

Examining Human T-Lymphotropic Virus Type 1 Infection and Replication by Cell-Free Infection with Recombinant Virus Vectors

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A sensitive and quantitative cell-free infection assay, utilizing recombinant human T-cell leukemia virus type 1 (HTLV-1)-based vectors, was developed in order to analyze early events in the virus replication cycle. Previous difficulties with the low infectivity and restricted expression of the virus have prevented a clear understanding of these events. Virus stocks were generated by transfecting cells with three plasmids: (i) a packaging plasmid encoding HTLV-1 structural and regulatory proteins, (ii) an HTLV-1 transfer vector containing either firefly luciferase or enhanced yellow fluorescent protein genes, and (iii) an envelope expression plasmid. Single-round infections were initiated by exposing target cells to filtered supernatants and quantified by assaying for luciferase activity in cell extracts or by enumerating transduced cells by flow cytometry. Transduction was dependent on reverse transcription and integration of the recombinant virus genome, as shown by the effects of the reverse transcriptase inhibitor 3'-azido-3'-deoxythymidine (AZT) and by mutation of the integrase gene in the packaging vector, respectively. The 50% inhibitory concentration of AZT was determined to be 30 nM in this HTLV-1 replication system. The stability of HTLV-1 particles, pseudotyped with either vesicular stomatitis virus G protein or HTLV-1 envelope, was typical of retroviruses, exhibiting a half-life of approximately 3.5 h at 37°C. The specific infectivity of recombinant HTLV-1 virions was at least 3 orders of magnitude lower than that of analogous HIV-1 particles, though both were pseudotyped with the same envelope. Thus, the low infectivity of HTLV-1 is determined in large part by properties of the core particle and by the efficiency of postentry processes.

The retrovirus human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia and of degenerative, neurological disorders termed tropical spastic paraparesis, or HTLV-1-associated myelopathy (9, 10, 27, 28, 38). Although disease is primarily associated with infection of CD4⁺ T cells (4, 11, 12, 28, 30), many other cell types can be infected with HTLV-1 in vivo and in vitro (2, 14, 15, 18, 20), suggesting that its receptor, which has yet to be identified, is widely expressed. HTLV-1 infection and replication have been difficult to examine in vitro, since the virus displays a very low infectivity and a tightly regulated gene expression program. In addition, some virus gene products appear to be detrimental to cell growth and proliferation, thus limiting detection of infected cells. These characteristics likely contribute to the observation that most established T-cell lines do not support productive infection. Therefore, previous studies have relied on the ability of HTLV-1 to infect and immortalize primary human T cells, to productively infect nonlymphoid cell lines, or to transiently infect T-cell lines. Although infections of primary lymphocytes have been initiated by cell-free infection (4, 8), infection is performed more frequently by cocultivating lethally irradiated HTLV-1 producer cells with target lymphocytes. This experimental system has proved useful for analysis of HTLV-1-mediated T-cell transformation, but due to the complexity of the cell mixture, the low efficiency of immortalization, and donor-dependent variations in primary lymphocyte targets, it is not reliable for quantitative analyses of early infection and replication events. For these reasons, many funda-

mental questions related to HTLV-1 infection and replication remain unanswered.

We previously described a spreading infection assay system for HTLV-1 with a fetal rhesus lung cell line (FRhL clone B5) and showed that infections could be initiated by cell-free virus stocks generated by transfection of 293 cells with wild-type and mutant provirus clones (6). This system provides a reproducible measure of the replication competence of proviruses through multiple rounds of infection; however, analysis of specific steps in the infectious cycle of HTLV-1 requires a more specific assay system. An alternative approach is to use recombinant viruses that encode selectable markers or reporter genes to identify infected cells. Such retrovirus and lentivirus vector systems have significantly advanced our understanding of the molecular mechanisms of virus entry, reverse transcription, and integration steps in the infectious cycle. This approach has been applied previously to HTLV-1 for the analysis of the virus envelope (3) and to assess virus replication fidelity (21). However, these studies employed coculture methods for virus transmission and selectable markers for detection, which impose limits on sensitivity and breadth of application.

In order to define individual events in the HTLV-1 infection process, particularly the steps involved in the early phase of the virus replication cycle, we have developed a single-round, cell-free infection assay. The HTLV-1-based vectors, encoding either firefly luciferase or enhanced yellow fluorescent protein (eYFP), and the cell-free infection methods described here provide a rapid, sensitive, and quantitative measure of virus infectivity and replication. We demonstrate the utility of this system for analyzing virion stability, examining the effects of antiviral agents, and characterizing determinants of the low infectivity of HTLV-1 compared to that of other retroviruses.

