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Specific ablation of human immunodeficiency virus Tat-expressing cells by conditionally toxic retroviruses

(ablation therapy/human immunodeficiency virus type 2 long terminal repeat/retroviral vectors/thymidine kinase)

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ABSTRACT **The identification of human immunodeficiency virus (HIV) as the etiologic agent of AIDS has led to the proposal of novel intervention strategies to block HIV infection and viral replication or eliminate HIV-infected cells. We have produced recombinant retroviruses for a molecular ablation system, whereby a toxin gene can be delivered to hematopoietic cells for the specific elimination of HIV Tat-expressing cells. For this cell-specific ablation, we have coupled the conditional toxin herpes simplex virus type 1 thymidine kinase (tk) gene to the HIV-2 promoter and Tat responsive region (TAR) in order that transcriptional activity be under the absolute control of HIV and simian immunodeficiency virus Tat trans-activator proteins. Since the HIV-2 promoter has a considerable level of basal expression in the absence of Tat, we constructed a number of modifications in the HIV-2 promoter to minimize the risk of cytotoxicity to cells not containing HIV Tat. We demonstrate that certain promoter modifications reduce basal transcription while maintaining high trans-activated levels of expression when transfected or transduced by retroviral vectors into several different cell lines. In mouse and human cells infected with HIV-2 tk retroviruses, we show that Tat-induced expression from the HIV-2 promoter results in differential ablation and a massive reduction in Tat-positive cells after ganciclovir treatment. Thus, the retroviruses produced in these studies may be applicable to HIV ablative therapy.**

Conventional strategies to combat acquired immunodeficiency syndrome (AIDS) (1, 2) have concentrated on the development of antiviral drugs and preventive vaccines against human immunodeficiency virus (HIV). However, the intracellular immunization approach (3) has prompted the development of molecular tactics for the inhibition of HIV infection and replication (4–6). One strategy designed for the specific elimination of HIV-infected, Tat-expressing cells involves the use the herpes simplex virus type 1 thymidine kinase (tk) toxin gene (7). The tk gene product, a conditional cell lethal, is toxic to mammalian cells only in the presence of nucleoside analogues such as ganciclovir (GCV). These analogues kill replicating cells because they possess high affinity for the tk gene product with only slight affinity for endogenous mammalian tk. Lymphocyte-specific lethality has been demonstrated *in vivo* by antiherpetic drug treatment of tk transgenic mice (8, 9). Upon withdrawal of the prodrug in these studies, mature lymphocytes can be restored to normal numbers. Thus, the *in vivo* ablative system is regenerative and reversible.

Previously, specificity for ablation of HIV Tat-expressing cells has been achieved through the use of the HIV-1 long terminal repeat (LTR) promoter element in transfected cells (10) or adenovirus transduced cells (11). The HIV-1 LTR promoter element is composed of cis-acting control sequences, enhancer, transcription start site, and the Tat responsive region (TAR) (12–14). This basic structure is also conserved for HIV-2 (15–17). The presence of Tat has been shown to dramatically increase the level of HIV LTR-directed mRNA transcription (12, 14). A common mechanism of Tat trans-activation through the TAR is shared by HIV-1, HIV-2, and simian immunodeficiency virus (SIV) (15–17). However, the three Tat gene products are not completely interchangeable in their effects. Tat protein from both HIV strains and SIV effectively trans-activates the HIV-2 promoter, whereas the HIV-1 promoter and SIV promoter are only maximally trans-activated by their own specific Tat protein (16, 17).

Whereas HIV infection is limited at the level of viral entry into T lymphocytes by CD4 (18), non-CD4 positive human cells can actively transcribe HIV sequences (19). Introduction of the Tat-controlled HIV-2 tk sequences into hematopoietic stem cells by retroviral vectors (thus creating a complete blood system with all precursor and mature cell types carrying the HIV-2 tk sequence) requires the presence of an HIV promoter with a very low level of basal transcription in uninfected (Tat-negative) cells. We describe here the construction and analysis of modified HIV-2 promoters designed for the reduction of basal transcription while maintaining the ability to be trans-activated to high levels of expression by HIV-1 Tat. We demonstrate the basal and trans-activated expression properties of these constructs in transient transfection assays and after retroviral-mediated gene transduction. Furthermore, we present evidence for the differential ablation of HIV-2 tk retrovirally infected cells after prodrug treatment.

