids.

TABLE 3. Effect of pEBV-ZIE deletions on transactivation

Plasmid	Fold stimulation of CAT activity	
	Expt 1	Expt 2
pM-CAT^a		
+pEBV-ZIE	17.0	91.1
+pEBV-ZIE (Δ Mst-BamHI)	29.7	104.4
+pEBV-ZIE (Δ HindIII-HindIII)	5.0	14.3
+pEBV-ZIE (Δ Sma-Pst)	1.0	1.9
pC-CAT^b		
+pEBV-ZIE	0.4	0.4
+pEBV-ZIE (Δ Mst-BamHI)	0.2	0.2
+pEBV-ZIE (Δ HindIII-HindIII)	1.9	0.9
+pEBV-ZIE (Δ Sma-Pst)	1.2	1.2

^a Constitutive activity was measured as percent acetylation of chloramphenicol without pEBV-ZIE and was 0.3 and 0.4% for experiments 1 and 2, respectively.

^b Constitutive activity was measured as percent acetylation of chloramphenicol without pEBV-ZIE and was 12.7 and 14.2% for experiments 1 and 2, respectively.

We also compared the ability of the pEBV-ZIE deletion mutants to disrupt viral latency when transfected into the Raji cell line. The pEBV-ZIE and pEBV-ZIE (Δ Mst-BamHI) plasmids (each of which contains the intact BZLF1 ORF) both clearly induce the expression of productive viral antigens when transfected into the normally tightly latent Raji cells (Fig. 6). However, we could not detect disruption of latency with the two plasmids containing deletions in either the amino-terminal portion [pEBV-ZIE (Δ HindIII-HindIII)] or carboxy-terminal portion of BZLF1 [pEBV-ZIE (Δ Sma-Pst)]. Thus, although the pEBV-ZIE (Δ HindIII-HindIII) plasmid retains the capacity for positive but not negative transactivation of the BMLF1 promoter, it has lost the ability to disrupt viral latency, suggesting that down regulation of latent promoters may contribute to the disruption of latency. Alternatively, the CAT assay may simply be a more sensitive method of detecting transregulatory function than is detection of antigens by serology.

DISCUSSION

Infection of lymphocytes with EBV results in immortalization and transformation of B cells in vitro and is associated with polyclonal lymphoproliferation and Burkitt's lymphoma in the human host (10). The transregulatory functions of the EBV BZLF1 gene product are likely to play an important role in viral regulation and cellular transformation. In this paper, we have examined the effect of the BZLF1 gene product on different classes of EBV promoters. We find that the BZLF1 gene product positively transactivates two early EBV promoters (BMLF1 and BMRF1) but down regulates the expression of three latent EBV promoters (the BamHI-W and BamHI-C Epstein-Barr nuclear antigen [EBNA] promoters and the latent membrane protein promoter). Both of these BZLF1 effects occur in EBV-negative as well as EBV-positive cells and therefore do not require the presence of additional EBV gene products. The increase in pM-CAT activity produced by the BZLF1 transactivator is accompanied by a correspondingly similar increase in CAT mRNA. In addition, we show that a portion of the amino-terminal half of the BZLF1 ORF, although not essential for up regulation of the BMLF1 promoter, may be required for down regulation of the latent promoters and disruption of viral latency.

FIG. 6. Disruption of viral latency by pEBV-ZIE deletion plasmids. The latently infected Raji cells were transfected with 5 μ g of pM-CAT and 5 μ g of pHD1013 vector DNA (A), pEBV-ZIE (B), pEBV-ZIE (Δ Mst-BamHI) (C), pEBV-ZIE (Δ HindIII-HindIII) (D), or pEBV-ZIE (Δ Sma-Pst) (E). Two days after transfection, CAT assay analysis in a sample of the cells showed that, as expected, the cultures cotransfected with the pEBV-ZIE, pEBV-ZIE (Δ Mst-BamHI), and pEBV-ZIE (Δ HindIII-HindIII) plasmids all had increased pM-CAT activity compared with the pHD1013 control condition. At 4 days after transfection, the cells from each condition were examined by indirect immunofluorescence (with pooled patient sera) for the expression of EBV productive antigens. Representative fields are shown for each condition. Only the cells transfected with pEBV-ZIE or pEBV-ZIE (Δ Mst-BamHI) had detectable EBV productive antigens. The results were identical in a repeat experiment examined by immunofluorescence at 6 days after transfection.

