Published in final edited form as:

Curr HIV Res. 2007 July ; 5(4): 394–402.

Rev-Dependent Indicator T Cell Line

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Abstract

Measuring virion infectivity is critical for studying and monitoring the process of HIV-1 infection. The easiest and the most common method utilizes reporter cell lines based on the HIV LTR promoter. The early HIV gene product Tat amplifies expression from the LTR; however, there is a background transcriptional activity that is independent of Tat. Furthermore, LTR activity can be influenced by cellular activation states. We have recently constructed a Rev-dependent expression vector, and as a test of this construct's functionality, we have integrated this vector into a continuous T cell line. This novel indicator cell has no measurable background signal, is not affected by elevated metabolic states, and yet responds robustly to the presence of HIV. The line is able to complete TCID₅₀ assays in 3–5 days, and appears sensitive to both CCR5- and CXCR4-utilizing viruses.

Keywords

Reporter; HIV; Rev; GFP

All existing HIV indicator cell lines are based on the HIV long terminal repeat promoter, LTR. An early HIV gene product, Tat, promotes HIV transcriptional activity that is initiated at the LTR; therefore, the placement of reporter genes downstream to the LTR makes expression responsive to the synthesis of Tat, a measure of HIV replication. However, inherent features of the LTR lead to limitations of this particular tool. First, the LTR is leaky. Following integration of the HIV provirus into the host chromatin, the LTR transcribes the early gene products Tat, Rev, and Nef. Tat and Rev are critical for productive viral transcription, but neither of the proteins is present in the infecting viral particle. As such, there is a required basal level of transcription that is Tat-independent. A second limiting feature of the LTR is its responsiveness in the absence of HIV to the activity state of cells, in particular, T cells. The HIV promoter possesses binding sites for transcriptional factors commonly induced in active T cells [6,13,17,27]. Thus, proliferating T cells typically yield higher background reporter expression from LTR-based vectors in the absence of HIV [23]. Furthermore, the presence of cytokines or mitogens further induces LTR-based transcriptional levels [1,27,29]. Nevertheless, T cells are a natural host for HIV, and support replication at a level higher than other cells used as HIV indicator lines. For example, infectivity measurements with human CD4 T cell lines, such as MT-2, MT-4, or CEM cells result in a 5- to 10-fold increase in infectivity of HIV over the adherent HeLa-CD4 cell line [10,31,32]. Thus, an increased capacity to report HIV infectivity with T cell indicator lines also comes with an increased background signal.

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Besides the selection of the reporter cell itself, there is also variation in the choice of the reporter gene. Some of the earliest HIV indicator approaches utilized enzyme reporters, such as chloramphenicol acetyltransferase, luciferase, or β -galactosidase, from lysed cell populations [1,5,12,26,32], where reporter yield is directly proportional to HIV replication within the population. These enzyme-based assays can be carried out in multi-well plates, permitting high sample number output, and are straightforward in their ability to measure neutralization or viral inhibition in the populations. These are all relatively objective measurements, but background levels of reporter in the populations must be determined and data must be normalized. There are some applications where these systems cannot function well; for example, low level (cell number-wise) infection is not easily discernable and accurate enumeration of infected cells is not possible. In addition to enzyme reporters, the use of fluorescent proteins has found utility in HIV indicator cells [3,8], where quantitation of reporter-positive cells is achieved by flow cytometry, and the average fluorescence intensity of cell populations or the percent of GFP-positive cells corresponds to the level of HIV infection.

Direct enumeration of primary infection events, that is the measurement of infectious particles, is better achieved with adherent reporter cells [12,24], where infectious foci can be easily determined. However, because of the inherent leakiness, only darkly stained cells are counted [33] or incubation of the fixed cells with substrate is held to a minimum time [12,24,33]. It has been noted that background in LTR-based reporter HeLa cells can be minimized by increased confluency [24].