MATERIALS AND METHODS

Plasmid Constructions. Regions of the HIV-2 LTR (15) were generated by PCR amplification using pHIV-2 LTR chloramphenicol acetyltransferase (−556/+156) (16). Amplified fragments were obtained using the 3′ primer 3′-CGTGAACCGGCCACGACCCGTTCATGACCTAG-GTG-5′, which contains HIV-2 LTR sequences +96 to +116 and *Sca* I and *Bam*HI restriction sites and the 5′ primers as follows (5′ → 3′) for constructs: 1, GGCCTGCCAGAGGAA-GAGTGG; 2, CCTCATATTCTCTGTATAAATATACC; 3, AGGGACTTTCCAGAAGGGGCTGTAACCACTCTG-TATAAATATACC; 4, GAGGGACATGGGAGGAGC; 5, TGGTGGGGAACGCCCTC; 6, TTCGCCCACATATTC-TCTG; 7, AGAAGGGGCTGTAACCAACGTACGTAT-GCTTCGCCCACATATTCTCTG; 8, AGAAGCGGCTG-TAACCAACGTACGTATGCTTCGCCCACATATTC-TCTG. The 5′ primers contained *Hin*dIII, *Bgl* II, and *Xho* I sites in addition to the HIV-2 LTR sequence. Amplified

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; tk, herpes simplex virus 1 thymidine kinase; GCV, ganciclovir; hGH, human growth hormone; SIV, simian immunodeficiency virus.

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products were digested and cloned into the *Hin*dIII and *Bam*HI sites of pθGH (20) containing a human growth hormone (hGH) gene. Amplified products (*Bgl* II–*Sca* I) were ligated with the tk gene (*Bam*HI–*Bgl* II fragment) to *Bam*HI-linearized pUC19.

Cell Culture, Transfection, and Expression Assays. COS-7 cells were transiently transfected using DEAE-dextran (21) and 10–15 μg of HIV-2 hGH DNA (4:1) with pSV2Tat (17) and 5 μg of actin *lacZ* (22). CEM cells were transfected by electroporation with 100 μg of HIV-2 hGH DNA, 50 μg of pSV2Tat, and 50 μg pCMV *lacZ* expression vector (23). hGH expression was measured 72 hr after transfection of COS and CEM cells by radioimmunoassay (Tandem-R HGH, Hybritech) and normalized by reference to the cotransfected *lacZ* expression vector (23, 24). 3T3, HeLa, and retroviral packaging cells were transfected by the calcium phosphate coprecipitation technique. Amphotropic packaging cells PA317 (25) and AM12 (26) were transfected with 12 μg of each retroviral vector construct. 3T3 cells were transfected with pSV2Tat and a hygromycin expression plasmid (from R. Zamoyska, National Institute of Medical Research, London) (10:1) and selected in 120 μg of hygromycin B per ml (Sigma). Infected HeLa and CEM cells were transiently transfected with pSV2Tat and transfection efficiency was tested with a pCMV *lacZ* by histochemical staining.

RNA and DNA Analysis. RNA was isolated from cells (27) and S1 analysis was performed (28) on 0.5–25 μg of total cellular RNA using ^{32}P-end-labeled probes. The probe for HIV-2 tk transcripts was a 442-bp *Xho* I–*Mlu* I fragment spanning the HIV-2 tk transcription start site giving a protected fragment of 207 bases. The β-actin probe (*Xho* I–*Ava* I fragment) from pHFβA-1 (29) protects a 145-base human and 112-base mouse fragment. Fold induction of transcripts was quantified by using a PhosphorImager (Molecular Dynamics). Restriction enzyme-digested genomic DNA was transferred to Nytran nylon filters (21) and probed with a labeled 2.8-kb *Bgl* II–*Bam*HI tk fragment.

