A Model System for Human Cytomegalovirus-Mediated Modulation of Human Immunodeficiency Virus Type 1 Long Terminal Repeat Activity in Brain Cells

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Received 19 September 1996/Accepted 10 January 1997

Previously, our laboratory showed that human cytomegalovirus (HCMV) activates human immunodeficiency virus type 1 (HIV-1) in brain-derived cells with limited HIV-1 gene expression but inhibits HIV-1 in cells fully permissive for replication of both viruses (F. M. Jault, S. A. Spector, and D. H. Spector, *J. Virol.* 68:959–973, 1994). To investigate these effects further, we developed a model system that uncouples HIV-1 gene expression from long terminal repeat (LTR) activity. Two monoclonal U373-MG astrocytoma/glioblastoma cell lines (LTRIG and LIGHIVDC) were generated, each containing an integrated copy of an LTR-chloramphenicol acetyltransferase (CAT) construct and the *Escherichia coli lacI* gene. LIGHIVDC also has an inducible HIV-1 genome controlled by a Rous sarcoma virus promoter with *lac* operator sequences. Basal LTR-mediated CAT activity is 65-fold higher in LIGHIVDC than in LTRIG, and this activity is further increased (20-fold) following incubation of LIGHIVDC with isopropyl- β -D-thiogalactopyranoside (IPTG). Tat protein can be detected by immunostaining in LIGHIVDC. However, Rev-mediated transport and subsequent translation of the singly spliced and unspliced HIV-1 mRNAs is inefficient. In the absence of Tat, HCMV stimulated CAT activity approximately 20-fold, and this activation required HCMV gene expression but not viral DNA replication. LTR-directed transcription was unaffected by HCMV infection in LIGHIVDC but was inhibited in these cells when they contained increased Tat levels following IPTG induction. These results support the hypothesis that HCMV can induce the HIV-1 LTR when HIV-1 gene expression is minimal and that a threshold level of HIV-1 gene products is necessary for HCMV to inhibit this promoter.

Human cytomegalovirus (HCMV) is an opportunistic pathogen that is found in the majority of individuals infected with human immunodeficiency virus type 1 (HIV-1). Although HCMV infection is usually asymptomatic in immunocompetent persons, it is the source of significant morbidity and mortality in AIDS patients, whose immune systems have been severely compromised. In AIDS patients, HCMV infection in the brain is commonly associated with encephalitis (2), and the virus has been found in astrocytes, neurons, oligodendrocytes, capillary endothelia, and microglia (30, 50). HIV-1 also infects the brain and often leads to AIDS dementia complex in adults and pediatric AIDS encephalopathy in children (18, 29). Several cell types in the brain, including microglia, capillary endothelia, astrocytes, oligodendrocytes, and neurons, have been shown to be infected by HIV-1 to various degrees (1, 4, 18, 31, 35, 36, 40, 46). However, the level of active HIV-1 infection seems to be inconsistent with the extent of the damage, and as a consequence, the mechanisms underlying the neuropathogenesis are controversial. One of several hypotheses is that the neuronal damage is elicited by neurotoxic factors secreted as a result of cell-to-cell interactions between infected macrophages, astrocytes, and neurons (8, 10, 14, 19, 28, 36, 39). Whether direct infection of neurons also plays a role in this process, however, has yet to be resolved.

The findings that a number of sites in vivo are infected with both HIV-1 and HCMV and that in the brain, retina, and lung

a single cell may be infected with both viruses (16, 33, 43) have led to the suggestion that both direct and indirect interactions between these two viruses could influence their replication and the resulting disease pathogenesis. In the brain, the majority of cells infected by both viruses appear to be microglia, although dual infection of capillary endothelia and glia (astrocytes and oligodendrocytes) has also been observed (5, 7, 32, 33). The complexity of the in vivo interactions between HIV-1 and HCMV has provided a strong impetus for the development of in vitro model systems involving dual infection of cultured cells and the use of HIV-1 transcription reporter constructs (see reference 44 for a review).

Previous studies from our laboratory examined coinfection of several brain-derived neuronal and glial cell lines with HIV-1 pseudotypes (amphotropic retrovirus [45]) and HCMV (22, 24). The results showed that HIV-1 did not influence the course of the HCMV infection but that HCMV had a marked effect on HIV-1 replication that appeared to depend on the permissiveness of the cells for each virus. In cells which fully supported both HCMV and HIV-1 replication, coinfection resulted in inhibition of the HIV-1 infection, and this inhibition appeared to be at the level of RNA transcription. When HCMV replication was limited but HIV-1 replicated well, HCMV had no effect on the HIV-1 infection. However, in cells which allowed only a low level of HIV-1 replication but were fully permissive for HCMV, HIV-1 gene expression was transiently stimulated by HCMV. These studies led to the hypothesis that the effect of HCMV on the HIV-1 infection depends on the overall activity of the HIV-1 promoter as well as the expression of key HCMV gene products. Further support for this hypothesis was provided by a subsequent study in which

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astrocytoma/glioblastoma cell lines were established with two different stably integrated HIV-1 proviruses. One was *tat* and *nef* minus and transcriptionally inactive, while the other was *env* and *nef* minus but expressed the other HIV-1 gene products at high levels (25). HCMV infection of these cell lines led to stimulation of HIV-1 gene expression from the *tat*-minus genome but inhibited the synthesis of HIV-1 gene products from the transcriptionally active genome.