In our own work on basic studies of the HIV infection process, including how cellular and viral components contribute to the efficiency of the process, it becomes critical to know the number of infectious particles. At low levels of virus, it becomes difficult to discern the presence of infectious virus from background signal. A reporter line that had high sensitivity to HIV infection, but lacked the non-specific noise seen with the LTR-based reporters, might permit a wider use of indicator cells to accurately measure HIV infectivity. We have recently constructed an HIV-dependent expression vector that is based on Tat and Rev activity [34] (see Fig. 1A). The vector is composed of various HIV sequences including both 5' and 3' LTRs, splice donor and acceptor sites, and a reporter gene coupled to a Rev response element (RRE). It is silent in the absence of HIV and appears to be an ideal expression system for an HIV reporter cell. As CEM cells have been shown to be highly sensitivity to HIV infection [8,32], we decided to examine the utility of the Rev-dependent vector stably expressed in this cell line (Fig. 1B).

RESULTS

We transduced the Rev-dependent green fluorescent protein (GFP) reporter expression vector [34] into the CEM-SS T cell line. None of the cells within the population transduced with the lentiviral vector displayed any GFP fluorescence, as defined by fluorescent microscopy examination or flow cytometry (data not shown). As there was no selection marker, individual clones were examined by exposure to HIV and scored for the presence of GFP expression. Initially, 129 clones were isolated, and 23 of these responded to HIV_{NL4-3} infection with GFP production. If the cloning was random, this suggested that minimally 18% of the CEM population was successfully transduced with a functional reporter vector. One of the clones, which yielded bright GFP-positive cells after HIV infection, is characterized in this report and is designated here as Rev-dependent CEM, or Rev-CEM. Characterization of this clonal line by flow cytometry demonstrated an expected presence of CD4 (Fig. 2A) and the co-receptor CXCR4 (Fig. 2B), as seen on the parental CEM-SS population; however, there was also the unexpected presence of a low level of CCR5 (Fig. 2C). The native expression of the CCR5 HIV co-receptor on CEM lines has previously been reported [14,15], with one of the papers noting that *in vivo* culturing resulted in enhanced expression [14]. Although the Rev-CEM also

has increased levels relative to the parental line, it was a fortuitous event without known causation. The expression of these receptors appeared stable over 3 months of continuous culture.

Given the presence of the two major HIV co-receptors, we then examined the cell for susceptibility to infection by various HIV-1 strains (see Table 1). As noted with previous GFP-based indicator cells [3,8], the expression of GFP in the Rev-CEM has an advantage of detection of on-going HIV infection in living cells without fixation or enzyme processing. We found that the Rev-CEM clone was susceptible to laboratory derived HIV-1, a range of HIV-1 primary isolates from subtype A to E, as well as macrophage tropic HIV_{Ba-L} and HIV-1_{NL4-3(AD8)}. Susceptibility to HIV infection of cells expressing very low CCR5 levels has been noted [20].

Although measurements of infectivity can be improved by using T cells, there are also disadvantages in the choice of T cell lines as indicator cells. As mentioned above, there is a correlation of T cell activity and induction of LTR activity in the absence of Tat. To address the susceptibility of the Rev-dependent reporter to non-HIV stimuli, we compared signals from the previously published LTR-based CEM-GFP [8] with the Rev-dependent Rev-CEM line. In the absence of any mitogen, the CEM-GFP cell (Fig. 3, dashed blue line) was found to possess a basal activity higher than the Rev-CEM (Fig. 3, dashed red line), whose background fluorescence was indistinguishable from the parental CEM-SS cells (data not shown). Following addition of the mitogen PMA (Fig. 3, solid lines), the Tat-dependent CEM-GFP cell displayed a forty-fold HIV-independent increase in mean fluorescence intensity, relative to the Rev-dependent cell, which was unaffected by this non-HIV stimuli. Cytokines were also without effect on the Rev-CEM cell (TNF, GM-CSF; data not shown).

