

# Large-Scale Manufacturing of Safe and Efficient Retrovirus Packaging Lines for Use in Immunotherapy Protocols

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Received: 23 November 1998  
Revised: 15 March 1999  
Accepted: 1 April 1999  
Published online: 16 April 1999

## Abstract

**Background** The use of gene modified T lymphocytes for immunotherapy in a cancer or AIDS clinical trial requires an efficient, safe *ex vivo* method for modification of these cells at manufacturing scale. Since retroviruses have been shown to be a moderately effective means of stably integrating therapeutic genes into T lymphocytes, we wanted to create packaging and producer cell lines that would produce replication competent retrovirus (RCR)-free supernatants, at large scale (>200 l), and transduce with high efficiency.

**Methods** cDNA expression plasmids containing only coding sequences for *gagpol* or *env* were built and sequentially transfected into human 293 cells. Packaging and producer clones were characterized for stability, titer and RCR. A producer clone delivering chimeric immune receptors was scaled-up and supernatants used to transduce patient T lymphocytes for clinical studies. PCR and RT-PCR assays were utilized to evaluate the transmission of HERV-H sequences. Relative infectivity of producer clones pseudotyped with different envelopes was determined by transduction and RT assays.

**Results** RCR-free, human 293 split-genome packaging lines, pseudotyped with amphotropic, xenotropic, or 10A1 envelopes, were created. A CC49 $\zeta$  producer clone was scaled-up to 5  $\times$  54 l lots and supernatants used to safely and efficiently transduce patient T lymphocytes with minimal *ex vivo* manipulation. While 293 cells express HERV-H mRNA, the transmission frequency in our packaging clones was less than 1 HERV-H sequence per 5  $\times$  10<sup>5</sup> proviral integrations. Additionally, 10A1 and xenotropic packaging lines had higher infectivities than the amphotropic clone.

**Conclusion** These packaging lines represent the safest configuration for the large-scale production of retroviral vectors, and are capable of producing high titer, RCR-free retroviral vector for large scale clinical use. While all three clones efficiently transduce human T lymphocytes, the 10A1 clone has the highest infectivity. These packaging cell lines will be valuable for use in human gene therapy protocols. Copyright © 1999 John Wiley & Sons, Ltd.

**Keywords** retrovirus vector; T lymphocyte; large-scale manufacture

## Introduction

The use of gene modified T lymphocytes and hematopoietic stem cells for immunotherapy of cancer, genetic and infectious diseases requires not only efficient *ex vivo* gene modification of these cells, but it must also be adaptable

to large scale manufacturing for clinical protocols. Retroviruses have previously been demonstrated to be an efficient means of stably integrating therapeutic genes into both T lymphocytes and hematopoietic stem cells [1,2].

Over the last ten years, significant improvements in both safety of retroviral packaging lines and the titer of producer clones have been achieved. The earliest MMLV packaging line,  $\psi$ 2, utilized a complete proviral genome on a single plasmid containing only a 300 bp deletion of the  $\psi$  or packaging signal [3]. The first improvement in packaging line technology was deletion of the remaining *cis*-acting sequences necessary for viral replication (PA317), but retained a single plasmid configuration [4]. Subsequently a new class of MMLV packaging lines was created (crip, cre, AM12) in which the viral genome was split between two plasmids and many of the *cis*-acting sequences were removed [5–7].

Although these efforts decreased the frequency of replication competent retrovirus (RCR) generation, these cell lines faced several limitations. First, these packaging cell lines were derived from murine fibroblasts and contained endogenous retroviral elements which could be co-packaged with the transfer vector by the virus particles [8], and recombination during reverse transcription could lead to the generation of RCR [9]. Although these recombination events would occur at a low frequency, large-scale production of retroviral vectors at a scale necessary for gene therapy clinical trials (20–100 l) increases the chance of detecting a rare recombinant. Once generated, RCR could spread rapidly through a production lot. Second, the viral titer of producer clones derived from these packaging lines was not sufficient to efficiently transduce T lymphocytes [2,10,11].

In order to improve safety and obtain viral titers sufficient for high efficiency gene transfer into human T lymphocytes, we and others developed both transient transfection and stable virus production systems using packaging cell lines derived from human cells. Several systems have been described over the last several years in which recombinant retrovirus have been produced in HT1080 or 293 cells [12–16].

Here we describe the development of three retroviral packaging lines derived from the human embryonic kidney line 293, which produce retrovirus pseudotyped with the 4070A amphotropic, the NZB9.1 xenotropic, and 10A1 envelopes. These packaging cell lines encode viral proteins necessary in *trans* for virus production on two expression plasmids in which only the coding sequence for *gagpol* or the appropriate *env* are expressed under the control of a heterologous promoter, with an intron and polyadenylation site. This represents the safest configuration for expression of viral packaging genes. We demonstrate that these packaging lines produce RCR-free, retroviral vector at a scale and quality sufficient for large-scale clinical use and for transduction of primary human T lymphocytes at high efficiency. In contrast to murine packaging lines which are capable of transmitting murine VL30 elements equally as efficiently as recombinant

retroviruses [8], we demonstrate that our human packaging lines transmit HERV-H elements at least  $5 \times 10^5$  less efficiently than our recombinant retroviruses. Additionally, although we show that all three cell lines produce virus that transduces primary T lymphocytes efficiently, the virus particles derived from the 10A1 cell line are the most efficient per particle at T lymphocyte transduction. These packaging cell lines will be valuable for use in human gene therapy protocols.