As an alternative approach for examining the effects of HCMV infection on HIV-1, many laboratories have used the HIV-1 long terminal repeat (LTR) promoter linked to a reporter gene, such as that for chloramphenicol acetyltransferase (CAT), in transient transfection assays. The results of these studies, including those performed in our laboratory, uniformly showed that HCMV dramatically stimulates transcription directed by the HIV-1 LTR promoter (6, 9, 11, 13, 21, 38, 49). Additionally, in transient expression assays, the HCMV major immediate-early transcriptional activator proteins alone were able to activate LTR-directed gene expression (for a review, see reference 20). Although the results of these assays were highly reproducible, they were difficult to reconcile with the observed effects of HCMV on HIV-1 replication in dually infected cells. To resolve these seemingly disparate effects, we focused first on the state of the viral genome, one of the primary differences between infection and transient transfection. During the normal course of an infection, HIV-1 proviral DNA integrates into the genome of the host and assumes the conformation of chromatin, while in a transient assay, the transfected DNA remains extrachromosomal. As described in a paper by Koval et al. (25), the relative response of the HIV-1 promoter to HCMV and HIV-1 regulatory proteins was markedly affected by the integration state of the DNA. In particular, it was found that HIV-1 infection or *tat* transfection activated an integrated LTR much more effectively than a transient LTR, while the reverse was true for HCMV. Moreover, the stimulation of the integrated LTR during HIV-1 infection was 100- to 1,000-fold higher than that achieved during HCMV infection. Since HIV-1 gene expression and replication require integration of the viral DNA into the genome, these results highlighted the importance of having the HIV-1 promoter in a biologically relevant context when one is studying the effects of HCMV infection.

On the basis of the studies described above, we proposed that during a productive HIV-1 infection, any stimulation of the HIV-1 LTR by HCMV would be obscured by the high level of transactivation by HIV-1 Tat. Thus, the stimulation by HCMV would be revealed only when the expression of HIV-1 genes, including *tat*, was low. We also reasoned that HCMV infection was able to both stimulate and inhibit HIV-1 transcription and that inhibition required a critical level of HCMV early-gene products. However, one difficulty with this model was that in cells fully permissive for HCMV replication, HCMV stimulated but did not subsequently inhibit HIV-1 gene expression from an integrated *tat*-minus genome (25). These results suggested that a threshold level of Tat, or of some HIV-1 protein whose expression required Tat, was necessary to observe the inhibitory effect of the HCMV infection.

During the HIV-1 infection, Tat activates LTR-directed transcription by binding to an RNA element called TAR, located just downstream of the start site, and functions to increase viral RNA synthesis at both the initiation and the elongation steps (for a review, see reference 17). Since all HIV-1 proteins (including Tat) are encoded by mRNAs directed by the LTR, there is a positive feedback loop between HIV-1 protein expression and LTR activity. Because of this autoregulation, the hypothesis that the net effect of HCMV on the

LTR was dependent upon a threshold level of some HIV-1 protein was difficult to test with the HIV-1 provirus.

To circumvent this problem, we have developed a model system in which the interactions between HCMV and HIV-1 can be studied under conditions in which HIV-1 gene expression is uncoupled from LTR activity and the levels of HIV-1 gene products can be regulated. We show here that the HCMV infection influences HIV-1 LTR activity negatively as well as positively, with the net effect clearly dependent on the levels of HIV-1 gene products. When the concentration of HIV-1 proteins is elevated and transcription is greatly activated by Tat, the overall effect of HCMV infection is inhibition. However, in the absence of HIV-1 proteins when LTR transcription is minimal, HCMV stimulates the LTR, and this stimulation requires HCMV gene expression but not viral DNA replication.

MATERIALS AND METHODS

Plasmids. All enzymes and linkers used in plasmid construction were obtained from GibcoBRL, New England Biolabs, or Stratagene. Plasmid DNA was prepared by using the Qiagen maxiprep kit.

p3'SS and pOPRSVICAT are components of the LacSwitch System (Stratagene). pOPRSVI was derived from pOPRSVICAT by digestion with *NotI* to remove the CAT gene and religation of the plasmid. Then, pOPRHIV was constructed by placing into the *NotI* site of pOPRSVI a 9-kb *SstI* fragment (to which *NotI* linkers had been added) from the proviral DNA of the HXB2D strain of HIV-1.

The pLTR-1CAT construct (referred to as LTRCAT) contains sequences between nucleotides (nt) -633 and +185 of the HIV-1 LTR upstream of the CAT gene and was a gift from P. Luciw (University of California, Davis). pD5tat (a gift from R. LaFemina, Merck Sharp & Dohme) expresses the full-length HIV-1 Tat protein. pHIV-1LUC contains nt -340 to +80 of the LTR from HIV-1 (ARV-2) linked to a luciferase reporter gene and was a gift from P. Sheridan and K. Jones (Salk Institute).

pHIVSDribo was generated by ligating the 1,612-bp *XmnI/SstI* fragment of pOPRHIV into *SmaI*- and *SstI*-digested pGEM4Z (Promega). When pHIVSDribo is digested with *NotI* and transcribed with T7 polymerase (Promega), a 617-nt antisense transcript that contains 601 nt of HIV-1 sequence is formed. The HIV-1 sequence begins 72 nt upstream of the major 5' splice donor site and extends downstream into the *gag* sequence.

Generation of cell lines. The human glioblastoma/astrocytoma-derived cell line U373-MG (ATCC HTB 17) was a gift from R. LaFemina (Merck Sharp & Dohme) and was maintained in Dulbecco's modified Eagle medium-high glucose (4.5 g/liter) containing 5% fetal bovine serum and 1 ml of Mito+ Serum Extender (Collaborative Research) per liter. All media were supplemented with (per ml) 0.26 mg of L-glutamine, 180 U of penicillin-streptomycin, 1.4 µg of amphotericin B (Fungizone), and 45 µg of gentamicin sulfate.