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MATERIALS AND METHODS

Plasmids. The initial packaging plasmid (pCMVHT1) was derived from the infectious molecular clone of HTLV-1 (pCS-HTLV) (5) by replacing the 5' long terminal repeat (LTR) and 5' untranslated region (positions 1 to 800) with a cytomegalovirus (CMV) promoter linked to a fragment from the R region of the LTR (positions 439 to 567). The minus-strand primer binding site and virion RNA-packaging elements are absent (6, 31). pCMVHT- Δ env was derived from pCMVHT1 by deletion of the *Xho*I fragment (positions 5779 to 6497) in the *env* gene. pCMVHT-Int⁻ was derived from pCMVHT- Δ env by site-directed mutagenesis to create a stop codon (nucleotide position 4700) in the integrase-coding region. The transfer vector, pHTC-luc, was derived from pCS-HTLV by replacing sequences between the *Nco*I and *Mlu*I sites at positions 1232 and 7482, respectively, with a cassette containing the CMV immediate early promoter joined to the firefly luciferase gene (Promega). In a later version, a fragment containing the HTLV-1 *tax/rex* splice acceptor site (positions 6731 to 7436) was inserted immediately upstream of the CMV promoter to generate pHTC-luc-tsa. The transfer vectors pHTC-eYFP and pHTC-eYFP-tsa were derived from pHTC-luc and pHTC-luc-tsa, respectively, by replacing the luciferase gene with the eYFP gene (Clontech). The HTLV-1 *env* expression plasmid pHT-envX-CMV contains the 3' half of the HTLV-1 provirus genome (positions 5095 to 9035) containing *env*, *tax*, and *rex* genes controlled by a CMV promoter. pCMV-VSV-G encodes the vesicular stomatitis virus G protein (VSV-G). The human immunodeficiency virus type 1 (HIV-1) transfer vector, pHR'-CMVlacZ, and packaging plasmid, pCMV- Δ R8.2, were generously provided by Luigi Naldini and have been described previously (25, 26). pHR'-CMVlacZ and pHR'-CMV-eGFP were made by replacing the *lacZ* gene in pHR'-CMVlacZ with the firefly luciferase gene and the enhanced green fluorescent protein (eGFP) gene, respectively.

Cell lines, transfections, and preparation of recombinant viruses. Human kidney (293 and 293T), human osteosarcoma (HOS), and fetal rhesus lung (FRhL clone B5) cell lines were maintained in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and antibiotics. Human T-cell lines MOLT4, HUT78, and Jurkat were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Virus stocks were generated by transfecting human 293 cells, seeded at 3×10^6 in 10-cm plates the previous day, with 10 μ g of plasmid DNAs by calcium phosphate coprecipitation. Medium was changed 16 h after transfection, and virus-containing supernatants were collected 12 h later. Transfected-cell supernatants were cleared by low-speed centrifugation and filtered through 0.45- μ m-pore-size low-protein-binding filters (Millipore). HTLV-1 p19 Gag antigen capture enzyme-linked immunosorbent assay (ELISA) reagents were purchased from ZeptoMetrix Corporation, and assays were performed according to the manufacturer's protocols. HIV-1 p24 Gag antigen capture ELISA kits were obtained from the AIDS Vaccine Program, SAIC-Frederick.

Infections and gene transduction analyses. Cells of the adherent lines 293T, HOS, and FRhL(B5) were seeded in six-well plates at 2×10^5 cells per well. On the following day, medium was removed and replaced with 2 ml of filtered supernatant. After 4 h of exposure to virus, medium was removed, the cells were rinsed with phosphate-buffered saline (PBS), and fresh medium was applied. MOLT4, HUT78, and Jurkat cells were maintained in log-phase growth; 10^6 cells were suspended in 0.5 ml of filtered supernatant containing 5 μ g of Polybrene per ml. Cells and virus were centrifuged at 1,500 rpm in a Sorvall RT6000D centrifuge for 2 h, rinsed with PBS, and suspended in 2 ml of fresh medium. To assay for luciferase activity, cells were harvested 72 h after infection, pelleted, and suspended in 0.1 ml of lysis buffer (1% Triton X-100, 50 mM NaCl, 10 mM Tris-HCl [pH 7.6], 5 mM EDTA). Luciferase assays were performed with 20 μ l of cell extract in the Promega luciferase assay system according to the manufacturer's protocol. For flow cytometry, cells were collected by trypsinization 72 h after infection, washed with PBS, and fixed in 1% paraformaldehyde for 20 min. Cells were then pelleted and suspended in 1 ml of 3 mM EGTA in PBS.

RNA purification and Northern blotting. Cellular poly(A)⁺ RNA was prepared from transfected cells with RNeasy and Oligotex (Qiagen) reagents according to the manufacturer's protocols. Virion RNA was prepared from concentrated virions by layering 7.5 ml of filtered supernatant on 2.5 ml of 10% glycerol in PBS and centrifuged at 30,000 rpm for 90 min in a 70.1 Ti rotor. Virus pellets were suspended in RNA STAT60 reagent (Bio 101), extracted, and precipitated by the manufacturer's protocol. Virion RNA was dissolved in RNA sample buffer (Ambion), run on agarose formaldehyde gels, transferred to nylon membrane, and hybridized to a ³²P-labeled DNA probe. Hybridized bands were visualized by autoradiography and quantified on an ABI Storm phosphorimager.

