

A *Trans*-Lentiviral Packaging Cell Line for High-Titer Conditional Self-Inactivating HIV-1 Vectors

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Lentiviral vector safety has been the impetus underlying the progress in packaging cell line development. The prospects of generating replication-competent lentiviruses (RCLs) and the potential for vector mobilization continue to be the driving force for the advancement of packaging cell lines. We have exploited the *trans*-lentiviral packaging system to develop the SODk3 packaging cell line for the generation of conditional self-inactivating (cSIN) vectors. Separating the *gag-pol* genome into two distinct expression cassettes (*gag-pro* and *vpr-RT-IN*) may reduce the potential for RCL formation, while concurrently employing cSIN vectors supports retention of the SIN phenotype in target cells and alleviates technical constraints associated with generating producer cell lines. Through development of the SODk3 packaging cell line we determined that the ratio of Gag/Pol in vector particles may be used as an indicator for packaging cell clones that yield high vector titers. Conditional SIN vector titers (1×10^7 TU/ml) were augmented through clonal selection. Distinct producer cell clones revealed a parallel between vector titer and transgene expression levels. We exploited this observation to demonstrate that incorporation of an internal ribosome entry site between the GFP marker and a relevant transgene affords efficient selection of high-titer producer cell lines. Furthermore, cSIN vectors generated from SODk3 packaging cells imparted efficient transduction of primary human fibroblasts, an indication of the future applicability of the SODk3 packaging cell line.

Key Words: lentivirus, vector, packaging cell, *trans*-lentiviral, conditional SIN vector, inducible gene expression, tetracycline, gene therapy

INTRODUCTION

The advent of stable packaging cell lines has provided an avenue by which to generate large quantities of vector in a safe and reproducible manner for gene delivery in animal models, as well as clinical applications. Concerns regarding the potential to generate replication-competent lentiviruses (RCLs) have led to recent improvements in packaging cell lines through codon optimization of the stably incorporated *gag-pol* genes to reduce homology between the packaging and the vector genetic elements [1,2]. Although codon optimization has been effective at limiting the risk, nonhomologous recombination within the poly(A) tracts of the packaging mRNA and vector mRNA during reverse transcription in target cells may facilitate formation of vector particles, which lack an envelope, in transduced target cells [3]. Furthermore, HIV-1 virus deficient in envelope protein has been shown to infect CD4⁻ epithelial cells in an envelope-independent manner [4]. Together, these data indicate that *de novo* generated *env*⁻ HIV-1-derived vector particles pose a risk

for transfer of genetic cargo that encodes viral genes in the absence of envelope.

Wu *et al.* demonstrated that recombination in target cells could be curtailed by using a *trans*-lentiviral vector packaging system in which the *gag-pol* genes were separated onto two distinct expression cassettes: one expressing the Gag/Gag-Pro products and the other expressing the Vpr-RT-IN products from the *pol* gene [3]. Exploiting the capacity of the Vpr protein to interact with the p6 product of the *gag* gene imparted the ability to achieve efficient packaging, and ensuing functional activity, of the reverse transcriptase and integrase proteins in vector particles [5–8]. We have combined the potential safety attributes of a *trans*-lentiviral vector packaging system and the use of conditional SIN (cSIN) vectors into a single stable packaging cell line to yield high vector titers.

Many of the current HIV-1 vector packaging cell lines have been placed under the control of a tetracycline (tet)-inducible system to minimize the cytotoxic and cytostatic effects associated with constitutive expression of vesicular

stomatitis virus G protein (VSV-G), protease, and Vpr proteins [9–11]. These packaging cells contain stable, constitutively expressed tetracycline transactivator (tTA) that is maintained in the “off state” in the presence of doxycycline (Dox; a tetracycline analog) [12]. Upon removal of Dox the tTA interacts with a minimal promoter comprising a heptameric repeat of tetracycline response elements driving expression of the vector packaging and envelope genes. Nearly all the existing tet-dependent HIV-1 vector packaging cell lines have been assessed by transduction with non-SIN, Tat-dependent [1,2,13], or CMV-based Tat-independent vectors [14]. Vectors produced from these packaging cell lines pose a safety hazard in that they may be mobilized in target cells upon inadvertent transfer of the *gag-pol* and *env* genes to the target cells or following superinfection with wild-type HIV-1 [15,16]. cSIN vectors harboring a minimal tet-inducible promoter (TRE) in the U3 region impart maintenance of the SIN phenotype in target cells, thereby obviating vector mobilization [17].

We determined that successful development of the SODk3 *trans*-lentiviral vector packaging system depends upon the levels of Gag, Pol, and vector mRNA, as well as the Gag/Pol ratio. Through clonal selection we demonstrate that producer cell clones showing high levels of transgene expression yield an ancillary increase in vector titer and that this can be exploited to isolate producer cells yielding high titers of vector particles that contain a gene of research or clinical relevance.

RESULTS AND DISCUSSION

Establishing a Tet-Dependent VSV-G Cell Line

We initiated generation of the *trans*-lentiviral vector packaging cell line with the SODk0 cell line, which are 293 cells that contain a stably integrated tTA expressed from the constitutively active CMV promoter [13]. We introduced the VSV-G protein stably into the SODk0 cells under the control of the TRE bidirectional promoter, pBIGFVG (construct is described in [13]), to preclude cytotoxicity associated with constitutive expression of VSV-G. GFP is expressed in the opposite orientation to monitor the Dox-dependent control of the system.

