The Effect of Viral Regulatory Protein Expression on Gene Delivery by Human Immunodeficiency Virus Type 1 Vectors Produced in Stable Packaging Cell Lines

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We describe the generation of stable human immunodeficiency virus type 1 (HIV-1)-packaging lines that constitutively express high levels of HIV-1 structural proteins in either a Rev-dependent or a Rev-independent fashion. These cell lines were used to assess gene transfer by using an HIV-1 vector expressing the hygromycin B resistance gene and to study the effects of Rev, Tat, and Nef on the vector titer. The Rev-independent cell lines were created by using *gag-pol* and *env* expression vectors that contain the Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE). Vector titers approaching 10^4 CFU/ml were routinely obtained with these cell lines, as well as with the Rev-dependent cell lines, with HeLa-CD4 cells as targets. The presence of Nef and Tat in the producer cell each increased the vector titer 5- to 10-fold. Rev, on the other hand, was absolutely essential for gene transfer, unless the MPMV CTE was present in the vector. In that case, by using the Rev-independent cell lines for packaging, Rev could be completely eliminated from the system without a reduction in vector titer.

Retroviral vectors based on oncoviruses, such as Moloney murine leukemia virus, have been used extensively for gene transfer with efficient packaging cell lines and vectors developed for this purpose (35). However, a major drawback in the use of these systems is that at least one round of cell division is required for proviral integration into the target cell (36, 47, 53). Thus, these vectors cannot be used for gene transfer to nondividing or growth-arrested cells (36). This has severely restricted their potential usefulness.

In contrast to the simpler oncornaviruses, the more complex human immunodeficiency virus (HIV) can infect nondividing cells, and integration of proviral DNA occurs without the need for cell division (30, 31). Indeed, HIV-based vector systems exploiting this property have recently been shown to transfer genes to nondividing cells (e.g., neurons) (39, 43), establishing "proof of principle."

Several features inherent to HIV create challenges for its development as a gene delivery vector. The fact that it is a known pathogen requires that the vector systems be completely free from even traces of replication-competent virus. Additionally, the complexity of the HIV genome and the key roles played by the viral regulatory (11) and accessory proteins (55) in modulating gene expression and viral infectivity make the development of efficient HIV-based vector systems a non-obvious venture.

A key HIV regulatory protein that has to be considered in the development of an HIV vector system is Rev. Rev is normally required to mediate nuclear-cytoplasmic transport of mRNAs encoding the HIV structural proteins (12, 15, 20, 33). In most circumstances, Rev would thus be expected to be needed in the packaging cell to allow the production of the structural proteins as well as for production of full-length RNA from an HIV vector.

We have previously described the use of a small fragment from the Mason-Pfizer monkey virus (MPMV) genome, called the constitutive transport element (CTE), that allowed HIV structural protein synthesis without the need for Rev (6). This element also enabled the production of infectious HIV when introduced into a proviral clone lacking a functional *rev* gene. It should thus be possible to utilize this element to create an HIV vector system that does not require Rev. Such a system would be simpler to manipulate, and the Rev-independent HIV vectors could be also be used to deliver Rev antagonists such as Rev M10 (32, 38) for the inhibition of HIV infection.

The other regulatory genes, *tat* and *nef*, might also be expected to affect the titer of HIV vectors produced from packaging cells. *tat* would be expected to be necessary for the transcriptional activation of the viral long terminal repeat (LTR) (11) and possibly also for efficient reverse transcription in the target cell (21a). *nef* might be expected to increase the infectivity of vector produced from the packaging cell, in analogy to its effect on infectious virus (2, 41, 48, 49). In addition, the other HIV accessory genes (*vif*, *vpr*, and *vpu*) (55) might also have effects on gene transfer efficiency.

In this paper, we describe the generation of stable HIV type 1 (HIV-1)-packaging cell lines that constitutively express the HIV-1 structural proteins. Cell lines which produce structural proteins that efficiently package vector RNA either with or without Rev expression are described. These lines were used to generate HIV-1 vector stocks capable of delivering a selectable marker to CD4-positive target cells. The packaging cell system was also used assess the effect of differential expression of *rev*, *tat*, and *nef* on the vector titer.

MATERIALS AND METHODS

Plasmid constructs. Each plasmid described below is referred to by both name and number in the form of pHRxxxx to facilitate identification.

The plasmid pCMV (pHR16) has been previously described (29). Except as noted, all of its derivatives described below have HIV sequences positioned

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immediately downstream of the promoter-enhancer from the simian cytomegalovirus (CMV) IE94 gene (bp 681 to 1349) (SCU38308 [GenBank Accession number M16019]) (24) and upstream of a spliceable intron and polyadenylation signal derived from the rabbit beta-globin gene (bp 906 to 1827) (RABHBB1A1 [GenBank accession number J00659]).

pCMVrev (pHR30) was described previously as pRev1 (29). It contains a *Bsu*36I fragment spanning the *rev* coding region from the HIV-1 cDNA clone pCV1 (bp 957 to 1678) (HIVPCV12 [GenBank accession number M11840]) (3) inserted into the plasmid pCMV (pHR16) as described above. pCMVtat (pHR136) is similar to pCMVrev except that it contains a *Sal*I-*Bam*HI fragment, spanning the *tat* coding region from pCV1 (bp 787 to 1142), in place of the *rev* sequences. pCMVnef (pHR1405) was derived from pCMVrev by cloning *nef* sequences from pNL4-3 (bp 8787 to 9410) (HIVNL43 [GenBank accession number M19921]) (1) in place of *rev* sequences between the ATG at the start of *rev* and the *Bam*HI site present near the 3′ end of the *rev* gene.

