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Human Immunodeficiency Virus Vectors for Inducible Expression of Foreign Genes†

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Tat-dependent expression of an endogenous lethal or deleterious foreign gene might be useful for abrogating the production of human immunodeficiency virus (HIV) from cells. This type of HIV-induced cellular killing, as well as other approaches to gene therapy for HIV infection, would be facilitated by simple HIV vectors that express introduced genes in a Tat-inducible manner. As part of studies to examine the feasibility of this concept, we constructed HIV-1 vectors that express the hygromycin B phosphotransferase gene (Hyg^r) in a Tat-dependent manner. Comparison of the efficiency of propagation of each vector indicates that sequences extending into the *gag* open reading frame are necessary in *cis* for efficient vector propagation. Southern blot analysis of genomic DNA isolated from vector-infected cells demonstrated that the vectors were capable of being propagated as expected without gross rearrangements or deletions. A fragment of the influenza A virus hemagglutinin (H5 HA) gene, capable of eliciting antibody and cytotoxic T-cell responses, was used as a marker for further characterization of the vector system. A Tat-dependent vector conferring the H5 HA^+ phenotype was assayed by indirect immunofluorescence, and cells which contained but did not express the H5 HA gene were isolated. The activation of H5 HA expression following HIV infection of Tat^- cells that stably contained but did not express the H5 HA construct was determined to be an efficient process.

A number of advances (for example, see references 37, 43, and 47) make the prospect of using gene therapy as a therapeutic regimen for a variety of human disease processes a realistic goal. Gene therapy could be used either to introduce functional copies of defective genes (for example, see reference 17) or to introduce foreign genes into cells to combat neoplastic or infectious diseases. Replication-defective retroviral vectors are currently the best-characterized method for introducing foreign genes into cells in a relatively controlled manner (reviewed in reference 35). Human trials using retroviral vectors to deliver the tumor necrosis factor or adenosine deaminase genes were recently instituted (3, 8) in efforts to assess their value as therapies for advanced melanoma and adenosine deaminase deficiency, respectively.

AIDS, caused by human immunodeficiency virus (HIV) (7), is a candidate for an infectious disease which might be combated by gene therapy. Because HIV predominantly infects cells of the hematopoietic system, pluri- or multipotent stem cells are potential targets for introduction of a foreign gene which might result in the presence of the gene in functional hematopoietic cells following proliferation and differentiation of the stem cells. Since HIV is a retrovirus, an HIV vector might be useful for delivery of genes of interest. In addition, the nature of specific structural and regulatory genes and of sequences necessary for viral replication suggests strategies for gene therapy that take advantage of HIV-specific *cis*- and *trans*-acting elements.

Relatively complex HIV-1 vectors that express marker genes have been developed to study viral replication (20, 26, 39). Although these could also be used for expression of other foreign genes in cells, it would be advantageous to use a simple vector in which expression is under control of an inducible promoter. The vectors described in this paper express the introduced gene only in the presence of the viral regulatory protein Tat, which is necessary for efficient expression from the HIV long terminal repeat (LTR) (11, 36, 46). Tat is an RNA-binding protein; its target sequence, the TAR region, is a 59-nucleotide sequence present in the newly transcribed R region of the 5′ LTR (42). Although many mechanisms of action have been proposed for Tat, it now appears that Tat acts, at least in part, by preventing premature termination of nascent HIV transcripts (13) in an as yet undetermined manner.

Since Tat is normally found only in cells infected with HIV, this type of vector might provide a means by which foreign genes that interfere with HIV replication could be expressed specifically in HIV-infected cells. This control of gene expression might decrease potential side effects associated with expression of the foreign gene in uninfected cells. Expression of foreign genes under the control of tissue-specific or inducible promoters has been used to ablate specific tissues in transgenic mice (9) and to interfere with viral replication (4, 21). In addition, induced expression of a foreign gene could be used to develop what has been termed intracellular immunity (5) but which, in fact, would more appropriately be viewed as a novel way to deliver therapy.

Examples of types of genes which might be useful in anti-HIV gene therapy include those encoding products that specifically interfere with the HIV life cycle or that are capable of contributing to an induced cytocidal effect. Such a cytocidal effect could result directly from a foreign gene product that is toxic to infected cells or indirectly from a gene product capable of eliciting a specific immune response which destroys infected cells. Genes encoding products to which a person typically has developed immunity (either via natural exposure or vaccination) are candidates for use in inducing a cytocidal effect. Cells that constitutively express hemagglutinin (HA) from one influenza virus strain can be targets for cytotoxic T cells (18). An inducible vector containing a fragment of the H5 HA gene from influenza virus

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† This article is dedicated to the memory of Delbert and Leona Buchschacher.

A/Ty/Ont/7732/66 (H5N9) was constructed. This vector was used in these studies as a marker to characterize further the Tat-inducible vector system and its usefulness for delivery of genes expressed from the HIV LTR.