We stably cotransfected SODk0 cells, in the presence of 1 μ g/ml Dox, with linearized pBIGFVG and pPUR (Clontech) to facilitate screening of clones by puromycin selection. We isolated three clones following selection in puromycin. All three were positive for GFP expression following withdrawal of Dox; however, only a single clone (PVG3) was viable upon subsequent growth in tissue culture. Presumably, the low efficiency is a consequence of the cytotoxicity associated with the VSV-G expression [11]. Even though difficulty in obtaining stable VSV-G cell lines is not unusual, a larger population of clones is achievable [13]. Nonetheless, as described below we found the PVG3 clone to be suitable for further development of the *trans*-lentiviral packaging cell line. Ensuing transient

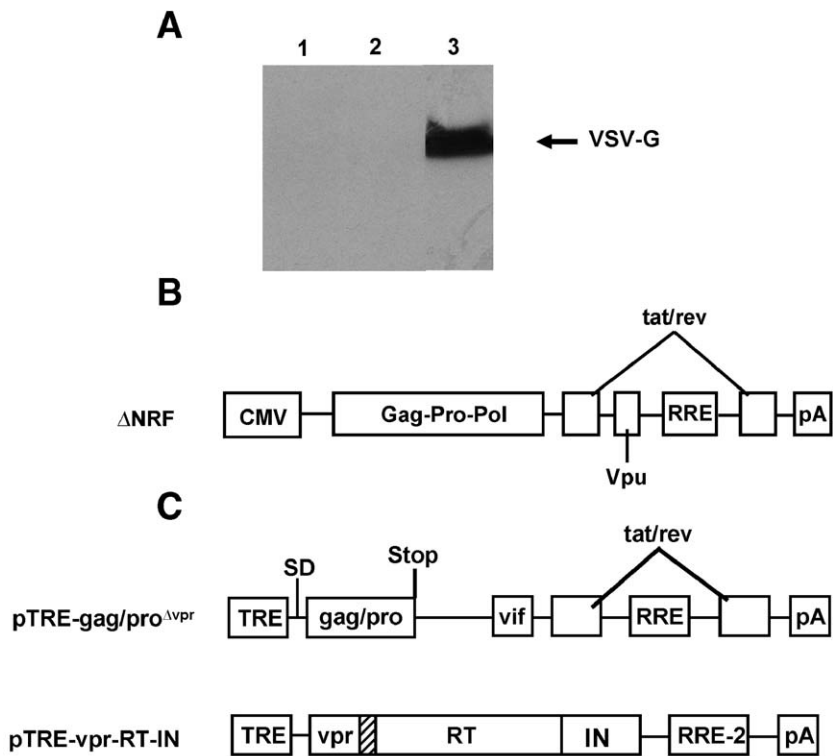
cotransfection of PVG3 cells with pTK113 (vector construct) and Δ NRF (packaging construct, Fig. 1B) produced vTK113 vector with titers of 2.1×10^6 TU/ml, confirming the capacity of clonal isolate PVG3 to engender viable vector upon induction. Expression of VSV-G protein in the PVG3 clone was tightly regulated by Dox as shown by Western blot analysis (Fig. 1A). We detected VSV-G protein only following withdrawal of Dox. Confident that tight control of VSV-G expression could be achieved, we commenced with generation of the packaging cell line by introducing the *trans*-lentiviral packaging system into PVG3.

Generation of the *trans*-Lentiviral Vector Packaging Cell Line

The *trans*-lentiviral packaging system comprises two tet-dependent expression cassettes: (i) the TRE-*gag/pro* ^{Δ vpr} cassette and (ii) the TRE-*vpr-RT-IN* cassette (Fig. 1C). Except for replacement of the CMV promoter with the TRE in each of the cassettes, the system is similar to that described previously [3]. Placing the system under tet-dependent regulation garners the advantage of minimizing putative cytotoxicity associated with the Vpr and Pro proteins [9,10]. The *vpr* and *tat* genes are typically removed from many third-generation packaging constructs. As discussed above, the Vpr protein facilitates packaging of the RT and IN proteins into a vector particle, and Tat protein provides a level of flexibility in the packaging cell line by retaining the option to use Tat-dependent vectors for the subsequent generation of producer cell lines, even though this is not our intended purpose for generating the *trans*-lentiviral vector packaging cell line.

We stably cotransfected the PVG3 cells, in the presence of Dox, with each of the linearized plasmids harboring either the TRE-*gag/pro* ^{Δ vpr} or the TRE-*vpr-RT-IN* expression cassette. The pTRE-*gag/pro* ^{Δ vpr} plasmid contains the neomycin resistance marker to facilitate the isolation of cell clones under neomycin selection. We isolated at least 50 cell clones under neomycin selection in the presence of Dox and expanded them. The *trans*-lentiviral packaging system presented us with the unique problem of determining which of the 50 clones would yield the greatest titers. Separating the RT/IN genes onto a cassette distinct from the *gag-pro* genes obviates the use of the inherent frameshift control that naturally maintains the appropriate ratio of intracellular Gag/Gag-Pro-Pol protein levels [18]. Until now, all HIV-1 packaging cell lines comprised a linear *gag-pol* packaging genome that utilizes the innate frameshift mechanism to preserve appropriate levels of the Gag and Gag/Pol gene products [1,2,13,14,19–23]. Similar to the traditional packaging systems, the frameshift is maintained within the Gag/Pro gene product of the *trans*-lentiviral packaging cell line described here; however, the Pol gene products are expressed independent of the inherent frameshift mechanism. Thus, the best packaging cell clones could not be