The stable cell lines LIGHIVDC, LTRIG, and LIGCON were derived from U373-MG cells following calcium phosphate transfection (42) with linearized and dephosphorylated expression vectors. LTRCAT was linearized with *XhoI*. pOPRHIV and pOPRSVI were linearized with *AatII*, and p3'SS was linearized with *NaeI*.

LTRIG was generated by transfecting U373-MG with LTRCAT and p3'SS at a ratio of 10:1. Cells were allowed to double twice, passaged one to four times, and then propagated in medium containing 250 µg of hygromycin B per ml (Boehringer Mannheim). Monoclonal cell lines were isolated with cloning rings after 3 weeks of selection. Next, the lines were screened for Tat activation of the integrated LTRCAT by transient transfection with pD5tat and subsequent assay of CAT activity. Lines were screened for Lac repressor expression by Western blot (immunoblot) using rabbit antiserum to the Lac repressor (Stratagene). The monoclonal cell line LTRIG has an integrated LTRCAT which is highly inducible by Tat and has high Lac repressor expression.

LIGHIVDC was generated by transfecting LTRIG with pOPRHIV. Cells were allowed to double twice, passaged one to eight times, and then grown in medium containing 250 µg of hygromycin B and 400 µg of G418 (geneticin) per ml. After 3 weeks of selection, cloning rings were used to isolate monoclonal lines. The polyclonal line LIGCON was generated in a manner similar to that used for LIGHIVDC except that pOPRSVI was transfected into LTRIG.

Transient transfection. Cell line LTRIG was screened for LTRCAT inducibility by transient transfection with pD5tat. Transfection was performed with Transfectam (Promega) according to the manufacturer's instructions, with a 2:1 ratio of Transfectam to DNA. Plasmid DNA (10 µg) was used, and Transfectam mix was left on 10⁶ cells for 4 h. The cells were harvested at 48 h, and CAT activity was determined.

Cell line LIGHIVDC was transfected with 3.3 or 1.65 µg of pHIV-1LUC per 3.3 × 10⁵ cells in the presence or absence of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Transfectam mix was left on for 4 h, cells were harvested at 48 h, and luciferase activity (per 20 µl of lysate) was determined with the

Luciferase Assay System (Promega) according to the manufacturer's instructions.

CAT assay. CAT assays were performed as previously described (42). Protein concentrations were determined with the Micro BCA kit (Pierce), with Triton X-100 added to a 0.1% final concentration. Results (percent acetylation per hour) were normalized to micrograms of total protein (percent acetylation per hour per microgram).

Southern blot. To isolate genomic DNA, cell monolayers were trypsinized, washed twice with phosphate-buffered saline (PBS), resuspended in lysis buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8], 25 mM EDTA, 0.5% sodium dodecyl sulfate, 0.1 mg of proteinase K per ml), and then incubated overnight at 37°C. Following phenol extraction, the sample was resuspended in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA), and treated with RNase-It Ribonuclease Cocktail (Stratagene) for 30 min at 37°C. The DNA was then digested to completion with restriction endonucleases and electrophoresed on a 0.8% agarose gel in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA). Transfer and hybridization were performed as described by Koval et al. (25) except that the hybridization buffer was 50% formamide, 5× Denhardt's solution, 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 0.5% sodium dodecyl sulfate, and 0.1 mg of salmon sperm DNA per ml. Double-stranded DNA probes were generated with the Prime-It II random priming kit (Stratagene) according to the manufacturer's protocol, using [α -³²P]dCTP (3,000 Ci/mmol; ICN).

Immunofluorescence. Tat-inducible LIGHIVDC cells and parental LTRIG cells were seeded onto poly-L-lysine-coated glass coverslips and allowed to settle overnight. Subsequently, cells were incubated in 2 mM IPTG for 72 h to induce Tat protein expression. The cells were washed twice in cold PBS and then simultaneously fixed and permeabilized in cold methanol at -20°C for 10 min. After two more washes with PBS, cells were incubated with polyclonal antiserum 705 directed against HIV-1 Tat (a gift from C. Rosen, Roche Institute of Molecular Biology) at a dilution of 1:500 in PBS-0.2% gelatin for 10 min at room temperature. After extensive washing in PBS, cells were incubated with fluorescein-conjugated goat anti-rabbit antiserum (Jackson Laboratory) at a dilution of 1:100 in PBS-0.2% gelatin for 10 min at room temperature. Cells were washed extensively with PBS, mounted in glycerol/paraphenylene diamine (an antiphotobleaching agent), and analyzed on a Zeiss fluorescence microscope.

RT-PCR. Cytoplasmic RNAs were isolated and DNase treated by the basic protocol as previously described (3). Nuclear RNAs were isolated from the nuclear pellet after one wash with lysis buffer and treated with DNase in the same manner as the cytoplasmic RNAs. RNasin was added to the lysis buffer at a final concentration of 1,000 U/ml (Ambion). The reverse transcription-PCR (RT-PCR) procedure is a modification of the method of Saksela et al. (41). Briefly, 2 µg of RNA in 20 µl of water was heated for 5 min at 80°C and then reverse transcribed for 1 h at 42°C in a total of 40 µl of 1× RT buffer (GibcoBRL) containing 0.4 mM deoxynucleoside triphosphates, 24 U of RNasin (Promega), 10 pM random hexamers (Boehringer Mannheim), 2.5 mM dithiothreitol (GibcoBRL), and 400 U of Moloney murine leukemia virus reverse transcriptase (GibcoBRL). Next, PCR was performed in 50 µl of mixture containing 2 µl of each reverse-transcribed sample, 1× PCR buffer II (Perkin-Elmer), 1.5 mM MgCl₂, 50 µM each unlabeled deoxynucleoside triphosphate (Perkin-Elmer), 100 ng of each primer, 0.25 µl of [α -³²P]dCTP (3,000 Ci/mmol; ICN), and 1.25 U of Taq polymerase (Perkin-Elmer). After overlay with mineral oil, PCR was run for 33 cycles with denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min. The amplified ³²P-labeled PCR products were analyzed subsequently by polyacrylamide gel electrophoresis and autoradiography. Quantitation was done with a Molecular Dynamics PhosphorImager.

