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An Epstein–Barr virus immediate-early gene product trans-activates gene expression from the human immunodeficiency virus long terminal repeat

(herpesvirus/acquired immunodeficiency syndrome/gene promoters/chloramphenicol acetyltransferase assay/S1 nuclease)

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ABSTRACT **Acquired immunodeficiency syndrome patients are frequently coinfected with Epstein–Barr virus (EBV). In this report, we demonstrate that an EBV immediate-early gene product, *Bam*HI *MLF1*, stimulates expression of the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the human immunodeficiency virus (HIV) promoter. The HIV promoter sequences necessary for trans-activation by EBV do not include the *tat*-responsive sequences. In addition, in contrast to the other herpesvirus trans-activators previously studied, the EBV *Bam*HI *MLF1* gene product appears to function in part by a posttranscriptional mechanism, since it increases pHIV–CAT protein activity more than it increases HIV–CAT mRNA. This ability of an EBV gene product to activate HIV gene expression may have biologic consequences in persons coinfected with both viruses.**

Although it is estimated that up to one million people in the United States are infected with the human immunodeficiency virus (HIV), only a portion of the infected persons develop acquired immunodeficiency syndrome (AIDS). At present, we do not understand the factors that may differentiate those HIV-infected individuals in whom active AIDS will develop from those who will remain only latently infected. One possibility is that other viruses may act as cofactors with HIV in producing disease.

An interesting mechanism whereby two viruses might interact at the molecular level is promoter trans-activation. HIV has a single promoter in the 5′ long terminal repeat sequence of the proviral genome (1, 2), which is stimulated in trans by the HIV-encoded trans-acting transcriptional activator (*tat*) (3, 4). HIV DNA sequences located 3′ of the mRNA start site are necessary for trans-activation by *tat*. Recently, it has been reported that a herpes simplex type I gene product can increase the expression of a heterologous gene linked to the HIV promoter (5, 6). In addition, several other DNA viruses, including three papovaviruses (JC, BK, and lymphotropic papovaviruses) also trans-activate the HIV promoter (6). These DNA virus trans-activators all function by increasing the level of HIV promoter-directed mRNA. In contrast, the mechanism by which *tat* itself activates HIV gene expression has been somewhat controversial. Some reports indicate that the trans-activation by *tat* can be explained totally by increased levels of mRNA, whereas other reports find greater stimulation at the protein level than can be accounted for by the observed increase in HIV-directed mRNA alone (7–11).

Because of the very high frequency of Epstein–Barr virus (EBV) infection in AIDS patients, with up to 96% showing serologic evidence of active infection (12), we have investigated whether EBV functions might also activate the HIV promoter. EBV is a human B-cell lymphotropic virus that is the etiologic agent of infectious mononucleosis. EBV also induces fatal B-cell lymphomas in immunocompromised patients (13) and is associated with both Burkitt lymphoma and nasopharyngeal carcinoma (14, 15). Homosexual men, one of the main groups at risk for HIV infection, have a 50% prevalence of active EBV infection (EBV early antigen, ≥1:40) even when seronegative for HIV, whereas in healthy heterosexuals the prevalence of active EBV infection is only 5% (16).

We have examined the ability of two different EBV immediate-early gene products, *Bam*HI *MLF1* and *Bam*HI *ZLF1*, to trans-activate the HIV promoter. We report here that the EBV gene product *Bam*HI *MLF1* stimulates expression of the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the HIV promoter, whereas the *Bam*HI *ZLF1* gene product does not. We show that the HIV promoter sequences necessary for trans-activation by *Bam*HI *MLF1* are different from those required for *tat* trans-activation. In addition, we demonstrate that the EBV trans-activator, *Bam*HI *MLF1*, increases pHIV–CAT activity disproportionately at the protein level, suggesting at least in part a posttranscriptional mechanism of trans-activation. The *Bam*HI *MLF1* EBV trans-activator thus appears to function by a mechanism different from that of other DNA viruses.

METHODS

Cell Lines. The experiments were all performed in an EBV-negative human Burkitt lymphoma B-cell line (B-JAB) and an EBV-positive Burkitt lymphoma B-cell line (AG876). Lymphoid cell lines were maintained in RPMI media with 10% fetal calf serum.

