The Epstein-Barr Virus Immediate-Early Gene Product, BMLF1, Acts in *trans* by a Posttranscriptional Mechanism Which Is Reporter Gene Dependent

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Received 17 February 1989/Accepted 16 May 1989

In DNA cotransfection experiments, the Epstein-Barr virus immediate-early gene product, BMLF1, stimulated the chloramphenicol acetyltransferase (CAT) activity of both latent and productive EBV promoters linked to CAT. This BMLF1-induced increase in CAT activity was out of proportion to the effect on CAT mRNA, suggesting a posttranscriptional mechanism. Furthermore, when growth hormone was used as a reporter gene instead of CAT, BMLF1 no longer functioned. Thus, the BMLF1 effect was reporter-gene dependent. The effect of the BMLF1 gene product does not then appear to be directed at promoter activation, but instead may function to increase the level of an as yet unidentified protein(s) required for Epstein-Barr virus infection.

A biologic hallmark of Epstein-Barr virus (EBV) infection, as with other herpesviruses, is latency (10, 27). The mechanisms governing latency are still obscure, but it is likely that control of viral gene expression is critical. The availability of latently infected, EBV-transformed B-cell lines provides the opportunity to study herpesviral gene regulation in a latent system and to examine factors important in disrupting latency.

Few viral genes are transcribed in latent EBV infection (26, 43). The most important latent gene products identified so far are Epstein-Barr nuclear antigen (EBNA)-1, EBNA-2, and the latent membrane protein (LMP) (12, 17-19, 25, 34, 36, 44, 47). The EBNA-1 gene product encodes a transactivating function which is crucial in maintaining episomal replication during latent infection (36, 47). In productive EBV infection, which can be induced from the latent state in vitro by various inducers (31, 34), many more genes are transcribed, including three EBV immediate-early (IE) genes (4, 39) (BZLF1, BRLF1, and BMLF1) whose products have been shown to encode transactivating functions (6, 7, 15, 16, 21, 23, 30, 41, 45). At least one of these IE gene transactivators, BZLF1, clearly plays a crucial role in the disruption of latency and activation of viral replication (15, 41). The BZLF1 gene product has been shown to stimulate the activity of several different EBV promoters (6, 7, 16), including the BMLF1 promoter (20a, 21).

The role of the BMLF1 IE gene product in EBV infection is less clear. The BMLF1 gene product appears to transactivate several different promoters when linked to the heterologous chloramphenicol acetyltransferase (CAT) gene (6, 30, 45). We reported recently that the BMLF1 gene product can increase CAT activity directed by the human immunodeficiency virus promoter (22) and demonstrated that the mechanism of transactivation by BMLF1 may be at least partly posttranscriptional, since the steady-state level of human immunodeficiency virus-CAT mRNA was not affected to the extent that CAT activity was increased.

In this report, we have examined the effect of the BMLF1

gene product on latent versus productive EBV promoters. Representative EBV promoters were chosen for study: latent (latent membrane protein and the *Bam*HI-W and *Bam*HI-C EBNA promoters [5, 29, 37]), IE (BMLF1 [38] and BZLF1 [4]), early (the early antigen diffuse gene, BMRF1 [8, 38]), and late (the major envelope glycoprotein, gp350/220 [2]). We show that cotransfection of each of the EBV-CAT constructs with the BMLF1 construct results in increased CAT activity. As in our previous report, the increase in CAT activity produced by BMLF1 is not accompanied by a significant increase in steady-state level CAT mRNA (22). However, we now demonstrate that the BMLF1 transactivation effect is reporter-gene limited. Although the activity of the different promoters linked to the CAT gene is increased by cotransfection with BMLF1, several of the same promoters linked to the human growth hormone gene are not transactivated. We present here the evidence that the ability of the BMLF1 gene product to transactivate CAT vectors appears to be posttranscriptionally mediated and dependent on some quality brought to the vectors by the CAT gene itself, rather than specific to a particular class of promoters.

