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# LuSIV Cells: A Reporter Cell Line for the Detection and Quantitation of a Single Cycle of HIV and SIV Replication

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A single cycle of viral replication is the time required for a virus to enter the host cell, replicate its genome, and produce infectious progeny virions. The primate lentiviruses, human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), require on average 24 h to complete one cycle of replication. We have now developed and characterized a reporter assay system in CEMx174 cells for the quantitative measurement of HIV/SIV infection within a single replication cycle. The $SIV_{mac}$239 LTR (−225 → +149) was cloned upstream of the firefly luciferase reporter gene and this reporter plasmid is maintained in CEMx174 cells under stable selection. This cell line, designated LuSIV, is highly sensitive to infection by primary and laboratory strains of HIV/SIV, resulting in Tat-mediated expression of luciferase, which correlates with viral infectivity. Furthermore, manipulation of LuSIV cells for the detection of luciferase activity is easy to perform and requires a minimal amount of time as compared to current HIV/SIV detection systems. The LuSIV system is a powerful tool for the analysis of HIV/SIV infection that provides a unique assay system that can detect virus replication prior to 24 h and does not require virus to spread from cell to cell. Thus these cells can be used for the study of replication-deficient viruses and the high throughput screening of antivirals, or other inhibitors of infection. 

## INTRODUCTION

The human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are members of the lentivirus family and are the causative agents for the acquired immunodeficiency syndrome (AIDS) in primates. Since the mechanisms of viral infection in primates are complicated by the interplay of the host immune responses, the study of lentivirus infection in tissue culture has proved to be a useful model for the analysis of viral replication and pathogenesis *in vivo*. The retroviral life cycle can be separated into two parts: the early events required for entry and establishment of productive infection and the late events responsible for assembly, persistence, and spread of infection (Varmus, 1982).

To study the mechanisms of the retroviral life cycle, it is crucial for a viral replication assay to detect a single round of infection, which is the time required for a virus to infect the host cell and produce the first round of progeny virions. For HIV/SIV a single replication cycle is approximately 24 h and is dependent on the number and the intrinsic infectivity of infectious particles used as the viral input (Varmus, 1982). To study the mechanisms of viral persistence and spread, an assay that can follow HIV/SIV infection over time is required. An ideal assay system would provide the ability to detect early and late events in a single round of infection as well as have the capability to measure infection over time. In addition, this system would be sensitive to infection by phenotypically diverse virus isolates.

Of the current techniques available for measuring viral infection, systems that measure the total number of viral particles such as reverse transcriptase assays or ELISA-based assays to detect viral antigen are useful for following infection over time. However, they are limited by the requirement for the production of threshold levels of viral particles as a readout, are unable to detect a single round of infection, or are unable to discriminate between infectious and noninfectious particles. Assays such as endpoint dilution that utilize syncytia formation or cytopathic effects (CPE) as readouts are useful for determining the number of infectious particles, yet are time consuming because of the requirement for viral spread and formation of syncytia. In addition, these techniques are biased against viral strains that do not cause fusion and syncytia formation during persistent infection and are also unable to detect a single round of infection.

Many of the cell-based assay systems designed to study HIV/SIV infection utilize the viral LTR activated by the viral transactivating protein Tat early in infection to drive expression of a reporter gene. These LTR-based assay systems employ the LTR fused to GFP (Gervaix *et*

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*al.,* 1997), β-galactosidase (Chackerian *et al.,* 1995; Kimpton and Emerman, 1992), CAT (Felber and Pavlakis, 1988), secreted alkaline phosphatase (SEAP) (Berger *et al.,* 1988), or luciferase (Aguilar-Cordova *et al.,* 1994). Of the cell-based HIV/SIV infection assay systems that employ these reporter constructs few have been successful in achieving a high level of sensitivity. To date, the most commonly used assays are the multinuclear activation of galactosidase indicator (MAGI/sMAGI) systems (Chackerian *et al.,* 1995; Kimpton and Emerman, 1992). The MAGI/sMAGI assays utilize the viral LTR from HIV and SIV, respectively, to drive expression of the β-galactosidase reporter gene. Although these cell systems may be capable of detecting a single round of HIV/SIV infection, β-gal activity is measured 2–3 days postinfection and may be affected by virus spread.

We have developed an LTR-based assay system in CEMx174 cells (Salter *et al.,* 1985) that is sensitive enough to detect a single round of HIV/SIV infection within 24 h of exposing cells to virus. Using a truncated portion of the $SIV_{mac}239$ LTR cloned upstream of the firefly luciferase reporter gene we generated a stable cell line containing this episomally maintained reporter plasmid. This cell line, named LuSIV, is sensitive to induction of luciferase expression by HIV and SIV Tat, and luciferase activity directly correlates with viral infectivity.

The LuSIV cells provide the advantage of detecting a single round of infection, while requiring only picogram quantities of p24/p27 as viral input, and the infectivity of primary and laboratory strains of HIV/SIV can be detected using these cells. The measurement of luciferase activity requires minimal amounts of time, is easily performed, and can be used for high throughput assays. The LuSIV system is an effective tool to study the general biology of HIV/SIV infection and can be extended to the study of replication-defective viruses used in vaccine development and the screening of inhibitors of viral infection.

