# The Epstein-Barr Virus BRLF1 Immediate-Early Gene Product Transactivates the Human Immunodeficiency Virus Type 1 Long Terminal Repeat by a Mechanism Which Is Enhancer Independent

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The Epstein-Barr virus (EBV) immediate-early gene product, BRLF1, transactivates the human immunodeficiency virus type 1 (HIV-1) long terminal repeat. BRLF1-induced transactivation of HIV-1 promoter constructs is accompanied by an increase in plasmid mRNA and is reporter gene independent. Previously, BRLF1 transactivation of EBV promoters has been mapped to regions which function as enhancer elements. Deletional analysis demonstrates that BRLF1 transactivation of the HIV-1 promoter does not require the HIV-1 enhancer. Thus, the EBV BRLF1 gene product may transactivate by at least two different mechanisms, one mechanism involving certain enhancer elements and another mechanism which is enhancer independent.

Epstein-Barr virus (EBV) is a 170-kilobase herpesvirus which can produce either latent or lytic infection in human B cells (3, 23), epithelial cells (31), and T cells (19, 30). Although each of the three EBV immediate-early (IE) genes (BZLF1, BRLF1, and BMLF1) has now been shown to possess transactivating functions (4–7, 13, 15, 17, 18, 20, 33, 36, 37), the effect of each IE transactivator on homologous and heterologous promoters is distinct. The BZLF1 IE gene product, which has homology to the c-*fos* gene product and can bind to the consensus AP-1 site (9), has been shown to transactivate several EBV early promoters and downregulate the *Bam*HI C and *Bam*HI W EBV nuclear antigen promoters (5, 14). The BZLF1 gene product alone (when placed under the control of a strong heterologous promoter) is sufficient to disrupt viral latency in EBV-infected lymphoid cells (33). The BMLF1 IE gene product has been demonstrated to increase the chloramphenicol acetyltransferase (CAT) activity driven by a variety of homologous and heterologous promoters (18, 20, 37), including the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). However, the BMLF1 effect has been recently shown to be mediated by a posttranscriptional mechanism which is reporter gene dependent (16, 21a) and whose function in EBV infection remains unknown.

The most recently described EBV IE transactivator, BRLF1, has been shown to transactivate three EBV promoters (the BMLF1 IE promoter, the BHRF1 early promoter, and the DR early promoter) (6, 13, 14) and is likely to mediate, at least in part, the switch from latent to productive EBV infection (4, 5, 14, 21, 35). Interestingly, the BMLF1, BHRF1, and DR promoters have each been recently shown to have upstream enhancer elements, and the BRLF1 response region in each promoter has been mapped to these enhancer elements. A 14-base-pair sequence (with a 1-base-pair mismatch) has been found in each of these three BRLF1-responsive enhancers (14). Consequently, the

BRLF1 gene product has been referred to as an enhancer factor (21, 35).

In this paper we report that a fourth promoter, the LTR of HIV-1, is also responsive to the EBV BRLF1 transactivator. We show that BRLF1-induced transactivation of HIV-1 promoter constructs is accompanied by a similar increase in plasmid mRNA and is reporter gene independent, unlike the previously reported effect of the BMLF-1 transactivator (16, 17, 21a). We demonstrate by deletional analysis that BRLF1 transactivation of the HIV-1 promoter does not require the presence of the HIV-1 enhancer. Thus, the EBV IE BRLF1 gene product may transactivate by at least two different mechanisms, one mechanism involving the activation of certain enhancer elements and another mechanism which is enhancer independent.

Transactivation of the HIV-1 LTR by the BRLF1 gene product was demonstrated in transient cotransfection assays using several different cell types. An HIV-1 LTR CAT construct (pU3R-III; a gift of C. Rosen and W. Haseltine) (32) was cotransfected with either the pGEM2 vector containing the cytomegalovirus IE promoter alone (pHD1013; gift of M. Davis and E.-S. Huang) (8) or a pUC18 vector containing the BRLF1 transactivator driven by the cytomegalovirus IE promoter (pEBV-RIE) (14). Transfection into all cell types was performed by the electroporation method (34) at 1,500 V with a Zapper electroporation unit from the Medical Electronics Shop, University of Wisconsin, Madison. In each experiment, $10^7$ cells were transfected with 5 µg of each plasmid. After 48 h, the cells were harvested and extracts of the cells were incubated at 37°C in the presence of $^{14}$C-labeled chloramphenicol and acetyl coenzyme A as previously described (12). The amount of extract used for each CAT assay was adjusted to keep the total CAT activity after cotransfection of the CAT plasmids with pEBV-RIE within the linear range. After thin-layer chromotography, the percent acetylation of chloramphenicol was quantitated by autoradiography and scintillation counting.

