4836–4842 Nucleic Acids Research, 1993, Vol. 21, No. 20

Recombinant retroviral systems for the analysis of drug resistant HIV

Roger K.Strair, Daniel J.Medina, Carol J.Nelson, Timothy A.Graubert and John W.Mellors[1]
Division of Medical Oncology, Yale University School of Medicine, New Haven, CT 06510 and
[1]Division of Infectious Diseases, University of Pittsburgh School of Medicine and the Veterans Affairs Medical Center, Pittsburgh, PA, USA

Received February 10, 1993; Revised and Accepted August 27, 1993

ABSTRACT

Two recombinant retroviral systems are described that can be used to analyze antiretroviral drug activity and HIV breakthrough (replication in the presence of the drug). The first system utilizes a recombinant HIV encoding beta-galactosidase as a reporter gene (HIV-LacZ). The defective HIV-LacZ virus is produced in COS cells after co-transfection of a plasmid encoding the HIV-LacZ genome with a plasmid encoding HIV proteins necessary for packaging and infectivity. Subsequent infection of CD4+ target cells, followed by assay for LacZ expression, permits the rapid identification of individual virus-infected cells. This system can be used to quantitate the inhibition of early events in the HIV replicative cycle and is suitable for the screening of compounds for anti-HIV activity. However, this system cannot be used to analyze HIV drug resistance because of the limited genetic heterogeneity of the virus that is produced in COS cells. To circumvent this problem, a second system has been developed in which heterogenous recombinant HIV is produced by rescue with replication-competent 'helper' HIV. This system required the production of CD4+ cell lines containing defective proviruses encoding either LacZ or guanosine phosphoribosyl transferase (gpt). The defective proviruses are rescued by infection of the cell lines with 'helper' HIV and used to infect target cells in the presence of antiretroviral agents. Subsequent reporter gene assay is used to identify virus-infected cells. This system has been used to detect rare HIV breakthrough infection of cells in the presence of the non-nucleoside reverse transcriptase inhibitor TIBO R82150. Similar analyses with other antiretroviral agents, alone and in combination, may help identify therapeutic strategies that minimize breakthrough replication of HIV.

INTRODUCTION

A significant obstacle to effective chemotherapy of viral disease is the continued replication of a subfraction of the virus population despite the presence of the antiviral agent (viral breakthrough). Even agents that are potent inhibitors *in vitro* (e.g. 3′-azidothymidine [AZT]) allow breakthrough viremia *in vivo* (1). There are several possible mechanisms responsible for this viral breakthrough. Viral variants encoding drug resistance mutations may pre-exist within the virus population and emerge as the dominant replicating species when the selective pressure of an antiviral agent is applied. In the case of HIV-1, variants resistant to nucleoside and non-nucleoside reverse transcriptase inhibitors have readily emerged after drug selection *in vitro* and *in vivo* (3,4,6,7,8,12,14). These resistant strains probably contribute to the breakthrough viremia and disease progression that has been observed in patients on long term antiretroviral therapy (2,18).

Another mechanism by which viral breakthrough can occur involves cellular heterogeneity with regard to drug uptake, activation or elimination. This 'cellular' drug resistance renders the antiviral agent ineffective in a subset of the host cell population. For example, the metabolism and antiretroviral activity of certain nucleoside analogs (e.g. AZT) is dramatically influenced by the growth and activation state of the host cell (15,17).

There are currently no experimental systems available to systematically quantitate and differentiate the mechanisms responsible for breakthrough replication of HIV-1 in the presence of various antiretroviral agents. There are also no published data available on the frequency with which drug-resistant mutants spontaneously exist within unselected virus populations. Such information is needed to design more effective combination antiviral therapies that minimize or eliminate viral breakthrough.

This report describes two novel recombinant HIV systems that allow the detection of rare virus-infected cells. In the first system a recombinant HIV encoding beta-galactosidase (HIV-LacZ) is used to study the antiretroviral effects of drugs that inhibit early stages of the HIV replicative cycle. This system uses recombinant HIV produced in COS cells after co-transfection of a plasmid encoding the defective HIV-LacZ genome with a plasmid encoding the complementing HIV genes necessary for the production of an infectious recombinant virus. The limited genetic heterogeneity of these recombinant viruses, however, does not allow an analysis of the contribution of drug-resistant variants

to HIV breakthrough events. To achieve this, a second system was used in which recombinant virus was rescued from CD4+ cell lines by rescue with replication-competent 'helper' HIV. Since the defective virus depends upon the helper HIV for many of the proteins necessary for infectivity, the rescued defective genome carrying a reporter gene can serve as an indicator of the drug sensitivity and resistance of the replication-competent population used as the source of helper virus.