## Methods

### Viral vectors

The retroviral transfer vector *rkat43.2* (Figure 1A) has been described previously [16–17]. *rkat43.2CD4 $\zeta$*  and *rkat43.2CC49 $\zeta$*  are retroviral transfer vectors encoding the chimeric immune receptors CD4 $\zeta$  [16–18] or CC49 $\zeta$  [19] respectively. The packaging constructs *pkat2gagpol* and *pkat2amenv* (Figure 1A) are plasmid-based expression vectors containing only coding sequences for the MMLV *gagpol* (GenBank #J02255, nucleotides 621–5872) or MMLV 4070A amphotropic envelope (GenBank #M33469, nucleotides 37–2001) proteins respectively, under the control of a hybrid CMV enhancer/MMLV promoter [16]. Plasmids *pkat210A1env* and *pkat2xenoenv* encode for the 10A1 (GenBank #M33470, nucleotides 65–2002) and NZB9.1 (GenBank #K02730, nucleotides 291–2225) envelope proteins respectively. The *pkat2gagpol* plasmid (Figure 1A), a MMLV *gagpol* expression plasmid, was constructed by replacing a 679 bp HindIII-XbaI DNA fragment containing the CMV enhancer/promoter within *pkat1gagpol* [16] with the corresponding CMV enhancer/MMLV promoter sequences from the *pkat2ampac* plasmid [16]. The *pkat2amenv* plasmid (Figure 1A), was derived from *pkat1amenvATGut $\Delta$*  by replacing the 679 bp HindIII-XbaI DNA fragment containing the CMV enhancer/promoter within *pkat1amenvATGut $\Delta$*  with the corresponding CMV enhancer/MMLV promoter sequences from the *pkat2ampac* plasmid. *pkat1amenvATGut $\Delta$*  was derived from the amphotropic envelope expression plasmid, *pkat1amenv* [16] by deleting the untranslated sequences 3' to the envelope ORF by replacing the 172 bp ClaI-NheI fragment of *pkat1amenv* with a 100 bp ClaI-NheI fragment generated by PCR in which the termination codon of the A-MMLV 4070A envelope gene was encoded as part of a new NheI site. *pkat210A1env* and *pkat2xenoenv* were derived from *pkat2amenv* by replacing the amphotropic envelope encoding NcoI-NheI fragment of *pkat2amenv* with the corresponding fragment from either the 10A1 encoding plasmid, pB6 (generous gift of Alan Rein) [20] or pCRUX (generous gift of Olivier Danos) [21] respectively, where the ATG start and TAG stop codons were incorporated into the NcoI and NheI sites by PCR cloning. Plasmid sequences available upon request.

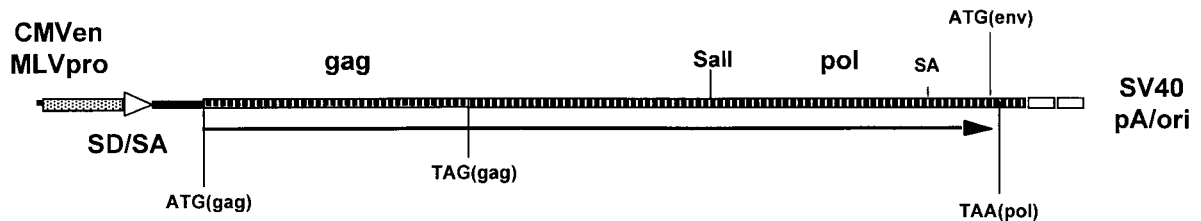
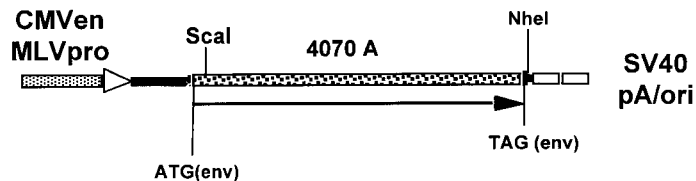
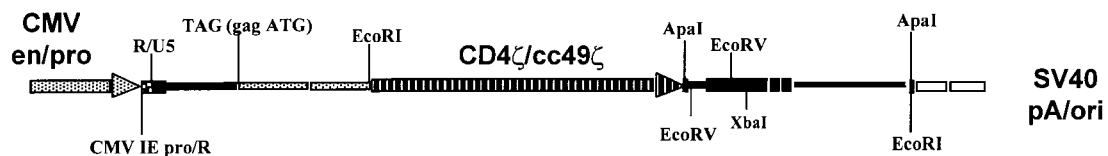
**pkat2gagpol****pkat2amenv****rkat43.2**

Figure 1A. Retroviral constructs. Packaging constructs: *pkat2gagpol*; *pkat2amenv*. Translation start (ATG) and stop (TAA or TAG) codons for MMLV *gagpol* and *env* are shown, as are splice donor (SD) and acceptor (SA) sites and selected restriction endonuclease cleavage sites. Sequences derived from ecotropic MMLV *gagpol*, striped bar. Sequences derived from amphotropic, xenotropic or 10A1 MMLV envelopes, stippled bar. SV40 pA and origin of replication (SV40pA/ori), open bar. CMV immediate early enhancer and MMLV promoter (CMVen/MLVpro) gray and white arrow. The *pkat2* intron (SD/SA) black bar. Retroviral construct *rkat43.2CD4ζ* or *rkat43.2CC49ζ*: CMV immediate early enhancer/promoter (CMVen/pro), gray arrow. MMSV viral sequences, black bar, MMLV viral sequences, shaded bars. CD4ζ or CC49ζ chimeric receptor sequences, striped arrow. 3' flanking genomic DNA, black line. SV40 pA and origin of replication (SV40 pA/ori), open bar. The junction between the following elements are indicated: CMV IE enhancer-promoter/MMLV-R and MMLV-R/U5

## Cell growth, DNA transfection, viral infection and FACS analysis

293S, a suspension culture adaptable variant of 293 cells (ATCC CRL-1593) [22], 293-derived tsA54 cells [23] and all 293-derived clones were grown in DMEM, 1.0 g/l glucose, and 10% donor calf serum. Mus dunni (ATCC CRL-2017) [24] and PG4 S+L- cells (ATCC CRL-2023) [25] were grown in McCoy's 5A medium with 10 and 15% fetal bovine serum respectively. NIH-3T3 (ATCC CRL-1658) and Cf2Th (ATCC CRL-1430) cells were both grown in DMEM, 4.0 g/l glucose with 10% donor calf serum and 20% fetal bovine serum respectively. All cells were split at three to four day intervals.

