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The Epstein-Barr Virus BMLF1 Promoter Contains an Enhancer Element That Is Responsive to the BZLF1 and BRLF1 Transactivators

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We have previously shown that the Epstein-Barr virus (EBV) immediate-early gene product, BZLF1, can activate expression of the EBV BMLF1 immediate-early promoter in EBV-positive, but not EBV-negative, B cells, suggesting that the BZLF1 effect may be mediated through another EBV gene product (S. Kenney, J. Kamine, E. Holley-Guthrie, J.-C. Lin, E.-C. Mar, and J. S. Pagano, J. Virol. 63:1729–1736, 1989). Here, we show that the EBV BRLF1 immediate-early gene product transactivates the BMLF1 promoter in either EBV-positive or EBV-negative B cells. Deletional analysis revealed that both the BZLF1-responsive region and the BRLF1-responsive region of the BMLF1 promoter are contained within the same 140-base-pair *FokI-PvuII* fragment located 300 base pairs upstream of the mRNA start site. This *FokI-PvuII* fragment functions as an enhancer element in the presence of the BRLF1 transactivator and contains the sequence CCGTGGAGA ATGTC, which is strikingly similar to the BRLF1-responsive region of the EBV DR/DL enhancer (A. Chevallier-Greco, H. Gruffat, E. Manet, A. Calender, and A. Sergeant, J. Virol. 63:615–623, 1989). The effect of BZLF1 on the BMLF1 promoter is likely to be indirect and mediated through the BRLF1 transactivator.

Epstein-Barr virus (EBV) infection of B lymphocytes is largely latent, with only rare cells producing infectious virus. The disruption of EBV latency in B cells is likely to involve a complex series of interactions between virally encoded transactivating proteins and *cis*-regulating functions of key viral promoters. Three different EBV immediate-early genes, BZLF1, BMLF1, and BRLF1, have now been shown to encode transactivating functions (2–5, 13–16, 19, 25, 27, 28; S. Kenney, J. Kamine, E. Holley-Guthrie, and J. C. Lin, *in* P. Levine, D. Ablashi, M. Nonoyama, G. Pearson, and R. Glaser, ed., *Epstein-Barr Virus and Human Disease*, in press). Of these, it appears likely that it is the BZLF1 gene product that provides the crucial first step in the disruption of latency, since high-level expression of the BZLF1 gene product (but not of the BMLF1 or BRLF1 gene product) is sufficient to disrupt latency (11, 22, 25). The BZLF1 gene encodes a 34- to 38-kilodalton nuclear protein (20) which has some sequence homology to the c-*fos* protein (8) and can bind to the consensus Ap-1 site.

We recently reported that the BZLF1 gene product activates expression of the EBV BMLF1 immediate-early promoter and showed that the stimulating effect of the BZLF1 transactivator on the BMLF1 promoter is much greater in EBV-positive than in EBV-negative B cells (14; Kenney et al., in press). The BMLF1 promoter contains a consensus Ap-1 binding site approximately 100 base pairs (bp) upstream of the mRNA start site, and it has therefore been suggested that the BZLF1 effect on BMLF1 promoter function may be direct, involving binding of the BZLF1 protein to the BMLF1 promoter Ap-1 binding site (8). However, the BMLF1 promoter sequences required for BZLF1-induced transactivation have not been previously mapped, and the contribution of the Ap-1 binding site in the promoter has only been inferred. Since the BZLF1 immediate-early gene product is a much more effective transactivator of BMLF1 in EBV-infected cells, it seemed likely that its action on the BMLF1 promoter was indirect and mediated through another EBV gene product.

In this study, we have constructed a series of 5′ deletion mutants in the BMLF1 promoter (linked to the heterologous chloramphenicol acetyltransferase [CAT] gene) and have examined the ability of each promoter mutant to respond to the BZLF1 transactivator. We found that the BZLF1-responsive element of the BMLF1 promoter could be localized to a 140-bp fragment several hundred nucleotides 5′ to the cap site, suggesting that BZLF1 binding to the potential Ap-1 site is not important for BMLF1 promoter activation. We demonstrate that, in contrast to the BZLF1 gene product, which stimulates the BMLF1 promoter in EBV-positive cells, the EBV immediate-early gene product, BRLF1, transactivates the BMLF1 promoter independent of EBV infection in transfected cells. The BRLF1-responsive region of BMLF1 is localized within the same 140-bp fragment responsive to the BZLF1 transactivator. This 140-bp fragment transfers BRLF1 responsiveness to a heterologous promoter and can function as an enhancer element. We conclude that the BZLF1 effect on the BMLF1 promoter is indirect and likely to be mediated through BRLF1.

