Inhibition of Human Immunodeficiency Virus Replication by the Herpes Simplex Virus Virion Host Shutoff Protein

TAREK HAMOUDA,¹ RODERICK MCPHEE,¹ SHAO-CHUNG HSIA,¹ G. SULLIVAN READ,²
THOMAS C. HOLLAND,¹ AND STEVEN R. KING^{1*}

Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan 48201,¹ and Division of Cell Biology and Biophysics, University of Missouri—Kansas City, Kansas City, Missouri 64110-2499²

Received 24 January 1997/Accepted 4 April 1997

The herpes simplex virus (HSV) virion host shutoff gene (*vhs*) encodes a protein which nonspecifically accelerates the degradation of mRNA molecules, leading to inhibition of protein synthesis. This ability to inhibit a critical cellular function suggested that *vhs* could be used as a suicide gene in certain gene therapy applications. To investigate whether *vhs* might be useful for treatment of AIDS, we tested the ability of both HSV type 1 (HSV-1) and HSV-2 *vhs* to inhibit replication of human immunodeficiency virus (HIV). Replication of HIV was substantially inhibited when an infectious HIV proviral clone was cotransfected into HeLa cells together with *vhs* under the control of the cytomegalovirus (CMV) immediate-early promoter. HSV-2 *vhs* was more active than HSV-1 *vhs* in these experiments, consistent with previously published studies on these genes. Since expression of *vhs* from the CMV promoter is essentially unregulated, we also tested the ability of *vhs* expressed from the HIV long terminal repeat (LTR) promoter to inhibit HIV replication. Wild-type HSV-1 *vhs* inhibited HIV replication more than 44,000-fold in comparison to a mutant *vhs* gene encoding a nonfunctional form of the Vhs protein. Production of Vhs in transfected cells was verified by Western blot assays. A larger amount of Vhs was observed in cells transfected with plasmids expressing *vhs* from the HIV LTR than from the CMV promoter, consistent with the greater inhibition of HIV replication observed with these constructs. Mutant forms of Vhs were expressed at higher levels than wild-type Vhs, most likely due to the ability of wild-type Vhs to degrade its own mRNA. The strong inhibitory activity of the *vhs* gene and its unique biological properties make *vhs* an interesting candidate for use as a suicide gene for HIV gene therapy.

Human immunodeficiency virus (HIV) is the causative agent of AIDS (2, 14, 30). Infection with HIV results in a slow, progressive degeneration of the immune system largely due to depletion of CD4-positive T cells (12). Current drug therapies inhibit the growth of wild-type virus, but drug-resistant mutants present in low amounts in the virus pool rapidly grow and become the dominant virus species (8). Recently, combination drug therapies including protease inhibitors have produced promising results; however, these drugs carry many side effects, and their long-term effect is not known yet. Therefore, it is important to pursue alternative types of treatment such as gene therapy.

Proposed genetic therapies for HIV type 1 (HIV-1) infection fall into two general categories. The first includes methods that confer some type of resistance to HIV-1 infection to the genetically altered cells, e.g., antisense RNA (6, 22, 32, 41), ribozymes (29, 32, 51, 53, 55), RNA decoys (31), and trans-dominant negative mutant HIV-1 genes (3, 33, 34, 36, 52). Resistance genes are intended to interrupt the HIV-1 replication cycle, but they do not eliminate virus from the infected cells. Once an HIV-1 provirus becomes integrated, none of the resistance genes currently under investigation can eliminate it.

The second category is the use of suicide genes that are turned on by HIV-1 infection. The rationale for the use of suicide genes is to sacrifice the infected cell in order to abort the viral replication cycle and thus prevent production of progeny virus. The strategy of killing infected cells is similar to

that used by the cell-mediated immune system. Suicide genes must be highly regulated to prevent inappropriate expression yet sufficiently inducible to be effective. This effective control over suicide gene expression must persist for a long period of time. There must not be any deleterious effects on cell functions or differentiation. The herpes simplex virus type 1 (HSV-1) thymidine kinase gene (4) and the diphtheria toxin A (DTA) gene (11) have been used under HIV long terminal repeat (LTR) control as suicide genes to kill HIV-infected cells. The use of viral host shutoff genes to inhibit HIV has been discussed (49). We have investigated the potential of the HSV virion host shutoff gene (*vhs*) as a suicide gene for the inhibition of HIV replication.

During lytic HSV infections, Vhs polypeptides enter cells as components of the infecting virions, facilitating the shutoff of host protein synthesis by accelerating the degradation of host cell mRNAs (45, 47). In addition, following the onset of viral transcription, the Vhs protein ensures rapid turnover of viral mRNAs, thereby facilitating the sequential transition between expression of different classes of viral genes (37, 38, 40, 47). Thus, although the *vhs* function is specific for mRNAs, it does not distinguish between different mRNAs in targeting them for degradation. The virion host shutoff function is mediated by a 58-kDa protein encoded by the viral UL41 open reading frame (28). Recent studies have shown that transfection of cells with the *vhs* gene from either HSV-1 or HSV-2 dramatically reduces the expression of a cotransfected reporter gene, demonstrating that *vhs* is the only viral gene required for virion host shutoff (21, 39). This finding, together with the ability of *vhs* to induce degradation of most if not all mRNAs, raised the possibility that appropriately regulated expression of the *vhs* gene could be used to inhibit HIV replication.

* Corresponding author. Mailing address: Wayne State University, Department of Immunology and Microbiology, 540 E. Canfield Ave., Detroit, MI 48201. Phone: (313) 577-6494. Fax: (313) 577-1155. E-mail: sking@cms.cc.wayne.edu.

We have investigated the ability of *vhs* to inhibit HIV replication. Our initial studies showed that *vhs*, when driven by the constitutive cytomegalovirus (CMV) promoter, caused substantial inhibition of HIV replication. When placed under the control of the HIV LTR, *vhs* inhibited HIV replication to an even greater extent. These studies indicate that it may be possible to develop a *vhs*-based gene therapy protocol for HIV.