Clearly the addition of Rev-dependency to an LTR-based vector offers greater stringency than vectors based only on the LTR, but as an additional restriction, the Rev-dependency might also diminish the level of response to HIV. To compare signal induction of a Rev-based reporter system to an LTR-based system, we infected the Rev-CEM and the LTR-based CEM-GFP cell line [8] with equivalent levels of HIV-1_{NL4-3} and measured subsequent GFP production by flow cytometry. A low multiplicity of infection was used to minimize multiple integrations into a single cell. We then measured GFP expression by setting a gate to capture events with above background FL1 intensities. This region for the CEM-GFP LTR-based system yielded cells with a 33-fold increase in mean fluorescence intensity over the average intensity of the uninfected population (compare Fig. 4A and B; also see legend). The Rev-CEM line responded with an 11-fold increase in average fluorescence intensity (Fig. 4C and D), defining a one-third reduction in intensity response compared to the LTR-based cell. The intensity of the brightest cells for both populations of HIV-infected cells were nearly equivalent, and unexpectedly the percent of the total population which reported infection (above background GFP fluorescence) following equivalent HIV exposure was 10-fold higher in the Rev-CEM cell (see Fig. 4 legend).

We then assessed the ability of Rev-CEM to determine viral titer. Suspension cells present limitations as indicator lines, as the enumeration by microscopy of reporter-positive suspension T cells can be imprecise, and one must prevent secondary spread of the virus. T cell suspensions are effectively used, however, in a second type of assay for viral infectivity, the TCID₅₀ (50% tissue culture infectious dose) assay. The TCID₅₀ value is defined by the reciprocal of the viral dilution at which there is a 50% probability of containing an infectious virion [22]. We compared human PBMC, CEM-SS cells, and the Rev-CEM cell in evaluating serial dilutions of NL4-3 HIV in a TCID₅₀ assay. The PBMC and CEM-SS cells were evaluated for HIV p24 production at 14 days post-infection, whereas Rev-CEM cells were evaluated by fluorescent microscopy at 5 days. At 5 days secondary infection has occurred and clusters of GFP-positive cells were evident. It is critical to appreciate that the scoring for TCID₅₀ assays only requires

a determination of the positive or negative presence of replicating HIV, and enumeration of GFP-positive cells is not necessary. With no background in the Rev-dependent system, the presence of any reporter expression was easily counted as positive. As shown in Table 2, Rev-CEM infection yielded comparable viral titers in the TCID₅₀ assay to those obtained from CEM-SS and PBMC infection, with these latter cells evaluated by p24 ELISA. In practice, we found the lack of background facilitated any analysis. Methodologies with LTR-based systems can involve attempts to minimize background activity, such as the counting of only darkly stained cells [33]. The TCID₅₀ assay with the CEM-Rev requires incubation of cells with serial dilutions of the viral suspension, and after a few days, examination by fluorescence microscopy. Direct enumeration of GFP-positive CEM-Rev cells is proportional to virus input (data not shown), but because of secondary infection, it will not yield titer values of the input solution. We have also compared the TCID₅₀ assay with the CEM-Rev to adherent cell infection with foci counting. When comparing a stock HIV_{NL4-3}, the titer derived from the Rev-CEM with the TCID₅₀ assay was 15-fold higher (15.0 ± 1.2 ; mean $\pm$ SE, n=3) than that determined by the LTR-based HeLa reporter cell foci assay, consistent with noted differences in infectivity with these different cell types [10,31,32]. We suspect that this reduction in infectivity measurements with the LTR-based cell may in part be due also to the exclusion, by limited substrate incubation, of truly infected cells with low-level reporter generation.