## RESULTS

### Construction of reporter plasmid that is transactivated by SIV and HIV Tat

We have engineered a pLuc reporter construct containing the $SIV_{mac}239$ LTR cloned upstream of the firefly luciferase reporter gene. Activation of pLuc requires the HIV/SIV viral transactivating protein Tat to drive expression of the luciferase reporter gene from the LTR. The truncated portion of the SIV LTR (−225 → +149) used to construct pLuc was previously shown to be the minimal region required for efficient transactivation of the SIV genome (Anderson and Clements, 1992). This region of the SIV LTR minimizes background activation from cellular transcription factors while maintaining optimal sensitivity to Tat. The HIV and SIV Tat proteins depend on the same consensus NFκB and SP1 sites within the LTR (Franza *et al.,* 1987; Garcia *et al.,* 1987; Jones *et al.,* 1986; Nabel and Baltimore, 1987) and have been shown to be interchangeable with respect to their ability to transactivate either the SIV or HIV genome (Viglianti and Mullins, 1988). The portion of the LTR in pLuc contains the NFκB and SP1 consensus sites and we anticipated that both SIV and HIV Tat would induce luciferase expression.

To test the reporter construct and confirm its sensitivity to HIV/SIV Tat, we cotransfected CEMx174 cells with equal amounts of pLuc and DNA encoding SIV *tat,* HIV *tat,* or visna virus *tat* as a negative control. Visna virus Tat does not activate transcription through the NFκB and SP1 sites that are within the HIV/SIV LTR but, instead, uses AP1 and AP4 consensus binding sites (Gabuzda *et al.,* 1989; Gdovin and Clements, 1992; Hess *et al.,* 1989; Saltarelli *et al.,* 1990). For this reason, we predicted that visna virus Tat would not transactivate the $SIV_{mac}239$ LTR. At 24 h posttransfection $2.0 \times 10^5$ cells from each transfection were analyzed for luciferase activity. The cells transfected with both the pLuc and SIV or HIV Tat constructs showed a >300-fold increase in luciferase activity as compared to those cells transfected with pLuc alone (Fig. 1A). In contrast, visna virus Tat did not induce luciferase activity. Whereas HIV and SIV Tat showed the highest level of induction at 24 h posttransfection, a 30-fold increase in activity could be detected as early as 5 h posttransfection (data not shown), indicating that Tat is a very efficient inducer of luciferase activity.

To ascertain whether this Tat-mediated induction of luciferase could also occur in the context of the entire viral genome, we next cotransfected CEMx174 cells with equal amounts of pLuc and infectious $HIV_{NL4-3}$ or $SIV_{mac}239$ DNA. At 48 h posttransfection a >700-fold increase in luciferase activity was observed for $SIV_{mac}239$ and a >450-fold induction with $HIV_{NL4-3}$ (Fig. 1B).

### Generation of LuSIV cells

To develop a stable cell line containing the pLuc construct, we chose CEMx174 cells, which are susceptible to infection by T-cell-tropic strains of HIV and by molecular clones and primary strains of SIV (Emau *et al.,* 1991; Sei *et al.,* 1990; Stefano *et al.,* 1993). The pLuc plasmid was constructed in the background of the pCEP4 plasmid, which carries the hygromycin B resistance gene for use in selecting stable cells and is maintained episomally through EBNA-1 (Sugden *et al.,* 1985; Yates *et al.,* 1985). We transfected CEMx174 cells with 12 μg of pLuc and selected a stable cell population with hygromycin B. The cells were cloned by limiting dilution under selection with hygromycin B (300 μg/ml) and screened for their ability to induce luciferase activity upon infection with SIV (data not shown). The final cloned cells that yielded the highest signal-to-noise ratio, pLuc6.3a, were used for all further experiments and are hereafter referred to as the LuSIV cells.