MATERIALS AND METHODS

Cells. HeLa-CD4⁺-LTR- β -gal cells (25) are CD4⁺ and contain one integrated copy of HIV-1 LTR linked to a β -galactosidase gene. HLtat cells (13, 46) contain the HIV-1 LTR promoter and produce a high level of a synthetic one-exon *tat* gene. HL2/3 cells (7) produce high levels of Gag, Env, Tat, Rev, and Nef proteins. They do not produce detectable viral reverse transcriptase or detectable amounts of mature virions. All cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: HeLa cells from Richard Axel, HeLa-CD4⁺-LTR- β -gal cells from Michael Emerman, and HLtat and HL2/3 cell lines from Barbara K. Felber and George N. Pavlakis.

HeLa, HeLa-CD4⁺-LTR- β -gal, HLtat, and HL2/3 cells were grown in Dulbecco's modified Eagle's medium with 4.5 g of glucose (Mediatech, Washington, D.C.) per liter. The medium was supplemented with 10% fetal bovine serum (Sigma Chemical Company, St. Louis, Mo.) and gentamicin (50 mg/liter; GIBCO/BRL, Life Technologies, Gaithersburg, Md.). The cells were grown at 37°C in 5% CO₂.

Plasmids. Plasmid pNL4.3, containing a full-length, replication-competent HIV proviral DNA in pUC18, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from Malcolm Martin (1). Plasmids containing wild-type and mutant HSV-1 *vhs* or wild-type HSV-2 *vhs* genes in the expression vector pCDNA1 have been described previously (39). Each of these plasmids expresses *vhs* from the CMV promoter. pKOScmv contains the wild-type *vhs* gene from HSV-1 strain KOS. p333cmv contains the wild-type *vhs* gene from HSV-2 strain 333. pI₂₃₇cmv contains an insertional mutant of the HSV-1 strain KOS *vhs* gene, with a 6-bp *Hind*III linker insertion at codon 237 (39). pCH110 (Pharmacia LKB Biotechnology, Piscataway, N.J.) contains a *lacZ* which is expressed from the simian virus 40 (SV40) early promoter (15). All the enzymes were obtained from Life Technologies (Gaithersburg, Md.).

Two plasmids containing *lacZ* as a reporter gene under the control of the HIV LTR promoter were constructed from the parent vector pLXSN, which was provided by Dusty Miller (The Fred Hutchinson Cancer Research Center, Seattle, Wash.) (35). pHZP contains the *lacZ* gene under the control of the HIV LTR followed by a synthetic poly(A) site. pHZRP is similar to pHZP but has a Rev-responsive element sequence between the *lacZ* gene and the poly(A) site. The HIV-1 LTR was amplified by PCR using pNL4.3 as a template with the forward primer 5'-CAC AAG GCT ACT TCC CTG ATT GG3' and the backward primer 5'-GGA TCC GCG GCC GCT CAC CAG TCG CCG CCC CT3', containing *Bam*HI and *Not*I sites. *lacZ* was amplified from pCH110 (Pharmacia LKB Biotechnology) by PCR using the forward primer 5'-GCG GCC GCG GGA CAC TTC ACA TGA GCG AAA AA3', which contains a *Not*I site, and the backward primer 5'-CTC GAG CAT GGC CTG CCC GGT TAT TA3', which contains an *Xho*I site.

The structures of the *vhs* transcriptional units are shown in Fig. 1. Two sets of plasmids containing these units were prepared, one using pcDNA1 as a vector and the other using pcDNA3 (Invitrogen, Carlsbad, Calif.) as a vector. The p3 series of *vhs* expression plasmids is based on pcDNA3. The others are based on pcDNA1. p3KOScmv and p3I₂₃₇cmv were prepared by excision of wild-type or mutant *vhs* from pKOScmv or pI₂₃₇cmv by using *Eco*RI and *Xba*I, followed by ligation of the excised *vhs* fragment into pcDNA3 digested with *Eco*RI and *Xba*I.

Plasmids which have no promoter driving *vhs* were prepared by excision of the CMV promoter. pKOS was prepared from pKOScmv by digestion with *Vsp*I, followed by religation. p3KOS was prepared by digestion of p3KOScmv with *Mlu*I and *Hind*III, followed by removal of single-stranded ends by using T4 polymerase and self-ligation with T4 ligase.

Plasmids which contain *vhs* under the control of the HIV LTR were prepared by digestion of pKOS or p3KOS with *Eco*RI, followed by treatment with calf intestinal alkaline phosphatase. An *Eco*RI fragment containing the HIV LTR was excised from pHZRP, gel purified, and then inserted into *Eco*RI-digested pKOS and p3KOS to create pKOSLTR and p3KOSLTR. The correct orientation of the LTR was confirmed by digestion with *Eco*RV and *Bgl*II, respectively.

Plasmids containing a nonfunctional *vhs* gene (I₂₃₇) were prepared by replacing the wild-type *vhs* in pKOSLTR and p3KOSLTR with the mutant *vhs* (I₂₃₇) excised from pI₂₃₇cmv by *Bst*EII digestion. This resulted in the production of pI₂₃₇LTR and p3I₂₃₇LTR.

pcDNA1-based plasmids were grown in MC1061 cells (Invitrogen), while pcDNA3-based plasmids were grown in DH5 α cells (Life Technologies). All of the plasmids were extracted by using the Wizard Plus Maxipreps DNA purification system (Promega, Madison, Wis.) as instructed by the manufacturer. Then the plasmids were purified by using a single cesium chloride ethidium bromide

FIG. 1. Construction of the pcDNA3-based effector plasmids. The name of each plasmid is indicated on the right. The marked enzyme sites indicate the site of cut for further cloning. The dark boxes indicate the newly cloned genes. Pcmv, CMV immediate-early promoter; PA, polyadenylation sequence; KOS *vhs*, the wild-type *vhs* gene of HSV-1 strain KOS; I₂₃₇, an insertional mutant of *vhs* which produces a nonfunctional protein.

gradient (20) for 18 h at 50,000 rpm. Ethidium bromide was removed with CsCl-saturated isopropanol. Cesium chloride was removed by repeated dialysis. DNA was extracted with phenol followed by chloroform-isoamyl alcohol; then the DNA was precipitated with ethanol and redissolved in Tris-EDTA buffer (pH 8).