The initial preliminary set of experiments revealed that the activity of the pU3R-III–CAT construct was increased in the presence of the EBV BRLF1 IE gene product in several different cell types, including the Jurkat T-cell line, the

TABLE 1. Effect of pEBV-RIE on pU3R-III 5' deletion and
site-directed mutant plasmids in Jurkat cell lines

| Plasmid type | Fold increase in CAT activity[a] | |
|---|---|---|
| | Avg | Range |
| 5' Deletion (n = 3) | | |
| pU3R-III (n = 2) | 6.8 | 5.1–8.6 |
| p−167/−17 | 3.6 | 2.3–5.0 |
| p−120/+80 | 8.5 | 6.7–10.2 |
| p−104/+80 | 5.4 | 4.6–5.0 |
| p−57/+80 | 3.2 | 2.4–4.2 |
| p−44/+80 | 4.6 | 2.0–7.4 |
| | | |
| Mutant (n = 2) | | |
| pU3R-III (n = 3) | 14.5 | 5.1–29.8 |
| pΔκB | 15.1 | 13.4–16.7 |
| pΔTATA | 5.7 | 3.8–7.5 |

[a] Calculated by comparing the percent acetylation obtained after cotransfection with the pEBV-RIE (BRLF1) plasmid with that obtained after using the pHD1013 vector. The average fold increases in the intact HIV-1 LTRs, determined in three different cell lines, were as follows: Jurkat (n = 3), 14.5 (range, 5.1 to 29.8); Louckes (n = 2), 12.4 (range, 7.5 to 15.7); HeLa (n = 1), 6.5.

EBV-negative Louckes B-cell line, and the HeLa epithelial-cell line (Table 1). Since the effect of the BRLF1 transactivator on HIV-1 promoter activity was greatest in Jurkat cells, Jurkat cells were used for the remainder of the experiments. In each cell type tested, the stimulatory effect of the BRLF1 transactivator on the HIV-1 promoter was less than its effect on the homologous EBV BMLF1 IE promoter (14). However, BRLF1-induced transactivation of the HIV-1 promoter was consistently observed, whereas a variety of other promoters (including the Rous sarcoma virus and the EBV BamHI W and BamHI C nuclear antigen promoters) were not found to be transactivated by BRLF1 (data not shown).

The ability of the BRLF1 gene product to transactivate the HIV-1 LTR at the mRNA level was confirmed using the primer extension method (Fig. 1). The pU3R-III plasmid (5 μg) was cotransfected into Jurkat cells with 5 μg of either pHD1013 or pEBV-RIE. Cytoplasmic RNA was harvested 24 h after transfection (10) and hybridized to a γ-$^{32}$P-labeled primer homologous to the CAT gene sequences 15 to 34 base pairs downstream of the ATG codon, and primer extension was performed (22). The expected primer extension product was 165 base pairs. In the presence of the BRLF1 transactivator (Fig. 1, lane C), the pU3R-III–CAT mRNA was shown to be initiated at the expected start site, and the quantity of CAT mRNA was increased. In contrast, the EBV BMLF1 transactivator was shown to increase HIV-1-directed CAT activity but did not significantly increase the level of CAT mRNA (17).

