

## A New-Generation Stable Inducible Packaging Cell Line for Lentiviral Vectors

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### ABSTRACT

We have successfully generated and characterized a stable packaging cell line for HIV-1-based vectors. To allow safe production of vector, a minimal packaging construct carrying only the coding sequences of the HIV-1 *gag-pol*, *tat*, and *rev* genes was stably introduced into 293G cells under the control of a Tet<sup>0</sup> minimal promoter. 293G cells express the chimeric Tet<sup>R</sup>/VP16 *trans*-activator and contain a tetracycline-regulated vesicular stomatitis virus protein G (VSV-G) envelope gene. When the cells were grown in the presence of tetracycline the expression of both HIV-1-derived and VSV-derived packaging functions was suppressed. On induction, approximately 50 ng/ml/24 hr of Gag p24 equivalent of vector was obtained. After introduction of the transfer vector by serial infection, vector could be collected for several days with a transduction efficiency similar or superior to that of vector produced by transient transfection both for dividing and growth-arrested cells. The vector could be effectively concentrated to titers reaching 10<sup>9</sup> transducing units/ml and allowed for efficient delivery and stable expression of a GFP transgene in the mouse brain. The packaging cell line and all vector producer clones described here were shown to be free from replication-competent recombinants, and from recombinants between packaging and vector constructs that transfer the viral *gag-pol* genes. The packaging cell line and the assays developed will advance lentiviral vectors toward the stringent requirements of clinical applications.

### OVERVIEW SUMMARY

The production of lentiviral vectors poses significant challenges due to the hybrid nature of the vectors, the complexity of the lentiviral genome, and the toxicity of several components of the vector particle. Accordingly, lentiviral vectors have been produced until now by transient co-transfection of the required constructs, an approach hardly amenable to the stringent characterization and scale-up required for clinical applications. Here we report the successful development and characterization of a stable inducible packaging cell line for HIV-1-derived lentiviral vectors. To allow safe and efficient production of vector we used a minimal set of HIV-derived genetic information and a tetracycline-dependent system to control expression of packaging and envelope functions. We selected clones with

undetectable basal expression and efficient release of vector particles on induction. By serial infection of the transfer construct, producer clones could be obtained that yielded vectors with titer, infectivity, and performance matching those of vector obtained by transient transfection. In addition, these vectors allowed for efficient delivery and stable transgene expression in the mouse brain. We also developed sensitive assays for replication-competent recombinants and replication-defective recombinants transferring viral packaging functions. The packaging cell line described here and all vector producer clones derived from it were shown to be free from either type of recombinant. The scaleable and safe vector production allowed by this new packaging cell line, and the assays developed for its characterization, should advance the applications of lentiviral vectors for gene therapy.

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## INTRODUCTION

LENTIVIRAL VECTORS are replication-defective, hybrid viral particles consisting of the core proteins and enzymes of a lentivirus and the envelope of a different virus. They transduce several types of cells and tissues independent of cell division and allow long-term expression of the transgene after direct injection into adult rodents (Naldini *et al.*, 1996a; Naldini, 1998).

Compared with conventional murine leukemia virus (MuLV)-based retroviral vectors, the production of lentiviral vectors poses significant challenges due to the hybrid nature of the vectors, the complexity of the lentiviral genome, and the toxicity of several components of the vector particle.

The hybrid nature of the vectors dictates the use of three different plasmids to produce infectious particles (Naldini, 1998). Two of the plasmids encode the proteins that assemble the particle, one plasmid expressing the core proteins and enzymes of human immunodeficiency virus type 1 (HIV-1) (packaging construct), and the other expressing the envelope protein of an unrelated virus, most often the protein G of the vesicular stomatitis virus (VSV-G). The third construct contains an expression cassette for the transgene flanked by HIV-1 *cis*-acting sequences required for encapsidation (McBride and Panganiban, 1996; McBride *et al.*, 1997), reverse transcription, and integration (transfer vector construct).

The complexity of lentiviral genomes is due to the presence, in addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, of two regulatory genes, *tat* and *rev*, whose products are essential for expression of the genome in the context of its native state, and a set of accessory genes that are not essential for viral replication but are critical for pathogenesis (Cullen, 1998). The Tat and Rev proteins act at the transcriptional and posttranscriptional level, respectively. HIV-1 Tat binds to a stem-loop structure (the *trans*-activation response [TAR] element) in the nascent long terminal repeat (LTR) RNA and tethers the cyclin T-CDK9 complex to polymerase II, promoting transcriptional elongation (Wei *et al.*, 1998; Bieniasz *et al.*, 1999). HIV-1 Rev binds to an RNA motif (Rev-responsive element, RRE) found in the envelope-coding region of the transcript and bridges it to the nuclear export factor exportin 1 (CREM), promoting cytoplasmic export of unspliced and singly spliced viral transcripts that express late viral proteins (Fornerod *et al.*, 1997; Neville *et al.*, 1997; Stade *et al.*, 1997). Additional components of the Rev-dependent switch of *gag-pol* expression are suboptimal splice acceptor sites downstream of the genes that facilitate accumulation of unspliced transcripts (Staffa and Cochrane, 1994), and inhibitory sequences within the *gag-pol* coding frames that promote degradation of unspliced transcripts in the absence of active export (Schneider *et al.*, 1997).

The design of HIV-1-derived vectors reflects and exploits the complexity of the viral genome. Tat is required to activate transcription from the transfer vector LTR. Rev is required to export from the nucleus unspliced transcripts both of the transfer construct, allowing encapsidation, and of the packaging construct, allowing expression of the Gag and Pol polyproteins. As Tat and Rev are expressed *in trans* in vector-producer cells, transcription from the vector LTR, and accumulation of transcripts that can be encapsidated, are restricted in transduced

cells (Naldini *et al.*, 1996a; Naldini, 1998; Dull *et al.*, 1998). The requirement for HIV-1 accessory genes in vector production has been investigated. We previously showed that vectors packaged by a construct from which all the HIV-1 accessory genes were deleted were as efficient as vectors made from wild-type constructs at transducing genes *in vitro* and *in vivo* (Zufferey *et al.*, 1997). Similar findings have since been reported by others (Kim *et al.*, 1998; Mochizuki *et al.*, 1998; Gasmi *et al.*, 1999). As the nonessential genes of HIV-1 are critical for pathogenesis (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995; Aldrovandi and Zack, 1996), the new "minimal" packaging constructs were adopted to increase the biosafety of vector production.

Several of the proteins required to assemble lentiviral vectors are toxic to cells when overexpressed, including VSV-G (Burns *et al.*, 1993), HIV-1 Vpr (Bartz *et al.*, 1996), Tat (Li *et al.*, 1995), Rev (Miyazaki *et al.*, 1995), and protease (Konvalinka *et al.*, 1995). Accordingly, vectors are currently produced by transient cotransfection of the required constructs into human kidney 293T cells (Naldini *et al.*, 1996a). The average transient producer culture yields the vector equivalent of 100–1000 ng/ml/24 hr of Gag p24 antigen for 2–3 days, corresponding to  $10^6$ – $10^7$  transducing units (TU)/ml as measured by end-point biological titration on HeLa cells (Zufferey *et al.*, 1997; Dull *et al.*, 1998). The average particle output per cell is approximately 50–500 fg of p24/cell/24 hr and the average particle infectivity is  $10^4$  TU/ng p24 (Dull *et al.*, 1998). Purification and concentration of vector to 1000-fold higher titer are easily achieved by ultracentrifugation. Transient transfection, however, is hardly amenable to the stringent characterization, standardization, and scale-up required by good manufacturing practices of vector production for human applications. Furthermore, cotransfection may increase the risk of recombination between plasmids, thus detracting from the biosafety devices built into the vector design. The availability of a stable producer system will assist in transferring the unique advantages of lentiviral vectors to the clinic.

Various attempts at generating stable packaging cell lines for HIV-derived vectors have been reported, including the use of inducible regulatory elements to limit expression of toxic components from early generations of packaging constructs (Carroll *et al.*, 1994; Yu *et al.*, 1996; Srinivasakumar *et al.*, 1997; Kaul *et al.*, 1998; Kafri *et al.*, 1999). While these studies have been helpful in evaluating the different approaches, they have not yielded a producer with satisfactory vector output, activity, stability, or biosafety. Here we describe the successful development and detailed characterization of a new second-generation, inducible Lentikat packaging cell line for HIV-1-derived vectors. On induction approximately 100 ng/ml/24 hr of Gag p24 equivalent of vector was obtained with infectious titers above  $10^6$  TU/ml and transducing activity indistinguishable from, or superior to, that of vector produced by transient transfection. By newly developed and highly sensitive assays, we demonstrated the absence of replication-competent and replication-defective recombinants that would have transferred packaging functions in vector batches from the stable producers. This packaging cell line and the assays developed for its characterization should be of significant value

for most applications of gene transfer mediated by lentiviral vectors.

