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Detection of Replication-Competent and Pseudotyped Human Immunodeficiency Virus with a Sensitive Cell Line on the Basis of Activation of an Integrated β-Galactosidase Gene

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We have constructed a HeLa cell line that both expresses high levels of CD4 and contains a single integrated copy of a β-galactosidase gene that is under the control of a truncated human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). This cell line, called CD4-LTR/β-gal, can be used to determine quantitatively the titer of laboratory-adapted HIV strains, and the method used to do so is as sensitive as the determination of viral titers in a T-cell line by end point dilution. Using this cell line as a titer system, we calculated that HIV-1 stocks contain only one infectious particle per 3,500 to 12,000 virions. Virus derived from a molecular clone of a macrophagetropic provirus will not infect this cell line. We have also cocultivated peripheral blood lymphocyte cultures from HIV-infected individuals with the CD4-LTR/β-gal indicator cells. In a majority of primary isolates (five of eight), including isolates from asymptomatic patients, rare virus-infected cells that can activate the β-galactosidase gene are present.

The ability to determine the number of infectious particles in a viral stock is essential for studying the viral life cycle and for determining the effects of drug inhibitors and mutations on the virus. Quantitative titers of human immunodeficiency virus (HIV) stocks can be determined by end point dilution of the virus, but these assays are time consuming and difficult to perform for a large number of samples. End point dilution assays, as well as plaque-forming assays (14), cytotoxicity assays (27), and focal immunoassays (7), rely on virus spread or production of viral structural proteins for detection of HIV.

We present a method of titrating HIV based on activation of an integrated LTR-β-galactosidase gene in a $CD4^+$ cell. This assay, called the multinuclear activation of a galactosidase indicator assay (MAGI assay), exploits the ability of the viral Tat protein to transactivate an integrated β-galactosidase gene driven by the HIV type 1 (HIV-1) long terminal repeat (LTR) promoter in $CD4^+$ cells (CD4-LTR/β-gal indicator cells). Individual infected cells or syncytia are counted in situ with a light microscope by virtue of their blue color after incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The MAGI assay is linear over at least 2 orders of magnitude and is as sensitive as end point dilution titration of virus in a T-cell line.

The assay described here relies on only one round of infection and, indeed, can detect the presence of replication-defective virus. The advantage of using an in situ assay over an assay that measures replication in bulk populations is that 1 infected cell in a background of 10^6 cells can easily be detected by its ability to turn blue. Routine determination of a quantitative titer of laboratory strains of HIV is completed in 2 days.

We also tested the ability of cell-free and cell-associated virus isolated from HIV-1-seropositive individuals with different degrees of immunosuppression to infect the CD4-LTR/β-gal indicator cells. These isolates very inefficiently infected or were unable to infect the CD4-LTR/β-gal indicator cells. Furthermore, virus derived from a molecular clone of an isolate that grows only on primary cells could not infect the CD4-LTR/β-gal indicator cells by either free-virus or cell-to-cell transmission. However, virus from five of eight seropositive individuals, including isolates from asymptomatic individuals, could be detected by infection of the CD4-LTR/β-gal indicator cells by cell-to-cell transmission.

MATERIALS AND METHODS

Plasmids. pJK2 is a derivative of pEQ222 (4). These plasmids contain a truncated HIV-1 LTR from −138 to +83 (*Sca*I to *Hin*dIII) 5′ to the complete β-galactosidase gene. The 5′ end of the β-galactosidase gene was modified in pJK2 by insertion of oligonucleotides that introduced the amino acid sequence MPKKKRK as the first seven amino acids of the β-galactosidase gene. These seven amino acids found in the T antigen of simian virus 40 are sufficient to direct heterologous proteins to the nucleus (16).

All HIV plasmids are based on the infectious clone of the LAI isolate (22, 33). The LAI isolate was previously called the BRU isolate or LAV-1. pME337 has a deletion in the envelope gene between the *Bgl*II sites at 6635 and 7215. pTMO was a gift of Eric Hunter and contains the envelope gene of simian retrovirus type 3 (SRV-3) derived from an infectious clone of SRV-3 (24). The SRV-3 envelope gene in this plasmid is driven by the LTR of the myeloproliferative sarcoma virus (15a). pJD1 has been previously described (8) and contains the envelope gene of amphotropic murine leukemia virus (A-MuLV) driven by an immediate early promoter of cytomegalovirus. pSP278 is an expression vector for the HIV type 2 (HIV-2) ROD isolate envelope gene (13). This plasmid contains HIV-2 sequences from nucleotides 6090 to 9672 driven by the HIV-2 LTR. pSP278 expresses HIV-2 envelope protein when cotransfected with a plasmid that expresses either HIV-1 or HIV-2 Rev (21a) protein and a plasmid that expresses Tat protein. The HIV-1 envelope gene was provided by transfection of pME239 (9),

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which contains the HIV-1 envelope gene driven by the HIV-2 LTR.

Cells. HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum. The T-cell line CEM clone A3.01 (10) was grown in RPMI medium with 10% fetal bovine serum. The CD4-LTR/β-gal indicator cells were constructed as described in Results. Fluorescence-activated cell sorting (FACS) for CD4 expression was done with about 10^7 cells that were detached from the monolayer with 1 mM EDTA. The cells were incubated for 30 min with 50 μl of fluorescein isothiocyanate-conjugated Leu3A (3 μg/ml; Becton Dickinson) at 4°C, washed three times with 2% calf serum, and resuspended at 2×10^6 cells per ml. The CD4-LTR/β-gal indicator cells were grown in DMEM with 10% calf serum that was supplemented with 0.1 mg of hygromycin B and 0.15 mg of G418 per ml and were passaged at a 1:10 or a 1:20 dilution once a week at confluence. We have occasionally observed that long-term passage of the cells reduces their ability to be infected (data not shown). Therefore, the cells were passaged no more than 15 times before a new aliquot was thawed and put in culture.