Given the added susceptibility to macrophage tropic HIV, the TCID₅₀ assay was also performed with primary human macrophages and Rev-CEM. Rev-CEM yielded a comparable titer in 5 days, when compared to 25 days with infection of primary macrophages (Table 2). Longer incubations of the Rev-CEM samples did not result in additional positive wells. When we determined HIV p24 from macrophages at shorter time periods, we derived lower TCID₅₀ values (Table 2), due to the lack of p24 detection in the most dilute samples. Thus the comparison of the times required for optimal performance of the two assays (5 versus 25 days) is reasonable.

The lack of positive cells by flow cytometry or fluorescent microscopy in HIV-negative populations of Rev-CEM cells does not give us an absolute sense of background reporter generation, especially since we assume that the LTR in a T cell line should have measurable background transcription. A more sensitive approach to access the reporter transcriptional activity in the Rev-CEM cell is RT-PCR analyses for HIV-1 induced expression of the reporter gene, as described in Fig. (5). We used two sets of primers that can amplify both the spliced and non-spliced transcripts from the lentiviral construct (Fig. 5A). We predicted that in the absence of HIV infection, Rev-CEM would generate some basal level of transcription from the integrated LTR promoter, similar to LTR-based indicator cells. As shown in Fig. (5B), in uninfected Rev-CEM cells, we observed basal levels of transcription from the LTR promoter (lane 2, spliced reporter RNA). This transcriptional activity would represent HIV-independent signal; however, in this Rev system the transcripts were all spliced (lane 2; compare spliced to unspliced reporter RNA) and thus lacked the capacity to generate the GFP reporter. The generation of a non-spliced, reporter-encoding message (lane 4) was dependent on HIV infection. Note that the vectors used to detect spliced transcript will also recognize HIV transcripts. As shown in Fig. (5B) HIV-specific transcript, consistent with the size of fully spliced transcript amplified with these primers (0.76 kb), appeared in the two cell populations infected with HIV (lanes 3 and 4). These data confirmed that expression of GFP in the Rev-CEM cell was HIV-dependent, and that the dependency corresponded to the lack of splicing of reporter message.

DISCUSSION

The inclusion of Rev-dependent elements to the classical LTR-based (Tat-dependent) expression system resulted in a lack of background signal that is associated with LTR-based

reporters. This highly stringent reporting has permitted the use of indicator T cells as tools to accurately quantitate infectious viral titer, and demonstrates the high stringency seen with the Rev-dependent expression vector. In this report we have utilized the term *Rev-dependence* to distinguish this construct from the existing LTR-based Tat-responsive HIV reporter systems; however, with the intact LTR, we would speculate that this Rev-dependent vector should maintain responsiveness to Tat expression as well. This dual dependency is likely to contribute to the higher stringency.

The Rev-CEM cell brings efficiency and a simplification to the standard TCID₅₀ assay. Operationally, the assay involves a determination of whether replication occurred in a well or not, where scoring is either positive or negative for the presence of HIV. Thus, the most critical measurements are at the greatest dilution of virus. This means that assays that involve slow viral replication, such as HIV infection of primary macrophages, may take two to three weeks. Additionally, measurement of p24 or reverse transcriptase is labor-intensive. While this assay performs well with the strains of HIV used here, examination of other virus, especially primary isolates, will require comparisons to PBMC cultures. In a previous work with a CXCR4- and CCR5-positive CEM indicator line, the CEM indicator cell and PBMCs were both infected by a wide range of primary HIV isolates; however, the two types of cells displayed differential measurements of infectivity to these individual isolates [28].

In a comparison of LTR-based expression to the Rev-based reporter CEM cells we find a reduction in the average fluorescence intensity of GFP-positive Rev-dependent cells, potentially due to the additional molecular requirements for expression in the Rev system. However, we may also be overestimating the robustness of the response (not in cell number, but in fluorescence intensity) by the LTR-based cell due to its wide range of background (GFP) fluorescence. That is, a ten-fold increase in fluorescence for approximately one half of the CEM-GFP cell population in Fig. (4A and B) would not result in a fluorescence intensity that would move into the GFP-positive window. Indeed, this might be the factor responsible for the reduction of response in the number of generated GFP-positive CEM-GFP cells, relative to the Rev-CEM cell, following HIV infection. This wide range of fluorescence in uninfected CEM-GFP cells may in part be due to cell cycle events. It has been demonstrated that transcripts driven by an LTR-reporter system in Jurkat T cells are most abundant in the G2 phase of the cell cycle, about 5-fold higher when compared to the same cells in the G1/S phase, in the absence of any HIV proteins [9].