MATERIALS AND METHODS

Cell lines. The Raji cell line is a latently infected EBV-positive Burkitt's lymphoma B-cell line. The B95-8 cell line is a productively infected marmoset B-cell line. The Jurkat line is a human T-cell line, and the BJAB cell line is an EBV-negative Burkitt's lymphoma B-cell line. The HeLa cell line is a human cervical epithelial cell line. The lymphoid cells lines were maintained in RPMI 1640 medium with 10% fetal calf serum. HeLa cells were grown in Dulbecco modified Eagle medium H with 10% fetal calf serum.

DNA transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. Electroporation (26) into lymphoid cells and HeLa cells was accomplished at

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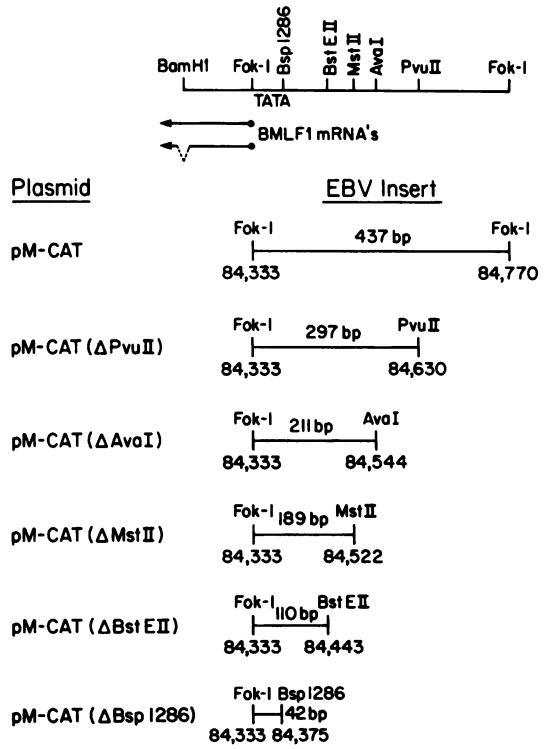


FIG. 1. Construction of pM-CAT deletions. The parent pM-CAT construct contains the 437-bp *Fok*I-*Fok*I fragment from *Bam*HI S (EBV nucleotides 84770 to 84333) linked to CAT. We have mapped the BMLF1-BSLF2 RNA cap site at approximately nucleotide 84330. Previous RNA mapping has shown that the BSLF2 open reading frame can be spliced into the BMLF1 open reading frame to form a 50- to 60-kilodalton protein (23). A series of 5′ deletion mutants were constructed by cutting at the indicated restriction endonuclease site, blunt ending with the T4 polymerase or Klenow enzyme, and religating. An additional plasmid, pS-CAT (not shown), was constructed and contains the entire EBV *Bam*HI S fragment linked to CAT.

1,500 V, using a Zapper electroporation unit from the University of Wisconsin, Madison, Medical Electronics Shop; 10^7 cells were transfected per condition for each CAT assay.

EBV-CAT plasmids. EBV promoter sequences were ligated into the *Bgl*II site of plasmid pCAT3M (18). Plasmid pCAT3M contains the bacterial CAT gene and a simian virus 40 (SV40) polyadenylation site but no eucaryotic promoter element. The exact genome map locations (1) of each construct are shown in Fig. 1. The pS-CAT plasmid (not shown) contains the entire *Bam*HI S fragment (EBV nucleotides 79537 to 84233) ligated into pCAT3M. The pM-CAT construct contains the *Bam*HI S fragment nucleotides 84770 to 84333 linked to CAT. Deletion mutants of pM-CAT were constructed by cutting with the restriction enzymes shown, blunt ending with T4 polymerase or the Klenow enzyme, and then religating. We have mapped the major mRNA start site of BMLF1 to the EBV map coordinate of approximately 84330. The pS-CAT construct (but probably not the pM-CAT construct) contains the endogenous mRNA cap site.