MATERIALS AND METHODS

Vector construction. Standard techniques were used in molecular cloning. The HIV vectors were made in the following manner. To construct pGB102, pAP907 (14), a derivative of the infectious HIV-1 proviral clone pNL4-3 (1), was digested with *Spe*I (in the *gag* open reading frame [ORF]) and *Xba*I (in a polylinker introduced 3′ to the *env* ORF) and the ends were ligated to produce the intermediate clone pGB109. To introduce the hygromycin B phosphotransferase gene (Hyg^r) into pGB109, the plasmid was digested with *Bss*HII and treated with the Klenow fragment. The Hyg^r gene was obtained from pJD214hy (15) by digesting the plasmid with *Acc*I and *Cla*I, treating it with the Klenow fragment, and isolating the appropriate DNA fragment. This fragment was then blunt end ligated into the *Bss*HII-digested pGB109 (regenerating *Bss*HII sites on both sides) to produce the vector pGB102. To construct pGB106, pGB109 was digested with *Xmn*I and *Sma*I and the ends were ligated to produce the intermediate clone pGB105. The Hyg^r gene was isolated from pGB102 by digestion with *Bss*HII and then introduced into the *Bss*HII site of pGB105 to produce the vector pGB106. pGB112 was constructed by digestion of pGB109 with *Bss*HII, treatment with the Klenow fragment, and addition of an *Eco*RI linker to generate the intermediate pGB111. Virginia S. Hinshaw, University of Wisconsin—Madison, provided an H5 HA gene fragment (nucleotides 1 to 1072 [40]) from influenza virus A/Ty/Ont/7732/66 (H5N9) which had been cloned into an SP64-based construct. This was digested with *Hin*dIII and treated with the Klenow fragment, and then an *Eco*RI linker was added. Following digestion with *Eco*RI, the H5 HA fragment was isolated and inserted into the *Eco*RI site of pGB111 to generate the construct pGB112.

Virus production and infections. COS cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HeLa T4 cells (32) were maintained at 37°C in the same medium supplemented with 7% calf serum, antibiotics, and 800 μg of G418 per ml; HeLa *env-c* cells (provided by Miguel A. Gama Sosa, Dana-Farber Cancer Institute [22]) were maintained in this same medium but without G418. Five micrograms of each DNA was transfected into 10^6 COS cells on 60-mm-diameter tissue culture dishes by the DEAE-dextran procedure (12). On the second day posttransfection, COS cell medium was changed and 4×10^5 HeLa T4 or HeLa *env-c* cells were plated onto 60-mm-diameter dishes for infection the following day. For the cell-free infection, medium was collected from the transfected COS cells and subjected to low-speed centrifugation to remove cell debris. The top portion of the supernatant was removed, and DEAE-dextran was added to a final concentration of 8 μg/ml. One-half milliliter of this supernatant was added to the HeLa cells. Following a 3-h incubation, fresh medium was added. Two days postinfection, selection was initiated for infected cells by fluid changing with medium that contained 200 μg of hygromycin B per ml. Cells were cultured for 7 to 10 days, and the virus titer was determined by fixing and staining the cells with 0.5% crystal violet in 50% methanol and counting resistant colonies.

HeLa cell transfection and selection of Hyg^r cells. For transfection of HeLa T4 or HeLa *env-c* cells, 5×10^5 cells were plated onto 60-mm-diameter dishes. The following day, cells were transfected by the calcium phosphate coprecipitation method (23) with a 15% glycerol shock for 4 min. For single transfections, 11 μg of DNA was used; for cotransfection of a vector and Tat-expressing construct, 8 μg of each DNA was used; for cotransfection of pJD214hy and pGB111 or pGB112, a 20:1 ratio of the two DNAs was used. To determine whether Hyg^r colonies were produced, selection was begun with 200 μg of hygromycin per ml 2 days posttransfection and 7 to 10 days later, if appropriate, resistant colonies were counted as described above. For cells cotransfected with pJD214Hy and pGB111 or pGB112, resistant colonies were pooled or isolated and analyzed by indirect immunofluorescence.

Determination of H5 HA expression. To determine H5 HA expression, indirect immunofluorescence was performed as described previously (51) by using rabbit antiserum prepared to the influenza virus A/Ty/Ont/7732/66 (H5N9), provided by V. S. Hinshaw, at a 1:100 dilution followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody at a 1:40 dilution. When H5 HA and HIV proteins were simultaneously visualized, either FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG antibody (1:32 dilution) was used for H5 HA visualization. For detection of HIV proteins, AIDS patient serum (1:100) and TRITC- or FITC-conjugated goat anti-human antibody (1:40) was used. Filters were used to avoid false-positive signals resulting from leakage of tetramethylrhodamine or fluorescein signals into inappropriate channels. Conjugated antibodies were purchased from Sigma Chemical Co., St. Louis, Mo. To determine H5 HA expression following transfection, 2×10^5 HeLa T4 or HeLa *env-c* cells were plated onto glass coverslips in 35-mm-diameter dishes for transfection as described above. To determine whether the H5 HA gene could be expressed from the HIV-1 vector following infection, virus production and infection were performed as above except that 2×10^5 cells plated on glass coverslips were infected. To determine whether HeLa T4 cells containing the H5 HA-expressing construct could be induced to express H5 HA, GB111- or GB112-containing cells were infected as described above with replication-defective HIV (GB107) pseudotyped with murine amphotropic envelope. Two days posttransfection or postinfection, cells were fixed in ice-cold methanol containing trace acetone.