Infectivity measurements of HIV are intended to quantitate the level of replicative competent virions. When this is attempted by classical indicator lines, there is the problem of background signal, which interferes with an accurate enumeration. While certain cell types can be utilized to minimize background, the choice can also minimize efficient infection, comparable to natural target cells such as T cells. One successful approach for determining viral titer has been the TCID₅₀ assay, and certainly one reason for using this assay is that it permits use of primary cells, such as PBMCs and macrophages, which can support wide ranges of viral isolates. The standard procedure, however, requires a demonstration of above background signal as generated from replicating virus. This can add significant time to the assay. The absolute absence of GFP expression from the Rev-CEM line (by microscopy, FACS, or RT-PCR) makes it uniquely applicable to the TCID₅₀ assay. The presence of GFP-positive cells implies replicative competent HIV. In practice we perform this assay in flat-bottom multi-well plates, and GFP-positive cells become apparent in 2–3 days, when we commonly add fresh media. Typically, within two more days, there is an increase in the number of GFP-positive cells, but only in those wells previously identified as being HIV-positive.

MATERIALS AND METHODS

Viruses and cells

Viruses and cells were obtained through the NIH AIDS Research and Reference Reagent Program: HTLV-III_B/H9 from Dr. Robert Gallo [21]; HIV-1_{Ba-L} from Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo [7]; HIV-2CBL-20/H9 from Dr. Robin Weiss [25]; HIV-1DJ263(GS003), HIV-1US1(GS004), HIV-1ETH2220(GS014), HIV-1SE365(GS017), HIV-1CM235(GS020) from Dr. Nelson Michael [11]; CEM-SS from Dr. Peter L. Nara [19]; CEM-GFP from Dr. Jacques Corbeil [8]; HeLa-CD4-LTR- β -gal (MAGI) from Dr. Michael Emerman [12]. HIV_{NL4-3}(AD8) is HIV_{NL4-3} in which a segment from the HIV_{AD8} *env* has been exchanged [4]. HIV-1AD8 is a macrophage-tropic molecular clone [30]. Macrophage derived monocytes (MDMs) were derived from peripheral blood monocytes from healthy donors from the Department of Transfusion Medicine at the National Institutes of Health [2]. Cells were cultured in RPMI media supplemented with 10% fetal bovine serum (Hyclone) and recombinant human macrophage colony stimulating factor (rH-MCSF) (R & D Systems) at 10 ng/ml. Media supplemented with rH-MCSF was changed every 48 hours for ten days. All other cells were grown in RPMI with 10% fetal bovine serum.

DNA constructs

pCMV_R8.2 and pMD.G have been described previously [18]. The development of pNL-GFP-RRE has been recently reported [34].

Virus generation

Stocks of the HIV-1_{NL4-3} and HIV-1_{NL4-3}(AD8) were prepared by transfection of HeLa cells with cloned proviral DNA. HIV-1 based lentiviruses carrying reporter genes were made by cotransfection of DNA constructs as follows: 2×10^6 293T cells were cotransfected with 10 μ g of pNL-GFP-RRE-SA, 7.5 μ g of pCMV Δ R8.2, and 2.5 μ g of pMD.G using the calcium phosphate method (Promega). Viruses were harvested 2 days after cotransfection and filtered through a 0.45 μ m filter and stored at -80° . Virus generated from the pNL-GFP-RRE-SA transfection (vNL-GFP-RRE(SA)) was used to produce the CEM-SS indicator cell (Rev-CEM).