MATERIALS AND METHODS

Cell lines. Loukes is an EBV-negative Burkitt's lymphoma B-cell line, Raji is a latently infected EBV-positive Burkitt's lymphoma B-cell line, and B95-8 is a virus-producing EBV-positive marmoset B-cell line. Jurkat, a T-cell line, the human cervical epithelial cell line HeLa, and a human glioblastoma multiforme line, U-373-MG, were also used. Lymphoid cell lines were maintained in RPMI medium with 10% fetal calf serum. The monolayer cells were maintained in minimal essential medium with 5% fetal calf serum.

DNA transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. Electroporation into lymphoid cells (42) was accomplished at 1,500 V with a Zapper electroporation unit from the University of Wisconsin Medical Electronics Shop, Madison; 10^7 lymphoid cells were transfected per condition for each CAT assay. Each of the lymphoid lines had an approximately 30 to 50% cell

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FIG. 1. Construction of seven EBV promoter-containing plasmids. The promoter regions of selected EBV genes were excised from the EBV genome and ligated into the *Bgl*II site of vector pCAT3M (29). The vector contains the bacterial CAT gene and an SV40 polyadenylation site, but no eucaryotic promoter element. Restriction sites were chosen based on previously published 5' mRNA start sites for each gene (2, 4, 5, 8, 20, 30, 38). Three promoters expressed during latent infection (the *Bam*HI-C and -W EBNA promoters and the latent membrane protein promoter), two promoters of IE genes (BZLF1 and BMLF1), one early promoter (BMRF1), and one promoter of a late gene (gp350/220) were chosen for study. An additional plasmid, pS-CAT (not shown), was constructed which contains the entire *Bam*HI S fragment (and hence the BMLF1 promoter) linked to CAT.

survival rate under these electroporation conditions. HeLa cells were transfected with the DNA-calcium phosphate coprecipitation method as described previously (14).

Plasmid constructions. (i) EBV-CAT plasmids. EBV promoter sequences were ligated into the *Bgl*II site of plasmid pCAT3M. Plasmid pCAT3M (a gift from the late George Khoury) contains the CAT gene and a simian virus 40 (SV40) polyadenylation site, but no eucaryotic promoter element (29). The exact genome map locations (1) of the EBV promoter inserts are shown in Fig. 1. The sequences cloned into the CAT expression vector were based on previously published 5' mRNA start sites (2, 4, 5, 8, 20, 30, 32, 38) and the EBV sequence (1). Seven different EBV-CAT plasmids were constructed: three latent EBV promoters, two IE promoters, and one early and one late promoter were chosen for study. The pC-CAT and pW-CAT constructs place two different putative EBNA promoters (5, 37) (from *Bam*HI-C and *Bam*HI-W) in front of the CAT gene. The pLMP-CAT plasmid has the promoter region of the latent membrane protein. The pZ-CAT and pM-CAT constructs contain the upstream regions of the two different EBV IE genes BZLF1 and BMLF1. An additional plasmid, pS-CAT (not shown), was also constructed which contains the entire *Bam*HI S fragment (and hence the BMLF1 promoter) linked to CAT. The pEA-CAT construct contains the BMRF1 promoter of the early antigen diffuse gene. The pLate-CAT construct contains the suspected promoter region of the late gene encoding the two major outer envelope glycoproteins gp350/220. We have confirmed the use of the expected 5' start site

in the pC-CAT and pS-CAT constructs; the other mRNA start sites are presumed from the work of previous investigators and the EBV sequence (1).

(ii) Plasmid expressing the BMLF1 gene product. We constructed plasmid pEBV-MIE (22) to place the BMLF1 gene product under the control of a strong promoter. To create pEBV-MIE, the 3.0-kilobase *Bam*HI-*Bgl*II J fragment (which contains the second exon of BMLF1) was excised from the pM-B2-J plasmid (35) (a gift from George Bornkamm) and ligated into the *Bam*HI site of pHD1013 (a gift from Michelle Davis and E. S. Huang). The pHD1013 plasmid is a pGem-2 (Promega)-based vector which contains the human cytomegalovirus IE promoter (9). Previous studies have shown that only the second exon of BMLF1 is required for transactivating effects (30).

