Infection with human T-cell leukemia virus type-I (HTLV-I) has been epidemiologically associated with the subsequent development of adult T-cell leukemia (ATL) as well as with the progressive neurologic disease tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP-HAM). In contrast to infection with another retrovirus, human immunodeficiency virus type-I (HIV-1), where the majority of those infected ultimately develop the acquired immunodeficiency syndrome, only a small percentage of those infected with HTLV-I will develop ATL or TSP-HAM, suggesting the role of other factors in determining the outcome of infection. In HIV-1 infection it has been suggested that other viral cofactors may be important determinants in viral activation and disease progression. The role of viral cofactors in the expression of other human retroviruses such as HTLV-I has not been extensively explored. Both HTLV-I and the human herpesvirus, cytomegalovirus (HCMV), have been shown to infect OKT4+ T lymphocytes in vitro and in vivo. The latter virus is quite common, with a range of 50% to close to 100% of African and Asian populations showing serologic evidence of infection depending on the geographic location. After acute infection with HCMV, the virus remains latent in the normal host with expression of viral genes limited to the immediate-early gene complex. The immediate-early gene complex of HCMV codes for at least two transactivation proteins, IE-1 and IE-2. IE-2 gene product(s) have been shown to be potent transactivators of heterologous promoters, including the long terminal repeat (LTR) of HIV-1 in both Hela and B-cell lines. Furthermore, HCMV and HIV-1 dual infection of HOS cells enhances expression of both viruses. We examined the interaction between the immediate-early region 2 gene product(s) and the LTR of HTLV-I.

We have studied the ability of HCMV IE-2 gene product(s) to modulate the expression of the bacterial gene chloramphenicol acetyl transferase (CAT) that is driven by the HTLV-I LTR. We report in this study that the HCMV IE-2 protein represses the HTLV-I promoter both in HTLV-I-producing cell lines where there is expression of the transactivating protein tax as well as in uninfected primary human peripheral blood lymphocytes (PBL) and cloned lymphocyte cell lines. By analysis of HTLV-I 5' and 3' LTR deletion mutants, a single region responsive to the IE-2 protein could not be identified. However, RNase protection assays indicated that this response was at least in part mediated at the RNA level.

MATERIALS AND METHODS

Cells. Established HTLV-I-producing T-cell lines, MT-226 and HUT-102, as well as the uninfected Jurkat T-cell line, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 2 mmol/L glutamine. PBL were prepared from whole fresh blood. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Pharmacia, Pleasant Hill, CA) cultured for 3 days in RPMI 1640 medium containing 20% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, 1% phytohemagglutinin, and recombinant interleukin-2 (30 U/mL). The nonadherent cells were used for transfection studies.

Construction of HTLV-I LTR-CAT deletion mutants. HTLV-I 3' deletion mutants were constructed from the pU3RCAT plasmid containing the U3, R, and 40 bases of the U5 region of the intact HTLV-I LTR. By the polymerase chain reaction method, fragments of 500-700 bp length were synthesized, the fragments digested with the restriction enzymes XhoI and HindIII, and ligated into an XhoI- and HindIII-digested pU3RCAT vector. The
sense oligonucleotide used for amplification of the specific fragments was the same (5'TCAAATCCGACCTTTACATTTTGATCCATCTC3'). The antisense primers were as follows: 3B(5'TACGAACTGTGCGGCGGTTAAGAGGAGA3'); 3C(5'TAGCAGCTTTGCGACTAACGGCGGTG3'); 3D(5'TACGAACTTTTAAACTCTACCTATAGCGCG3'); 3E(5'TACGAGCTGTGGCTATGAGCTG3'); and 3F(5'TACGAACTTTGCGACTAACGGCGGTG3').

HTLV-I LTR 5' deletion mutants were a gift from John Brady (National Cancer Institute, Bethesda, MD).23 Fragments of 200 to 700 bp were ligated into the pCAT3M vector at the unique BgII site.

HCMV plasmids. HCMV IE-2 activity was obtained using plasmid pHD101-4 (pHD4), a gift from Eng-Shang Huang and Michelle Davis (University of North Carolina, Chapel Hill). This plasmid expresses the IE-2 gene product(s) under the control of the HCMV major immediate-early promoter. The IE-1 gene product is not expressed. A control plasmid designated pHD4-R was constructed by placing exon 5 in the antisense orientation using the BamHI site.