pCMVgagpol-RRE (pHR354) contains HIV sequences derived from the BH10 clone (19, 42) (bp 229 to 5332 followed by bp 7168 to 8021), and pCMVenv contains HIV sequences derived from the HxB2 clone (42) (bp 5550 to 8443). In both cases, the numbering system is given in terms of the HxB2 genome (HIVHXB2R [GenBank accession numbers K03455 and M38432]). These plasmids are similar to the previously described pGAGPOL-RRE-r (pHR561) (51) and pSVSX1Δ1 (pHR278) (20) except that they contain the simian CMV immediate-early promoter in place of the simian virus (SV40) sequences.

pCMVgagpol-CTE (pHR1361) is similar to pCMVgagpol-RRE except that the Rev response element (RRE) sequences (HIV bp 7620 to 8474) and betaglobin intron have been replaced by the MPMV CTE and polyadenylation signal (bp 8007 to 8557) (SIVMPCG [GenBank accession number M12349]) (6). pCMVenv-CTE (pHR1374) was created from pCMVenv by insertion of the MPMV CTE and polyadenylation signal (bp 8007 to 8540) in place of the beta-globin intron.

The HIV vector pTR167 (pHR1266) has been previously described (45) and was a gift from Nito Panganiban, University of Wisconsin, Madison. To make this vector, pNL4-3 (HIVNL43 [GenBank accession number M19921]) (1) was cleaved to completion with *Nsi*I and religated. This deleted the central portion of the HIV genome between nucleotides 1251 and 6742. A cassette containing the hygromycin B resistance gene under the control of the SV40 early promoter was then inserted into the *Nhe*I site near the middle of the *env* gene at nucleotide 7250. pTR167-ΔXho (pHR1572) was derived from pTR167 by cleavage and T4 DNA polymerase repair of its unique *Xho*I site corresponding to nucleotide 8887 of pNL4-3. This inactivated *nef* by the introduction of a frameshift mutation near the start of the open reading frame. pTR167-CTE contains the MPMV CTE (SIVMPMV) (bp 8007 to 8240) inserted into the repaired *Xho*I site as well as a small deletion of pNL4-3 nucleotides (8790 to 8886) immediately upstream of the CTE. These modifications also inactivated *nef*.

pHyg (pHR392), a plasmid which confers hygromycin B resistance (54), and pRSVneo (pHR1265) (SYNPRSVNEO [GenBank accession number M77786]), a plasmid which confers G418 resistance (17), have been previously described. All plasmids and further details about their construction are available on request.

Maintenance of cell lines. Cell lines were maintained in Iscove's medium (GIBCO) containing 10% calf serum (Hyclone). CMT3-COS cells are a line of African green monkey kidney cells expressing SV40 large T antigen under the control of the mouse metallothionein gene promoter (16). HeLa-CD4 cells (8) were kindly provided by D. Camerini, University of Virginia, Charlottesville, and were maintained in Iscove's medium containing G418 (1.5 mg/ml).

Creation of cell lines stably expressing HIV structural proteins. Cells were stably transfected by using a modification of the calcium phosphate method (18). In preparation for the transfection, cells were first plated onto 100-mm-diameter plates at a density of about 2×10^6 cells per plate and incubated overnight. The transfection was performed the next day, when the plates were 70 to 80% confluent. At this time, the cells were washed twice with 10 ml of Tris-buffered saline (TS) (pH 7.4) (137 mM NaCl, 20 mM KCl, 25 mM Trizma base, 0.7 mM Na_2HPO_4 [anhydrous], 0.9 mM $CaCl_2$ [anhydrous], and 0.5 mM $MgCl_2$ [6-hydrate]) prior to the addition of the DNA.

A 1.5-ml suspension containing the DNA was added per 100-mm-diameter plate. The DNA suspension was prepared from two separate solutions. For each 1.5 ml of suspension, the first solution (solution A) contained 0.15 ml of 1.25 M $CaCl_2$, 0.15 ml of DNA mixture in H_2O, and 0.45 ml of H_2O. The second solution (solution B) contained 0.6 ml of H_2O and 0.15 ml of 10× HEPES buffer, pH 7.25 (1.37 M NaCl, 5 mM KCl, 7 mM Na_2HPO_4 [anhydrous], 10 g of glucose per liter, and 210 mM HEPES). Solution A was added slowly to solution B under continuous bubbling to form a fine precipitate. To create B4.14 cells, 8 µg of pCMVgagpol-RRE, 2 µg of pCMVrev, and 2 µg of pHyg were added per 100-mm-diameter plate of CMT3-COS cells. To create 5BD.1 cells, 8 µg of pCMVenv and 2 µg of pRSVneo were added per 100-mm-diameter plate of B4.14 cells. To create 2A.22 cells, 8 µg of pCMVgagpol-CTE, 8 µg of pCMVenv-CTE, and 2 µg of pRSVneo were added per 100-mm-diameter plate of CMT3-COS cells.

The 1.5 ml of DNA suspension was left on the cells for 2 min, and then 1.5 ml of complete medium was added. After an additional 15 min of incubation at room temperature, 6 ml more of medium was added, and the cells were then incubated for an additional 5 h at 37°C in a 5% CO_2 incubator. The precipitate and medium were then removed, and 4 ml of 20% glycerol in TS was added. The glycerol solution was left on the cells for precisely 70 s. The cells were then washed twice with 10 ml of TS before 10 ml of complete medium was added. The cells were then incubated at 37°C in 5% CO_2 overnight.