In an attempt to detect specific sites in the HIV-1 promoter required for BRLF1 responsiveness, the pEBV-RIE plasmid was next cotransfected into Jurkat cells with a series of HIV-1 LTR 5' deletion mutants linked to the reporter gene CAT (a gift from Craig Rosen and W. Haseltine) (29). The deletion plasmids tested, which were named to reflect the number of base pairs present relative to the mRNA start site, were as follows: p−167/−17 (5' deletion of 286 nucleotides and deletion of the 3' tat-responsive region); p−120/+80 (5' deletion of 333 nucleotides); p−104/+80 (5' deletion of 349 nucleotides); p−57/+80 (5' deletion of 396 nucleotides, which removed the two κB enhancer sites and two of the three Sp1 sites); and p−44/+80 (5' deletion of 408 nucleotides, which removed the last Sp1 site). Each deletion
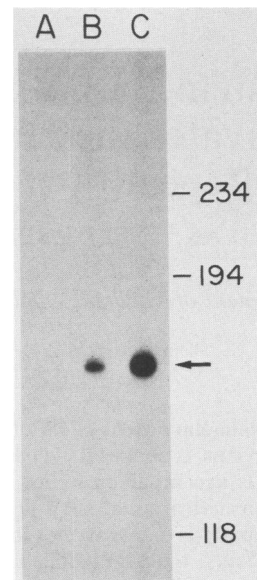


FIG. 1. Primer extension detection of pHIV-CAT mRNA levels. The pHIV-CAT plasmid (5 μg) was cotransfected into 10$^7$ Jurkat cells with 5 μg of either pHD1013 (lane B) or pEBV-RIE (lane C). Lane A contains mock-transfected cells. Cytoplasmic RNA was harvested as previously described (2). Lysis of the cells by Nonidet P-40 was followed by digestion with proteinase K in the presence of sodium dodecyl sulfate, extraction with phenol-chloroform, and DNAase treatment. A 20-base oligonucleotide sequence complementary to the CAT gene in the region 15 to 34 bases downstream of the ATG codon was synthesized and labeled with [γ-$^{32}$P]ATP. Labeled oligonucleotide (10$^5$ cpm) 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 with avian myeloblastosis reverse transcriptase as previously described (22). After RNase digestion, the primer extension products were electrophoresed on a 6% acrylamide-urea gel. Numbers on the right indicate size in base pairs. The expected size of the primer extension product is shown (arrow).

mutant was cotransfected with pHD1013 or pEBV-RIE, and the CAT activity was determined. We did not demonstrate that a single specific region of the HIV-1 promoter is essential for BRLF1 responsiveness (Table 1). Each of the series of 5' deletions, including the plasmids which did not contain the κB enhancer sequence (p−57/+80 and p−44/+80), was activated 3.2- to 8.5-fold by the BRLF1-containing plasmid, pEBV-RIE.

Two additional plasmids containing mutant sequences of the HIV-1 LTR produced by site-directed mutagenesis (gifts from G. Nabel) within the two κB sites (pΔκB) (25) or the TATA sequence (pΔTATA) (26) were also tested for the ability to respond to the BRLF1 transactivator (Table 1). The TATA mutation, which contains GCGC in place of the TATA sequence, was transactivated 5.7-fold (mean of two experiments) by BRLF1. The κB mutation, which affects both κB sites and abolishes the phorbol myristate acetate responsiveness of the promoter, was transactivated 15-fold. Thus, neither the κB enhancer element nor the TATA box element is essential for BRLF1-induced transactivation of the HIV-1 promoter.

We also used a β-galactosidase (GAL)–HIV-1 LTR construct (gift of D. Markovitz) to evaluate reporter gene dependence of the BRLF1 transactivator. The HIV-1 LTR–GAL construct (pHIV/GAL) contains the lacZ gene (cut

TABLE 2. Effect of BRLF1 transactivator (pEBV-RIE) on intact HIV-1 LTR as determined by CAT and GAL assays

| Expt no. | CAT activity (% acetylation) with transactivator plasmid | | GAL activity ($\Delta A$/min) with transactivator plasmid | |
|---|---|---|---|---|
| | pHD1013 | pEBV-RIE (fold increase) | pHD1013 | pEBV-RIE (fold increase) |
| 1 | 5.6 | 74.6 (13.3) | 0.0004 | 0.0041 (10.2) |
| 2 | 5.5 | 50.5 (9.2) | 0.0005 | 0.0057 (11.6) |
| 3 | 1.2 | 17.6 (14.7) | 0.0004 | 0.0065 (16.0) |