HIV plasmids. The plasmids pCD15CAT and pCD12-CAT were a gift from Steven Josephs (Baxter, Chicago, IL). The plasmid pCD15-CAT contains the HIV-1 IIIB U3-R region of the LTR as well as a portion of nef cloned into the HindIII site of pSVoCAT. The plasmid pCD12-CAT is an identical construct without the nef sequences.25 The plasmid ptat contains the tat coding sequence driven by the HIV-1 LTR.26

Transfection of cells. MT-2, HUT-102, PBL, and Jurkat cells were transfected by electroporation with the BioRad Gene Pulser (Richmond, CA). The cells were washed once with growth media and resuspended at a concentration of 4 × 10^7 cells/mL in ice-cold medium. Fifty micrograms of DNA was added to 0.25 mL of cell suspension (containing 10^7 cells), mixed gently, and incubated on ice for 10 minutes. A single pulse of 960 microfarads, 250 V was applied to each sample, followed by an additional 10 minutes of incubation on ice. Cells were resuspended in 10 mL of fresh growth media and incubated at 37°C. Twenty-four hours after electroporation, cells were harvested for CAT assay.

CAT assay. Cell extracts were prepared by standard methods and assayed for CAT activity.24 MT-2, Jurkat, and HUT-102 extracts were assayed as follows: (35)Cl choramphenicol (0.2 μC) was incubated with 0.7 mmol/L acetyl coenzyme A and 50 μL of cell extract in a final volume of 150 μL of 0.25 mol/L Tris-HCL (pH 7.8) for 12 hours at 37°C. (35)Cl choramphenicol was extracted with ethyl acetate and separated by chromatography on thin-layer plates. Each transfaction was performed in duplicate and the results expressed as change in the percent acetylation of choramphenicol as determined by densitometry.

PBL extracts were assayed using the scintillation method.26 Cell extract (50 μL) was heated to 65°C for 5 minutes and added to a 7-mL glass miniscintillation vial containing 50 μL 0.25 mol/L Tris (pH 7.8), 0.5 mmol/L aqueous solution, and labeled acetyl CoA (0.1 μCi). The final reaction volume was 0.25 mL with final concentrations of 100 mmol/L Tris-HCL (pH 7.8), 1.0 mmol/L chloramphenicol, and 0.1 mmol/L acetyl CoA. The reaction mixture was gently overlaid with 4 mL of Eononfour II (Dupont, Wilmington, DE) and incubated at 37°C. At 1 and 12 hours the individual vials were counted for 6 seconds. Transfections were performed in triplicate and the results expressed as total (35)acetylcchloramphenicol activity in cpm × 10^6.

RNA isolation. Total cellular RNA was isolated from 5 × 10^6 cells PBL by the acid guanidinium thiocyanate method.29

RESULTS

Products of HCMV IE-2 region repress pU3RCAT. We assayed the effect of the HCMV IE-2 region on HTLV-I promoter function by transfecting 40 μg of either pU3RCAT or pCD15CAT in the presence of pHD4 or pCMV. After 24 hours, 50% of the cells were removed for CAT assay and total cellular RNA was prepared from the remaining cells. A 500-bp region of the CAT coding sequence was cloned into the plasmid pTT73 (Pharmacia, Piscataway, NJ) downstream from the T7 polymerase promoter. A 500-bp region of the β-actin sequence was cloned downstream from the T7 polymerase promoter as well. 3P-labeled antisense RNA probes were synthesized using the T7 polymerase. The gel-purified RNA probes (5 × 10^6 cpm) were hybridized to the total cellular RNA (20 μg). Digestion of the hybrid RNA was performed with 40 U/mL of RNase A and 2 μg/mL of RNase T1 at 37°C for 1 hour. After termination of the digestion reactions with the addition of 10% sodium dodecyl sulfate and 50 μg/mL proteinase K, the reactions were extracted with phenol-chloroform and the 3P-RNA/RNA hybrids were precipitated with ethanol. The protected hybrid RNA was analyzed by autoradiography on a 6% acrylamide gel containing 7 mol/L urea. Band intensity was quantified by densitometry. The quantity of CAT RNA was compared with β-actin RNA.

