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The Epstein-Barr Virus DNA Polymerase Transactivates the Human Immunodeficiency Virus Type 1 5' Long Terminal Repeat

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SUMMARY: We have demonstrated in transient expression assays that the Epstein-Barr virus (EBV) DNA polymerase transactivates expression of the bacterial chloramphenicol acetyltransferase (CAT) gene linked to the human immunodeficiency virus (HIV) type 1 5' long terminal repeat (LTR). The evidence was provided by two sets of experiments. Transfection of Raji cells with HIV LTR-CAT followed by superinfection with EBV resulted in a 150-fold increase in CAT activity. In the presence of viral DNA inhibitor 3'-azido-3'-deoxythymidine (AZT), the CAT activity was inhibited by approximately 70%, suggesting that EBV DNA polymerase was involved in the transactivation of HIV LTR. The direct proof came from the cotransfection of HIV LTR-CAT with expression plasmid containing EBV polymerase gene; depending on the polymerase gene construct cotransfection with both plasmids resulted in a 23- to 38-fold increase of HIV LTR-CAT activity. The interaction between EBV polymerase and HIV may contribute to the role of EBV as a cofactor in the pathogenesis of AIDS.

The World Health Organization estimated that up to 2.5 million people contracted human immunodeficiency virus (HIV) in the first six months of this year; almost half of these infections have developed among women. Although most individuals infected with HIV eventually develop AIDS (1), only a small portion of the total individuals infected by HIV actually develop clinical AIDS each year. At present, it is not known whether or how cofactors or host susceptibility factors increase the risk of AIDS in infected persons. One possibility is that other viruses may act as cofactors with HIV in producing clinical disease. AIDS patients have a high frequency of active infection by members of the herpesvirus group, in particular with EBV, human cytomegalovirus (HCMV), herpes simplex virus types 1 and 2 (HSV-1 and -2), varicella-zoster virus (VZV), and human herpesvirus 6 (2). Active EBV infection is common in AIDS patients (3) and may contribute to its pathogenesis as a cofactor. EBV-containing B-cell lymphoma is the second most common malignancy in AIDS (2). Some studies indicated that the disease in those infected with a second virus progresses more rapidly than that in those infected with HIV alone (2).

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The regulation of transcription and replication in HIV infected cells is extremely complex and still not fully understood. It is possible that the complex scheme of HIV regulation might be perturbed by other viruses, particularly members of the herpesvirus group. Recent data have shown that HSV-1, EBV, and HCMV can activate transcription of genes linked to the HIV long terminal repeat (LTR) (4-8). Since opportunistic viral infections, especially EBV, HCMV and HSV are prevalent in AIDS patients (2), it was postulated that the productive infections of herpesviruses might enhance HIV expression. Since viral DNA polymerase is the key enzyme responsible for productive viral DNA replication, inhibitors of viral DNA polymerase (9-11) should block the transactivation of HIV by herpesvirus infections. Based on this premise, the following experiments were undertaken. The results clearly indicated that EBV polymerase have a positive regulatory effect on HIV-LTR-directed expression.

Materials and Methods

Cell cultures and virus preparation. The Raji cells were propagated as suspension cultures in RPMI 1640 medium. Virus was prepared from the uninduced P3HR-1(LS) cell culture fulids (12).
Plasmids. The pHIV LTR-CAT plasmid (pUR-III CAT) was kindly provided by C.A. Rosen; pHD101SV1 (pHD1013 vector contains HCMV IE regions 1 and 2) by E-S Huang; pEBV M IE (pHD1013 vector conatins EBV BMLF1) by S. Kenney. The plasmid pHD1013 is a pGEM2 containing HCMV enhancer sequence cloned at BamHI site. The cloning of EBV polymerase gene (BALF5) into pHD1013 expression vector was detailed (13).
Cell transfectionss and CAT assays. Raji cells (10^6) were transfected by electroporation with pHIV LTR-CAT (5 μg) alone, or in combination with expression plasmid (10 μg) containing transactivator genes. In some experiments, viral infections were initiated 16 hr after transfection by adding the appropriate volume of viral suspension to obtain the desired m.o.i. per cell. Cell extracts were prepared 48 hr after transfection and assayed for CAT activity.