AZT inhibition. 3'-Azido-3'-deoxythymidine (AZT) (Sigma) was dissolved in water to give a 5 mM stock solution. 293T cells were treated with various

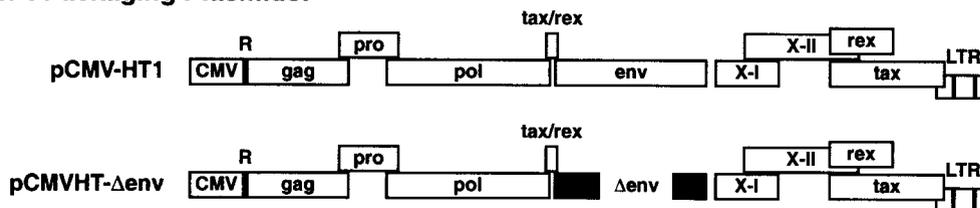
concentrations of drug 3 h prior to infection and were continuously exposed to the drug during infection and up to the time of cell harvest for luciferase assays.

RESULTS

Construction and expression of HTLV-1 vectors. The single-round assay system for HTLV-1 is similar to other retroviral gene transfer systems where recombinant viruses are generated from cells transiently transfected with three plasmids. These include (i) a packaging plasmid which encodes regulatory, structural, and enzymatic proteins for virus gene expression, assembly, and replication; (ii) a transfer vector containing a promoter-reporter gene cassette, whose RNA is encapsidated and subsequently replicated and expressed in the infected cell; and (iii) an envelope expression plasmid. The HTLV-1 packaging plasmid, pCMV-HT1, was derived from an infectious molecular clone of HTLV-1 by replacing the 5' LTR with a CMV immediate early promoter joined to a small fragment from the R region of the LTR which contains the major splice donor site (Fig. 1). Both the RNA packaging signal and the tRNA primer binding site are absent from the recombinant provirus to prevent specific incorporation of its RNA into virus particles or its subsequent replication in infected cells. To allow pseudotyping of recombinant viruses with various envelope proteins, pCMVHT- Δ env was constructed by deleting the *env* gene in pCMVHT1. Both pCMVHT- Δ env and pCMVHT1 expressed high levels of virus proteins after transfection into 293 cells. Immunoblotting showed that viral structural proteins were assembled and appropriately processed in virus particles (31). Concentrations of recombinant virus in transfected-cell supernatants varied between experiments with an average yield of approximately 75 ng of HTLV-1 p19 matrix protein per ml. Transfer vectors were constructed by replacing HTLV-1 provirus sequences between *gag* and *pX* genes (positions 1232 to 7482) with a cassette containing the CMV immediate early promoter and either the firefly luciferase gene or the eYFP gene (Fig. 1). The transfer vectors retain *cis*-acting elements necessary for HTLV-1 RNA encapsidation and replication. Second-generation transfer vectors were constructed by inserting fragments containing the splice acceptor site from the third exon of the *tax/rex* mRNA upstream of the CMV promoter (Fig. 1). The internal CMV promoter, rather than the HTLV-1 LTR, is necessary to drive reporter gene expression in this system, since the latter requires the viral Tax protein, which is not expressed in vector-transduced cells. Finally, expression plasmids that encode either the HTLV-1 envelope protein or VSV-G were used in the experiments described here.

Recombinant HTLV-1 vectors transduce genes by cell-free infection. To determine whether the HTLV-1 vectors generate infectious virus particles, filtered supernatants from cells transfected with pCMVHT- Δ env, pHTC-luc, and pCMV-VSV-G plasmids were used to infect various cell lines. For comparison, another set of cells was infected with an analogous HIV-1-based vector (25, 26), generated by cotransfecting 293 cells with pCMV- Δ R8.2, pHR'-luc, and pCMV-VSV-G. Recombinant viruses were pseudotyped with VSV-G to focus on postentry and replication events and to avoid effects that could be attributed to the HTLV-1 envelope. Monolayer cultures of 293T, HOS, and FRhL clone B5 cells were infected with filtered supernatants, and luciferase activity was measured 72 h

HTLV-I Packaging Plasmids:



HTLV-I Transfer Vectors:

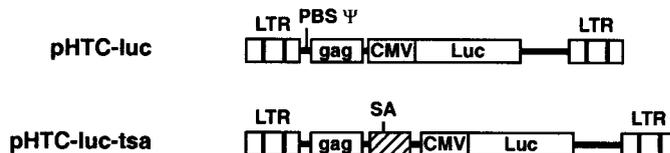


FIG. 1. HTLV-1 packaging plasmids and transfer vectors. pCMV-HT1 was constructed from an infectious molecular clone, pCS-HTLV, by replacing the 5' LTR, tRNA primer binding site, and RNA encapsidation elements with a CMV promoter joined to the major splice donor site from the HTLV-1 R region. A *Xho*I fragment was deleted from the *env* gene of pCMV-HT1 to produce pCMVHT-Δ*env*. HTLV-1 transfer vectors were constructed by replacing sequences between the *gag* and *pX* genes in pCS-HTLV with promoter/reporter gene cassettes. pHTC-luc contains a CMV promoter joined to the firefly luciferase gene. pHTC-luc-tsa was made by inserting a fragment that contains the splice acceptor site (SA) from the third exon of the *tax/rex* gene immediately upstream of the CMV promoter into pHTC-luc. Analogous transfer vectors were constructed in which the luciferase gene was replaced with the eYFP gene to give pHTC-eYFP and pHTC-eYFP-tsa.