Indicator cell production

CEM-SS cells were infected with lentivirus, vNL-GFP-RRE(SA) (see above). Infected CEM-SS cells were diluted and aliquoted into 96 well plates and grown for two weeks. Initially, 129 clones were isolated. 23 of them responded to HIV infection with GFP production. None of the transduced cells expressed detectable GFP in the absence of HIV infection. One of the clones, G11.1.16, designated here as Rev-CEM, was selected and further purified by three repeated clonings.

HIV infection of GFP indicator cells

Rev-CEM cells and CEM-GFP T cells were seeded into 6 well format at 10^6 cells per well. HIV_{NL4-3} was added to each well at a multiplicity of infection of approximately 0.01 and incubated at 37° for 3 hours, followed by an addition of medium to a final volume of 1.5 ml RPMI supplemented with 10% fetal bovine serum and gentamicin. Cells were incubated for 3 days then fixed with 1 ml of CytoFix CytoPerm (Becton Dickinson) for 20', washed, then examined by flow cytometry. Gains were set to move control populations to full visibility at the lowest FL1 intensity and compensation was achieved with GFP-positive cells [16]. Gates were set to capture GFP expression where background fluorescent events of uninfected control populations did not exceed 0.1%.

TCID₅₀ assay

TCID₅₀ assays were carried out using pre-activated peripheral mononuclear blood cells (PBMC, 3 ug/ml PHA, 10U/ml IL-2), monocyte-derived macrophage (MDM), CEM-SS, or the Rev-CEM indicator cell line. 2×10^5 PBMC were infected with serially diluted virus for 2 hrs. Following three washings, cells were cultured (RPMI + 10% FCS, 2 ml) in 24 well plates for 2 weeks to measure p24 release. CEM-SS or Rev-CEM cells were infected as described above for PBMC and viral replication was measured by p24 release or by fluorescence microscopy, respectively. For MDM, 5×10^5 monocytes were cultured in 24 well plates in media plus rh-MCSF (10 ng/ml) for two weeks, as described above, and then infected with serially diluted virus. Infected cells were washed and cultured in RPMI plus 10% FCS up to one month with fresh medium additions. Viral replication was measured by p24 release. Following enumeration of positive and negative wells, the TCID₅₀ was determined by the method of Reed and Muench [22].

For routine measurements of infectivity with the Rev-CEM cells, we now perform this assay in 96 well plates: 2×10^5 cells are infected with serially diluted virus in flat-bottom 96 well plates (final volume 200 μ l RPMI + 10% FCS) and incubated for 3 to 5 days with fresh medium added as needed. The presence of GFP-positive cells was identified by fluorescent microscopy.

RT-PCR analysis

Total cellular poly(A⁺) mRNA was purified from cells by MicroPoly(A)Pure mRNA isolation kit (Ambion) as recommended by the manufacturer. Reverse transcription was accomplished using the RETROscript First-Strand Synthesis Kit (Ambion) with random decamers as the first-strand primers. Following cDNA synthesis, PCR was carried out using primer 5' TAATCGGCCGAACAGGGACTTGAAAGCGAAAG3' and 5' CAGGCACAAGCAGCATTGTTAG 3' to amplify spliced lentiviral transcripts, and primer 5' TAATCGGCCGAACAGGGACTTGAAAGCGAAAG 3' and 5' ATCGTGTACGGCCGAATTGGGTACACTTACCTG 3' to amplify non-spliced GFP transcripts. The PCR condition was: 1x PCR buffer, 125 μ M dNTPs, 1.5 mM Mg²⁺, 50 pmol of each primer, 1 U SuperTaq Plus (Ambion) in 50 μ l, with 30 cycles of 20 sec at 94°, and 180 sec at 68°. One fifth of the product was analyzed on 2% agarose gel. Cellular β -actin transcripts were amplified using Quantum-RNA β -actin Internal Standards (Ambion) with similar conditions as above except using 20 pmol of each *actin* primer and runs at 20 sec at 94°, 30 sec at 55°, and 40 sec at 68°.