DNA Transfection. Plasmid DNA was purified through two sequential cesium chloride gradients. Electroporation into lymphoid cells was accomplished by using a "Zapper" electroporation unit from the University of Wisconsin medical electronics shop at 1500 V.

Plasmid Constructions. *EBV plasmids.* Two EBV trans-activating plasmids were constructed. The plasmid pEBV–MIE places the second exon of the EBV immediate-early gene, *Bam*HI *MLF1*, under the control of the cytomegalovirus (CMV) immediate-early promoter. Other investigators (17) have shown that only the second exon of the *Bam*HI *MLF1* gene product is required for trans-activation effects. To construct pEBV–MIE, the EBV DNA sequence from positions 81,253–84,238 (18) was excised from a vector containing the EBV *Bgl* II J fragment (19) (a gift from Georg Bornkamm, University of Freiburg, Freiburg, F.R.G.) and then ligated into the vector phD1013 (a gift from Michelle Davis and E. S. Huang, University of North Carolina,

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Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase.

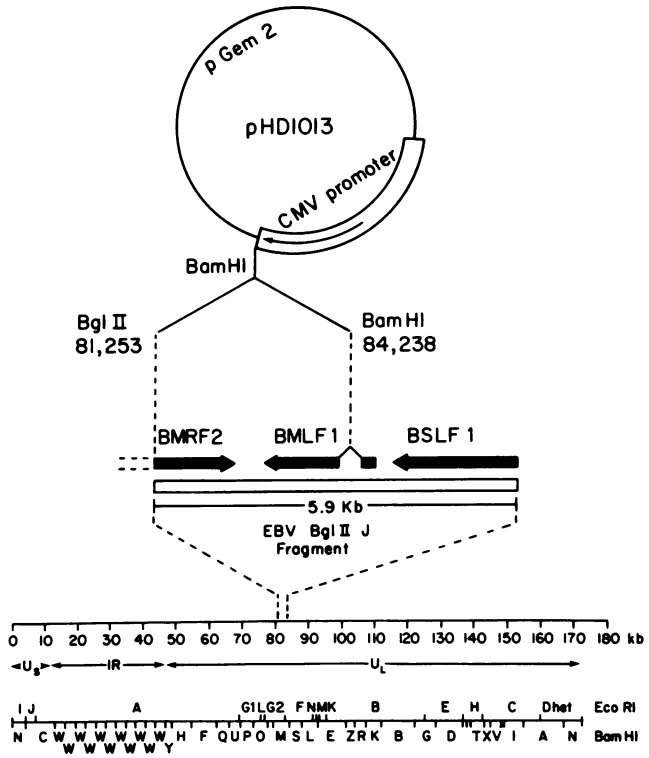


FIG. 1. Construction of pEBV–MIE. The EBV DNA from sequences 81,253–84,238 (18) was excised from the *Bgl* II J fragment of the EBV genome, then ligated into the *Bam*HI site of the phD1013 vector (a gift from Michelle Davis and E. S. Huang), which has the human CMV immediate-early promoter. The pEBV–MIE thus contains the second exon of the EBV immediate-early gene product, *Bam*HI *MLF1*, under the control of the CMV immediate-early gene promoter.

Chapel Hill, NC), under the control of the CMV promoter (Fig. 1). In a second plasmid, pEBV–ZIE, the EBV immediate-early gene product located within the *Bam*HI Z fragment of the genome, was placed under control of the CMV immediate-early gene promoter in phD1013.

HIV–CAT plasmids. Two HIV–CAT plasmids were constructed. The pHIV–CAT plasmid contains the intact HIV promoter, including the *tat*-responsive region, linked to the bacterial CAT gene (Fig. 2). The second plasmid, pHIV ΔCAT, is identical to pHIV-CAT except that the HIV TAR sequences from +29 to +77 (relative to the mRNA start site) have been deleted, so the resultant plasmid is no longer responsive to trans-activation by *tat*.

HIV tat *plasmids.* Two different *tat*-expressing plasmids were tested. The plasmid pBenn-2 (a gift from Malcolm Martin, National Institute of Allergy and Infectious Diseases, Bethesda, MD) (20) contains the portion of the HIV genome from the 5′ long terminal repeat through nucleotide 8052. The phD101–*tat* plasmid (a gift from Michelle Davis and E. S. Huang) contains the HIV sequences from positions 5331–8052 under the control of the CMV immediate-early promoter. Neither plasmid encodes a functional *art* gene product.