later. FRhL clone B5 and 293T cells yielded similar levels of luciferase activity after infection with recombinant HTLV-1, while HOS cells gave approximately 10-fold-lower values (Table 1). By comparison, levels of luciferase activity transduced by the recombinant HIV-1 vector were 3 to 4 logs higher than those obtained with HTLV-1. Transduction of Jurkat, HUT78, and MOLT4 cells was accomplished by suspending cells in 0.5 ml of filtered supernatant followed by low-speed centrifugation for 2 h. The human T-cell lines were efficiently transduced with both recombinant HTLV-1 and HIV-1 vectors, and again the latter yielded about 1,000-fold-higher levels of luciferase activity than HTLV-1 (Table 1). This difference in transduction

efficiency most likely reflects distinct properties of the HTLV-1 virion or inefficient postentry processes, since (i) both recombinant viruses were pseudotyped with the same envelope protein, (ii) both HTLV-1 and HIV-1 transfer vectors contain the same CMV promoter-luciferase gene cassette and express similar levels of luciferase activity when directly transfected into cells (unpublished observation), and (iii) virus concentrations, deduced from virion core protein ELISA measurements, indicated that recombinant HTLV-1 particles were present at a threefold excess compared to HIV-1 particles.

Gene transduction by recombinant HTLV-1 is dependent on reverse transcription and integration. Transduction of luciferase activity by cell-free infection with the HTLV-1 vector was abolished when the packaging plasmid, transfer vector, or envelope expression plasmid was omitted. To further establish that transduction was dependent on virus replication, we examined the effects of the reverse transcriptase inhibitor AZT. Cells were grown in the presence of various concentrations of AZT for 3 h prior to infection and continuously exposed to AZT during and after infection. There was a linear decrease in luciferase activity with respect to the log of the AZT concentration yielding a 50% inhibitory concentration (IC₅₀) of 30 nM (Fig. 2). This value is in good agreement with those previously reported for HTLV-1 in peripheral blood mononuclear cells (PBMCs) (16, 19, 22). The inhibitory effect of AZT was identical with HTLV-1 envelope and VSV-G (data not shown). This assay system thus provides a sensitive and rapid method for examining HTLV-1 reverse transcriptase inhibitors. To determine the relationship between luciferase transduction and integration of the viral genome, we constructed a packaging plasmid (pCMVHT-Int⁻) with a premature stop codon in the integrase gene. The integrase mutant and wild-type packaging plasmids yielded similar levels of virus core proteins in transfected-cell supernatants. The integrase-negative packaging

TABLE 1. Transduction of the luciferase gene by cell-free infection with recombinant HTLV-1 and HIV-1 vectors^a

Cell line	Luciferase activity (RLU) per ml of supernatant ^b	
	HTLV-1 (10 ³)	HIV-1 (10 ⁶)
293T	102 ± 7.0	400 ± 32
FRhL clone B5	85 ± 1.1	16.2 ± 1.9
HOS	1.3 ± 0.1	21.5 ± 1.0
Jurkat	61.1 ± 2.3	70.0 ± 4.0
HUT78	52.4 ± 4.1	81.6 ± 1.6
MOLT4	34.8 ± 1.4	2.4 ± 0.2

^a Filtered supernatants (2 ml) from 293 cells cotransfected with pHTC-luc, pCMVHT-Δ*env*, and pCMV-VSV-G (HTLV-1) or pCMV-ΔR8.2, pHR'-CMV-luc, and pCMV-VSV-G (HIV-1) were used to infect 2 × 10⁵ 293T, FRhL clone B5, or HOS cells in six-well plates. The T-cell lines Jurkat, HUT78, and MOLT4 were infected by suspending 10⁶ cells in 0.5 ml of filtered supernatant containing 5 μg of Polybrene per ml. The p19 ELISA titer for HTLV-I supernatant was 138 ng/ml, and the p24 ELISA titer for HIV-1 supernatant was 42 ng/ml.

^b Cells were lysed 72 h after infection; 20% of each sample was used for determination of luciferase activity, which is expressed as relative light units (RLU) and normalized per milliliter of filtered supernatant used for infection. Infections were performed at least three times, and mean values with standard deviations are shown.

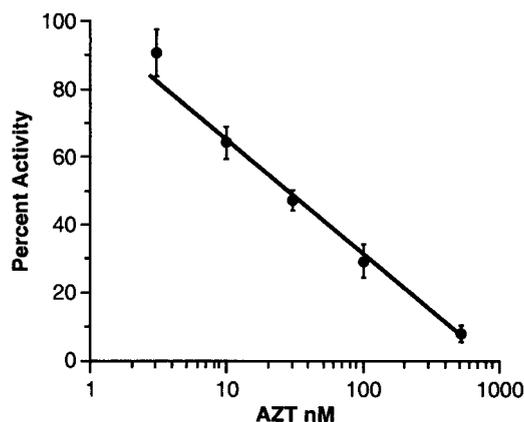


FIG. 2. AZT inhibits HTLV-1-mediated gene transduction. Recombinant virus was generated from 293 cells transfected with pHTC-luc, pCMVHT Δ env, and pCMV-VSV-G. Human 293T cells were treated with no drug or with 3, 10, 30, 100, or 500 nM AZT for 3 h prior to infection and then maintained in AZT until lysis at 72 h postinfection. Luciferase activities are expressed relative to the no-drug control. The data are the averages of two experiments.

plasmid transduced luciferase activity at 6% of the wild-type level, indicating a dependence on integrase gene function. This residual level of expression could be due to transcription of unintegrated viral DNA or to nonspecific integration events. In summary, cell-free infection with recombinant HTLV-1 vectors was dependent on both reverse transcription and integration steps of the virus infectious cycle.