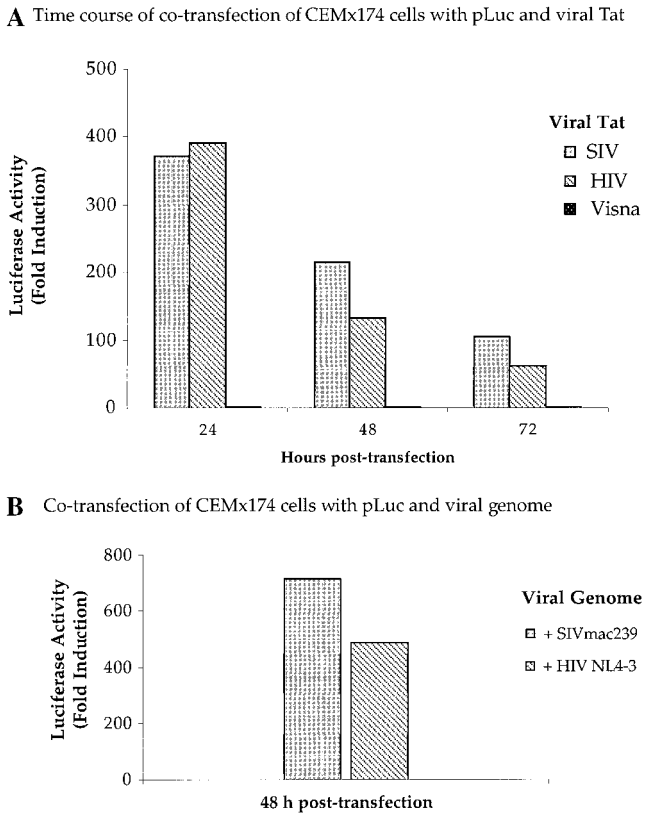


FIG. 1. Luciferase activity from the pLuc reporter plasmid. CEMx174 cells were cotransfected with pLuc and HIV, SIV, visna virus *tat* (A), or with HIV or SIV viral genomes (B). For each time point the luciferase assay was performed as described under Materials and Methods using $2.0 \times 10^5$ viable cells. Values are shown as fold induction of luciferase activity over background activity, and the data are representative of three independent experiments.

## Optimization and infection of LuSIV cells

Once a stable cell line was isolated, it was necessary to optimize the conditions of infection to give the best induction of luciferase activity. To lower the background in the LuSIV cells, we reduced the FBS concentration in the tissue culture medium from 10 to 2%. Although this change did have a modest effect on the background luciferase activity, we suspected that something in the viral inoculum was inhibiting the infection process, since increasing amounts of viral input did not result in an increase in luciferase activity.

To test this hypothesis, we pelleted varying amounts of SIV/17E-Fr (calculated on the basis of RT activity) and resuspended the virus for inoculation in conditioned medium (the supernatant from which it was pelleted), serum-free cRPMI or cRPMI with 2% FBS. Using these virus preparations, we infected LuSIV cells ($2.0 \times 10^5$ cells/well) in 96-well flat-bottom dishes, and assayed them for luciferase activity at 24 h postinfection. The maximum luciferase induction of the inoculum containing the conditioned medium was 4.5-fold; however, this induction was not as robust as that observed for the serum-free (18-fold) or 2% cRPMI (42-fold) samples, nor was the induction titratable with increasing amounts of virus input (Fig. 2). As a consequence, in all subsequent experiments involving infection with SIV the virus was pelleted as described under Materials and Methods and resuspended in fresh RPMI with 2% FBS.

## Detection of a single round of HIV and SIV infection

To examine the capability of LuSIV cells to detect a single replication cycle, we infected the LuSIV cells ($1.5 \times 10^5$) with SIV/17E-Fr for 6 h, washed off the virus inoculum, and treated the cells with the RT inhibitor AZT (10 $\mu$M) for the remainder of the infection. Infection with SIV/17E-Fr resulted in significant luciferase induction in both untreated and AZT-treated samples (Fig. 3). Since AZT inhibits RT activity, the luciferase activity detected 24 h postinfection reflects the infectivity of those virions that entered and initiated reverse transcription prior to AZT treatment. The difference in luciferase induction observed between the untreated and AZT-treated samples is likely the result of the activity of virions that either just entered the cells or were in the process of fusion at the time the virus inoculum was removed (Fig. 3). However, in the AZT-treated samples, virions in this state would be inhibited by the drug and result in an overall lower-fold induction of luciferase activity as compared to the untreated samples. Furthermore, to confirm the inhibitory effect of AZT one of the samples was treated with AZT at the time of infection and did not demonstrate a significant induction of luciferase activity (Fig. 3). The ability to detect a significant induction of luciferase 24 h postinfection in AZT-treated samples provides strong evidence that the LuSIV cells do measure a single cycle of SIV/HIV infection.

To evaluate single-cycle HIV/SIV infection with a panel of virus strains, LuSIV cells were infected with a variety

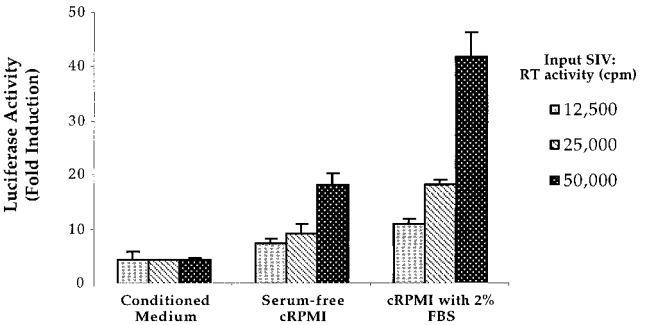


FIG. 2. SIV infection of LuSIV cells. LuSIV cells were infected with SIV/17E-Fr that was pelleted and resuspended in conditioned medium (the supernatant from which it was pelleted), serum-free cRPMI, or cRPMI with 2% FBS. Viral input was normalized by increasing quantities of reverse transcriptase (RT) activity. The LuSIV cells were assayed for luciferase activity 24 h postinfection, and values are represented as fold induction of luciferase activity (± SD) over background activity. Data are mean values from duplicate infections.