Southern blotting. Isolated or pooled GB102-infected hygromycin-resistant HeLa *env-c* colonies and pooled GB106-infected hygromycin-resistant colonies were grown, and genomic DNA was isolated by conventional methods. Fifteen micrograms of DNA was used in restriction enzyme digestion. Following electrophoresis through a 0.7% agarose gel, blotting and hybridization were performed as previously described (45) except that salmon sperm or testis DNA was used as the blocking DNA. A double-stranded DNA fragment containing the Hyg^r gene was used as a probe following labeling with [α-^{32}P]dCTP using the random hexamer Prime-a-Gene Labeling System (Promega Biotec, Madison, Wis.). Approximately 7×10^6 cpm of denatured probe per ml was used in the hybridization reaction; incubation was carried out at 65°C for 8 h. The filters were washed with 0.1× SSC (NaCl plus sodium citrate)–0.1% sodium dodecyl sulfate for 2 h at 55°C with agitation, the wash buffer being changed every 20 min. The filters were air dried and exposed to Kodak XAR-5 X-ray film, using a Du Pont Cronex Lightning-Plus intensifying screen.

RESULTS

Development of a Tat-dependent vector system. To construct a simple vector that would be dependent upon Tat for expression of foreign genes, large deletions were made in the infectious proviral clone pNL4-3 (1). Observations with other retroviruses (6, 29, 50) and previous mutational analysis of HIV (2, 10, 30) demonstrate that the untranslated leader region of the genomic-length viral RNA is necessary for efficient packaging. Since the HIV leader region is relatively short, it seemed likely that this encapsidation sequence extends into the *gag* ORF. For this reason, we constructed two vectors (GB102 and GB106; Fig. 1) that contain different lengths of the 5′ *gag* gene. GB106 contains sequences that extend 53 bp into the gene, whereas GB102 contains the first 722 bp of the *gag* ORF. In both vectors the hygromycin B phosphotransferase gene (Hyg^r) gene was placed upstream of the splice donor site so that it would be expressed directly from the LTR. This location of the expressed gene would be likely to prevent expression of the truncated *gag* genes and also results in localization of the putative encapsidation site to near the 3′ end of the genome.

Because Hyg^r expression from these vectors was predicted to be Tat dependent, both HeLa T4 (32) (Tat$^-$) cells and HeLa *env-c* cells (22), which express Tat, were used as target cells for infection. Since HeLa *env-c* cells do not express the major HIV receptor, CD4, a murine amphotropic retrovirus envelope protein was used to pseudotype the vector virus and thereby allow entry into these cells. Virus was produced by transfecting COS cells with vector DNA, a plasmid that expresses all viral proteins necessary for particle production except the envelope glycoprotein (pGB107; Fig. 1), and a plasmid that expresses the envelope glycoprotein of an amphotropic murine leukemia retrovirus (pJD1; Fig. 1). Virus was then harvested, and target cells were infected. The cells were then selected with hygromycin B, and the titer of the vector virus produced was determined by fixing, staining, and counting resistant colonies.

When tested by this assay, GB102 consistently gave a modestly higher titer than GB106 when HeLa *env-c* cells were infected (Table 1). Use of the GB102 vector resulted in a titer of about 100 hygromycin-resistant CFU/ml of supernatant harvested from transfected cells. The GB106 vector resulted in a titer of about 20 CFU/ml. The GB102 vector therefore results in an approximately fivefold higher titer than GB106. No Hyg^r colonies resulted from infection of HeLa T4 cells with GB102 or GB106, demonstrating the Tat dependence of the Hyg^r phenotype following introduction of a single copy of the vector into cells (Table 1). Infection with an HIV-1 vector that expresses the Hyg^r gene from a Tat-independent promoter (GB108; Fig. 1 [20]) resulted in hygromycin-resistant colonies of both cells types. Additional experiments involving introduction of the vector into HeLa T4 cells in the presence or absence of a plasmid that expresses Tat indicated that the level of Tat independence of the vector was approximately 5% (data not shown).

Since GB102 is propagated more efficiently than GB106, it is likely that the 5′ end of the *gag* gene is an ancillary *cis*-acting sequence that facilitates virus propagation. The difference in titer between GB102 and GB106 was not a result of a difference in the ability to express the Hyg^r gene or the ability of the constructs to be transfected; when DNAs were transfected into HeLa *env-c* cells (Tat$^+$) or cotransfected with a Tat-expressing construct into HeLa T4 cells with subsequent hygromycin selection, no difference in the number of Hyg^r colonies was detected. In addition, the fact