RNase protection analysis. Protection analysis was used to quantitate CAT RNA transcripts. PBL (10^6) were electroporated with 40 μg of either pU3RCAT or pCD15CAT in the presence of pHD4 or pCMV. After 24 hours, 50% of the cells were removed for CAT assay and total cellular RNA was prepared from the remaining cells. A 500-bp region of the CAT coding sequence was cloned into the plasmid pTT73 (Pharmacia, Piscataway, NJ) downstream from the T7 polymerase promoter. A 500-bp region of the β-actin sequence was cloned downstream from the T7 polymerase promoter as well. 3P-labeled antisense RNA probes were synthesized using the T7 polymerase. The gel-purified RNA probes (5 × 10^6 cpm) were hybridized to the total cellular RNA (20 μg). Digestion of the hybrid RNA was performed with 40 U/mL of RNase A and 2 μg/mL of RNase T1 at 37°C for 1 hour. After termination of the digestion reactions with the addition of 10% sodium dodecyl sulfate and 50 μg/mL proteinase K, the reactions were extracted with phenol-chloroform and the 3P-RNA/RNA hybrids were precipitated with ethanol. The protected hybrid RNA was analyzed by autoradiography on a 6% acrylamide gel containing 7 mol/L urea. Band intensity was quantified by densitometry. The quantity of CAT RNA was compared with β-actin RNA.

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RNA isolation. Total cellular RNA was isolated from 5 × 10^6 cells PBL by the acid guanidinium thiocyanate method.29
pHD4 and pU3RCAT was analyzed for specific CAT transcripts using RNase protection. In three separate experiments, RNA from PBL cotransfected with pHD4 and pU3RCAT showed a twofold reduction in CAT message compared with pU3RCAT cotransfected with pCMV (as determined by densitometry). This corresponded to a twofold reduction in CAT activity (Fig 2). β-Actin expression was comparable in the same RNA sample.

**Effect of HCMV IE-2 gene products on 5' and 3' HTLV-I LTR deletion mutants.** The three 21-bp imperfect repeats required for transactivation of the HTLV-I promoter by tat<sup>+</sup> are not required for the repressive effects of HCMV IE-2. When 5' deletion mutants in which these sequences were sequentially eliminated were cotransfected with pHD4 into MT-2 cells there was a significant reduction in CAT activity when compared to transfection of the mutants with the control plasmid, pCMV (Fig 3). As the deletions involved regions close to the mRNA start site the reduction persisted despite a decrease in basal expression. Because there was no discrete responsive elements by 5' deletion analysis, 3' deletion mutants were examined to determine if there was an HCMV IE-2 responsive region downstream of the mRNA start site. The results of the cotransfection experiments are shown in Fig 4. In all experiments there was a significant repression of HTLV-I LTR-driven CAT activity.

**Table 1. Effect of the HCMV IE-2 Region on CAT Expression in Primary Blood Lymphocytes**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein Expressed</th>
<th>pU3RCAT Activity</th>
<th>pCD15CAT Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV</td>
<td>None</td>
<td>136,140</td>
<td>5,050</td>
</tr>
<tr>
<td>pHD4</td>
<td>HCMV IE-2</td>
<td>68,930 (+2-fold)</td>
<td>12,050 (+2.3-fold)</td>
</tr>
<tr>
<td>pCMV + ptat</td>
<td>HIV-1 tat</td>
<td>28,365</td>
<td></td>
</tr>
<tr>
<td>pHD4 + ptat</td>
<td>HCMV IE-2, HIV-1 tat</td>
<td>56,410 (+2-fold)</td>
<td></td>
</tr>
</tbody>
</table>

*The indicator plasmids pU3RCAT (40 μg) and pCD15CAT (30 μg) were cotransfected into primary blood lymphocytes with 10 μg of the control plasmid pCMV or pHD4 with or without 10 μg p tat. The total (μCi) acetylchlamphenicol activity is expressed in cpm x 10<sup>4</sup> and represents the mean of two to three transfections. The fold change in parentheses is the mean change in the presence of pHD4 compared with the baseline activity in the presence of the control plasmid pCMV.
**HTLV-I PROMOTER IS REPRESSED BY HCMV**

Fig 3. 5' Deletion analysis. (Top) Schematic of the HTLV-I LTR and the promoter 5' deletion mutants (gift of John Brady). The numbering is relative to the mRNA cap site. (Bottom) Results of cotransfection experiments with the HTLV-I 5' deletion mutants. Forty micrograms of pU3RCAT or each deletion mutant was cotransfected with 10 μg of the control plasmid pCMV (−) or 10 μg of the IE-2-expressing plasmid, pHD4 (+). The fold change represents the mean of two separate transfections and is relative to the baseline activity seen with pCMV.