MATERIALS AND METHODS

Construction of recombinant proviral DNA

The HIV constructs encoding LacZ are shown schematically in Figure 1. The HIV-gpt and HXB2env plasmids were provided by Kathleen Page (University of California, San Francisco, CA) (9). The HIV-gpt plasmid contains an HXB2 provirus into which an SV40 promoter-gpt gene was inserted into the env region. The HXB2 env plasmid contains the HXB2 gp160 gene driven by an SV40 promoter. The BH10 plasmid was obtained from F. Wong-Staal and R. Gallo (NCI, Bethesda, MD.). The CMV/HIV-gag-pol-env (CGPE-) plasmid was provided by A. Brodsky and A. Nienhuis (NIH, Bethesda, MD.) and contains a CMV promoter driving the expression of an HIV genome from which the LTRs and putative packaging sequence have been deleted (13).

An infectious proviral clone of HIV-1(pHIV-1) was reconstructed from the HIV-gpt and BH10 plasmids by ligating the internal Sal I-Bam HI fragment from BH10 into Sal I and Bam HI digested HIV-gpt. HIV(Bal I-Bal I)H4 LacZ and HIV(Bal I-Bal I)CMVLacZ were made by digesting pHIV-1 with Bal I and inserting a 3.6 kb DNA fragment containing either the histone H4 promoter or the CMV promoter adjacent to the LacZ gene (16). These constructs also had an SV40 origin of replication inserted into the plasmid backbone. The HIV(Bal I-Bam HI)SV40LacZ, HIV(Bal I-Bam HI)CMVLacZ and HIV(Bal I-Bam HI) CMVLaczpA(ro) constructs were prepared by digesting

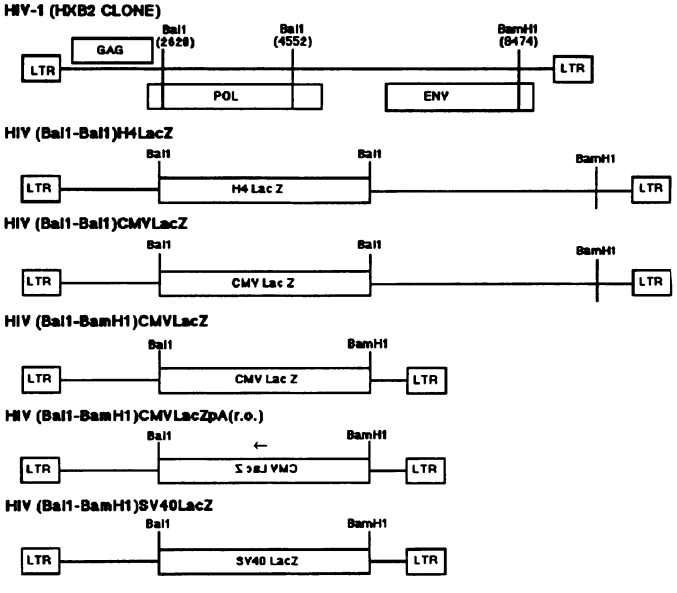

Figure 1. Schematic representation of recombinant retroviral constructs. Plasmids were constructed as described in Materials and Methods.

pHIV-1 with Bal I and Bam HI and inserting a 3.6 kb DNA fragment containing either the CMV early promoter or the SV40 promoter adjacent to the LacZ gene. The HIV (Bal I-Bam HI) CMVLacZpA(ro) construct contained the CMV promoter adjacent to the LacZ gene and a 3′ SV40 polyadenylation site inserted in reverse orientation (ro) to the HIV backbone. These HIV(Bal I-Bam HI) constructs also had an SV40 origin of replication inserted into the plasmid backbone.

Production of 'plasmid derived' recombinant retroviruses

All transfections and cell culture were performed in an approved BSL-2 facility with BSL-3 techniques. Plasmid DNA co-transfections into COS cells were performed as described by Page et al (9). Supernatants from COS cells were collected 40 hours after transfection and assayed for infectious recombinant HIV-LacZ virus by inoculating 2×10^5 HeLa-T4 cells (5) with 0.1 ml of filtered (0.45 μm) supernatant. Cells were stained for beta-galactosidase activity with X-gal 48 hours after infection as described (16). To assay for infectious recombinant HIV-gpt virus, the infected HeLa-T4 cells were trypsinized and split 1:10 into gpt selective media as described (9). Medium changes were performed every 3 days and colonies were counted 14 days post-infection after staining with 1% crystal violet in 10% formalin.

Production of cell lines containing defective HIV-gpt and HIV-LacZ

The H9/HIV-gpt cell line was prepared by infecting H9 cells with HIV-gpt followed by selection in gpt selective media. The HeLa-T4/HIV-LacZ cell line was prepared by calcium:phosphate co-transfection of HeLa-T4 cells with the HIV(Bal I-Bam HI) SV40LacZ and PSV2GPT plasmids (10:1) followed by selection of clones in gpt selective media. Individual colonies were isolated, expanded and assayed for beta-galactosidase expression with X-gal staining (16). Isolated colonies containing beta-galactosidase were infected with HIV-1_{IIIB} at an m.o.i. of 0.1 and five to seven days later the culture supernatant was harvested, filtered (0.45μm) and assayed for infectious HIV-LacZ by infecting HeLa-T4 cells with 0.1 ml of supernatant followed by staining for beta-galactosidase activity after 48 hours. One HeLa T4/HIV-LacZ cell line consistently produced $> 1\times10^3$ infectious HIV-LacZ/ml of culture media after rescue infection with HIV-1_{IIIB}.