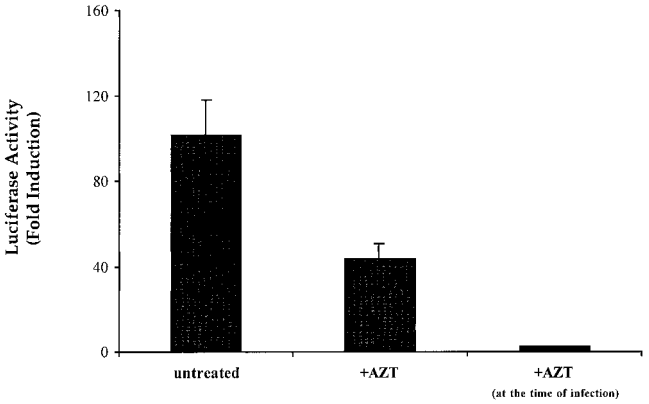


FIG. 3. Detection of single-cycle SIV replication in LuSIV cells. LuSIV cells were infected with 25 ng of SIV/17E-Fr for 6 h, washed, and either treated with AZT (10 $\mu$M) to prevent viral spread or left untreated. To control for the inhibitory effect of AZT, one sample was treated with AZT at the time of infection. The LuSIV cells were assayed for luciferase activity 24 h postinfection, and values are represented as fold induction of luciferase activity (± SD) over background activity. Data are representative of three independent experiments and values are the means from quadruplicate infections.

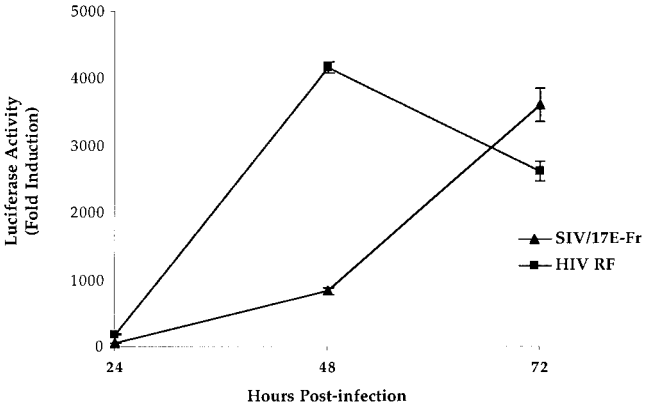


FIG. 4. Infection of LuSIV cells with SIV and HIV. LuSIV cells ($2.0 \times 10^5$) were infected with 25 ng of SIV/17E-Fr p27 and $HIV_{RF}$ p24. At 24, 48, and 72 h postinfection the LuSIV cells were assayed for luciferase activity. The values are represented as fold induction of luciferase activity (± SD) over background activity and are the mean values of infections performed in triplicate.

of HIV/SIV stocks and measured for luciferase activity at 24 h postinfection. The standard input for each infection was 25 ng of HIV (p24) or SIV (p27) viral core antigen. Whereas the extent of the induction varied for each strain, there was a significant induction associated with each virus after a single round of infection (Table 1). In other studies we have detected a robust luciferase activity in as few as 16 h after exposing LuSIV cells to HIV (data not shown). Furthermore, when the infection proceeded for 48 h the fold induction for each virus increased significantly, demonstrating the ability of the LuSIV cells to detect virus spread. The variability in the extent of the induction between strains is an indication of the difference in relative infectivity between the viruses studied. These data indicated that LuSIV cells are highly susceptible to HIV/SIV infection and are capable of detecting a relatively small amount of virus input within a single replication cycle.

TABLE 1

Luciferase Activity in LuSIV Cells at 24 and 48 h Postinfection with Various Strains of HIV and SIV

| Strain | Luciferase activity postinfection (fold induction) | |
|---|---|---|
| | 24 h | 48 h |
| HIV | | |
| IIIb | 38.6 ± 8.76 | 2880 ± 572 |
| RF | 187 ± 10.9 | 4190 ± 78.8 |
| MN | 44.4 ± 1.15 | 426 ± 4.77 |
| SIV | | |
| 17E-Br | 161 ± 21.8 | 1660 ± 142 |
| 17E-Fr | 115 ± 14.8 | 1390 ± 337 |
| mac239 | 8.65 ± 2.21 | 191 ± 47.9 |
| mac251 | 21.3 ± 1.35 | 161 ± 11.4 |
| Delta B670 | 3.02 ± 0.48 | 41.3 ± 15.8 |

*Note.* Virus input was 25 ng of SIV p27 or HIV p24 viral core antigen. Luciferase activity is represented as the fold induction of luciferase activity over background.