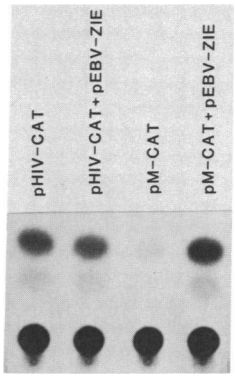
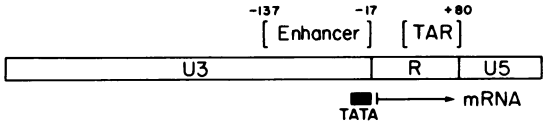


FIG. 3. Effect of the EBV *Bam*HI Z immediate-early gene product on pHIV–CAT. Five micrograms of the pHIV–CAT plasmid was cotransfected into the EBV-positive AG876 cell line with either 5 μg of phD1013 vector DNA, or with 5 μg of the pEBV–ZIE plasmid. CAT activity was measured from cell extracts 48 hr after transfection. The CAT activity of pHIV–CAT was not affected by cotransfection with the EBV *Bam*HI Z immediate-early gene product. The M-CAT plasmid, which has an EBV early promoter (BMLF1) fused to the CAT gene, is trans-activated by the pEBV–ZIE plasmid in AG876 cells and serves as the positive control.

CAT Assays. Five micrograms of the pHIV–CAT plasmid DNA was cotransfected with either 5 μg of phD1013 DNA or with 5 μg of the test plasmid. Forty-eight hours after transfection, an extract of the cells was prepared and incubated at 37°C with [^{14}C]chloramphenicol in the presence of acetyl coenzyme A, as described (21). The percentage acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by autoradiography and scintillation counting. All CAT assays were repeated with smaller amounts of extract if results were not in the linear range (<50% chloramphenicol acetylation).

RNA Analysis. Total cellular RNA was prepared 2 days after transfection by a modification of the Favaloro method (22, 23). A 700-base-pair (bp) DNA fragment containing the HIV promoter plus 250 bp of the CAT gene was excised from pHIV–CAT and cloned into the pGem 2 vector (Promega), which has the SP6 promoter. Total cellular RNA (35 μg) was hybridized overnight to a complementary ^{32}P-labeled single-stranded RNA probe (6.5×10^4 cpm) generated by SP6 polymerase. The hybrid probe was then digested with S1 nuclease (24) and the S1 nuclease-protected fragment was electrophoresed on a 6% acrylamide gel with 7 M urea.



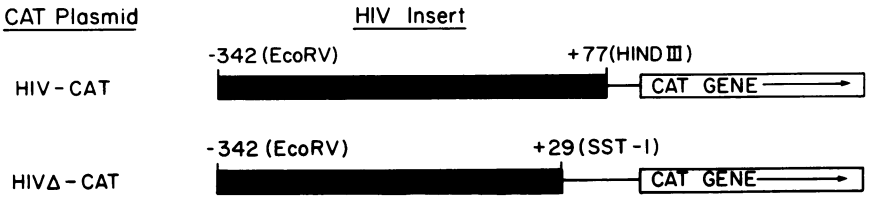


FIG. 2. Construction of pHIV–CAT and pHIVΔCAT. The pHIV–CAT plasmid contains the intact HIV promoter sequences (−342 to +77 relative to the mRNA start site), linked to the bacterial CAT gene. The pHIVΔCAT is identical to pHIV–CAT except that the HIV *tat*-responsive sequences (TAR) from +29 to +77 have been deleted.