FIG. 1. Establishing the *trans*-lentiviral packaging cell line. The SODk0 cell line was transfected with pBIGFVG to create the PVG3 cell line, which contains a Dox-inducible VSV-G envelope protein. (A) Dox-dependent regulation of VSV-G expression was demonstrated by standard SDS-PAGE and Western blot analysis in the presence (lane 2) or absence (lane 3) of Dox. SODk0 cells served as a negative control (lane 1). (B) The Δ NRF packaging construct comprises the *gag*, *pol*, *vpu*, *rev*, and *tat* genes expressed from the CMV promoter. (C) The *trans*-lentiviral packaging expression cassettes that were introduced into the PVG3 cell line are depicted. Both pTRE-gag/pro ^{Δ vpr} and pTRE-vpr-RT-IN comprise a minimal TTA-dependent promoter (TRE) driving expression of each respective cassette. pTRE-gag/pro ^{Δ vpr} consists of the *gag-pro* genes harboring the frameshift, *vif*, *tat-rev*, the HIV-1 RRE, and the poly(A) tail. pTRE-vpr-RT-IN contains the *vpr* gene, a protease recognition site (hatch mark), the reverse transcriptase-integrase genes, the HIV-2 RRE, and the poly(A) tail.



identified solely as a result of the Gag or Pol expression levels by Western blot, ELISA, or RT assay. Therefore, selecting a packaging cell clone based simply on the levels of Gag-Pro and/or RT-IN products may not result in the production of functional vector.

Considering this added complication the most relevant method for screening packaging cell clones was to assess 10 of the clones randomly for vector yield following transduction of each clone with a cSIN vector. We transduced each clone with the cSIN vector TK136 [17]. After induction we harvested the vectors from each of the

packaging cell clones and evaluated the titers on 293T cells (Table 1). Only a small number of packaging cell clones (1, 7, 9, and 20) produced discernable titers, yet even these titers differed between the cell clones. The disparate vector titers could be attributed to various factors including the (i) levels of Gag-related proteins in vector particles, (ii) levels of Pol-related proteins in vector particles, (iii) ratios of Gag/Pol proteins, and (iv) availability of full-length vector mRNA for packaging. We analyzed the levels of Gag and Pol proteins in vector particles from each clone by p24 ELISA and reverse

TABLE 1: Assessment of *trans*-lentiviral packaging cell clones^a

Packaging cell clone	Titer (TU/ml) ^b	p24 (ng/ml) ^c	RT (ng/ml) ^d	p24/RT	Specific activity (TU/ng p24) ^e
Clone 1	2.3×10^6	1836	243	8	1253
Clone 6	0	0	0	0	NA
Clone 7	2.4×10^6	4379	385	11	548
Clone 9	1.3×10^4	1702	94	18	8
Clone 10	0	204	0	0	N/A
Clone 20	4.5×10^5	137	11	13	3285
Clone 23	0	0	0	0	N/A
Clone 29	0	241	0	0	N/A
Clone 40	0	0	0	0	N/A
Clone 47	0	189	0	0	N/A

^a Each of the cell clones was transduced with the vTK136 cSIN vector. Vector was produced by withdrawal of Dox and addition of 5 mM sodium butyrate.

^b Titer was determined by serial dilution on 293T cells and scoring for positive cells by fluorescence microscopy.

^c p24 was determined by ELISA.

^d RT was determined by a functional chemiluminescence assay.

^e N/A indicates that the specific activity could not be determined from conditioned media lacking measurable levels of RT and/or p24.

transcriptase assay, respectively (Table 1). In all cases in which the vector titers were not measurable the amount of Pol protein in the vector particle was not detectable, even in the instances in which Gag protein was detectable. Clones 9 (1.3×10^4 TU/ml), 20 (4.5×10^5 TU/ml), 1 (2.3×10^6 TU/ml), and 7 (2.4×10^6 TU/ml) exhibit a gradual increase in vector titers; however, there is not a direct correlation with the Gag or Pol protein levels in the vector particles. Apparently, the ratio of Gag/Pol proteins is more a measure of functional vector particles than are the amounts of Gag or Pol proteins present in the particles (Table 1). As packaging cell clones 9, 20, 1, and 7 yield vector with increasing titer there was an ancillary decrease in the Gag/Pol ratios. Most dramatic was the vector from clone 9, which differs from clone 1 vector only by the levels of Pol, and the Gag/Pol ratio, yet there is nearly a 200-fold difference in the vector titers. Presumably, effective vector titers are more a consequence of correct Gag/Pol ratios, rather than the levels of Gag or Pol proteins in the vector particles. It is possible, though, that the titer differences are a consequence of differences in the amount of full-length vector mRNA that is packaged into the particles, as we demonstrate below using the SODk3/TK731 producer cell line. Moreover, the vector titers from clone 1 are nearly equivalent to those from clone 7; however, due to the high amount of Gag from clone 7 the specific activity is much lower than that of clone 1. Clone 1, hereafter referred to as SODk3, is more amenable to subsequent manipulation since the specific activity indicates that fewer empty vector particles are being generated.

