The Epstein-Barr Virus (EBV) BMRF1 Promoter for Early Antigen (EA-D) Is Regulated by the EBV Transactivators, BRLF1 and BZLF1, in a Cell-Specific Manner

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The Epstein-Barr virus early antigen diffuse component (EA-D) is essential for Epstein-Barr virus DNA polymerase activity, and its activity is suppressed during latent infection. We investigated the regulation of the promoter (BMRF1) for this early gene by studying its responsiveness in vitro to two immediate-early viral transactivators, BZLF1 (Z) and BRLF1 (R), focusing on the differences in response in lymphoid cells and epithelial cells. In lymphoid cells, Z or R alone produced only small increases in EA-D promoter activity, whereas both transactivators together produced a large stimulatory effect. In epithelial cells, the Z transactivator alone produced maximal stimulation of the EA-D promoter; the effect of R and Z together was no greater than that of Z alone. Deletional analysis and site-directed mutagenesis of the EA-D promoter demonstrated that in epithelial cells the potential AP-1 binding site plays an essential role in Z responsiveness, although sequences further upstream are also important. In lymphoid cells, only the upstream sequences are required for transactivation by the Z/R combination, and the AP-1 site is dispensable. These data suggest that EA-D (BMRF1) promoter regulation by Z and R is cell type specific and appears to involve different mechanisms in each cell type.

Epstein-Barr virus (EBV) is a herpesvirus that infects primarily B cells and epithelial cells (17, 28, 32). EBV infection of B cells is predominantly latent, whereas epithelial-cell infection is often productive (28), raising the question of whether there are different mechanisms for regulation of viral expression in lymphoid and epithelial cells. Overexpression of the immediate-early (IE) EBV gene product, BZLF1, in EBV-infected B cells is sufficient to convert latent expression to productive infection (10, 13, 29, 33). The BZLF1 gene (Z) encodes a 34- to 38-kilodalton nuclear protein (the Z protein) which has sequence similarity to the DNA-binding site of the *c-fos* protein and binds to the AP-1 binding site (7), although there is no potential leucine zipper (36) in the Z protein. Exactly how the Z protein binds to the AP-1 site, or whether binding of Z to AP-1 or closely related sites is always required for transactivation of target promoters, is still unknown. Although the Z protein appears to play a key role in determining whether EBV infection is latent or productive, the disruption of latency is likely to involve other EBV transactivators.

Two other EBV IE gene products have been shown to encode transactivator functions. The BMLF1 gene product has been shown by several groups to transactivate a number of homologous and heterologous promoters (4, 14, 16, 24, 37) linked to chloramphenicol acetyltransferase (CAT). However, we have shown that the BMLF1 effect is reporter gene dependent and does not result in significantly increased levels of mRNA (14). The role of the BMLF1 gene in induction of lytic viral infection remains unknown.

The BRLF1 gene also encodes an IE transactivator function (1, 3, 4, 11, 12). The BRLF1 gene product is a 94- to 98-kDa protein which localizes to the nucleus and cytoplasm (5, 26). As shown by cDNA analysis, the BRLF1 protein (R) is derived from a bicistronic mRNA which also encodes the

Z protein (25), suggesting that the regulation of Z and R is tightly linked. The R transactivator, in contrast to Z, cannot disrupt viral latency when transfected and overexpressed in cells (5).

Several EBV early promoters are transactivated by either Z or R. The genes activated by Z include BMLF1, BMRF1, the BZLF1 gene itself, and the divergent promoter (DR/DL) (1-5, 12, 13, 23, 25, 31, 35). Z-responsive elements in these promoters have been mapped within 100 base pairs (bp) of the TATA box, and the response elements have included AP-1 (or closely related) binding sites. The R transactivator activates the DR promoter and the BMLF1 promoter (1-3, 5, 12). The R-responsive regions in both of these promoters reside within upstream enhancer elements, and both of these R-responsive elements contain the same 14-bp sequence motif, CCGTGGAC/GAATGTC. Whether the R transactivator binds to DNA is unknown at present.