Retroviral Vector Construction, Virus Production, and Infections. Retroviral vectors pLA1, -4, and -5 were produced by cloning *Xho* I–*Bam*HI HIV-2 tk sequences into pLA. pLA was constructed by ligating *Pvu* I–*Bam*HI-linearized pLJ (30) to a *Sma* I/*Bam*HI/*Xho* I adapter and a *Pvu* I–*Xho* I (residues 419–1560) gag region fragment of Moloney murine leukemia virus. Packaging cell lines PA317 and AM12 were transfected and selected in 0.4 mg of G418 per ml (GIBCO/BRL). Higher titer clones were identified by analysis of viral RNA in cell supernatants (31). 3T3 and HeLa cells were infected with supernatants of cloned packaging cells in 8 μg of Polybrene per ml and selected in 0.6 mg of G418 per ml. CEM cells were infected by cocultivation with packaging cells (32) and selected for 4 weeks in G418 at 1–1.5 mg/ml. All cells were found to be free of replication-competent virus after extensive culture (26).

GCV Treatment. 3T3 cells (3×10^4 per well) were plated in 24-well dishes (Costar). After 2 days, GCV (Syntex) was added to the medium at various molar concentrations and changed every 2 days. After 6 days, the cells were washed and stained (80% methanol/5% methylene blue). HeLa cells (2×10^4) were plated and grown under the same conditions except that they were fixed in formyl calcium before staining.

RESULTS

Eight modified HIV-2 promoter constructs were generated using PCR, creating deletions and consensus sequences in the factor binding sites of the wild-type HIV-2 LTR so as to decrease the level of basal transcription but retain trans-activation via Tat. These constructs all contain the HIV-2 TATA sequence and a single TAR but vary in 5′ sequences as shown in Fig. 1.

Basal Activity of HIV-2 Promoter Constructs Is Decreased. The ability of the HIV-2 promoters to direct basal transcription was tested by transient expression of the hGH reporter gene (20) in transfected COS and CEM (CD4-positive human T) cells. As shown in Table 1, the basal levels of hGH expression were decreased in both cell lines with all of the modified promoter constructs. In COS cells the levels of hGH protein ranged from 0.52 to 1.2 ng/ml and are at least 4-fold lower than from the wild-type promoter 1 (4.9 ng/ml). The basal levels of hGH protein produced in CEM cells from the modified constructs were 0.25–1.9 ng/ml, with the levels decreased 2- to 14-fold in comparison to the wild-type promoter (3.5 ng/ml). Overall, the lowest level of basal activity was found with construct 4. Deletion of either NF-κB or Sp1 sites effectively decreased basal activity. When basal activity was measured by analysis of mRNA in an S1 protection assay (33), low levels of reporter gene transcripts were found to correspond to the reduced basal levels of hGH protein.

HIV-2 Promoter Constructs Can Be Trans-Activated to Varying Levels. The HIV-2 promoter constructs were tested for their ability to be Tat trans-activated in transfected COS and CEM cells. Our results indicate that all promoter sequences can be trans-activated by HIV-1 Tat (but to varying degrees) in both cell lines (Table 1). In COS cells the wild-type promoter transfectant was optimally trans-activated for hGH expression by a factor of 20, whereas transfectants receiving constructs 3, 4, 5, and 7 were trans-activated 4- to 10-fold. The levels of hGH protein were decreased from 100 ng/ml, as produced by construct 1, to 3, 5, 12, and 3.4 ng/ml from constructs 3, 4, 5, and 7, respectively. In CEM cells, Tat trans-activation greatly increased hGH production. Wild-type promoter-driven hGH production was increased to 426 ng/ml and the levels of hGH expression from constructs 3, 4, and 5 were raised to 32, 43, and 69 ng/ml, respectively. Surprisingly, upon trans-activation, COS and CEM cells containing construct 5 (only one Sp1 site) produced higher levels of hGH than construct 4 (three Sp1 sites). Also, the degree of trans-activation in CEM cells of constructs 4 and 5 (153-fold and 138-fold) was higher than the Tat-induced trans-activation produced by the wild-type construct 1 (123-fold). Construct 3 with two NF-κB sites was trans-activated only by a factor of 43.