We evaluated the SODk3 packaging cell line for Dox-dependent expression of the TRE-gag/pro^{Δvpr} and TRE-

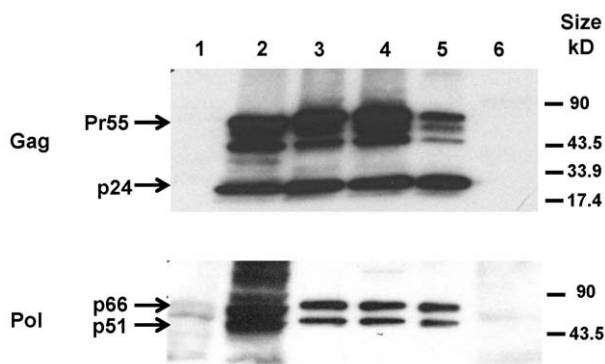


FIG. 2. Dox-dependent regulation of the SODk3 packaging cell line. Extracts from SODk3 *trans*-lentiviral packaging cells transduced with vTK136 were examined for control of Gag and Pol expression by SDS-PAGE and Western blot analysis in the presence (lane 1) and absence (lane 2) of Dox. Vector TK136 generated either from the induced SODk3/TK136 producer cell line (lane 3) or by transient cotransfection of SODk0 cells with pTRE-gag/pro^{Δvpr}, pTRE-vpr-RT-IN, pBIGFVG, and pTK136 (lane 4) was compared to vTK136 packaged with ΔNRF via transient transfection of 293T cells (lane 5). 293T cells served as a negative control (lane 6). The following proteins are indicated: Pr55 (precursor) and p24 for Gag and p66 and p51 representing Pol.

vpr-RT-IN cassettes by Western blot analysis (Fig. 2). Clearly, in the presence of Dox the Gag and RT proteins are virtually undetectable (Fig. 2, lane 1). In contrast, withdrawal of Dox and addition of 5 mM sodium butyrate (SB) induced expression of Gag and RT to significantly high levels (Fig. 2, lane 2). The use of SB to induce vector production from producer cell lines was previously demonstrated to alleviate epigenetic suppressive effects on tetracycline-regulated promoters [13], presumably through its action as an inhibitor of histone deacetylases. We compared the vTK136 vector prepared from conditioned media of induced SODk3/TK136c21 producer cells to vector packaged with either the *trans*-lentiviral packaging system or the ΔNRF packaging system following transient transfection of SODk0 cells (Fig. 2, lanes 3–5). Gag and RT were readily detected in each of the vectors, nonetheless processing of the Pr55 Gag precursor to its constituent proteins was much more efficient when TK136 was packaged by ΔNRF (lane 5) rather than with the *trans*-lentiviral packaging system (lanes 3 and 4). Apparently, altering the context of the PR gene product within the Gag–Pol proteins impairs the autoprocessing capacity of the PR on the Pr55 product; however, the PR remains competent for processing Pr55 Gag molecules supplied *in trans* [24]. In line with this reasoning, separating the Pol gene products from the Gag–PR products alters the natural context of the PR protein and thereby may interfere with efficient autoprocessing of the Pr55 Gag. Despite the differences in processing there was only a minor influence on vector titers obtained from transient transfection of the *trans*-lentiviral packaging system (4×10^7 TU/ml) compared to that obtained with the ΔNRF packaging system (5.8×10^7 TU/ml). As is evident from Dox-dependent regulation of Gag and RT protein expression, strict control of vTK136 vector production was attained in SODk3 packaging cells.

Augmenting the Titer of the cSIN vector (vTK136) from SODk3 Packaging Cells

A persistent problem with packaging cell lines has been the inability to achieve vector titers that rival those obtained from transient transfection methods ($>10^7$ TU/ml), prior to concentration. Transduction of the SODk3 *trans*-lentiviral packaging cell line with vTK136 (cSIN vector) conferred vector titers of 2.3×10^6 TU/ml, as described above. Even though these are effective titers, reaching the levels of vector production obtained with other packaging cell lines is not beyond the scope of the SODk3 packaging cell line. We evaluated SODk3 packaging cells maintained in the presence of Dox for GFP expression by FACScan analysis before and after transduction with vTK136 (Fig. 3A). The FACScan profile revealed a heterogeneous population of SODk3 cells transduced with vTK136. Based on this it was reasonable to conclude that the heterogeneity in GFP expression is a consequence of cell-to-cell differences in the number of