Transfection. The cells were seeded in six-well plates at 2×10^5 /well. The following day, the cells were transfected by using cationic liposomes (LipofectAMINE; Life Technologies) as instructed by the manufacturer. To estimate transfection efficiencies, cells transfected with *lacZ* expression vectors were stained for β -galactosidase activity by using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; U.S. Biological, Swampscott, Mass.). To quantify the amount of β -galactosidase expressed in cells transfected with *lacZ* expression vectors, cell lysates were harvested 48 h posttransfection and assayed by using the Promega β -galactosidase enzyme assay system as instructed by the manufacturer. Briefly, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS); then $1 \times$ reporter lysis buffer was added to the plates for 15 min at room temperature with repeated rocking. The plates were then scraped, and the cell lysate was collected in a microfuge tube, vortexed for 10 to 15 s, and centrifuged at 4°C for 2 min. The supernatant was transferred to fresh tubes and used for assay of β -galactosidase. A standard curve was generated by using purified β -galactosidase. The amount of β -galactosidase activity present in the cell lysates was determined from this standard curve.

HIV assays. Forty-eight hours posttransfection, the supernatants were harvested and titrated by MAGI assay (25) to determine the number of infectious units of HIV per milliliter. Briefly, HeLa-CD4⁺-LTR- β -gal cells were infected with serial dilutions of the transfection supernatants. Two days later, the cells were fixed and stained with X-Gal for 50 min, and the blue-stained infectious centers were counted. For evaluation of the accuracy of the MAGI assay, the supernatant obtained from cotransfection of HeLa-CD4⁺-LTR- β -gal cells was also assayed for the HIV core antigen, p24, using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Coulter, Hialeah, Fla.) as instructed by the manufacturer.

Western blotting. Forty-eight hours posttransfection, cell extracts were analyzed by Western blotting to detect Vhs. The cells were washed with PBS and then dislodged with 1 ml of 1 mM EDTA in PBS. The cells were pelleted in a microfuge; the pellet was resuspended in 50 μ l of 1.2% octyl- β -thiogluco-pyranoside (Pierce, Rockford, Ill.) and incubated on ice for 15 min. All centrifugation steps were performed at 4°C in screw-capped biohazard containment vessels. The cytoplasmic fraction of the cell lysate was separated from the cell nuclei by centrifugation through a 0.2- μ m Z-spin column (Gelman Sciences, Ann

Arbor, Mich.). The eluate was harvested, and the proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (44). Briefly, each sample was resuspended in an equal volume of 2× SDS gel-loading buffer and boiled for 3 min, and the entire amount of each cytoplasmic extract was electrophoresed on an SDS-polyacrylamide gel composed of a 5% stacking gel and a 10% separating gel at 50 V for 3 to 4 h. A sample of HSV-1 KOS strain containing 1.4×10^7 PFU (as a positive control) and a protein molecular weight marker (Rainbow colored high-molecular-weight protein markers; Amersham, Arlington Heights, Ill.) were electrophoresed on each gel along with the cytoplasmic extract samples. Following electrophoresis, the gel was equilibrated in transfer buffer and electrophoreted to a membrane (Rad-Free nylon membrane; Schleicher & Schuell, Keene, N.H.) overnight at 30 V, using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, Calif.) as specified by the manufacturer.

The following day, the blot was probed according to Schleicher & Schuell recommendations. Briefly, the membrane was blocked in 1% Rad-Free blocking solution (Schleicher & Schuell) for 1 h at room temperature. The membrane was probed for Vhs with a 1:500 dilution of rabbit antiserum against a UL41-LacZ fusion protein (50) for 30 min at room temperature. The membrane was washed and probed with a 1:7,500 dilution of goat anti-rabbit (Fc)-alkaline phosphatase conjugate (Promega) for 30 min at room temperature. Then the membrane was washed, and the substrate, 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT; Promega) was added to the membrane and allowed to develop for 20 to 60 min. When the desired color had developed, the reaction was stopped with 0.5 M EDTA in PBS.

RESULTS

HSV *vhs* constitutively expressed from a CMV promoter inhibits HIV-1 production. To determine whether the Vhs of HSV could inhibit HIV-1 replication, HeLa cells were cotransfected with an infectious molecular clone of HIV-1 (pNL4.3) and *vhs* expression vectors. These vectors contain either wild-type *vhs* or mutant *vhs* genes placed under the control of the human CMV immediate-early promoter (39). To ensure that any inhibition of HIV replication was not due to effects of the strong CMV promoter, the total molar amount of CMV promoter in each transfection was held constant by the addition of vector without *vhs* (pcDNA1). Cell supernatants were harvested 48 h posttransfection, and the amount of infectious HIV-1 was determined by plating on HeLa-CD4⁺-LTR-β-gal cells, using the MAGI assay (25).

Cotransfection of pNL4.3 and an HSV-1 strain KOS *vhs* expression plasmid (pKOScmv) resulted in a substantial decrease in HIV titers in a dose-dependent manner (Fig. 2). HIV titers were decreased to 5% of the control level by cotransfection with 0.5 μg of pKOScmv. Cotransfections with the HSV-2 strain 333 *vhs* (p333cmv) produced even greater decreases in HIV titers. Cotransfection with only 0.02 μg of p333cmv resulted in 97% inhibition of HIV replication, whereas complete inhibition was observed in the presence of 0.1 μg of p333cmv. HIV titers were not reduced when pNL4.3 was cotransfected with an HSV-1 *vhs* insertion mutant, *vhs*_{I237}, which has a 6-bp insertion located after codon 237 of *vhs*. These results were reproduced in assays using a different HeLa-derived cell line, HeLa-CD4⁺-LTR-β-gal. Cotransfection of pNL4.3 with either pKOScmv or p333cmv decreased the amount of infectious HIV recovered to 13 or 2% of the control level, respectively.