(iii) Growth hormone plasmids. To test for reporter-gene dependence of the BMLF1 transactivating effect, we constructed two plasmids (pM-GH and pC-GH) which insert the identical EBV promoter sequences contained within pM-CAT or pC-CAT into the *Bam*HI site of plasmid pφGH (40). Vector pφGH contains the human growth hormone gene and a polyadenylation site, but has no eucaryotic promoter element.

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 ¹⁴C-labeled chloramphenicol in the presence of acetyl coenzyme A, as described previously (13). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by autoradi-

ography and scintillation counting. All CAT assays were repeated with smaller amounts of protein extract if results were not in the linear range (<70% chloramphenicol acetylation).

RNA analysis. An Sp6-generated antisense RNA probe complementary to the pLate-CAT plasmid was made by cloning a fragment from pLate-CAT containing the intact promoter region plus 250 base pairs (bp) of the CAT gene into the pGem-2 vector (Promega) next to the Sp6 promoter. Total cellular RNA was prepared 2 days after transfection by a modification of the Favaloro method (11, 24); then, 35 µg was hybridized overnight to the ³²P-labeled antisense RNA probe (6.5 × 10⁴ cpm). The hybrid probe was digested with S1 nuclease (3), and the S1-protected fragment was electrophoresed on a 6% acrylamide gel with 7 M urea. Autoradiogram bands were analyzed by laser densitometry.

To determine the actual 5' start site(s) in the pS-CAT and pC-CAT plasmids, we used the primer extension method. A 20-bp oligonucleotide sequence homologous to the CAT gene in the region either 15 to 34 or 66 to 85 bp downstream of the ATG codon was synthesized and labeled with [γ-³²P] ATP. Labeled oligonucleotide (10⁵ counts) was hybridized overnight with 50 µg of RNA at 30°C in a solution containing 80% formamide, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4), 400 mM NaCl, and 1 mM EDTA. Primer extension was then performed at 42°C for 90 min, using avian myeloblastosis virus reverse transcriptase as described previously (33). After RNase digestion, the primer extension products were electrophoresed on a 6% acrylamide-urea gel.

RESULTS

Constitutive activity of EBV promoters in different cell types. Prior to assessing the effect of the EBV BMLF1 gene product on the different EBV-CAT constructs, we determined the constitutive activity of each EBV promoter in a variety of cell types. In these experiments, the activity of the EBV-CAT constructs was compared in each cell type with the activities of the pCAT3M plasmid, which has the CAT gene without any eucaryotic promoter; the pRSV-CAT plasmid (13), which has the strong Rous sarcoma virus promoter; and plasmid pA10CAT (28), which has the weak SV40 early promoter (but not enhancer) linked to CAT. The CAT constructs were transfected into cells, using either electroporation (lymphoid lines) or the calcium phosphate technique (HeLa and U-373-MG cells); CAT activity produced by each plasmid was assayed 2 days later.

The two EBNA promoters, pW-CAT and pC-CAT, each produced significantly more CAT activity (relative to the control plasmids pCAT3M and pA10CAT) in lymphoid versus nonlymphoid lines (Table 1). In both latently and productively infected EBV-positive B-cell lines, the pC-CAT construct produced more CAT activity than did the pW-CAT plasmid. The activity of the pLMP-CAT plasmid was quite weak in all cell lines tested. Other than the strong activity produced by the pM-CAT plasmid in the productively infected B95-8 cell line, the constitutive activity of the four productive promoters was also quite weak in all cell lines tested.