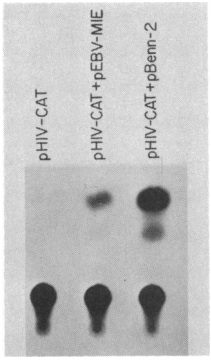


FIG. 4. Effect of the EBV *Bam*HI M immediate-early gene product on pHIV-CAT. Five micrograms of the pHIV-CAT was cotransfected into EBV-negative B-JAB cells with either 5 μg of pEBV-MIE or 5 μg of pBenn-2 (a *tat*-producing plasmid) and CAT activity was measured 48 hr later. Both the pEBV-MIE and the pBenn-2 plasmids increase the CAT activity of pHIV-CAT, although the degree of trans-activation produced by pBenn-2 is greater than that observed with pEBV-MIE. In the experiment shown, 5 μl (of a total of 200 μl) of the extract was incubated at 37°C for 1 hr. Under these conditions, pHIV-CAT by itself produced 0.3% acetylation, whereas the sample cotransfected with pEBV-MIE produced 9.7% acetylation, and the sample cotransfected with the pBenn-2 plasmid produced 51.3% acetylation.

RESULTS

The EBV *Bam*HI Z Immediate-Early Gene Product Does Not Affect pHIV-CAT Activity. Two different EBV immediate-early gene products were tested for the ability to stimulate the activity of the pHIV-CAT plasmid. When the electroporation technique was used to introduce plasmid DNA into B-JAB cells, the baseline activity produced by pHIV-CAT alone was significantly higher than that produced by the negative control plasmid pCAT3M. Incubating 100 μl (of a total of 200 μl) of the pHIV-CAT extract for 1 hr at 37°C generally resulted in 20–50% acetylation, whereas <1% acetylation was produced by the same amount of pCAT3M extract. Therefore, the cotransfection experiments were generally performed with only a small amount (5–20 μl) of the total 200-μl extract so that the CAT activity of the conditions cotransfected with active transactivators remained within the linear range. Although the transfection efficiency varied from experiment to experiment, within each experiment the CAT activity produced by duplicate transfections was very similar (coefficient of variation averaged 8.7%).

The *Bam* HI Z immediate-early gene product has been shown (25, 26) to disrupt viral latency when transfected into the latently EBV-infected Raji cells. In this laboratory, cotransfection of the *Bam*HI Z immediate-early gene product (plasmid pEBV-ZIE) into an EBV-infected cell line (AG876) with different EBV promoters linked to the CAT gene produces increased CAT activity from two different early EBV promoters but does not affect the activity of several latent EBV promoters (S.K., unpublished data). The pHIV-CAT activity was not affected by cotransfection with the *Bam*HI Z immediate-early gene in either EBV-positive or -negative cell lines (Fig. 3). Therefore, the EBV *Bam*HI Z immediate-early gene product does not appear to stimulate HIV expression.

The EBV *Bam*HI M Immediate-Early Gene Product Stimulates Activity from the pHIV-CAT Plasmid. The second EBV gene tested for potential of trans-activating pHIV-CAT was the *Bam*HI *MLF1* immediate-early gene product (plasmid pEBV-MIE). This EBV gene product has been previously shown to trans-activate several different heterologous promoters (17, 27) but, unlike the *Bam*HI Z immediate-early gene product, does not disrupt viral latency when transfected into the latently EBV-infected Raji cell line (26). Fig. 4 shows the results of cotransfection experiments with pEBV-MIE and pHIV-CAT. As expected, the activity of pHIV-CAT, which contains the intact HIV promoter plus the *tat*-responsive sequences, is dramatically increased when cotransfected with the *tat*-producing plasmid pBenn-2. The plasmid pEBV-MIE also reproducibly increases CAT activity when cotransfected with pHIV-CAT (5- to 30-fold in seven separate experiments; $P < 0.05$ by the Wilcoxin signed-rank test). The phD1013 vector alone has no effect on pHIV-CAT activity. Thus, pEBV-MIE encodes a trans-activator capable of stimulating pHIV-CAT expression. The pEBV-MIE effectively trans-activated pHIV-CAT in all cell lines tested (HeLa, baby hamster kidney cells, Raji) (data not shown). The effect of cotransfecting both the pBenn-2 and pEBV-MIE plasmids with pHIV-CAT was additive.