In the work described in this paper, we have studied the regulation of the EBV BMRF1 early promoter, EA-D. The BMRF1 gene encodes the early antigen diffuse (EA-D) protein, which is an essential component of the viral DNA polymerase activity (22). EA-D activity is suppressed in latent infection and reactivated during productive infection (6). We show here that the EA-D promoter is differentially regulated in lymphoid and epithelial cells. In epithelial cells, the Z transactivator alone is sufficient to transactivate the EA-D promoter. In contrast, in lymphoid cells, Z transactivation of the EA-D promoter cells is inefficient unless the R transactivator is also present. We demonstrate here that the potential AP-1 binding site in the EA-D promoter is essential for Z-induced transactivation in epithelial cells, but is not required for the Z/R combined effect in lymphoid cells.

MATERIALS AND METHODS

Cell lines. The cell lines used include the EBV-negative Burkitt's lymphoma B-cell line (Louckes), a T-cell line

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FIG. 1. Construction of pEA-CAT deletions. The parent pEA-CAT construct contains the EBV *Bam*HI-*Rsa*I fragment (EBV nucleotides 79537 to 79898) linked to CAT. A series of 5' deletion mutants were constructed by cutting at the indicated restriction endonuclease site, blunt ending with T4 polymerase or Klenow enzymes, and religating. The TATA box and a potential AP-1 binding site are shown.

(Jurkat), a cervical epithelial-cell line (HeLa), and a laryngeal epithelial-cell line (HEp-2). All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa and HEp-2 cells were maintained in Dulbecco modified Eagle medium H supplemented with 10% fetal calf serum.

DNA transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. DNA was transfected into both lymphoid and epithelial cell lines by electroporation (34). For each condition, 10^7 cells were shocked at 1,500 V with the Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Epithelial cells were harvested and suspended into RPMI 1640 medium for electroporation.

EBV-CAT plasmids. EBV-CAT plasmids were constructed by inserting EBV promoter sequences into the *Bgl*II site of the plasmid vector pCAT3M (21). The plasmid pCAT3M contains the bacterial CAT gene and the simian virus 40 polyadenylation site but no eucaryotic promoter element. The plasmid pM-CAT (12), as previously described, contains the promoter of the EBV IE gene, BMLF1, linked to the CAT gene. The pEA-CAT plasmid, which has the promoter of the early EA-D gene, contains the 333-bp EBV *Bam*HI-*Rsa*I fragment (nucleotides 79537 to 79898) linked to the CAT gene. 5' deletion mutants of pEA-CAT were made by cutting with the restriction enzymes illustrated in Fig. 1, blunt ending with T4 polymerase or the Klenow enzymes, and religating to pCAT3M. The genome map locations of each construct are shown in Fig. 1. The plasmid pEA-BS-CAT was made (for use in *in vitro* site-directed mutagenesis) by inserting the *Xba*I-*Bam*HI fragment from pEA-CAT (which contains the complete EA promoter sequences, the CAT gene, and the polyadenylation site) into the *Xba*I-*Bam*HI site of the M13 Bluescript SK+ plasmid (Stratagene Inc.).

EBV transactivating plasmids. The pEBV-ZIE plasmid (Fig. 2) contains the *Bam*HI Z gene fragment (nucleotides 101741 to 103947) in the pGem2-based vector pHD1013 (15), such that the BZLF1 gene is under the control of the strong human cytomegalovirus (CMV) IE promoter. The pEBV-RZ

FIG. 2. Construction of the EBV transactivator plasmids. The pEBV-ZIE transactivator plasmid was made by placing the *Bam*HI Z fragment into the *Bam*HI site of pHD1013. This places the BZLF1 gene under control of the strong human CMV IE promoter. The pEBV-RZ transactivator construct contains the *Bgl*II J fragment inserted into the *Bam*HI site of pHD1013. pEBV-RZ places both the R and Z transactivating proteins under the control of the CMV IE promoter. The pEBV-RIE plasmid contains a *Hind*III-*Hind*III fragment from pEBV-RZ containing the BRLF1 gene linked to the CMV IE promoter ligated into a pUC18 vector.