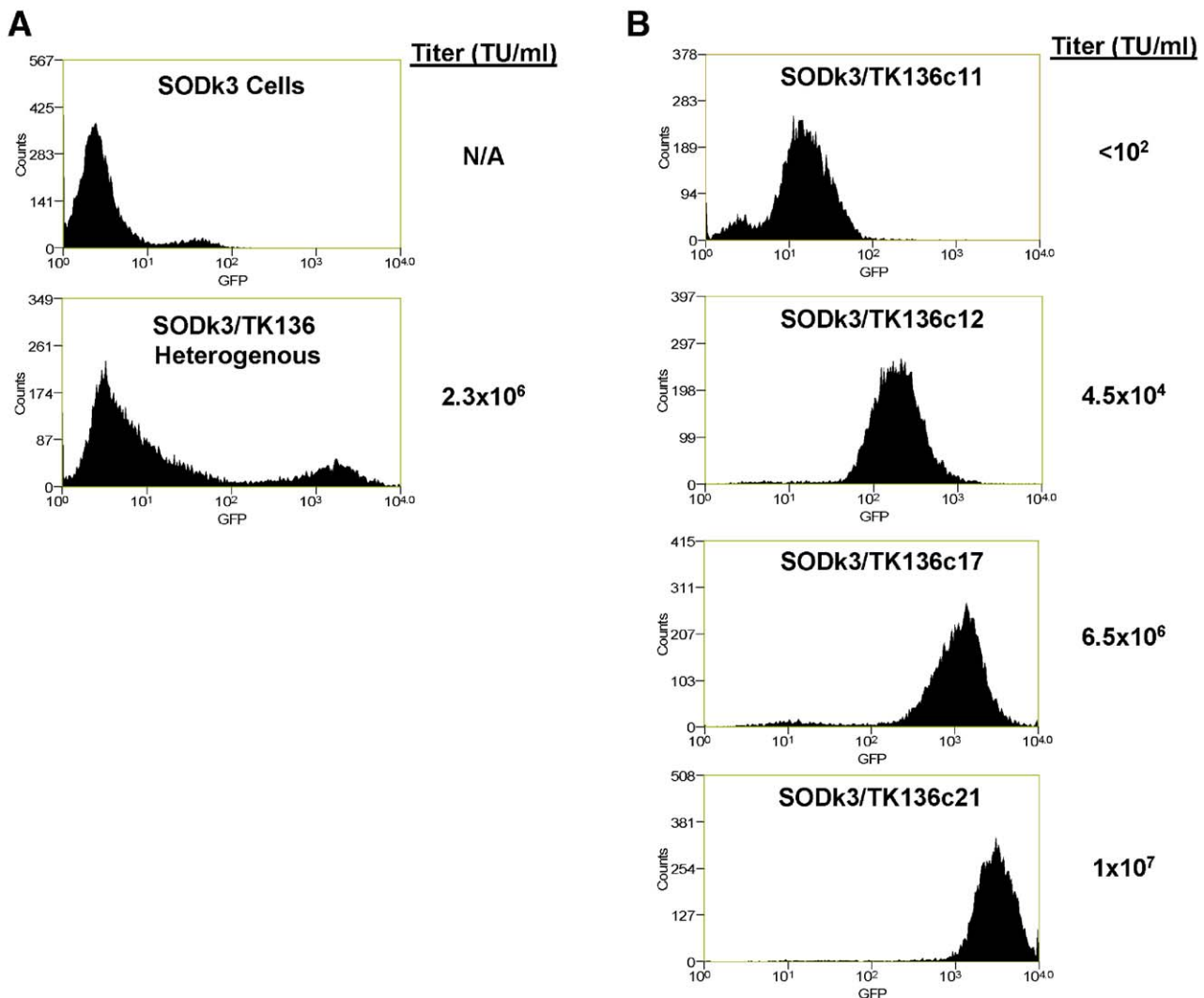


FIG. 3. Augmenting vector titers through clonal selection. (A) SODk3 packaging cells maintained in the presence of Dox were evaluated for GFP expression by FACScan analysis before and after transduction with vTK136. (B) Single-cell clones were isolated following limiting dilution of the SODk3/TK136 heterogeneous cell population and assessed for GFP expression by FACScan analysis. In parallel experiments vTK136 was harvested from conditioned medium of each indicated cell population. Vectors were titered on 293T cells by serial dilution and scored for GFP-positive cells.

integrated vector copies. Therefore, vTK136-transduced SODk3 producer cells with higher GFP expression may also express more full-length vector mRNAs for packaging, which would manifest itself in transduced target cells as an increase in vector titer. We examined this possibility by isolating/selecting single-cell clones by limiting dilution of the heterogeneous SODk3/TK136 cell population in the presence of Dox. We expanded 10 GFP-positive clones in the presence of Dox and evaluated them for GFP expression levels by FACScan analysis. In parallel, we harvested TK136 vector from each induced packaging cell clone and titered it on 293T cells. Even though only 4 of the clones are presented (Fig. 3B), the FACScan profiles for each of the 10 clones portrayed a

level of GFP expression that was commensurate with the titer obtained from each packaging cell clone (data not shown). Indeed, as the level of GFP expression elevates through SODk3/TK136c11 < SODk3/TK136c12 < SODk3/TK136c17 < SODk3/TK136c21 there is an analogous increase in the vector titers (Fig. 3B). Through clonal isolation we could augment the vector titers to 1×10^7 TU/ml, which approaches titers routinely obtained by transient transfection methods. Importantly, the vTK136 vector obtained from the SODk3/TK136c21 producer cell was concentrated to 6.9×10^9 TU/ml and examined for RCLs by the *gag*-transfer, *tat*-transfer, and marker rescue assays [13]. All assays were negative for replication-competent vector (data not shown). Furthermore, titers