For stable transfections 293 cells were plated at  $1 \times 10^6$  cells on 10 cm plates 24 h prior to transfection by calcium phosphate precipitation [26]. Medium was changed 18–24 h post transfection, and after an additional 24 h the cells were diluted into appropriate selective medium. Clones were picked for characterization after 10–14 days. Transient transfections of stable clones or tsA54 cells were performed as described above. Supernatants were harvested, filtered through 0.45  $\mu$ m filters, and flash frozen on dry ice and stored at  $-70^\circ\text{C}$ . For viral infection of packaging clones, cells were plated at  $3\text{--}4 \times 10^6$  cells

per 10 cm plate overnight. Cells were then fed with 4 ml of complete media and infected with 1 ml of viral supernatant and 8  $\mu$ g/ml polybrene for 24 h. Producer clones were then isolated by dilution cloning in 96-well plates and characterized for virus production. For viral titration on NIH-3T3 or Cf2Th cells,  $1 \times 10^6$  cells were infected in 5 ml of complete media containing viral supernatant and 8  $\mu$ g/ml polybrene. Polybrene-free media was added 24 h after transduction, followed 24 h later by analysis for CD4 expression by flow cytometry as follows. Cells were trypsinized, resuspended and washed 2x in phosphate buffered saline plus 5% fetal bovine serum (FBS/PBS). The pellet was resuspended in 100  $\mu$ l of 50% FBS in PBS and 10  $\mu$ l of (FITC or PE)-conjugated OKT4A (DAKO) for 40 min at  $4^\circ\text{C}$ . The cells were washed 3x in FBS/PBS, and resuspended in 0.1% formaldehyde and analyzed by flow cytometry (Becton Dickinson FACScan).

Amphotropic, xenotropic and 10A1 envelope expression was analyzed by flow cytometry using rat anti-gp70 antibody 83A25 [27]. Cells were trypsinized, washed with PBS plus 2% FBS, incubated with 47  $\mu$ g antibody at  $4^\circ\text{C}$  for 30 min. Following antibody incubation cells were washed 3x in FBS/PBS, incubated with goat anti-rat IgG-PE at 0.5  $\mu$ g/tube (Biosource International), washed 2x

with FBS/PBS and resuspended in 0.1% formaldehyde. Cells were then analyzed by flow cytometry.

## CD8+ T lymphocyte growth and transduction

Human CD8+ T lymphocytes were purified from peripheral blood lymphocytes (PBL) on day 0 and grown as described by Morecki *et al.* [11].  $0.5 \times 10^6$  purified and activated human CD8+ T lymphocytes were plated in 2 ml of media in 24-well plates. On day 1, 1.5 ml of medium was removed from each well and the cells incubated with 0.5 ml of fresh medium, 1.0 ml of viral supernatant, 2 µg/ml polybrene, and 10% IL-2 (Chiron). On day two medium was gently removed and replaced with fresh medium. T lymphocytes were analyzed for expression of human CD4 or CC49 extracellular domains by flow cytometry on day three using (FITC or PE)-conjugated OKT4A (DAKO) or CC49 anti-idiotypic antibody (gift of J. Schlom), respectively.

Human T lymphocytes used for clinical studies were obtained by lymphapheresis using an automated cell separator. The mononuclear cells were stimulated with anti-CD3/anti-CD28 coated beads for three days [28], followed by growth for an additional two days in AIM-V medium (GIBCO/BRL) containing 200 IU/ml of IL-2. On day five the cells were pelleted and resuspended in AIM-V medium containing 50% vector and 0.2% polybrene and centrifuged for 1 h at 12 500 RPM (1400 g) [29], resuspended and incubated overnight at 37°C. In some studies the transduction procedure was repeated on day seven by exposing the cells to 25% vector and 0.1% polybrene for an additional day. Cells were washed by centrifugation and grown for a total of 12–14 days. Transduced cells were then analyzed for surface expression of the relevant protein by flow cytometry as described above. Transduction efficiency is represented as the average number of integrated vector genomes per cell and was determined assuming that the frequency or number of integrated vector genomes in a target cell population follows a Poisson distribution where  $p_r = (x^r/r!)e^{-x}$ , ( $p_r$  = the fraction of cells that contain  $r$  integrated proviral genomes and  $x$  = the average number of integrated vector genomes per cell). The fraction of nontransduced cells,  $p_0$ , can be used to calculate the vector titer ( $a$ ) according to equation  $a = (-\ln p_0)N$  where  $N$  is the number of target cells initially exposed to vector and  $p_0$  is measured by subtracting the % number of transduced cells (determined by FACS analysis) from 100 and multiplying by 0.01. Having calculated the vector titer  $a$ , the equation  $x = a/N$  can be used to determine the average number of integrated vector genomes per cell ( $x$ ).

## Quantitative reverse transcriptase assay

Quantitative reverse transcriptase assays [30] were performed by mixing 20 µl of viral supernatant with 5 µl of Solubilization Buffer (120 mM NaCl, 1% Triton

X-100, 50 mM Tris pH 8.3) and incubating for 15 min at room temperature. 25 µl of fresh 2x Substrate Buffer (50 mM Tris pH 8.3, 10 mM DTT, 1.2 mM MnCl<sub>2</sub>, 10 µg/ml poly(rA), 10 µg/ml oligo(dT) 12–18, 5 µCi per 25 µl [<sup>3</sup>H]TTP) was added and the mixture incubated for 2 h at 37°C. DNA was blotted onto DEAE filtermats in a 96-well format (LKB Wallac) with a TomTech Mach II harvesting apparatus, rinsed in 1% Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.0 buffer, and read on UV max kinetic microplate reader (Molecular Devices). MMLV reverse transcriptase standards were set up with quantitated preparations of MMLV reverse transcriptase (Advanced Biotechnology).

## Large scale vector manufacture

JC1-L1 cells were scaled up to production lots of  $90 \times 850$  cm<sup>2</sup> roller bottles containing 200 ml of media each. Media was collected 3x at 24 h intervals. 900 ml samples and  $10^8$  production cells were removed from the last collection of each lot for RCR testing by expansion on *M. dunnii*, followed by an S+L- culture on PG4 cells.

## Replication competent retrovirus assays

Test supernatants from laboratory scale or 5% of the vector manufacturing for clinic were inoculated onto cultures of *M. dunnii* cells. Cells were passaged twice at 1:20 and grown to confluency. Culture media from the second passage was inoculated onto PG4 S+L- cells, grown for six days and stained with crystal violet in order to assess focus formation. Following completion of a manufacturing run,  $10^8$  producer cells were co-cultivated with *M. dunnii* cells for five passages. Culture media was harvested at passage five and tested for RCR on PG4 S+L- cells.