The EBV IE gene product, BMLF1, transactivates each of the seven EBV-CAT constructs. We next analyzed the effect of the EBV IE gene product, BMLF1, on the seven different EBV-CAT constructs. Table 2 shows the results of repre-

TABLE 1. Activity of EBV-CAT plasmids in different cell types

Plasmid	% Acetylation ^a					
	Loukes	Raji	B95-8	Glial	HeLa	Jurkat
Control						
pRSV-CAT	3.7	6.06	12.77	2.2	3.77	6.14
pCAT3M	0.2	0.10	0.34	0.05	0.03	0.11
pA10-CAT	0.1	0.05	0.52	0.06	0.05	0.10
Latent promoters						
pW-CAT	0.7	0.36	0.42	0.05	0.03	2.90
pC-CAT	1.8	1.98	2.66	0.04	0.13	2.88
pLMP-CAT	0.1	0.17	0.60	0.04	0.07	0.37
Productive promoters						
pZ-CAT	0.2	0.03	0.19	0.08	0.03	0.07
pM-CAT	0.1	0.04	1.55	0.04	0.07	0.05
pEA-CAT	0.1	0.04	0.93	0.05	0.03	0.12
pLate-CAT	0.1	0.04	0.10	0.08	0.04	0.02

^a Calculated as percentage of the ratio of counts of acetylated chloramphenicol products over total chloramphenicol counts per 10 µg of protein extract. Values represent the averages of at least two experiments in each cell type.

sentative cotransfection experiments done in the EBV-negative Loukes and the EBV-positive Raji B cells. In either line, the CAT activity of each of the seven EBV-CAT constructs was increased after cotransfection with the

TABLE 2. Effect of BMLF1 gene product on different EBV-CAT constructs

Plasmid	Expt	Fold increase in CAT activity ^a	Avg increase ± 2 SD
Control			
pCAT3M	1	6.1	6.4 ± 1.1
	2	5.4	
	3	7.6	
Latent promoters			
pW-CAT	1	15.3	16.5 ± 1.7
	2	15.7	
	3	18.6	
pC-CAT	1	54.3	29.7 ± 20.3
	2	14.4	
	3	20.3	
pLMP-CAT	1	38.2	35.8 ± 17.6
	2	45.2	
	3	24.0	
Productive promoters			
pZ-CAT	1	11.3	6.3 ± 4.1
	2	3.5	
	3	4.0	
pM-CAT	1	13.8	16.8 ± 6.3
	2	12.1	
	3	24.5	
pEA-CAT	1	12.5	6.3 ± 5.1
	2	3.2	
	3	3.1	
pLate-CAT	1	3.8	3.9
	2	4.0	

^a Based on a comparison of percent acetylation (per microgram of protein extract) after cotransfection with pEBV-MIE versus cotransfection with pHD1013. Experiments 1 and 2 were performed in Loukes cells, and experiment 3 was done in Raji cells.

BMLF1 gene product. Somewhat surprisingly, the activity of the negative control plasmid, pCAT3M, which contains the CAT gene but no eucaryotic promoter element, was also increased by cotransfection with BMLF1. The effects of the BMLF1 transactivator were similar in the latently infected Raji cells and the EBV-negative Loukes cells. The increase in CAT activity produced by the BMLF1 gene product was consistently greater when BMLF1 was cotransfected with the three latent promoter plasmids (pW-CAT, pC-CAT, and pLMP-CAT) or the pM-CAT plasmid versus the other EBV-CAT plasmids (pZ-CAT, pEA-CAT, and pLate-CAT). However, the BMLF1 transactivator nonspecifically increased the activity of both latent and productive EBV promoters linked to CAT.