Rescue of defective retroviruses

Rescue of defective retroviruses from the H9/HIVgpt cell line and the HeLa T4/ HIV-LacZ cell line were performed as described above except that the specific virus population used for rescue was varied to include known drug-resistant and drug-sensitive viruses. Following each rescue infection, the resultant titer of HIV-LacZ or HIV-gpt was was determined and the inoculum used to infect HeLa-T4 cells was adjusted depending upon the number of infectious events to be analyzed.

RESULTS

Production and characterization of recombinant HIVs encoding beta-galactosidase

A previous study demonstrated the utility of murine retroviruses encoding beta-galactosidase in characterizing the activity of antiretroviral compounds (15). To extend these studies to HIV we sought to develop an HIV based recombinant retrovirus containing a LacZ reporter gene. The strategy used to generate infectious recombinant HIV was similar to that previously

described by Page et al. for the production of HIV-gpt (9). A recombinant HIV plasmid encoding LacZ was co-transfected into COS cells with one or more complementing plasmids containing the HIV regulatory and structural genes necessary for production of infectious recombinant virus. The HIV-LacZ plasmids constructed are shown schematically in Figure 1. In initial experiments, the HIV(Bal I-Bal I)H4LacZ plasmid was complemented with the HIV-gpt plasmid. Transfection of the HIV(Bal I-Bal I)H4LacZ plasmid without a complementing plasmid served as the control.

The titer of infectious HIV-LacZ produced by COS cell co-transfection was quantitated by counting the number of beta-galactosidase positive HeLa-T4 cells per 35-cm^2 well following infection with 0.1 ml of COS cell supernatant. As shown in Table I, initial co-transfections of HIV(Bal I-Bal I)H4LacZ with the HIV-gpt plasmid produced $2-4\times10^2$ infectious units/ml of HIV-LacZ. Further complementation of HIV(Bal I-Bal I)H4LacZ with the HXB2env plasmid in addition to the HIV-gpt plasmid increased the titer of HIV-LacZ to approximately 1×10^3/ml (Table I).

Several other HIV-LacZ constructs were made in an attempt to increase the efficiency of virus production (Figure 1 and Table I). In the HIV(Bal I-Bal I) CMVLacZ construct, the H4 promoter was replaced with a CMV promoter. Although this increased the intensity of X-gal staining of individual infected cells, the number of LacZ positive cells present was not increased (Table I). Reducing the size of the HIV-LacZ construct by replacing the Bal I- Bam HI HIV fragment with CMVLacZ or SV40LacZ also did not improve the virus yield. However, use of CGPE- as the complementing plasmid increased the titer of several HIV LacZ constructs to as high as 1.5×10^3/ml (Table I). Using CGPE- as the complementing plasmid, the HIV(Bal l-Bam HI)SV4OLacZ construct was found to produce the highest titers of HIV-LacZ of all the constructs studied. To assess whether replication-competent virus had been generated by recombination during co-transfections of CGPE- and HIV(Bal I-Bam HI)SV40LacZ, 5 ml of COS cell supernatant containing 7.5×10^3 infectious HIV-LacZ was used to inoculate the MT-2 cell line. Culture of the MT-2 cells for one month failed to reveal any cytopathological evidence of replication-competent virus indicating that less than 1.33×10^{-4} of the infectious virus produced in this system is replication-competent. Nevertheless, the potential for production of replication-competent virus exists and all materials were handled in the same manner as infectious HIV-1.

Table I. The indicated plasmids were co-transfected into COS cells and the supernatant was assayed for infectious HIV-LacZ in HeLa-T4 cells as described in Materials and Methods.

PRODUCTION OF RECOMBINANT HIV-LacZ		
Lac Z Plasmid	Complementing Plasmid(s)	Viral Titer (per ml)
HIV(Bal1-Bal1) H4LacZ	-	0
HIV(Bal1-Bal1) H4LacZ	HIV-gpt	4.4 x 10²
HIV(Bal1-Bal1) H4LacZ	HIV-gpt + HXB2env	1.0 x 10³
HIV(Bal1-Bal1) CMVLacZ	HIV-gpt + HXB2env	6.8 x 10²
HIV(Bal1-BamH1) CMVLacZ	HIV-gpt + HXB2env	3.6 x 10²
HIV(Bal1-BamH1) CMVLacZpA(ro)	HIV-gpt + HXB2env	2.5 x 10²
HIV(Bal1-BamH1) SV40LacZ	HIV-gpt + HXB2env	9.3 x 10²
HIV(Bal1-BamH1) SV40LacZ	CGPE-	1.5 x 10³
HIV(Bal1-Bal1) SV40LacZ	CGPE-	1.3 x 10³