The upstream sense primer RPM (5'-TCT CGA CGC AGG ACT CGG CTT-3') is located in exon 1 of HIV-1 and was used for detecting both *tat* and *ypu* RNAs. Tat2 (5'-ACG CCT ATT CTG CTA TGT CG-3') and VPU2 (5'-GGT CCA CAC AAC TAT TGC TA-3') are the downstream primers. VPU2 is located in exon 5E (37), and the RPM/VPU2 primer set produces a 219-bp product. The Tat2 primer is within exon 4 (37), and the RPM/Tat2 primer set produces several amplicons corresponding to the different spliced forms of *tat* RNA. The 92-bp amplicon representing the splice between exons 1 and 4 was used for evaluation of *tat* RNA.

Infection procedures. The AD169 and Towne strains of HCMV were prepared as previously described (24). Cell lines were infected at a multiplicity of infection (MOI) of 2 or 3 in the presence or absence of 2 mM IPTG, and the medium, containing fresh IPTG, was changed at 16 h. Protein or RNA was harvested at 48 or 72 h postinfection (p.i.). Some experiments contained 0.4 mM ganciclovir throughout the infection. Ganciclovir was a gift from Roche Bioscience (Palo Alto, Calif.). Slot blot analysis was performed on viral DNA and showed that DNA replication was inhibited by the addition of ganciclovir (data not shown). UV inactivation was performed by exposing virus for 1 h to the UV light in a BioGuard cell culture hood (at a distance of 60 cm) with shaking every 15 min; under these conditions, no infectious virus remained.

RNase protection. Cellular RNA was isolated by the RNagents Total RNA Isolation System (Promega). All [α -³²P]UTP (3,000 Ci/mmol; ICN)-labeled riboprobes were prepared using the MAXIScript in vitro transcription kit (Ambion) according to the manufacturer's protocol. The riboprobe for CAT RNA was generated from p456GemCAT following cleavage with *Ava*I (48), using 5 µl of [α -³²P]UTP supplemented with unlabeled UTP for a final UTP concentration

of 3.3 µM. The riboprobe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was generated from pTRIGAPDH (Ambion) following cleavage with *Dde*I, using 1 µl of [α -³²P]UTP with a final UTP concentration of 120 µM. The riboprobe for HIV-1 unspliced *gag* RNA was generated from pHIVSDribo after cleavage with *Not*I, using 8.5 µl of [α -³²P]UTP with a final UTP concentration of 5.2 µM. RNase protection assays were performed with the RPA II kit (Ambion) according to the manufacturer's standard procedure. Briefly, hybridization was performed overnight at 42°C, and each RNA sample was hybridized to all three riboprobes (10⁵ cpm each) in the same tube. Digestion was performed with 20 U of RNase T₁ in a final volume of 220 µl for 30 min at 37°C.

RESULTS

Generation of cell lines with inducible HIV-1 gene expression. To examine the molecular basis of the effects of HCMV on HIV-1 LTR-mediated transcription in the presence of different levels of HIV-1 gene products, it was first necessary to generate cell lines in which HIV-1 gene expression was uncoupled from LTR-directed transcription. This was accomplished with the use of the Stratagene Lac Switch system, in which the synthesis of HIV-1 gene products is directed by a Rous sarcoma virus (RSV) promoter with *lac* operator sequences. Thus, in the presence of the Lac repressor, transcription is inhibited, and this inhibition can be relieved by the addition of IPTG.

The strategy for constructing the cell lines was as follows (see Fig. 1 and 2). U373-MG astrocytoma/glioblastoma cells were cotransfected with the LTRCAT construct (Fig. 1A) and the plasmid p3'SS, which contains the *lacI* gene and the gene for hygromycin resistance (Fig. 1B). Fourteen hygromycin-resistant monoclonal cell lines were isolated and assayed by Western blot analysis with an antibody specific for the Lac repressor protein. Subsequently, cell lines which highly expressed the Lac repressor were tested for activation of the integrated LTRCAT construct following transient transfection of a Tat expression vector. From this analysis, we were able to select a cell line, designated LTRIG, which had a high level of Lac repressor and a low background level of CAT activity (0.007%/µg/h) which could be induced by Tat 107-fold. This cell line thus serves as a model for basal HIV-1 LTR-mediated transcription in the absence of HIV-1 proteins.

Then, the monoclonal cell line LTRIG was additionally transfected with pOPRHIV to generate the cell line LIGHIVDC. pOPRHIV was constructed using the plasmid vector pOPRSVI, which contains an RSV promoter with *lac* operator sequences upstream of a *Not*I site as well as the neomycin resistance gene (Fig. 1C). A 9-kb *Sst*I fragment from the HIV-1 HXB2D proviral genome was placed into the *Not*I site. Since this fragment lacks the 5' LTR but contains all of the HIV-1 coding sequences, transcription of the HIV-1 genes is mediated by the inducible RSV promoter. The vector pOPRHIV was linearized and transfected into LTRIG. Five monoclonal cell lines resistant to G418 were selected and screened for IPTG induction of Tat from pOPRHIV as measured by an increase in LTR-mediated CAT activity. The monoclonal cell line designated LIGHIVDC showed a 20-fold induction of CAT activity when the cells were maintained in the presence of IPTG for 72 h (Table 1) and was used for subsequent studies. Note, however, that the noninduced level of CAT activity in LIGHIVDC was still 65-fold higher than that in the parental cell line LTRIG, indicating that the modified RSV promoter is leaky, even in the presence of the Lac repressor.