Peripheral blood mononuclear cells (PBMC) were obtained from a healthy HIV-1-seronegative donor and stimulated for 24 h with 0.8 μg of phytohemagglutinin P (Difco) per ml prior to cocultivation with cells from HIV-infected individuals. Cells from the same donor were used for all experiments. Cells from seropositive individuals were donated with informed consent as part of ongoing projects at the University of Washington Retrovirus Laboratory. PBMC (10^6) were mixed with phytohemagglutinin P-stimulated PBMC (10^6) from a seronegative donor and cultured for 4 to 21 days until syncytia were observed and $p24^{gag}$ assays were positive (see below). The cells were then either frozen or used directly in the infectivity assays.

For generation of virus stocks, 2×10^5 HeLa cells per 35-mm plate were transfected (5) with 2 μg of an HIV-1 provirus. One day after transfection, the cells were washed and the medium was replaced with 1.5 ml of DMEM with 10% fetal calf serum. Virus was collected 24 to 48 h later. When HIV-1 stocks were prepared with the envelope of another retrovirus, 2×10^5 HeLa cells were cotransfected with 1.5 μg of an *env*-deleted HIV-1 provirus and 8.5 μg of either pTMO (SRV-3 *env*), pJD1 (A-MuLV *env*), or pSP278 (HIV-2 *env*).

Virus assays. The CD4-LTR/β-gal indicator cells were plated in 12-well plates (22-mm diameter) at 8×10^4 cells per well in DMEM with 10% calf serum the day before infection. On the next day, the cells were generally about 20% confluent. The cells were infected by removing the medium from each well and replacing it with dilutions of virus in a total volume of 300 μl in the presence of 20 μg of DEAE-dextran (Pharmacia) per ml. The plates were rocked every 30 to 45 min, and 1 ml of DMEM with 10% calf serum per well was added after 2 hours. Two days later, the medium was removed and the monolayer was fixed at room temperature with 2 ml of a solution of 1% formaldehyde–0.2% glutaraldehyde in phosphate-buffered saline (PBS) for exactly 5 min. Time course experiments indicate that virus spread is minimal when the assay is terminated after 2 days (data not shown). The cells were then washed three times with PBS and incubated for exactly 50 min at 37°C in 500 μl of a solution of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg of X-Gal per ml. The reaction was stopped by removing the staining solution and washing the cells twice with PBS. Blue cells were counted under a microscope at a magnification of 100×.

The amounts of $p24^{gag}$ in cell-free supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (Coulter Immunology) by using the instructions and standards supplied by the manufacturer. Samples were diluted 500- to 2,000-fold in DMEM with 10% calf serum so that they were in the linear range of the assay. The fluorometric β-galactosidase assay was done as described elsewhere (11) with 10% of an extract of 10^5 infected cells in a 20- to 60-min reaction.

Virus was prepared for the electron microscope as follows. One milliliter of HIV-1 stock was spun at 10,000 rpm for 5 min in a microfuge (Epindorf). The clarified supernatant was then layered on a 20% sucrose cushion and spun at 43,000 rpm for 2 h in an SW41 rotor. The pellet was resuspended in 5 ml of PBS and spun similarly at 43,000 rpm. The pellet was then resuspended in 500 μl of PBS, and an equal volume of fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.2 M cacodylate) was added. Virus particles were then counted on grids with latex beads as standards as described elsewhere (2).

RESULTS

Construction of a cell line for titration of HIV. In order to develop a sensitive test for HIV infection that would allow the titer of an HIV stock to be directly determined, CD4 was introduced into HeLa cells by infection with an amphotropic retrovirus vector (20) that contained the human CD4 cDNA driven by the MuLV LTR and the *neo* gene driven by the simian virus 40 promoter. Individual G418-resistant clones did not express high levels of CD4 (data not shown). Therefore, about 700 G418-resistant colonies were pooled and analyzed for CD4 expression by FACS. As expected, the majority of the cells in the population did not express high levels of CD4 on the cell surface, although a population that expressed CD4 at levels significantly over that of the background could be identified (Fig. 1A). In order to purify this subpopulation, the cells were sorted by FACS in each of four successive passages, and the 15 to 20% of the cells expressing the most CD4 were selected for further passage. After the fourth sort, the amount of CD4 expression could not be further improved by additional sorting, and the cells were cloned. A single cell clone that expressed high levels of CD4 was isolated (Fig. 1B).

The HeLa-CD4 cells were then cotransfected with plasmid pJK2 and a plasmid that encodes resistance to hygromycin B (pCMV-hph). pJK2 is a plasmid that contains HIV-1 LTR sequences from −138 to +80 (a minimal promoter and the Tat-responsive region, TAR) upstream of the β-galactosidase gene, followed by the poly(A) signal from and the enhancer region of simian virus 40. Expression of the β-galactosidase gene in pJK2 is responsive to Tat (data not shown). Truncation of the LTR lowered the amount of expression of β-galactosidase in HeLa cells in the absence of Tat without affecting the level of expression in the presence of Tat (data not shown). The β-galactosidase gene in pJK2 had been altered at its 5′ end so that the protein was targeted to the nucleus (see Materials and Methods). Nuclear localization of β-galactosidase allows easy visualization of expression of the introduced β-galactosidase gene as opposed to the faint blue cytoplasmic stain that is sometimes seen with dead or dying cells.