The HIV titers obtained by the MAGI assay paralleled results obtained when the supernatants from cotransfected cells were analyzed for the presence of HIV CA protein (p24) by ELISA. Constitutive expression of HSV-1 *vhs* resulted in an overall dose-dependent decrease in HIV-1 p24 expression to 15% of that observed with the parent vector, pcDNA1. HSV-2 Vhs also inhibited HIV-1 p24 expression in a dose-dependent manner, with the final inhibition being similar to that seen with HSV-1 Vhs; however, the effect of HSV-2 Vhs was greater at lower amounts of plasmid (i.e., 0.5 and 1.0 μg). The ability of wild-type HSV-1 and HSV-2 Vhs to dramatically decrease both the HIV titer and the amount of p24 indicates that Vhs

FIG. 2. Effect of *vhs* expression on HIV-1 titer. HeLa cells were transfected with 0.1 μg of pNL4.3 and 0.02, 0.1, 0.5, 1.0, or 1.5 μg of each of the three effector CMV expression plasmids. pKOScmv contains wild-type *vhs* from HSV-1, pI237cmv contains an inactive *vhs* mutant from HSV-1, and p333cmv contains wild-type *vhs* from HSV-2 (39). HIV titers in supernatants were determined by MAGI assay. The data from different experiments were normalized to the mean number of infectious units/milliliter obtained in each experiment by transfecting pNL4.3 and the control vector pcDNA1. Each point represents the mean of the normalized data, with error bars showing the standard deviations of the means. The actual means were 2,290 and 644 infectious units/ml in experiments 1 and 2, respectively.

was able to inhibit the production of infectious HIV, probably by limiting the synthesis of viral proteins.

HSV *vhs* under the control of the HIV promoter inhibits HIV-1 production. To create *vhs* expression vectors for HIV gene therapy, regulated expression of *vhs* is necessary. Constitutive expression of *vhs* from the unregulated CMV promoter is likely to be deleterious due to either the decreased survival or diminished function of cells. A good candidate vector for HIV gene therapy should express Vhs in HIV-infected cells but express little or no Vhs in uninfected cells. The promoter in the HIV-1 LTR was used to obtain regulated expression. In uninfected cells, the HIV LTR promoter will be only weakly active due to the absence of Tat. In cells that become infected with HIV-1, Tat will transactivate the LTR, resulting in high levels of Vhs expression.

We used PCR to amplify from pNL4.3 a fragment that contained nearly the entire LTR and the untranslated leader region of HIV-1. To determine the promoter activity of this HIV DNA fragment, we ligated this fragment to the bacterial *lacZ* reporter gene to produce two different vectors so that we could monitor expression driven from the LTR promoter. The two resulting LTR-*lacZ* vectors were called pHZRP and pHZP. Few HeLa cells transfected with either pHZRP or pHZP produced enough β-galactosidase activity to stain positive with X-Gal (Table 1). However, many more β-galactosidase-expressing cells were observed when either pHZRP or pHZP was transfected into HLtat or HL2/3 cells. Both HLtat and HL2/3 are HeLa cell derivatives that have been transformed to express HIV Tat (7, 13, 46). The increased number of *lacZ*-expressing cells in the presence of Tat cannot be explained by increased transfection efficiency compared to HeLa cells. Transfection of pCH110 (15), which contains the *lacZ* gene under the control of the SV40 promoter, was only about

TABLE 1. Expression of *lacZ* under the control of the HIV LTR

Plasmid ^a	No. of transfected cells expressing <i>lacZ</i> ^b		
	HeLa	HL2/3 ^c	HLtat ^c
None	0	0	0
pHZP	14	623	967
pHZRP	22	546	884
pCH110	945	2,250	1,895

^a Plasmids pHZP and pHZRP express the *lacZ* gene from the HIV LTR. *lacZ* expression is driven by the SV40 early promoter in pCH110 (15).

^b Determined by staining with X-Gal.

^c Stable HeLa cell derivatives that expresses HIV *tat* (7, 13, 46).

twofold lower in HeLa cells than in either HLtat or HL2/3 cells. These results indicate that the PCR-amplified HIV LTR fragment showed little basal expression and that the expression under the control of this promoter was transactivated by HIV Tat.

To obtain regulated expression of Vhs, we excised the LTR fragment from the LTR-*lacZ* vector pHZRP and used it to replace the CMV promoter in the *vhs* expression vectors pKO-Scmv and pI₂₃₇cmv. The resulting vectors, pKOSLTR and pI₂₃₇LTR, contain the vector sequences from pcDNA1. The same series of plasmids was also generated in a pcDNA3 background with *vhs* driven by either the CMV promoter (p3KOScmv and p3I₂₃₇cmv) or the HIV LTR promoter (p3KOSLTR and p3I₂₃₇LTR). As additional control vectors, plasmids that lack a promoter to drive *vhs* expression were created (pKOS and p3KOS).

To determine the ability of the HIV LTR to regulate *vhs* activity, we cotransfected these *vhs* vectors with the LTR-*lacZ* reporter plasmid (pHZRP) into either HeLa or HLtat cells (Fig. 3). In HeLa cells, only background amounts of β -galactosidase activity were detected. Due to the lack of Tat transactivation in the HeLa cells, neither the *lacZ* gene nor the *vhs* genes were efficiently expressed. However, in the presence of Tat, high levels of β -galactosidase activity were expressed in cells cotransfected with vectors containing either mutant *vhs* genes (p3I₂₃₇cmv and p3I₂₃₇LTR) or the promoterless *vhs* construct (p3KOS). Wild-type *vhs* activity in HLtat cells inhibited the expression of β -galactosidase. Expression of *vhs* under the control of the CMV promoter resulted in a twofold reduction in *lacZ* expression. The *vhs* gene driven by the Tat-transactivated HIV LTR promoter reduced the amount of β -galactosidase expressed over 10-fold. These results indicate that *vhs* activity can be expressed from the HIV promoter even more efficiently than from the CMV promoter.