plasmid contains both the BRLF1 (R) and BZLF1 (Z) transactivators under the control of the CMV-IE promoter (both gene products can be made from a bicistronic mRNA). This plasmid has the 4,061-bp *Bgl*II-*Bgl*II fragment (EBV nucleotides 101351 to 105412) inserted into the *Bam*HI site of the vector pHD1013. In the pEBV-RIE plasmid, a *Hind*III-*Hind*III fragment from pEBV-RZ (which contains the BRLF1 gene linked to the CMV-IE promoter) was ligated into the pUC18 vector. The construction of these EBV transactivator plasmids is illustrated in Fig. 2.

CAT assays. Cells were transfected with 10 μ g of plasmid DNA as described above. At 48 h after transfection, a cell extract was prepared and incubated at 37°C with [14 C]chloramphenicol in the presence of acetyl coenzyme A as described previously (9). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by autoradiography and scintillation counting. If results were not in the linear range (less than 70% chloramphenicol acetylation), CAT assays were repeated with less extract.

RNA analysis. To quantitate CAT mRNA, we prepared cytoplasmic RNA 24 h after transfection as previously described (8). 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 end 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 (18) was performed at 42°C for 90 min with avian myeloblastosis virus reverse transcriptase (27). The primer extension products were electrophoresed on a 6% acrylamide-7 M urea gel.

In vitro site-directed mutagenesis. Mutants were made by using the Bio-Rad Muta-Gene phagemid *in vitro* mutagenesis kit, based on a method described by Kunkel et al. (19, 20).

FIG. 3. Effect of the Z and R transactivators on the BMRF1 (EA-D) early promoter versus the BMLF1 IE promoter. A 5- μ g portion of either the pM-CAT plasmid (12) (which contains the primarily R-responsive BMLF1 promoter) or the pEA-CAT plasmid (-331 to +1), which contains the early EA-D promoter, was cotransfected with 5 μ g of either pEBV-ZIE (which encodes Z), pEBV-RIE (which encodes R), or both transactivators together. CAT assays were performed as described previously (9). CAT assay results are presented as fold induction over basal levels. In lymphoid cells (Louckes and Jurkat), the combination of R and Z is required for maximal stimulation of the EA-D promoter, whereas R alone is sufficient for maximal stimulation of the BMLF1 construct. In epithelial cells (HeLa and HEP-2), Z alone produces maximal transactivation of the EA-D promoter, whereas R alone is sufficient for maximal stimulation of the BMLF1 promoter.

Mutants were made by using a synthetic oligonucleotide complementary to the region to be altered except for a limited internal mismatch. For screening possible mutants, synthetic oligonucleotides were created such that *in vitro* mutations would create a new *PvuII* restriction site which allows each possible mutant to be analyzed by digestion with restriction endonucleases. Following initial screening, potential mutants were sequenced with the Sequenase 2.0 enzyme sequencing kit (United States Biochemical Corp.) to identify the mutation.

RESULTS

The EA-D promoter is differentially regulated in lymphoid and epithelial-cell types. We have previously reported that the EA-D promoter can be stimulated in both EBV-positive and EBV-negative cell lines by cotransfection with the Z transactivator plasmid (13). In this study we examined the effects of the Z and R transactivators alone and in combination on EA-D promoter activity in different cell types. Figure 3 illustrates representative CAT assays in which the pEA-CAT plasmid was cotransfected into various cell types with either the pEBV-ZIE plasmid (which encodes BZLF1) or the pEBV-RIE plasmid (which encodes BRLF1), or both transactivators together.