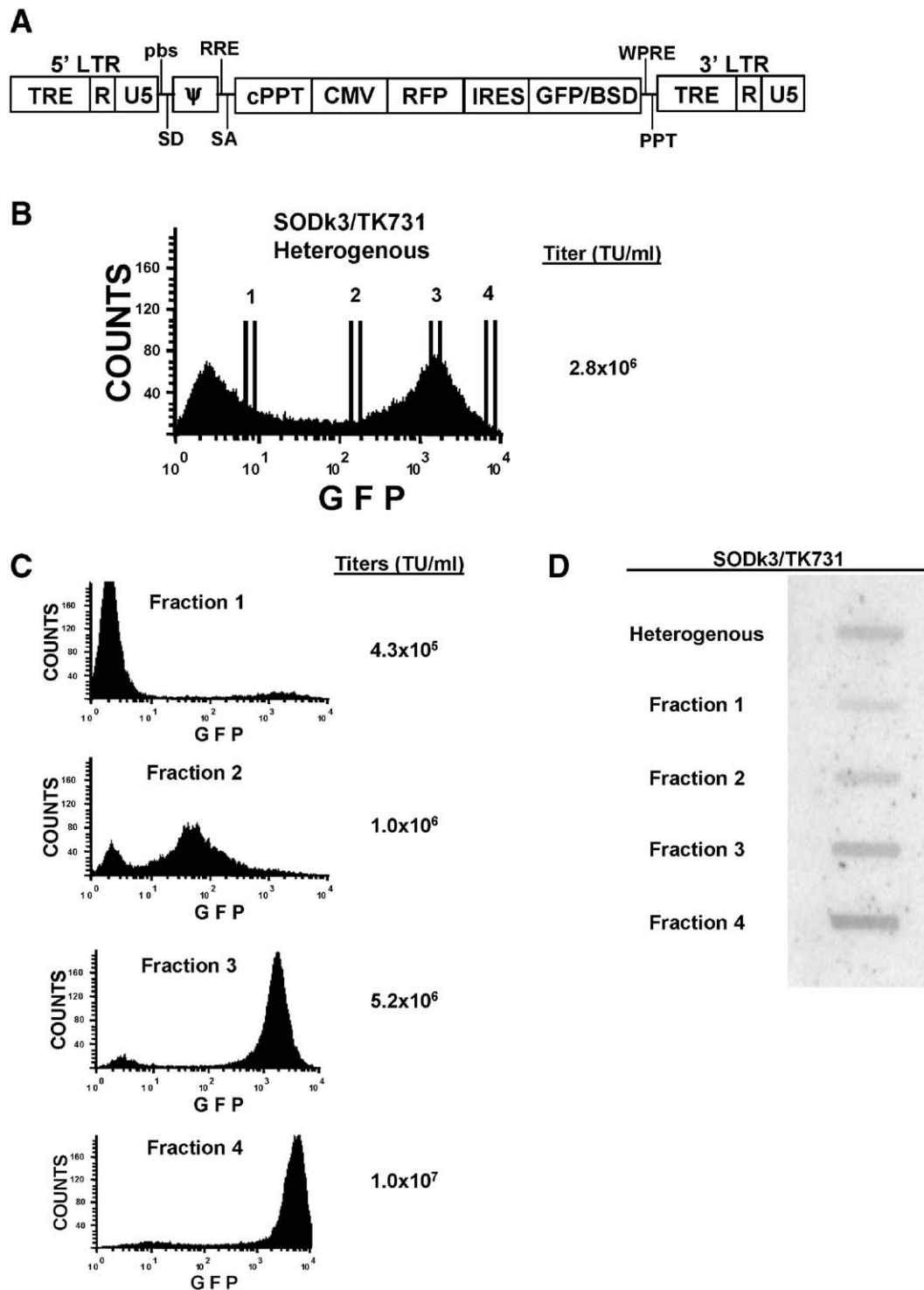


FIG. 4. Efficient marker gene selection for SODk3 producer cell lines that yield high-titer vector carrying a gene of interest. (A) The transgene cassette of the vTK136 vector was altered by inserting an internal ribosome entry site (IRES) between the gene of interest (red fluorescent protein in this case, RFP) and the GFP for use as the selectable marker, resulting in the vTK731 construct. (B) SODk3 packaging cells maintained in the presence of Dox were evaluated for GFP expression by FACScan analysis after transduction with vTK731. (C) Four distinct fractions as defined in the heterogeneous SODk3/TK731 cell population were isolated by FACS sorting and assessed by FACScan analysis. In parallel experiments vTK731 was harvested from conditioned media and titered on 293T cells by serial dilution and scored for GFP-positive cells. (D) Vector particles from each of the cell populations were further assessed for the amount of RNA in the vector particles by slot-blot analysis. Equivalent amounts of vector particles (as determined by the p24 levels) were transferred to a Hybond-H+ membrane by slot blot and probed for the CMV promoter.

were maintained after freezing and thawing, indicating that the stability of this cell line has not been altered (data not shown). These data implicate GFP as a useful marker for isolating high-titer producer cell lines from a heterogeneously transduced population of SODk3 packaging cells.

A Streamlined Method of Marker Gene Selection for High-Titer SODk3 Producer Cell Lines That Yield Vector Carrying a Relevant Transgene

In the absence of a marker gene, selecting producer cell clones (as described above) would be an arduous task. We sought to make the vector more amenable to this process by incorporating an internal ribosome entry site between the relevant transgene and the GFP marker, vTK731 (Fig. 4A). Although only a marker itself, dsRed (RFP) was incorporated to substitute as the relevant transgene. We evaluated SODk3 packaging cells, maintained in the presence of Dox, for GFP expression by FACScan analysis after transduction with vTK731 (Fig. 4B). The FACS profile revealed a heterogeneous population of producer cells, similar to that observed with the SODk3/TK136 cell line above. Furthermore, the SODk3/TK731 cell line produced vTK731 titers (2.8×10^6 TU/ml) similar to those of the SODk3/TK136 cell line. Accordingly, these titers could be enhanced to levels comparable to those acquired from clonal selection of the SODk3/TK136 producer cell line. Exploiting the fact that there is a strong correlation between GFP expression and the potential titers of a producer cell line, we used fluorescence-activated cell sorting to isolate four cell fractions from the heterogeneous SODk3/TK731 cell population (Fig. 4B). Sorting for populations of cells reduced the time needed to procure producer cell lines by nearly 1 month, eliminating the task of isolating cell clones, as done for the SODk3/TK136 producer cell line. We assessed each of the individual expanded fractions by FACScan analysis while they were maintained in Dox and induced them to produce vector (Fig. 4C). Concomitant with an increase in vTK731 titer there was a rise in GFP expression from fraction 1 through fraction 4. There was an analogous increase in RFP expression (data not shown). The vTK731 yield from fraction 4 (1×10^7 TU/ml) was equivalent to the highest titer obtained from clonal selection of SODk3/TK136c21 producer cell lines (Fig. 3B), signifying that cell sorting is the method of choice to secure producer cell lines bearing high titers. Furthermore, we concentrated fraction IV of the SODk3/TK731 producer cell line to 6.5×10^9 TU/ml and determined it to be negative for RCLs by the assays indicated previously. Notably, the stability of the SODk3 packaging cell line did not seem to be greatly altered, since the SODk3/TK731 producer cells were maintained in tissue culture for up to 2 months and, after repeated freeze/thaw cycles, yielded titers of vTK731 ± 2 -to 3-fold of the 1×10^7 TU/ml obtained for fraction 4 (data not shown).