from pCH110; Pharmacia LKB) directed by the HIV-1 promoter (21a). The pHIV/GAL plasmid was cotransfected into Jurkat cells with the pU3R-III–CAT construct or pHIV-CAT (17) and either the pHD1013 vector or the pEBV-RIE plasmid. Two days after transfection, a portion of the extract was used to determine the amount of GAL activity and another portion was used to determine CAT activity. GAL activity in cell lysates was determined by hydrolysis of the substrate chlorophenol red β-D-galactopyranoside (Boerhinger-Mannheim Biochemicals) measured at 574 nm by using the kinetic mode of the DU-70 spectrophotometer (Beckman Instruments, Inc.) (21a). Transfection of the HIV-CAT constructs with the BRLF1 transactivator resulted in a 9- to 15-fold increase in CAT activity (Table 2). A similar rise in GAL activity in the same extracts was also detected in the presence of the BRLF1 transactivator. Therefore, the level of transactivation of the HIV-1 LTR was comparable when either reporter gene was used; we conclude that BRLF1-induced transactivation of the HIV-1 promoter is not due to a reporter gene effect.

We have demonstrated that the EBV BRLF1 IE gene product is able to transactivate the HIV-1 LTR. We have shown that transactivation of the HIV-1 promoter by the BRLF1 gene product, in contrast to that by BMLF1, is independent of the reporter gene used and produces the expected increase in CAT mRNA (16). Thus, if EBV-induced transactivation of the HIV-1 promoter in cells infected by both viruses does play a role in regulating HIV-1 expression, it is likely that such an effect is modulated by BRLF1 rather than BMLF1.

A variety of different HIV-1 promoter deletions, including 5' deletions and site-directed mutations within the κB sites and TATA box, retained the ability to be transactivated by the BRLF1 gene product. Although some decreases in the responses to the BRLF1 transactivator were noted in a number of different deletion mutants, including those with deletions involving the TATA box (pΔTATA) and the TAR region (p−16/−17), no single deletion totally abolished BRLF1 responsiveness. Computer-assisted homology scanning did not reveal any sequence homology between the HIV-1 LTR and the BRLF1-responsive region of the BMLF1 promoter. These results suggest that optimal transactivation by BRLF1 may involve the combined effects of more than one region of the HIV-1 promoter. The BRLF1 effect on HIV-1 promoter activity is thus comparable to the previously described effects of the cytomegalovirus IE and herpes simplex virus (ICP0 and ICP4) transactivators. Each of these herpesvirus transactivators can also increase HIV-1 LTR-directed CAT activity (and CAT mRNA). No single region of the HIV-1 promoter has yet been shown to be essential for transactivation by the ICP4, ICP0, or cytomegalovirus IE transactivators, although the combined removal of the NF-κB and Sp1 binding sites can markedly diminish the transactivating effect (1, 8, 11, 21a, 24, 26, 27).

The EBV BRLF1 gene product is likely to mediate, at least in part, the switch from latent to productive EBV infection induced by BZLF1 (4, 5, 14, 35). The BRLF1 gene is encoded in a region just upstream of the BZLF1 transactivator (2), and the BRLF1 protein appears to be made from bicistronic mRNAs containing both BRLF1 and BRLF1 open reading frames (21). Although the HIV-1 promoter is responsive to the BRLF1 transactivator and not the BZLF1 transactivator (17), several EBV promoters have both BRLF1- and BZLF1-responsive elements (4, 5, 14), and the combined effects of both BZLF1 and BRLF1 are required for optimal activation of certain EBV promoters (S. Kenney, E. Holley-Guthrie, and E.-C. Mar, 14th Int. Herpesvirus Workshop Abstr. p. 214, 1989). It has been previously suggested that the BRLF1 gene product may function as an enhancer transactivator while the BZLF1 gene product transactivates promoters (21). We therefore anticipated that BRLF1 transactivation of the HIV-1 LTR might be mediated by the κB enhancer sequence. However, since we have demonstrated that the enhancer region of the HIV-1 promoter is not required for the BRLF1 response, it is likely that BRLF1 transactivation can occur by at least two different mechanisms, one of which is enhancer independent.

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