Several randomly picked hygromycin B-resistant clones were then infected with HIV-1 (LAI strain) or mock infected. Two days later, the cells were fixed and incubated with the chromophore X-Gal, incubated at 37°C, and ob-

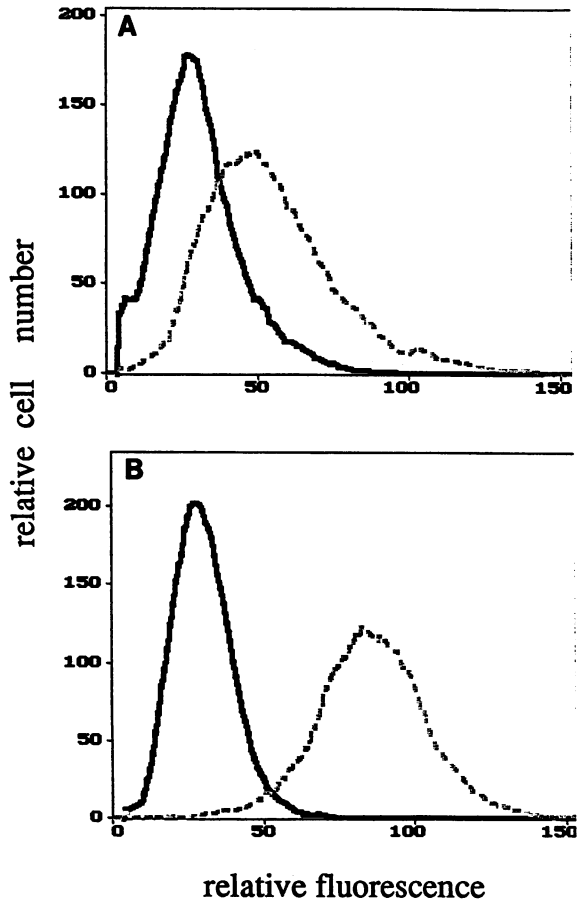


FIG. 1. Selection of CD4+ HeLa cells. HeLa cells were infected with an amphotropic retrovirus expressing the human CD4 cDNA in the vector pLXSN (20). The G418-resistant colonies were sorted for surface expression of CD4 with fluorescein isothiocyanate-labelled Leu3A antibody. Dashed lines, G418-resistant HeLa cells infected with the CD4 retrovirus; solid lines, uninfected HeLa cells. (A) The G418-resistant population before sorting for CD4 expression; (B) the population of cells after selecting the 20% of the cells expressing the highest amount of cell surface CD4 in three successive sorts in the FACS.

served under a light microscope every 10 min. Of seven clones that were screened, three expressed high levels of β-galactosidase activity in the absence of HIV infection, two expressed no β-galactosidase activity regardless of HIV infection, and two expressed much-elevated levels of β-galactosidase activity after infection. Intense-blue nuclei could be observed in the infected cells but not in the uninfected cells of these last two clones after 50 min of incubation with X-Gal (Fig. 2A and B).

When replication-competent virus fuses with the CD4-LTR/β-gal indicator cells, envelope synthesized in the infected cell should cause it to fuse with its CD4+ neighbors. At high multiplicities of infection (0.1 or greater), we find that this leads to formation of syncytia containing up to 20 nuclei (Fig. 2D). In these syncytia, each nucleus is stained blue. For this reason, the assay is called the multinuclear activation of a galactosidase indicator (MAGI) assay. At low concentrations of virus, syncytia are rare (Fig. 2C). Occasionally, very large blue nuclei are seen, which we presume are fusions of several nuclei within a multinucleated cell.

Further incubation of these clones in X-Gal for 1.5 h resulted in no increase in the number of blue cells in the infected cells but did result in a few blue cells in the mock control. Incubation for 3 h resulted in many blue cells in the mock-infected control. Therefore, the assay is routinely stopped in our laboratory after 50 min of incubation with X-Gal. One clone in particular was chosen for further investigation because the difference in the amount of β-galactosidase activity between the infected and noninfected controls was the greatest. This clone was found to contain a single integrated copy of the LTR-β-galactosidase plasmid by restriction enzyme analysis and Southern blotting (data not shown).

To determine whether the MAGI assay could be used to quantitate the titers of HIV stocks, it was necessary to determine the linear range of the assay. A virus stock of HIV-1_{LAI} was serially diluted, and each dilution was used to infect 1 well of a 12-well plate. The numbers of blue cells in each well were counted (Table 1). Syncytia were counted as one cell. At high concentrations of virus, there are too many blue cells to count and nearly every cell in the well is blue. At lower dilutions, the number of blue cells or syncytia is directly proportional to the amount of virus added (Table 1). Therefore, this assay can be used to determine the number of infectious particles per given volume of virus stock. Wells with 20 to 100 blue cells are the most informative because they are always in the linear range of the assay (Table 1 and data not shown). The titer of the stock determined in the experiment in Table 1 is about 1.4×10^5.

We also measured the extent of activation of the β-galactosidase gene by determining the amounts of β-galactosidase activity in extracts of the CD4-LTR/β-gal indicator cells, compared with an extract of noninfected CD4-LTR/β-gal indicator cells. This assay makes it possible to detect activation of infected cells at dilutions of virus that give too many blue cells to count microscopically (Table 1). However, the in situ assay of counting cells is more sensitive than the fluorometric assay of β-galactosidase activity. At the highest dilution of virus, we could count 13 blue cells (the noninfected cells contained none), while the β-galactosidase activity at this dilution was only twofold the amount of β-galactosidase activity in noninfected cells (Table 1).