Recombinant HIVs producing cytopathic effect

Some of the HIV-LacZ constructs shown in Figure 1 encode both LacZ and the envelope glycoproteins of HIV-1 (gp120/41). When these viruses infect cells, expression of both LacZ and Gp120/41 is possible. Expression of gp120/41 in cells bearing CD4 produces cytopathology characterized by syncytium formation. Indeed, as shown in Figure 2A, when the HIV(Bal l-Bal l)CMVLacZ plasmid, which encodes the HIV env gene, was co-transfected into COS cells with the HXB2env and HIV-gpt plasmids, the resultant virus produced occasional syncytia in Hela-T4 cells that also stained intensely with X-gal, indicating co-expression of LacZ and gp120/41. In contrast, when the HIV(Bal l-Bam HI)CMVLacZ plasmid, which does not contain the env gene, was transfected with the same complementing plasmids, the resultant virus produced LacZ positive HeLa-T4

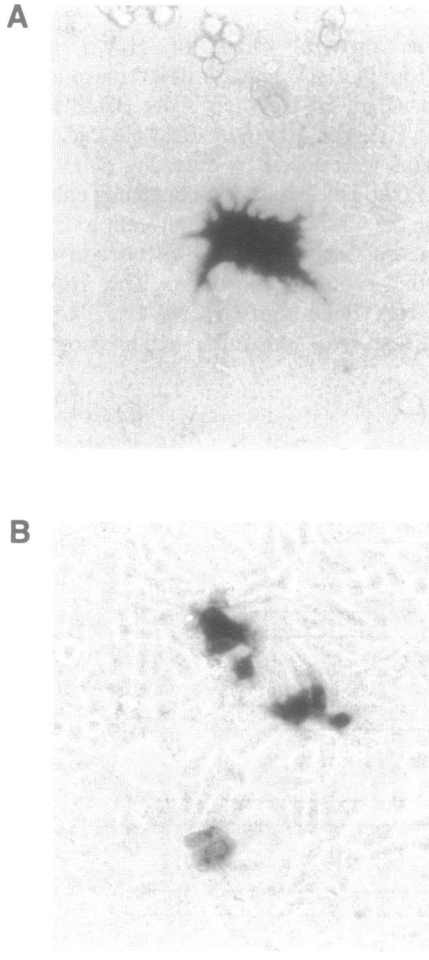

Figure 2. Cytopathological effects of recombinant HIV. Recombinant retroviruses were prepared by transfection of COS cells as described in Material and Methods. Syncytium formation following infection of HeLa-T4 cells was seen with HIV(Bal l-Bal l)CMVLacZ (panel A) but not with HIV(Bal l-Bam HI)CMVLacZ (panel B).

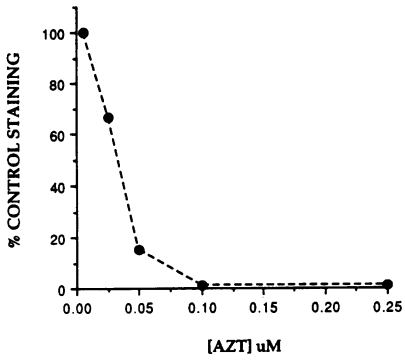

Figure 3. AZT concentration-inhibition curve using HIV-LacZ. Recombinant HIV(Bal l-Bam HI)SV4OLacZ was prepared by transfection of COS cells as described in Materials and Methods and used to infect HeLa-T4 cells in the presence of various concentrations of AZT. Forty eight hours later the infected cells were stained with X-gal and the number of blue cells counted. The abscissa shows the AZT concentration and the ordinate shows the number of blue cells as a percentage of a control infection in the absence of AZT. In this experiment the control (no AZT) corresponded to approximately 100 blue cells.

cells but no syncytia (Figure 2B). Hence, infection of target cells by certain recombinant HIVs can be detected both by expression of a reporter gene and by production of characteristic cytopathology.

Use of recombinant HIVs to assess the activity of antiretroviral compounds

The HIV-LacZ constructs described above have potential utility in determining the effects of antiretroviral compounds on early replicative events following HIV infection. For example, the inhibitory activity of AZT was examined by infecting HeLa-T4 cells with HIV(Bal I-Bam HI)SV4OLacZ (produced in COS cells after co-transfection with CGPE-) in the presence of increasing concentrations of AZT. The number of HeLa-T4 cells staining with X-gal was determined 48 hours after infection. Figure 3 shows that the concentration-dependent inhibitory activity of AZT could be readily demonstrated using LacZ expression as the indicator of retroviral infection. This initial study suggests that the antiretroviral activity of compounds can be rapidly screened with recombinant HIV-LacZ.

Use of HIV-gpt to study antiretroviral drug resistance

While the retroviruses described above can quickly provide information about the antiviral activity of specific compounds, the limited genetic heterogeneity of these recombinant viruses does not permit the detection of rare drug resistant viral variants that might exist within a replication-competent viral population. To overcome this limitation a system was needed that introduced the genetic heterogeneity of replication-competent viruses into the recombinant virus population. This was achieved by using 'helper' viruses to rescue the defective recombinant viruses described above.