In either B or T lymphoid cell lines (Louckes and Jurkat), the EA-CAT promoter activity was only slightly increased

on cotransfection with either Z or R. However, when pEA-CAT was cotransfected with both transactivators together in lymphoid cells, a large stimulatory effect was obtained. This synergistic effect of Z and R together was observed with either the pEBV-ZIE and pEBV-RIE plasmids in combination or the pEBV-RZ plasmid alone, which expresses the bicistronic Z/R mRNA. In contrast, the plasmid pM-CAT, which contains the promoter of the EBV BMLF1 gene and has been previously shown to be primarily R responsive (12), is transactivated equally well by either R alone or the combination of Z and R in Jurkat cells.

In the epithelial-cell lines HeLa and HEP-2, the Z transactivator alone produced a large stimulatory effect on pEA-CAT activity. As in lymphoid cells, the R transactivator alone produced only a small effect. When both transactivators were cotransfected into epithelial cells, the effect of Z and R together upon EA-CAT activity was no greater than that of Z alone. The pM-CAT plasmid, as expected, was efficiently transactivated by either R alone or the R and Z combination in HeLa cells. These data suggest that the Z transactivator alone is sufficient to activate EA-D promoter activity maximally in epithelial cells. In contrast, both Z and R are required for efficient EA-D promoter transactivation in lymphoid cells.

RNA analysis. Previous studies have shown that both the Z- and R-induced transactivation of reporter gene constructs is associated with a similar increase in steady-state RNA levels (12, 13). In this study we analyzed the effect of the Z/R transactivator combination on the steady-state level of pEA-CAT RNA in lymphoid cells. In the experiment shown in Fig. 4, 5 μ g of the pEA-CAT construct was cotransfected into Jurkat cells with 5 μ g of either pHD1013 (lane B) or the pEBV-RZ transactivator construct (lane C). Lane A contains untransfected Jurkat RNA. Cytoplasmic RNA was harvested 24 h after transfection and hybridized to a γ -³²P-labeled primer homologous to the CAT gene sequence 15 to 34 bp downstream of the ATG codon, and primer extension was performed as described in Materials and Methods. Primer extension analysis clearly demonstrated an increase in the steady-state level of pEA-CAT mRNA after cotransfection with the Z/R transactivators. The mRNA start site, which mapped to EBV sequence 79897, was not altered in the presence of the two transactivators.

Localization of the Z- and R-responsive regions in the EA-D promoter. To define the Z- and R-responsive region(s) of the EA-D promoter, we created a series of 5' deletion mutants of the pEA-CAT plasmid. These deletions are illustrated in Fig. 1. The parent pEA-CAT plasmid contains EBV nucleotides 79537 to 79898 (-331 to +1) relative to the RNA start site. Four deletion plasmids were constructed (Fig. 1) which delete progressively larger fragments of the upstream 5' promoter sequences. The smallest construct, pEA-CAT (-62 to +29) contains only 62 bp upstream of the cap site. A potential AP-1 binding site is located between -57 and -64 relative to the start site.

The effects of the Z, R, and Z/R transactivating proteins on these pEA-CAT 5' deletions are summarized in Tables 1 and 2. In lymphoid cells (Table 1), the data indicate that maximum transactivation of the parent pEA-CAT is achieved only when both Z and R are present. The effect of the Z transactivator alone could be significantly increased by deleting the sequences from -331 to -265, although even with this construct the effect of Z and R together was greater than that of Z alone (85.3-fold transactivation versus 18.8-fold). A progressive loss of response to the Z transactivator alone, or the Z-plus-R combination, occurred as 5'

TABLE 1. Effect of EBV-IE transactivators on pEA-CAT deletion plasmids in the Jurkat cell line