The EBV Trans-Activating Effect Does Not Require the HIV TAR Sequences. The HIV *tat* gene does not increase pHIV-CAT activity unless the *tat*-responsive sequence, located 3′ of the mRNA start site, is present. We tested the ability of the EBV immediate-early gene product to stimulate CAT activity from the HIV promoter when the *tat*-responsive sequence (TAR) is deleted. Fig 5 shows the results of cotransfection experiments using the pHIVΔCAT plasmid, which contains the HIV promoter without the *tat*-responsive sequence. The pHIVΔCAT plasmid, as expected, is not stimulated by cotransfection with the pBenn-2 plasmid (which encodes *tat*). However, the EBV trans-activator in plasmid pEBV-MIE still stimulates CAT activity from pHIVΔCAT. The activity of the CAT gene alone, with no HIV promoter elements, is not significantly increased by cotransfection with pEBV-MIE (data not shown). Therefore, the EBV and HIV trans-activators require different HIV sequences for their effect and may function through a different mechanism.

EBV Trans-Activates pHIV-CAT by a Posttranscriptional Mechanism. We analyzed the ability of the HIV *tat* and EBV *Bam*HI *MLF1* trans-activators to increase the steady-state level of HIV long terminal repeat-directed mRNA. The pHIV-CAT mRNA was quantitated by hybridizing 35 μg of total cellular RNA to a ^{32}P-labeled single-stranded RNA probe and then digesting with S1 nuclease. Only those experiments that showed a similar amount of actin mRNA (determined by RNA blot analysis) in each sample of harvested RNA were used for quantitation of pHIV-CAT mRNA with S1 nuclease (data not shown).

Table 1 compares the effect of the different trans-activating plasmids on the level of CAT activity versus the increase in pHIV-CAT mRNA. Note that cotransfection with the pEBV-MIE plasmid always resulted in an increased level of pHIV-CAT mRNA; therefore, the trans-activating effect must occur at least partly at the level of mRNA. However, the observed increase in pHIV-CAT mRNA could account for only 14–33% of the increase in CAT activity. This suggests that the EBV *Bam*HI *MLF1* gene product may have more than one mechanism of trans-activation, with at least one of these mechanisms occurring at the posttranscriptional level.

Two different plasmids were used to test the mechanism of the HIV *tat* trans-activator, and, somewhat surprisingly, the two plasmids gave different results. Cotransfection with the plasmid pBenn-2, which contains the *tat* gene product directed by its own promoter, stimulated pHIV-CAT mRNA to a similar degree as CAT activity. On the other hand, cotransfection with the phD101-*tat* plasmid, which has *tat* under the control of the CMV promoter, always produced significantly more CAT activity than explained by the observed increase in pHIV-CAT mRNA alone. These data suggest that subtle differences in the level of *tat* expression may influence the degree to which trans-activation occurs at the RNA versus protein level. As only one vector (pEBV-MIE) was used to determine the effect of the EBV trans-activator on pHIV-

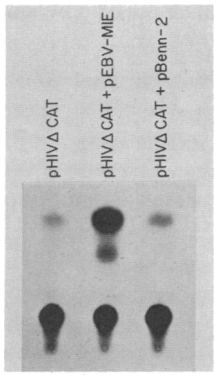


FIG. 5. The HIV *tat*-responsive sequences are not necessary for trans-activation by pEBV–MIE. The pHIVΔCAT plasmid, which has the *tat*-responsive sequence deleted, was cotransfected into B-JAB cells with either pEBV–MIE or pBenn-2. The pEBV–MIE still increases the CAT activity of pHIVΔCAT, whereas the *tat*-producing pBenn-2 plasmid no longer trans-activates. In the experiment shown, 20 μl of the extract was incubated at 37°C for 1 hr. The pHIVΔCAT alone produced 2.7% acetylation, the pHIVΔCAT cotransfected with pEBV-MIE produced 33.4% acetylation, and pHIVΔCAT cotransfected with pBenn-2 produced 2.8% acetylation.