## MATERIALS AND METHODS

### *Plasmid construction and transient transfections*

*Plasmids for packaging and producer cell lines.* pHR2CMVGFP is a lentiviral transfer vector carrying the EGFP gene (Clontech, Palo Alto, CA) under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter. The parental transfer vector plasmids that formed pHR2CMVGFP were described previously (Dull *et al.*, 1998). pHR2CMVGFP was constructed by ligating the 10.1-kb *ClaiI/BamHI* fragment of pHR2hPGK.GFP to the 827-bp *BamHI* fragment containing the CMV enhancer/promoter of pMDLg/p. A *ClaiI*-to-*BamHI* linker consisting of synthetic oligonucleotides 5'-CGATTTATGG-3' and 5'-GATCCCATAAAT-3' completed the construction by spanning the fragments at the 5' end of the CMV enhancer/promoter. pCMV $\Delta$ R8.75 differs from the previously described packaging plasmid pCMV $\Delta$ R8.74 (Dull *et al.*, 1998) by containing an optimized Kozak sequence at the ATG of the *gag-pol* gene (Kozak, 1997). It was constructed by joining the 5.3-kb *NgoMI/SstII* and 6.6-kb *NcoI-NgoMI* fragments of pCMV $\Delta$ R8.74 with an *SstII*-to-*NcoI* linker. The linker contained the HIV 5' major splice donor sequence and the Kozak consensus sequence and consisted of synthetic oligonucleotides 5'-GGGACTGGTGAGTGAATTCGAGATCTGC-CGCCGC-3' and 5'-CATGGCGGCGGCAGATCTCGAATTC-CACTCACCAGTCCCGC-3'. pTetCMV $\Delta$ R8.75 was derived from pCMV $\Delta$ R8.75 by swapping the CMV enhancer/promoter with the TetO7-controlled CMV promoter in the following way. The 4.6-kb *NgoMI-PstI* fragment of pCMV $\Delta$ R8.74 was blunted with Klenow enzyme at the *PstI* site and ligated to the 450-bp *XhoI*-blunted *SstII* fragment of pTetSplice (GIBCO-BRL, Grand Island, NY) and the 6.6-kb *SstII/NgoMI* fragment of pCMV $\Delta$ R8.74. The construction of the TetO7/CMV-controlled VSV-G envelope expression plasmid, pMDtet.G, was previously described (Ory *et al.*, 1996).

*Plasmids for recombinant assays.* Plasmid pR8.7 $\Delta$ Env was generated by insertion of a 4.3-kb *BclII-XhoI* fragment of plasmid pCMV $\Delta$ R8.74 containing deletions of all accessory and *env* viral genes into the corresponding region of pR8 plasmid (Gallay *et al.*, 1995). To restore a full-length *env* gene in pR8.7 $\Delta$ Env, the *SallI-BamHI* fragment of pR8.7 $\Delta$ Env was replaced by a corresponding fragment of pR7 (Kim *et al.*, 1989).

*Vector generation by transient transfection.* 293T cells were plated at  $5 \times 10^6$  cells per 10-cm plate for 24 hr and refed with 10 ml of fresh medium 2 hr prior to transfection. Precipitate was formed by adding the appropriate plasmids to a final volume of 450  $\mu$ l of 0.1 $\times$  TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0] diluted 1:10 with deionized H<sub>2</sub>O [dH<sub>2</sub>O]) and 50  $\mu$ l of 2.5 M CaCl<sub>2</sub>, mixing well. Five hundred microliters of 2 $\times$  HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.12) was then added while aerating the so-

lution with a pipette. The mixture was then added dropwise to 293T cells and swirled gently. Medium was replaced with fresh growth medium 16 hr posttransfection, and after an additional 24 hr the medium was collected, filtered through a 0.22- $\mu$ m pore size filter, and flash frozen on dry ice.

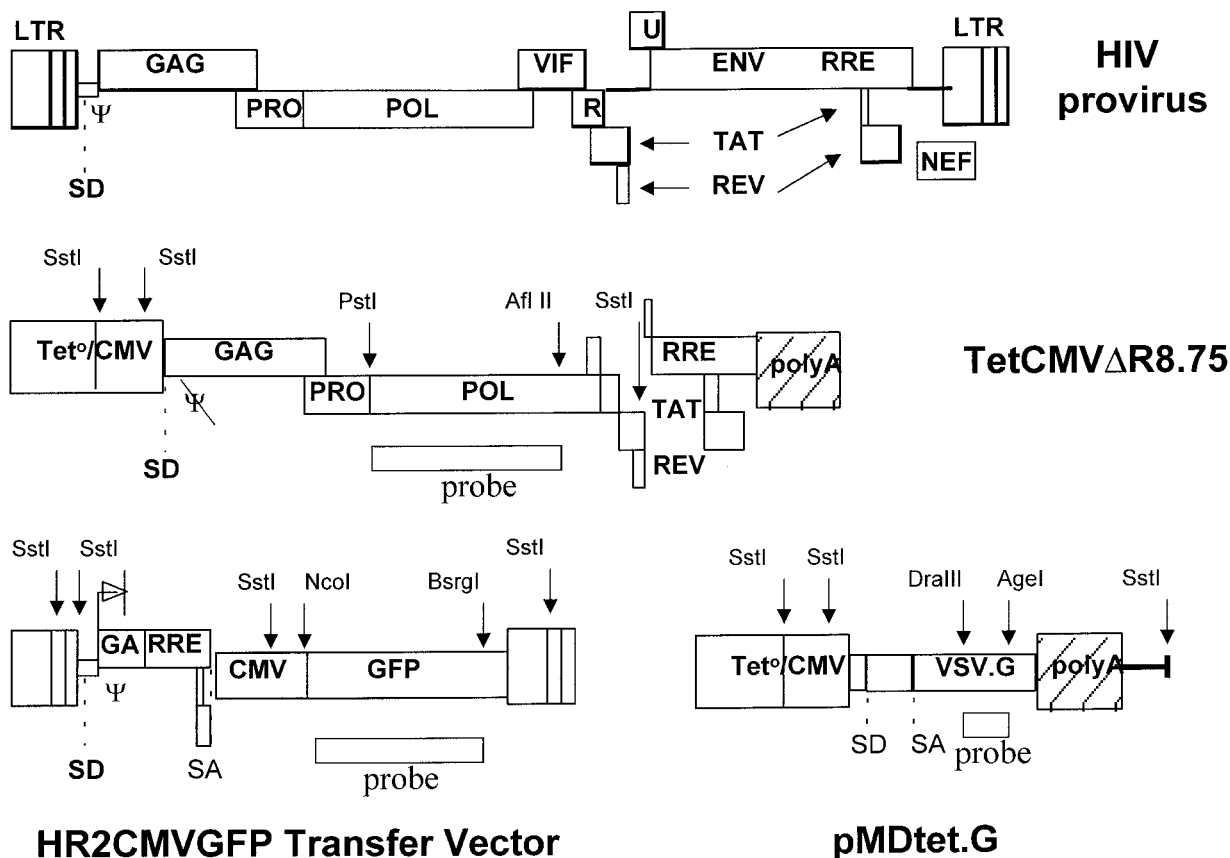
### *Cells, stable packaging and producer clones, inductions, titrations*

*Cells.* 293G cells (Ory *et al.*, 1996) and all 293G-derived clones were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% calf serum and tetracycline (1  $\mu$ g/ml) or doxycycline (0.7  $\mu$ g/ml). 293T cells were grown in IMDM supplemented with 10% fetal bovine serum. HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM)-glucose (4.0 g/liter) with 10% fetal bovine serum. All cells were split at 3 to 4-day intervals.

*Generation of Lentikat2.54.* 293G cells were plated at  $0.65 \times 10^6$  cells per 10-cm dish 48 hr prior to use. Medium was changed 2 hr prior to transfection. A total of 20  $\mu$ g of plasmid DNA was used per 10-cm dish: 18  $\mu$ g of the packaging plasmid pTet $\Delta$ CMVR8.75 and 2  $\mu$ g of the zeocin resistance plasmid, pZeoSV2 (Invitrogen, Carlsbad, CA) and precipitated as described above. Medium was changed 14–16 hr posttransfection, and after an additional 24 hr the cells were split into medium supplemented with zeocin (250  $\mu$ g/ml) (Invitrogen) and tetracycline (1  $\mu$ g/ml). Colonies were picked 3–4 weeks later and characterized for p24 expression after induction in six-well plates. When cells were 90% confluent the medium was changed and after 24 hr a noninduced sample was collected, filtered through a 0.45- $\mu$ m pore size filter, and flash frozen on dry ice. The medium was replaced by induction medium, DMEM-glucose (1.0 g/liter) with 10% calf serum, and collected daily as needed. All conditioned media were filtered and frozen before assay. Conditioned media were assayed for p24 by immunocapture (Du Pont, Wilmington, DE). Packaging clones were evaluated for their ability to produce vector after two infections at 3.5–5 multiplicities of infection (MOIs) with transiently produced HR2CMVGFP vector in the presence of Polybrene (8  $\mu$ g/ml). Cells were expanded and noninduced and induced conditioned media were collected as described above. Conditioned media were assayed for p24 by immunocapture and titered for green fluorescent protein (GFP) as described below.

*Producer clones.* Producer populations were made by the serial transduction (infections A–E) of Lentikat2.54 packaging clone with transiently produced VSV-G-pseudotyped HR2CMVGFP vector in the presence of Polybrene (8  $\mu$ g/ml). Populations were expanded, induced, and evaluated for p24 and GFP titers as described. Lentikat2.54 population E was subcloned by dilution cloning, and producer clones were induced and characterized for p24 and GFP titers as described below.