To verify that the enhanced CAT activity in the IPTG-treated LIGHIVDC cells was due to activation of the LTR promoter in response to increased levels of Tat, we transfected into these cells a vector (HIV-1LUC) in which the gene for luciferase was under the control of the HIV-1 LTR. Figure 3 shows that in transient expression assays, there was a signifi-

FIG. 1. Plasmids used to construct the model cell lines. (A) pLTRCAT contains the CAT reporter gene expressed by the HIV-1 LTR. (B) p3'SS constitutively expresses the *lacI* gene and the hygromycin resistance gene. (C) The *SstI* fragment of HIV-1 strain HXB2 is expressed by the modified RSV promoter in pOPRHIV. Op represents the *lac* operator sequence, and the HIV-1 sequence numbers shown are relative to the start site of transcription.

cant induction of luciferase activity when the cells were maintained in IPTG, thus indicating that the increased expression of both CAT and luciferase was likely due to the effects of Tat on the LTR. As another control, it was assessed whether the enhanced levels of CAT activity might be due to the backbone plasmid pOPRSVI. For this experiment, pOPRSVI was transfected into LTRIG, and a G418-resistant polyclonal cell line

TABLE 1. IPTG induction of CAT activity in cell lines^a

Cell line	CAT activity (%/μg/h)		Induction (fold)
	-IPTG	+IPTG	
LTRIG	0.0060	0.0067	1.1
LIGCON	0.012	0.016	1.3
LIGHIVDC	0.39	7.8	20

^a Cells were incubated with or without 2 mM IPTG for 72 h.

FIG. 2. Selection scheme for model cell lines.

(designated LIGCON) was generated (Fig. 2). As expected, CAT activity in this cell line was minimal, was not induced by IPTG, and was comparable to that in the parental cell line LTRIG (Table 1).

Analysis of the genomic structures of the integrated LTRCAT and pOPRHIV vectors in cell line LIGHIVDC. To characterize further the monoclonal cell line LIGHIVDC, the integration site of the LTRCAT plasmid was examined by Southern analysis. Figure 4A shows a Southern blot of the LIGHIVDC genomic DNA cleaved with either *KpnI* and *NotI* (lane K) or

FIG. 3. Activity of a transfected LTR in LIGHIVDC. The LTR luciferase reporter construct HIV-1LUC was transiently transfected into LIGHIVDC with or without 2 mM IPTG for 48 h. Luciferase activity is presented as fold induction relative to activity in the absence of IPTG. Data are the averages of two experiments.

FIG. 4. Analysis of pLTRCAT integration in LIGHIVDC. (A) Genomic DNA from LIGHIVDC was analyzed by Southern blotting, with the *ScaI* fragment from LTRCAT as the probe. Lane K is a *KpnI* and *NotI* double digest, and lane K/E is a *KpnI*, *EcoRI*, and *NotI* triple digest. *NotI* cuts only pOPRHIV and was used to reduce the sizes of LTR sequences from pOPRHIV that might cross-hybridize with the probe. The positions and sizes (in kilobases) of the molecular weight standards are shown on the left. (B) Map of the integrated pLTRCAT, with the locations of restriction sites labeled. LTRCAT nucleotide sequence numbers are indicated, and the wavy lines represent cellular DNA. Also shown is the location of the *ScaI* probe, which contains sequence from -139 of the HIV-1 LTR to +716 of the CAT gene.

with *KpnI*, *NotI*, and *EcoRI* (lane K/E) and probed with the *ScaI* fragment from LTRCAT. A restriction map of the LTRCAT vector in its integrated form is shown in Fig. 4B along with the location of the *ScaI* probe. *EcoRI* digests the LTRCAT plasmid at two sites (one within the CAT gene and the other in plasmid sequences upstream of the LTR). *KpnI* digests at one site within the LTR, and *NotI* has no sites. To exclude the possibility that the observed hybridization might be due to the 3' HIV-1 LTR sequences present in the integrated copy of pOPRHIV, *NotI* was first used to cleave the DNA. *KpnI* and *NotI* together cleave the pOPRHIV 3' LTR into a 563-bp fragment which overlaps 174 bp of the *ScaI* probe; the 563-bp fragment was not detected, as can be seen in Fig. 4A. In the *KpnI/NotI* digest (lane K) of the integrated LTRCAT construct, the *ScaI* probe should detect a fragment from the internal *KpnI* site to the first *KpnI* or *NotI* site downstream in the cellular DNA. The presence of a single 2.7-kb band in lane K shows that the monoclonal cell line contains only one copy of the integrated LTRCAT. In the *KpnI/NotI/EcoRI* digest (lane K/E), the *ScaI* probe should detect a 1-kb internal *KpnI*-to-*EcoRI* band and another band from the *EcoRI* site to the next enzyme site in the downstream cellular DNA. Lane K/E has a doublet between 1 and 1.4 kb, indicating that the plasmid is intact.