It has been reported that Tat can diffuse from cells and transactivate an LTR in other cells at a low level (15). However, such diffusion is below the level of detection in our system, because no blue cells were observed when CD4-LTR/β-gal indicator cells were cocultivated with HeLa cells that constitutively express Tat (data not shown). On the other hand, blue cells were observed when HeLa cells expressing the Tat, Rev, and HIV-1 envelope proteins were cocultivated with the CD4-LTR/β-gal indicator cells (data not shown). This indicates that diffusion of Tat within a syncytium is sufficient to induce the β-galactosidase gene but that diffusion of Tat between cells is not.

Sensitivity of the MAGI assay. We compared the titer of a laboratory-adapted HIV-1 strain (HIV-1_{LAI}) obtained by using the CD4-LTR/β-gal indicator cells with that obtained by limiting dilution of an established T-cell line, CEM clone A3.01. A stock of HIV-1 was grown in CEM cells and then diluted with medium. The amount of $p24^{gag}$ protein in each dilution was determined by an ELISA, and 100 μl of each dilution was used either to infect the indicator cells or to infect CEM cells in quadruplicate (Table 2). The viral titers on CD4-LTR/β-gal indicator cells were assayed by staining the monolayers with X-Gal 48 h after infection and counting the number of blue cells for each well. The titer on CEM

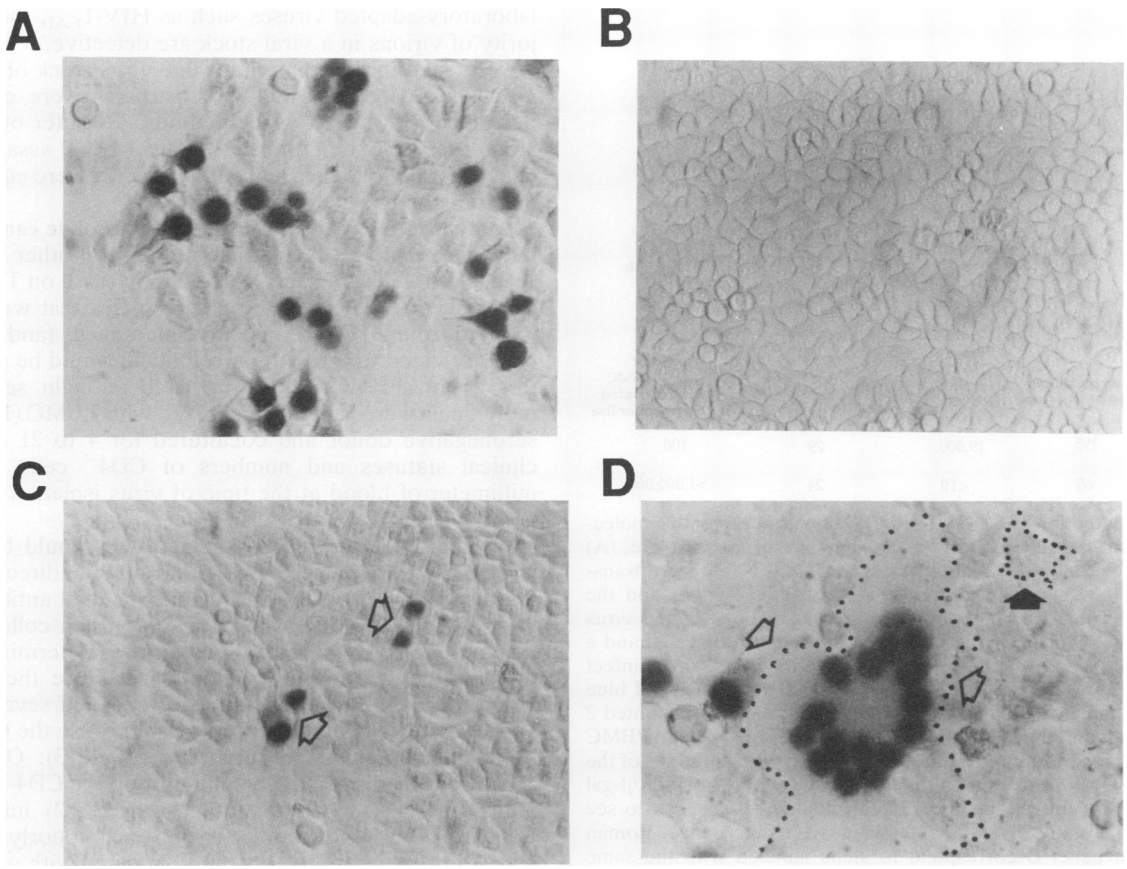


FIG. 2. The MAGI assay. CD4-LTR/β-gal indicator cells were fixed and stained as described in Materials and Methods. Magnification, ×100 (panels A, B, and C) or ×320 (panel D). (A) Infection of CD4-LTR/β-gal indicator cells with a high-titer HIV-1_{LAI} virus stock. Note that about 50% of the cell nuclei are darkly stained. These nuclei are dark blue. (B) Mock infection of the CD4-LTR/β-gal indicator cells. (C) Infection of CD4-LTR/β-gal indicator cells at a low dilution of virus. The virus titer can be calculated by counting the number of blue cells in a field such as this. Each set of darkly staining nuclei (open arrowheads) is counted as one syncytium. (D) Large syncytium of the type observed at high multiplicities of infection. The syncytium (open arrowheads) is outlined by black dots and contains 17 nuclei. The size of an uninfected cell is also outlined and is marked by the solid arrowhead.

cells was assayed by $p24^{gag}$ expression 1 week after infection. The dilution of virus in which half of the CEM cultures became infected (the 50% tissue culture infective dose) in one week was 10^5. By comparison, the titer of this same HIV-1 stock determined by counting the number of blue

TABLE 1. MAGI assay at different dilutions of HIV-1_{LAI}

Amt of virus (μl)[a]	No. of blue cells[b]	Fold activation of β-galactosidase[c]
100	TMTC[d]	68.9
10	1,538	10.4
1	140	3.5
0.1	13	2
0	0	1

[a] Amounts of virus stock used for infection in a total volume of 300 μl in one well of a six-well plate (22-mm diameter). These are data from a representative experiment. The titer of this virus stock as measured by the MAGI assay is about 1.4×10^5 U/ml.