Transactivator plasmids. The pEBV-ZIE plasmid, as previously described (14; Kenney et al., in press), contains the BZLF1 gene product within the pGem-2-based vector, phD1013 (6), under the control of the strong human cytomegalovirus immediate-early promoter. The pEBV-RIE plasmid has the 2,332-bp *Bgl*II-*Hin*dIII fragment (EBV nucleotides 105413 to 103080) inserted into the *Bam*HI site of the phD1013 vector. The resultant plasmid contains the EBV BRLF1 gene under the control of the human cytomegalovirus immediate-early promoter.

CAT assays. Cells were transfected with plasmid DNA, and 48 h later an extract of the cells was prepared and incubated at 37°C with ^{14}C-labeled chloramphenicol in the presence of acetyl coenzyme A, as previously described (10). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography, followed by autoradiography and scintillation counting. All CAT assays were repeated, using smaller amounts of extract, if results were not in the linear range (less than 70% chloramphenicol acetylation).

RNA analysis. To quantitate CAT mRNA, cytoplasmic RNA was prepared 24 h after transfection, as previously described (9). A 20-bp oligonucleotide primer homologous to the CAT gene in the region 15 to 34 bp downstream of the ATG codon was synthesized and labeled with [γ-^{32}P]ATP. Labeled oligonucleotide (10^5 cpm) was hybridized overnight with cytoplasmic RNA in a solution containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 400 mM NaCl, and 1 mM EDTA. Primer extension was performed at 42°C for 90 min with avian myeloblastosis virus reverse transcriptase (21); the primer extension products were electrophoresed on a 6% acrylamide–7 M urea gel.

Sequence analysis. Computer searches of the EBV genome were performed on a VAX computer, using the Wisconsin Genetics Computer group sequence analysis software (7).

RESULTS

Constitutive activities of BMLF1 deletion mutants in different cell types. To study the BMLF1 promoter sequences required for *cis* and *trans* regulation, we built a series of 5′ deletion mutants in the pM-CAT plasmid, as illustrated in Fig. 1. The pM-CAT plasmid contains the EBV nucleotides 84770 to 84333. Five plasmids were constructed from pM-CAT [pM-CAT(ΔPvuII), pM-CAT(ΔAvaI), pM-CAT(ΔMstII), pM-CAT(ΔBstEII), and pM-CAT(ΔBsp1286)] which delete progressively larger fragments of the upstream promoter sequences.

The effects of 5′ deletions on constitutive activity of the BMLF1 promoter in several cell types are shown in Table 1. Plasmid pCAT3M, which has no eucaryotic promoter element, served as a negative control, and plasmid pRSV-CAT (10), which has the strong Rous sarcoma virus promoter, served as a positive control for transfection efficiency. In the latently infected, EBV-positive Raji B-cell line, in which the endogenous BMLF1 gene is not normally transcribed, neither the pS-CAT nor pM-CAT plasmids, nor any of the deletion plasmids, produced significant CAT activity in comparison with the negative control plasmid, pCAT3M. Similar results were obtained in the other cell lines.

Localization of the BZLF1- and BRLF1-responsive regions. In preliminary experiments, we found that the levels of pS-CAT and pM-CAT activities could be greatly increased by cotransfection with either the pEBV-ZIE (which encodes the BZLF1 gene product) or the pEBV-RIE (which encodes the BRLF1 gene product) constructs. To identify the BMLF1 promoter region responsive to the BZLF1 and BRLF1 transactivators, the pM-CAT deletion plasmids (5 μg) were cotransfected with 5 μg of either the phD1013 vector, pEBV-ZIE (which encodes the BZLF1 gene prod-

TABLE 1. Constitutive activities of BMLF1 promoter deletion plasmids in different cell types