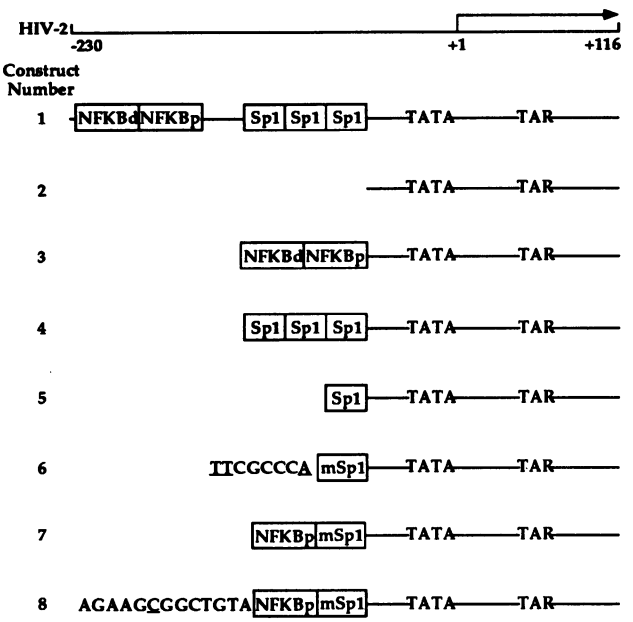

FIG. 1. HIV-2 LTR promoter constructs. NFκBd and NFκBp refer to the distal and proximal NF-κB sites, respectively, and mSp1 refers to a mutated Sp1 site.

Table 1. HIV-2 directed hGH expression in COS and CEM cell transfectants

Cell line	HIV-2 construct	− Tat hGH, ng/ml	Fold decrease in basal transcription	+ Tat hGH, ng/ml	Fold trans-activation
COS	1	4.90 ± 1.8	—	98.9 ± 49.7	20.2
COS	2	0.97 ± 0.15	5	2.66 ± 1.10	2.7
COS	3	0.76 ± 0.19	6	3.17 ± 0.6	4.2
COS	4	0.52 ± 0.24	10	4.79 ± 1.95	9.5
COS	5	1.20 ± 0.27	4	11.8 ± 7.5	9.8
COS	6	0.76 ± 0.29	6	2.49 ± 1.05	3.3
COS	7	0.62 ± 0.30	8	3.38 ± 1.82	5.5
COS	8	0.68 ± 0.34	7	1.51 ± 0.22	2.2
CEM	1	3.5 ± 0.2	—	426.0 ± 65.0	123.0
CEM	2	0.25 ± 0.08	14	1.1 ± 0.1	4.5
CEM	3	0.74 ± 0.25	5	31.9 ± 1.2	43.0
CEM	4	0.28 ± 0.07	12	42.9 ± 8.8	153.0
CEM	5	0.5 ± 0.2	7	68.9 ± 10.1	138.0
CEM	6	0.6 ± 0.2	6	7.1 ± 2.5	12.8
CEM	7	1.3 ± 0.6	3	4.1 ± 0.5	3.1
CEM	8	1.9 ± 0.7	2	0.8 ± 0.1	—

Basal and Tat trans-activated levels of hGH expression after transient transfection in COS and CEM cells. Cells were cotransfected with promoter constructs 1–8 cloned 5′ to a hGH gene, pCMVLacZ and pSV2Tat. The presence (+) and absence (−) of Tat are indicated. Each transfection was carried out in duplicate or triplicate on at least three separate occasions. The concentration of hGH produced was calculated from a standard curve after normalization. Average values ± standard deviations are shown.