The precise mechanism by which the BZLF1 gene product disrupts latency is still unproven, but may well be related to its transregulatory effects on the promoters of key EBV genes. Our data demonstrate that the BZLF1 transactivator increases the activity of the promoters of two EBV regulatory genes, BMLF1 and BMRF1. Since both the BMLF1 and BMRF1 gene products also encode known transactivating functions (7, 26, 29, 35), the ability of BZLF1 to disrupt latency could well be mediated through its stimulation of the BMLF1 or BMRF1 promoters, or both. However, although we find that the BZLF1 gene product alone (under the control of the CMV IE promoter) is sufficient to disrupt

latency in Raji cells, we do not find that either the BMLF1 or BMRF1 gene products (when likewise placed under the control of the CMV IE promoter) can disrupt latency in Raji cells (S. Kenney, unpublished data). The inability of high expression of either the BMLF1 or BMRF1 transactivator alone to disrupt latency suggests that the combined expression of both of these transactivators, and perhaps the BamHI-R transactivator (16) as well, may be required for disruption of latency.

Alternatively, the ability of the BZLF1 to down regulate expression of latent EBV genes may also contribute to the disruption of latency. We find that the deletion mutant

pEBV-ZIE (Δ HindIII-HindIII), which retains the ability to up regulate the BMLF1 productive promoter but has lost the ability to down regulate the BamHI-C EBNA promoter, no longer disrupts viral latency. As previously suggested by Baichwal and Sugden (2), the EBNA 1 transcript could theoretically act as an antisense RNA to the BZLF1 transcript, thereby reducing translation of BZLF1. A reduction in EBNA transcription may thus be required if adequate synthesis of the BZLF1 gene product is to occur for disruption of latency.

Countryman et al. (9) have previously analyzed the sequences from defective EBV virions required for disruption of latency. These investigators found that a particular DNA clone, WZhet, which contains the BamHI Z fragment linked to a portion of the BamHI W fragment, is capable of disrupting viral latency. Deletion analysis of the WZhet fragment showed that it is the BZLF1 ORF that is required for disruption of latency, whereas the BamHI-W sequences are dispensable. These investigators reported that a site-directed mutation in the upstream HindIII site of BZLF1 destroyed the ability of BZLF1 to disrupt latency. However, in contrast to our own HindIII-HindIII deletion, this mutation disrupted the BZLF1 ORF.

The BZLF1-induced increase in pM-CAT mRNA as well as CAT activity suggests that the increase in CAT activity may be mediated by a transcriptional mechanism. Absolute proof of a transcriptional mechanism would require the use of nuclear run-on studies, which we have not yet performed. In comparing the sequences of the two promoters stimulated by BZLF1 (the BMLF1 and BMRF1 promoters), we have been unable to identify any common motif. Likewise, we have not identified any sequence similarity in the three latent promoters down regulated by BZLF1. At this point, it is unknown whether the BZLF1 transactivator exerts its effect directly by binding to promoter sequences or indirectly through an effect on cellular proteins. However, it is clear that the BZLF1 gene product does not produce indiscriminant transactivation of all EBV productive promoters, since we find that only two of the four productive EBV promoters tested are up regulated by BZLF1.

It will now be of particular interest to study the regulation of the BZLF1 gene product itself. To maintain a latent infection of lymphoid cells, the expression of BZLF1 must be tightly controlled, since overexpression of the gene leads irreversibly to productive infection and death of the host cell, which is perhaps a disadvantage to the virus. We have previously shown that the presumed BZLF1 promoter region functions very poorly in latently infected B-cell lines, although its activity can be increased by 12-*O*-tetradecanoyl-phorbol-13-acetate (23), which also disrupts viral latency (27). A major control mechanism in EBV may be directed at preventing the expression of BZLF1 in B cells (in which viral infection is largely latent), while allowing the expression of BZLF1 within the epithelial cells that are permissive for EBV replication.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants I K08 CA 01 229-01, NIAID A10751, and NIAID 1 VOI A123634-01 from the National Institutes of Health and by the Jefferson Pilot Blake Newton Award.

We thank Michele Davis and Eng-Shang Huang for use of the pH1013 vector, Bruce Howard for pRSV-CAT, the late George Khoury for pCAT3M, Nancy Raab-Traub for cloned EBV fragments, and Gloria Majette for preparation of the manuscript.

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