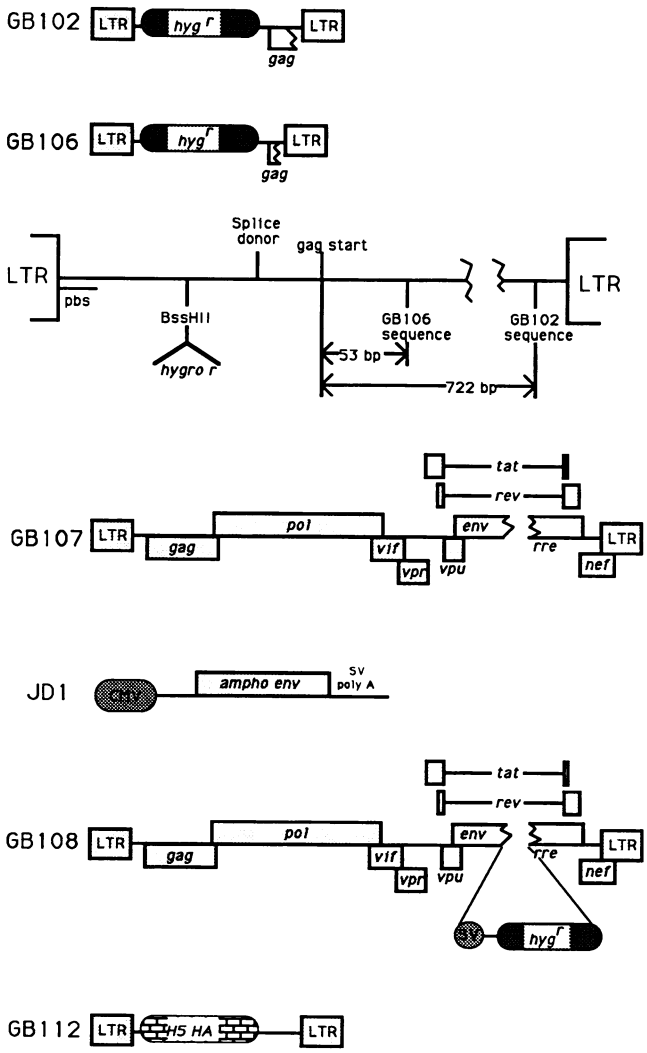

FIG. 1. HIV-1 vectors and expression constructs. Modifications of the infectious molecular clone pNL4-3 were made to generate the HIV vectors and protein-expressing constructs shown. GB102 and GB106 are HIV-1 vectors capable of expressing the Hyg^r gene. Details of their structures are shown below. GB102 and GB106 are identical except for the extent to which *gag* sequences are left intact. GB102 extends 722 bp and GB106 extends 53 bp into the *gag* ORF. The replication-defective helper construct GB107 (20) contains a 426-bp in-frame deletion in the *env* ORF but is capable of expressing other proteins necessary for viral propagation. GB108 (20) is a HIV-1 vector that expresses the Hyg^r gene from the simian virus 40 (SV) early promoter. The murine amphotropic envelope used for pseudotyping HIV-1 core particles is expressed from the cytomeyalovirus (CMV) intermediate-early gene promoter (on the plasmid pJD1 [16]). GB112 is a vector that contains HIV sequences identical to those of GB102; it is capable of expressing a fragment of the influenza virus H5 HA gene directly from the LTR.

that both vectors could be propagated to some degree indicates that placement of the HIV-1 encapsidation region to the 3′ end of the genome still allows RNA packaging, as has been seen with other retroviruses (34).

To determine whether the vectors had been propagated as expected, the structures of proviruses in Hyg^r cells were examined. Genomic DNA from infected, selected HeLa *env-c* cells was prepared from either pooled (GB102 and

TABLE 1. Relative titers of GB102 and GB106 vectors following infection of HeLa *env-c* cells

Expt no.	Hyg^r CFU/ml[a]		
	GB102	GB106	GB102/GB106
1	80	20	4.0
2	100	20	5.0
3	104	24	4.3
4	100	20	5.0
Mean	96 ± 10	21 ± 2	4.6 ± 0.5

[a] The results of four separate experiments are shown. Control values (seen when attempts were made to harvest virus following transfection of COS cells with no DNA, pGB102, pGB106, pGB107, pGB102 plus pGB107, pGB106 plus pGB107, or pGB107 plus pJD1) were all zero. Also, values in experiments using HeLa T4 cells were all zero.

GB106) or isolated (GB102) colonies and used in Southern blot analysis. Following digestion with *Hin*dIII, which cuts once in each proviral LTR, the DNA was electrophoresed, blotted, and probed with a double-stranded DNA fragment of the Hyg^r gene labeled with [α-^{32}P]dCTP. Following autoradiography, bands approximately 2.7 kb in size for GB102 and 2.0 kb for GB106, corresponding to the expected sizes of the respective proviruses, were observed (Fig. 2). Further Southern blot analysis using other restriction enzymes confirmed that the vectors had been propagated without gross rearrangements or deletions. Recombination between vector and defective helper constructs would be expected to result in a Hyg^r-expressing construct with intact HIV ORFs, which would have resulted in the presence of a band of a discrete size. However, no such recombinants were observed. This result indicates that the increased titer of GB102 was not due to recombination resulting from additional *gag* sequences on that vector compared with GB106 but was due to a real difference in ability to be propagated.

We wanted to ensure that the virus was undergoing only a single replication cycle. In addition, we tried to determine whether detectable replication-competent HIV might have been formed by recombination following transfection. Medium was harvested from infected HeLa *env-c* cells, and attempts were made to infect HeLa T4 cells, which are susceptible to infection by virus containing HIV-1 envelope glycoprotein. Also, virus-containing medium from transfected COS cells was used in attempts to infect the HeLa T4 cells. No cytopathic effects were observed in either case. When wild-type HIV produced by transient transfection of COS cells was used to infect these cells, approximately 5,000 syncytium-forming units per ml were observed by a focal immunofluorescence assay.