The ability of *vhs* under the control of the HIV LTR promoter to inhibit the production of HIV was tested by cotransfection of the *vhs* expression vectors with infectious HIV proviral DNA (pNL4.3) into HeLa cells (Fig. 4). Forty-eight hours posttransfection, the culture supernatants were collected and the production of HIV in each culture was determined by the MAGI assay. When the supernatants were collected, the cells from the transfection were harvested for analysis of Vhs expression by Western blotting. Expression of *vhs* under the control of the HIV LTR promoter almost completely inhibited HIV production; only a single infectious unit of HIV was observed in 4 ml of harvested supernatants. This represented a more than 44,000-fold reduction in HIV titers compared to control cotransfections. This inhibition in HIV titers could not be attributed to any competition due to sequences contained in the HIV promoter because cotransfection with similar vectors that express the mutant *vhs* (pI₂₃₇LTR and p3I₂₃₇LTR) re-

FIG. 3. Tat-transactivated *vhs* expression inhibits expression of a reporter gene driven by the HIV promoter. HeLa cells (open bars) or *tat*-expressing HLtat cells (black bars) were transfected with 0.5 μ g of pHZRP and 1.0 μ g of the *vhs* expression plasmids indicated below the bars. Wild-type *vhs* was expressed by p3KOScmv and p3KOSLTR under the control of the CMV promoter and the HIV LTR promoter, respectively. The corresponding control vectors containing mutant *vhs* genes are p3I₂₃₇cmv and p3I₂₃₇LTR. Plasmid p3KOS contains a wild-type *vhs* gene but lacks a promoter necessary for *vhs* expression. Cells were harvested 48 h posttransfection, lysed, and assayed for β -galactosidase activity, using *o*-nitrophenyl- β -D-galactopyranoside. The β -galactosidase activities were converted to units by comparison with the linear regression analysis ($r = 0.99$) of a standard curve. Each bar represents the mean activity of duplicate samples, and error bars show the standard deviations of the means.

duced HIV titers an average of 1.6-fold. The difference in HIV titers observed in cells transfected with either pI₂₃₇LTR or p3I₂₃₇LTR was not due to differences in the amount of Vhs protein expressed, which could have partial *vhs* activity. We did not observe a difference in the amount of the mutant protein expressed from pI₂₃₇LTR and p3I₂₃₇LTR by Western blotting (data not shown), and previous studies have established the null phenotype of the I₂₃₇ mutation (39). Thus, most of the inhibition of HIV production can be attributed to the expression of a wild-type *vhs* gene. By comparison, expression of wild-type *vhs* from the CMV promoter reduced HIV titers more than 10-fold compared to cotransfection with control vector (pcDNA1 or pcDNA3). These results suggest that *vhs* driven by the HIV LTR was expressed at higher amounts than when expressed by the CMV promoter and that this amount of Vhs was extremely efficient in inhibiting the production of infectious HIV.

The amount of Vhs expression driven by the HIV LTR when cotransfected with HIV proviral DNA into HeLa cells was examined by Western blotting (Fig. 5). Vhs was readily detectable in a small sample of purified HSV-1 (strain KOS) virus preparation, as shown by the presence of a prominent band that migrates with an apparent molecular mass of 55 kDa. In samples cotransfected with the *vhs* expression vectors, two protein bands were observed. The predominant band migrates with an apparent molecular weight that is slightly greater than that of the band observed in purified virus (Fig. 5; compare HSV-1_{KOS} to p3I₂₃₇LTR). A smaller amount of Vhs in cotransfected cell lysates that comigrates with the viral Vhs was also observed. The slower-migrating form of Vhs observed in transfected cells is due to an upstream ATG in the recombinant *vhs* gene that was introduced during the cloning process (40a). Initiation of protein synthesis at this upstream AUG

FIG. 4. Inhibition of HIV production by *vhs* expressed from the HIV LTR. HeLa cells were transfected with 0.1 μ g of pNL4.3 and 1.0 μ g of the *vhs* expression plasmids shown below the bars. Wild-type *vhs* was expressed by pKOScmv and pKOSLTR under the control of the CMV promoter and the HIV LTR promoter, respectively, in pcDNA1-derived vectors. The corresponding control vectors containing mutant *vhs* genes are pI₂₃₇cmv and pI₂₃₇LTR. The p3 series plasmids contain *vhs* transcription units identical to their pcDNA1-derived vector counterparts but are cloned in a pcDNA3 background. Culture supernatants were harvested 48 h posttransfection, and the HIV titers were determined by MAGI assay. The titers from different experiments were normalized to the titers obtained by cotransfection with the appropriate control vector (pcDNA1 or pcDNA3). The actual titers for each control cotransfection were 1.7×10^5 and 6.2×10^4 infectious units/ml for pcDNA1 and 8.4×10^4 infectious units/ml for pcDNA3. Bars represent the relative normalized mean titers, and error bars show the standard deviations of the means.

would result in the in-frame addition of 32 amino acids to the amino terminus of Vhs. No Vhs was detected in samples cotransfected with either the vector that lacks a promoter to drive *vhs* (p3KOS) or the control vector that lacks a *vhs* gene (pcDNA3). More Vhs was expressed in samples cotransfected with the vectors containing the mutant *vhs* (p3I₂₃₇LTR and p3I₂₃₇cmv) than in samples cotransfected with the corresponding wild-type *vhs* gene (p3KOSLTR and p3KOScmv). These results are consistent with the ability of the wild-type Vhs to cause the degradation of its own mRNA and thus limit *vhs* expression. The amount of Vhs expressed from the HIV LTR (p3I₂₃₇LTR and p3KOSLTR) was consistently greater than the amount driven by the CMV promoter (p3I₂₃₇cmv and p3KOScmv). The greater amount of Vhs expressed from the HIV LTR than from the CMV promoter is in good agreement with observed amounts of Vhs inhibitory activity discussed above.

DISCUSSION

The HSV virion host shutoff gene (*vhs*) was investigated for the ability to inhibit HIV-1 replication. Both HSV-1 and HSV-2 *vhs* expressed from a CMV promoter significantly inhibited HIV-1 gene expression and replication in a dose-dependent fashion. When HSV-1 *vhs* was expressed by transactivation of the HIV promoter by Tat, even higher amounts of Vhs were produced, which resulted in almost total inhibition of HIV replication. In addition to inhibiting HIV replication, Vhs inhibited its own expression.