*Titer assay.* Titrations were done on HeLa cells plated at  $5 \times 10^4$  cells per well of a 6-well plate 24 hr prior to infection. Infections were done in the presence of Polybrene (8  $\mu$ g/ml), and refed with growth medium 24 hr later. After an additional 2 to



**FIG. 1.** Schematic diagrams of the HIV provirus and the three constructs used to create the *Lentikat* packaging and producer cell lines. The viral LTRs, the reading frames of the viral genes, the major 5' splice donor site (SD), the encapsidation signal ( $\Psi$ ), and the Rev response element (RRE) are boxed and indicated in boldface type. pTetCMV $\Delta$ R8.75 expresses the *gag*, *pol*, *tat*, and *rev* genes under the control of the hybrid tetracycline operator-minimal CMV promoter (Tet<sup>o</sup>/CMV). The transfer construct HR2CMVGFP contains HIV-1 *cis*-acting sequences and an expression cassette for the transgene. pMDtet.G encodes the heterologous envelope VSV-G under control of the Tet<sup>o</sup>/CMV promoter to pseudotype the vector. Splice sites are from the  $\beta$ -globin gene. Restriction sites utilized in the Southern analysis and in generating the <sup>32</sup>P-labeled probes are indicated over each diagram. Probed regions are indicated by boxes beneath each diagram. The 3' *SstI* site shown in the diagram of pMDtet.G is in the 293 cellular genome flanking the integration site of the transfected expression cassette approximately 6.6 kb away from the internal *SstI* site.

10 days the HeLa cells were evaluated for GFP expression by FACScan (Becton Dickinson, Mountain View, CA).

#### *Southern analysis of packaging and producer cell lines*

Genomic DNA (10  $\mu$ g) from each packaging or producer cell line was digested with *SstI* and electrophoresed on a 0.7% 2-mercaptoethanol (2-ME) agarose gel, and Southern blots were transferred to Zetabind filter paper (CUNO Laboratory Products, Meriden, CT). Sequences of the *gag-pol* expression plasmid pTetCMV $\Delta$ R8.75 were probed with a <sup>32</sup>P-labeled, 1.9-kb *pol* fragment isolated by *PstI* and *AflIII* digestion, and a <sup>32</sup>P-labeled, 0.3-kb fragment isolated from *rev* intron 2 by *BamHI* and *XhoI* digestion. Sequences of the envelope plasmid, pMDtet.G, were probed with a <sup>32</sup>P-labeled, 990-bp fragment isolated by *EcoRI* and *AgeI* digestion. Sequences of the transfer vector, HR2CMVGFP, were probed with a <sup>32</sup>P-labeled, 711-bp, GFP-coding region fragment isolated from the plasmid pEGFP (Clontech) by *NcoI* and *BsrGI* digestion. The *SstI*-cut genomic DNAs

were also probed with a <sup>32</sup>P-labeled 382-bp *MluI*-to-*XbaI* fragment containing coding sequences of the tet<sup>R</sup>/VP16 *trans*-activator from the plasmid pTet-on (Clontech). The resultant bands on the Southern blots were as follows: TetCMV $\Delta$ R8.75, 4.7 kb with *PstI*-*AflIII* probe, 6.1 kb with the *BamHI*-*XhoI* probe; VSV-G *env*, 6.6 kb; HR2CMVGFP, 1.4 kb; tet<sup>R</sup>/VP16 *trans*-activator, 1.2 kb with the *MluI*-*XbaI* probe. Figure 1 displays the locations of the restriction enzyme sites and probes. The stability of each viral gene in each packaging and producer cell line was examined by exposing the probed filters to phosphorimager plates for 18 hr. Densitometry was performed after scanning with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The band intensities of the early-passage cells were set at 1.0 and the band intensities of the late-passage cells were expressed as fractions thereof. Background was subtracted from all bands analyzed. All lanes were normalized for differences in loading by probing the filters with a 1.1-kb *BglII*-to-*SpeI* fragment of the human factor VIII cDNA to yield a band of 8.5 kb (GenBank accession number M14113).

### *Sodium butyrate treatment*

Lentikat producer clones were plated in doxycycline (0.7  $\mu\text{g/ml}$ ) and at 90% confluency were induced with fresh growth medium minus doxycycline. Three days postinduction duplicate plates of each clone were refed with induction medium with or without 10  $\mu\text{M}$  sodium butyrate. Twenty-four hours later all plates were refed with fresh induction medium minus sodium butyrate. Medium was changed and collections taken on days 5–14. Conditioned medium collections were characterized for p24 and GFP titers.

### *Assays for the detection of recombination*

*Assay for replication-competent retrovirus.* To select the detector cells most permissive for amplification of R8.7 control virus, C8166, CEM-SS, SupT1 and HeLa T4 cell lines were tested. Cells of each cell type ( $2 \times 10^5$ ) were mixed with serially diluted stock of R8.7 virus to a final volume of 1 ml per well in 24-well plates. Infected cultures were incubated for 8–10 days, during which cells were split to avoid overconfluent culturing conditions. Cultures were scored for establishment of productive viral replication by monitoring syncytium formation and production of p24 antigen by enzyme-linked immunosorbent assay (ELISA). In spiking experiments the R8.7 virus stock was diluted into media containing variable amounts of vector generated by transient transfection. Median tissue culture infective dose (TCID<sub>50</sub>) determination was performed by the method of Reed and Muench.

*Assay for envelope-defective recombinant.* 293G detector cells were seeded in 6-well plates at  $5 \times 10^4$  cells per well in medium containing doxycycline 24 hr prior to addition of test samples. Cells were then washed with doxycycline-free medium, and test-conditioned media were added and incubated for 48 hr. Plates were refed with medium containing doxycycline and incubated for 2–3 weeks. Medium was replaced every 2–3 days and undiluted conditioned media were assayed for p24 in duplicate by immunocapture. To monitor the reproducibility of the assay detection limits serial dilutions of positive control R8.7 $\Delta$ Env viral stock were run in parallel with every assay.

### *Treatment/infection of growth-arrested cells*

HeLa cells were seeded at  $10^5$  cells per well of a 6-well plate and 2 hr after plating aphidicolin (15  $\mu\text{g/ml}$ ) was administered to cells. After 20–22 hr cells were infected with serial dilutions of either Lentikat2.54 population B-conditioned medium transferring the pHR2CMVGFP expression cassette or transiently generated MuLV, VSV-G-pseudotyped vector transferring the 43.2GFP expression cassette (Finer *et al.*, 1994) in the presence of aphidicolin (15  $\mu\text{g/ml}$ ) and Polybrene (8  $\mu\text{g/ml}$ ). Twenty-four hours postinfection the cells were rinsed with  $\text{Mg}^{2+}/\text{Ca}^{2+}$ -free phosphate-buffered saline (PBS) and refed with 2 ml of HeLa growth medium. Cells were harvested after 72 hr and assayed for GFP expression by FACScan.

### *Concentration of vector*

Producer clones 14 and 262 were induced and 36 ml of each was harvested, filtered, and centrifuged in a Beckman (Fuller-

ton, CA) SW28 rotor at 19,500 rpm for 140 min ( $50,000 \times g$ ). Supernatant was aspirated, tubes were inverted to drain off residual liquid, and pellets were resuspended in 1% fetal bovine serum (FBS) in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (FBS–PBS). To disrupt particle aggregates each suspension was shaken (lowest speed; table-top vortexer) at 4°C for 2 hr, and then diluted with FBS–PBS and centrifuged as described above. Supernatant was aspirated and the pellets were resuspended in 36  $\mu\text{l}$  of FBS–PBS, shaken for 2 hr at 4°C, and then flash frozen on dry ice and stored at  $-80^\circ\text{C}$ .

### *In vivo gene delivery*

For vector injection, C57BL/6 mice were anesthetized with an intraperitoneal injection of tribromoethanol (1.25%; Sigma, St. Louis, MO) and positioned in a stereotactic frame (David Kopf Instruments, Tujunga, CA), and the skull was exposed by a small incision. One microliter of vector concentrate, previously resuspended by slow vortexing for 1 hr at room temperature and adjusted to a p24 concentration of 100  $\text{ng}/\mu\text{l}$ , or vehicle only, was injected by a Hamilton (Reno, NV) syringe with a 33-gauge blunt-tip needle into the right hippocampus (stereotactic coordinates: AP  $-1.70$ , ML 2, and DV  $-2.5$  from the skull surface) at a rate of 0.2  $\mu\text{l}/\text{min}$ . The needle was left in place for an additional 5 min before slow removal. At 4 and 16 weeks postinjection, mice were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde-0.1 *M* sodium phosphate buffer, pH 7.4. The brain was removed, post-fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer for 8–12 hr at 4°C, followed by a cryoprotective soaking in 30% sucrose at 4°C overnight. Brains were frozen in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrance, CA) on dry ice and 10- $\mu\text{m}$ -thick cryostat sections were cut and scored for GFP expression by direct fluorescence microscopy.

## RESULTS

### *Generation of inducible packaging cell lines*

Our strategy to obtain stable packaging cell lines for HIV-derived vectors was to limit expression of both the packaging and envelope constructs in producer cells to the time of vector collection by a tetracycline-regulated system. We first modified the minimal packaging construct pCMV $\Delta$ R8.74 by replacing the CMV promoter with a minimal CMV promoter linked to tandem repeats of seven tetracycline operator sites (Tet<sup>o</sup>) as described by Gossen *et al.* (1995). We then deleted all HIV-1-derived sequence upstream of *gag*, except for the splice donor site, and optimized the sequence around the *gag* translation initiation site according to Kozak (1997) (Fig. 1). We derived the packaging clones from 293G cells (Ory *et al.*, 1996), which constitutively express the Tet<sup>R</sup>/VP16 *trans*-activator from the CMV promoter and also contain the VSV-G envelope gene under the control of a Tet-responsive promoter element, so that in medium containing tetracycline the VSV-G envelope is not expressed (Tet-off system) (Gossen *et al.*, 1995).