## Increase in luciferase induction in LuSIV cells over time

In addition to their capacity for detecting a single round of infection, the LuSIV cells are also useful for detecting virus spread. If the standard virus input used to infect the LuSIV cells is low (<1 multiplicity of infection), not all of the LuSIV cells will be infected in the first round; therefore, there will be additional susceptible cells for subsequent infection. In addition to virus spread, luciferase will accumulate over time in those cells that have an established infection. To confirm that the luciferase induction increases over time as infection proceeds, we infected $2.0 \times 10^5$ LuSIV cells in triplicate with 25 ng of SIV/17E-Fr (p27) or $HIV_{RF}$ (p24). The cells were assayed for luciferase activity at 24, 48, and 72 h postinfection (Fig. 4). $HIV_{RF}$ causes a robust infection that rapidly increases by 48 h postinfection (>4000-fold induction), but starts to diminish in 72 h. The decrease in luciferase activity at 72 h is most likely the result of a decrease in the viability of the LuSIV cells as they succumb to the cytopathic effects of virus infection. SIV/17E-Fr induction increased exponentially over the time period studied (exceeding a 3000-fold induction). It is likely that this trend would continue until the infection reached a plateau and the cells begin to die as observed with $HIV_{RF}$. The ability of the LuSIV cells to reflect the growth kinetics of a particular HIV/SIV virus strain makes this assay a useful tool for following HIV/SIV infection *in vitro* and monitoring inhibitors of viral replication.

TABLE 2

Sensitivity of LuSIV Cells to HIV and SIV Infection at 24 and 48 h Postinfection

| p24/p27 (pg) | Luciferase activity postinfection (fold induction) | | | |
|---|---|---|---|---|
| | $HIV_{RF}$ | | SIV/17E-Fr | |
| | 24 h | 48 h | 24 h | 48 h |
| 40 | 3.78 ± 1.09 | 81.2* ± 17.2 | 0 | 7.02* ± 2.17 |
| 200 | 5.24* ± 1.20 | 472 ± 26.9 | 2.03 ± 0.66 | 31.9 ± 0.77 |
| $1.0 \times 10^3$ | 10.2 ± 1.39 | 1560 ± 172 | 11.0* ± 0.62 | 160 ± 14.0 |
| $5.0 \times 10^3$ | 27.4 ± 1.91 | 1630 ± 264 | 60.2 ± 2.96 | 790 ± 26.2 |
| $2.5 \times 10^4$ | 166 ± 10.7 | 3890 ± 350 | 225 ± 11.1 | 4100 ± 129 |

*Note.* The values designated by * represent the input sensitivity for the LuSIV cells.

### Sensitivity of LuSIV cells to HIV/SIV infection

We were interested in determining the lower limit of virus input that would yield a significant increase in the induction of luciferase activity over background in the LuSIV cells. Starting with 25 ng of p27/p24, we made fivefold serial dilutions of SIV/17E-Fr or $HIV_{RF}$ and infected $2.0 \times 10^5$ LuSIV cells in triplicate. The cells were analyzed for luciferase activity at 24 and 48 h postinfection (Table 2). A fourfold increase in luciferase activity over background was considered the lower limit of sensitivity for virus input. In a single round of replication (24 h), the assay was remarkably sensitive: as shown in Table 2, the LuSIV cells could be used to detect infection with 200 pg of $HIV_{RF}$ p24 and 1 ng of SIV/17E-Fr p27 input, and, after allowing the infection to proceed for 48 h, the LuSIV cells were able to detect 40 pg of $HIV_{RF}$ p24 and 40 pg of SIV/17E-Fr p27.

To further demonstrate the sensitivity and usefulness of the LuSIV assay we directly compared SIV infection in LuSIV and sMAGI cells by performing parallel infections with SIV/17E-Fr. The LuSIV cells were infected as described under Materials and Methods and the sMAGI cells were infected as previously described (Chackerian *et al.,* 1995). For each infection the virus inoculum (25 ng of SIVp27) remained on the cells for the duration of the infection, and the cells were analyzed for luciferase or $\beta$-gal activity 24 h postinfection. Consistently, the LuSIV cells were at least three times more sensitive than the sMAGI and as high as eight times more sensitive in some instances (Fig. 5). In addition to the greater sensitivity, the LuSIV assay required a third of the time that the sMAGI assay required to set up for infection and process for $\beta$-Gal activity.

## DISCUSSION

We have developed a very sensitive and quantitative LTR-based infectivity assay system in CEMx174 cells that can detect infection by a broad range of HIV/SIV viral strains. The LuSIV cells described in this study are capable of detecting a single round of HIV/SIV infection. The extent of viral infection in the LuSIV cells directly reflects the viral input when used to measure a single replication cycle. Since virus does not spread in this time frame, the luciferase signal is indicative of the virus that was able to enter the cells and produce the Tat protein.

Several elements in the design of the LuSIV system have contributed to its high sensitivity to viral input, low background luciferase activity, and high signal-to-noise ratio. The pLuc plasmid derived from the pCEP4 plasmid is episomally maintained and, unlike plasmids that integrate into genomic DNA, is unlikely to be influenced by endogenous promoters that may alter the expression of luciferase in the absence of Tat. The portion of the $SIV_{mac}239$ LTR ($-225 \rightarrow +149$) used to generate the pLuc plasmid has been previously shown to be the minimal region required for efficient transactivation by SIV Tat (Anderson and Clements, 1992). The choice of luciferase as a reporter contributed to the sensitivity of the assay, as it can be detected at concentrations in the picomolar range (Wood, 1991). Finally, cloning of the LuSIV cells by limiting dilution after stable selection allowed us to select a single clone representing the highest signal-to-noise ratio of luciferase in response to infection.