Constitutive expression of HSV-1 and HSV-2 *vhs* from the

FIG. 5. Western blot of Vhs in transfected cells. HeLa cells were cotransfected with the plasmids indicated above the lanes and an HIV proviral clone (pNL4.3). Cells were lysed 48 h posttransfection, and the amount of total protein in each lysate was determined. Equal amounts of protein (2.5 μ g) of each sample were boiled in SDS sample buffer, and the proteins were separated by SDS-10% polyacrylamide gel electrophoresis. Proteins were transferred to a nylon membrane and probed with rabbit anti-Vhs serum. Bound antibody was detected by using alkaline phosphatase-conjugated anti-rabbit immunoglobulin antibody and visualized by using BCIP-NBT substrate. The positions of molecular weight markers are shown at the right in kilodaltons.

CMV immediate-early promoter inhibited virus production, as determined by virus titers and p24 expression. HSV-2 Vhs had a greater effect on HIV-1 replication at lower amounts of transfected DNA than did HSV-1 Vhs. These data confirm and extend results reported by Pak et al. (39). Those investigators observed that wild-type HSV-1 Vhs inhibited expression of a cotransfected chloramphenicol acetyltransferase (CAT) reporter gene in a dose-dependent fashion by reducing the amount of CAT mRNA. The maximum inhibition of CAT expression observed was fourfold. We observed that the HSV-1 *vhs* expression vector used by Pak et al. (pKOScmv) decreased HIV-1 titers approximately 10-fold and p24 expression nearly 8-fold. These findings point out the strong inhibitory effect of Vhs, since the Tat-transactivated HIV-1 LTR has been reported to drive gene expression at a level 68-fold higher than that for the SV40 promoter in HeLa cells (48). We have observed 20-fold more β -galactosidase activity when the *lacZ* gene is under control of the HIV LTR than when expression is driven by the SV40 promoter (data not shown). The ability of Vhs to inhibit *lacZ* expression driven by the HIV LTR (Fig. 3) indicated that the strong inhibitory activity of Vhs cannot be overcome by the extremely active HIV LTR promoter. Both the mechanism of Vhs activity and the regulation of genes expressed from the HIV promoter may contribute to the hypersensitivity of HIV gene expression to Vhs inhibition.

The specific mechanism of this Vhs-mediated degradation of mRNA has only recently been investigated. Karr and Read (23) studied HeLa cells infected with either wild-type HSV-1 or mutant HSV-1 lacking host shutoff function. They found that in cells infected with the wild-type virus, the 5' end of mRNA disappeared more rapidly than did the 3' end. In cells infected with a mutant virus known to be defective in host shutoff function, the overall rates of disappearance of the 5' and 3' ends were significantly lower and very similar to each other. This finding suggests that Vhs may initiate mRNA degradation at the 5' end (23).

Recent studies by Zelus et al. suggest that Vhs possesses intrinsic RNase activity which is responsible for its ability to

degrade mRNA and inhibit protein synthesis (56). By translating *vhs* mRNA in vitro and incubating the product with β -globin mRNA, these authors demonstrated a 30-fold increase in the degradation rate of the mRNA in the presence of wild-type *vhs*. This finding indicated that in the absence of other viral or cellular proteins, Vhs was capable of degrading mRNA, suggesting that this activity was directly attributable to Vhs.

Expression of HIV proteins could be inhibited by the Vhs-induced RNase activity at multiple stages of the virus replication cycle. Early in infection, the regulatory proteins of HIV (Tat and Rev) must be expressed before any of the viral structural proteins. Tat functions to upregulate transcription driven by the LTR promoter. In the absence of Tat, the HIV promoter has only a weak basal level of expression due to both a low rate of transcription initiation and a block in transcription elongation (9). Tat binds to its RNA target sequence, called TAR, located in the nascent transcript. The TAR region has been mapped to the first 44 nucleotides of the HIV-1 RNA (19, 43). The binding of Tat to TAR results in an increase in subsequent transcription initiation events and makes the transcription unit more processive, thus relieving the block in transcription elongation. Tat functions in a positive feedback loop to increase the amount of all viral RNA expression. Since Vhs functions in the cytoplasm (42) whereas the effect of Tat on transcription occurs in the nucleus, Vhs probably does not function by degrading TAR sequences on nascent transcripts. Vhs is more likely to degrade the mRNAs that encode both Tat and Rev, which are required for HIV late gene expression.

Rev changes the pattern of viral mRNA splicing in infected cells. The viral RNAs detected about 12 h after infection are the 2-kb, multiply spliced mRNAs that encode the regulatory proteins (24). Thus, Tat and Rev are among the first viral proteins expressed following HIV infection. Rev binds to the RNA target sequence called the Rev-responsive element, facilitating the transport of viral mRNA from the nucleus into the cytoplasm and increasing the ratio of unspliced and singly spliced mRNA to doubly spliced mRNA (10). The splicing pattern induced by Rev favors the expression of the viral structural proteins and the viral genome, which are both required for the assembly of progeny virus. Presumably, any of these late viral mRNAs produced in *vhs*-transfected cells will be susceptible to Vhs degradation, further reducing overall HIV production. If Vhs inhibited the synthesis of host factors that may be required for virion assembly and release, then HIV titers would be even further decreased. The inhibition of both early and late HIV gene expression by Vhs may explain the almost total block in HIV replication.

Using Western blot analysis of cells cotransfected with infectious HIV proviral DNA and each of the various *vhs* expression plasmids, we demonstrated an inverse correlation between the amount of Vhs expressed and the amount of HIV-1 inhibition. The most Vhs was detected in cells transfected with the *vhs* mutant expression plasmid pI₂₃₇-LTR, which had no inhibitory effect on HIV-1 gene expression. Mutant Vhs was detected in smaller quantities when expressed from the CMV promoter (pI₂₃₇-cmv). These results are in good agreement with those of other studies that examined the relative promoter strengths of the HIV LTR and the CMV immediate-early promoter. Subramanian and Chinnadurai found that the HIV-1 LTR expresses approximately fivefold more of an indicator gene than does the CMV promoter in HeLa cells (48). The wild-type Vhs was expressed in much lower amounts than mutant Vhs. Pak and coworkers have reported similar results, suggesting that Vhs activity is not only capable of inhibiting the synthesis of HIV proteins but also able to inhibit its own synthesis (39). This conclusion is supported by numerous re-

ports that wild-type Vhs functions by degrading host mRNA as well as all classes of HSV mRNA (26, 38, 47).