Tat-dependent expression of a foreign viral gene fragment. We wanted to determine whether a gene on an endogenous Tat-dependent vector could be induced by infection with HIV-1. Moreover, we attempted to construct a vector carrying a gene that could inhibit the generation of progeny virus particles by signalling the immune system to destroy the cell upon infection by HIV. The H5 HA gene of influenza virus A/Ty/Ont/7732/66 (H5N9) is known to elicit murine antibody and cytotoxic T-lymphocyte responses that can result in specific destruction of influenza virus-infected cells (28). Therefore, we inserted a fragment of the H5 HA gene (40) known to contain T- and B-cell antigenic sites (27) in place of the Hyg^r gene of GB102 to generate a vector designated GB112 (Fig. 1). The inserted H5 HA gene encodes the entire HA1 subunit and the first 5 amino acids of

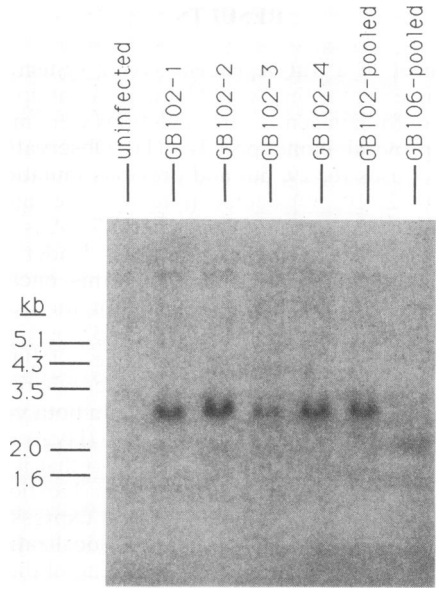

FIG. 2. Southern blot analysis of proviruses from GB102- and GB106-infected HeLa *env-c* cells. Genomic DNA was obtained from isolated (for GB102) or pooled (GB102 or GB106) colonies and digested with *Hin*dIII, which cuts once in each LTR. A ^{32}P-labeled double-stranded DNA fragment of the Hyg^r gene was used as a probe. Fragments of 2.7 kb for GB102 and 2.0 kb for GB106, corresponding to the predicted sizes, were seen, indicating that the vectors had been propagated as expected without gross rearrangements, deletions, or recombination. The structures of the proviruses were confirmed by further Southern blot analysis following digestion with other restriction enzymes.

HA2. In these studies, this construct was used as a marker for expression from the HIV LTR. Eventual evaluation of its use as a functional inducer of cell death via cytotoxic T cells will be dependent upon isolation of an appropriate target cell line which contains the construct. Since at this time the anti-H5 HA cytotoxic T cells which are available are murine major histocompatibility complex restricted (28), a system in which the vector's effect on both HIV infection and the response of cytotoxic T cells can be evaluated is not available.

We first investigated the ability of the H5 HA fragment to be expressed in a Tat-dependent manner. Either pGB112 or a control HIV vector without a foreign gene (pGB111) was transfected in parallel into HeLa *env-c* cells by the calcium phosphate coprecipitation method. In a complementary experiment, an adherent Tat$^-$ cell line (HeLa T4) and a human T-cell line (A3.01) were transfected with various combinations of pGB112 and the Tat-expressing plasmids pSV$_2$tat72 (19) or pGB107. Two days posttransfection, cells were fixed in methanol containing trace acetone and H5 HA expression was determined by indirect immunofluorescence using rabbit antiserum to influenza virus A/Ty/Ont/7732/66. Only cells that had been transfected with both GB112 and Tat-expressing constructs were positive for H5 HA. Examples of H5 HA$^+$ HeLa T4 and HeLa *env-c* foci are shown in Fig. 3A. To determine whether H5 HA expression could be detected following infection with GB112, virus was produced via COS cell transfection (as described above) by using GB111 or GB112 as the vector and was used to infect HeLa *env-c* cells. H5 HA expression, observed 2 days later following indirect immunofluorescence, indicates that the H5 HA gene frag-