To establish a system that would allow packaging of HIV-gpt by rescue with replication-competent HIV-1, the H9/HIV-gpt cell line containing an integrated HIV-gpt was produced by infection of H9 cells with HIV-gpt virus followed by selection in gpt selective media. This cell line was then superinfected with well characterized drug-sensitive and drug-resistant laboratory strains of HIV as schematically shown in Figure 4. The strains chosen

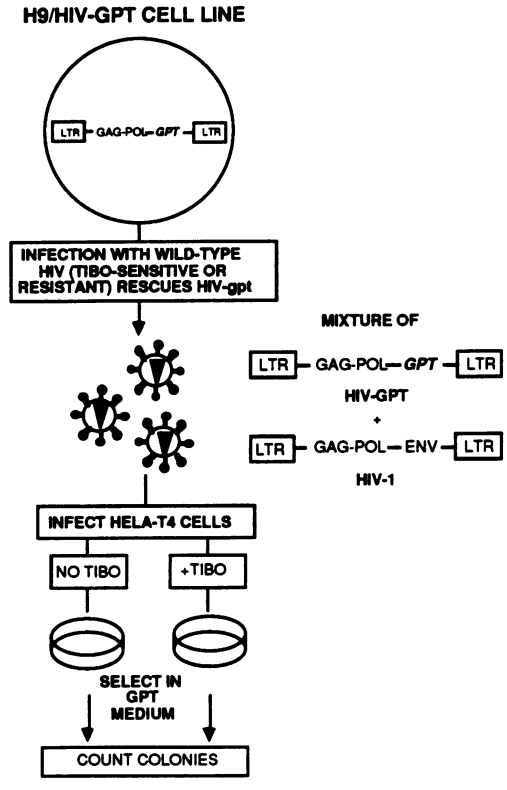

Figure 4. Schematic representation of experiments in which HIV-gpt is rescued from the H9/HIV gpt cell line by superinfection with replication-competent helper virus. Rescue of the integrated defective virus produces recombinant virus which can be studied for drug resistance (see Table II).

for these studies were HIV-1_{IIIB} and a variant of HIV-1_{IIIB} that is highly resistant (>100-fold) to the tetrahydroimidazo-(4,5,1-jk)(1,4)-benzodiazepine-2(1H)thione (TIBO) derivative R82150 (7,11). The TIBO-resistant virus encodes a single nucleotide change in reverse transcriptase causing a leucine to isoleucine mutation at amino acid 100 (7,11). Seven days after infection of H9/HIV-gpt with the TIBO-resistant and TIBO-sensitive viruses, supernatants were harvested and used to infect HeLa -T4 cells in the presence and absence of 1μM TIBO (~25 times the IC_{50}). Three days later the HeLa -T4 cells were split into gpt selective media and colonies were counted after 10 days. As shown in Table II, TIBO-resistant HIV-gpt was generated when HIV-gpt was rescued with TIBO-resistant HIV-1_{IIIB}. Similarly, TIBO-sensitive HIV-gpt resulted from rescue with sensitive helper virus. These results demonstrate that the drug sensitivity phenotype of the rescued HIV-gpt reflects the phenotype of the helper virus.

To estimate the frequency of TIBO-resistant virus within an unselected population of replication competent HIV-1, the H9/HIV-gpt cell line was infected with a standard laboratory stock of HIV-1_{IIIB} and the rescued virus was harvested 10 days later. This virus preparation was used to infect HeLa-T4 cells in the presence of 1μM TIBO. Infection of HeLa-T4 with HIV-gpt generated by plasmid co-transfections of COS cells ('plasmid derived' virus) was performed in parallel. Table III provides a summary of these results, which demonstrate that TIBO-resistant HIV breakthrough was approximately twenty times more frequent with the HIV-gpt virus generated by HIV-1_{IIIB} rescue as

Table II. Rescue of HIV-gpt from H9/HIV-gpt with TIBO-sensitive and TIBO-resistant HIV-1_{IIIB}

	Number of Colonies	
Rescue Virus	No TIBO	TIBO (1 uM)
No Rescue	0	0
HIV-1_{IIIB}	178	0
TIBO-Resistant HIV-1	289	269

H9/HIV-gpt cells were infected at an m.o.i. of 0.1 with TIBO-sensitive and TIBO-resistant strains of HIV-1_{IIIB}. Seven days after infection, supernatants were harvested and used to infect HeLa-T4 cells in the presence and absence of 1μM TIBO. Two days later the HeLa-T4 cells were split into gpt media and colonies were counted after an additional 10 days.

Table III. Detection of TIBO-resistance within an unselected stock of HIV-1

	Number of Colonies		
Source of HIV-gpt	No TIBO	TIBO (1 uM)	Frequency
Plasmid-derived	8.4×10^4	4	4.8×10^{-5}
Rescue with HIV-1_{IIIB}	6.4×10^4	72	1.1×10^{-3}

HIV-gpt virus was prepared by either COS cell transfections ('plasmid derived virus') or by rescue infection of the H9/HIV-gpt cell line with HIV-1_{IIIB} as depicted in Figure 4 and described in the text. The two virus preparations were used to infect HeLa-T4 cells in the presence and absence of 1μM TIBO. Two days later the HeLa-T4 cells were split into gpt media and colonies were counted after an additional 10 days. The inoculum of each virus preparation was adjusted to generate between 5×10^4 and 1×10^5 colonies.