Flow cytometry

Cells were resuspended into 100 μ l Hank's staining buffer (Hank's buffer plus 0.1% bovine serum albumin, 0.1% sodium azide, and 25 mM HEPES, pH 7.2) for staining with antibodies at concentrations recommended by manufactures. Following staining for 30 min on ice, cells were either washed with Hank's buffer or, in the case of HIV infected cells, fixed with 500 μ l of Cytofix/Cytoperm (Becton Dickinson) for 20 min on ice for flow cytometry analysis on a FACScan analyzer (Becton Dickinson). Antibodies to CD4, CXCR4, and CCR5 were from Becton Dickinson, and mCD24 antibody was from Southern Biotechnology.

Acknowledgements

We thank Drs. Michael Emerman, Tomris Mustafa, and Sundararajan Venkatesan for their comments and criticisms concerning this manuscript. This research was supported by the Intramural Research Program of the NIMH/NIH and by the NIH grant 5R21NS51130-2 awarded to YW.

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Fig (1).

Rev-dependent reporter construct. **(A)** The vector includes four separate segments of the HIV genome with no intact HIV genes. The 5' end of the vector consists of the HIV 5' LTR, the splice donor 1 site, D1, and a portion of the *gag* open reading frame that includes the packaging signal. The second HIV segment is from the *tat1/rev1* exon that includes splice acceptor site 5, A5, and splice donor site 4, D4. The third segment of HIV DNA is from the *env* exon and encompasses the RRE, and the splice acceptor site 7, A7. The last segment includes the entire 3' LTR along with a small portion of the *nef* reading frame 5' to the LTR. In the absence of HIV infection the reporter provirus undergoes basal transcription generating a single message that is rapidly spliced, and results in the removal of the GFP reading frame. In the presence of HIV Rev, singly and non-spliced transcript are delivered to the cytosol, and the reporter gene is expressed. **(B)** CEM-SS cells were infected with the Rev-dependent green fluorescent protein (GFP) reporter construct incorporated into a lentivirus. A clone was isolated and infection by HIV resulted in generation of GFP.

Fig (2).

Flow cytometry analyses of Rev-CEM cells for surface expression of HIV-1 receptor and co-receptors. Rev-CEM and control CEM-SS cells were stained with R-phycoerythrin (A,C) or FITC (B) conjugated monoclonal antibodies (solid lines) against CD4 (A), CXCR4 (B) or CCR5 (C). The isotype antibody staining of the cells is shown in dotted lines. The isotype and CCR5 cytometry analysis of the CEM-SS cells overlapped so that the two runs are not discernable. Twenty thousand cells were examined in each run. Only viable cells are displayed. Two independent analyses yielded the same result.

Fig (3). HIV-independent induction of GFP expression in LTR-based CEM-GFP and Rev-CEM cells. To examine non-specific induction of the reporter CEM-GFP (blue lines) or Rev-CEM (red lines) cells were stimulated (solid line) or not (dashed line) with PMA (100ng/ml) for 24 hrs, then analyzed by flow cytometry for GFP expression. Twenty thousand cells were examined in each run.

Fig (4).

Comparison of reporter generation by CEM-GFP and Rev-CEM cells following low level HIV infection. The LTR-based CEM-GFP (**A** and **B**) and Rev-CEM (**C** and **D**) cell populations were either not infected (**A** and **C**) or were infected (**B** and **D**) with equivalent levels (see Methods) of HIV_{NL4-3}. After 3 days the cells were fixed and fluorescence from GFP (FL1) was determined. Gates were positioned to minimize background events from the non-HIV control populations (less than 0.1%). The fold-increase in fluorescence intensity (geometric mean) of non-infected cells (**A** and **C**) compared to the gated populations of GFP-positive cells in HIV-infected cells (**B** and **D**) was determined for both the CEM-GFP (n=2, 30.6- and 35.7-fold) and the Rev-CEM line (n=4, 11.6 ± 1.5 ; mean $\pm$ SE).