Luciferase activity is proportional to the number of infected cells. In order to determine the relationship between luciferase activity, which is averaged over the infected-cell population, and the number of infected cells, we compared transduction of cells with recombinant HTLV-1 vectors encoding either luciferase or eYFP. Recombinant viruses were pseudotyped with VSV-G, and cells were infected with serial twofold dilutions of filtered supernatants. At 72 h after infection, luciferase activity was determined in cell lysates, or eYFP-expressing cells were enumerated by flow cytometry. Luciferase activity was proportional to the number of infected cells determined over the range of virus dilutions tested (Fig. 3). The infectious titer for recombinant HTLV-1, deduced from flow cytometry, was 2.4×10^3 infectious units per ml of filtered supernatant. We next examined analogous HIV-1 vectors encoding eGFP and pseudotyped with VSV-G by flow cytometry and calculated a titer of 1.0×10^7 infectious units per ml of filtered supernatant (Fig. 3). As was observed in comparisons of luciferase activities transduced by HTLV-1 and HIV-1 vectors (Table 1), flow cytometry indicated a 4,000-fold-higher infectious titer for HIV-1 than for HTLV-1. Taking into account the viral core protein concentrations determined by antigen capture ELISA of filtered supernatants and assuming 2,000 Gag proteins per virion, we calculated the number of infectious units per virus particle of 1 in 3×10^5 for recombinant HTLV-1 and 1 in 75 for recombinant HIV-1. This value for HIV-1 is in close agreement with published values (13). The value for recombinant HTLV-1 is also consistent with the ratio of 1:10⁶ determined by PCR analysis of nascent proviral DNA formed after infection of primary human lymphocytes with virus from MT2 cells (8).

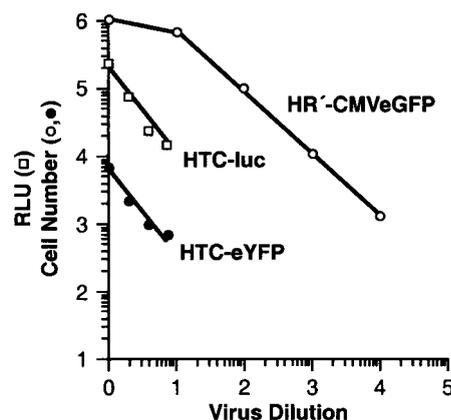


FIG. 3. Luciferase transduction is proportional to the number of infected cells. Human 293T cells were infected with serial twofold dilutions of virus generated from cells transfected with HTLV-1 vectors pCMVHT Δ env, pCMV-VSV-G, and either pHTC-luc or pHTC-eYFP. In a parallel experiment, cells were infected with serial 10-fold dilutions of filtered supernatants from cells transfected with HIV-1 vectors pCMV- Δ R8.2, pHR'-CMVeGFP, and pCMV-VSV-G. At 72 h after infection, cells infected with pHTC-luc were assayed for luciferase activity, and cells infected with pHTC-eYFP or with pHR'-CMVeGFP were enumerated by flow cytometry. Luciferase activity (relative light units [RLU]) and the number of fluorescent cells are expressed per 10^6 target cells and are plotted versus the \log_{10} virus dilution factor. The experiment was performed three times, and data from a typical experiment are shown.

HTLV-1 transfer vector synthesis and encapsidation. To address how the sequence and organization of regulatory elements in the HTLV-1 transfer vector might affect transduction efficiency, we constructed several variants of pHTC-luc. In pHTC-luc-tsa, a splice acceptor site was inserted upstream of the CMV promoter to provide an intron analogous to the organization of the HIV-1 transfer vector, pHR'-CMVluc (26) (Fig. 4A). Transduction efficiency with pHTC-luc-tsa was almost fivefold higher than with pHTC-luc (Table 2). The effect of replacing the strong CMV promoter with a simian virus 40 promoter/enhancer element was examined with the transfer vector pHTSV-luc. We reasoned that the weaker simian virus 40 promoter might allow higher levels of expression and encapsidation of the full-length transfer vector mRNA than the CMV promoter. However, pHTSV-luc yielded much lower levels of luciferase activity than pHTC-luc in transduced cells (Table 2). Thus, inclusion of an intron improved transduction efficiency, and the internal CMV promoter did not appear to interfere with transfer vector expression.