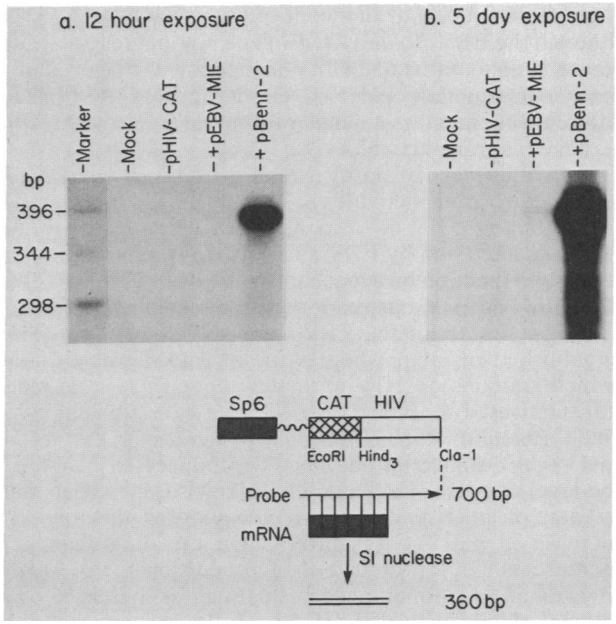


FIG. 6. RNA analysis. Total cellular RNA (35 μg) was hybridized overnight to a 700-bp complementary ^{32}P-labeled single-stranded RNA probe produced by SP6 polymerase. The RNA hybrid was then digested with S1 nuclease, and the protected fragment was electrophoresed on a 6% acrylamide/7 M urea gel. The expected protected fragment is 360 bp. The autoradiograph shown is from Table 1, Exp.1. In this experiment, pHIV–CAT was stimulated at the level of CAT activity 29-fold by cotransfection with pEBV–MIE and 151-fold by cotransfection with pBenn-2. Densitometry analysis showed a 4-fold increase in the level of pHIV–CAT mRNA after cotransfection with pEBV–MIE, and a ≥100-fold increase in mRNA after cotransfection with pBenn-2. The quantitative mRNA value for each band shown was arrived at by first normalizing the densitometry value of each band to another band on the same gel (data not shown) of intermediate intensity after short and long autoradiographic exposures of the gel.

CAT, we cannot comment on whether different levels of this trans-activator might similarly affect the degree of RNA versus protein stimulation.

Fig. 6 shows the results of a representative S1 nuclease experiment. In this particular experiment, cotransfection with the pEBV–MIE plasmid produced a 29-fold increase in CAT activity, whereas the mRNA increase (as measured by laser densitometry) was only 4-fold. In the same experiment, the cotransfection with the pBenn-2 plasmid resulted in a similar degree of stimulation at both the mRNA and CAT levels.

DISCUSSION

We demonstrate that an EBV immediate-early gene product, *Bam*HI *MLF1*, can activate the expression of a heterologous gene linked to the HIV promoter. In addition, we show that the trans-activating function of this EBV *Bam*HI M immediate-early gene product appears to occur, at least partly, at the posttranscriptional level, since the degree of mRNA stimulation observed cannot account for the level of protein stimulation produced. The molecular interaction between EBV and HIV suggested by our observations may have biologic significance since both EBV and HIV can coinfect B cells in patients with AIDS (28).

The function of the *Bam*HI *MLF1* gene product within EBV-infected cells is not clearly understood. We have shown that a number of different EBV promoters, including that of the *Bam*HI *MLF1* gene itself, are trans-activated by the *Bam*HI *MLF1* gene product (29). In addition, the EBV *Bam*HI *MLF1* gene product can trans-activate other promoters, including the simian virus 40 early promoter and the herpes simplex thymidine kinase gene promoter (17, 27). The *Bam*HI *MLF1* gene might thus play a role in EBV analogous to that of the herpes simplex immediate-early 110-kDa protein, which serves as a general trans-activator for a number of different herpes simplex virus promoters (30) and has also recently been shown to activate the HIV promoter (31).

A major distinction, however, between these two herpesvirus trans-activators is that the herpes simplex virus immediate-early 110-kDa protein trans-activating function appears to occur primarily at the level of increased mRNA (5, 31), whereas the EBV *Bam*HI *MLF1* increases pHIV–CAT protein activity out of proportion to the increase induced at the level of pHIV–CAT mRNA. In collaboration with E.-S. Huang, we have recently found that a gene product of the human CMV major immediate-early gene region also trans-activates the pHIV–CAT plasmid (32). The CMV immediate-early gene trans-activator, like that of herpes simplex virus

Table 1. Stimulation of RNA versus CAT activity by the HIV *tat* and EBV *Bam*HI M immediate-early gene product