Transduction of HIV-2 tk Sequences by Retroviral Vectors. Since the promoter constructs 1, 4, and 5 were the most highly trans-activated while having decreased basal activity, these promoters were cloned with the tk gene in the reverse orientation into the retroviral vector pLA to produce pLA1, pLA4, and pLA5, respectively (Fig. 2*A*). Amphotropic producer clones LA1, LA4, and LA5 were found to have titers of 5×10^3, 1×10^3, and 5×10^3 colony-forming units/ml, respectively, as assayed by G418 resistance of infected 3T3 cells. When the proviral integrants of LA1-, 4-, and 5-infected 3T3, HeLa, and CEM cells were tested, we found complete, intact viral (8.1 kb) sequences present in the genomic DNA (Fig. 2). In addition, at a low frequency, smaller tk-hybridizing bands (7.3 kb and 6.4 kb) were found, indicating some rearrangement of the retroviral genome. The 7.3-kb proviral integrant was found to delete a sequence (0.8 kb) immediately 3′ to the poly(A) signal of the tk gene (data not shown).

Trans-Activated tk Expression Occurs After Retroviral-Mediated Gene Transduction. To examine the effects of retroviral-mediated gene transduction on expression of the HIV-2 tk sequences, we examined basal and trans-activated levels of HIV-2 tk mRNA in LA1-infected 3T3, HeLa, and CEM cells. The results of S1 analysis on the 3T3 clone (31.1), CEM population (C1), and three HeLa clones (H1.2, H1.4, and H1.5) are shown in Fig. 3. We tested transcriptional trans-activation of the infected 31.1 clone by analysis of three stably Tat-transfected subclones (31.1A, 31.1B, and 31.1C) and found a 3-fold increase in tk expression (Fig. 3*A*). Previous data suggest this to be the maximum level of Tat trans-activation expected in 3T3 cells (23). Upon transient transfection of a Tat expression vector into H1.4 and H1.5 clones we observed a 5- to 6-fold increase in transcripts. The H1.2 clone can also be trans-activated but to a lesser extent, about 2-fold (Fig. 3*C*). CEM cells are also trans-activated by a factor of 4 (Fig. 3*B*). Using this transient assay only a fraction of the transfected HeLa and CEM cells actually take up the Tat expression vector. The efficiency of uptake as measured by transfection with a CMV-lacZ plasmid and β-galactosidase staining was determined to be 2–6% (data not shown). This indicates that the levels of trans-activation in these cells should be increased by an additional 16- to 50-fold.

Differential Ablation of HIV-2 tk Infected Cells. To determine whether differential killing of retrovirally infected cells occurs *in vitro*, the cytotoxic effect of GCV was tested on the LA1-infected 3T3 and HeLa cells after stable transfection of a Tat expression vector. The basal expression of the HIV-2 tk construct 1 resulted in complete ablation of the 3T3 cells (31.1) at GCV concentrations >4 μM (Fig. 4 *A* and *B*), whereas <0.3 μM GCV was completely cytotoxic to Tat-transfected subclones 31.1A and 31.1B (Fig. 4*A*). HeLa cells (H1.4) infected with the same retrovirus were 40-fold more