Distinct differences in GFP levels from fraction 1 to fraction 4 indicate that the heterogeneous cell population contains several subpopulations of packaging cells that acquired different copy numbers of integrated vTK731 upon initial transduction. As a consequence these differences are reflected in the vector titers, implying that the vTK731 titers may show a correlation with the amounts of vector mRNA packaged into the particles. As expected the Gag/Pol ratio was not altered from any of the SODk3/TK731 cell populations since these were all derived from the same SODk3 packaging cells (data not shown). Therefore, the differences in titer must be a consequence of the mRNA levels. We evaluated this by isolating mRNA from equivalent amounts of vector generated from induction of the heterogeneous cell population and each of the producer cell fractions. Based on equivalent amounts of p24 we loaded the RNA onto the slot blot and probed it for the internal CMV promoter (Fig. 4D). Indeed, the levels of mRNA from fraction 1 to fraction 4 coincide with the elevation in vector titer. Although it has not been ascertained by our experiments further addition of vTK731 to the packaging cells may impart greater enhancement of vector titers. Furthermore, the use of a selectable marker as an efficient method to isolate producer cell lines was substantiated by an analogous report portraying a streamlined method for retroviral producer cell lines [25]. Loew *et al.*, however, incorporated the Cre/Lox system to excise the marker gene following isolation of the producer cell line, so as not to include unwanted gene elements in the vector stocks. A similar modification in the vTK731 vector would be warranted to remove GFP, which has been identified as a potential immunogen [26].

In vitro assessment of vTK136c21 and vTK731 on primary hEFs demonstrated their ability to transduce biologically relevant cells and the capacity to express two genes concurrently in the same cell (Supplemental Fig. 1). Additional modifications of the vTK731 vector will include replacement of RFP with a transgene of clinical relevance to examine the efficacy of the vectors in an animal model. In accordance with this, we have initial evidence from ongoing studies that vTK136c21 can transduce rat brain neurons upon *in vivo* administration (data not shown).

MATERIALS AND METHODS

Lentiviral Vector Constructs

The vector cassettes pTK113 and pTK136 and the Δ NRF packaging cassette were all described previously [17]. The pBIGFVG construct contains the VSV-G envelope gene and GFP gene expressed in opposite directions from a bidirectional tet-inducible promoter [13]. The pPUR (Clontech) construct harbors an expression cassette encoding the gene for puromycin resistance. The pTRE-gag/pro ^{Δ vpr} and pTRE-vpr-RT-IN *trans*-lentiviral packaging constructs (Fig. 1B) were kind gifts from Tranzyme, Inc. Except for substitution of the 5' promoters with the TREs, the *trans*-lentiviral packaging cassettes are the same as those reported previously [3].

The pTK731 vector construct was derived from pTK136 and a *NotI/SacII* fragment from pTK643. The *NotI/SacII* fragment from pTK643 is composed of the HIV-1 RRE-cPPT genetic elements, CMV-RFP-IRES-GFP/

BSD cassette, and the WPRE. This was ligated into the *NotI/SacII* site between the LTRs of pTK136. The resultant pTK731 vector consisted of an internal CMV promoter driving expression of the full-length vector RNA with the TRE in the U3 of the 3' LTR for subsequent expression of full-length RNA in tet-permissive cells and retains the SIN phenotype in cells lacking the tetracycline transactivator.

Vector Production via Transient Transfection and Assays for RCL

Vesicular stomatitis virus G glycoprotein-pseudotyped HIV-1 vectors were generated by transient transfection as described [27]. Regarding vectors packaged with Δ NRF the following plasmid amounts were used: 15 μ g vector, 10 μ g Δ NRF, and 5 μ g of the VSV-G envelope plasmid pMD.G. Transient vector production with the *trans*-lentiviral packaging system was executed in the SODk0 cell line [13], which harbors a constitutively expressed tTA. SODk0 cells were transiently transfected with the following plasmids: 15 μ g vector, 10 μ g pTRE-gag/pro ^{Δ Vpr}, 3 μ g pTRE-vpr-RT-IN, and 5 μ g pMD.G. Vector titers were assessed on 293T cells following serial dilutions and scored for GFP expression by fluorescence microscopy. Vectors were analyzed for RCLs by the *tat* transfer, *gag* transfer, and marker rescue assays [13].

trans-Lentiviral Packaging Cell Line Development/Maintenance and Vector Production via Producer Cell Lines

All cell types were maintained in DMEM-H (Sigma) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), and 25 μ g/ml amphotericin B (Invitrogen).

PVG3 cell line. The entire *trans*-lentiviral packaging cell line was derived from the SODk0 cell line [13]. To generate the PVG3 cell line subconfluent SODk0 cells were cotransfected with 25 μ g pBGFVG linearized with *FspI* and 5 μ g pPUR by calcium phosphate precipitation. Cells were maintained in 1 μ g/ml Dox, unless otherwise stated. The medium was changed at 12–16 h posttransfection and the cells were grown to near confluence. Cells were split 1:10 onto 10-cm dishes and sustained in culture for 2 weeks under 1 μ g/ml puromycin (Clontech). A single colony was expanded (PVG3) and tested for pseudotyping of HIV-1 vector by transient transfection. Confluent PVG3 cells were induced by washing twice with 1 \times PBS and split 1:3 in the absence of Dox. The cells were expanded to confluence with replacement of the medium daily and split 1:3 onto dishes pretreated with 0.001% polylysine (Sigma). In the absence of Dox PVG3 cells were cotransfected with 15 μ g pTK113 and 10 μ g Δ NRF. At 12–16 h posttransfection the medium was replenished with the addition of 5 mM sodium butyrate (Sigma). Dox-free medium containing 5 mM sodium butyrate was replenished 24 h later, and conditioned medium was harvested 72 h later. The conditioned medium was filtered through a 0.45- μ m filter and titered on 293T cells by serial dilution and scored for GFP expression by fluorescence microscopy.