To be useful for gene therapy, the expression of suicide genes such as *vhs* must be tightly regulated to prevent inappropriate activity in uninfected cells. The importance of tight regulation is pointed out by two groups that have used the HIV regulatory sequences to express the DTA fragment in HIV-infected cells (5, 11, 18). DTA is a potent bacterial toxin that kills cells by enzymatically inhibiting elongation factor 2 to prevent protein synthesis. Both groups used transient transfection assays to show that HIV regulatory proteins can induce DTA expression from the HIV LTR and inhibit protein synthesis (5, 18). However, only one group successfully obtained stable cell lines that contained an integrated copy of their DTA expression clone (11, 16, 17). Caruso et al. (5) produced 20 cell clones by transfection with their DTA expression vector, but the DTA gene was deleted in all 20 cell clones. These results presumably indicate that there is a strong selection against low amounts of toxin expressed from the uninduced HIV LTR promoter. This conclusion is supported by earlier studies that reported that one DTA molecule in a cell is sufficient to inhibit protein synthesis and produce cell death (54).

At present it is uncertain how many molecules of Vhs can be tolerated by a cell without deleterious effects. It has been suggested that there may be a threshold for *vhs* activity (27). To examine the effect of basal and induced *vhs* expression on cell functions, we are currently attempting to produce cell lines capable of induced *vhs* expression. The present study demonstrates that HSV-1 Vhs and HSV-2 Vhs are capable of inhibiting HIV-1 replication in vitro, thereby making these proteins candidates for use in HIV-1 gene therapy.

ACKNOWLEDGMENTS

We thank David Everly (University of Missouri—Kansas City) for helpful discussions and Robert Lyons (University of Michigan Department of Biological Chemistry and Molecular Biology Core Facility) for assistance with DNA sequencing.

This work was supported in part by NIH grants awarded to T.C.H. (KO4 AI00875) and G.S.R. (RO1 AI21501).

REFERENCES

- Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**:284–291.
- Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868–871.
- Berchtold, S., J. Ries, U. Hornung, and C. Aepinus. 1994. Exchange of functional domains between Rev proteins of HIV-1 and SIVmac239 results in a dominant negative phenotype. *Virology* **204**:436–441.
- Caruso, M., and D. Klatzmann. 1992. Selective killing of CD4+ cells harboring a human immunodeficiency virus-inducible suicide gene prevents viral spread in an infected cell population. *Proc. Natl. Acad. Sci. USA* **89**:182–186.
- Caruso, M., G. Tsikas, M. Roussel, M. Alizon, and D. Klatzmann. 1992. Can diphtheria toxin be used for gene therapy of human immunodeficiency virus infection? *AIDS Res. Hum. Retroviruses* **8**:1949–1950.
- Chatterjee, S., P. R. Johnson, and K. K. Wong, Jr. 1992. Dual-target inhibition of HIV-1 in vitro by means of an adeno-associated virus antisense vector. *Science* **258**:1485–1488.
- Ciminale, V., B. K. Felber, M. Campbell, and G. N. Pavlakis. 1990. A bioassay for HIV-1 based on Env-CD4 interaction. *AIDS Res. Hum. Retroviruses* **6**:1281–1287.
- Cohen, J. 1993. Early AZT takes a pounding in French-British 'Concorde' trial. *Science* **260**:157.
- Cullen, B. R. 1993. Does HIV-1 Tat induce a change in viral initiation rights? *Cell* **73**:417–420.
- Cullen, B. R. 1994. RNA-sequence-mediated gene regulation in HIV-1. *Infect. Agents Dis.* **3**:68–76.