Southern analysis was also used to examine the integrated form of pOPRHIV in LIGHIVDC. Figure 5A shows a Southern blot using the 1.3-kb *NdeI* fragment (depicted in Fig. 5B) for a probe. Total cellular DNA was digested with *NdeI*, which cleaves the plasmid DNA at two sites, or with *MscI*, which cleaves at three sites (Fig. 5B). As expected, there is a single

band of 1.3 kb in the *NdeI* digest (lane N) which corresponds to the internal *NdeI* fragment. When the *NdeI* probe is used, the only *MscI* fragment which should be detected contains cellular sequence at the 3' end. The presence of a single band of approximately 7.5 kb in the *MscI* digest (lane M) indicates that there is a single site of integration and that the cellular DNA contains an *MscI* site close to the point of integration.

Expression of HIV-1 gene products in LIGHIVDC. To analyze LIGHIVDC further, the expression patterns of HIV-1 proteins and RNAs were examined in this cell line. The level of the Gag proteins was below the limits of detection of Western blot and p24 enzyme-linked immunosorbent assays (data not shown). However, the Tat protein could be detected by immunofluorescence and was found primarily in the nuclei of the IPTG-induced LIGHIVDC cells (Fig. 6A). Consistent with the observed leakiness of the inducible RSV promoter (Table 1), a similar pattern of staining was also observed in the absence of IPTG induction (data not shown). Only a background level of faint cytoplasmic staining was visible in LTRIG, which does not express Tat (Fig. 6B).

To examine *tat* RNA expression and Rev function in LIGHIVDC, nuclear and cytoplasmic RNAs were isolated from LIGHIVDC after incubation in the presence or absence of IPTG, and RT-PCR was used to determine the distribution of singly spliced *ypu* RNA and multiply spliced *tat* RNA. Figure 7 shows that both RNAs were induced by IPTG, but only the *tat* RNA is present in the cytoplasm at significant levels, while the *ypu* RNA is present predominantly in the nucleus. This type of distribution is characteristic of nonfunctional Rev, since Rev directs the expression of late proteins by facilitating transport of the unspliced and singly spliced forms of HIV-1 RNA to the cytoplasm (15, 27). A lack of Rev function is also consistent with the fact that Gag proteins, which are expressed from unspliced RNA, were below the limits of detection. Thus,

FIG. 5. Analysis of pOPRHIV integration in LIGHIVDC. (A) Genomic DNA from LIGHIVDC was analyzed by Southern blotting, with the *NdeI* fragment from pOPRHIV as a probe. Lane M is an *MscI* digest, and lane N is an *NdeI* digest. Numbers indicate the locations and sizes (in kilobases) of the molecular weight standards. (B) Map of the integrated pOPRHIV, with the restriction sites indicated along with the pOPRHIV nucleotide sequence numbers. The *NdeI* probe is also indicated, and the wavy lines are cellular DNA.

FIG. 6. Analysis of Tat expression in LIGHIVDC. Cells were incubated in 2 mM IPTG for 72 h, fixed, and then stained with polyclonal antiserum 705 directed against HIV-1 Tat. (A) Nuclear localization of the Tat protein expressed in the cell line LIGHIVDC. (B) Parental cell line LTRIG, which does not express Tat. Magnification is 2,000-fold in both panels.

it is likely that only proteins encoded by multiply spliced Rev-independent RNA species are expressed at detectable levels in the LIGHIVDC cells. The cause of the Rev defect is unclear, but the defect may be due to low Rev expression.

HCMV gene expression, but not DNA replication, is required for stimulation of HIV-1 LTR-mediated transcription. Previously, we generated one polyclonal and two monoclonal cell lines that each contained a single integrated copy of an HIV-1 LTRCAT construct and showed that HCMV activated the integrated promoter 2.8- to 54-fold (25). To extend these results, we assessed the effect of HCMV infection on LTR-mediated CAT expression in the cell line LTRIG, which contains an integrated copy of LTRCAT as well as the Lac repressor. The cells were infected with the Towne strain of HCMV, and CAT activity was determined at 72 h p.i. In accord with the previous results, HCMV activated CAT expression approximately 20-fold over background levels (Fig. 8). Thus, the ability of HCMV to activate the HIV-1 LTR in the absence of any HIV-1 gene products is not affected by the presence of the Lac repressor.

To determine whether HCMV gene expression was required for stimulation, LTRIG was infected with UV-inactivated

A. TAT

B. VPU

FIG. 7. Expression of pOPRHIV RNA in LIGHIVDC. RT-PCR was performed on nuclear (N) and cytoplasmic (C) RNAs isolated from LIGHIVDC incubated for 72 h in the presence or absence of 2 mM IPTG. The products were separated by polyacrylamide gel electrophoresis and subjected to autoradiography. The numbers 0, -1, -2, -3, and -4 indicate 10-fold dilutions of cDNA prior to PCR. CEM represents the RT-PCR in which template RNA from unfractionated HIV-infected CEM cells was used as a positive control. (A) The TAT amplicon represents the *tat* multiply spliced RNA. (B) The VPU amplicon represents the *vpu* singly spliced RNA. Note that relative levels can be compared only for reactions that used the same primer set.

HCMV, and CAT activity was measured at 72 h p.i. As can be seen in Fig. 8, CAT activity remained at basal levels when the cells were incubated with UV-inactivated HCMV, thus indicating that HCMV gene expression was necessary to activate the HIV-1 promoter. To assess whether HCMV viral DNA replication was required for the stimulation, we used the drug ganciclovir, a specific inhibitor of the HCMV-encoded DNA polymerase. As shown in Fig. 8, the fold activation of HIV-1 LTR-directed CAT expression was the same in the presence and absence of ganciclovir, indicating that neither HCMV DNA replication nor late-gene expression is required for stimulation to occur. Similar results were obtained using the AD169 strain of HCMV and the LIGCON control cell line, which is derived from LTRIG and contains the backbone plasmid pOPRSVI lacking HIV-1 sequences (data not shown).