Trans-activating plasmid	Exp.	Extract used, μl	Acetylation, % pHIV–CAT alone	Acetylation, % pHIV–CAT plus trans-activator	CAT stimulation	RNA stimulation	CAT/RNA
pEBV–MIE	1	5	0.34	9.74	29×	4×	7.3
	2	5	1.32	11.75	9×	3×	3.0
	3	20	5.16	27.91	5×	1.5×	3.3
pBenn-2	1	5	0.34	51.3	151×	>100×	≤1.5
	2	2	0.16	16.05	100×	>100×	≤1.0
phD101–*tat*	1	5	1.35	33.35	25×	8×	3.2
	2	5	1.32	24.50	19×	3×	6.3

1, operates entirely at the level of increased mRNA. Thus, although the EBV *Bam*HI *MLF1* gene product resembles the herpes simplex virus and CMV immediate-early genes in its ability to stimulate pHIV–CAT, it appears to utilize a different functional mechanism from that used by the other herpesvirus trans-activators.

The exact mechanism by which the EBV *Bam*HI *MLF1* gene product trans-activates pHIV–CAT is still unclear. The finding that the HIV *tat*-responsive sequence is not essential for trans-activation by EBV suggests that the *Bam*HI *MLF1* is likely to function by a mechanism different from that of the HIV *tat*. The HIV upstream negative regulatory element is also dispensable for the EBV trans-activating effect (S.K., unpublished results), whereas the pCAT3M plasmid alone (which contains no HIV promoter sequences) is no longer trans-activated by *Bam*HI *MLF1*. The EBV trans-activator could function at least partly by increasing the rate of pHIV–CAT transcription, since it produces an increase in the level of pHIV–CAT mRNA. Likewise, increased stabilization of mRNA could also account for this observed increase in pHIV–CAT mRNA. On the other hand, the increase in CAT protein out of proportion to the mRNA invokes an additional mechanism, such as increased translational efficiency of pHIV–CAT mRNA or increased stability of the CAT protein itself. We think it very unlikely that this greater increase in CAT protein stimulation versus CAT RNA is artifactual and secondary to instability of CAT mRNA, since in our hands two other trans-activating genes (the human cytomegalovirus immediate-early gene and the EBV BZLF1 immediate-early gene) have been shown to function entirely at the level of increased mRNA using an identical system. (S.K., unpublished data; ref. 32).

Since patients with AIDS have an increased incidence of EBV-infected lymphomas (33, 34), this raises the possibility that HIV-encoded gene products might also affect EBV molecular processes. We have fused a number of promoters from different classes of EBV genes to the bacterial CAT gene. To date, however, we have been unable to demonstrate activation of any EBV promoter by HIV gene products, including *tat* (S.K., unpublished results).

The biological relevance of the molecular interaction between EBV and HIV remains to be determined. *A priori*, one might expect that the ability of the EBV *Bam*HI M immediate-early gene to increase the level of gene expression of HIV could result in increased virus production. In many HIV-infected individuals, the HIV genome is integrated within the host-cell DNA but is not actively producing infectious virus. The exposure of the HIV genome to the EBV *Bam*HI *MLF1* gene product might result in increased levels of viral gene products (including *tat*) and consequently more virus production. However, any effect of EBV on HIV replication would be limited to B cells (and perhaps epithelial cells). Given the ability of HIV to infect a number of different cell types (in particular, the helper T cell), the clinical relevance of the HIV/EBV interaction has yet to be established.

It is now known that at least two HIV gene products other than *tat* are also important in HIV regulation. The HIV gene *art* (or *trs*) appears to be required for the synthesis of the gag and env proteins (10, 35). *art* may function by relieving an inhibition to expression of the *gag* and *env* genes (35). Another HIV gene product, termed *3'orf* (or *orfB*), may function to promote viral latency, since deletions in the *3'orf* consistently result in more viral replication than occurs with wild-type virus (36, 37). The posttranscriptional effect of the EBV trans-activator *Bam*HI *MLF1* might affect certain of these HIV proteins more than others. In addition, EBV might increase the *3'orf* gene product to the extent that HIV replication might actually be decreased. In any event, the biologic interactions between HIV and EBV are likely to be important since these two viruses commonly coexist within the human host, which may allow one virus to affect the pathogenesis of the other.

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