Plasmid	% Acetylation in cell line (avg and range)[a]			
	Raji	BJAB	Jurkat	HeLa
pS-CAT	0.7 (0.5–0.9)	0.6 (0.6–0.6)	1.2 (0.7–1.4)	1.8 (1.7–1.8)
pM-CAT	0.4 (0.3–0.5)	0.4 (0.3–0.5)	0.7 (0.6–0.7)	0.3 (0.2–0.3)
pM-CAT(ΔPvuII)	0.4 (0.3–0.5)	0.4 (0.4–0.5)	0.7 (0.5–0.8)	0.4 (0.3–0.4)
pM-CAT(ΔAvaI)	0.8 (0.4–1.4)	0.5 (0.4–0.5)	1.6 (0.8–4.4)	0.5 (0.4–0.6)
pM-CAT(ΔMstII)	0.5 (0.4–0.5)	0.6 (0.4–0.7)	1.3 (1.9–1.5)	0.4 (0.3–0.4)
pM-CAT(ΔBstEII)	0.7 (0.5–0.9)	0.8 (0.6–1.0)	2.4 (2.0–2.7)	0.4 (0.4–0.4)
pM-CAT(ΔBsp1286)	0.2 (0.2–0.3)	0.6 (0.3–0.8)	0.4 (0.3–0.5)	0.3 (0.2–0.3)
pCAT3M	0.5 (0.4–0.6)	0.4 (0.3–0.4)	0.8 (0.7–0.8)	0.3 (0.3–0.3)
pRSV-CAT	51.0 (27.0–66.2)	18.7 (16.4–21.0)	92.3 (87.5–97.0)	98.0 (97.0–99.0)

[a] The values shown are derived from three experiments in Raji cells and two experiments in other cell types, using 100 μl of cell extract.

uct), or pEBV-RIE (which encodes the BRLF1 gene product). These results are shown in Tables 2 and 3.

As we have previously reported (14), the BZLF1 transactivator significantly increases pM-CAT activity in the EBV-positive B-cell line Raji but has little effect in EBV-negative cell types. In Raji cells, BZLF1 transactivation of the pM-CAT plasmid requires a specific region of the BMLF1 promoter, within the 140-bp *Fok*I-*Pvu*II fragment located 300 to 440 bp upstream of the mRNA start site (Table 2). This fragment appears to be essential for BZLF1 transactivation, since all pM-CAT deletion plasmids not containing this region of the promoter were unresponsive to the BZLF1 transactivator.

We also examined the ability of each pM-CAT deletion plasmid to respond to the BRLF1 transactivator (Table 3). Since preliminary experiments had shown that the BRLF1 transactivator affects pM-CAT activity in either EBV-positive or EBV-negative cell types, the experiments shown were performed in EBV-negative cell lines to avoid the possibility of indirect effects induced through other EBV gene products. As was found with the BZLF1 transactivator, maximal response to the BRLF1 transactivator also required the BMLF1 promoter sequences within the 140-bp *Fok*I-*Pvu*II fragment. Thus, the BZLF1- and BRLF1-responsive elements of the BMLF1 promoter are located within the same 140-bp fragment. Since the BZLF1 effect occurs in EBV-positive, but not EBV-negative, B cells, the effect of BZLF1 on BMLF1 promoter activity may be mediated through BZLF1-induced activation of another EBV gene product, presumably BRLF1.

RNA analysis. We have previously shown that the BZLF1 transactivator increases the level of BMLF1-directed CAT mRNA (14). In this study, we used the primer extension method to confirm that the BRLF1-transactivating effect also increases CAT mRNA levels and that the mRNA start site does not change in the presence of the BRLF1 transactivator. These results are shown in Fig. 2. In the experiment shown, the pS-CAT plasmid (5 μg) was cotransfected into Jurkat cells with 5 μg of either phD1013 (lane A) or pEBV-RIE (lane B). Cytoplasmic RNA harvested 24 h after transfection was hybridized to a γ-^{32}P-labeled primer homologous to the CAT gene sequences 15 to 34 bp downstream of the ATG codon, and primer extension was performed. The expected primer extension product is 170 bp, if the TATA box is used and no splicing occurs. Using the pS-CAT plasmid, only one major 5′ start site was seen, which maps to approximately 25 bp downstream of the TATA box. Cotransfection with the BRLF1 transactivator clearly increases the level of pS-CAT mRNA and does not alter the mRNA start site.