FIG. 8. Effect of HCMV infection on LTR activity in the absence of HIV-1 proteins. The cell line LTRIG was infected with HCMV (MOI = 3) under various conditions. Protein was harvested at 72 h p.i., and CAT assays were performed. CAT activity relative to that in mock infection is shown. Mock, uninfected cells; GAN, cells incubated in the presence of 400 μ M ganciclovir; HCMV, infection with the Towne strain; HCMV UV, infection with UV-inactivated Towne strain; HCMV GAN, infection with Towne strain in the presence of ganciclovir.

FIG. 9. Effects of HCMV infection on RNA levels in LIGHIVDC. LIGHIVDC cells were incubated in the presence or absence of 2 mM IPTG and infected with the Towne or AD169 strain of HCMV (MOI = 2). Total RNA was harvested at 72 h p.i. and analyzed by RNase protection assay. The GAG riboprobe is complementary to the HIV-1 *gag* gene and represents full-length HIV-1 RNA; the protected fragment is 601 nt. The CAT riboprobe is complementary to the CAT gene and yields a protected fragment of 273 nt. The GAPDH riboprobe is complementary to the cellular GAPDH gene; the protected fragment is 148 nt. All three riboprobes were hybridized to 8 to 30 μ g of RNA, digested with RNase T₁, separated on a 5% acrylamide-8 M urea gel, and quantitated by PhosphorImager analysis. The data were normalized to GAPDH.

The effect of HCMV on HIV-1 LTR-mediated transcription is related to the level of HIV-1 gene expression. The effects of HCMV infection on LTR-directed transcription in the presence of different levels of Tat were analyzed following infection of the LIGHIVDC cell line in the presence or absence of IPTG. At 72 h p.i., total RNA was harvested and analyzed by an RNase protection assay using three riboprobes in each sample. A CAT riboprobe was used to measure HIV-1 LTR-directed transcription, while a GAG riboprobe was used to determine the amount of HIV-1 RNA. To ensure that similar amounts of cellular RNA were present in each sample, a riboprobe to GAPDH RNA was also included. The protected RNA fragments were subjected to gel electrophoresis and quantified by PhosphorImager analysis. Figure 9 shows that in uninfected cells, IPTG induced the expression of HIV-1 RNA, resulting in a corresponding 10-fold increase in CAT RNA levels that was due to induction of Tat (compare lanes 2 and 3). This increase in CAT RNA was consistent with the CAT enzyme activities shown in Table 1. In the absence of IPTG, HIV-1 RNA levels remained low, and HCMV had no effect on LTR-directed transcription in the LIGHIVDC cells (compare lanes 1 and 2 in Fig. 9). Furthermore, HCMV does not affect CAT enzyme levels in the absence of IPTG (data not shown). In contrast, when *tat* gene expression was induced by IPTG, infection with either strain of HCMV had an inhibitory effect on the LTR promoter, leading to a threefold reduction in CAT RNA levels (compare lane 3 with lanes 4 and 5 in Fig. 9). As expected, the level of HIV-1 RNA directed by the modified RSV promoter was not affected by the HCMV infection. These

results suggest that HCMV inhibits the LTR promoter but only when Tat is at a threshold amount greater than that present in the uninduced cells.

DISCUSSION

The importance of HIV-1 infection and the prevalence of HCMV infections in AIDS patients have led to many studies of the interactions between these two viruses, but the present picture is complicated and incomplete (see reference 44 for a review). At the cellular level, it appears that HCMV can both stimulate and inhibit HIV-1 replication, with the outcome depending on several factors, including the permissiveness of the cell to each virus, the order of infection, and the MOI. Here we have examined HIV-1 and HCMV interactions in the brain-derived cell line U373-MG, which is fully permissive to HCMV and Amphi-1B pseudotyped HIV-1 (which bypasses CD4-dependent viral entry [45]). This model is representative of a single cell infected by both viruses and thus differs from the model used in other studies, in which the MOI is sufficiently low that all cells are not infected and an infected cell likely contains only a single virus (44).

Previous work suggested that HIV-1 LTR transcription can be either positively or negatively regulated by HCMV in U373-MG cells and that the net effect is dependent upon a threshold of HIV-1 protein expression (25). The model presented here was designed to observe the effect of HCMV on LTR-directed transcription in the presence of three different levels of HIV-1 proteins. The cell line LTRIG expresses no HIV-1 proteins, while in LIGHIVDC, the HIV-1 protein concentrations are controlled by the inducible RSV promoter. Our results document that the effect of the HCMV infection on LTR expression varies with the level of HIV-1 gene products (Fig. 10 gives a schematic representation). HCMV stimulates HIV-1 LTR expression in the absence of HIV-1 proteins, and no effect is observed at intermediate levels. However, when HIV-1 gene expression in LIGHIVDC is elevated because of IPTG induction, HCMV inhibits LTR transcription, indicating that inhibition requires a threshold of HIV-1 protein expression. Since the HIV-1 Tat protein activates the LTR, there is a

FIG. 10. Summary of HCMV effects on LTRCAT activity. The net effect of HCMV infection and the relative LTRCAT expression levels in the model cell lines are shown relative to LTRIG mock. Note that the scale is split.

direct correlation between the level of HIV-1 gene products expressed by the heterologous promoter and LTR activity. Thus, the protein threshold could also be considered a threshold of LTR activity, which determines the switch from activation to inhibition. This is consistent with previous work which showed that HCMV stimulates a restricted HIV-1 infection but inhibits a productive infection (22).