A critical determinant of transduction with recombinant viruses is the level of transfer vector mRNA synthesis and its encapsidation (7, 8). The transfer vectors express mRNAs that are initiated either in the internal CMV promoter or in the 5' LTR (Fig. 4A). The former are constitutively expressed and direct the synthesis of reporter proteins in infected cells, whereas the latter are expressed in response to viral *trans*-activator proteins supplied by the packaging plasmid and are destined for virion incorporation. In addition to the full-length mRNA initiated in the 5' LTR, vectors such as pHTC-eYFP-tsa and pHR'-CMVeGFP, which contain an intron, produce a spliced mRNA (Fig. 4A). Expression and packaging of transfer vector mRNAs generated with the HTLV-1 vectors were ex-

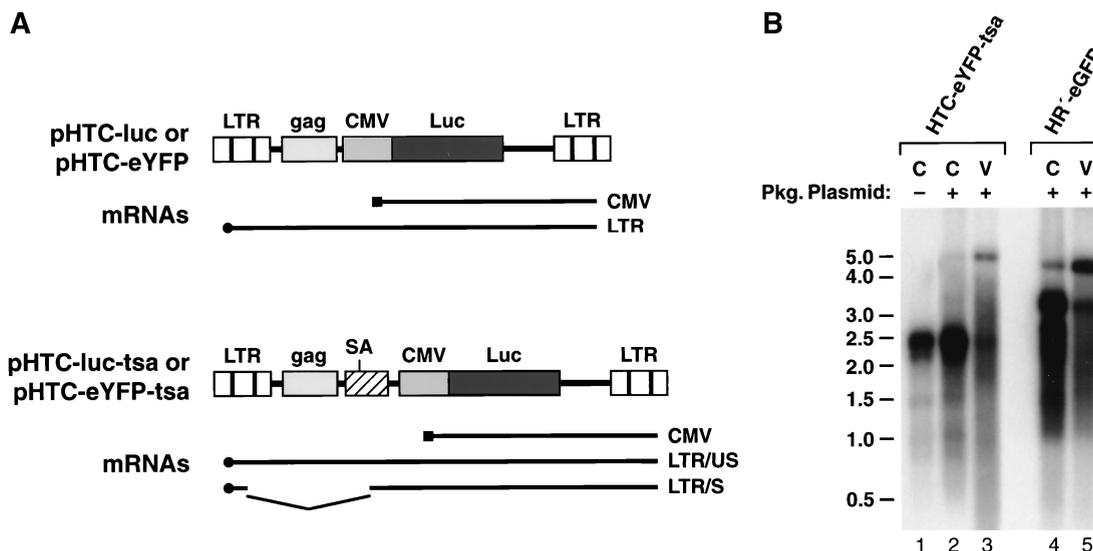


FIG. 4. Synthesis and encapsidation of transfer vector mRNAs. (A) mRNAs expressed from pHTC-luc or pHTC-eYFP and pHTC-luc-tsa or pHTC-eYFP-tsa transfer vectors are depicted. The mRNA initiating in the CMV promoter is constitutively expressed and is predicted to be approximately 2.5 kb. The mRNA initiating in the 5' LTR (LTR/US) is expected to be 4.8 kb and is expressed only in response to *trans*-regulatory proteins supplied by the packaging plasmid. Transfer vectors containing a splice acceptor site (SA) are predicted to generate an additional spliced mRNA (LTR/S) of 3.5 kb. The transcription pattern for the HIV-1 transfer vector, pHR'-CMVeGFP, is analogous to pHTC-eYFP-tsa, but the mRNAs are smaller. (B) Northern blot analysis of poly(A)⁺ mRNAs extracted from cells transfected with pHTC-eYFP-tsa (lane 1), pHTC-eYFP-tsa plus pCMVHT- Δ env (lane 2), or pHR'-CMVeGFP plus pCMV- Δ R8.2 (lane 4). Virion RNAs were extracted from concentrated virus particles produced by cells transfected with HTLV-1 vectors pHTC-eYFP-tsa plus pCMVHT- Δ env (lane 3) or with HIV-1 vectors pHR'-CMVeGFP plus pCMV- Δ R8.2 (lane 5). The amounts of virion RNA loaded on the gel are equivalent to 7.5 ml of supernatant for recombinant HTLV-1 (lane 3) and 0.75 ml of supernatant for recombinant HIV-1 (lane 5). RNAs were resolved on a 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized to a ³²P-labeled eYFP probe. Positions of RNA size markers (Ambion Millennium Markers) are indicated. The experiment was performed five times, and representative results are shown.