Plasmid	Constitutive activity ^a	Fold increase in CAT activity ^b		
		pEBV-ZIE	pEBV-RIE	pEBV-RZ
pEA(-331/+1)-CAT	1.3 (0.7-2.3)	3.7 (2.1-6.4)	3.1 (2.6-3.7)	33.7 (22.8-61.0)
pEA(-265/+1)-CAT	0.7 (0.6-1.0)	18.8 (4.0-31.2)	4.0 (2.2-5.3)	85.3 (50.5-119.5)
pEA(-214/+29)-CAT	0.9 (0.6-1.1)	4.2 (1.9-8.2)	2.5 (1.9-3.7)	23.3 (20.4-27.8)
pEA(-79/+1)-CAT	2.0 (1.3-2.4)	1.6 (0.5-2.4)	3.4 (1.2-4.5)	4.9 (4.4-5.8)
pEA(-62/+29)-CAT	4.7 (3.0-7.6)	1.5 (0.7-2.8)	1.6 (0.6-2.4)	1.7 (0.4-2.7)

^a The numbers shown represent the average percent acetylation from four experiments. Ranges are given in parentheses.

^b The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with either pEBV-ZIE, pEBV-RIE, or pEBV-RZ with respect to the activity with the pHD1013 vector. Ranges (in parentheses) are those from three experiments for pEBV-RIE and four experiments for pEBV-ZIE and pEBV-RZ.

sequences between -265 and -62 were deleted, although the region located between -265 and -214 appeared to play the greatest role in the total transactivation. Neither the intact pEA-CAT plasmid nor any of the 5' deletion mutations were significantly transactivated with the R-gene product alone in the Jurkat cell line.

In HeLa cells, maximum stimulation of the parent, pEA-CAT, occurred with the Z transactivation alone (Fig. 3). Likewise, the effect of Z and R together on each mutant was no greater than that of Z alone (data not shown). As in lymphoid cells, a significant portion of the Z effect in HeLa cells was lost when the sequences between -265 and -214 were deleted (Table 2). In contrast to the situation in lymphoid cells, total Z response actually was increased in epithelial cells when the sequences between -214 and -79

were deleted. In epithelial cells, the 17-bp region between -79 and -62 (which contains a portion of the potential AP-1 binding site) also contributed to Z responsiveness, since the pEA construct (-79 to +1) was 13-fold more responsive to Z than the pEA construct (-62 to +29). The effect of R alone in HeLa cells was too small to be accurately mapped.

Role of the AP-1 consensus site in Z and R transactivation of the EA-D promoter. To better define the role of the AP-1 consensus site in Z- and Z/R-induced transactivation of the EA-D promoter in different cell types, we constructed a series of site-directed mutants of the EA-CAT construct by using in vitro mutagenesis. To facilitate these studies, we transferred the EA-D promoter sequences and CAT gene from the pEA-CAT construct into a Bluescript M13 SK+ phagemid vector to create the construct pEA-BS-CAT. Using the method of Kunkel et al. (19, 20), the 6 bp defining the AP-1 binding site in pEA-BS-CAT was specifically altered as shown in Table 3, leaving the rest of the promoter unchanged. In addition, since analysis of the 5' deletion mutants suggested that sequences further upstream from the potential AP-1 site may also contribute to Z/R transactivation, three other site-directed mutants were constructed which each remove potential AP-1-like binding sites lying between -197 and -240. One additional mutant, which alters a sequence (-185 to -198) containing a 10-of-14-bp similarity to the shared R-response sequence region of the DR and BMLF1 enhancers (1-3, 12) was also constructed. The effects of the Z, R, and Z/R transactivators on these site-directed mutants are shown in Tables 4 and 5.

The AP-1 site-directed mutation in pEAΔAP1-CAT decreased Z-induced transactivation in HeLa cells by more than 20-fold, confirming that the AP-1 consensus site plays a major role in EA-D regulation in epithelial cells. However, in lymphoid cells (Table 5), the AP-1 mutation produced little

FIG. 4. Primer extension analysis of pEA-CAT RNA. The pEA-CAT plasmid was cotransfected into Jurkat cells with 5 µg of either the pHD1013 vector (lane B) or the Z/R transactivating plasmid, pEBV-RZ (lane C). RNA (50 µg) from untransfected Jurkat cells (lane A) or pEA-CAT transfected cells was hybridized overnight with a γ -³²P-labeled oligonucleotide primer homologous to CAT gene sequences 15 to 34 bp downstream of the ATG codon. Primer extension (18) was performed with reverse transcriptase at 42°C for 90 min (27). The mRNA start site mapped at EBV sequence 79897 and was not altered in the presence of the two transactivators. Cotransfection with the R-plus-Z transactivator demonstrated an increase in the steady-state level of EA-CAT mRNA (lane C), as indicated by the arrow.