Results

The effect of AZT on the transactivation of HIV LTR-CAT activity by herpesvirus infection. Figure 1 shows that HIV LTR-directed CAT activity was very low in mock-infected Raji cells (lanes 1 and 5), but was markedly increased (150-fold) by superinfection with EBV (lane 3) and HSV-1 (134-fold) (lane 2). To examine the ability of the EBV polymerase to activate the HIV LTR, an EBV DNA polymerase inhibitor, 3'-azido-3'-deoxythymidine (AZT), which has been shown to inhibit EBV replication (14), was specifically selected for this study. At the concentration (60 μM) 2-fold of 90% inhibitory dose (30 μM) for EBV replication (14), approximately 70% inhibition of CAT activity was consistently observed (Fig. 1, lane 6). In contrast, transactivation of the HIV LTR by HSV-1 could not be inhibited by AZT at the same concentration (lane 4), in agreement with our previous findings that AZT was a unique inhibitor of EBV but had virtually no effect on HCMV, HSV-1, HSV-2, and VZV (14). These results were reproducible in

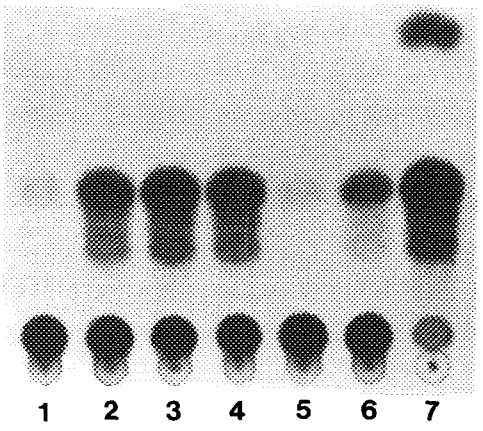

Figure 1. The effect of AZT on the transactivation of HIV LTR-CAT activity by herpes virus infection. Raji cells (10^6) were transfected by electroporation with pHIV LTR-CAT (5 μg) followed by superinfection with EBV or infection with HSV-1 (pfu of 0.1). The infected cells were cultured in the presence or absence of AZT and CAT activity was measured 48 hr later. Lane 1, mock infection, 0.32% acetylation (0.18-0.53%); lane 2, HSV-1 infection, 43% (28-54%); lane 3, EBV superinfection, 48% (33-57%); lane 4, HSV-1 infection plus AZT (60 μM), 45% (39-58%); lane 5, mock infection plus AZT (60 μM), 0.28% (0.13-0.46%); lane 6, EBV superinfection plus AZT (60 μM), 13% (9-15%); lane 7, pSV2CAT, 95% (93-98%). The values of % acetylation were the average of three separate experiments.

several experiments and indicated that viral DNA polymerase may be involved, either directly or indirectly, in transactivation of HIV gene expression.

To establish that the inhibition of HIV LTR-CAT activity was not limited to a single drug concentration, and that the inhibition did not result from the cytotoxicity of the drug, cells were treated with various concentrations of AZT after superinfection with EBV. At the noncytotoxic concentrations of the drug (0.1 to 100 μM) (14), we observed a dose-dependent reduction of HIV LTR-CAT activity. The CAT activity decreased with increasing drug concentrations, but never reduced to the level of mock-infected cells (data not shown). To study further the inhibition of HIV-LTR CAT activity by antiviral drugs, we tested a more potent DNA inhibitor 9([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine (Ganciclovir). At the concentration (10 μM) of more than 3-fold of 90% inhibitory dose (3 μM) for EBV replication (12), we consistently observed approximately 70% inhibition of CAT activity (data not shown).

EBV polymerase activates HIV LTR-directed expression in trans. To assess that EBV DNA polymerase is directly involved in transactivating the HIV promoter, we have cloned BALF5 (13) into an expression vector (pHD1013) under the control of HCMV immediate-early gene promoter (4). Three different constructs were cloned (13): 1) pol 1, the SphI fragment (3.9 kbp) containing the entire BALF5 ORF, 2) pol 2,

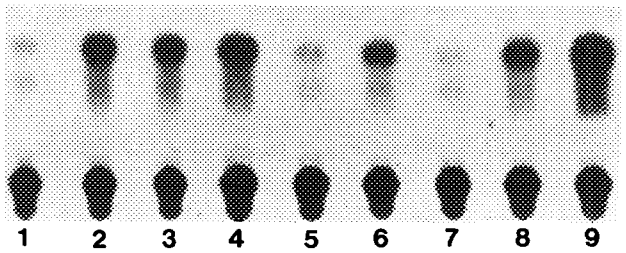

Figure 2. Effect of EBV BALF5 gene product on the HIV LTR-CAT. Raji cells were cotransfected with pHIV LTR-CAT (5 μg) and expression plasmid (10 μg) containing transactivator genes specified: lane 1, none, 0.4% acetylation (0.15-0.8%); lane 2, TPA, 14% (9-21%); lane 3,pHD101SV1 (contains HCMV IE regions 1 and 2), 12% (8-15%); lane 4, pEBV M IE, 16% (13-21%); lane 5, pHD1013, 0.5% (0.2-1%); lane 6, pEBV pol 1, 9% (7-12%); lane 7, pEBV pol 3, 0.4% (0.2-0.7%); lane 8, pEBV pol 2, 15% (12-21%); lane 9, pSV2CAT, 40% (30-50%).