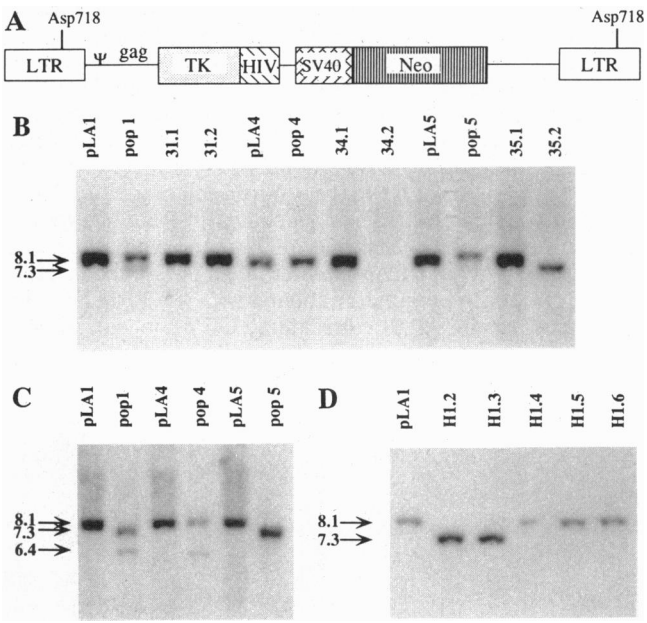

FIG. 2. HIV-2 tk retroviral vector design and viral passage analysis of cells infected with recombinant retroviruses. (*A*) pLA retroviral vector with HIV-2 tk sequences cloned in the opposite orientation to the simian virus 40 (SV40) promoter and the 5′ LTR. *Asp*718 sites are as indicated. (*B*) Southern blot analysis on DNA from 3T3 cells infected with retroviruses LA1, LA4, and LA5. Plasmid controls are indicated as pLA1, pLA4, and pLA5. DNA from populations and clones of infected 3T3 cells are indicated as pop 1, 31.1, and 31.2 (LA1 infected), pop 4, 34.1, and 34.2 (LA4 infected), and pop 5, 35.1, and 35.2 (LA5 infected). (*C* and *D*) Southern blot analysis on DNA from populations of CEM cells (pop 1, pop 4, and pop 5) infected with LA1, LA4, and LA5 (*C*) and LA1-infected HeLa clones, H1.2–H1.6 (*D*). *Asp*718-digested DNA was probed with a tk gene fragment.

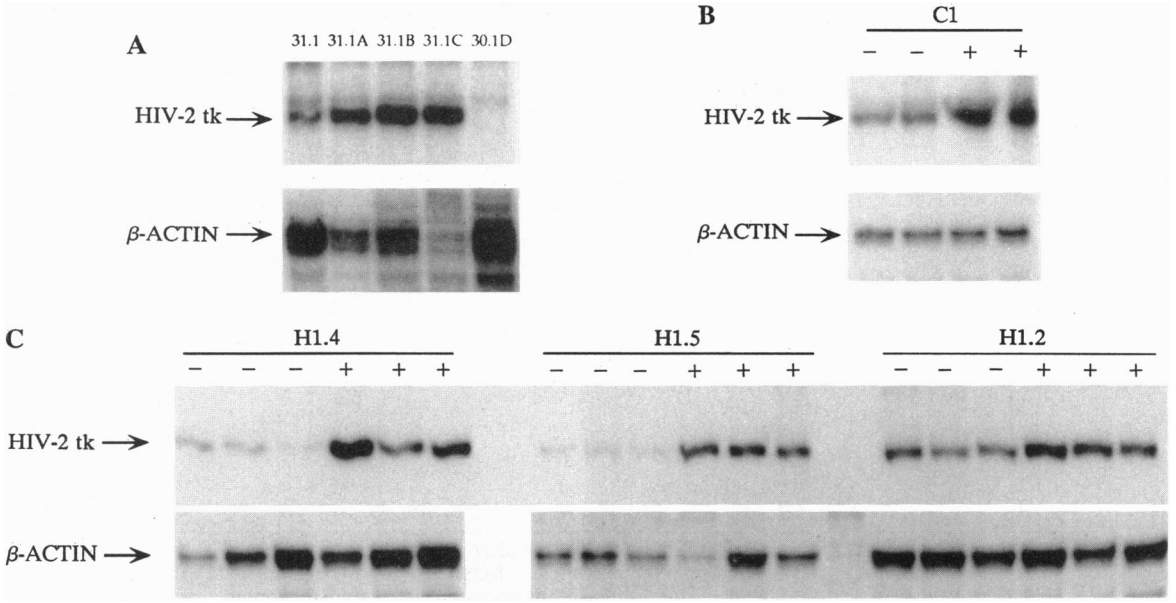

FIG. 3. Differential Tat trans-activated expression of tk in cells infected with LA1. (*A*) S1 analysis of RNAs from an infected 3T3 clone without Tat (31.1) and three subclones containing Tat (31.1A, *B* and *C*) hybridized with HIV-2 tk and β-actin probes. 30.1D is a Tat transfected, uninfected 3T3 clone. (*B* and *C*) S1 analysis of infected CEM cell population (*B*; C1) and infected HeLa clones (*C*; H1.4, H1.5, and H1.2) before (–) and after (+) duplicate or triplicate transient transfections with pSV2Tat.