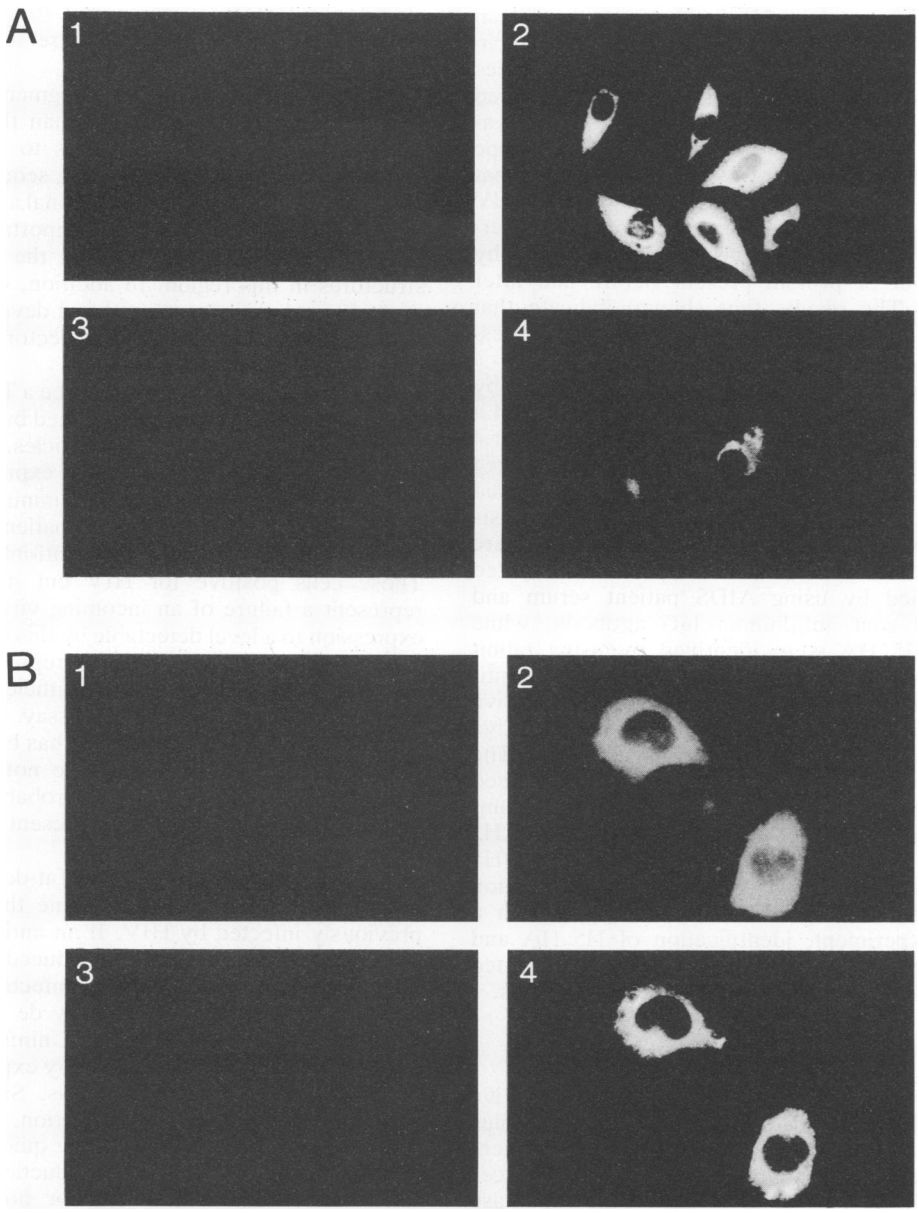

FIG. 3. Immunofluorescence analysis of cells containing H5 HA constructs. (A) Cells (2×10^5) on glass coverslips were transfected by the calcium phosphate coprecipitation method and analyzed for H5 HA expression 2 days later by using rabbit anti-influenza A virus serum and FITC-conjugated goat anti-rabbit IgG antibody following methanol-acetone fixing of the cells. For panels 1 and 3, no positive foci were seen when the entire coverslip was examined. Panel 1, HeLa *env-c* (Tat^+) cells transfected with GB111 (HIV vector with no H5 HA sequences present); panel 2, HeLa *env-c* (Tat^+) transfected with GB112 (expresses H5 HA from HIV LTR); panel 3, HeLa T4 (Tat^-) cells transfected with GB112; panel 4, HeLa T4 cells cotransfected with GB112 and pSV_2tat72. (B) Activation by infection with GB107 (defective HIV construct) of GB112 which is stably maintained but not expressed in HeLa T4 cells (HeLa T4/112 cells) shows coexpression of HIV and H5 HA proteins. Two days following infection with GB107 (approximately 5×10^3 infectious units per ml), HIV protein expression was monitored by using AIDS patient serum and TRITC-conjugated goat anti-human IgG antibody. H5 HA expression was monitored by using rabbit anti-influenza A virus serum and FITC-conjugated goat anti-rabbit IgG antibody. For uninfected cells, no positive H5 HA or HIV foci were seen when the entire coverslip was examined. Panels 1 and 3 and panels 2 and 4 show the same cells photographed with two different filters. Panel 1, HeLa T4/112 uninfected cells, HIV protein expression; panel 2, HeLa T4/112 cells infected with GB107, HIV protein expression; panel 3, HeLa T4/112 uninfected cells, H5 HA protein expression; panel 4, HeLa T4/112 cells infected with GB107, H5 HA expression.

ment is capable of being propagated on the vector and expressed following infection of Tat^+ cells.

For this type of vector system to be useful in abrogation of progeny virus production, it must be possible to introduce the construct into Tat^- cells where it can be stably maintained but not expressed until activated by infection. To determine whether this would be possible, we generated a population of HeLa T4 cells that harbor GB112 (HeLa T4/112 cells) but do not express the H5 HA gene. HeLa T4 cells were cotransfected with either pGB111 or pGB112 and

a construct that expresses the Hyg^r gene from the spleen necrosis virus LTR (pJD214hy) (15). Following hygromycin selection, resistant GB111- or GB112-containing colonies were pooled. These HeLa T4/112 cells were then infected with GB107 (an env-minus, Tat-expressing HIV-1 derivative) that had been pseudotyped with amphotropic envelope glycoprotein. H5 HA expression was seen only in cells that stably contained GB112 and had been infected with the HIV construct. We have also studied the expression of an alternate foreign gene introduced into cells in a similar manner by examining the level of protein present before and after induction by Tat. The results (not shown) indicate that without Tat there is an undetectable level of protein expression when analyzed by immunoprecipitation.