the 5'-truncated BALF5 fragment (3.65 kbp) containing three intact ATGs, and 3) pol 3, the 5'-truncated BALF5 with deletion of the first ATG. Cotransfection of the first and second constructs with plasmid containing HIV LTR-CAT resulted in 23- to 38-fold enhancement of CAT activity (Fig. 2, lanes 6 and 8), as compared with cells transfected with the HIV LTR-CAT plasmid alone (lane 1). In contrast, no appreciable enhancement of CAT activity was observed when cells were cotransfected with the third construct (Fig. 2, lane 7). The inability of the third construct to transactivate the HIV LTR-CAT could be due to inefficient translation of the mRNA (13), or to the truncated polymerase polypeptide (95 kDa as compared with the full length product of 110 kDa) (13). The activity of HIV LTR-CAT in Raji cells was low (Fig. 2, lane 1). However, the CAT activity was markedly induced (35-fold) by treatment of the cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (lane 2). For positive control experiments, two expression vectors containing HCMV and EBV immediate-early genes, which had been shown to transactivate HIV LTR-CAT (4,6), were used in this study. The activation levels (23- and 38-fold) observed with plasmids containing the EBV polymerase gene (Fig. 2, lanes 6 and 8) were comparable with that of plasmids containing HCMV (30-fold) (lane 3) and EBV immediate-early genes (40-fold) (lane 4), but were consistently lower than that of superinfection with EBV (150-fold). (Fig. 1). The vector (pHD1013) used for cloning was devoid of transactivating activity (Fig. 2, lane 5). To assess whether EBV polymerase can transactivate homologous promoter, we tested its effect on EBNA-2 (W-promoter) and early antigen (BMRF1) promoters. No appreciable enhancement of CAT activity was observed in both cases (data not shown). Furthermore, EBV polymerase failed to transactivate γ-globulin gene promoter (data not shown).

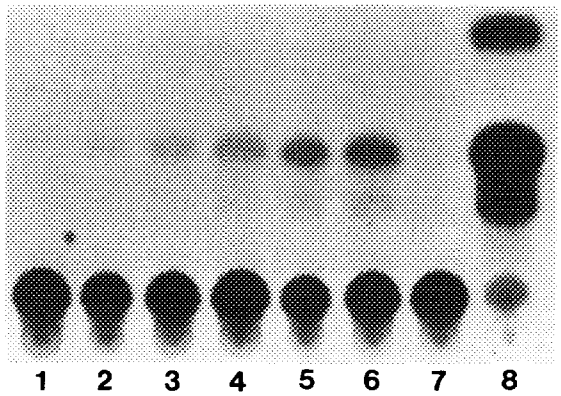

Figure 3. Dose-dependent effect of transactivation of HIV LTR-CAT by EBV polymerase. Raji cells were cotransfected with pHIV LTR-CAT (5 μg) and various amounts of pEBV pol 2. The amount of pEBV pol 2 for lane 1 to lane 6 were 0, 0.5, 1, 3, 6, and 10 μg, respectively. Lane 7, pCAT3M (negative control); lane 8, pSV2CAT. The % of acetylation for lanes 1-8 were 0.2 (0.1-0.3), 0.8 (0.4-1), 1.5 (0.9-2), 2.6 (1.4-3.5), 4.5 (2.8-6), 12 (9-14), 0.18 (0.1-0.3), 96% (94-98), respectively.

Dose-dependent effect of transactivation. To more precisely quantitate the effect of EBV polymerase on HIV-LTR activation, we cotransfected Raji cells with pHIV-LTR CAT and various amounts of the pEBV pol 2 DNA. A linear increase in CAT activity was observed with increasing amounts of pEBV pol 2 DNA used (Fig. 3). These results, taken together, clearly indicate that EBV DNA polymerase is capable of transactivating HIV gene expression.