sensitive to GCV than the 3T3 clone and were killed at GCV concentrations >0.1 μM (Fig. 4*C*). Concentrations of GCV as low as 20 nM were sufficient to kill Tat-expressing HeLa subclones H1.4A and H1.4B. These results demonstrate predictable Tat-induced trans-activation of the HIV-2 promoter and differential GCV sensitivity in mouse and human cells after gene transduction by retroviral vectors.

As a functional test of diminished basal activity from the modified HIV-2 promoter constructs, we compared the GCV sensitivity of LA1-infected 3T3 cells with LA4-infected cells (Fig. 4*B*). LA1-infected 3T3 cells (31.1) were completely ablated with >4 μM GCV, whereas LA4-infected 3T3 cells (34.1) were less sensitive to the prodrug and required >16 μM GCV for complete ablation. As shown in Fig. 4*D*, we also compared the GCV sensitivity of LA1-infected HeLa cells with that of LA4- and LA5-infected cells. LA1-infected HeLa cells (H1.4) were completely ablated with >0.1 μM GCV but LA4- and LA5-infected cells required GCV concentrations of >1 μM for cytotoxicity. Thus, the cytotoxic effect of GCV on the basal expressing cells is greatly reduced by the use of the modified HIV-2 promoters in the LA4 and LA5 retroviruses.

DISCUSSION

We have described the development of a retroviral-mediated intracellular molecular ablation method for the specific elimination of HIV Tat-expressing cells. Previously, an HIV-1 tk transfected cell line treated with acyclovir was shown to effectively ablate HIV-infected cells and arrest the spread of infectious HIV (10). Our use of modified HIV-2 promoters to direct tk conditional toxin gene expression has advantages over this method. It can be effective in HIV-1-, HIV-2-, or SIV-infected cells and, additionally, the decrease in the basal expression from the modified HIV-2 promoters should protect uninfected cells (especially lymphocytes) from ablation. We have shown that HIV-2 constructs containing various arrangements of the Sp1 sites and NF-κB binding regions