The BMLF1 transactivator does not significantly affect mRNA levels. We demonstrated previously that the BMLF1 transactivator effect on pHIV-CAT activity is more than its effect on pHIV-CAT mRNA (22). In this study, we analyzed the effect of the BMLF1 transactivator on the level of RNA produced by two different EBV promoters. To perform S1 nuclease analysis, a 32 P-labeled RNA probe complementary to pLate-CAT mRNA was generated by using SP6 polymerase. The pM-CAT plasmid (5 μ g) was cotransfected into Raji cells with 5 μ g of either the pH1013 vector DNA or pEBV-MIE. Cytoplasmic RNA was prepared from each condition 48 h after transfection, hybridized to the 32 P-labeled RNA probe, and digested with S1 nuclease, and the S1 nuclease-protected fragment was run on an acrylamide-urea gel. A sample of cells from each condition was also analyzed for CAT activity. We used the pLate-CAT probe to quantitate pM-CAT RNA so that any increase in CAT RNA could be detected in this experiment regardless of whether this RNA originated from the EBV promoter or a cryptic plasmid promoter.

The S1 nuclease results are shown in Fig. 2. In the experiment shown, the pEBV-MIE transactivator increased the level of CAT activity produced by pM-CAT by 20-fold. However, this increase in CAT activity was not accompanied by a similar increase in steady-state level of CAT mRNA. Since the pCAT3M plasmid is also affected by cotransfection with BMLF1, one explanation for our inability to detect a significantly increased level of CAT mRNA could have been that in the presence of BMLF1 most of the CAT mRNA is initiating from a cryptic plasmid promoter, rather than the authentic EBV promoter. However, this explanation is unlikely since the total amount of CAT mRNA (regardless of start site) is not influenced by the presence or absence of the BMLF1 transactivator.

In addition to S1 nuclease analysis, we used the primer extension method to examine the effect of the BMLF1 transactivator on the level of CAT mRNA initiating at the correct start site (Fig. 3). In the first experiment, the pC-CAT plasmid (5 μ g) was cotransfected into Raji cells with either the pH1013 vector or the pEBV-MIE plasmid. Cytoplasmic RNA was harvested at 24 h and hybridized with a 32 P-labeled oligonucleotide sequence homologous to the CAT gene in the region 66 to 85 bp downstream of the ATG codon, and primer extension was performed. The pC-CAT mRNA was shown to originate from the expected start site (lane A), and cotransfection with pEBV-MIE did not alter the pC-CAT start site or increase the level of CAT mRNA (lane B).

In a second experiment, the pS-CAT plasmid was cotransfected into Jurkat cells with the pH1013 vector (lane C), the pEBV-MIE plasmid (lane D), or plasmid pEBV-R1E, which encodes the EBV-BRLF1 transactivator (lane E). Cytoplas-

FIG. 2. S1 nuclease analysis of CAT mRNA. A 5- μ g portion of the pS-CAT plasmid was cotransfected with 5 μ g of either the pH1013 vector or pEBV-MIE. Two days after transfection, a sample of each was analyzed for CAT activity, demonstrating a 20-fold increase in CAT activity after cotransfection with pEBV-MIE. Cellular RNA was prepared from the remainder of the cells. A 35- μ g amount of RNA was hybridized overnight with a 32 P-labeled antisense LATE-CAT RNA probe and then digested with S1 nuclease. Since the probe and the pM-CAT construct are homologous only in the CAT-encoding sequences, the protected RNA includes CAT transcripts originating from all potential upstream start sites.

mic RNA was hybridized with a primer homologous to the CAT gene sequence 15 to 34 bp downstream of the ATG codon. The pS-CAT mRNA was shown to originate from the expected start site (lane C). Once again, cotransfection with the BMLF1 transactivator did not affect the level of CAT mRNA, in spite of a large increase in CAT activity (lane D), whereas the BRLF1 transactivator clearly increased CAT mRNA in the same experiment (lane E). The pS-CAT (rather than pM-CAT) construct was used in the primer extension