SODk3 *trans*-lentiviral packaging cell line. The SODk3 packaging cell line was generated by cotransfecting subconfluent PVG3 cells with 25 μ g pTRE-gag/pro ^{Δ Vpr}, linearized with *PvuI* and 5 μ g pTRE-vpr-RT-IN, linearized with *FspI*. Construct pTRE-gag/pro ^{Δ Vpr} contains a cassette that expresses the neomycin resistance gene. Cells were maintained in 1 μ g/ml Dox, unless otherwise stated. The medium was changed at 12–16 h posttransfection and the cells were grown to near confluence. Cells were split 1:10, 1:100, and 1:1000 onto 10-cm dishes and sustained in culture for 2 weeks under 600 μ g/ml neomycin (Invitrogen). Clones were isolated and expanded. Several clones were selected and screened for vector production following transduction with vTK136 that was prepared by transient transfection. The producer clones were expanded to confluence and induced as described above. The induction was the same as described above except that the producer clones were not transfected. Vectors were harvested and titered as described.

SODk3 *trans*-packaging cells were transduced with vTK731 as described above. The cells were sorted into four distinct populations by FACS sorting as described below. Inductions for vTK731 production were executed as described above.

Further concentration of vector from the SODk3/TK136c21 or SODk3/TK731 producer cell lines was implemented by scaling up the number of cells for induction. SODk3/TK136c21 or SODk3/TK731 cells were induced as outlined above. Harvested vector was centrifuged at 3000 rpm for 5 min in a Sorvall SL-250T rotor, to remove cellular debris, and passed through a 0.45- μ m filter. The vector was purified over two consecutive sucrose gradients (20, 30, 60, and 70%) at 20,000 rpm for 2 h in a Beckman SW28 rotor, collecting the banded vector from between the 30 and the 60% sucrose. The collected vector was diluted with 1 \times PBS and concentrated to a pellet at 20,000 rpm for 2 h. The vTK136c21 or vTK731 was resuspended in 1 \times PBS and titered on 293T cells as described above.

FACS Analysis

Cells were prepared for FACS analysis by being removed from the dishes through treatment with 0.05% trypsin/EDTA and pelleted at 300g at 4°C. Cells were resuspended in cold 1 \times PBS and pelleted. Cells were then resuspended in 2% formaldehyde/0.2% glutaraldehyde in 1 \times PBS to 1 \times 10⁶ cells/ml, pelleted, and resuspended in 1 \times PBS. All cells were analyzed for GFP expression by FACSscan or FACSCalibur systems (Becton-Dickinson).

FACS sorting was executed by the UNC-CH Flow Cytometry Core Facility. Cells were removed from dishes by 0.05% trypsin/EDTA and pelleted at 300g at 4°C. Cells were resuspended in DMEM containing 0.1% FBS and 10 μ g/ml ciprofloxacin. Cells were collected in DMEM as described above with an addition of 10 μ g/ml ciprofloxacin. The cells were pelleted; resuspended in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 μ g/ml amphotericin B; and plated.

HIV-1 p24 and Reverse Transcriptase Assays

The Gag (p24) measurements for vectors were executed by a commercially available HIV-1 p24 ELISA kit (Perkin-Elmer). ELISA was performed according to the manufacturer's instructions.

Vectors were assayed for reverse transcriptase activity using a commercially available chemiluminescence assay (Reverse Transcriptase Assay; Roche). The RT assay was executed as described by the manufacturer's protocol. The levels of reverse transcriptase activity were correlated directly to picogram amounts of RT as extrapolated from a known standard.

Western Blot Analysis

Cell lysates were examined by standard denaturing SDS-PAGE on 4–20% gradient gels and Western blot analysis. Blots were probed with a murine anti-VSV-G monoclonal antibody (Ab) (1:1000; Sigma), murine anti-HIV-1 RT (1:200, 8C4; NIH AIDS Research & Reference Reagent Program), and murine anti-HIV-1 p24 Gag (1:5000, 6457; NIH AIDS Research & Reference Reagent Program) followed by a polyclonal goat anti-murine Ab labeled with horseradish peroxidase (1:5000; Pierce). Signals were detected by using ECL (Amersham Pharmacia Biotech).

RNA Slot-Blot Analysis

Total RNA was extracted from vector particles using the RNeasy kit (Qiagen). Briefly, RNA extracts were prepared from an equivalent number of vector particles as determined by p24. Immediately prior to preparation of RNA extracts equivalent numbers of vector particles were mixed with 293T cells, for use as carrier RNA. The RNA was transferred to a Hybond-H + membrane (Amersham Pharmacia Biotech) using a slot-blot apparatus (Bio-Rad) and analyzed with a probe to the CMV promoter, which was labeled by random priming (Roche). The membrane was washed and the image was developed by phosphorimaging (Molecular Dynamics Storm System).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ymthe.2005.12.015](https://doi.org/10.1016/j.ymthe.2005.12.015).

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