The plate of transfected cells was then split into six plates which were incubated in complete medium until the next day, when the appropriate drug selection was started. During the selection period, medium containing the drug was changed every 3 to 4 days. G418 was used at 1.5 mg/ml. Hygromycin B was used at 200 µg/ml. In either case, small resistant colonies were observed starting at about 8 to 10 days after the start of selection. The cell colonies were picked into 24-well plates at about day 14, expanded further as necessary, and screened for *gag* expression by p24 enzyme-linked immunosorbent assay (ELISA) or *env* expression by Western blotting. Each transfection yielded numerous positive clones.

Transient transfection of packaging cells and infection of target cells. Packaging cells were transfected to produce a vector stock, using a DEAE-dextran transfection method previously described (21). Typically, a 100-mm-diameter plate of cells was transfected with 5 µg of HIV vector (pTR167 and its derivatives) and 2 µg of each of the pCMV-based vectors expressing the various HIV regulatory proteins. When pCMVenv was cotransfected, 20 µg was used. Supernatants were harvested at 72 h posttransfection and cleared of cells by centrifugation at 2,500 rpm in an IEC Centra-8R centrifuge at 4°C for 15 min.

To perform the infection of HeLa-CD4 cells with the HIV vector stocks, 10-fold serial dilutions of the cleared supernatant were made in complete medium. DEAE-dextran was then added to each dilution at a concentration of 8 µg/ml to facilitate viral absorption, and 1 ml of each dilution was added to a 60% confluent 60-mm-diameter dish of cells, which had been subcultured the previous day. The vector was allowed to adsorb for 4 h at 37°C, at which time 4 ml of complete medium was added and the incubation was continued. The medium was replaced 2 days later with medium containing 200 µg of hygromycin B per ml. This medium was changed every 2 to 3 days. After about 14 days, the resultant colonies were fixed and stained with 0.5% crystal violet in 50% methanol. All experiments were carried out in duplicate.

To test for replication-competent virus that might be produced from the packaging cells, medium was collected from the transfected cell cultures and 6 ml of the collected medium was used to infect 5×10^6 MT-4 cells. Virus was allowed to absorb for 24 h in the presence of 8 µg of DEAE-dextran per ml. The cells were then spun down, washed one time in phosphate-buffered saline, and resuspended in 10 ml of RPMI plus 10% fetal calf serum. Thereafter, at 3- to 4-day intervals, two-fifths of the culture (4 ml) was collected for p24 assay and replaced with fresh medium.

Western blots and p24 assays. Western blotting was performed as previously described (20, 21) either with a monoclonal antibody directed against p24 (9), Rev (40), or Vif (50) or with a rabbit polyclonal antiserum directed against gp120. The rabbit serum was produced by immunization with a fragment of gp120 (amino acids 343 to 512) produced in *Escherichia coli*. p24 assays were performed with a commercial kit (DuPont) according to the directions of the manufacturer.

RESULTS

Creation of packaging cell lines that constitutively express the HIV-1 structural protein genes. The first step in the generation of a helper-free vector system is the establishment of cell lines that can be used to package vector-derived RNA. To create such cell lines for use with HIV vectors, one must take into account the fact that expression of the HIV-1 structural protein genes normally requires the presence of the RRE in *cis* and coexpression of the HIV-1 *rev* gene in *trans*.

We have previously described transient-expression systems containing these components which allowed production of large quantities of HIV-1 pseudovirions (51) and envelope proteins (44). Using these systems, we demonstrated that virus-like particles could be produced from transfected CMT3-COS cells and that cell surface envelope protein expression could be readily obtained. Subsequently, we showed that expression of structural protein genes from these vectors could be made Rev independent by insertion of the MPMV CTE into the respective expression vectors (6). This element consists of a 168-nucleotide fragment derived from the 3′ end of the MPMV genome (13, 14). The CTE overcomes the need for Rev coexpression, presumably by interacting directly with a cellular protein involved in RNA export.

Our original plasmid expression systems were based on SV40 late replacement vectors that contained the entire SV40 early region and required SV40 replication for HIV-1 protein expression. Because SV40 replication kills transfected cells,

A. Rev-Containing Packaging Cells

Transfect
pCMVgagpol-RRE
pCMVrev
pHyg

Transfect
pCMVenv
pRSVneo

CMT3 ─────────→ B4.14 ─────────→ 5BD.1
gag,gagpol,rev   *gag,gagpol,rev,env*

B. Rev-Independent Packaging Cells

Transfect
pCMVgagpol-CTE
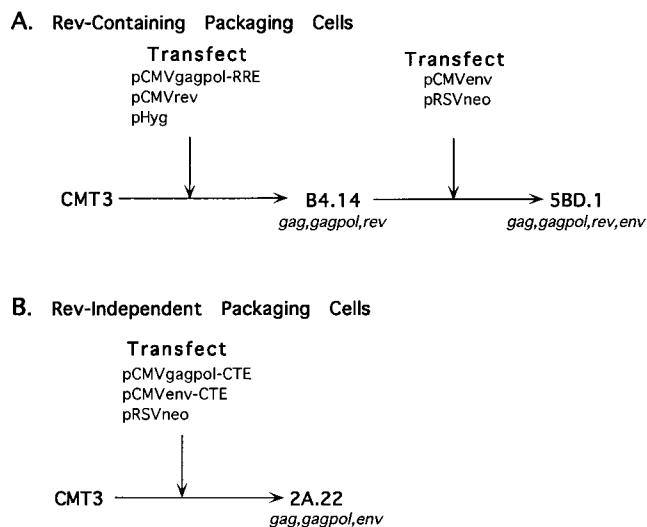pCMVenv-CTE
pRSVneo