Fig 4. 3' Deletion analysis. (Top) Schematic of the HTLV-I LTR 3' deletion mutants. (Bottom) Results of cotransfection experiments with the HTLV-I 3' deletion mutants. Forty micrograms of each deletion mutant was cotransfected with 10 μg of control plasmid pCMV (−) or 10 μg of the IE-2-expressing plasmid, pHD4 (+). The fold change represents the mean of two separate transfections and is relative to the baseline activity seen with pCMV.
expression in the presence of the IE-2-expressing plasmid. A specific HCMV IE-2 responsive element was not detected by deletion analysis.

**DISCUSSION**

We have shown that products from the HCMV IE-2 gene region specifically repress HTLV-I LTR-directed CAT gene expression in both cell lines as well as primary PBL cells known to harbor both viruses in vivo. The observed unique repressive effect of one viral gene product on the promoter of another introduces another potential role of viral cofactors in possibly altering disease expression in the host. Because only a small percentage of HTLV-I-infected individuals ultimately manifest clinical disease, it is possible that host factors such as another viral infection might account for the differential phenotypes expressed by infected individuals. In asymptomatic individuals infected with HCMV, virus expression may be limited to the expression of the immediate-early gene products. Thus, this HCMV product could play an inhibitory role in expression of HTLV-I in patients that are coinfected with HTLV-I and HCMV.

The repression of the HTLV-I LTR by HCMV IE-2 gene product(s) was associated with a decrease in specific level of CAT mRNA, which may reflect either a decrease in transcription or a decrease in the stability of the message. We have now shown in primary lymphocytes, and others have shown in cell lines, that products from this same HCMV region enhance gene expression from other heterologous promoters including HIV, thus suggesting that these observed effects represent specific interactions between the IE-2 gene products and the promoters. The previously reported enhanced expression of the HIV-1 promoter in the presence of IE-2 is also associated with an increase in specific RNA.

The LTR of HTLV-I has previously been shown to be trans-activated by Tax, the HTLV-I transactivator protein encoded by the pX region of HTLV-I genome. At least two of the three 21-bp imperfect repeats contained in the U3 region of the LTR are necessary for an optimal response to tax. Recent evidence suggests that tax interacts indirectly with LTR sequences -117 to -163, located between two 21-bp repeats by binding to other cellular transcription factors. Our deletion analysis showed that the specific tax-responsive elements of the HTLV-I LTR are not required to demonstrate repression by HCMV IE-2 in the MT-2 cell line. Using 3' deletions as well, there was no distinct region responsive to HCMV IE-2. Similar results were initially reported with attempts to map the regions of the HIV-1 and -2 promoters that are responsive to the upregulation associated with HCMV IE-2 proteins. However, more recently the IE-2-responsive element in the HIV promoter has been mapped between -6 and +7. Our deletion analysis does not exclude the possibility that a specific HCMV IE-2-responsive element does lie within the region of the mRNA initiation site. The HCMV IE-2 product(s) effects on the promoter may be mediated through cellular intermediaries that have multiple binding domains.

Deletion analysis also showed a putative negative element between +45 and +105 relative to the cap site that has not been previously described. As noted in Results (Fig 4), baseline CAT activity was markedly decreased with deletion 3D, but further deletion resulted in a return to baseline CAT activity. This finding suggests the presence of a negative element(s) within this region that will be further characterized.

While the potential molecular interactions between different viruses have been clearly shown in vitro, only limited studies have supported the importance of these interactions in vivo. Nevertheless, such molecular studies provide the basis for more extensive epidemiologic studies of factors that might play a role in disease manifestations. This is particularly relevant in the case of HTLV-I-associated ATL and TSP-HAM, where factors such as genetic variability of virus isolates is minimal, and therefore other host factors must be examined to determine why the vast majority of infected patients do not develop disease.

**ACKNOWLEDGMENT**

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The promoter of human T-cell leukemia virus type-I is repressed by the immediate-early gene region of human cytomegalovirus in primary blood lymphocytes

RB Gartenhaus, F Wong-Staal and ME Klotman