## Lymphocyte DNA Isolation and PCR

$5 \times 10^6$  cells from each patient sample were pelleted at 500 xg for 2 min, washed with PBS and repelleted. DNA was immediately prepared using DNAzol (Molecular Research Center, Inc.) according to manufacturer's protocol. PCR reactions were performed in 0.2 ml thin wall optical PCR tubes (Perkin Elmer/ABI) in a total volume of 50 µl. Primer F was located in the 3' untranslated region of  $\zeta$ s 10 $\zeta$  on the *rkat43.2CC49 $\zeta$*  vector, 80 bp downstream of the TAG stop codon (nucleotides 6063–6081), and primer R was located in the very 5' end of the 3' LTR (nucleotides 6254–6273). The primer pair F/R yielded a 211 bp product. 2 µl of the appropriate template was mixed with 48 µl of the 'master mix' (24.84 µl sterile deionized water, 5 µl 10X TaqMan Buffer A, 9 µl 25 mM MgCl<sub>2</sub>, 1 µl each of 10 mM dATP, 10 mM dGTP, 10 mM dCTP and 20 mM dUTP, 2 µl each of 10 pmol/µl Primer F and 10 pmol/µl Primer R, 0.5 µl 1 U/µl AmpErase UNG, 0.058 µl 167 pmol/µl TM1F probe, and 0.6 µl Amplitaq Gold from Perkin Elmer).

Samples were amplified in a 7700 Thermal Cycler (PE Applied Biosystems) with one hold 2 min at 50°C, a second hold 10 min at 95°C, and 40 cycles at 15 s at 95°C followed by 60 s at 60°C. Results were analyzed according to the method detailed in the 7700 Sequence Detector User's Manual.

## HERV-H PCR and RT-PCR protocols

High molecular weight DNA was prepared from 293 and NIH-3T3 cells by a method adapted from Maniatis [31]. DNA was measured on a Hoefer TKO 100 fluorimeter. Based on the assumption that a diploid human cell contains 6.6 pg of genomic DNA, PCR was carried out on the DNA mass equivalent of one 293 cell to  $10^{-4}$  293 cells spiked into the DNA mass equivalent of  $10^5$  NIH-3T3 cells. Primers used were as follows. HERV-H primers: H5, 5'actataggcaactttccaccctcc3'; H10, 5'gctacttggtcctctactctat3' (Genbank # M18048). Primers H5/H10 yield a band of 905 base pairs. Actin primers: Upper; 5'cgagcatccccaaagtccaca3', Lower; 5'cccagccacaccacaagtcaca3'. Actin primers yielded 480 bp and 520 bp bands from cDNA. The following cocktail was added to the genomic DNA mixes; 25 pmol each primer, 1X PCR Buffer, 1.5 mM MgCl<sub>2</sub>, 50 μM dATP, dGTP, dTTP, 20 μM cold dCTP, 33 μM<sup>32</sup>P dCTP, 1.0 U/reaction tam pol (Promega), 0.2 μl/reaction Taq Start antibody (Clontech), 1X Taq Start ab buffer, H<sub>2</sub>O 50 μl. A thermocycle program of 94°C, 30 s; 60°C, 30 s; 72°C, 45 s was repeated 30 times. After thermocycling, 10 μl of each PCR reaction was run on a precast 6% acrylamide gel (Novex) in TBE at 100 volts for one h. Gels were dried onto Whatman 3 mm paper at 80°C under vacuum for 15 min and exposed to X-ray film for 1–2 h or a phosphorimager plate for 10 min.

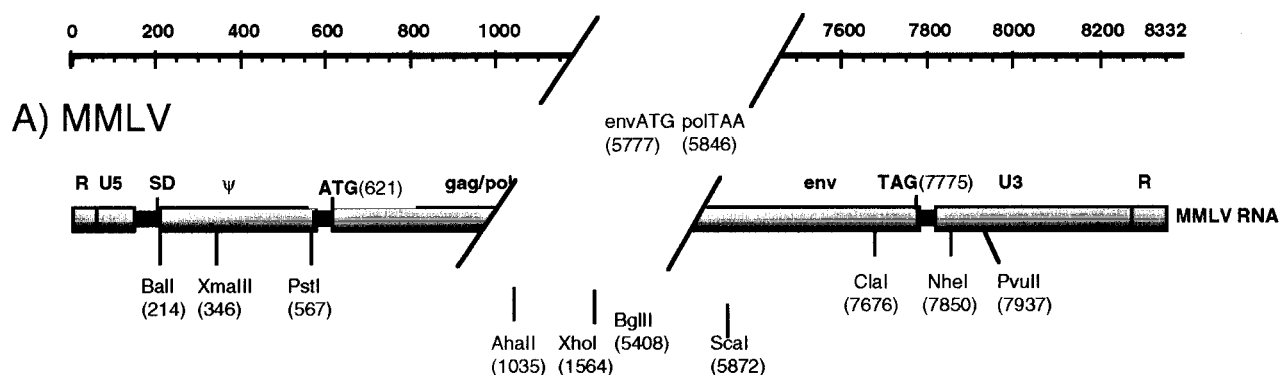
For RT-PCR total genomic RNA from 293, NIH-3T3, and NT2 cells was prepared by the RNazol B method (Tel-Test, Inc). All RNA samples were treated with DNase I, RNase-free according to manufacturer's protocol (Boehringer Mannheim), followed by phenol/chloroform extraction and ethanol precipitation. cDNA was synthesized by reverse transcription in the following manner: 0 μg, 0.25 μg, and 1 μg RNA was mixed into the reaction cocktail below to a final volume of 25 μl. Duplicate reactions were set up without RT enzyme to

control for DNA contamination of the RNA preparation. RNA, pdN6, and H<sub>2</sub>O were heated to 65°C for 10 min prior to adding master mix. Final concentrations: 1X 1st strand buffer, 1 mM DTT, 3 mM MgCl<sub>2</sub>, 1.5 mM dNTP's (Pharmacia), 37.5 ng/μl pd(N)6 (Pharmacia), 2 U/μl Superscript RT (GIBCO/BRL), 0.1 U/μl RNAsin (Promega), H<sub>2</sub>O to 25 μl. Tubes were incubated 10 min at 25°C, 1 h at 37°C, 10 min at 95°C. 2 μl of each RT reaction was transferred to a 50 μl PCR reaction. All reagents were added in the following master mix (final concentrations: 25 pmol each primer, 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 1U/reaction Taq polymerase (Promega), 0.2 μl/reaction Taq-start antibody (Clontech), 1X Taq Start ab buffer, H<sub>2</sub>O to 50 μl). For H5/H10 and actin primers a thermocycle program of 94°C, 30 s; 60°C, 30 s; 72°C, 45 s was repeated 34 times on a Perkin/Elmer 9600 PCR machine. After thermocycling, 10 μl of each RT-PCR reaction was run on a 0.8% agarose gel stained with ethidium bromide.