To determine the efficiency of H5 HA gene activation by HIV infection, we isolated and maintained individual HeLa T4/112 clones in culture without hygromycin selection for 4 months. Such clones were identified by screening isolated colonies by transfection with a Tat-expressing plasmid (pSV_2tat72) and subsequent immunofluorescence analysis with anti-HA antiserum. Three HeLa T4/112 clones were expanded and infected with HIV (GB107) particles. Infected cells were identified by using AIDS patient serum and TRITC-conjugated goat anti-human IgG antibody, while cells expressing H5 HA were identified by using rabbit anti-influenza virus antibody and FITC-conjugated goat anti-rabbit IgG antibody. Figure 3B shows a representative example of H5 HA activation. One hundred HIV-positive foci were examined for H5 HA protein expression. The results demonstrated that, on average, 94% of foci expressed both HIV and H5 HA proteins. Approximately equal numbers of the remaining foci were positive for only HIV or H5 HA signals. Variation in the strength of both HIV and H5 HA signals was seen, although there was no correlation between the relative strengths of the two signals. In a complementary experiment, identification of H5 HA and HIV proteins was also done by using FITC-conjugated anti-human and TRITC-conjugated anti-rabbit antibodies.

DISCUSSION

Comparison of two HIV-1 vectors that vary in the putative RNA encapsidation site indicate that the leader region plus approximately 50 nucleotides of the *gag* gene are sufficient for viral nucleic acid propagation. However, the sequences extending further into the *gag* gene significantly increase vector propagation. Although the reason for the difference in vector propagation is not known, the most likely explanation, based on comparison of encapsidation sequences of other retroviruses (6, 29, 50), is that there is a difference in the abilities of the RNAs to be incorporated into virus particles. This interpretation is supported by recent work that indicates that regions of the HIV genome that extend into the *gag* gene are capable of binding purified Gag protein (31). A segment of one RNA sequence that binds to Gag is present in both GB106 and GB102. A second sequence with the ability to bind Gag is present only in GB102. In addition, these sequences have the potential to form complex stem-loop structures which might function in encapsidation and/or RNA-RNA dimer formation. Our observations are consistent with a situation analogous to the *psi* and *psi*$^+$ encapsidation signals of the murine amphotropic retrovirus (6). A vector with *cis*-acting HIV-1 sequences similar to GB106 but with an internal murine retroviral LTR, an internal heterologous polyadenylation site, and an alternative selectable gene which is constitutively expressed has been described elsewhere (41). This vector was successfully propagated, indicating that these sequences are sufficient for propagation.

Since the vector with a longer segment of the 5′ *gag* region was replicated more efficiently than the shorter vector, it would probably be advantageous to use this vector and derivatives to study the *cis*-acting sequences necessary for virus propagation. Further mutational analysis could be used to identify the exact sequences important for encapsidation of HIV and the significance of the potential stem-loop structures in this region. In addition, ways to increase the titer of vector virus produced and development of an alternate system for delivery of HIV vectors are currently being investigated.

It was possible to stably introduce a Tat-responsive vector into cells such that it was maintained but not expressed until infected by exogenous HIV-1 particles. Infection resulted in efficient (about 95%) induction of expression from the vector, on the basis of double-label immunofluorescence with antisera against the virus (AIDS patient serum) and against the Tat-inducible gene product (anti-influenza virus serum). Those cells positive for HIV but negative for H5 HA represent a failure of an incoming virus to activate vector expression to a level detectable by this assay. Those positive for H5 HA but negative for HIV represent either spontaneous activation of the vector or insufficient expression of HIV proteins for detection in the assay. However, since no spontaneous activation of GB112 has been observed in cells that contain the vector but have not been exposed to a Tat-expressing construct, it is probable that the H5 HA-positive, HIV-negative cells represent inapparent infection by HIV.

The construction of a simple Tat-dependent HIV vector might be useful for anti-HIV gene therapy of individuals previously infected by HIV. If an antiviral gene expressed from the HIV LTR can be introduced into a subset of the HIV target cells prior to HIV infection, gene expression might be specifically activated by de novo Tat expression from an incoming virus (Fig. 4). Uninfected cells would not express, or would very inefficiently express, the gene, since Tat is not present in those cells. Since Tat would also promote wild-type virus production, the efficacy of this therapy would depend upon how quickly the infected cell could be destroyed or virus production could be inhibited relative to the time required for progeny virions to be formed. However, if some virus particles are released, this would also lead to propagation of the vector to other, uninfected cells. This would benefit an individual by increasing the number of cells which contain the antiviral gene.

Since this type of strategy involves the expression of genes which are potentially toxic, stringent control of gene expression may be necessary for such an approach to be successful as a therapeutic modality. In the experiments presented here, we measured expression by the production of an easily recognizable phenotype. The significance of background (Tat-independent) expression is unclear and would likely depend on the particular foreign gene on the vector, the cell type infected (unpublished observations and reference 25), or the presence of other factors known to affect LTR expression (for example, see reference 38). It is conceivable that minimal expression of particular genes has no detrimental effect while a similar level of expression of an alternative gene is toxic to the cell. Addition of the *cis* acting Rev-responsive element to the vector, as has been done with other inducible systems (25, 49) using partial HIV-1 LTRs,