CMT3 ─────────→ 2A.22
gag,gagpol,env

FIG. 1. Scheme for the production of stable packaging cells. (A) Rev-containing packaging cells were produced from the parental CMT3 cell line in two steps by transfection with the indicated plasmids. These cells are hygromycin B and G418 resistant. (B) Rev-independent packaging cells were produced in a single step by transfection with the indicated plasmids. These cells are G418 resistant. The HIV genes contained in each cell line are given in italics under the cell line name.

these plasmids were not suitable for the creation of stable cell lines. To create expression plasmids that could be used for this purpose, we modified our original vectors by exchanging the SV40 sequences for the simian CMV immediate-early promoter. This led to the creation of four novel plasmids: CMVgagpol-RRE and pCMVenv, which require Rev coexpression for HIV-1 structural gene expression, and pCMVgagpol-CTE and pCMVenv-CTE, which do not. (see Materials and Methods for plasmid details). These plasmids were structurally similar to pCMVrev, a plasmid which expresses HIV-1 Rev, which has been previously described (29). Similar vectors that expressed Tat (pCMVtat) and Nef (pCMVnef) were also made.

RNA produced from these plasmids would not be expected to be packaged into viral particles, since sequences from the 5′ end of the genome, which are required for packaging, are lacking in every case. The constructs expressing *env*, *nef*, *tat*, and *rev* are all devoid of any of the sequences that have been implicated in packaging. The plasmids expressing *gag-pol* do contain some of the region thought to be required for packaging but lack the first 228 nucleotides of the HIV genome. This includes all of R, all of U5, and the primer binding site. Several studies have shown that these sequences form part of the RNA packaging signal (5, 34, 56), and HIV RNA lacking these sequences packages at least 15 times less well than the wild type (22). The expression plasmids also lack some of the sequences required for reverse transcription.

To create Rev-containing and Rev-independent packaging cell lines, CMT3 cells were transfected with vectors expressing Gag, Gag-Pol, and Env, using a calcium phosphate transfection procedure. The lineages of these cell lines are shown in Fig. 1. Rev-containing B4.14 cells were made by cotransfection of pCMVgagpol-RRE, pCMVrev, and pHyg, a plasmid that confers resistance to hygromycin. This cell line was then used as transfection recipient to create the 5BD.1 cell line. To make 5BD.1 cells, B4.14 cells were cotransfected with pCMVenv and pRSVneo, a plasmid that confers resistance to G418. 5BD.1 cells are thus resistant to both hygromycin and G418. The

Rev-independent cell line 2A.22 was made by cotransfection of pCMVgagpol-CTE, pCMVenv-CTE, and pRSVneo.

All three cell lines were initially assessed for HIV-1 structural protein gene expression. HIV-1 envelope protein expression was determined by analysis of cell lysates on Western blots, using a gp120-specific antiserum (Fig. 2A). As expected, both 5BD.1 and 2A.22 cells expressed high levels of gp160 and gp120. A similar analysis of the cell lines with an anti-Rev antiserum demonstrated that B4.14 and 5BD.1 cells also expressed Rev (data not shown).

A quantitative p24 (CA) assay was performed to analyze the levels of HIV-1 Gag expression in the cell lines. To do this, a confluent plate of each cell line was split 1:5 and allowed to grow at 37°C for 3 days. Samples of media were taken at 1-day intervals during this time period and assayed for p24 by ELISA. The results of this assay are shown in Fig. 2B. All three cell lines excreted large amounts of p24, reaching levels of between 10 and 30 ng/ml after 3 days in culture. The 5BD.1 cell line consistently showed threefold-higher levels than the other two. In addition, Western blot analyses with either a human
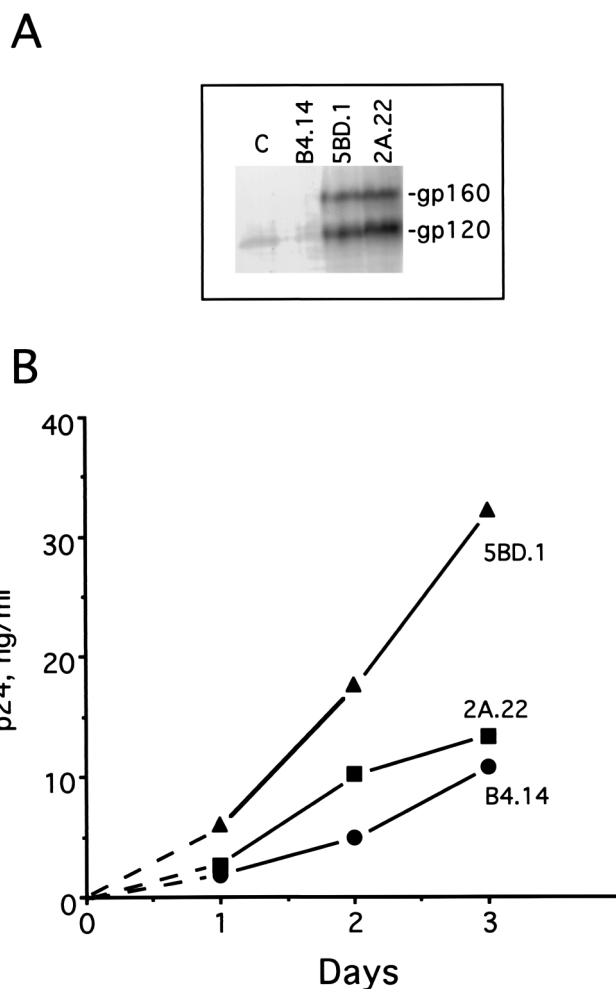
A