The BZLF1- and BRLF1-responsive region in the BMLF1 promoter functions as an enhancer element. Because the BZLF1- and BRLF1-responsive element of the BMLF1 promoter could be localized to a specific region contained within the 140-bp *Pvu*II-*Fok*I fragment, we tested the ability of this fragment to transfer BZLF1 and BRLF1 responsiveness to the SV40 early promoter. The 140-bp fragment was inserted into the pA10CAT plasmid (17) (Fig. 3) directly upstream of the SV40 early promoter (*Bgl*II site) in either orientation or downstream of the SV40 promoter (*Bam*HI site). In addition, the 140-bp piece was divided into two smaller fragments (the 83-bp *Fok*I-*Sau*3A piece and the 57-bp *Sau*3A-*Pvu*II piece), each of which was also cloned into the *Bgl*II site of the pA10CAT plasmid.

TABLE 2. Effect of BZLF1 transactivator on pM-CAT deletion plasmids

Plasmid	Fold increase in CAT activity in cell line (avg and range)[a]		
	Raji	Jurkat	BJAB
pS-CAT	15.5 (12.6–18.3)	1.2 (1.1–1.3)	NT
pM-CAT	46.8 (34.2–54.7)	1.9 (1.3–3.3)	1.4 (1.0–1.8)
pM-CAT(ΔPvuII)	1.3 (1.2–1.3)	1.5 (0.5–3.2)	1.8 (0.9–2.6)
pM-CAT(ΔAvaI)	1.1 (0.4–1.4)	0.5 (0.3–0.8)	0.8 (0.6–0.9)
pM-CAT(ΔMstII)	1.4 (0.8–2.2)	0.6 (0.3–1.1)	1.3 (0.9–1.6)
pM-CAT(ΔBstEII)	1.0 (0.4–1.1)	1.1 (0.6–1.4)	1.1 (0.6–1.6)
pM-CAT(ΔBsp1286)	1.0 (0.8–1.3)	0.7 (0.7–0.8)	0.8 (0.8–0.8)

[a] The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with the pEBV-ZIE plasmid versus the phD1013 vector. The constitutive activity produced by each CAT vector is as shown in Table 1. NT, Not tested. Ranges are those seen in four experiments in Raji cells, three experiments in Jurkat cells (pS-CAT not used in one experiment with each cell type), and two experiments in BJAB cells.

TABLE 3. Effect of the BRLF1 transactivator on pM-CAT deletion plasmids

Plasmid	Fold increase in CAT activity in cell line (avg and range)[a]	
	BJAB	Jurkat
pS-CAT	35.4 (32.4–38.3)	53.6 (39.4–67.7)
pM-CAT	45.6 (12.7–81.7)	63.1 (26.6–119.1)
pM-CAT(ΔPvuII)	1.6 (1.3–1.9)	2.8 (0.7–6.6)
pM-CAT(ΔAvaI)	2.6 (2.1–3.3)	2.6 (1.6–3.8)
pM-CAT(ΔMstII)	2.4 (1.9–3.4)	1.7 (1.3–2.3)
pM-CAT(ΔBstEII)	1.0 (0.7–1.6)	2.1 (1.0–3.8)
pM-CAT(ΔBsp1286)	1.0 (0.9–1.1)	1.3 (0.9–1.9)

[a] The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with the pEBV-RIE plasmid versus the phD1013 vector. The amount of extract used for each CAT plasmid was adjusted to keep the total CAT activity after cotransfection with pEBV-RIE within the linear range. Ranges are those seen in three experiments (one experiment with each cell type did not include the pS-CAT construct).