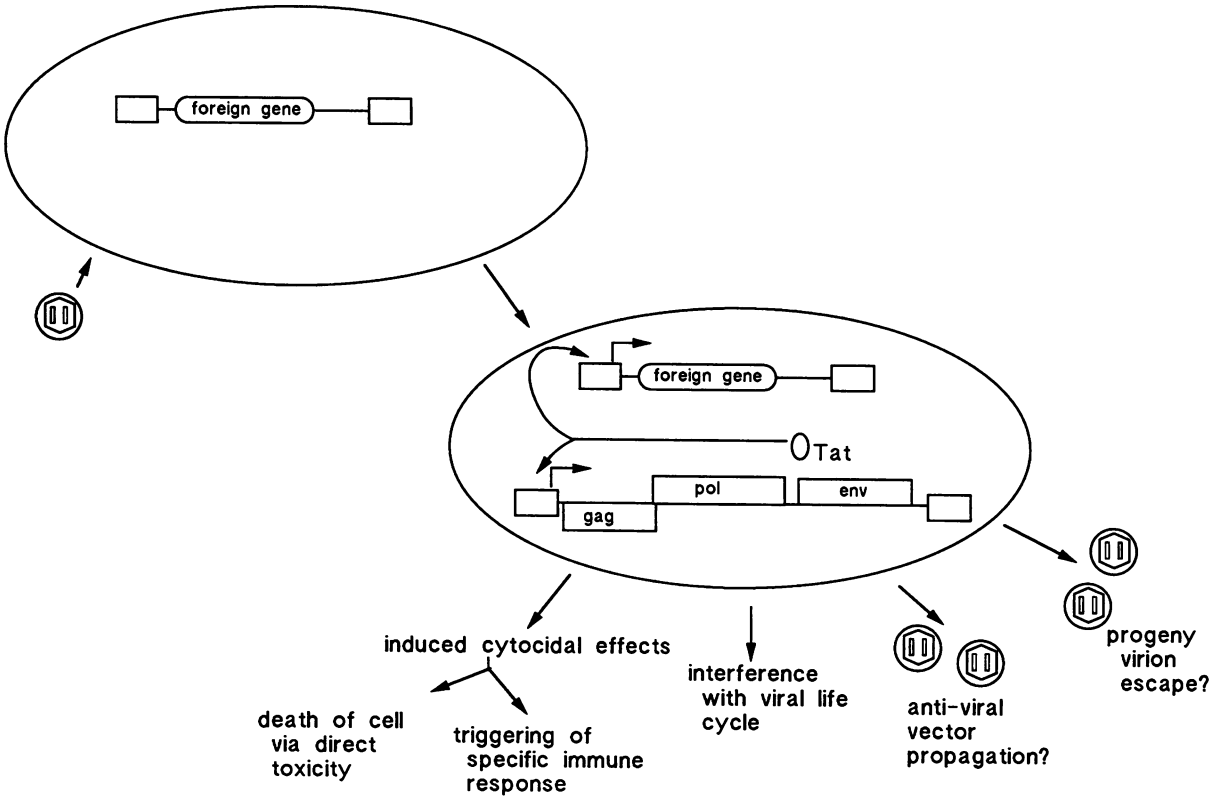

FIG. 4. How inducible gene therapy for HIV infection might work. A construct which contains a foreign gene under control of the HIV LTR is introduced into and stably maintained in a cell susceptible to HIV infection. Because such a cell would not contain the Tat protein necessary for efficient expression from the LTR, the foreign gene would not be expressed to a significant degree. This might decrease potential side effects associated with foreign gene expression. Foreign gene expression is induced by wild-type virus infection. The wild-type virus produces Tat which activates expression from both vector and virus LTRs. The choice of the foreign gene used would determine the effect on the cell. Infected cells could be specifically destroyed by using genes which cause an induced cytocidal effect through either a direct or an indirect mechanism. Alternatively, the release of progeny virus from infected cells might be prevented by using genes which specifically interfere with the HIV life cycle. In either case, the goal of the therapy would be to prevent or slow the spread of HIV within an individual. Because Tat would also be increasing expression of viral proteins, the efficiency of this therapy would depend upon how quickly the infected cell could be destroyed or viral replication could be interfered with relative to the time that it would take for progeny virions to be released. One benefit of using the HIV vector is that any wild-type virus escape which may occur might also be accompanied by vector virus release which could spread to other, uninfected cells, thereby increasing the number of cells containing the antiviral gene.

may offer increased control of gene expression and act as an additional safeguard against inappropriate gene expression.

Types of antiviral genes that might be used to eliminate or slow the spread of HIV include those coding for proteins which directly inhibit the production of infectious HIV. Examples are dominant negative mutants (20, 24, 33, 48) or ribozymes specific for viral RNA sequences (44, 52). For these cases, although the infected cell would remain in the body, virus production would be decreased or prevented. Another class of antiviral genes is those that would directly or indirectly eliminate infected cells through an induced cytocidal effect. Activation of expression of this gene by an incoming virus would induce the cell to commit suicide. Induction of a gene coding for a protein which is toxic to the cell, for example, diphtheria toxin (25) or herpes simplex thymidine kinase (49), would have a direct cytocidal effect. A gene coding for a protein that signals the immune system to destroy the cell (for example, HA) would be an example of an indirect cytocidal effect. Activation of expression of this gene by an incoming virus would result in the production of a protein that the immune system would recognize as foreign. A gene of one of the infectious agents against which human populations are routinely immunized might serve as a suitable foreign gene.

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