FIG. 3. Primer extension analysis of CAT mRNA. Primer extension analysis was used to determine the effect of the BMLF1 transactivator on mRNA originating from the expected start site. In the first experiment, the pC-CAT plasmid was cotransfected into Raji cells with either the pHD1013 vector (lane A) or the pEBV-MIE plasmid (lane B). Cytoplasmic RNA was hybridized with a γ - 32 P-labeled primer homologous to the CAT gene sequences 64 to 85 bp downstream of the ATG codon, and primer extension was performed. The expected primer extension product from pC-CAT is 125 bp. Cotransfection with pEBV-MIE had no effect on the level of pC-CAT mRNA or the use of the start site. In the second experiment, the pS-CAT plasmid was cotransfected into Jurkat cells with the pHD1013 vector (lane C), the pEBV-MIE plasmid (lane D), or a plasmid encoding the EBV BRLF1 transactivator (lane E). In this experiment, CAT mRNA was hybridized to a primer homologous to the CAT sequences 15 to 34 bp downstream of the ATG codon, and the expected primer extension product is 170 bp. Cotransfection with the BRLF1 transactivator resulted in an increase in the CAT mRNA (lane E), whereas the BMLF1 transactivator had no effect (lane D).

experiment since the pS-CAT plasmid contains additional 3'-promoter sequences, including the endogenous cap site. The CAT activity produced by the pS-CAT and pM-CAT constructs was shown to be identical in numerous cell types (data not shown).

The BMLF1 transactivator is reporter-gene dependent. To determine the plasmid sequences required for transactivation by the BMLF1 gene product, we systematically removed different portions of the pM-CAT plasmid and tested for the ability of the resultant plasmids to respond to the BMLF1 transactivator.

The results of these experiments are summarized in Table 3. As already shown by the previous set of data, the EBV promoter element in pM-CAT is not required for transactivation by BMLF1, since pCAT3M (which removes the EBV promoter) is also transactivated. To determine whether the BMLF1 transactivator is specifically recognizing a cryptic promoter element within the pCAT3M vector sequences, we built two plasmids (pC-PUC9A and C-PUC9B) which remove the CAT gene and the SV40 polyadenylation signal from the pBR322 vector and placed these sequences into the PUC-9 vector in either orientation (A or B). Since both of the

TABLE 3. Analysis of pM-CAT sequences required for transactivation by BMLF1 gene product

Plasmid	Promoter	Vector	Reporter	Poly(A)	Transactivation
pM-CAT	BMLF1	pBR322	CAT	+	+
pCAT3M	None	pBR322	CAT	+	+
pC-PUC9A ^a	None	PUC-9	CAT	+	+
pC-PUC9B ^a	None	PUC-9	CAT	+	+
pM-C-PUC9+ ^b	BMLF1	PUC-9	CAT	+	+
pM-C-PUC9- ^b	BMLF1	PUC-9	CAT	-	+
pM-GH ^c	BMLF1	PUC-12	GH	+	-

^a pC-PUC9A and pC-PUC9B have the CAT reporter gene and SV40 polyadenylation sequences in a PUC-9 vector (in either orientation A or B) with no eucaryotic promoter element.

^b pM-C-PUC9+ and pM-C-PUC9- have the BMLF1 promoter driving the CAT gene in a PUC-9 vector, the only difference between the two plasmids being the presence or absence of the SV40 polyadenylation sequences.

^c pM-GH has the BMLF1 promoter inserted upstream of the human growth hormone gene in the pGH vector (40).

resultant plasmids responded equally well to the BMLF1 transactivator, it seems unlikely that the BMLF1 transactivator recognizes a specific promoter element within either the pBR322 or the PUC-9 sequences. To exclude the possibility of a response region within the SV40 polyadenylation sequences, we built two plasmids (pM-C-PUC9+ and pM-C-PUC9-) which are identical except for the presence or absence of the SV40 polyadenylation sequence. The SV40 polyadenylation sequence proved not to be required for response to BMLF1, since the M-C-PUC9- plasmid (which has no SV40 polyadenylation site) still responds to the BMLF1 transactivator.