In two separate experiments 293G cells were cotransfected with a 9:1 DNA ratio of pTetCMV $\Delta$ R 8.75 and pZeoSV2 and

TABLE 1. CHARACTERIZATION OF *Lentikat* PACKAGING CLONES FOR VIRUS PRODUCTION

Clone	Nontransduced		Transduced with GFP lenti vector					Infectivity <sup>d</sup>
	Non-induced p24 (ng/ml)	Induced p24 (ng/ml)	Non-induced p24 (ng/ml)	Total induced p24 <sup>a</sup> (ng)	Average induced p24 <sup>b</sup> (ng/ml)	Total titer <sup>c</sup> (TU × 10 <sup>6</sup> /ml)	Average titer <sup>b</sup> (TU × 10 <sup>6</sup> /ml)	
2.8	<LD <sup>e</sup>	24.60	0.01	360	9.0	2.50	0.06	6944
2.54	<LD	3.42	0.14	2958	42.25	27.39	0.39	9257
20.12	<LD	15.65	0.02	1236	30.90	7.75	0.19	6290
20.36	<LD	14.95	<LD	1755	35.10	3.08	0.06	1761
20.76	0.25	12.41	4.34	798	11.40	1.11	0.01	1375
20.81	<LD	19.80	0.01	1333	22.21	1.27	0.02	977
20.85	<LD	20.33	0.02	2109	52.70	20.48	0.51	9716
20.87	<LD	12.65	0.01	2550	36.42	25.23	0.36	9882
20.98	<LD	14.96	0.05	670	9.57	6.39	0.09	9552
20.104	0.2	184.71	0.19	2096	69.86	2.15	0.07	1025
20.116	<LD	29.71	0.34	765	25.48	2.08	0.07	2719
20.253	<LD	14.75	0.23	1706	56.85	8.45	0.28	4971

<sup>a</sup>Sum of p24 collected during induction of 10-cm plate, 10 ml of medium per plate.

<sup>b</sup>Sum of transducing units collected during induction of 10-cm plate, 10 ml of medium per plate.

<sup>c</sup>Average daily value over the 8-day collection.

<sup>d</sup>Total titer/total p24.

<sup>e</sup>LD, limit of detection. The limit of detection of p24 was 0.03 ng/ml.

clones were selected in the presence of zeocin (250 µg/ml) and tetracycline (1 µg/ml). We screened the clones for regulated production of vector particles and transduction efficiency of vector produced after introduction of a GFP transfer construct by infection. The culture medium of 366 clones was screened for expression of the mature Gag protein p24 by immunocapture assay before and after 3 days of induction in tetracycline-medium. Although 43 clones yielded p24 levels greater than 1 ng/ml postinduction, only 34 of these had nondetectable levels of p24 in the noninduced state. While clone 20.104 yielded p24 at 184 ng/ml on induction, most were in the 3 to 30-ng/ml range and 12 clones were chosen to test vector production.

The selected clones were infected twice with transiently produced vector containing a CMV-GFP expression cassette (Fig. 1) at an MOI of 5. Conditioned media from the transduced clones were collected prior to induction and at 24-hr intervals for 8 days after induction in tetracycline-free medium, and assayed for GFP transfer to HeLa cells by FACS and for particle output by p24 ELISA (Table 1). We found that the p24 induction curve was unique for each clone and that significant vector output was maintained over several days. Since there was no collection window that was common to all producer populations, the data in Table 1 are expressed as the total GFP transducing units (TU) and total nanograms of p24 collected per 10-cm plate after induction. The average titer, in TU per milliliter of culture medium, and vector infectivity are also shown. Vector infectivity, defined as the ratio of TU to physical particles measured as nanograms of p24, gives a reliable measure of the potency of a vector preparation. The data in Table 1 show that levels of p24 from the initial screen were not predictive of vector production. This could also be expected from the variability of the production kinetics after withdrawal of tetracycline. Five of the 12 producer populations made better than 10<sup>5</sup>

TU/ml, and three of those populations (2.54, 20.85, and 20.87) had cumulative titers of 2 × 10<sup>7</sup> GFP transducing units and infectivity levels approaching those seen in transient transfections. The good infectivity of vector produced by these clones indicated the viability of our strategy to build stable packaging cells.

In the course of these experiments we noted that the conditioned medium from some producer populations, while apparently allowing for a high level of GFP transfer, could not be titered by serial dilution in the HeLa assay, and that GFP expression in cells transduced by these media decreased over time. This pseudotransduction was possibly dependent on the excess GFP protein expressed in producer cells. In contrast, GFP expression in target cells for the other clones was dependent on the vector dose and remained constant with time. Accordingly, all target cells were maintained in culture for at least 10 days prior to analysis to verify transduction.

#### Generation of vector producer clones

The packaging cell line *Lentikat*2.54 was selected for further characterization to determine its maximum potential for vector output and its stability in long-term culture. As the expression level of the transfer vector RNA is a crucial limiting factor for the titer of vector produced by transient transfection (Dull *et al.*, 1998), we added vector genomes to clone *Lentikat*2.54 by serial rounds of infection with transiently produced GFP vector (VSV-G pseudotyped) for a total of five infections (populations A to E). After each infection a portion of the population was set aside for characterization before the next infection was performed. FACS analysis proved that each round of infection increased the average level of transgene expression and consequently, vector copy number, in each producer cell

(Fig. 2A). Populations from infections B to E were then simultaneously induced and the conditioned media collected for 8 days and assayed as described above (Fig. 2B and C). We found that each new round of infection produced an increase in the titer of output vector. Infection E titer peaked at  $6.6 \times 10^6$ /ml, and remained over  $10^6$ /ml for at least 6 days. Infectivity of vector also increased with each infection and reached levels comparable and superior to those obtained by transient transfection. We also noted that the kinetics of induction of particle release (measured by p24) were faster in the transduced clones, reaching higher maximal levels and yielding a higher total output in the collection window when compared with the values observed for the original nontransduced *Lentikat2.54* cells (Fig. 2C). We ruled out the outbreak of replication-competent and replication-defective recombinants, as shown below. Similar increases in p24 output were observed in other packaging clones after transduction of the transfer vector (see Table 1; and data not shown). The possible reasons for the increased p24 output after vector transduction are discussed below.

We then isolated individual producer clones from population E by limiting dilution for further characterization. We were surprised to find that of the 286 clones assayed, only 66 had induced p24 levels above 1 ng/ml, which corresponded to the 66 clones with HeLa titers of greater than  $10^5$ /ml. Only 23% of the clones had retained p24 expression, which suggested that the population was highly unstable or not clonal. We then subcloned an early passage of *Lentikat2.54* and probed 24 clones for HIV-1 *gag-pol* sequences by polymerase chain reaction (PCR) and found that only 7 of the 24 clones were positive, suggesting that the instability or oligoclonality was a feature of the original *Lentikat2.54* packaging cell line isolate. We derived and characterized new producer cell populations from individual *gag-pol*-positive subclones of *Lentikat2.54* cells (not shown). These experiments reproduced the above-described results in satisfactory yield of infectious vector, and increased titer with increasing round of vector infection into the producer cell population. Ongoing studies will clarify whether the new subclones have gained an advantage in stability.

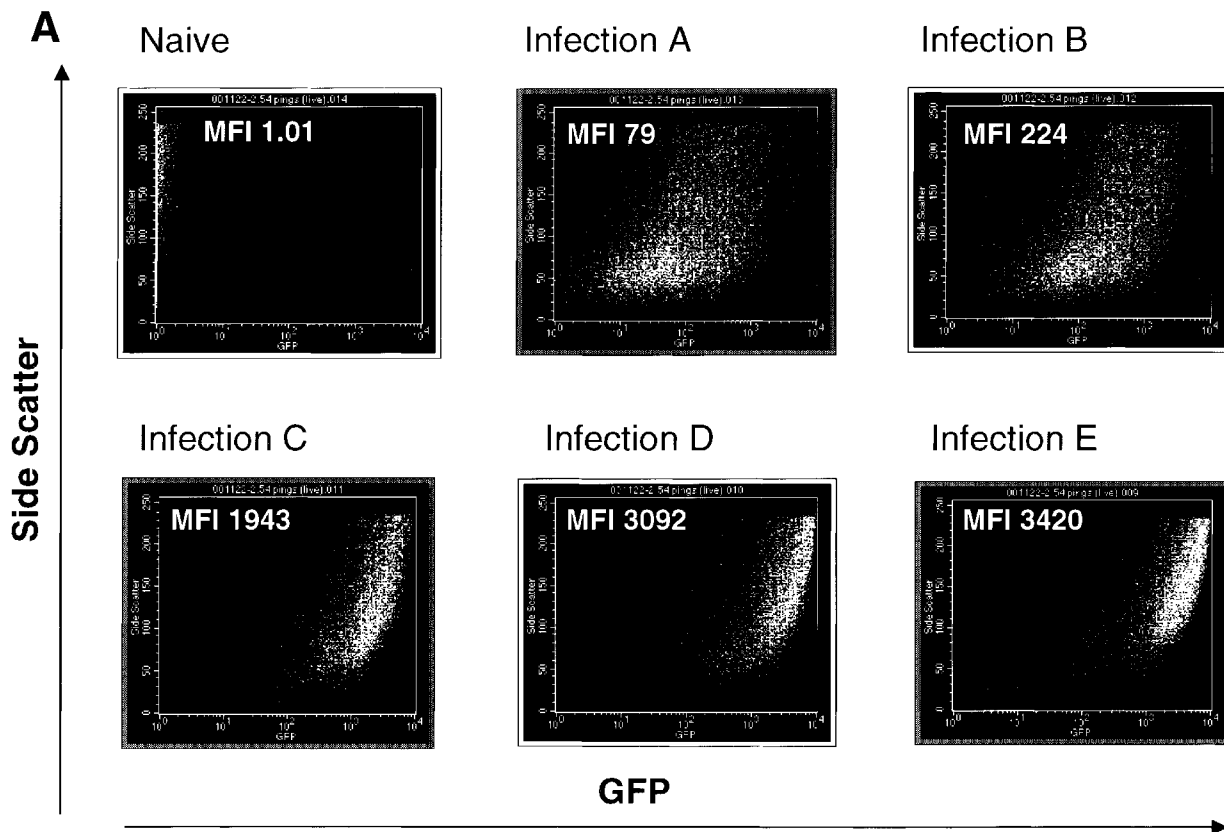
#### Stability in culture of vector producer clones