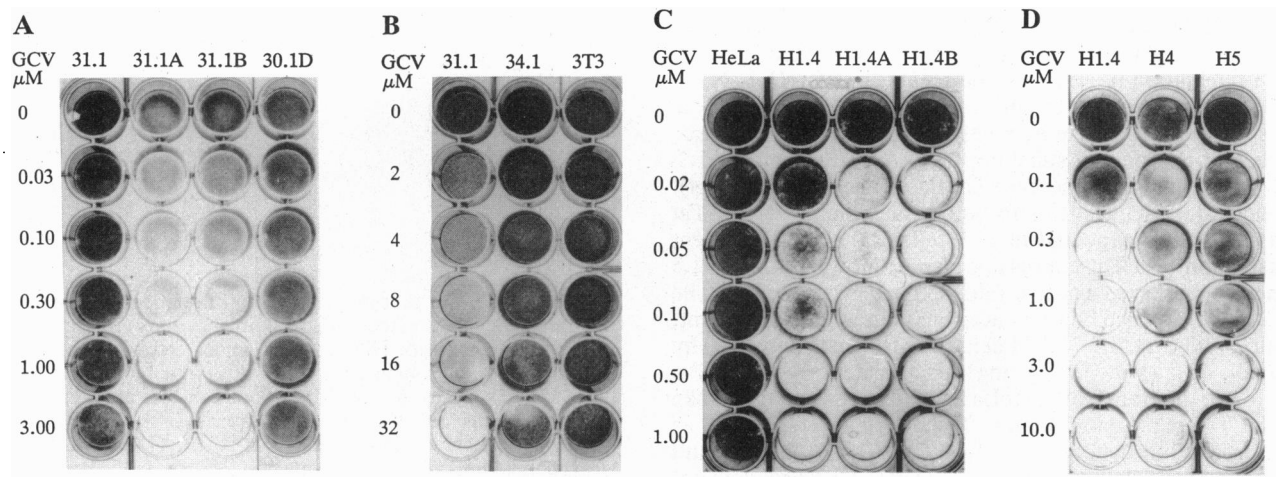

FIG. 4. Differential Tat trans-activated ablation of HIV-2 tk retrovirus-infected 3T3 and HeLa cells. GCV treatment was performed on LA1-infected Tat-negative 3T3 cells (31.1), LA1-infected Tat-positive (31.1A and 31.1B), uninfected Tat-positive (30.1D), and 3T3 cells (*A*) and LA1-infected (31.1), LA4-infected (34.1), and 3T3 cells (*B*). GCV was also administered to LA1-infected HeLa cells without Tat (H1.4) and with Tat (H1.4A and H1.4B) as well as uninfected HeLa cells (*C*) and LA1-infected (H1.4), LA4-infected (H4), and LA5-infected (H5) HeLa cells (*D*). Molar concentrations of GCV are as indicated.

linked to reporter genes in COS and CEM transfectants are reduced in their basal levels of expression between 4- and 10-fold from the wild-type HIV-2 promoter. Importantly, two of the constructs (nos. 4 and 5) retained the ability to be trans-activated to high levels as compared to the wild-type promoter in COS and CEM cells.

We can conclude from the transfection studies that Sp1 sites (in constructs 4 and 5) are more important to the Tat trans-activation of the HIV-2 promoter than the NF-κB binding regions, although promoter construct 3 (containing the NF-κB regions without any Sp1 sites) was Tat inducible, but to a lesser degree. This is in agreement with previous work on the HIV-1 LTR (34) and therefore suggests that the NF-κB binding region can substitute for the Sp1 sites and allow Tat trans-activation in T cells. This region in HIV-2, however, is probably not equivalent to the two HIV-1 NF-κB sites, which are a direct repeat (35). In HIV-2, the distal NF-κB site is identical but the proximal site differs by four bases from HIV-1 (15, 36), rendering it nonfunctional (37).

In the context of a retroviral vector for toxin gene delivery, our studies show that HIV-2 promoter sequences retain low levels of basal expression and can be efficiently Tat trans-activated in mouse and human cells, even though two other promoters (Moloney murine leukemia virus and simian virus 40) are present, which could have disrupted the transcriptional properties of the HIV-2 promoter. A 100- to 200-fold induction of HIV-2 tk was found in infected human T cells when normalized for Tat transfection efficiency, corresponding to the trans-activation observed in transfection studies. Furthermore, we found that GCV treatment of LA1-infected 3T3 cells and HeLa cells produced a clear differential in cell killing between Tat-positive and Tat-negative cells. In addition, the decrease in the basal transcriptional levels due to HIV-2 promoter modification rendered LA4- and LA5-infected cells 4- to 5-fold less sensitive to GCV than LA1-infected cells. The use of modified HIV-2 promoters could allow for clinically tested doses of GCV (mean plasma levels, 0.8–7 μM; refs. 38 and 39) to specifically ablate HIV Tat-positive cells, preserving the HIV Tat-negative cells harboring HIV-2 tk sequences.

Future research must be directed toward infecting the CEM clones with HIV to confirm GCV-mediated ablation and to test for the arrest of viral spread. New, higher titer HIV-2 tk retroviruses for infection of primary human hematopoietic cells must also be constructed. Given the current dilemmas with anti-HIV treatment using vaccines or drugs (40), it may well be that this ablative therapy could, in conjunction with other treatment regimens (41), help restore partial function of a patient's immune system.

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