Finally, to exclude a responsive region within the CAT gene itself, we replaced the CAT gene with another reporter gene, the human growth hormone gene (40), and named this plasmid pM-GH. Table 4 shows the results of several different growth hormone experiments with either pM-GH or pC-GH (which has the *Bam*HI-C promoter linked to the growth hormone gene). In each of these experiments, pM-GH and pM-CAT (or pC-GH and pC-CAT) were cotransfected into the same cells with the pHD1013 vector, the pEBV-MIE construct, pEBV-ZIE (which has the EBV BZLF1 transactivator) (21), or pHD101SV1 (which has the cytomegalovirus IE transactivator) (9). Three days after transfection, the cells were harvested for CAT activity, while the supernatant fluid was analyzed for growth hormone activity by using a radioimmunoassay (40). The simultaneous analysis of the effect of BMLF1 on both CAT and growth hormone activities within the same cells was facilitated by the fact that, whereas CAT activity is contained within cells, growth hormone is secreted into the media. The extreme sensitivity of the growth hormone assay used (Allegro HGH) allows reproducible detection of as little as 0.06 ng of human growth hormone per ml.

In each experiment the CAT activity produced by pM-CAT or pC-CAT was significantly increased by cotransfection with BMLF1 (Table 4), whereas the growth hormone activity produced by the identical promoter was not increased by cotransfection with BMLF1. On the other hand, the pM-GH plasmid responded as expected to the EBV BZLF1 transactivator. Likewise, the pC-GH plasmid responded to the cytomegalovirus IE transactivator. When cellular extracts (instead of supernatant fluids) were analyzed for growth hormone activity, there was still no stimulation observed with the BMLF1 transactivator. We have recently observed that an additional reporter gene, *gal*, is

TABLE 4. Effect of different transactivators on CAT versus growth hormone reporter genes

Expt	Plasmid	Transactivator	CAT activity ^a			Growth hormone ^b		
			Constitutive (%)	Transactivator (%)	Fold increase	Constitutive (ng/ml)	Transactivator (ng/ml)	Fold increase
1	pM-GH/pM-CAT	pEBV-MIE	2.08	10.84	5	0.2	0.1	<1
		pEBV-ZIE	2.08	14.39	7	0.2	1.4	7
2	pM-GH/pM-CAT	pEBV-MIE	2.30	10.36	5	0.1	0.1	1
		pEBV-ZIE	2.30	16.02	7	0.5	0.5	5
3	pC-GH/pC-CAT	pEBV-MIE	2.0	58.20	29	0.1	0.1	1
		pHD101SV1	2.0	32.40	16	0.1	1.7	17
4	pC-GH/pC-CAT	pEBV-MIE	1.18	19.91	17	0.4	0.1	<1

^a All experiments were performed in B95-8 cells. CAT activity is expressed as percent acetylation produced per 10 µg of protein extract.

^b Growth hormone activity secreted into the media was measured by using radioimmunoassay (40), while CAT activity was measured in cellular extracts from the same experiment.

also unresponsive to the BMLF1 transactivator (D. Markovitz et al., unpublished data).

DISCUSSION

The differential regulation of viral promoters expressed during latent versus productive EBV infection may be a key element in governing the switch from the latent to the productive state. A major objective of the present work was therefore to determine whether the EBV BMLF1 transactivator could specifically activate a particular class of EBV promoter. Our own work, and that of others, had previously indicated that certain promoters linked to the heterologous bacterial CAT gene can be activated by cotransfection with the BMLF1 gene product (7, 22, 30, 45). Somewhat surprisingly, further study has in fact demonstrated that the primary effect of the BMLF1 transactivator appears to be directed at the CAT gene itself, with the role of the promoter being, at most, secondary. Therefore, the BMLF1 "transactivator" is unlikely to activate a specific promoter(s) in EBV and more likely functions to increase the level of an unidentified viral or cellular protein.