TABLE 2. Effect of the BZLF1 transactivator on pEA-CAT deletion plasmids in the HeLa cell line

Plasmid	Constitutive activity ^a	Fold increase in CAT activity ^b
pEA(-331/+1)-CAT	0.7 (0.7-0.7)	65.0 (33.0-96.5)
pEA(-265/+1)-CAT	0.5 (0.4-0.6)	111.9 (94.5-129.2)
pEA(-214/+29)-CAT	0.7 (0.5-0.9)	2.5 (1.0-4.0)
pEA(-79/+1)-CAT	0.5 (0.4-0.6)	13.0 (12.0-14.9)
pEA(-62/+29)-CAT	0.5 (0.4-0.5)	1.1 (0.6-1.5)

^a The numbers shown represent the percent acetylation produced by each construct and are derived from two experiments. Ranges are given in parentheses.

^b The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with pEBV-ZIE with respect to the activity with the pHD1013 vector. Ranges (in parentheses) are results of two experiments.

TABLE 3. Construction of in vitro site-directed mutations in pEA-CAT

Plasmid	Altered sequences relative to mRNA start site	Mutations
pEAΔAP1-CAT	-57 to -63	TGACTCA ^a → TCAGCTG
pEAΔZA1-CAT	-240 to -246	TGGCTCG ^b → TCAGCTG
pEAΔZB2-CAT	-230 to -236	AACTGCT ^b → ACAGCTG
pEAΔZA3-CAT	-197 to -203	TCACCCA ^b → CAGCTGA
pEAΔZB4-CAT	-190 to -196	ACCAATG ^c → CAGCTGG

^a The mutation ΔAP1 alters the perfect consensus AP-1 binding site located between -57 and -63.

^b Mutations ΔZA1, ΔZB2, and ΔZB3 each alter sequences containing a 4-of-8-bp similarity to the consensus AP-1 site.

^c The mutation ΔZB4 alters a sequence which shares a 10-of-14-bp similarity with the previously described shared R-response regions in the EBV DR and BMLF1 enhancers (2, 3, 12).

effect on the ability of Z and R together to transactivate the EA-D promoter, suggesting that the ability of R to interact synergistically with Z in lymphoid cells is not mediated by increased Z binding to the consensus AP-1 site. None of the other site-directed mutants significantly affected Z or Z-plus-R transactivation in either cell type.

DISCUSSION

We have demonstrated in these studies that EA-D (BMLF1) promoter regulation by the Z and R IE transactivators is cell type specific and appears to involve different mechanisms. In epithelial cells, the Z gene product efficiently transactivates the promoter for the EA-D early gene, whereas effective transactivation of the same promoter in lymphoid cells requires the addition of the R transactivator. These differences in transactivation efficiency correlate with different mechanisms of transactivation. In epithelial cells, the potential AP-1 binding site, in addition to promoter sequences further upstream (-214 to -265), is required for Z response. In lymphoid cells, the AP-1 site is dispensable for Z-plus-R transactivation, although the upstream regulatory sequences (-214 to -265) are essential. Therefore, the ability of the R and Z transactivators to synergistically activate the EA-D promoter in lymphoid cells appears to involve a mechanism independent of Z binding to the AP-1 site.