One potential limitation of this assay system is that CEMx174 cells are in the lymphocyte lineage and support infection by primary/molecular strains of SIV and T-cell-tropic (T-tropic) strains of HIV, but are unable to be infected with most macrophage-tropic (M-tropic) strains of HIV-1 (Chen *et al.,* 1997; Jolly, 1997; Stefano *et al.,* 1993). The inability of CEMx174 cells to support infection by M-tropic strains of HIV-1 results from the lack of expression of the necessary chemokine coreceptors,

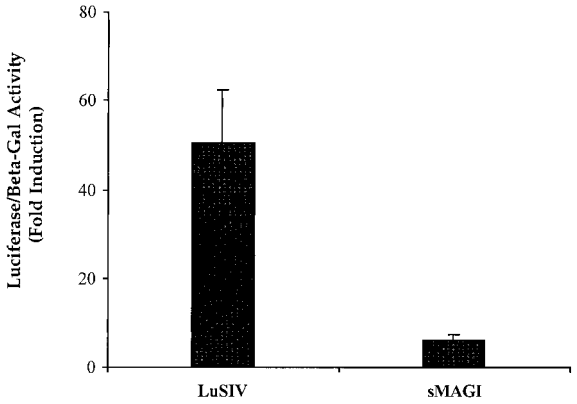


FIG. 5. SIV infection in LuSIV and sMAGI cells. The sensitivities of LuSIV and sMAGI cells were directly compared by parallel infection with 25 ng of SIV/17E-Fr. Each assay system was prepared for infection as described under Materials and Methods. At 24 h postinfection the LuSIV and sMAGI cells were analyzed for luciferase and $\beta$-Gal activity, respectively. The values are represented as fold induction of luciferase or $\beta$-Gal activity (± SD) over background activity and are the mean values of infections performed in quadruplicate.

that is, CCR5 (Chen *et al.,* 1997). Although this may be a shortcoming of the LuSIV cells, we are in the process of developing the assay system in a cell line susceptible to M-tropic HIV-1. Another approach to circumvent this problem is to transfect LuSIV cells with CCR5 and select stable cells using a different selectable marker. Despite these limitations, the LuSIV assay system is a useful tool to study the infectivity of a broad range of HIV and SIV viral strains.

The use of luciferase as a reporter gene in LTR-based assay systems such as ours is not unique. Aguilar-Cordova *et al.* (1994) have described an LTR-luciferase assay system in Jurkat cells, although the assay requires 4–5 days before a significant activation of luciferase is realized. Several other assays utilize LTR-based reporter constructs for detection of HIV/SIV infection; however, these systems do not offer the level of sensitivity we have demonstrated with the LuSIV system (Aguilar-Cordova *et al.,* 1994; Chackerian *et al.,* 1995; Felber and Pavlakis, 1988; Gervaix *et al.,* 1997; Kimpton and Emerman, 1992).

At present, two of the more common cell-based assays used to quantitate HIV/SIV infection are the MAGI and sMAGI systems. These cell assay systems can detect a single round of infection and determine the infectious titer of a viral stock. However, the cell lines containing the integrated LTR-$\beta$-Gal reporter construct used in both MAGI systems are of the fibroblast lineage and are not representative of the HIV/SIV host cells *in vivo.* These cells require the coexpression of CD4 by transfection and may not provide the best model for viral infection. In contrast, CEMx174 cells naturally express CD4 and are a common cell line used to study HIV/SIV infection. The ability to detect a single round of infection in MAGI/sMAGI assay systems often requires the addition of viral inhibitors such as AZT to prevent viral spread and allow for accumulation of $\beta$-gal; therefore, the MAGI/sMAGI cells are not processed for $\beta$-gal activity until 2 to 3 days postinfection. However, we can readily detect significant luciferase activity 24 h postinfection with the LuSIV cells that is indicative of a single round of HIV/SIV infection. When we directly compared the sMAGI and LuSIV assays, the LuSIV assay was three to eight times more sensitive using the same virus input and conditions. It is important to note that not only is the LuSIV assay more sensitive, but it also requires considerably less time to set up and quantitate the result. In addition, as a result of the intrinsic properties of each enzyme, the measurement of $\beta$-gal activity can be time consuming, whereas the time required to measure luciferase activity is minimal.

We believe the usefulness of the LuSIV cell system extends beyond its ability to quantitate HIV/SIV infection. CEMx174 cells are a common cell line used to titer viral stocks and perform neutralization assays. Both of these techniques often use cytopathic effect (CPE) or p24/p27 production as an indicator of infection. With an adaptation to the LuSIV cells these assays would be easier to perform without the requirement of visual inspection to ascertain CPE or the high costs associated with p24/27 assays. Furthermore, the LuSIV cells are not biased against those strains of HIV/SIV that do not cause CPE upon infection.