The insertion of the intact 140-bp *Sau*3A-*Pvu*II fragment or its two subfragments into pA10CAT had no effect on the constitutive promoter activity produced by pA10CAT in a variety of cell types (BJAB, Jurkat, Raji, HeLa, and HEp-2; data not shown). Thus, in the absence of the BRLF1 and BZLF1 transactivating functions, the 140-bp *Fok*I-*Pvu*II fragment cannot function as an enhancer element, at least in the five cell types tested. However, when the pA10CAT plasmids containing the 140-bp *Fok*I-*Pvu*II fragment were cotransfected into Raji cells with pEBV-ZIE, these plasmids showed a much greater increase in CAT activity in response to the BZLF1 transactivator than did the parent pA10CAT plasmid (Table 4). Whereas the activity of the parent pA10CAT plasmid was increased threefold in the presence of pEBV-ZIE, the addition of the 140-bp BZLF1-responsive region from the BMLF1 promoter to pA10CAT resulted in increases of 27- to 67-fold in the presence of BZLF1. Since this increased responsiveness to the BZLF1 transactivator occurred regardless of the orientation or position of the 140-bp fragment in pA10CAT, this fragment functions as an enhancer element. The 83-bp subfragment of the 140-bp region also increased the pA10CAT response to the BZLF1 transactivator but was not nearly as effective as the intact 140-bp fragment. The BMLF1 enhancer element was stimulated by the BZLF1 transactivator in the EBV-positive Raji cells but not in the EBV-negative Jurkat cells.

The BMLF1 enhancer fragment can also be transactivated by the BRLF1 gene product (Table 5). BRLF-1 induced transactivation (in contrast to the BZLF1 effect) occurred in either EBV-negative or EBV-positive cell types. Whereas the BRLF1 transactivator had a slight stimulating effect on the parent plasmid, pA10CAT (2.1- and 5.7-fold in two separate experiments), this effect was increased greatly (up to 134-fold) in the presence of the BMLF1 enhancer fragment, regardless of the position or orientation of the fragment. The intact 140-bp enhancer element also responded to the BRLF1 transactivator in nonlymphoid cells, such as HeLa and HEp-2 (data not shown). As with the BZLF1 transactivator, only the 83-bp subfragment, and not the 57-bp subfragment, was able to function independently as an enhancer element, although once again the intact 140-bp piece was the most effective enhancer.

DISCUSSION

High-level expression of the EBV immediate-early gene product, BZLF1, is sufficient to drive previously latent infection of B lymphocytes into the productive mode of EBV infection (11, 22, 25). The BZLF1 gene encodes a regulatory protein which shares homology with the c-*fos* gene product (8) and has been shown to enhance the expression of several different EBV early promoters (2–4, 13, 14), but little is known about the promoter sequences required for BZLF1 response. In this article, we demonstrate that the BZLF1-responsive sequence in the BMLF1 immediate-early promoter is contained within a 140-bp enhancer element. We show that this enhancer element is also transactivated by the EBV BRLF1 immediate-early gene product. We conclude that BZLF1 transactivation of the BMLF1 promoter is likely to be indirect and mediated through the BRLF1 transactivator, since a strong BZLF1 effect is seen in EBV-positive but not EBV-negative cell types.

In many respects, our findings concerning the BMLF1 immediate-early promoter are similar to the studies on the EBV DR/DL (duplicated right and left) promoter recently published by Chevallier-Greco et al. (2, 3). These investigators concluded that BZLF1-induced transactivation of the DR/DL promoter in EBV-positive cells is mediated through the BRLF1 transactivator and demonstrated that the

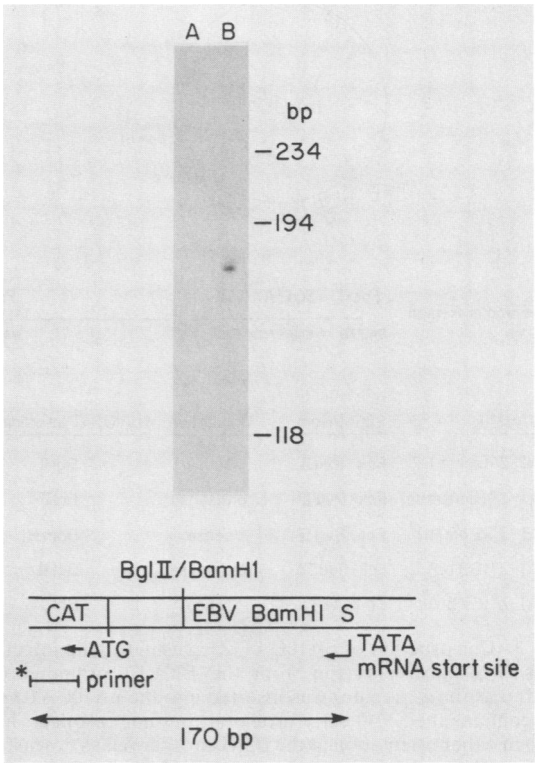
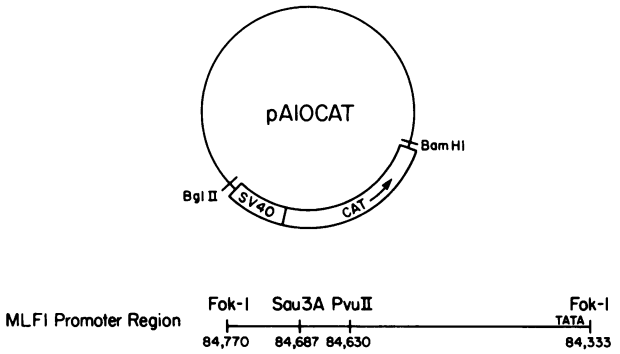