It was then important to assess the stability of the individual producer clones. The parental *Lentikat2.54*, the vector producer population E, and 10 vector producer clones with induced titers in excess of  $10^6$ /ml were grown in culture for 12 weeks in the presence of doxycycline. At 4-week intervals cells were removed from the cultures and induced in doxycycline-free medium. The use of doxycycline rather than tetracycline delayed the induction windows by several days. Media were collected at 24-hr intervals for at least 9 days and assayed for titer and p24. Total titer and p24 production were calculated for days 5–9 (Fig. 3). As expected from the subcloning experiments described above, producer population E was highly unstable: after 12 weeks in culture the induced particle output and titer had dropped significantly from a total production of 6200 ng of p24 to only 400 ng of p24, and total transducing units had fallen 50-fold from  $5.7 \times 10^7$  to  $1 \times 10^6$  TU. We found, however, that despite the instability of the parental population, 5 of the 10 producer clones were stable (clones 14, 68, 223, 262, and 281) over the 12 weeks of the experiment. Clones 16, 104, 244,

and 260 had lost titer and p24 output by passages 8 and 12, and clone 111 appeared to be in decline. We observed that clones that maintained particle production also maintained titer, and that initial p24 values were in the same range for all stable clones.

To identify possible reasons for the instability observed in half the producer clones we monitored by Southern analysis the maintenance of the different constructs introduced into the packaging and producer cells. Genomic DNA was prepared from each of the parental cell lines 293 and 293G, from *Lentikat2.54*, the vector producer population E, two stable producer clones (14 and 262), and one unstable clone, 244 (Fig. 4). DNA was sampled from noninduced cells after short (week 1) or long-term culture (week 14), and from cells induced for 9 days after long-term culture. All samples were digested with *SstI*, electrophoresed on an agarose gel, Southern blotted onto nylon filters, and scored by radiolabeled probes specific for the sequences in pTetCMV $\Delta$ R8.75, VSV-G, GFP, Tet<sup>R</sup>/VP16 *trans*-activator, and the endogenous factor VIII gene. Restriction by *SstI* was selected because it released well-recognized fragments from the original constructs, and because theoretical recombinants between transfer and packaging vectors would maintain two *SstI* sites and release a fragment of a size similar to that labeled by the *pol* probe from pTetCMV $\Delta$ R8.75 (Fig. 1). In this way we could screen for either the loss or the possible amplification of *gag-pol* genes by a recombinant spreading in the culture. The expected bands were produced for all four types of DNA: VSV-G at 6.6 kb, TetCMV $\Delta$ R8.75 at 4.7 kb, GFP at 1.4 kb, and Tet<sup>R</sup>/VP16 *trans*-activator at 1.2 kb. In addition, by probing downstream of the 3' *SstI* site in TetCMV $\Delta$ R8.75 with the *Bam*HI-*Xho*I probe we were able to demonstrate a single, identical integration event of the packaging vector (data not shown). This 6.1-kb band was common to *Lentikat2.54* and all its descendants. However, the intensity of this common band was less in *Lentikat2.54* than it was in population E and the producer clones. All bands were evaluated by PhosphorImager analysis after normalizing for DNA loading with an 8.5-kb band generated by a probe specific for the endogenous factor VIII gene. For each cell line, the density of each band was compared with the value at passage 0 in order to detect differences in copy number after 14 weeks of continuous culture and/or after 9 days of induction.

In all cases, except that of *Lentikat2.54*, the bands corresponding to the *gag-pol* genes appeared to be stable, and thus there was no detectable evidence of either amplification or loss of *gag-pol* during the course of 14 weeks of culture and induction. We consistently observed that the *gag-pol* copy number for *Lentikat2.54* was less than that of the producer clones and that it varied with long-term culture, which supported our earlier observation that the original isolate of this cell line was not clonal. The GFP transfer vector also appeared to be stably integrated. However, the Southern analysis showed that the VSV-G gene was lost during culture, and this loss correlated with those clones or populations that lost titer during passage. *Lentikat2.54*, population E, and clone 244 all lost VSV-G DNA. Clones 14 and 262 appeared stable in doxycycline, but lost a small fraction of VSV-G DNA during the induction. Instability of VSV-G DNA was also reflected in its different content among the cell populations tested at the initial time of the experiment. An interesting observation is that those cells that lost



**FIG. 2.** Analysis of *Lentikat2.54* packaging clone before and after serial infection with a GFP transfer vector. *Lentikat2.54* was infected serially five times (A to E) with VSV-G-pseudotyped HR2CMVGFP transfer vector. Nontransduced *Lentikat2.54* and transduced populations A–E were analyzed by flow cytometry for GFP expression (MFI, mean fluorescence intensity of GFP) (A), populations B–E were induced in tetracycline-free growth media, and the conditioned media analyzed daily (B) for end-point titer on HeLa cells (TU/ml; columns) and infectivity (TU/ng p24; lines). (C) Total yield of particles, in nanograms of p24, and of infectious vector, in transducing units (TU), and average particle concentration (ng p24/ml), titer (TU/ml), and particle infectivity (TU/ng p24) for the induction period.

VSV-G DNA were also the same that lost p24 expression, although the pTetCMV $\Delta$ R8.75 DNA appeared to be intact by Southern analysis. This could be because the expression plasmid for VSV-G was cotransfected with the plasmid expressing the Tet<sup>R</sup>/VP16 *trans*-activator during generation of 293G cells (Ory *et al.*, 1996). Since the DNA incorporated in stable transfectants is often concatemeric, we postulated that a selective pressure against the VSV-G DNA would also coselect against the *trans*-activator gene and lead to loss of both genes and, consequently, lack of expression of the pTetCMV $\Delta$ R8.75 DNA. Therefore we ran the same samples on a second Southern blot, probed for the Tet<sup>R</sup>/VP16 *trans*-activator, and found a pattern identical to that of VSV-G, thus demonstrating the loss of both VSV-G and *trans*-activator genes in the unstable clones and populations (Fig. 5), leading to the lack of expression of the pTetCMV $\Delta$ R8.75 DNA.

We also tested whether silencing of transcription from the transfected DNA was responsible for some of the decay in performance of the producer clones. It was previously reported that treatment with sodium butyrate reactivated transcription from Tet-responsive promoters after long-term culture of 293 cells expressing a first-generation packaging construct for lentiviral

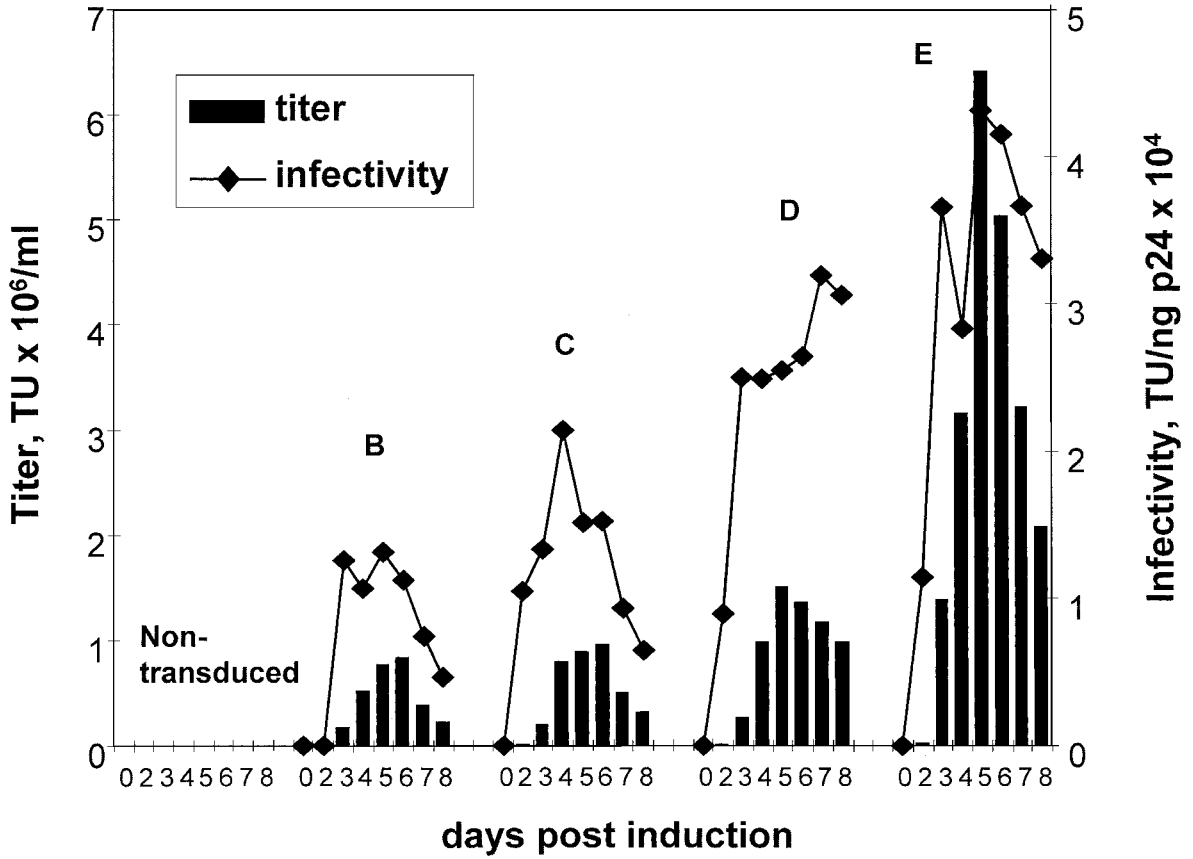
vectors (Kafri *et al.*, 1999). Butyrate treatment did not rescue vector output from our clones that had lost it. In the clones that had maintained output the treatment changed the kinetics of induction by shortening the induction time, but did not improve the total yield of vector (Fig. 6).