amined by Northern blot analysis of total RNA from transfected cells and from virus particles concentrated from transfected-cell supernatants. Hybridization of blots with a labeled eYFP fragment revealed expression of the CMV-directed mRNA in cells transfected with pHTC-eYFP-tsa alone (Fig. 4B, lane 1). Cotransfection of pHTC-eYFP-tsa with its packaging plasmid, which supplies Tax and Rex proteins, increased the level of expression of the mRNA initiated in the CMV promoter and activated expression of the 5' LTR mRNA at low but detectable levels (Fig. 4B, lane 2). By comparison, cotransfection of the HIV-1 transfer vector, pHR'-CMVeGFP, with the HIV-1 packaging plasmid, pCMV- Δ R8.2, resulted in the accumulation of full-length and spliced mRNAs that originate in the 5' LTR (lane 4) at much higher levels than with the HTLV-1 vectors (Fig. 4B, compare lanes 2 and 4). Quantitative

phosphorimage analysis revealed that the level of the unspliced 5' LTR mRNA in cells cotransfected with pHTC-eYFP-tsa and pCMVHT- Δ env was 43-fold lower than the level of the corresponding mRNA synthesized in cells cotransfected with analogous HIV-1 vectors. Analysis of virion-associated RNA levels revealed that from equal volumes of supernatant, the HIV-1 transfer vector mRNA was 50-fold more abundant than the HTLV-1 counterpart (Fig. 4B, lanes 3 and 5; note that 10-fold more HTLV-1 supernatant than HIV-1 supernatant is loaded on the gel). Based on ELISA determinations of HTLV-1 and HIV-1 core proteins in the supernatants, HTLV-1 particles were present in 2.5-fold excess compared to HIV-1. Therefore, there was a 125-fold-lower level of recombinant HTLV-1 than HIV-1 virion RNA per particle. The lower virion RNA levels for recombinant HTLV-1 particles might contribute in part to the lower transduction efficiency compared to other retrovirus systems. Whether the differences in virion RNA content observed here are unique to these vector systems or reflect intrinsic properties of the native viruses remains to be determined.

The stability of recombinant HTLV-1 particles is similar to that of other oncoretroviruses. An important question that has not been examined previously for HTLV-1 relates to virion stability. A labile virus particle could account for the low cell-free infectivity observed here and elsewhere and perhaps explain why the virus appears to be more efficient in coculture infections. Filtered supernatants containing recombinant HTLV-1 particles encoding the luciferase gene were incubated

TABLE 2. Transduction efficiencies of various HTLV-1 transfer vectors

Transfer vector ^a	Luciferase activity (10 ³ RLU) ^b	p19 ELISA titer (ng/ml)
pHTC-luc	89.8 ± 15.8	43
pHTC-luc-tsa	482 ± 114	36
pHTSV-luc	0.5 ± 0.1	53

^a Virus-containing supernatants were produced from 293 cells cotransfected with the indicated transfer vector, pCMVHT- Δ env, and pCMV-VSV-G.

^b 293T cells were infected with 2 ml of filtered supernatant; 72 h later, cells were lysed and 20% of each sample was assayed for luciferase activity. The data are expressed as relative light units (RLU) and are the averages of two experiments ± standard deviations.

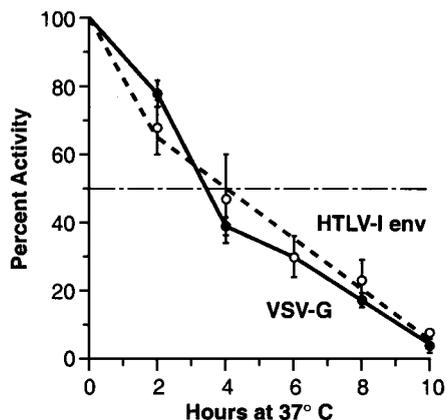


FIG. 5. HTLV-1 particle stability is typical of oncoretroviruses. The stability of HTLV-1 virions pseudotyped with either VSV-G (solid circles) or HTLV-1 envelope (open circles) was determined by incubating filtered supernatants at 37°C for 0, 2, 4, 6, 8, and 10 h prior to infection of 293T cells. Luciferase activities were determined in cell extracts prepared 72 h after infection and are expressed relative to the no-preincubation control. The data are the averages of two experiments.

for various times at 37°C prior to infection. Luciferase activities expressed in infected cells were plotted against the incubation time to estimate the point at which 50% of the infectivity was lost (Fig. 5). The half-life of HTLV-1 virions under these conditions and pseudotyped with either HTLV-1 envelope or VSV-G was determined to be approximately 3.5 h. We also examined the stability of recombinant HIV-1 particles under the same experimental conditions, and calculated a half-life of 4 h (data not shown). The half-life for recombinant HTLV-1 particles was similar to that of HIV-1 particles and is in close agreement with values obtained for other oncoretroviruses (13), indicating that the HTLV-1 virion is not unusually unstable.

DISCUSSION

We have developed vectors and cell-free infection methods that provide a rapid and quantitative replication assay for HTLV-1. The packaging plasmid expresses high levels of virus structural, enzymatic, and regulatory proteins in transfected cells, while the envelope deletion allows pseudotyping of the HTLV-1 core particles. Recombinant virus containing the firefly luciferase gene provided a sensitive measure of HTLV-1 infection which was proportional to the number of cells infected with recombinant virus encoding eYFP. Transduction was absolutely dependent on coexpression of envelope protein and the HTLV-1 packaging vector and could be inhibited by mutation of the integrase gene or by inhibiting reverse transcriptase with AZT. This experimental system, limited to a single round of infection, now enables studies of virus entry, replication, and integration steps in the virus infectious cycle. In addition, it provides a method for examining properties of the HTLV-1 virion. The short time frame of the experiments and the ability to quantify infection levels will complement and extend existing methods for studying HTLV-1 replication *in vitro*.