B

FIG. 2. Analysis of *env* and *gag-pol* expression in the stable packaging cell lines. (A) Western blot of lysates from about 10^6 cells of each indicated cell line. The primary antibody consisted of a 1:300 dilution of polyclonal rabbit antiserum directed against gp120. A goat anti-rabbit secondary antibody, conjugated to alkaline phosphatase, was used to develop the blot. C, CMT3 cell line control. (B) p24 production by the stable cell lines as a function of days in culture. Supernatants from each cell line were analyzed for p24 antigen by using a DuPont ELISA test kit as described in the text.
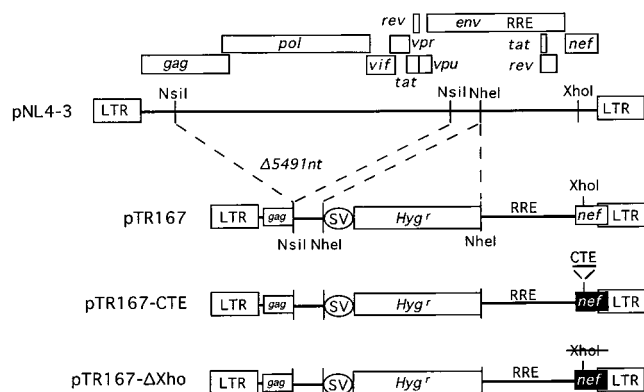
FIG. 3. HIV vectors used to transfer hygromycin B resistance. The top portion is a representation of the pNL4-3 genome showing the open reading frames, 5′ and 3′ LTRs, and restriction enzyme sites relevant to the construction of the vectors. The proviral portion of pTR167 and its derivatives are shown below this diagram. The inactive *nef* gene of pTR167-CTE and pTR167-ΔXho is shown as a filled box (see text for details).

HIV-positive antiserum or a monoclonal antibody directed against p24 (CA) showed good expression of the Gag and Gag-Pol gene products within all three cell lines (data not shown).

The Gag-Pol expression plasmids used to the make cell lines also contained the Vif open reading frame and were thus theoretically capable of expressing Vif from a spliced mRNA. To assay for Vif expression, a Western blot analysis was performed with each cell line by using an anti-Vif monoclonal antibody obtained from Michael Malim (University of Pennsylvania). Low-level Vif expression could be detected in the 5BD.1 cell line, but Vif expression was not detectable in either the B4.14 or 2A.22 cell line (data not shown). This result was surprising because 5BD.1 is derived from B4.14. However, since our p24 analysis demonstrated that 5BD.1 cells express higher levels of Gag-Pol than B4.14 cells, Vif may be expressed in B4.14 at levels below detection by the method used.

The B4.14, 5BD.1, and 2A.22 cell lines can be used to package HIV-1 vector-derived RNA without production of replication-competent virus. The cell lines were next tested for their abilities to package viral RNA derived from an HIV vector and promote the production of virus particles capable of delivering a gene to target cells. To do this, we utilized a previously described HIV vector, pTR167 (45). pTR167 is derived from the infectious HIV-1 proviral clone pNL4-3 (1), and a schematic diagram of the important portion of this vector is shown in Fig. 3. pTR167 was created by deletion of 5,491 nucleotides from the central region of pNL4-3, followed by the insertion of an SV40 early promoter-driven hygromycin resistance cassette in the middle of the *env* region. The vector retains both LTRs, the *nef* gene, the 3′ end of the *env* gene including the RRE, and sequences from the 5′ end of the genome just into the start of p24.

Transfection of packaging cells with pTR167, together with vectors that produce Tat and Rev (e.g., pCMVrev and pCMVtat), would be expected to produce three pTR167-derived RNA species. The largest of these would be a full-length RNA produced from the viral LTR. This RNA should be capable of being packaged into HIV particles and would also be expected to be spliced into a subgenomic species, since the major 5′ splice donor and 3′ *tat-rev* splice acceptor sites present in the HIV genome remain intact. The spliced mRNA should encode Nef. Indeed, Western blot analysis of cells transiently

transfected with pTR167, pCMVrev, and pCMVtat confirmed that Nef was abundantly produced (data not shown). The third RNA species would be expected to be an mRNA encoding hygromycin resistance produced from the internal SV40 early promoter. Infection of target cells with HIV particles containing full-length pTR167 RNA would be expected to lead to integration of a vector-derived provirus and expression of hygromycin resistance, assuming that expression of the SV40 promoter-driven mRNA was Tat and Rev independent.

To test the packaging cell-vector system, pTR167 and pCMVtat were transfected into 2A.22, B4.14, or 5BD.1 cells, using DEAE-dextran. In some cases pCMVenv was also cotransfected. pCMVrev was also added to the transfections involving the 2A.22 cells, since this cell line did not contain endogenous Rev. Medium from each transfected plate was harvested at 72 h posttransfection. One milliliter of the collected medium was then used to infect HeLa or HeLa-CD4 cells, according to the procedures described in Materials and Methods. The infected cells were then subjected to hygromycin selection for 14 days, at which time they were stained and colonies were counted. The results of this experiment are presented in Table 1.