These results indicate that clonal variation allows the isolation of stable vector producer clones with satisfactory output from *Lentikat2.54* cells. Moreover, the instability of vector producer clones appeared to be an intrinsic feature of the parental cell line from which they were derived, and may not be necessarily linked to the design or type of vector. Our data also point to possibly more effective strategies for building stable packaging cell lines that aim at improving the stringency of VSV-G expression control, removal of counterselective pressure, and sequential introduction of the different types of DNA.

#### *Screening for recombination of vector and packaging constructs*

To screen our vector producer cells for the absence of replication-competent recombinants (RCRs) we needed to establish an assay in which we could incubate aliquots of the conditioned

**B**

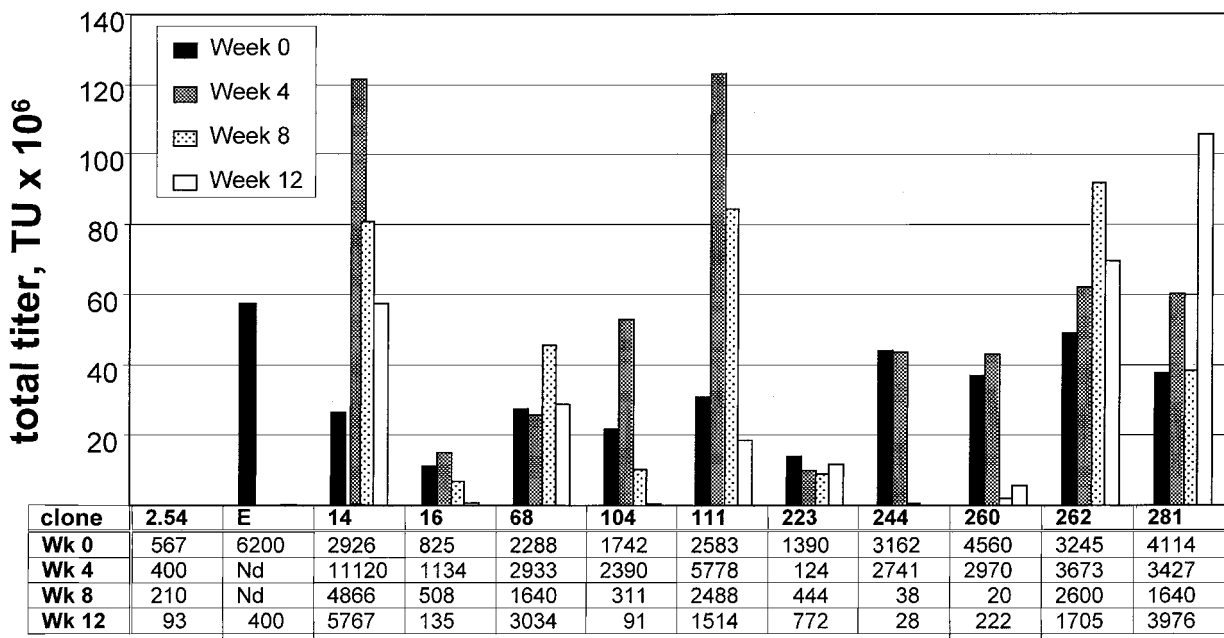


**C**

Infection	Non-induced		Induced				
	P24 ng/ml	Titer <10 <sup>4</sup> TU/ml	Total ng p24 (1)	Average induced p24 ng/ml	Total titer, TU x 10 <sup>6</sup> (2)	Average titer, TU x 10 <sup>6</sup> /ml	Infectivity (3)
Non-transduced	0.01		729	12.15			
B	0.12	0	2958	49.20	27.39	0.46	9257
C	0.57	0	2777	46.29	36.67	0.61	13201
D	0.00	0	2295	38.24	62.65	1.04	27261
E	0.27	0	5694	94.91	212.88	3.55	37351

- (1) Sum of p24 collected during induction of 10cm plate, 10mls media per plate.
- (2) Sum of transducing units collected during induction of 10cm plate, 10mls media per plate.
- (3) Total titer / total p24

FIG. 2. Continued.



### total ng induced p24

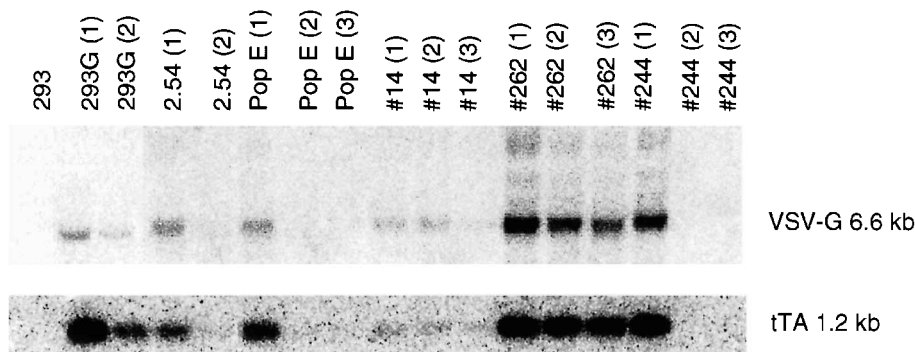
**FIG. 3.** Characterization of *Lentikat2.54* producer clones for stability of vector output with time. Ten producer clones of GFP vector (indicated by the clone number) and 2 controls (*Lentikat2.54* and transduced parental population E) were induced at 4-week intervals by removal of doxycycline. Medium was collected at 24-hr intervals and analyzed for end-point transduction of GFP into HeLa cells (bar graph) and for the content of p24 (chart). Data are expressed as the total yield of p24 and transducing units for the induction period of each clone. Nd, Not done.

medium with a cell line shown to be highly permissive to HIV-1 infection and then monitor p24 released in the culture supernatant. Cultures would be grown for a minimum of 3 weeks for clearance of the input p24 and amplification of any RCR. For a positive control RCR that mimicked one derived from the minimal packaging construct introduced into *Lentikat2.54* cells, we deleted the four accessory genes of an infectious clone of HIV-1, R8 (Gallay *et al.*, 1995), and cotransfected this plasmid with the expression construct for VSV-G into 293T cells to match the conditions of vector production for the initial infection. After the first round of infection this attenuated *vpr<sup>-</sup> vpu<sup>-</sup> vif<sup>-</sup> nef<sup>-</sup>* HIV-1 clone, referred to as R8.7, replicated by its own envelope. This approach was dictated by the biosafety concerns of generating a genotypic VSV-G-pseudotyped HIV-1. On the other hand, the adoption of a multiply attenuated packaging construct for vector production reduced correspondingly the biohazard of any possible RCR, including that represented by growing the positive RCR control even when phenotypically pseudotyped by VSV-G. The R8.7 virus grew efficiently in several tested cell lines, and as expected from its multiply deleted genome (Cullen, 1998), it did not grow detectably in primary human lymphocytes (data not shown).

Among the target cell lines tested, the human T cell line C8166 supported virus replication most efficiently as evidenced

by the fact that the minimal infectious dose required to establish productive infection of the culture was at least one order of magnitude lower than for any other cell line tested (data not shown). Consistent with our previous observation that VSV-G-pseudotyped vector particles were found to be significantly more infectious than their counterparts incorporating the gp120 glycoprotein of HIV-1 (Bukovsky *et al.*, 1999), we found that the minimal viral infectious dose was lowered another 1000-fold when detector cell cultures were infected with R8.7 particles pseudotyped with VSV-G (data not shown). The TCID<sub>50</sub> of C8166 cells by VSV-G-pseudotyped R8.7 virus was 1 fg of p24 equivalent of virus, as determined by the method of Reed and Muench (data not shown). Assuming that each virion contains approximately 2000 molecules of p24 capsid protein and that these molecules are assembled in particles of relatively homogeneous size, this would correspond to approximately 12 physical particles, indicating the high sensitivity of this assay. In spiking experiments we also established that the addition of a nonreplicating control vector at a maximum MOI of 5–10 did not increase the median infective dose (ID<sub>50</sub>) of the positive control (data not shown). Thus we used these experimental conditions to screen vector batches for RCR. To date we have not found a positive sample (more than 60 samples from more than 30 producer cell cultures were tested one or two times).