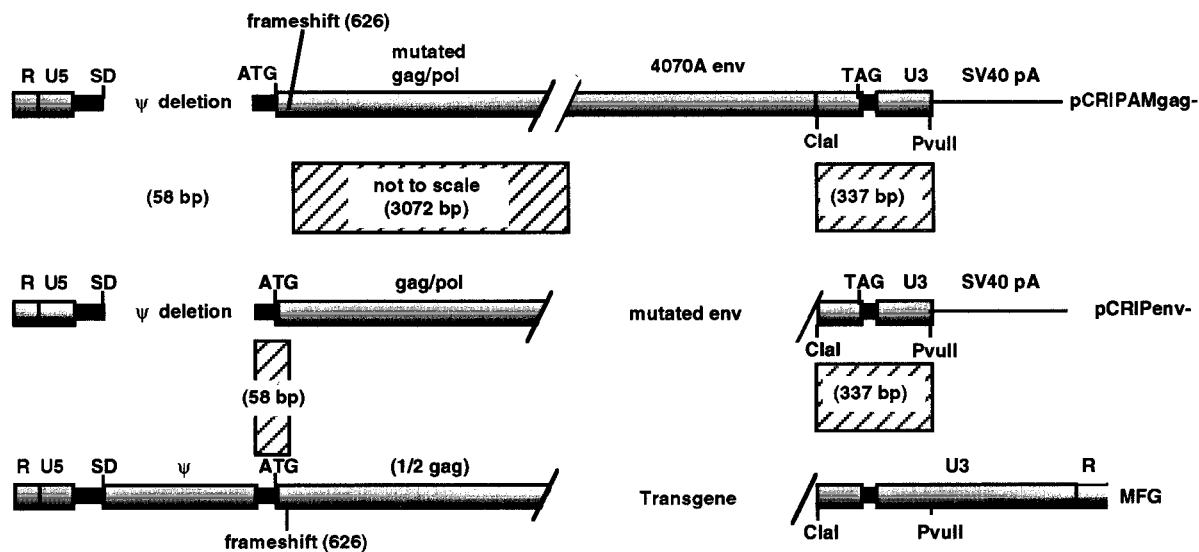
## Results

### Packaging Line Construction

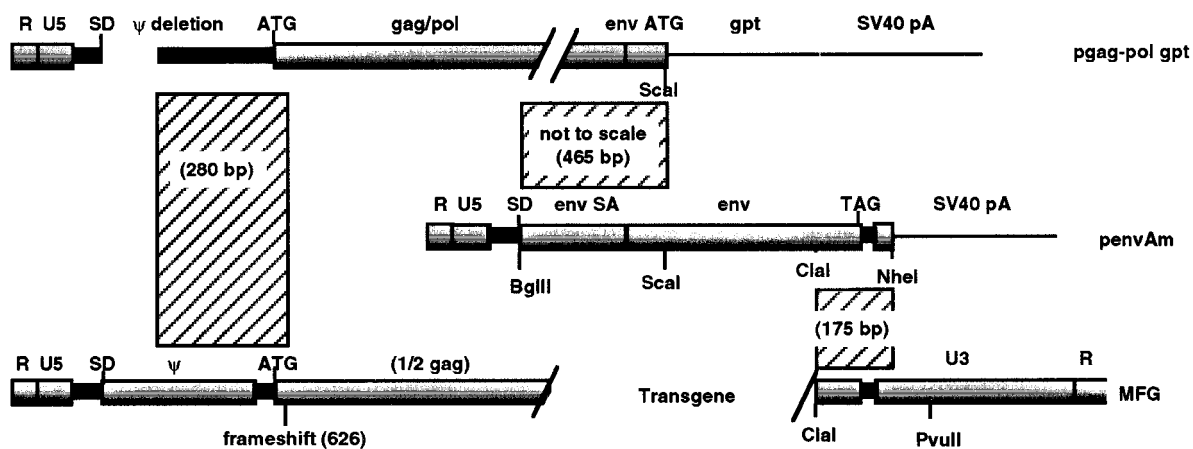
Our goal was to construct split genome packaging lines with the lowest potential for the generation of replication competent virus (RCR) when produced at large-scale for use in human gene therapy clinical trials. We surveyed existing packaging and producer cell lines used for virus production and evaluated the overlap between the transfer and packaging vectors (Figure 1B). In every packaging line there is homology between the packaging and commonly used transfer vectors 5' of the *gag* ATG such that a recombination event upstream of the ATG would restore  $\psi$  to the *gagpol* construct. These homologies range from 53 nucleotides between transfer vectors such as LXSN and the pPAM3 packaging vector used in PA317 cell line, to 280 nucleotides between transfer vector MFG and the p*gag-polgpt* packaging construct used in the AM12 packaging cell line. Additionally, the *gagpol* vectors used to construct most cell lines have 3' homology with commonly used transfer vectors (with the exception of a pPAM3 and LXSN combination). These 3' homologies range from 92 out of 99 nucleotides between