11. Curiel, T. J., D. R. Cook, Y. Wang, B. H. Hahn, S. K. Ghosh, and G. S. Harrison. 1993. Long-term inhibition of clinical and laboratory human immunodeficiency virus strains in human T-cell lines containing an HIV-regulated diphtheria toxin A chain gene. *Hum. Gene Ther.* **4**:741-747.
12. Curran, J. W., W. M. Morgan, A. M. Hardy, H. W. Jaffe, W. W. Darrow, and W. R. Dowdle. 1985. The epidemiology of AIDS: current status and future prospects. *Science* **229**:1352-1357.
13. Felber, B. K., C. M. Drysdale, and G. N. Pavlakis. 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *J. Virol.* **64**:3734-3741.
14. Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500-503.
15. Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee. 1983. Expression and regulation of *Escherichia coli* lacZ gene fusions in mammalian cells. *J. Mol. Appl. Genet.* **2**:101-109.
16. Harrison, G. S., C. J. Long, T. J. Curiel, F. Maxwell, and I. H. Maxwell. 1992. Inhibition of human immunodeficiency virus-1 production resulting from transduction with a retrovirus containing an HIV-regulated diphtheria toxin A chain gene. *Hum. Gene Ther.* **3**:461-469.
17. Harrison, G. S., C. J. Long, F. Maxwell, L. M. Glode, and I. H. Maxwell. 1992. Inhibition of HIV production in cells containing an integrated, HIV-regulated diphtheria toxin A chain gene. *AIDS Res. Hum. Retroviruses* **8**:39-45.
18. Harrison, G. S., F. Maxwell, C. J. Long, C. A. Rosen, L. M. Glode, and I. H. Maxwell. 1991. Activation of a diphtheria toxin A gene by expression of human immunodeficiency virus-1 Tat and Rev proteins in transfected cells. *Hum. Gene Ther.* **2**:53-60.
19. Hauber, J., and B. R. Cullen. 1988. Mutational analysis of the *trans*-activation-responsive region of the human immunodeficiency virus type 1 long terminal repeat. *J. Virol.* **62**:673-679.
20. Heilig, J. S., K. Lech, and R. Brent. 1994. Large-scale preparation of plasmid DNA, p. 1.7.1-1.7.15. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York, N.Y.
21. Jones, F. E., C. A. Smibert, and J. R. Smiley. 1995. Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that Vhs functions in the absence of other viral proteins. *J. Virol.* **69**:4863-4871.
22. Joshi, S., A. Van Brunschot, S. Asad, I. van der Elst, S. E. Read, and A. Bernstein. 1991. Inhibition of human immunodeficiency virus type 1 multiplication by antisense and sense RNA expression. *J. Virol.* **65**:5524-5530.
23. Karr, B. M., and G. S. Read. 1995. The HSV virion host shutoff function induces degradation of the 5'-terminus of the target mRNA prior to the 3'-end, p. 202. In *Abstracts of the 20th International Herpesvirus Workshop*, vol. 1.
24. Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore. 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. *J. Virol.* **63**:3708-3713.
25. Kimpton, J., and M. Emerman. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β -galactosidase gene. *J. Virol.* **66**:2232-2239.
26. Kwong, A. D., and N. Frenkel. 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc. Natl. Acad. Sci. USA* **84**:1926-1930.
27. Kwong, A. D., and N. Frenkel. 1989. The herpes simplex virus virion host shutoff function. *J. Virol.* **63**:4834-4839.
28. Kwong, A. D., J. A. Kruper, and N. Frenkel. 1988. Herpes simplex virus virion host shutoff function. *J. Virol.* **62**:912-921.
29. Leavitt, M. C., M. Yu, O. Yamada, G. Kraus, D. Looney, E. Poeschla, and F. Wong-Staal. 1994. Transfer of an anti-HIV-1 ribozyme gene into primary human lymphocytes. *Hum. Gene Ther.* **5**:1115-1120.
30. Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-842.
31. Lisiewicz, J., J. Rappaport, and R. Dhar. 1991. Tat-regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. *New Biol.* **3**:82-89.
32. Lo, K. M., M. A. Biasolo, G. Dehni, G. Palu, and W. A. Haseltine. 1992. Inhibition of replication of HIV-1 by retroviral vectors expressing tat-antisense and anti-tat ribozyme RNA. *Virology* **190**:176-183.
33. Malim, M. H., S. Bohnlein, J. Hauber, and B. R. Cullen. 1989. Functional dissection of the HIV-1 Rev trans-activator—derivation of a trans-dominant repressor of Rev function. *Cell* **58**:205-214.
34. Malim, M. H., W. W. Freimuth, J. Liu, T. J. Boyle, H. K. Lyerly, B. R. Cullen, and G. J. Nabel. 1992. Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. *J. Exp. Med.* **176**:1197-1201.
35. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7**:980-982.
36. Nabel, G. J., B. A. Fox, L. Post, C. B. Thompson, and C. Woffendin. 1994. A molecular genetic intervention for AIDS—effects of a transdominant negative form of Rev. *Hum. Gene Ther.* **5**:79-92.
37. Oroskar, A. A., and G. S. Read. 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. *J. Virol.* **61**:604-606.
38. Oroskar, A. A., and G. S. Read. 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. *J. Virol.* **63**:1897-1906.
39. Pak, A. S., D. N. Everly, K. Knight, and G. S. Read. 1995. The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral gene products. *Virology* **211**:491-506.
40. Read, G. S. 1996. Control of mRNA stability during herpes simplex virus infections, p. 311-322. In J. B. Harford and D. R. Morris (ed.), *mRNA metabolism and post-transcriptional gene regulation*. John Wiley & Sons, New York, N.Y.
- 40a. Read, G. S. Unpublished data.
41. Rhodes, A., and W. James. 1990. Inhibition of human immunodeficiency virus replication in cell culture by endogenously synthesized antisense RNA. *J. Gen. Virol.* **71**:1965-1974.
42. Roizman, B., and A. E. Sears. 1991. Herpes simplex viruses and their replication, p. 849-895. In B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), *Fundamental virology*. Raven Press, New York, N.Y.
43. Rosen, C. A., J. G. Sodroski, and W. A. Haseltine. 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. *Cell* **41**:813-823.
44. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. Schek, N., and S. L. Bachenheimer. 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. *J. Virol.* **55**:601-610.
46. Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis. 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J. Virol.* **64**:2519-2529.
47. Strom, T., and N. Frenkel. 1987. Effects of herpes simplex virus on mRNA stability. *J. Virol.* **61**:2198-2207.
48. Subramanian, T., and G. Chinnadurai. 1992. A high-level expression vector for human cells. *Gene* **120**:287-289.
49. Sun, X. H., and D. Baltimore. 1989. Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation. *Proc. Natl. Acad. Sci. USA* **86**:2143-2146.
50. Vaerman, J. L., C. Lammineur, P. Moureau, P. Lewalle, F. Deldime, M. Blumenfeld, and P. Martiat. 1995. BCR-ABL antisense oligodeoxyribonucleotides suppress the growth of leukemic and normal hematopoietic cells by a sequence-specific but nonantisense mechanism. *Blood* **86**:3891-3896.
51. Weerasinghe, M., S. E. Liem, S. Asad, S. E. Read, and S. Joshi. 1991. Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human CD4⁺ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme. *J. Virol.* **65**:5531-5534.
52. Woffendin, C., Z. Y. Yang, Udaykumar, L. Xu, N. S. Yang, M. J. Sheehy, and G. J. Nabel. 1994. Nonviral and viral delivery of a human immunodeficiency virus protective gene into primary human T cells. *Proc. Natl. Acad. Sci. USA* **91**:11581-11585.
53. Yamada, O., G. Kraus, M. C. Leavitt, M. Yu, and F. Wong-Staal. 1994. Activity and cleavage site specificity of an anti-HIV-1 hairpin ribozyme in human T cells. *Virology* **205**:121-126.
54. Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15**:245-250.
55. Yu, M., J. Ojwang, O. Yamada, A. Hampel, J. Rappaport, D. Looney, and F. Wong-Staal. 1993. A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**:6340-6344.
56. Zelus, B. D., R. S. Stewart, and J. Ross. 1996. The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. *J. Virol.* **70**:2411-2419.