**FIG. 5.** Concomitant loss of VSV-G and Tet<sup>R</sup>/VP16 *trans*-activator DNAs with long-term culture of vector packaging and producer cells, leading to the lack of expression of the packaging construct. Southern analysis of *Sst*I-digested DNA from the indicated cells grown in culture for 1 week (1), 14 weeks (2), and 14 weeks plus 1 week of induction (3). The upper panel was probed for VSV-G sequences, and the lower panel for the Tet<sup>R</sup>/VP16 *trans*-activator. Band sizes are indicated.

after 16 days. The limit of detection of p24 by the immunocapture assay was 2.5 pg/ml. To reproduce more accurately the actual conditions encountered in an assay, dilutions of the R8.7ΔEnv recombinant were added in the presence or absence of excess vector generated by transient transfection (Fig. 7B). This did not alter the sensitivity of the assay. The conditioned media from *Lentikat2.54* and all 10 producer clones induced at passage 12, were then examined for contamination by recombinants expressing the HIV-1 *gag-pol* genes. In all cases we observed a steady decline in p24 levels in the culture. Figure 7C shows a representative panel of clones and the decline in p24 levels from day 5 to day 16 of the assay. Considering the amount of vector tested and the limit of detection of the assay, we can conclude that the vector batches tested contained less than 1 particle in the  $1.6 \times 10^7$  tested containing a recombinant between transfer and packaging construct capable of transferring *gag-pol* packaging functions to target cells.

#### Performance of vector from stable producer cells

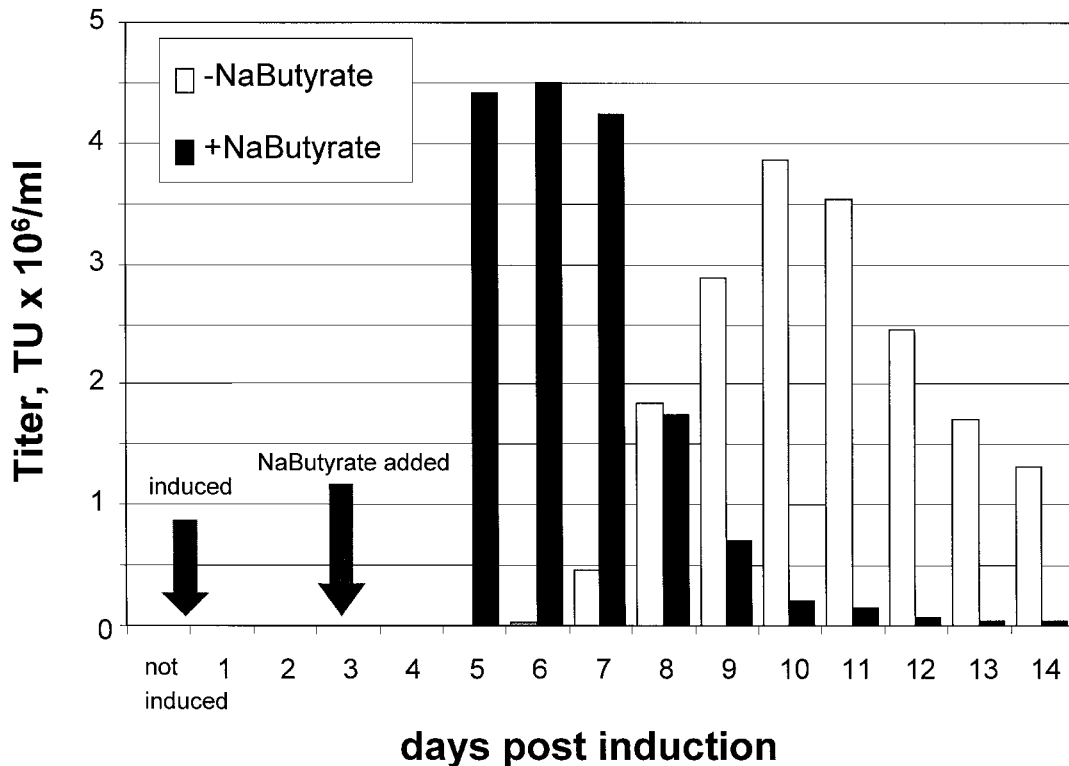
Having established the safety of the stable packaging system we wanted to functionally characterize the output vector to verify that it performed equivalently to that produced by transient transfection. Conditioned media from *Lentikat2.54* producer population B were evaluated for their ability to transduce growth-arrested cells (Naldini *et al.*, 1996a). HeLa cells were treated with aphidicolin (15 μg/ml) 24 hr prior to infection with population B conditioned medium or a VSV-G-pseudotyped MuLV vector delivering GFP. Growing HeLa cells were readily transduced with vectors made either by a lentiviral or oncoretroviral core, whereas the aphidicolin-treated cells were transduced only by the lentiviral vector in the population B conditioned medium. In addition, we demonstrated that the conditioned media from producer clones 14 and 262 could be efficiently concentrated to obtain vector stocks displaying high titer and transduction frequency. Clone 14 and 262 conditioned media were concentrated 1000-fold to titers reaching  $1 \times 10^9$  TU/ml, with an average recovery of 80%. When p24-matched

doses of vector before and after concentration were used to transduce HeLa cells we observed similarly high transduction frequencies by FACS analysis, indicating that the concentration step increased the transduction efficiency without concentrating contaminants that interfere with transduction (data not shown).

The concentrated vector stock from clone 14 was tested for efficient delivery *in vivo* of the GFP expression cassette. One microliter of the stock was injected into the brain of C57BL/6 mice, into the right hippocampus, under stereotactical guidance. Four and 16 weeks after the injection, mice were killed and 10-μm-thick cryostat sections were cut from the brain and analyzed for GFP expression by direct fluorescence microscopy. As shown in Fig. 8, remarkable levels of transduction of cells with the morphological features of hippocampal neurons were demonstrated in the injected side of the brain, as previously shown for vector produced by transient transfection (Naldini *et al.*, 1996b).

## DISCUSSION

We have successfully generated and characterized a stable packaging cell line for HIV-1-based vectors. The minimal HIV-derived packaging construct introduced into the cells allowed for safe production of vector. By serial infection of the transfer construct, producer clones could be obtained yielding vectors with titer, infectivity, and performance matching or exceeding those of vector obtained by transient transfection. The titration of vector genomes by serial transduction of the packaging cell line indicated that, similar to what was observed in transient transfection, the level of expression of vector RNA in producer cells is a major rate-limiting factor for the transduction efficiency of the output vector. Furthermore, the achievement of optimal vector infectivity indicates that the packaging cell line did not produce excess defective particles. This was shown by the nearly quantitative concentration of the vector to high titers allowing efficient gene transfer *in vivo*, such as into



**FIG. 6.** Sodium butyrate treatment of *Lentikat* vector producer clone 14. Cells were induced on day 1 by refeeding with doxycycline-free medium, and treated with (solid columns) or without (open columns) 10  $\mu$ M sodium butyrate for 24 hr on day 3. Conditioned media were analyzed daily for end-point transduction of GFP into HeLa cells.

hippocampal neurons after injection of the vector into the mouse brain.

The packaging cell line was derived from 293G cells (Ory *et al.*, 1996). Some of the advantages of the 293 host cell are the human origin, good transfectability and adaptation to growth in suspension, a safe track record in the production of retroviral vectors (Burns *et al.*, 1993; Pear *et al.*, 1993; Finer *et al.*, 1994; Davis *et al.*, 1997; Yang *et al.*, 1999), and moderate dependence on the accessory genes *vpu*, *vif*, and *nef* of HIV-1 to generate infectious virus.

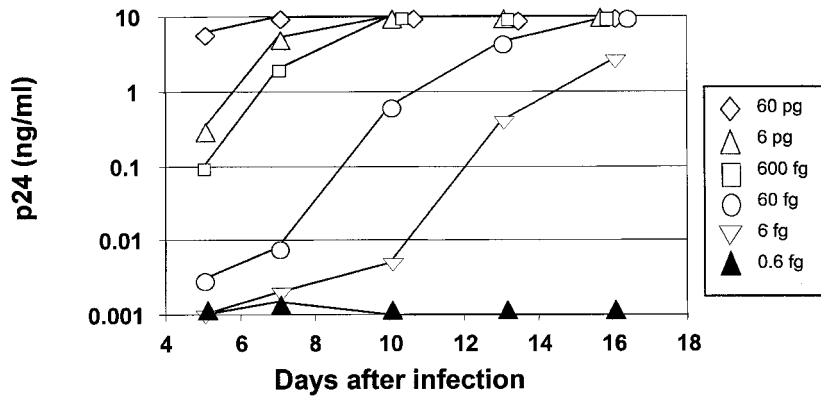
An unexpected observation was that the kinetics of induction and the total output of vector particles increased after transduction of the vector construct into the packaging cells. There are several possible explanations for this phenomenon. We ruled out that the observed increases were due to an amplification of the packaging DNA in the producer population, as it could occur by the outbreak and spread of a recombinant between the transfer and packaging construct. It is possible that the RNA-binding activity of Gag proteins responsible for encapsidation of the viral genome interferes with efficient translation, both *in cis* and *in trans*. A residual interaction could occur between the Gag protein and its mRNA in packaging cells, particularly in the absence of RNA carrying an intact encapsidation signal. When the transfer vector RNA is titered up into packaging cells by serial transduction it may progressively out-

compete the Gag mRNA from binding sites in the Gag proteins, and increase its translational output (A. Bukovsky and L. Naldini, unpublished observation).