Other applications for the LuSIV cells include high throughput screening of antiviral agents and replication-deficient viruses. The LuSIV assay utilizes a 96-well format that allows for the screening of multiple antivirals on a single plate in minimal time. Since the LuSIV cells detect both a single round of replication and virus spread, they can be used to monitor inhibitors of viral entry, maturation, specific viral proteins, and other essential steps in the viral life cycle. A strategy used by several groups in the field of vaccine development is to generate replication-deficient viruses that are able to enter the host cell but are unable to establish a productive infection, usually because of the absence of necessary viral gene(s) or protein(s). The ability of LuSIV cells to detect small quantities of virus input would make our assay useful for screening replication-deficient viruses for safety purposes and/or general infectivity. In addition, the LuSIV assay system has been used in a recent study to demonstrate increased infectivity of HIV particles adsorbed to specific adhesion molecules expressed on the surface of 293 cells (Liao *et al.,* 2000). We believe the LuSIV assay will allow for the development of new strategies to understand HIV/SIV infection that have currently been thwarted because of the inability of current techniques to achieve the high sensitivity observed with LuSIV cells.

## MATERIALS AND METHODS

### Plasmid constructions

The pLuc reporter construct was made by inserting the $SIV_{mac}239$ ($-225 \rightarrow +149$) long terminal repeat (LTR) upstream of the firefly luciferase reporter gene (de Wet *et al.,* 1985) into the pCEP4 plasmid (Invitrogen, Carlsbad, CA). The LTR region was amplified by polymerase chain reaction (PCR) with primers derived from the 5′ and 3′ regions of the $SIV_{mac}239$ LTR ($-225 \rightarrow +149$).

The 5′ primer (5′CTAGGTACCGATGTCGACAGTATGAGGCATATGTTAGATACCC-3′) was engineered with *Kpn*I and *Sal*I restriction sites upstream of the LTR, and the 3′ primer (5′-TCTCCATGGCACGAGGGCTTTAAGCAAGCGTGG-3′) contained an *Nco*I restriction site downstream of the LTR. The amplified LTR region was digested with *Kpn*I and *Nco*I (Life Technologies, Grand Island, NY) and subcloned into the pGL3 basic plasmid (Promega, Madison, WI) directly 5′ to the luciferase reporter gene. The subclone was digested with *Sal*I (Life Technologies) to excise the entire expression cassette containing the $SIV_{mac}239$ LTR, luciferase reporter gene,

and poly(A) signal. This expression cassette was subcloned into pCEP4, which had previously been digested with *Sal*I to remove the CMV promoter, multicloning site, and poly(A) signal. The final construct was referred to as pLuc.

The plasmids containing the SIV Tat gene p11 (Anderson and Clements, 1992), the HIV Tat gene RSV-HIV Tat (NIH AIDS Research and Reference Reagent Program, Rockville, MD), and the visna virus Tat gene CMV-Tat (Carruth *et al.,* 1996) have been described.

### Cell culture

The B-cell/T-cell hybrid CEMx174 (Salter *et al.,* 1985), a generous gift from Dr. James Hoxie (University of Pennsylvania), was maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM sodium pyruvate, 10 mM HEPES, 0.5 mg/ml gentamicin and 2 mM L-glutamine (cRPMI). The LuSIV cells were maintained in cRPMI supplemented with 300 $\mu$g/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN) for maintenance of the pLuc plasmid.

To generate the LuSIV cells, CEMx174 cells ($5 \times 10^5$) were transfected by electroporation with 12 $\mu$g pLuc plasmid DNA. The cells were maintained in cRPMI supplemented with 1 mg/ml hygromycin B to select for stable transfectants. Once a stable cell line was generated, the hygromycin concentration was lowered to 300 $\mu$g/ml for maintenance of the pLuc plasmid. These cells were cloned by limiting dilution and the final clone selected had the highest signal-to-noise ratio of luciferase activity upon infection.

The CMMT-CD4-LTR-$\beta$-Gal (sMAGI) cells were obtained from Dr. Julie Overbaugh through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (Chackerian *et al.,* 1995). The sMAGI cells were maintained in DMEM supplemented with 10% FBS (Atlanta Biologicals), 2 mM sodium pyruvate, 10 mM HEPES, 0.5 mg/ml gentamicin, and 2 mM L-glutamine (cDMEM). For maintenance of the CD4 and LTR-$\beta$-Gal plasmids in the sMAGI cells, cDMEM was supplemented with 700 $\mu$g/ml G418 (Boehringer Mannheim) and 140 $\mu$g/ml hygromycin B.