FIG. 2. RNA analysis. Jurkat cells were transfected with 5 μg of the pS-CAT plasmid and 5 μg of phD1013 (lane A) or with 5 μg of pS-CAT and 5 μg of pEBV-RIE (lane B). Cytoplasmic RNA was harvested 24 h after transfection and hybridized overnight to a [^{32}P]ATP-labeled primer homologous to the CAT gene in the region 15 to 34 bp downstream of the ATG codon. Primer extension (21) was performed, and the primer extension products were run on a 6% acrylamide–7 M urea gel. The size of the expected primer extension product is 170 bp, assuming that the TATA box is used and no splicing occurs (splicing is not expected with the pS-CAT construct, since no splice acceptor sequences are included within the EBV insert). Cotransfection of the pS-CAT plasmid with the BRLF1 transactivator (pEBV-RIE) increased the level of S-CAT mRNA (lane B).



PLASMID	EBV Insert	Orientation	pAIOCAT Insertion Site
pAIOCAT-Z*(sense)	Fok-PvuII	sense	BglII
pAIOCAT-Z*(antisense)	Fok-PvuII	antisense	BglII
pAIOCAT-Z*(BamHI)	Fok-PvuII	sense	BamHI
pAIOCAT-Z*(83 bp)	Fok-Sau3A	antisense	BglII
pAIOCAT-Z*(57 bp)	Sau3A-PvuII	sense	BglII

FIG. 3. Construction of pA10CAT-Z* plasmids. The intact 140-bp BZLF1-responsive region from the BMLF1 promoter (here referred to as the Z* region) was inserted into the pA10CAT vector (which contains the SV40 early promoter, but not enhancer, linked to CAT) in either orientation at the *Bgl*II site, as well as downstream at the *Bam*HI site. In addition, the 83-bp *Fok*I-*Sau*3A fragment and the 57-bp *Sau*3A-*Fok*I fragment were inserted into the *Bgl*II site of pA10CAT.

BRLF1-responsive region of the DR/DL promoter can function as an enhancer element. In addition, the 28-bp sequence CTGTGCCTTGTCCCGTGGACAATGTCCC found in the DR/DL promoter was sufficient to confer BRLF1 responsiveness in a heterologous promoter.

Strikingly, the 83-bp subfragment of the BMLF1 enhancer, which is also sufficient to confer BRLF1 responsiveness to a heterologous promoter (albeit with reduced efficiency compared with the intact 140-bp enhancer), contains the sequence CCGTGGAGAATGTC, which has a 13- to 14-bp identity to the DR/DL BRLF1 response element, CCGTGGACAATGTC. In addition, imperfect copies of the sequence CTGGGC are repeated multiple times within the 140-bp BMLF1 enhancer fragment. When we searched the entire EBV genome in both orientations for the DR/DL promoter sequence CCGTGGACAATGTC (allowing 1-bp mismatch), this sequence was found only twice: in the BHRF1 promoter, which contains the left-hand copy of the DR/DL promoter and responds to the BRLF1 transactivator (13), and in the BMLF1 enhancer. Therefore, the finding that three different BRLF1-responsive enhancers each contain the sequence CCGTGGA(C/G)AATGTC strongly suggests that this sequence plays a crucial role in conferring BRLF1 responsiveness. At this point, it is not known whether the BRLF1 protein can bind to this sequence directly or acts by stimulating the binding of cellular transcription factors.