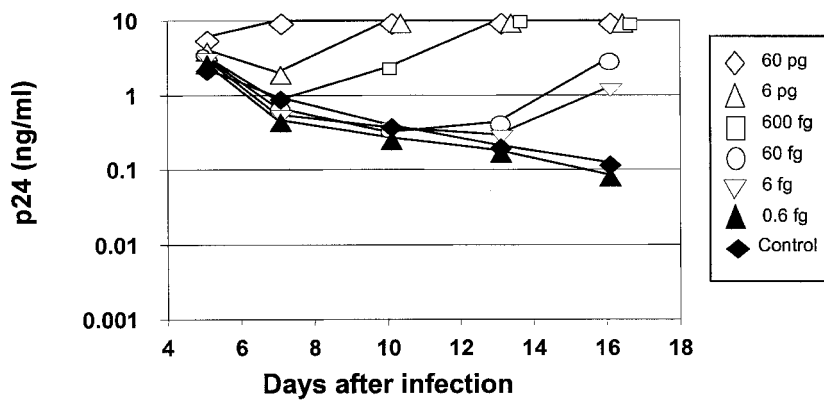
An important safety issue was raised by the use of the VSV-G envelope in stable producers. We showed that cells expressing high levels of VSV-G do not exhibit interference with superinfection, that is, they could be infected efficiently by VSV-G-pseudotyped vector or virus. Self-infection of a producer clone is one undesirable feature that may compromise its stability and increase the risk of generation of recombinants even by such unlikely mechanisms as gene capture. The vector producer system that we have developed has a built-in mechanism that limits self-infection. Expression of the packaging and envelope functions to adequate levels for release of infectious particles is limited to a short window of time after induction. While we could not detect infectious vector in the noninduced state, we observed, however, a basal level of expression of the VSV-G protein. This "leaky" expression appeared significant enough to induce a selective pressure against the maintenance of the VSV-G DNA in long-term culture, and it was a major factor in limiting the stability of the cell line. It thus appears that a more stringent control of VSV-G expression would improve the reliability and performance of stable packaging cell lines.

The packaging cell line and all vector producer clones de-

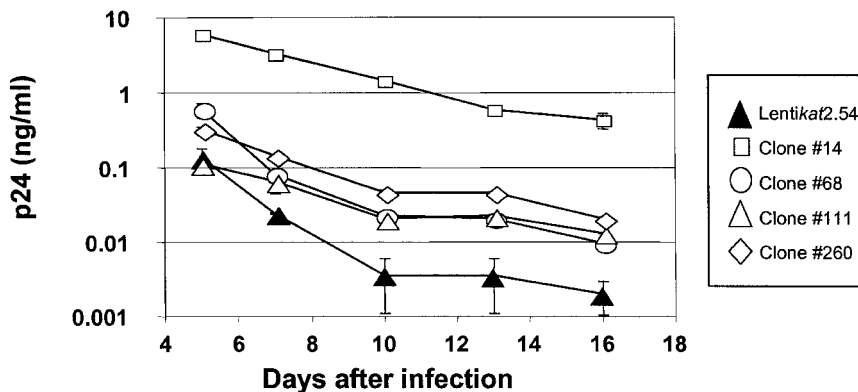
### A. R8.7 $\Delta$ Env



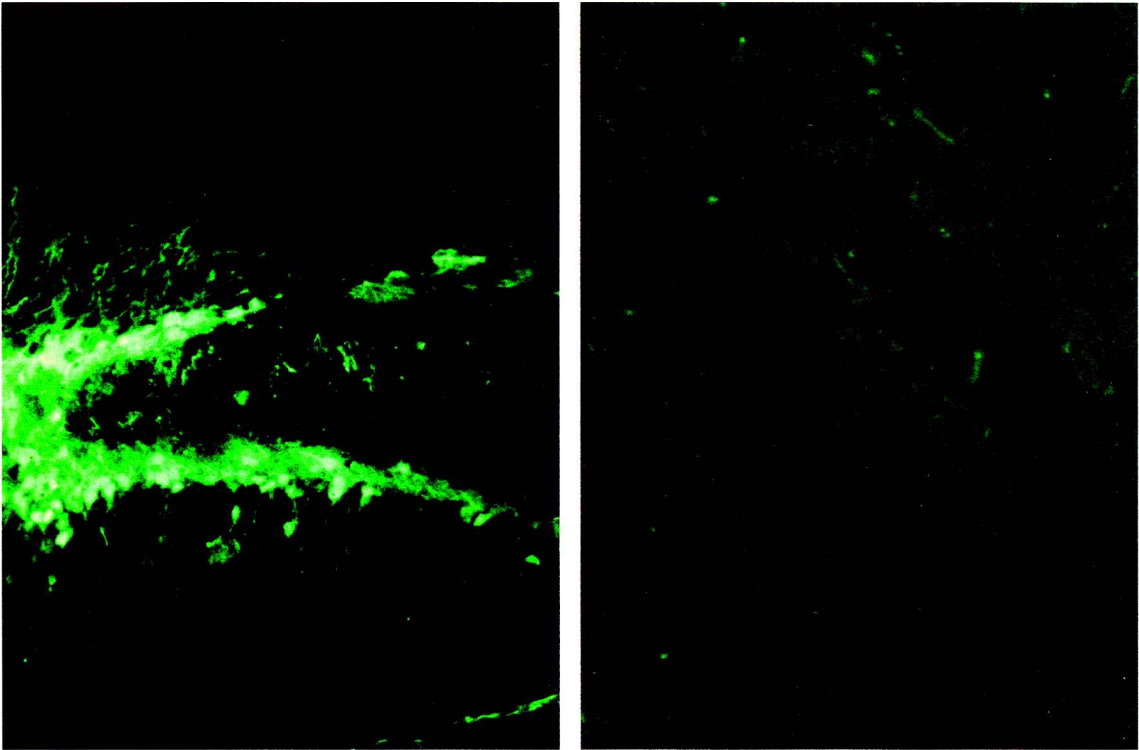
### B. R8.7 $\Delta$ Env + Control Vector



### C. Vector producing clones



**FIG. 7.** Assay for replication-defective recombinants that transfer HIV-1 *gag-pol* packaging functions to target cells. 293G reporter cells were induced and exposed to control and test vector samples from the indicated *Lentikat2.54* producer clones. Conditioned media from the assay were monitored for p24 levels at designated intervals. Assay sensitivity: (A) reporter cells were infected with the indicated serial dilutions of the positive control vector R8.7 $\Delta$ Env, VSV-G pseudotyped, as described in Materials and Methods. The envelope-defective recombinant was amplified efficiently, starting from the inoculum of 6 fg of p24. (B) The same dilutions of VSV-G-pseudotyped R8.7 $\Delta$ Env shown in (A) were spiked into lentiviral GFP vector (200 ng of p24) produced by transient transfection. The assay sensitivity was unchanged in the presence of excess vector particles. Note that the slow clearance of input p24 antigen does not prevent detection of amplifying spiked recombinant. (C) Test of vector samples. Reporter cells were exposed to 1 ml of the conditioned medium from the indicated late-passage, induced *Lentikat2.54* and producer clones. No p24 amplification was observed. The limit of detection of p24 by the immunocapture assay was 2.5 pg/ml.



**FIG. 8.** Concentrated vector stock from *Lentikat* cells efficiently transduces cells with the morphological features of hippocampal neurons after direct delivery into the mouse brain. Cryostatic sections from the hippocampus of a GFP vector-injected (*left*) and control-injected (*right*) mouse were examined 4 months after injection for GFP fluorescence. Similar results were obtained in four mice examined 4 and 16 weeks postinjection.

scribed here were shown to be free from replication-competent recombinants by a highly sensitive assay. However, the outbreak of a replication-competent recombinant is a remote event given the hybrid composition and the split design of the lentiviral vector system. While this is a crucial safety feature of this type of vector, it also makes it difficult to compare the quality of different vector batches and different vector production systems. We thus developed an assay to monitor the appearance of recombinants between packaging and vector constructs that transfer the viral *gag-pol* genes without having to rely on the extremely unlikely outcome of an RCR outbreak. Such recombinants are replication defective but they may be propagated in vector producer cells that do not exhibit envelope-dependent interference with superinfection as is the case with VSV-G. The recombinants would interfere with transduction of the vector construct because they would compete for encapsidation by the viral particles. Moreover, they may be harmful to a vector recipient as they could transfer vector-packaging functions, and may cause toxicity or immune reactions. Most importantly, the possible amplification of such a recombinant in a producer cell line increases the risk of additional recombination events eventually leading to generation of a replication-competent retrovirus. Sensitive detection of enve-

lope-defective recombinants expressing the viral *gag-pol* genes is thus crucial to validate and maintain the performance of a vector producer system, and to prove the purity and safety of a vector batch. The packaging cell line described here and all vector producer clones derived from it did not yield any detectable recombinants of such a type.

An additional improvement in the safety of vector production could be achieved by a self-inactivating (SIN) transfer vector (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998; Iwakuma *et al.*, 1999). This would not only reduce the risks associated with reinfection of the producer system, but also enhance the safety of the output vector, alleviating the risks of recombination or mobilization in the recipient (Bukovsky *et al.*, 1999). However, as SIN vectors cannot be infected to generate producer cells, obtaining a good producer clone may require the screening of a large population of transfectants, or the adoption of other strategies such as targeted recombination of a preselected vector integration site (Vanin *et al.*, 1997), or the use of self-maintaining episomal constructs (Kinsella and Nolan, 1996).

The second-generation packaging clone described in this work, and the assays developed for its characterization, should enable scaling-up and advancing the applications of gene transfer mediated by lentiviral vectors.

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Received for publication April 7, 2000; accepted after revision March 13, 2001.