The regulation of the EA-D promoter by Z and R is distinct from that of other EBV promoters previously studied. We have previously shown that another EBV promoter, BMLF1, responds to the R transactivator in either

TABLE 5. Effect of the combined BRLF1/BZLF1 transactivators on pEA-CAT site-directed mutants in Jurkat cells

Plasmid	Fold increase in CAT activity ^a
pEA-BS-CAT	43.1 (30.8-52.0)
pEAΔAP1-CAT	50.3 (36.8-74.8)
pEAΔZA1-CAT	110.3 (52.5-213.6)
pEAΔZB2-CAT	76.9 (22.8-163.2)
pEAΔZA3-CAT	99.3 (61.4-138.0)
pEAΔZB4-CAT	91.1 (37.2-197.6)

^a The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with the pEBV-RZ plasmid with respect to the activity with the pHD1013 vector. Ranges are those from two experiments.

EBV-positive or EBV-negative lymphoid and epithelial cells, and mapped the R-response region in this promoter to a 140-bp enhancer element (12). Several investigators have reported that in addition to R, the BMLF1 promoter can be activated (although to a much lesser extent) by the Z transactivator, and they have mapped the Z-response region to an AP-1 site (1, 12, 30, 35).

Our studies suggest that the regulation of the BMLF1 and BMRF1 (EA-D) promoters by the Z and R transactivators is clearly different. The BMLF1 promoter is primarily R responsive, and the effect of R and Z together is no greater than that of R alone (at least when levels of R and Z are not limiting) (12). Furthermore, no tissue specificity in the effects of R or Z on BMLF1 promoter activity has been observed (1, 12). Unlike the case with BMRF1, in the BMLF1 promoter the R-responsive and Z-responsive regions of the promoter clearly map to separate regions.

Studies involving the EBV divergent promoter (BHLF1 and BHRF1) have shown that the R and Z transactivators are both involved in the regulation of this complex element. The R-responsive region of this promoter element can be clearly mapped to an enhancer region which contains a 14-bp sequence similar to a sequence in the R-responsive enhancer of the BMLF1 promoter (1-3, 12). The Z transactivator also activates the BHLF1 promoter. Lieberman et al. (23) have reported that regulation of the BHLF1 promoter by Z is tissue specific, with the response region to Z being located in sequences between -7 and -155 in Vero cells but localized within sequences further upstream in EBV-positive lymphoid cells. However, it is possible that the Z effect observed in EBV-positive cells was actually mediated by the R transactivator and did not represent true tissue-specific differences in Z response elements.

Although the DR enhancer region can be activated by R alone and not by Z alone, Cox et al. have recently reported that the combination of R and Z activates the DR enhancer synergistically (5). This synergistic effect of the Z-plus-R combination on the DR enhancer element is different from the EA-D promoter effect described here in several ways. The EA-D effect is tissue specific, with synergy occurring in lymphoid cells but not epithelial cells. Although the EA-D promoter appears to be primarily Z responsive in epithelial cells, at least when the Z and R transactivators are tested separately, the DR enhancer is primarily R responsive (2, 3, 5, 11). The DR enhancer has a clearly mapped R response region which contains the 14-bp element CCGTGGACAATGTC (similar to the R response element in the BMLF1 promoter) and can transfer R responsiveness to heterologous promoters. In contrast, the effect of R alone on the EA-D promoter is minimal and does not map to a single region of the promoter. Interestingly, the EA-D promoter does con-

TABLE 4. Effect of the BZLF1 transactivator on pEA-CAT site-directed mutants in different cell types

Plasmid	Fold increase in CAT activity ^a in:	
	Jurkat cells	HeLa cells
pPEA-BS-CAT	2.3 (1.4-3.2)	156.9 (60.6-326.0)
pEAΔAP1-CAT	0.4 (0.3-0.5)	7.0 (5.3-7.6)
pEAΔZA1-CAT	2.8 (0.9-4.6)	71.5 (47.8-95.4)
pEAΔZB2-CAT	3.0 (2.0-4.0)	68.1 (36.9-117.5)
pEAΔZA3-CAT	2.0 (2.0-2.1)	46.3 (41.6-52.1)
pEAΔZB4-CAT	1.8 (1.1-2.6)	71.4 (49.2-93.5)