Although AZT was previously shown to inhibit HTLV-1

infection, it was difficult to obtain an accurate and reproducible measure of dose response in these systems. In one study, the effects of AZT were examined by measuring HTLV-1 Gag protein expression, viral mRNA synthesis, and proviral DNA formation after coculture of human PBMCs with lethally irradiated HTLV-1 producer cells; IC_{50} s for AZT were estimated to be between 10 and 80 nM (19). In an earlier study using a similar approach, the lowest concentration of AZT tested was 3 μ M, so an IC_{50} was not determined (22). An IC_{50} between 50 and 500 nM was determined in rabbit PBMCs by measuring cell immortalization after coculture with HTLV-1 producer cells (16). The effects of AZT on HIV-1 replication *in vitro* have been examined in a variety of cell lines, with both wild-type virus and recombinant virus vectors giving IC_{50} s that ranged between 5 and 50 nM (1, 23, 24). AZT is also an effective inhibitor of murine retroviruses, with inhibitory effects in the nanomolar range (29, 33). Thus, the IC_{50} for AZT defined here agrees well with previous estimates for HTLV-1 and other retroviruses and establishes this assay system as a valuable method for future studies of antiviral agents directed against HTLV-1.

The unusually low infectivity of HTLV-1 compared to other retroviruses has been an obstacle to understanding basic aspects of its replication cycle; conversely, the underlying events that determine this characteristic have been elusive. Factors that contribute to this property might include (i) poor virus attachment and entry mediated by an atypical envelope glycoprotein; (ii) the composition, maturation, or stability of the virus particle; or (iii) postentry steps, such as inefficient reverse transcription and integration processes. The envelope protein is essential for virus attachment and entry into the cell and thus was suspected to be a major determinant of HTLV-1 infectivity. Early pseudotyping experiments with other viruses and viral vectors suggested that the HTLV-1 envelope was less efficient than other viral envelopes (17, 32, 36, 37). However, the extent to which HTLV-1 envelope differed from other envelopes varied depending on the cell lines used, the virus that was pseudotyped, and the sensitivity of the assay. In recent studies with recombinant HIV-1 particles, VSV-G pseudotypes were the same as or about 20-fold better than HTLV-1 envelope in transducing reporter gene activity (34, 35). Using recombinant HTLV-1 particles, we observed approximately 20-fold-higher transduction levels with VSV-G than with the HTLV-1 envelope (data not shown), consistent with results reported for the HIV-1 pseudotypes. Furthermore, the HTLV-1 envelope did not appear to confer a unique instability to the virus, since HTLV-1 particles pseudotyped with VSV-G or HTLV-1 envelope had similar sensitivities to incubation at 37°C. The 3.5-h half-life of HTLV-1 particles at this temperature is comparable to the 3-h half-life reported for murine leukemia virus particles (13). These and other studies indicate that HTLV-1 envelope alone does not account for the low infectivity of the virus and suggest that factors responsible for this characteristic probably lie elsewhere.

In side-by-side comparisons of transduction levels obtained with recombinant viruses pseudotyped with the same envelope protein and generated by analogous vectors, we observed particle/infectivity ratios of approximately 3×10^5 :1 and 75:1 for HTLV-1 and HIV-1, respectively. The specific infectivity for recombinant HTLV-1 observed here is in close agreement with

the value of $10^6:1$ obtained by quantitative PCR analysis of PBMCs infected with cell-free HTLV-1 produced from MT-2 cells (8), and the value determined for recombinant HIV-1 is consistent with published values (13). Since both recombinant HTLV-1 and HIV-1 were pseudotyped with VSV-G, the differences observed in specific infectivity appear to reflect intrinsic differences in core particle composition that would affect subsequent formation of an active replication complex. Comparison of the relative levels of transfer vector mRNAs revealed that HIV-1 vectors accumulated 40-fold-higher levels of transfer vector mRNA in transfected cells and encapsidated approximately 125-fold higher levels of transfer vector mRNA in virions than HTLV-1 vectors. This difference correlates with relative promoter activities of the HIV-1 and HTLV-1 LTRs in the presence of their cognate *trans*-activators (data not shown). The disparity in transfer vector mRNA synthesis could contribute to differences in the specific infectivities of the two recombinant viruses. Whether the virion RNA deficit is unique to the recombinant HTLV-1 particles studied here or is also a property of wild-type virus is an important question that needs to be addressed in future studies. A consequence of these observations is that it may be possible to modify the HTLV-1 transfer vector to achieve higher levels of mRNA synthesis and encapsidation, thereby improving infectious titer and increasing the sensitivity of this assay.

In summary, the recombinant HTLV-1 vectors recapitulate properties of the wild-type virus, including its low infectivity. As yet, no single step in the infection process explains the latter characteristic, suggesting that it is the product of several sub-optimal infection and replication processes. The assay system described here will facilitate future studies of these properties and processes. In addition, optimization of this assay system should provide better methods for studying HTLV-1 infection of primary lymphocytes *in vitro*.

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