Fig (5).

Transcriptional analyses of Rev-CEM in response to HIV-1 infection. **(A)** diagram of PCR primers used. Two sets of primers were used to detect the spliced (black arrows) and unspliced (green arrows) transcripts by reverse transcriptase PCR (RT-PCR) as described in Methods. The mRNA molecules from HIV-infected (82 ng p24; HIV_{NL4-3}/0.5 million cells) and uninfected cells were extracted and analyzed by RT-PCR for the spliced transcripts and unspliced transcripts **(B)**. CEM-SS cells were used as a control for the specificity of PCR amplification. The cellular β -actin transcript was also amplified to ensure that comparable numbers of cells were being used for the analyses.

Table 1**Susceptibility of Rev-CEM to HIV Strains**

HIV Strain	Subtype	Tropism
HIV-1 _{NI 4-3}	laboratory strain, subtype B	CXCR4
HTLV-III _B /H9	laboratory strain, subtype B	CXCR4
HIV-1 _{Ba-L}	laboratory strain, subtype B	CCR5 (CCR3, STRL33)
HIV-1 _{NI 4-3(AD8)}	laboratory strain, subtype B	CCR5
DJ263(GS003)	primary strain, subtype A(A/G) ^a	unknown
US1(GS004)	primary strain, subtype B	unknown
ETH2220(GS014)	primary strain, subtype C	unknown
SE365(GS017)	primary strain, subtype D	unknown
CM235(GS020)	primary strain, subtype E(A/E) ^a	unknown

Rev-CEM cells plus virus were incubated for 3 days.

^a recombinant strains.

Table 2
TCID₅₀ Assay Using Rev-CEM, CEM-SS, Primary T Cells and Macrophages

Cell ^a	Virus ^b	Infection Time ^c	Assay	TCID ₅₀ ^d
Rev-CEM	HIV-1 _{NL4-3}	5 days	GFP observation	10 ^{5.5} /ml
CEM-SS	HIV-1 _{NL4-3}	14 days	p24 ELISA	10 ^{4.8} /ml
PBMC	HIV-1 _{NL4-3}	14 days	p24 ELISA	10 ^{5.3} /ml
Rev-CEM	HIV-1 _{NL4-3 (AD8)}	5 days	GFP observation	10 ^{6.3} /ml
Macrophage	HIV-1 _{NL4-3 (AD8)}	14 days	p24 ELISA	10 ^{4.5} /ml
Macrophage	HIV-1 _{NL4-3 (AD8)}	25 days	p24 ELISA	10 ^{6.3} /ml

^a Human peripheral mononuclear cells (PMBC) were purified from peripheral blood as described in Materials and Methods. Macrophages were derived from human primary monocytes and differentiated for two week in rH-MCSF (10 ng/ml).

^b Equal amounts of serially diluted HIV-1_{NL4-3} or HIV-1_{NL4-3(AD8)} were used to infect cells as described in Materials and Methods.

^c Cells infections were incubated for the period indicated, with the addition of fresh medium, if necessary. For macrophages, when the infection was extended from 14 to 25 days, an increased titer resulted.

^d The 50% tissue culture dose values, TCID₅₀, a measure of viral titer, were determined according to Reed-Muench (see Materials and Methods), where wells were defined as positive or negative for HIV by microscopic observation for the presence of GFP positive cells, or by measuring levels of viral p24 antigen in the supernatant. For GFP observation, single clusters or multiple GFP positive cells were present in a well. Positive p24 ELISA results were registered when p24 concentrations were equal or greater than 0.1 ng/ml in a well.