^a The numbers shown represent the average fold increase in CAT activity when each CAT plasmid was cotransfected with the pEBV-ZIE plasmid with respect to the activity with the pHD1013 vector. Ranges (in parentheses) are those from two experiments with Jurkat cells and three experiments with HeLa cells.

tain the sequence GCCTGACCAATGTC (from -185 to -198), which has a 10-of-14-bp similarity to the 14-bp element shared by the BMLF1 and DR enhancers. However, the site-directed mutant EAΔZB4-CAT, which mutates 6 bp of the above sequence, continues to respond to the Z-plus-R combination. These data together suggest that the ability of Z and R to act synergistically upon the EA-D promoter and DR enhancer elements may occur through different mechanisms.

It is not clear why the Z effect in epithelial cells is greater with the pEA(-79/+1) construct than with the pEA(-214/+29) construct. There may be two separate Z response elements, one located between -214 and -264 and the other located between -79 and -62. In this regard, we have found that the *Bst*EII-*Bst*XI fragment (-79 to -264) is not sufficient to transfer Z or Z/R responsiveness to the simian virus 40 early promoter in pA10CAT (21) (E. Guthrie et al., unpublished results), suggesting that both the upstream and downstream response elements may be required for maximal transactivation. In epithelial cells, the ability of Z to transactivate the downstream response element may be inhibited by sequences between -79 and -214. Interestingly, the BHLF1 promoter sequences located between -155 and -417 have been shown to exert a similar negative effect in the ability of Z to transactivate this promoter in Vero cells (23). Alternatively, the increased length of RNA leader sequences in the -214/+29 and -62/+29 constructs may inhibit the effect of Z. In either case, the importance of the potential AP-1 binding site (located between -57 and -64) for Z transactivation in epithelial cells was confirmed by site-directed mutagenesis of the AP-1 site.

Exactly why the Z protein alone is sufficient to activate the R promoter in epithelial cells, yet both the Z and R proteins are required for maximum activation of lymphoid cells, is still speculative. Perhaps there is a cellular factor expressed in epithelial cells that replaces the function of the R protein. If so, the mechanism of this hypothetical epithelial-cell factor must be different from the mechanism of R, since the AP-1 site is dispensable for the Z-plus-R interaction, but not when Z alone is used in epithelial cells. There may be a factor present in lymphoid cells, but lacking in epithelial cells, that inhibits efficient binding of Z to the AP-1 site. In this case the helper role of R in lymphoid cells might involve binding by Z to alternative upstream binding sites. Alternatively, the effect of Z plus R in lymphoid cells might involve direct EA-D binding by the R protein (with Z providing a helper function in this case). A particularly interesting possibility is that Z and R might act synergistically through protein-protein interactions. Distinguishing between these possibilities will require further protein-DNA binding studies.

The findings presented here with Jurkat and HeLa cells do not necessarily correlate with the regulation of EBV infection *in vivo*, since neither of these cell types can actually be infected by EBV. It is possible that the differences observed in EA-D promoter regulation in Jurkat cells and HeLa cells are due to differences in the degree of cell differentiation (since EBV tends to be more productive in more differentiated cells) rather than to tissue-specific differences. Nevertheless, these findings may well have biological relevance in EBV infection. The tendency for EBV infection to remain latent in lymphoid cells may relate to the requirement in this cell type for both transactivating proteins, Z and R, to be expressed if transactivation of certain crucial promoters such as EA-D and the DR enhancer element is to occur. Likewise, the ability of Z alone to activate certain promoters

in epithelial cells, but not lymphoid cells, may contribute to the relative permissiveness of this cell type for productive viral infection. The requirement for R transactivation (in addition to Z) for activation of certain promoters in lymphoid cells suggests that R function is likely to be crucial in the disruption of viral latency in lymphoid cells. However, whether the R transactivating function is also required for productive infection of epithelial cells remains to be determined.

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