### Virus stocks

Virus stocks of the molecular clones SIV/17E-Fr (Flaherty *et al.,* 1997), $SIV_{mac}239$ (Daniel *et al.,* 1985; Kestler *et al.,* 1990; Naidu *et al.,* 1988), and $SIV_{mac}251$ (Daniel *et al.,* 1985; Naidu *et al.,* 1988) were prepared by transfection of CEMx174 cells with infectious viral DNA. The cells were cultured in cRPMI until the reverse transcriptase (RT) activity was >80,000 cpm/ml, as determined by a standard RT assay (Clabough *et al.,* 1991). At the time of harvest, cell-free supernatants were filtered through a 0.45-$\mu$M syringe filter (Millipore, Bedford, MA), aliquoted, and stored at −80°C. SIV/17E-Br and SIV/Delta B670 are virus swarms prepared as previously described (Sharma *et al.,* 1992). HIV-1 strains (IIIB, RF, MN) were all obtained from the AIDS Research and Reference Reagent Program. The viruses were used to establish chronically infected PM1 cells as previously described (Orentas and Hildreth, 1993). Supernatants from infected cells were used as virus stocks after passage through 0.45-$\mu$M filters. HIV-1 was quantitated by p24 ELISA (Orentas and Hildreth, 1993).

### Cotransfection of CEMx174 with pLuc

CEMx174 cells ($5 \times 10^5$) were cotransfected with 6 $\mu$g of pLuc and 6 $\mu$g of the plasmids containing SIV Tat, HIV Tat, visna Tat, or the full -length viral clones HIV/pNL4–3 and $SIV_{mac}239$ by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) set at 200 V and 960 $\mu$F. At various times posttransfection $2 \times 10^5$ viable cells were analyzed in the luciferase assay.

### LuSIV infections

Virus was prepared for infection by pelleting 1 ml of the desired virus input through 100 $\mu$l of 20% sucrose/25 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM EDTA (TNE) in a Sorvall 5CRC high-speed centrifuge with an SH-MT rotor at 13,500 rpm for 1 h at 4°C. The supernatant was poured off and the virus pellet resuspended in phenol red-free RPMI 1640 (prfRPMI; Life Technologies) supplemented with 2% FBS at the desired inoculum volume. The LuSIV cells were counted by trypan blue exclusion, washed one time in prfRPMI, and resuspended in prfRPMI with 2% FBS to $2.0 \times 10^5$ cells/well. The cells were then added to the appropriate number of wells in a 96-well flat-bottom plate (Costar, Corning, NY), the virus inoculum was mixed with the cells and the plate was incubated at 37°C. For the experiments using the RT inhibitor, 3′-azido-3-deoxythymidine (AZT; Sigma, St. Louis, MO) LuSIV cells were infected for 6 h, washed two times with PBS, and resuspended in 200 $\mu$l of prfRPMI with 2% FBS containing AZT (10 $\mu$M). Control wells were treated with AZT (10 $\mu$M) at the time of infection.

### Luciferase assay

At the desired time postinfection the LuSIV cells and medium were transferred from the flat-bottom 96-well plate to a round-bottom 96-well plate (Costar). The cells were pelleted at 2500 rpm, the supernatant was removed, and the cell pellet was lysed in 50 $\mu$l of 0.5% Triton X-100 in PBS. After 5 min at room temperature, 25 $\mu$l of the lysate was analyzed in a Labsystems Fluoroskan Ascent FL with injector apparatus. To the lysate, 100 $\mu$l of luciferase assay substrate [20 mM Tricine, 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 $\mu$M coenzyme A, 470 $\mu$M D-luciferin (JBL, San Luis Obispo, CA), 530 $\mu$M ATP, and 33.3 mM DTT (Boehr-

inger Mannheim)] (Fortin *et al.,* 1997) were added by injection. Each well was analyzed individually for 5 s at the maximum photomultiplier tube (PMT) voltage. The output was relative light units (RLU), where an RLU is arbitrarily determined by the instrument and correlates with the photons of light detected in a given sample.

### sMAGI assays

The day before infection, sMAGI cells were plated into a 96-well flat-bottom plate such that the confluency was 40 to 50% for the infection. The next day the media was removed and the cells were inoculated with 50 $\mu$l of SIV/17E-Fr (25 ng p27) diluted in cDMEM containing 15 $\mu$g/ml DEAE-Dextran (Amersham Pharmacia Biotech, Piscataway, NJ). After incubation with the virus inoculum for 2 h at 37°C, an additional 150 $\mu$l of cDMEM was added to the cells and incubated at 37°C. After 24 h the cells were fixed and stained for $\beta$-Gal activity as previously described (Chackerian *et al.,* 1995). The extent of viral infection was determined by visual inspection and manual quantitation of infectious blue centers. The data are represented as fold induction of $\beta$-Gal activity over background.

### Virus sensitivity assays

The standard virus input for each assay was 25 ng of SIV p27 or HIV p24 antigen, as determined by enzyme immunoassay (Coulter, Miami, FL; Organon Teknika, Durham, NC). LuSIV cells ($2 \times 10^5$) were infected with serial fivefold dilutions, starting with 25 ng of SIV p27 or HIV p24 in triplicate. The samples were analyzed in the luciferase assay at 24 and 48 h postinfection. The data are represented as fold-induction over background, and the lowest virus input yielding a fourfold induction was considered the sensitivity limit.

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