TABLE 4. Effect of BZLF1 transactivator on pA10CAT-Z* plasmids

Plasmid	Fold increase in CAT activity[a] in cell line	
	Raji	Jurkat
pA10CAT	3.1	2.1
pA10CAT-Z*(sense)	66.6	1.3
pA10CAT-Z*(antisense)	57.6	1.0
pA10CAT-Z*(*Bam*HI)	26.9	2.1
pA10CAT-Z*(83 bp)	9.6	3.6
pA10CAT-Z*(57 bp)	2.3	1.7

[a] The numbers shown are the fold increase in CAT activity seen when each CAT plasmid was cotransfected with the pEBV-ZIE plasmid versus the pHD1013 plasmid.

TABLE 5. Effect of the BRLF1 transactivator on pA10CAT-Z* plasmids in Jurkat cells

Plasmid	Fold increase in CAT activity[a]	
	Expt 1	Expt 2
pA10CAT	2.1	5.7
pA10CAT-Z*(antisense)	35.6	66.3
pA10CAT-Z*(*Bam*HI)	69.3	134.2
pA10CAT-Z*(83 bp)	16.5	20.7
pA10CAT-Z*(57 bp)	4.9	3.1

[a] The numbers represent the fold increase in CAT activity produced by cotransfection with the pEBV-RIE plasmid versus the pHD1013 vector.

The potential Ap-1 site, located within the *Bsp* 1286-*Bst*EII fragment, was not demonstrated to be important for *trans*-acting regulation of the BMLF1 promoter in this study. Conceivably, the low-level transactivation induced by the BZLF1 gene product in EBV-negative cells might involve binding to this Ap-1 site. We have found that simultaneous cotransfection of the BMLF1 promoter with both the BRLF1 and BZLF1 transactivators into EBV-negative cells produces no greater activation than that seen with the BRLF1 transactivator alone, provided that the BRLF1 transactivator is driven by a strong heterologous promoter. Therefore, at this point we do not believe that the BZLF1 gene product plays an important role in regulating the BMLF1 promoter, other than indirectly through its presumed effect on BRLF1 promoter activity. On the other hand, we have previously shown that the promoter of the EBV BMRF1 gene, which encodes the early antigen diffuse protein, can be transactivated by the BZLF1 gene product equally well in EBV-negative and EBV-positive cell types (14). This result suggests that the BZLF1 effect on the EBV BMRF1 promoter is direct and not mediated through other EBV gene products.

The demonstration of an enhancer element within BMLF1 may help to explain the efficiency with which the defective P3HRI (het) virions disrupt viral latency. We have previously shown that the endogenous promoter of the BZLF1 gene is extremely weak, although its activity can be increased somewhat with 12-*O*-tetradecanoylphorbol-13-acetate (16). We have also shown that the BZLF1 promoter is not significantly transactivated by its own gene product (14). However, in the defective P3HRI (het) virions, the normal genomic locations of BMLF1 and BZLF1 have been altered such that the BMLF1 enhancer region is now located within 5 kilobases downstream of the BZLF1-encoding region (22). This juxtaposition of the BMLF1 enhancer with the BZLF1 gene presumably allows the BZLF1 gene product to enhance its own expression (through BRLF1-induced transactivation of the adjacent BMLF1 enhancer region), an event which probably cannot occur in the intact EBV genome.

Two EBV enhancers have now been identified which respond to the BRLF1 immediate-early transactivator. One

of these, the DR/DL enhancer, is present in two copies in most strains of EBV. The DR/DL enhancer can *cis* activate expression of the cytoplasmic early antigen (BHRF1) gene (13) and was recently shown to play an essential role in the EBV lytic origin of replication (12). The other enhancer function, BMLF1, could potentially *cis* activate expression of both the BMLF1 gene, which encodes a transactivating function, and the early antigen diffuse gene, which is located only 5 kilobases upstream of the BMLF1 enhancer. The crucial first step in BZLF1-induced disruption of viral latency is likely to involve activation of BRLF1 expression, which then leads to activation of the BRLF1-responsive enhancers. Regulation of BRLF1 expression by BZLF1 will be a key area for future investigation.

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