[b] Total numbers of blue cells or blue syncytia in one well.

[c] β-galactosidase activity was measured in a cell extract from one well by using the fluorographic substrate for β-galactosidase, 4-methylumbelliferyl-β-D-galactosidase. Fold activations are the fluorescence units of each infected well divided by the fluoroscence units of each noninfected well.

[d] TMTC, too many to count.

TABLE 2. Sensitivity of delection of a laboratory-adapted HIV-1 isolate by the MAGI assay and by end point dilution

Dilution of virus stock (fold)[a]	Amt of $p24^{gag}$ by ELISA (pg)[b]	No. of blue cells by MAGI assay[c]	Amt of $p24^{gag}$ at $TCID_{50}$ for CEM cells for each well[d]			
			1	2	3	4
10^3	48	64	+	+	+	+
10^4	5.5	6	+	+	+	+
10^5	1<	2	+	+	−	−
10^6	1<	0	−	−	−	−

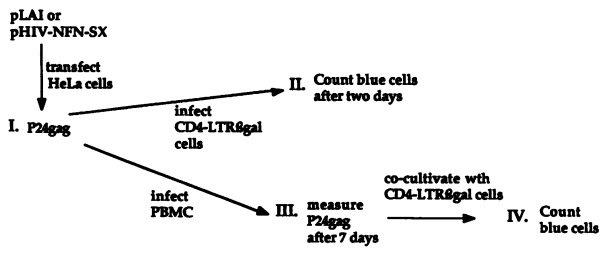
[a] An HIV-1_{LAI} stock was serially diluted 1,000- to 1,000,000-fold. The same dilutions were used for each assay.

[b] Amounts of $p24^{gag}$ (in picograms) in each of the virus dilutions were determined by ELISA (see Materials and Methods).

[c] Numbers of blue cells per milliliter of dilution observed after infection of a 22-mm well of 10^5 CD4-LTR/β-gal indicator cells. The apparent titer on CD4-LTR/β-gal indicator cells is the number of blue cells multiplied by the dilution. In this experiment, the titer of virus by the MAGI assay was 6.4×10^4.

[d] Each dilution of virus was used to infect 2×10^5 CEM cells in quadruplicate wells of a 24-well plate. One week later, the supernatant was diluted 1/100 and the amounts of $p24^{gag}$ (in nanograms) were determined. +, greater than 3 ng/ml; −, less than 0.01 ng/ml. The apparent titer of virus on CEM cells is the dilution at which half of the infections at a given dilution are positive ($TCID_{50}$). In this experiment, the titer of virus with CEM cells was 10^5.

A.



B.

Provirus	I. P24gag/ml (ng) after transfection	II. Titer of virus on CD4-LTRßgal cells in MAGIC assay (IU/ml)	III. P24gag/ml (ng) one week after infection of PBMC	IV. Number of infected PBMC needed to infect the indicator line
LAI	195	19,000	79	100
NFN-SX	65	<10	24	>1,000,000

FIG. 3. Infection of CD4-LTR/β-gal indicator cells with a molecular clone that contains the envelope gene of a primary isolate. (A) Flowchart of the experiment. LAI or pHIV-NFN-SX were transfected into HeLa cells, cell-free medium was collected, and the amount of $p24^{gag}$ was determined. Various dilutions of the virus were then used to infect the CD4-LTR/β-gal indicator cells, and a volume corresponding to 6.5 ng of each virus was used to infect activated PBMC from a seronegative donor. The numbers of blue cells in the infected CD4-LTR/β-gal indicator cells were counted 2 days after infection, and the amounts of $p24^{gag}$ in the infected PBMC were determined 7 days after infection. At that time, dilutions of the infected PBMC were added to a monolayer of CD4-LTR/β-gal indicator cells, and the numbers of infected PBMC needed to see three or more blue cells in the monolayer were determined. Roman numerals in panel B correspond to steps labelled with the same numerals in panel A.

cells after infection of the CD4-LTR/β-gal indicator cells was 6.4×10^4 (Fig. 3). Thus, the infectious titers in the two assays with this viral strain are nearly equivalent. We have also used this assay to determine titers of stocks of HTLV-IIIb and MN strains of HIV-1 (data not shown).