The data in Table 1 demonstrate that both the 2A.22 and 5BD.1 cell lines supported the production of infectious vector which could deliver the hygromycin resistance gene to the HeLa-CD4 target cells with titers greater than 10^3 CFU per ml. Gene delivery was clearly HIV envelope protein mediated, since infection of HeLa cells lacking CD4 did not occur. The B4.14 cell line also produced vector capable of delivering the marker gene to HeLa-CD4 cells at reasonably high titers, but only when a source of HIV-1 envelope protein was provided by cotransfection with pCMVenv. pCMVenv cotransfection had little effect on vector production from the 2A.22 cell line.

We next performed an experiment designed to determine if replication-competent virus was produced in the packaging cell lines transfected with pTR167. It seemed unlikely that this would occur, since the plasmids used to create the packaging cells lacked viral LTRs and had no regions of overlap (except for the RRE or CTE). Multiple recombination events between the inserted genes and pTR167 would thus have to take place to create a replication-competent virus (see Discussion). Nevertheless, because of the safety issues involved, we wanted to formally rule out this possibility.

To test for replication-competent virus production, a 100-mm-diameter plate of each of the three cell lines was cotransfected with pTR167, pCMVrev, pCMVtat, and pCMVnef. pCMVenv-CTE was also added to the cotransfection for the B4.14 cell line. After 72 h, the medium (10 ml) from each plate was collected. In each case we also collected medium from a plate that was not subjected to the transfection procedure. Six

TABLE 1. Titers of pTR167 packaged in different cell lines with or without added HIV envelope protein

Cell line	pCMVenv	Titer (CFU/ml)[a] on:	
		HeLa cells	HeLa-CD4 cells
2A.22[b]	−	0	2,480 ± 50
	+	0	1,840 ± 110
B4.14	−	0	0
	+	0	800 ± 200
5BD.1	−	ND[c]	9,000 ± 1,800

[a] Results are means ± standard deviations for duplicate determinations.
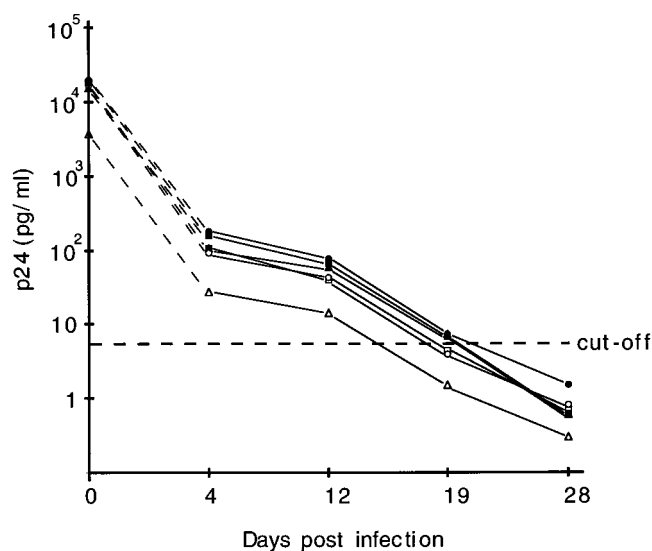[b] Transfections also contained pCMVrev.
[c] ND, not determined.

FIG. 4. p24 antigen assay to detect replication-competent virus. MT-4 cells were infected with supernatants from various cell lines that either were transfected with pTR167 and the additional expression vectors described in the text (closed symbols) or were not transfected (open symbols). p24 was assayed throughout the experiment at the days indicated. Supernatants were from B4.14 (circles), 2A.22 (triangles), or 5BD.1 (squares) cells. The dashed line represents the calculated sensitivity cutoff as specified by the ELISA kit's manufacturer.

milliliters of the collected medium from each plate was then used to infect 5×10^6 MT4 cells as described in Materials and Methods. The MT4 cell line is a line of human T-cell leukemia virus type 1-transformed CD4-positive T cells that are extremely permissive for growth of HIV isolates containing an HIV-1$_{\text{IIIB}}$ envelope protein (data not shown). The infected cells were passaged for 28 days under conditions that would support the growth of virus. At regular intervals, medium was collected and assayed for p24 by ELISA. The results of this experiment are shown in Fig. 4.

Analysis of Fig. 4 reveals that all six infected cutures behaved essentially the same. There was no significant difference between the infections derived from the cell lines that were cotransfected with the collection of plasmids and those that were not. In all cases, input p24 was reduced by about 100-fold at 4 days postinfection (the initial infection procedures washed away some of the input p24). The decline continued to below detectable levels by about 19 days. In no case could replication-competent virus be detected.

The presence of Tat in the producer cell enhances vector titer. Expression of the full-length RNA in pTR167 is directed by the viral LTR. It was therefore of interest to determine if the presence of Tat in the producer cell influenced vector titers, since Tat would be expected to transactivate the viral LTR. Tat has recently also been shown to be required for efficient reverse transcription in newly infected cells (21a).

To test for the consequence of Tat expression on the vector titer, 5BD.1 and 2A.22 cells were transfected with pTR167 with or without pCMVtat cotransfection. For this experiment, pCMVrev and pCMVnef were also added to all of the transfections. This controlled for any possible effect that Rev or Nef may have on the vector titer and allowed us to examine only the effect of Tat. Medium was harvested at 72 h posttransfection and used to infect HeLa-CD4 cells, which were subjected to hygromycin selection for 14 days. Colonies were then counted, and the results are tabulated in Table 2. When pCMVtat was present in the producer cells, titers of about $5 \times$

TABLE 2. Effect of Tat expression on vector titers in Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml)[a]
5BD.1 cells, pTR167	+	+	+	4,500 ± 100
	+	−	+	650 ± 250
2A.22 cells, pTR167	+	+	+	5,250 ± 450
	+	−	+	800[b]

[a] Results are means ± standard deviations for duplicate determinations.
[b] Single determination.