EBV polymerase transactivation is HIV LTR-specific. To determine whether EBV polymerase interacts specifically with the HIV LTR, we carried out an *in vivo* competition assay (15). A fixed amount of pHIV LTR-CAT and pEBV pol 2 DNA were cotransfected with increasing concentrations of competitor DNA (pHIV LTR) into Raji cells. Fig. 4 shows that increasing the concentration of pHIV LTR DNA relative to HIV LTR-CAT DNA decreased the level of CAT activity. In constrast, cotransfection of the plasmid pW containing promoter sequence of EBV nuclear antigen 2 with pHIV LTR-CAT DNA did not affect the level of CAT activity. The result of this competition assay suggests that the HIV LTR was the target for the EBV polymerase.

Discussion

We have demonstrated in this study that the EBV DNA polymerase can transactivate the expression of a heterologous gene linked to the HIV promoter. The evidence was derived from two sets of expermients. The study with the viral DNA polymerase inhibitor only provided the clue of the involvement of EBV DNA

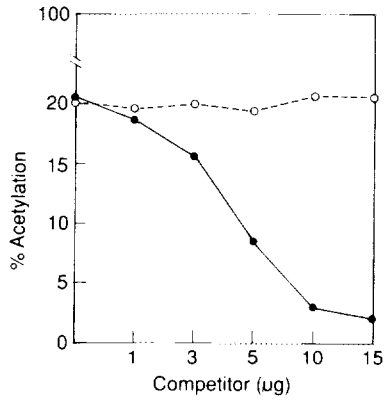

Figure 4. Competition assay for HIV LTR-CAT expression. CAT activity in Raji cells cotransfected with pHIV LTR-CAT (5µg) and pEBV pol 2 (10 µg), and increasing amounts of competitor pHIV LTR DNA (●) or pW DNA (○). All transfections were carried out with DNA adjusted to a total of 30 µg with pBR 322 DNA. Results represent an average of three independent experiments.

polymerase in the superinfection system. However, the direct proof came from the cotransfection of HIV LTR-CAT DNA with expression plasmid containing EBV polymerase gene. Superinfection of Raji cells resulted in a cascade of events which modulate EBV gene expression leading to lytic cycle (16). In this sequence of events, the immediate-early gene products such as BZLF1 (ZEBRA), BRLF1, and BMLF1(EA-D), in addition to the early gene (polymerase), were induced. The first three gene products have been shown to enhance HIV LTR-CAT activity (6,7). Thus, it is expected that superinfection with whole virus would result in an additive or synergistic effect on activation of HIV LTR. As has been shown in Fig. 1, superinfection of Raji cells with whole virus (EBV or HSV-1) resulted in a 150-fold increase in HIV LTR-CAT activity compared with only a 23- to 38-fold enhancement by the polymerase gene alone. The inability of AZT to completely block the HIV LTR-CAT activity in superinfected Raji cells (Fig. 1) was caused by the immediate-early genes' not being sensitive to the inhibition of most nucleoside analogs (see 11 for a review). It should be noted that AZT or Ganciclovir requires initial phosphorylation by the viral encoded thymidine kinase to be active. Virus-specific thymidine kinase was not induced in cells cotransfected with cloned EBV polymerase gene. Thus, in the cotransfection system the drug has essential no inhibitory effect.

The specificity of activation by EBV polymerase on HIV-LTR was indicated by the fact that both homologous (EBNA-2 and BMRF1) and heterologous (γ-globulin gene) promoters were not activated. A recent report showed that EBV latent membrane protein was an efficient transactivator of HIV-LTR and the effect was mediated through the NF-kB elements (17). The sequences within the HIV-LTR

recognized by cellular transcription factors such as SP1, AP1, and NF-kB may not be involved in the transactivation of EBV polymerase, since there are several copies of imperfect such sequences scattered around the EBNA-2 promoter (pW), which were unable to compete with the binding site of EBV polymerase (Fig. 4). Analyses of the deletion mutants of HIV LTR would locate the target sequence responsible for transactivation by EBV polymerase.

The interaction between EBV and HIV shown in this study may have biologic significance since both viruses can coinfect B cells in patients with AIDS (18-20). Since patients with AIDS have an increased incidence of EBV-associated B-cell lymphomas (21-24), this raises the possibility that HIV-encoded gene products might also affect EBV replication. It is interesting, therefore, to speculate that HIV might have a role in the pathogenesis of both African and AIDS EBV-positive lymphomas, and might act directly on B cells containing EBV. The biologic consequences of the molecular interaction between EBV polymerase and HIV remains to be determined. Whether the exposure of the HIV genome to the EBV BALF5 gene product might result in increased levels of tat expression and consequently more virus production is under investigation.

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