The ratio of the number of blue cells to the amount of $p24^{gag}$ is an indication of the relative infectivity of a virus stock. Wild-type $HIV\text{-}1_{LAI}$ grown in T cells typically gives a ratio of 1 to 3.5 blue cells per pg of $p24^{gag}$ (Fig. 3, Table 3, and data not shown). The majority (over 80%) of extracellular $p24^{gag}$ is associated with particles (data not shown). Therefore, we can calculate the approximate number of infectious particles per total number of extracellular particles with the following formula:

$$\begin{aligned}\text{Infectious units per total number of virions} &= (\text{molecules of } p24^{gag} \text{ per virion}) \\ &\times (\text{grams of } p24^{gag} \text{ per mole}) \\ &\times (\text{infectious units per gram of } p24^{gag})/ \\ &(\text{molecules of } p24^{gag} \text{ per mole})\end{aligned}$$

It has been estimated for an avian type C retrovirus that there are 2×10^3 molecules of capsid ($p24^{gag}$ is the capsid protein in HIV) per virion (31). With these numbers, the equation can be solved as follows:

$$(2 \times 10^3) \times (2.4 \times 10^4) \times (1 \times 10^{12} \text{ to } 3.5 \times 10^{12})/(6.02 \times 10^{23}) = 8 \times 10^{-5} \text{ to } 2.8 \times 10^{-4}$$

Thus, there is only 1 infectious virion per 3,500 to 12,300 virions. This indicates that even for the very infectious laboratory-adapted viruses such as $HIV\text{-}1_{LAI}$, the vast majority of virions in a viral stock are defective.

We tested this prediction for one virus stock of $HIV\text{-}1_{LAI}$ grown in H9 cells. Total viral particles were counted as described in Materials and Methods. The titer of the same stock was also determined by the MAGI assay. In this experiment, we found that 4,100 particles were equal to one infectious unit (data not shown).

A minority of virus in a fresh patient isolate can infect the indicator cells. The LAI strain and several other laboratory strains of HIV have been selected for growth on T-cell lines. We wished to determine whether viruses that were freshly isolated from HIV-1-seropositive individuals (and not previously selected for growth on cell lines) would be positive in this assay. PBMC (10^6) from each of eight seropositive individuals were isolated and mixed with PBMC (10^6) from a seronegative donor and cocultured for 4 to 21 days. The clinical statuses and numbers of $CD4^+$ cells per cubic millimeter of blood at the time of virus isolation were also noted (Table 3).

In each coculture, virus-infected cells could be demonstrated by the presence of syncytia and by indirect immunofluorescence with a Gag-specific monoclonal antibody (data not shown). Cell-free supernatant was then collected, and the amount of $p24^{gag}$ in the medium was determined (Table 3). Dilutions of the supernatant virus were then used to infect the CD4-LTR/β-gal indicator cells. Of seven primary virus isolate samples tested, two could cause the CD4-LTR/β-gal indicator cells to turn blue (Table 3). One isolate (NRO3143) had a relatively high titer on the CD4-LTR/β-gal indicator cells, while the other (NRO1762) infected the CD4-LTR/β-gal indicator cells only very poorly. Thus, at least some patients are apparently infected with viruses that can infect the indicator cell line without prior selection on T-cell lines.

In addition to testing cell-free virus from each of the primary isolates, we cocultured the infected PBMC directly with the CD4-LTR/β-gal indicator cells. We reasoned that cell-to-cell transmission of the patient isolate might be more efficient than cell-free transmission. The PBMC from seropositive individuals that had been cocultured with normal activated PBMC were washed and counted, and 10-fold dilutions of the cells were layered in a small volume onto the indicator cell monolayer.

One day after coculture, the monolayer was washed and stained with X-Gal. In this assay, five of eight of the primary isolates could form syncytia that stained blue with the indicator cell monolayer (Table 3). However, the infection was again very inefficient. In two cases, 10^6 cells were required to detect any infection of the indicator cell line, and in two other cases, 10^5 cells were required to detect infection. There was no strict correlation between the amount of $p24^{gag}$ that the cells produced and the number of infected PBMC needed to coculture with the CD4-LTR/β-gal indicator cells to obtain fusion with the CD4-LTR/β-gal indicator cells (Table 3). However, the primary isolate that scored positive in the cell-free assay (NRO3143) required the fewest cocultured cells (10^4) to infect the monolayer (Table 3). This isolate was from an asymptomatic patient (Table 3).

Primary isolates are a mixture of viruses with different sequences (12). Therefore, we next considered the possibility that the patient isolates consisted of a population of viruses that contained very few members that could infect the CD4-LTR/β-gal indicator cells. In order to test this hypothesis, we tested the ability of a molecular clone containing sequences from a primary isolate to infect the CD4-

TABLE 3. MAGI assay of patient isolates

HIV isolate or seronegative	$p24^{gag}$ (ng/ml of medium)[a]	No. of blue cells[b]	No. of PBMC[c]	No. of $CD4^+$ cells (status)[d]
NRO3143	8.7	2.3×10^3	10^4	386 (ASY)
NRO1578	6.6	0[e]	10^6	130 (AIDS)
NRO1419	4.7	0	$>10^6$[f]	310 (ASY)
NRO1800	4.6	0	10^6	80 (AIDS)
NRO3217	4.1	ND[g]	10^5	207 (ARC)
NRO1762	2.3	7×10^1	10^5	210 (ARC)
NRO1625	2.2	0	$>10^6$	361 (ASY)
NRO1319	0.6	0	$>10^6$	NA[h]
Seronegative	<0.01	0	$>10^6$	ND
LAI	400	1.4×10^6	10^2	ND

[a] Amounts of $p24^{gag}$ in cell-free medium of patient PBMC cocultivated with activated normal PBMC. Patient cells were cocultivated with normal PBMC for 4 to 21 days. The amounts of $p24^{gag}$ are from the time at which the cocultivation of the lymphocytes with the CD4-LTR/β-gal indicator cells was initiated.

[b] Numbers of blue cells per milliliter of cell-free virus stock after infection of the CD4-LTR/β-gal indicator cells.