10^3 CFU/ml were achieved with both cell lines. In the absence of Tat, the vector titers were approximately sevenfold lower.

Rev is required in the producer cell in *trans* for good vector titers but can be replaced by providing the CTE in the vector in *cis*. To test whether good vector titers were dependent on the presence of Rev in the producer cell, a transfection-infection experiment similar to the one described above was performed. In this instance, pTR167 was cotransfected into the packaging cell line 5BD.1 or 2A.22 with or without pCMVrev. For this experiment, pCMVtat and pCMVnef were also added to all of the transfections. This controlled for any possible effect that Tat or Nef may have on vector titer and allowed us to examine only the effect of Rev. Results from the subsequent infection of the HeLa-CD4 cells with the producer cell supernatants are shown in Table 3.

The deletion of pCMVrev from the transfection of 5BD.1 cells did not have any effect on the titer of the vector produced from these cells. However, in the case of 2A.22 cells, deletion of pCMVrev reduced the vector titer to near zero. These results suggested that the vector required Rev but that 5BD.1 cells contained enough endogenous Rev to obtain good titers.

To further establish this, we created a modified version of pTR167, pTR167-CTE, which had the MPMV CTE cloned into the *Xho*I site of Nef. The relevant portion of this plasmid is shown in Fig. 3. When this vector was tested in the transfection-infection protocol, good titers were obtained in both 5BD.1 and 2A.22 cells, with or without added Rev. Thus, we can conclude that expression from pTR167 is Rev dependent but that the need for Rev coexpression can be completely overcome by insertion of the CTE into the vector. It is interesting that the titers obtained from the system completely lacking Rev (2A.22/pTR167-CTE) are roughly equivalent to the titers obtained with Rev.

TABLE 3. Effect of Rev expression on vector titers in Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml)[a]
5BD.1 cells				
pTR167	+	+	+	4,500 ± 100
	−	+	+	5,600 ± 900
pTR167-CTE	+	+	+	3,300 ± 200
	−	+	+	2,950 ± 550
2A.22 cells				
pTR167	+	+	+	5,250 ± 450
	−	+	+	1.5 ± 0.5
pTR167-CTE	+	+	+	3,950 ± 650
	−	+	+	3,400 ± 700

[a] Results are means ± standard deviations for duplicate determinations.

TABLE 4. Effect of Nef expression on vector titers in
Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml)[a]
5BD.1 cells				
pTR167	+	+	+	4,500 ± 100
	+	+	−	3,400 ± 300
pTR167-CTE	+	+	+	3,300 ± 200
	+	+	−	650 ± 350
pTR176-ΔXho	+	+	+	1,850 ± 550
	+	+	−	700 ± 400
2A.22 cells				
pTR167	+	+	+	5,250 ± 450
	+	+	−	3,100[b]
pTR167-CTE	+	+	+	3,950 ± 650
	+	+	−	505 ± 25
pTR167-ΔXho	+	+	+	7,700 ± 1,200
	+	+	−	550 ± 150

[a] Results are means ± standard deviations for duplicate determinations.
[b] Single determination.

The presence of Nef in the producer cell enhances vector titer. As mentioned above, pTR167 contains an intact *nef* gene and expresses a functional Nef protein. To determine the effect of Nef expression on vector titer, we used two *nef*-negative derivatives of pTR167. The first of these, pTR167-ΔXho, was made by cleavage of pTR167 with *Xho*I followed by subsequent repair with T4 DNA polymerase. This causes a frameshift to occur near the start of the *nef* open reading frame. The relevant portion of this plasmid is shown in Fig. 3. We also used pTR167-CTE in these experiments, since it contains a nonfunctional *nef* gene due to the insertion of the CTE into the *nef* open reading frame.

To examine the consequence of Nef expression in the producer cell on the subsequent vector titer, 5BD.1 or 2A.22 cells were transfected with either pTR167, pTR167-CTE, or pTR167-ΔXho, with or without pCMVnef cotransfection. For this experiment, pCMVrev and pCMVtat were also added to all of the transfections. This controlled for the effects that Rev or Tat have on the vector titer and allowed us to examine only the effect of Nef. The results of this experiment are shown in Table 4.

As expected, pCMVnef had little effect on the vector titer in the case of pTR167. However, for pTR167-CTE as well as pTR167ΔXho, pCMVnef cotransfection increased vector titers significantly (3- to 14-fold). This was true in both the Rev-dependent and Rev-independent cell lines.

DISCUSSION

We have described cell lines that constitutively express HIV-1 structural proteins and have demonstrated that they can be used to package HIV vector RNA. These lines were readily created by standard transfection protocols. In contrast to the ease with which we made these lines, others have shown that constitutive expression of the HIV-1 envelope protein (52) and protease (25, 27) is toxic and have concluded that stable lines expressing these proteins are not easily obtainable (27). A recent report described the creation of an inducible packaging cell line which was designed to overcome this perceived problem (58).

It is hard to reconcile our ability to easily create cell lines constitutively expressing the HIV structural proteins with the difficulties encountered by others. There does not appear to be anything unique about the parental cell line used to establish these packaging cells that would explain this. Also, we have recently created similar packaging lines from a different parental line (293-T) by the same method (data not shown).

One possible reason for our success in creating packaging lines may be that the selectable marker was introduced on a plasmid separate from the ones expressing the structural protein genes. It is possible that this more readily allows for the selection of cells producing tolerable levels of the structural proteins, since it enables covariation. Consistent with this notion, another cell line that constitutively expresses HIV structural proteins has recently been described (10). To create this, a full-length proviral clone lacking only 37 nucleotides from the packaging region was cotransfected with a plasmid expressing a *neo* marker. This line was also shown to package a minimal HIV vector with an efficiency similar to that of our line.