Our finding that the BMLF1 transactivator does not significantly increase the level of CAT mRNA suggests a posttranscriptional mechanism. In addition, the reporter-gene dependence of the BMLF1 transactivator also argues for a posttranscriptional mechanism, since any transcriptional effect would be expected to be independent of the reporter gene used. At this point, however, we do not know whether the effect of BMLF1 is to stabilize CAT mRNA, to increase its translational efficiency, to stabilize the CAT protein itself, or to increase CAT enzyme efficiency. Since the increase in CAT activity produced by cotransfection with the BMLF1 gene product is sometimes accompanied by a small increase in mRNA (22) (although this CAT mRNA increase is less than the increase in CAT activity), it is possible that the BMLF1 gene product acts to stabilize CAT mRNA. Conceivably, a very small difference in CAT mRNA stability might result in much larger differences in CAT activity, if the CAT mRNA is very unstable and CAT protein is particularly stable. However, the inability of the BMLF1 gene product to activate the growth hormone constructs suggests that any such effect on mRNA stability would have to be limited to a certain subset of host or viral RNAs.

Alternatively, the BMLF1 transactivator might enhance translation of CAT mRNA. Again, this effect would have to be limited to a certain subset of viral or cellular mRNAs. A

third possibility is that the BMLF1 gene product stabilizes the CAT protein itself or increases the enzymatic activity of the CAT protein. The consistent differences in the response of each of the EBV-CAT constructs to the BMLF1 effect (i.e., the relatively low responses of the pLate-CAT and pEA-CAT constructs versus the very high response of the pC-CAT and pLMP-CAT constructs) suggest that the EBV insert sequences have some effect on the ability of the CAT gene to respond to the BMLF1 transactivator. This effect of insert sequences suggests that the function of BMLF1 is more likely to occur at the level of RNA stability or translational efficiency, since EBV insert sequences would be less likely to influence CAT protein stability or enzyme function. We are currently in the process of determining by which mechanism BMLF1 gene product increases CAT activity.

In any event, our finding that the BMLF1 transactivator is reporter-gene dependent has clear relevance to other investigators employing the "CAT assay" to study promoter regulation. The BMLF1 effect is directed at the CAT gene itself, rather than the promoter, and there are likely to exist other viral or cellular gene products which can use a similar mechanism to affect CAT activity. Therefore, it is prudent to use at least two different reporter genes before concluding that an observed increase in CAT activity is necessarily due to an effect on promoter activity.

Previous investigators have shown that the BMLF1 gene product is of heterogeneous size (molecular weights, 45,000 to 70,000), with the major product being a phosphorylated 60-kilodalton protein that localizes to the nucleus (32, 46). At this point, the role of the BMLF1 gene product in EBV infection remains unknown. Clearly, the purpose of this gene in EBV infection is not directed at affecting the activity of the bacterial protein CAT. The effect of BMLF1 on the CAT activity may be entirely fortuitous and may not relate to the mechanism by which the BMLF1 IE gene product presumably promotes EBV productive infection. Nevertheless, it is tempting to speculate that the BMLF1 gene product might also increase the level of cellular or viral protein(s) required for viral infection by using the same mechanism by which it increases CAT activity.

Previously, the BMLF1 transactivator has been described as "promiscuous" (30). However, since all previous studies examining the effect of BMLF1 on different promoters have used CAT vectors, it is now clear that BMLF1 may not be promiscuous at all in its effect; rather, the CAT gene may

fortuitously resemble in some aspect the intended target gene(s) of BMLF1. We believe that the reporter-gene dependence and posttranscriptional mechanism of BMLF1 make it an extremely novel regulatory protein. It will now be important to determine the intended target gene(s) of the BMLF1 gene product.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants 1 KO8 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 Michelle Davis and Eng-Shang Huang for use of the pH1013 vector, Bruce Howard for pRSV-CAT, George Khoury for pCAT3M, George Bornkamm for pM-B2-J, Nancy Raab-Traub for cloned EBV fragments, Richard Selden for p ϕ GH, and Gloria Majette for preparation of the manuscript.

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