[c] Numbers of PBMC that had to be added to the CD4-LTR/β-gal indicator cells in order to infect the indicator line and to obtain blue syncytia. PBMC were washed and layered onto the monolayer of 10^5 CD4-LTR/β-gal indicator cells. Two days later, the cells were fixed and stained for β-galactosidase activity.

[d] Numbers of $CD4^+$ cells per microliter of blood at the time that the virus was isolated. Statuses are clinical diagnoses at the time that the virus was isolated: ASY, asymptomatic; ARC, AIDS-related complex.

[e] The value 0 means that no blue cells were observed for an entire well after infection with 250 μl of virus.

[f] The value $>10^6$ means that no blue cells were observed when 10^6 PBMC were added to the indicator cells.

[g] ND, not done.

[h] NA, not available.

LTR/β-gal indicator cells. pHIV-NFN-SX is a recombinant provirus that contains a portion of the envelope genes of the primary isolate JR-FL and the regulatory and other virion genes of a laboratory-adapted virus, NY4.3 (21). This virus, like the JR-FL strain, grows on primary T cells and macrophages but does not grow on T-cell lines (21). The ability to enter the cells should be determined by the envelope sequences from the primary isolate, but the expression of the provirus, once in the cells, will be determined by the regulatory gene of the laboratory isolate.

One day after transfection of HeLa cells with pHIV-NFN-SX, supernatant was filtered, diluted, and used to infect either the CD4-LTR/β-gal indicator cells or unfractionated PBMC (Fig. 3A). A provirus of an infectious clone of LAI (22) was transfected in parallel as a positive control. Transfection of pHIV-NFN-SX and pLAI gave approximately equivalent amounts of $p24^{gag}$ in the supernatant (Fig. 3B). As expected, both the LAI and the NFN-SX stocks could grow on PBMC as determined by the production of $p24^{gag}$ (Fig. 3B). The titer in the MAGI assay of the LAI stock resulting from this transfection was 1.9×10^4 infectious units per ml (Fig. 3B). This corresponds to about 100 infectious units per ng of $p24^{gag}$ in the supernatant. The ratio of infectious virions per unit of $p24^{gag}$ of virus prepared by transfection is 1 to 10% of that of infectious virions per unit of $p24^{gag}$ of virus stocks prepared from infected cells (compare Fig. 3 with Tables 2 and 3).

In contrast to the infectivity of virus from the LAI transfection, there was fewer than 0.15 infectious units per ng of $p24^{gag}$ of the NFN-SX stock when titers were determined by the MAGI assay (Fig. 3B). Likewise, the PBMC infected with NFN-SX were unable to fuse with the CD4-LTR/β-gal indicator cells (Fig. 3B). Therefore, this macrophagetropic virus, which is unable to spread in continuous T-cell lines (21), is unable to undergo a single round of infection and *trans* activation of the CD4-LTR/β-gal indicator cells. These data suggest that in the primary isolates, the majority of viruses in the heterogeneous population of viruses that emerge from the blood of seropositive individuals cannot infect the CD4-LTR/β-gal indicator cells, although rare viruses in this virus population have acquired the ability to infect these cells.

Generation and detection of defective HIV. One of the reasons that we constructed the CD4-LTR/β-gal indicator cells was to detect HIV in a single round of infection (that is, in the absence of viral spread). This would be useful to determine the effects of mutations and antiviral agents on particular stages of the viral life cycle. In order to test whether a single round of infection was sufficient for detection of HIV by the MAGI assay, we generated viruses that would be defective in the *env* gene. pME337 is an HIV-1-based plasmid (see Materials and Methods) with a deletion in the envelope gene that includes sequences necessary to bind CD4. This deletion precludes synthesis of the transmembrane protein by changing the reading frame. However, pME337 encodes all of the other HIV genes.

In order to generate a population of defective viruses, we produced virions that would contain the envelope protein of another retrovirus and the Gag and Pol proteins and genome of HIV (pseudotypes). Pseudotypes between HIV and the envelopes of mouse amphotropic and xenotropic viruses have previously been described (17, 30). Pseudotypes of the HIV-1 *env*-deleted genome with the envelope of A-MuLV, the envelope of the Mason-Pfizer monkey virus (also known as SRV-3), and the envelope of HIV-2 were created by cotransfecting expression vectors for each envelope with pME337 into HeLa cells. Receptors for both A-MuLV and SRV-3 exist on human cells (29).

The cell-free supernatant was then used to infect the CD4-LTR/β-gal indicator cells. Cotransfection of pME337 (HIV-1 *env* deleted) with the amphotropic envelope, the SRV-3 envelope, and the HIV-2 envelope produced virus that was able to induce the β-galactosidase gene. There were about 10^3 blue cells per ml of medium in the supernatant of cells cotransfected with pME337 with either the A-MuLV envelope or the SRV-3 envelope (Table 4). There were over 10^4 blue cells per ml of medium in the supernatant of cells cotransfected with pME337 with the envelope of HIV-2. Medium from cells transfected with pME337 alone gave no blue cells, indicating that ability to induce the β-galactosi-

TABLE 4. Detection of defective viruses by the CD4-LTR/β-gal indicator cells[a]

HIV-1 provirus	*env* gene[b]	$p24^{gag}$ (ng/ml)[c]	No. of blue cells/ml of virus[d]	Infectivity ratio[e]
pME337 (*env* minus)	A-MuLV	61	1.2×10^3	20
pME337	SRV-3	102	1.6×10^3	16
pME337	HIV-2	192	13.5×10^3	69
pME337	None	205	<5	
pME301 (*gag* minus)	HIV-2	<1	<5	

[a] A total of 2×10^5 HeLa cells containing the gene expressing Tat were transfected with 2 μg of either pME337 or pME301. pME337 has a deletion in the *env* gene, while pME310 has a deletion in the *gag* gene. Two days after transfection, the medium was clarified, filtered, and serially diluted in DMEM containing 10% calf serum. Each dilution was used to infect 0.8×10^5 CD4-LTR/β-gal indicator cells in a 12-well plate.