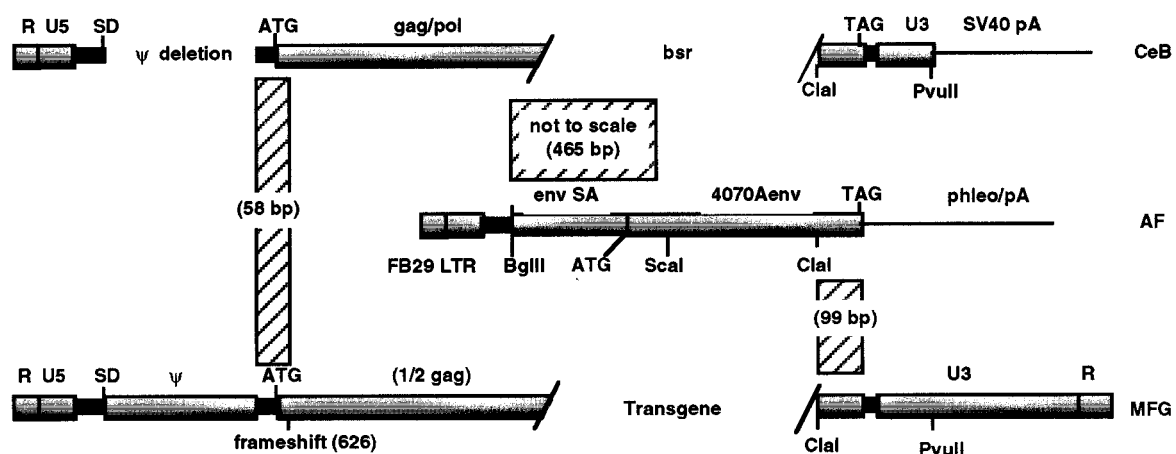
### B) $\psi$ CRIP(BING) vs. MFG



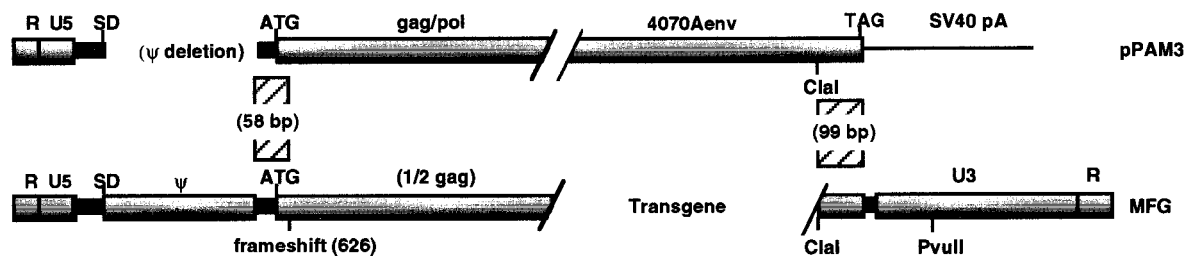
### C) AM12 vs. MFG



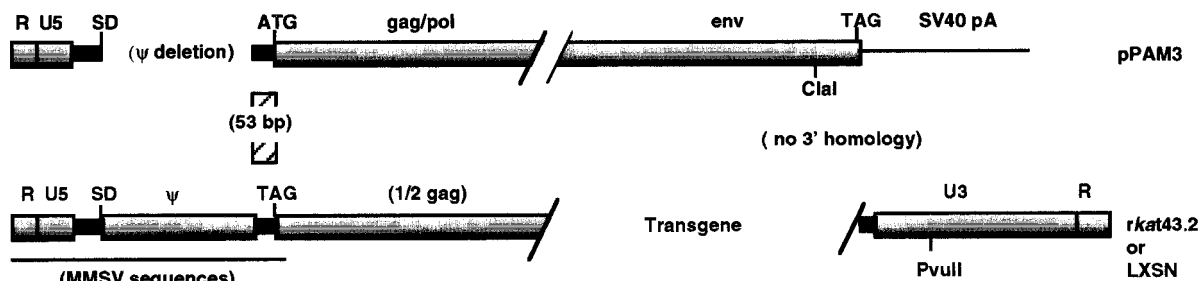
### D) FLY (ampho) vs. MFG



### E) PA317 vs. MFG



### F) PA317 vs. LXS orrrkat43.2



### G) PUZKat2 vs. rkat43.2

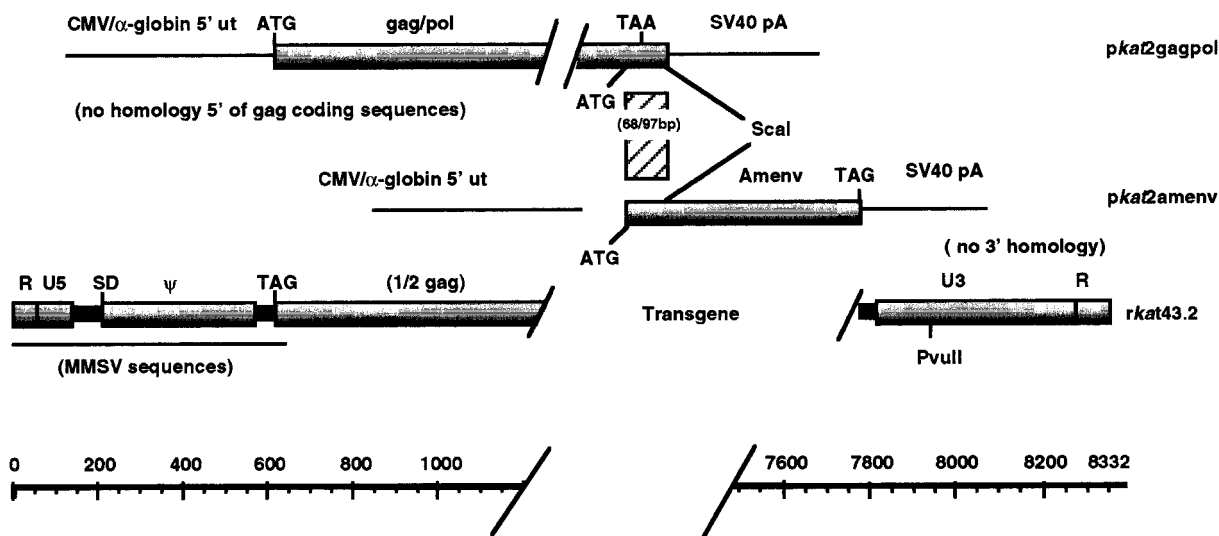


Figure 1B. Homology diagram. Homologies that could lead to partial or functional RCR between expressed and potentially co-packaged RNA transcripts from packaging and transfer vectors commonly used to create MMLV producer cell lines are shown. Vectors are displayed as un-spliced RNAs. MMLV sequence numbering is according to Shinnick *et al.* [49]. Regions of homology between vectors are shown as crosshatched boxes with sizes indicated. Selected restriction sites used for vector constructions and corresponding map locations are shown below MMLV wild type sequence. MMLV sequences are shown as gray or black boxes. Heterologous sequences are shown as black lines. (A) Wild type MMLV; (B) Homologies between pCRIPenv-, pCRIPAMgag- (5) representing *gag/pol* and *env* expressing plasmids respectively, used in packaging lines  $\psi$ CRIP [5] and BING [50] cell lines, and MFG transfer vector [39] commonly used to generate producer clones in these cell lines; (C) Homologies between *pgag-polgpt*, *penvAm*, plasmids used to generate packaging cell line AM12 [51] and MFG; (D) Homologies between CeB and AF, plasmids used to generate the FLY packaging cell line [12] and MFG; (E) Homologies between pPAM3 [4], used to generate packaging cell line PA317 and MFG; (F) Homologies between pPAM3 [4], used to generate packaging cell line PA317 and LXS [52] and (G) Homologies between *pkat2gagpol*, *pkat2amenv* and *rkat43.2* used in this study

pPAM3 and MFG to 337 bp between pCRIPenv- and MFG. In addition to the homology between *gagpol* and transfer vectors, all of the *gagpol* and *env* vectors in split genome packaging cell lines ( $\psi$ CRIP, BING, FLY, AM12) share significant homology in the *gagpol* or *env* splice acceptor regions such that multiple homologous recombination events could lead to full RCR. These homologies range from 465 nucleotides between the CeB and ALF/AXF/AF vectors used in FLY cell lines to at least 3072 nucleotides between the pCRIPenv- and pCRIPAmgag- vectors used in  $\psi$ CRIP and BING cell lines. Therefore we intended to construct cell lines with no vector overlap.

We previously described the plasmids *pkat2gagpol* and *pkat2amenv* [16]. In these vectors *gagpol* and *env* are expressed as cDNAs in which expression is under control of CMV enhancer-promoter, a heterologous intron and polyA site. Here we have replaced the CMV enhancer-promoter with a hybrid enhancer-promoter containing the CMV enhancer and the MMLV promoter (Figure 1A) since the hybrid promoter-promoter consistently gave higher titers in the transient system and the expressed message is the same in either vector. Our previous retroviral transfer vector, *rkat3*, has also been modified by the replacement of the MMLV sequences from the R region through the ATG of *gag* with sequences derived from MMSV to generate *rkat43.2*, and by the deletion of viral *env* sequences between the transgene and the polypurine tract. Although the vector *rkat43.2* contains *gag* sequences and overlaps with *pkat2gagpol*, the ATG of *gag* in the vector has been converted to a TAG. Therefore, since any recombination with *pkat2gagpol* would have to occur downstream of the TAG, the resulting recombinant would be unable to express *gagpol*. The *env* plasmid shares no overlap with the retroviral vector *rkat43.2* (Figure 1B). Therefore, the use of these constructs significantly reduces the potential for the generation of RCR.