The cell line LIGHIVDC appears to have very low Rev activity, possibly due to low *rev* transcription and insufficient Rev levels for function. Hence, the Rev-dependent late HIV-1 proteins, coded for by unspliced and singly spliced RNAs, are not expressed in detectable amounts in these cells, and HIV-1 gene expression is probably limited to the early HIV-1 proteins Tat, Rev, and Nef. It is unlikely that this pattern of expression is due to cell-determined suppression of Rev-RRE function, as reported for the TH4-7-5 astrocytoma cell line (34), since we have previously shown that the U373-MG cells infected with HIV-1 (amphotropic retrovirus) pseudotypes produce high levels of HIV-1 proteins and virus (22, 24, 45). A more likely explanation is that the integrated RSV promoter driving expression of the HIV-1 genes is not highly active in these cells, possibly because of the chromatin context.

In view of the lack of detectable late HIV-1 gene products in the LIGHIVDC cells, we propose that Tat, Rev, and Nef are more likely to be responsible for the threshold effect. Nef, however, is probably not essential, since we previously showed that a HIV-1 proviral mutant lacking the *nef* gene is still inhibited by HCMV (25). Moreover, the lack of a known transcriptional role for Rev argues against it playing a major role. The most likely candidate for the threshold protein is the transcriptional activator Tat. However, the question of how HCMV infection might interfere with Tat-mediated activation of the LTR remains to be answered. It is possible that the Tat-binding site on the promoter is blocked by an HCMV gene product or an induced cellular factor. Alternatively, the infection-associated factor might impair Tat function by binding directly either to Tat or to some other protein necessary for Tat activity.

In our model system, Tat expression is independent of HIV-1 LTR transcription, which differs from an *in vivo* dual infection, in which Tat controls its own expression by activating the LTR. In the latter case, a small inhibition of Tat-mediated LTR activation will lead to less Tat expression, which results in even lower LTR activity and further reduction of Tat expression. Through such a feedback loop, a relatively small Tat inhibition can cause a large decrease in HIV-1 transcription and eventually replication. Moreover, in a dual infection, effects on transcription may be only one of several pathways of modulation.

The current picture of HCMV-mediated inhibition of HIV-1 transcription in the U373-MG cell line is as follows. Previous work has shown that some immediate-early or early HCMV gene product(s) is able to inhibit HIV-1 replication in a dually infected cell (22, 25). This paper demonstrates that HCMV represses LTR-directed transcription but only when HIV-1 protein levels and LTR activity reach a certain threshold. In the model presented here, HCMV is presumably inhibiting high-level Tat activation of the LTR.

Other studies, in addition to our own, have shown that HCMV can upregulate HIV-1 replication and that stimulation probably occurs through several mechanisms involving both intracellular and extracellular factors (12, 22, 26, 47) (see reference 44 for earlier citations). Furthermore, in cells dually infected by both viruses, stimulation could be mediated by a direct interaction between HCMV and HIV-1 viral components or indirectly through some cellular intermediate. Extracellular mechanisms may include release of soluble factors

from HCMV-infected cells which increase HIV-1 replication in neighboring cells. We have previously shown that HCMV infection activates an integrated LTRCAT in a U373-MG cell line that is not expressing HIV-1 proteins, and in this report we demonstrate that HCMV gene expression is required for this effect. However, it is conceivable that gene expression may not be necessary for other pathways of stimulation. For example, activation of HIV-1 has been shown to occur when macrophages are exposed to either infectious or heat-treated HCMV (26). Thus, the mere interaction of the cells with HCMV particles may stimulate HIV-1 synthesis in macrophages.

In our experiments, HCMV-mediated stimulation of the integrated LTRCAT probably occurs through an intracellular mechanism, since we used a high MOI to ensure infection of every cell. In addition, the finding that HCMV gene expression, but not viral DNA replication, is required for the activation suggests that HCMV immediate-early- or early-gene products must be involved. These results are also consistent with the observation that immediate-early proteins can stimulate an integrated LTRCAT in transfection experiments (23). We further show that upregulation of the LTR by HCMV can be detected only when the LTR is relatively inactive, as evidenced by the fact that stimulation is observed in LTRIG but not in LIGHIVDC, which has higher levels of LTR-directed transcription. Taken together, the results of the *in vitro* experiments showing that HCMV can stimulate basal transcription of the HIV-1 LTR provide support for the hypothesis that an HCMV infection may contribute to the activation of a latent HIV-1 genome *in vivo*. Nevertheless, there is always the caveat that the effects observed in a simplified system may not accurately reflect the *in vivo* situation.

In summary, the studies presented here provide further insights into the molecular interactions that occur between HIV-1 and HCMV in dually infected cells. We have shown that HCMV can modulate HIV-1 transcription and that the net effect, whether positive or negative, depends on the activation state of the HIV-1 LTR, which is determined in turn by the level of the HIV-1 Tat protein. HCMV can stimulate basal transcription, but this stimulation is masked if the LTR is already activated by Tat. Once transcription rises to a certain threshold, HCMV causes a net inhibition. It is clear that multiple interactions are occurring in a dual infection and that the outcome of these interactions is likely cell type specific. The challenges are to decipher the molecular and biochemical bases of these effects and to use this knowledge to develop strategies for modulating the pathogenesis of both viruses.

ACKNOWLEDGMENTS

We thank R. Schwartz, C. Morello, M. Sommer, L. Cranmer, V. Koval, F. Jault, S. Rodems, and C. Clark for many helpful discussions throughout this work and V. Jayaran for assistance in screening cell lines. We also thank J. Lathey for critical reading of the manuscript.

This research was supported by Public Health Service grant AI28270 from the National Institute of Allergy and Infectious Diseases. T. N. Moreno is a Howard Hughes Medical Institute Predoctoral Fellow.

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