[b] The A-MuLV envelope was supplied by cotransfection of 8 μg of plasmid pJD1. The SRV-3 envelope was supplied by cotransfection of 8 μg of plasmid pTMO. The HIV-2 envelope was supplied by transfection of plasmid pSP278. See Materials and Methods for descriptions of the plasmids.

[c] Amounts of $p24^{gag}$ in the filtered supernatanat after transfection were determined by an ELISA kit (Coulter) with standards supplied by the manufacturer.

[d] Numbers of blue cells per well divided by the dilution. Numbers are the averages of two wells.

[e] Numbers of blue cells per milliliter divided by the amounts of $p24^{gag}$ (in ng/ml).

dase gene in the CD4-LTR/β-gal indicator cells depended on the envelope. Medium from cells transfected with a *gag*-deleted mutant of HIV and the envelope of HIV-2 also gave no blue cells, indicating that the ability to induce the β-galactosidase gene in the CD4-LTR/β-gal indicator cells depends on particle formation (Table 4). These results indicate that the CD4-LTR/β-gal indicator cells are capable of detecting HIV-1 in the absence of any viral spread. The pseudotype made with the HIV-2 *env* is more infectious than either the A-MuLV or the SRV-3 *env* (Table 4).

DISCUSSION

We have constructed a cell line that allows the quantitative titers of laboratory stocks of HIV to be determined. Because detection of HIV after infection of the CD4-LTR/β-gal indicator cells does not depend on viral replication, we were able to detect HIV proviruses that had been pseudotyped with the envelope genes of several other retroviruses. This cell line will be useful for determining the effects of mutations on various stages of the virus life cycle, the effects of antiviral agents, and the infectious titers (as opposed to the physical numbers of particles) of virus stocks.

The CD4-LTR/β-gal indicator cell line described here differs from one that we previously derived (26) because the integrated HIV sequences are not rearranged and do not contain an internal promoter, the mock-infected controls have no background, and the cells do not have to be infected near confluency to achieve syncytia. While this work was in progress, another $CD4^+$ cell line that contained an LTR-β-galactosidase gene that can be activated by infection was described (1). The β-galactosidase gene in this cell line is present on a multicopy Epstein-Barr virus-based plasmid. Our cell line differs in that the LTR-β-galactosidase gene is stably integrated and is a single-copy gene. Presumably, this lowers the background of our assay.

We tested eight viral isolates from patients for their ability to infect the CD4-LTR/β-gal indicator cells. None of the viruses could efficiently infect the cells. On the other hand, each of the isolates grew by coculture in PBMC as judged by the production of nanogram quantities of $p24^{gag}$ antigen in the medium. Virus from a molecularly cloned recombinant provirus with an envelope gene from a macrophagetropic isolate was also unable to infect the CD4-LTR/β-gal indicator cells. Because the other genes of this virus are encoded by a laboratory strain of HIV which can infect the indicator line, we presume that the block to infection is at the level of viral entry. Therefore, it is likely that the inability of the patient isolates to infect the CD4-LTR/β-gal indicator cells is also a property of their envelope genes.

Although we found evidence for viruses that infect the CD4-LTR/β-gal indicator cells with cell-free virus from only two individuals, we find that most of the primary isolates (five of eight) could activate the integrated β-galactosidase gene after coculture of infected cells with the monolayer. This indicates that a very small population of virus-infected cells that are capable of fusing with the CD4-LTR/β-gal indicator cells exists in each of these isolates. Such viruses would therefore be rare in vivo, although their increased tropism may have more profound effects on the course of the disease. Because we found the presence of such viruses in asymptomatic patients (Table 3) before the dramatic drop in CD4 cells late in the disease, the appearance of viruses with an expanded tropism is not simply a result of immune suppression. One hypothesis is that the presence of viruses such as those that can infect the CD4-LTR/β-gal indicator cells is a direct cause of the loss of CD4 cells later in the disease. Prospective studies on primary isolates from a larger number of healthy seropositive individuals are under way and will determine whether the MAGI assay is useful as a prognostic tool. Previous studies have found that viruses capable of replicating in cell lines are isolated more frequently from patients in the later stages of AIDS (3, 6, 32). However, the assays used in those studies are less sensitive than the MAGI assay because they rely on virus spread.

We calculate that only one in several thousand virions of $HIV\text{-}1_{LAI}$ is infectious (see above). In comparison, about 1 in 100 virions of biologically cloned Pr-C strain of Rous sarcoma virus is infectious (28). Defective HIV-1 proviruses need only to express the *tat* gene and to contain envelope protein in the virion to be detected in the MAGI assay. Because the error rate of HIV reverse transcriptase has been estimated to be 10 times that of type C retrovirus transcriptase (23, 25), some of the defective particles are predicted to be due to defective *tat* genes. These mutations have been shown to occur with high frequency in primary isolates (19). A more likely source of the majority of defective HIV particles is particles that have spontaneously shed gp120 from their surfaces (18) or particles that never incorporated envelope onto their cores. Differential ability to incorporate or maintain envelope on the virion might account for the differences in cell-to-cell versus cell-free infections in primary isolates. Moreover, the production of large numbers of defective particles in vivo might explain the continuous presence of an immune response to viral antigens throughout the course of infection and could serve to provide decoys to allow infectious virus to escape neutralization.

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