compared with plasmid-derived virus. This difference in TIBO breakthrough is likely due to the presence of TIBO-resistant viral variants within the stock of HIV-1_{IIIB} used for rescue. In contrast, virus packaged in COS cells contains viral proteins (e.g. reverse transcriptase) encoded by plasmids and thus drug-resistant variants are likely to be less frequent than in a genetically heterogenous replication-competent helper virus population such as HIV-1_{IIIB}. An alternative explanation for the observed difference in TIBO breakthrough is that packaging of virus in COS cells alters its TIBO susceptibility relative to virus packaged in H9 cells. However, control experiments have demonstrated no difference in the TIBO concentration-inhibition curves for COS cell-derived and H9-derived HIV-gpt except at the extreme end of the curve above the 99.5% inhibitory concentration. This difference in breakthrough at the far end of the concentration-inhibition curve is likely due to a higher frequency of high level TIBO-resistant variants in the HIV-gpt population produced by rescue with HIV-1_{IIIB}. If cellular-encoded resistance was the dominant mechanism responsible for HIV-gpt breakthrough, then the frequency of breakthrough would be similar for the two populations of HIV-gpt.

Production of 'rescued' recombinant HIV-1 encoding LacZ

Limitations of the HIV-gpt system presented above include the long time required for colony formation in gpt selective media (approximately 10–14 days) and the presence of the reverse transcriptase (RT) gene in the HIV-gpt genome. The RT gene in HIV-gpt will result in virions that are chimeric for the origin of RT after rescue with helper virus. For example, infection of the H9/HIV-gpt cell line with a helper virus that encodes a drug-resistant RT will result in the production of HIV-gpt virions that are likely to contain both drug-sensitive RT encoded by the HIV-gpt genome and drug-resistant RT, encoded by the helper virus. Although this did not alter the drug sensitivity phenotype of well characterized stocks of virus with known TIBO-sensitivity, it confounds quantitative analysis of drug-resistance in unselected stocks of helper virus. This problem of RT chimerism does not exist with several of the HIV-LacZ viruses. For example, the HIV(BalI-BamHI)SV40LacZ plasmid does not encode RT (Figure 1). Therefore, a cell line containing an integrated HIV(BalI-BamHI)SV40LacZ was prepared as described in Materials and Methods. This cell line, HeLa T4/HIV-LacZ, was infected with unselected (TIBO-sensitive) and TIBO-resistant HIV-1_{IIIB} and the drug sensitivity phenotype of the rescued virus was determined by infection of HeLa-T4 cells in the presence of 1 and 5 μM TIBO, followed by X-gal staining 48 hours later. The results of this experiment, shown in Table IV, indicate that HeLa-T4/HIV-LacZ can be used to rapidly detect and quantitate drug-resistant variants within the rescuing population without the potential problem of RT chimerism.

Table IV. Detection of TIBO-resistant HIV with an HIV-LacZ recombinant system

	Number of Lac Z Positive Cells		
Virus	No TIBO	1.0 μM TIBO	5.0μM TIBO
HIV-1_{IIIB}	3.2×10^3	17	6
TIBO-Resistant HIV	2.9×10^3	2.2×10^3	3.1×10^3

A cell line with an integrated defective HIV containing the LacZ gene was prepared as described in Materials and Methods and in the text. The defective HIV-LacZ was rescued from the cell line by infection with unselected (TIBO-sensitive) HIV-1_{IIIB} or TIBO- resistant HIV-1_{IIIB}. The rescued HIV-LacZ populations were used to infect HeLa-T4 cells in the presence and absence of the indicated concentrations of TIBO. Two days after infection the cells were stained for LacZ expression and the number of blue cells counted.

DISCUSSION

We have previously reported the use of Moloney leukemia viruses (MLV) encoding LacZ to rapidly quantitate retroviral infection and its inhibition by antiretroviral compounds (15). Extension of these studies to HIV required the construction of novel recombinant HIVs. To this end, we have constructed several HIV-based plasmids encoding LacZ that produce infectious recombinant virus when complemented with the necessary HIV structural and regulatory genes.

The plasmid-derived recombinant HIV-LacZ system described here holds promise as a screening tool for the identification of compounds that inhibit any of the early steps in the HIV replicative process. For example, drugs that block viral

attachment, penetration or reverse transcription could be identified with several of the recombinant viruses depicted in Figure 1. In addition, agents that block syncytia formation could be identified using recombinant viruses that also encode HIV env [e.g. HIV (Bal l-Bal l)CMVLacZ]. Although we have not proven that integration is necessary for LacZ expression 48 hours after infection, we have not noted any reduction in the number of clusters of blue cells when the X-gal staining is delayed until 7 days after infection, indicating that most of the LacZ expression detected at 48 hours is stable. Advantages of this recombinant HIV-LacZ system include (1) rapid and unequivocal identification of virus-infected cells within 48 hours of infection, (2) quantitation of the antiretroviral effects of drugs on a single cycle of virus infection (3) use of a replication defective HIV which reduces but does not eliminate the potential for biohazard (i.e.recombination leading to replication competent HIV could occur) and (4) the ability to use several different target cell types for infection. In addition, the LacZ gene is a component of the virus genome therefore making LacZ expression a direct indicator of virus infection. Therefore, this system should complement other available indicator systems such as the HeLa CD4-LTRβ-gal cell line, which detects tat transactivation of an LTR driven beta-galactosidase gene (19).