*kat* packaging cell lines were constructed in 293S cells by sequential transfection of *gagpol* and *env* plasmids to eliminate any possibility of recombination during transfection. Initially, a stable *gagpol* clone was created in order to provide the option of developing a panel of packaging clones expressing different retroviral envelopes. 293S cells were cotransfected with *pkat2gagpol* and the neomycin resistance plasmid, and clones were selected in geneticin. 100 *gagpol* transfectants were

picked, grown to confluency in 24-well plates, and the medium collected for reverse transcriptase (RT) assay. The 12 clones displaying the highest RT activity were further evaluated for production of viral particles by transient cotransfection with *pkat2amenv* and *rkat43.2CD4 $\zeta$* , a transfer vector encoding CD4 $\zeta$  [17]. The four clones with the best titers were then evaluated for stability by transient transfection with *pkat2amenv* and *rkat43.2CD4 $\zeta$*  at regular intervals over 12 passages (six weeks).

The *gagpol* clone designated 'TOM*kat*' was selected for further stable transfection with envelope plasmids since it was determined to be stable and RCR-free in long term passage, had the best growth characteristics, and had transient titers when cotransfected with *pkat2amenv* and *rkat43.2CD4 $\zeta$*  of  $1-2 \times 10^6$  transducing units (TU)/ ml on NIH-3T3 cells. To develop the amphotropic split plasmid packaging clone, TOM*kat* was cotransfected with *pkat2amenv* and a hygromycin resistance marker plasmid. Hyg B resistant transfectants were analyzed by FACScan for envelope protein expression with a primary rat anti-gp70 antibody 83A25 [27] and supernatants of the envelope-positive clones were assayed for reverse transcriptase (RT). Clones positive for reverse transcriptase activity and amphotropic envelope were grown up and assayed for production of viral particles by transient transfection with *rkat43.2CD4 $\zeta$* . The four clones with highest titers were passaged twice a week for six weeks without selection, and transiently transfected with *rkat43.2CD4 $\zeta$*  for virus production. These transient supernatants were routinely screened for RCR on *M. dunnii* and PG4 cells as described in methods, and all were negative. The clone with the highest titer of  $5-10 \times 10^5$ / ml on NIH-3T3 cells became the split-genome amphotropic packaging clone 'PUZ*ikat2*'. During six weeks of continuous culture of PUZ*ikat2* without selection we were able to produce transient viral supernatants with relatively consistent titers (Table 1). It should be pointed out that transient transfection is a highly variable testing method and the three-fold differences in titer seen in Table 1 over the course of six weeks are not unexpected. We later demonstrate that producer clones derived from this packaging clone are stable for six weeks without selection. Two additional split-genome packaging clones with different tropisms were constructed in a similar fashion. ALL*ikat2* (NBZ9.1 xenotropic envelope) [32] or