Several properties of our packaging cells probably account for the fact that we were unable to detect replication-competent virus. First, the cell lines were constructed by using a split genome approach, with the *gag-pol-vif* coding region on one plasmid and the *env* region on another. There is also virtually no overlap between the two plasmids except for the RRE or CTE. In the 2A.22 and 5BD.1 cell lines, one or the other of these sequences is present on both constructs, but the sequences are positioned such that a recombination event within them would not generate a complete genome. Both plasmids also lack almost all of the *cis*-acting signals needed for viral replication, including the viral LTRs. In addition, with the exception of *vif*, the viral regulatory and accessory genes are made from additional separate plasmids. Thus, multiple recombination events would have to occur between the stably transfected DNA and vector DNAs to produce an infectious recombinant.

The vector titer obtained for HIV-1 Env-mediated gene transfer to CD4-positive cells is comparable to titers recently published by others (10^3 to 10^4 CFU/ml) (10, 58). We obtained this titer without using any of the titer enhancement protocols that have been described in the literature, such as harvest at 32°C (26), spin infection (26), or pseudotyping with vesicular stomatitis virus envelope glycoprotein (VSV G) followed by concentration by ultrafiltration (43). It seems likely that incorporation of some of these methodologies into our protocols will lead to higher vector titers. In this regard, preliminary experiments using the B4.14 cell line have demonstrated that it can be used to create HIV pseudotypes that could be concentrated and used to expand the host range of our vectors.

Our experiments also assessed the role of Tat, Nef, and Rev in vector titer. The presence of Tat and Nef in the producer cell each increased vector titer about 5- to 10-fold. Rev, on the other hand, was absolutely essential for any titer, unless the MPMV CTE was included in the vector.

The increase in titer observed in the presence of Tat could be due to transactivation of the viral LTR present in the vector or to the recently described effect of Tat on reverse transcription (21a), or both. Activation of transcription from the viral LTR would be expected to lead to increased amounts of full-length RNA for packaging. The rather low magnitude of the effect (5- to 10-fold) compared to that in other reports describing Tat transactivation (23, 37) may be due to the fact that our HIV vector (pTR167) contained an SV40 origin of replication and our packaging cell line contained SV40 T antigen. Under these conditions, we have found the HIV LTR to be only partially dependent on Tat (unpublished data). The enhancement by Tat is also considerably lower than its reported effect on reverse transcription (21a). It is possible that the low enhance-

ment is partially the result of the fact that Tat was supplied by cotransfection that was not 100% efficient. Expressing Tat as a stable gene in a cell line might be expected to increase the titer further.

On the other hand, Tat is not needed in the target cell for the expression of the selectable marker, since the internal SV40 early promoter present in our plasmid is not dependent on Tat. It may thus be possible to distinguish between an effect of Tat on transcription in the producer cell and an effect of Tat on reverse transcription in the target cell by designing a vector with a modified LTR. Modifications which alter the HIV LTR to make it constitutively active at high levels have been described (46). We are presently examining whether such modifications can lead to increased vector titers in the absence of Tat.

The mechanism by which Nef increased the vector titer is not known, although our observation of a 5- to 10-fold increase is consistent with reports in the literature that Nef increases viral infectivity (2, 41, 48, 49). Those reports suggest that Nef may play a role early in the virus life cycle, since Nef seems to enhance the reverse transcription process. However, it is at present unclear whether Nef exerts its effect in the producer cell to modify the viral structural proteins or whether Nef functions in the target cell. The latter possibility is supported by recent reports that have demonstrated small amounts of Nef in virus particles (7, 41, 57). The vector system described here is likely to provide a useful tool for examining this aspect of Nef function.

By creating an HIV vector which contained the MPMV CTE (pTR167-CTE) and a packaging cell line which expressed the HIV structural proteins in a Rev-independent fashion (2A.22), we were able to obtain an HIV vector system that functions completely without Rev. The titer of the vector obtained from this system was essentially the same as that obtained from a parallel system which contained Rev. In this context the CTE seemed to substitute completely for Rev-RRE function, similar to what was previously observed in transient-expression assays with Rev-dependent constructs (6). This is in contrast to situations where several rounds of HIV replication were measured. In those cases, titers from CTE-containing viruses were always reduced by at least 1 log unit compared to viruses utilizing Rev and the RRE (6, 59). We presently do not have an explanation for this difference.

Our ability to create an HIV vector system that works in the absence of Rev opens the possibility of using it as a delivery vehicle for intracellular immunization (4) against Rev function. This is not possible to do with a vector system that is dependent on Rev, since the antagonist would inhibit vector production. Several Rev antagonists that have dramatic inhibitory effects on HIV replication, such as Rev M10 (32, 38) or RRE decoys (28), have been described. These genes could be readily introduced into an HIV vector and put into cells normally infectable by HIV. Expression of the "anti-Rev" gene would be expected to dampen HIV infection. Any residual HIV replication should lead to activation of the vector LTR (by Tat) and create a vector-derived RNA that would be packaged by proteins derived from the infectious virus. In this scenario, the wild-type virus would act as a helper that may allow the spread of vector particles to previously nonimmunized cells. Because of the additional vector spread, it is likely that this type of scheme will be more effective in modulating HIV infection in vivo than one based on traditional retrovirus vectors. We are currently testing this approach in model systems.

ACKNOWLEDGMENTS

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