The virus titers obtained with the plasmid-derived recombinant HIV-LacZ systems (10^2-10^3/ml) are lower than those obtained with recombinant MLV systems or other HIV systems (10,13). The absence of a packaging cell line as well as the greater complexity of HIV with respect to regulatory proteins and the RNA processing necessary for virion production may explain some of the differences in titer in comparison to MLV-based systems. Compared with other HIV-based systems, the lower titers of HIV-LacZ are likely related to differences in packaging and/or reverse transcription of viruses containing the LacZ gene. Despite lower titers, the incorporation of LacZ into recombinant HIV has the advantage of allowing very rapid assay without the need for prolonged propagation or manipulation of cells.

In addition to the use of defective viruses packaged in COS cells, we have also prepared defective recombinant HIV by rescue with replication-competent HIV. Helper virus rescue increases the recombinant virus titer and introduces the heterogeneity of replication-competent viruses into the recombinant virus population, allowing the detection of rare viral variants. This system is well suited for the analysis of resistance to antiretroviral agents active against the early stages of the retroviral infectious cycle.

Using this system with two different recombinant HIV-1 retroviruses we have been able to make preliminary conclusions concerning the nature of viral breakthrough events occurring *in vitro*. Data presented in Table II indicate that rescue of HIV-gpt with characterized TIBO-sensitive and TIBO-resistant stocks of HIV_{IIIB} produces HIV-gpt with the TIBO-sensitivity phenotype of the helper virus used for rescue. Thus, although HIV-gpt encodes RT and the rescued HIV-gpt virus is likely to be chimeric with respect to origin of RT, the HIV-gpt phenotype reflects the TIBO-resistance of the viral stock when well characterized stocks of virus are used for rescue.

HIV-gpt generated by rescue with an unselected population of HIV-1_{IIIB} resulted in colony formation in the presence of TIBO at a much greater frequency than virus packaged in COS cells. This difference was not due to intrinsic differences in drug sensitivity when virus is produced in two different cell types (COS cells as opposed to H9 cells) as demonstrated by control experiments which detected no differences in the TIBO sensitivity of the two virus populations except at TIBO concentrations producing greater than 99.5% inhibition. Therefore, this difference is presumed to be secondary to a higher frequency of TIBO-resistant variants within the helper virus population used for rescue in comparison to the 'plasmid-derived' virus packaged in COS cells. The exact frequency of these TIBO-resistant variants in the unselected helper virus population is difficult to determine with the HIV-gpt system because of the chimerism of RT. Thus, the frequency of TIBO-resistant variants determined with the HIV-gpt system is likely to be an underestimate, representing the minimum frequency of TIBO-resistant variants in the helper virus population. Similar experiments with recombinant virus rescued from the HeLa T4/HIV-LacZ cell line are not confouded by the production of RT by the recombinant genome. In fact, HIV-LacZ rescued from this cell line had a 4–5 fold higher frequency of TIBO-resistant virus. This difference in the frequency of TIBO-resistant HIV-gpt and TIBO-resistant HIV-LacZ after rescue with same helper virus (HIV-1_{IIIB}) may in part be explained by the RT chimerism of the HIV-gpt virus. That is, drug-sensitive RT molecules encoded by the HIV-gpt genome can dilute or negate the effects of co-packaged drug-resistant RT encoded by the helper virus.

Additional experiments have used an identical approach to study HIV breakthrough in in the presence of AZT (data not shown). These experiments utilized the same plasmid-derived and HIV-1_{IIIB} rescued virus stocks used in the TIBO breakthrough experiments described above. However, unlike the TIBO experiments, there was no difference in HIV breakthrough in the presence of 1μM, 5μM or 10μM AZT when plasmid-derived virus was compared with virus produced by rescue with unselected HIV-1_{IIIB}. The differences between TIBO and AZT breakthrough in this system is consistent with the hypothesis that we are detecting TIBO-resistant virus in the unselected helper virus population, since only a single point mutation is necessary for TIBO resistance. In contrast, spontaneous AZT resistance in an unselected virus population is likely to occur at a much lower frequency since multiple mutations in RT are necessary to induce high level AZT resistance (4). The sensitivity of the current assay does not allow detection of spontaneously AZT-resistant viruses in an unselected laboratory stock of HIV-1_{IIIB}. Efforts to increase the sensitivity of these assays are currently underway.

A particular advantage of the use of recombinant viruses to analyze breakthrough events in the presence of antiviral agents is the ability to study a single cycle of infection. This allows detection of breakthrough events in an unselected population without concern about spread of the virus and amplification of breakthrough events following exposure to the antiviral agent. Using recombinant viruses we can estimate that at least .001 of an unselected HIV population is spontaneously resistant to TIBO R82150.

ACKNOWLEDGEMENTS

We thank K.Page, A.Brodsky, A.Nienhuis, F.Wong-Staal and R.C.Gallo for providing us with the indicated plasmids. HeLa-T4 cells were provided by the AIDS Research and Reference Reagent Program, Bethesda, MD. R.S. is supported by grants R29CA49047 and RO1CA54547 from the N.C.I. J.W.M. is supported by the Medical Research Service of the Department of Veterans Affairs and RO1A134301.

REFERENCES

1. Fischl, M.A., D.D. Richman, M.H. Grieco, M.S. Gottlieb, P.A. Volberding, O.L. Laskin, J.M. Leodom, J.E. Groopman, D. Mildvan, R.T. Schooley, G.G. Jackson, D.T. Durack, and D. King. 1987. The efficacy of azidothymidine AZT in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N.Engl. J. Med. 317:185–191.
2. Fischl, M.A., D.D. Richman, M.H. Grieco, Y. Bryson, D. Mildvan, O.L. Laskin, J.E. Groopman, P.A. Volberding, R.J. Schooley, G.G. Jackson, D.T. Durack, J.C. Andrews, S. Nusinoff-Lerman, and D.W. Barry. AZT Collaborative Working Group. 1989. Prolonged zidovudine therapy in patients with AIDS and advanced AIDS-related complex. J. Am. Med. Assoc. 262:2405–2410.
3. Larder, B.A., G. Darby, and D.D. Richman. 1989. HIV with reduced sensitivity to zidovudine(AZT) isolated during prolonged therapy. Science. 243:1731–1734.
4. Larder, B.A. and S.D. Kemp. 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine(AZT). Science. 246:1155–1158.
5. Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell. 47:333–348.
6. Mellors, J.W., G.E. Dutschman, G-J. IM, E. Tramontano, S.R. Winkler, and Y.C. Cheng. 1992. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. 41:446–451.
7. Mellors, J.W., G-J. IM, E. Tramontano, D.J. Medina, S.R. Winker, C. Gonzales, G. Piras, H. Bazmi, and Y.C. Cheng. 1993. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to TIBO R82150. Mol. Pharmacol. 43:11–16.
8. Nunberg, J.H., W.A. Schlief, E.J. Boots, J.A. O'Brien, J.C. Quinlero, J.M. Hoffman, E.A. Emini, and M.E. Goldman. 1991. Viral resistance to human immunodeficiency virus type 1–specific pyridinone reverse transcriptase inhibitors. J. Virol. 65:4887–4892.
9. Page, K.A., N.R. Landau, and D.R. Litman. 1990. Construction and use of a human immunodeficiency virus vector for anallysis of virus infectivity. J. Virol. 64:5270–5276.
10. Poznansky, M., A. Lever, L. Bergeron, W. Haseltine, and J. Sodroski. 1991. Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. J. Virol. 65:532–536.
11. Pauwels, R., K. Andries, J. Desmyter, D. Schols, M.J. Kukla, H.J. Bresline, A. Raeymaekers, J. van Gelder, R. Woeslenborghs, J. Heykants, K. Schellenkens, M.A.C. Janssen, E. DeClerq and P.A.J. Janssen. 1990. Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. Nature. 343–470–473.
12. Richman, D.D., C.L. Shin, I. Lowy, J. Rose, P. Prodarovich, J. Goff, and J. Griffin. 1991. Human immunodeficiency virus type 1 mutants resistant to non-nucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. U.S.A. 88:11241–11242.
13. Schimada, T., H. Funjii, H. Mitsuya, and A.W. Nienhuis. 1991. Targeted and highly efficient gent transfer into CD4 + cells by a recombinant human immunodeficiency virus retroviral vector. J. Clin. Invest. 88:1043–1047.
14. St. Clair, M.H., J.L. Martin, G. Tudor-Williams, M.C. Bach, C.L. Vavro, D.M. King, P. Kellam, S.D. Kemp, and B.A. Larder. 1992. Resistance to ddl and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science. 253:1557–1559.
15. Strair, R.K., C.J. Nelson, and J.W. Mellors. 1991. Use of recombinant retroviruses to characterize the activity of antiretroviral compounds. J. Virol. 65:6339–6342.
16. Strair, R.K., M.T. Towle, P.W. Heald, and B.R. Smith. 1990. Retroviral mediated transfer and expression of exogenous genes in primary lymphoid cells: Assaying for a viral-like transactivator. Blood. 76:1201–1208.
17. Tornevik, Y., B. Jacobsson, S. Britton, and S. Eriksson. 1991. Intracellular metabolism of 3′-azidothymidine in isolated human peripheral blood mononuclear cells. AIDS Res. Human Retroviruses. 7:751–759.
18. Tudor-Williams, G., M.H. St. Clair, R.E. McKinney, M. Maha, E. Walter, S. Santacroce, M. Mintz, K. O'Donnell, T. Rudoll, C. Vavro, E.M. Connor, and C.M. Wilfert. 1991. HIV-1 sensitivity to zidovudine and clinical outcome in children. Lancet. 339:15–19.
19. Kimpton, J. and M. Emerman. 1992. Detection of replication competent and pseudotyped HIV with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virology 66:2232–2239.