**Table 1. Tropism stability of split-genome packaging clones**

clone	env	vector	host range	Stability <sup>1</sup>			
				passage 1	passage 4	passage 8	passage 12
TOM <i>kat</i>	—	<i>pkat2gagpol</i>	—	100	276.1	178.0	210.9
PUZ <i>ikat</i>	ampho	<i>pkat2gagpol</i> <i>pkat2amenv</i>	h,p,c,d,m	100	142.0	41.0	63.0
ALL <i>ikat2</i>	xeno	<i>pkat2gagpol</i> <i>pkat2xenoenv</i>	h,p,c,d	100	n.d.	n.d.	116.1
STRA <i>kat</i>	10A1	<i>pkat2gagpol</i> <i>pkat210Aenv</i>	h,p,c,d,m	100	163.9	275.0	236.1

<sup>1</sup>Percent of titer or transduction at passage 0

STRAkat2 (10A1) [20] packaging cell lines were constructed by transfection of TOMkat with *pkat2xenoenv* or *pkat210A1env*, respectively. Clones were characterized as described above, except that ALLIkat2 transient supernatants were titered on Cf2Th cells, and STRAkat2 supernatants were characterized by transduction of human T lymphocytes. As was seen with PUZIk2, ALLIkat2 and STRAkat2 packaging lines were also stable when tested by transient transfection with *rkat43.2CD4 $\zeta$*  for virus production, and RCR-free when maintained continuously in selection-free medium for six weeks (Table 1).

## Generation of Producer Clones

Increasing the proviral copy number of the transfer vector within the packaging line by serial infection with supernatants derived from transient transfection has been shown to generate producer clones of high viral titer [33]. Supernatants from ecotropic producer lines have been used to infect amphotropic packaging lines to generate high titer amphotropic producer lines for human clinical trials. Even though both ecotropic and amphotropic packaging lines derived from 293 cells have been generated [13], human cells lack a functional ecotropic retrovirus receptor and producer clones cannot be made by ecotropic infection. Therefore, we generated high titer retroviral producer clones by serial transduction of the PUZIk2 packaging clone with transient, high-titer xenotropic retroviral supernatants produced by the cotransfection of tsA54 cells with *pkat2gagpol*, *pkat2xenoenv*, and *rkat43.2CD4 $\zeta$* . We generated producer clones encoding CD4 $\zeta$  [17] or CC49 $\zeta$  [19], chimeric immune receptor molecules that enable human primary CD4 $^{+}$  or CD8 $^{+}$  T cells to be redirected toward HIV infected cells or tumor cells expressing the antigen TAG72 [34]. Xenotropic CD4 $\zeta$  or CC49 $\zeta$  supernatants were generated by transient co-transfection and used to serially infect PUZIk2 cells six times (pings A through F). The population at ping F was subcloned and supernatants were evaluated for viral transduction of primary human CD8 $^{+}$  T lymphocytes rather than NIH-3T3 cells based upon our observation that NIH-3T3 titer was not predictive of human T lymphocyte transduction. The ten clones with the most efficient T cell transduction were then expanded and evaluated for stability and RCR for 10–11 passages. The viral titer of nine out of the ten clones was stable for the six week period (Figure 2). S+L– assays for RCR on PG4 cells did not detect RCR production by any of the clones.

## Human Endogenous Retrovirus (HERV) Evaluation

Retroviral packaging lines for MMLV vectors derived from murine cell lines co-package and transmit VL30 elements to target cells at levels equal to or greater than the viral vector [8]. Recombination with co-packaged *gagpol* transcripts during reverse transcription could lead to

the generation of partial recombinants [12]. An advantage of using human cells to package murine retroviral vectors is the absence of sequences homologous to VL30 elements. However, human cells express human endogenous retroviral elements (HERVs) [35]. Therefore, the potential mobilization of HERVs during MMLV retrovirus production in the 293 packaging cells was examined. The HERV-H family was evaluated based upon its presence at  $10^3$  copies per diploid genome and its expression as mRNA in 293 cells [36,37]. A sensitive PCR assay was developed to detect HERV-H genomes integrated in cellular DNA following infection of murine NIH-3T3 cells with recombinant MMLV generated by our human 293 producer cells. To maximize the potential identification of a HERV-H provirus,  $10 \times 2$  ml of high titer ( $2 \times 10^7$  TU/ml) retroviral supernatants of a prototype of the *rkat43.2CD4 $\zeta$*  retroviral vector were collected from 293 producer cells and used to serially transduce murine NIH-3T3 cells. After each serial transduction, an aliquot of cells was used for isolation of high molecular weight DNA, serial passage for the subsequent transduction, or generation of a frozen cell stock. Thus a series of ten populations of NIH-3T3s was generated which had undergone from one to ten transductions (A–J). Southern blot analysis of high molecular weight DNA prepared from each of the A–J transductions demonstrated increasing number of proviral integrations per cell, from approximately five per cell to greater than 50 per cell in F–J (Figure 3A).

PCR was then performed on genomic DNA using primers specific for HERV-H near the 5' primer binding site that generated a 905 base pair product (Figure 3B). The data show that we detected HERV-H sequences to 0.001 of a 293 genome equivalent in  $10^4$  naive NIH-3T3 cells, which (assuming  $10^3$  HERV-H copies per 293 cell) would be equivalent to a single copy HERV-H sequence in a total of  $10^4$  cells. The control naive NIH-3T3 cells were negative for HERV-H sequences. Transductions A–J were also negative for HERV-H (Figure 3C). Given that F–J contained more than 50 copies of CD4 $\zeta$  per cell, if the retroviral supernatants produced in 293 cells mobilized HERV-H sequences, the transmission frequency was less than one HERV-H sequence per  $5 \times 10^5$  proviral integrations. In contrast to murine packaging lines which are capable of transmitting murine VL30 and recombinant retroviruses at a 1:1 ratio, we demonstrate that our human packaging lines transmit HERV-H elements at a ratio at least as low as  $1:5 \times 10^5$ .

To verify the existence of transcribed HERV-H sequences in our 293 cells, we analyzed total cellular RNA by Reverse Transcription PCR (RT-PCR). RNA from NT2 cells, a teratocarcinoma cell line known to express high levels of HERV-H, was used as a positive control [36]. As shown in Figure 4A, the 293 RNA gave rise to a 905 base pair RT-PCR product identical to that produced by NT2 RNA. No bands were present in RT-PCRs performed on NIH-3T3 RNA. The relevant PCR controls for these experiments are displayed in Figure 4B. These data

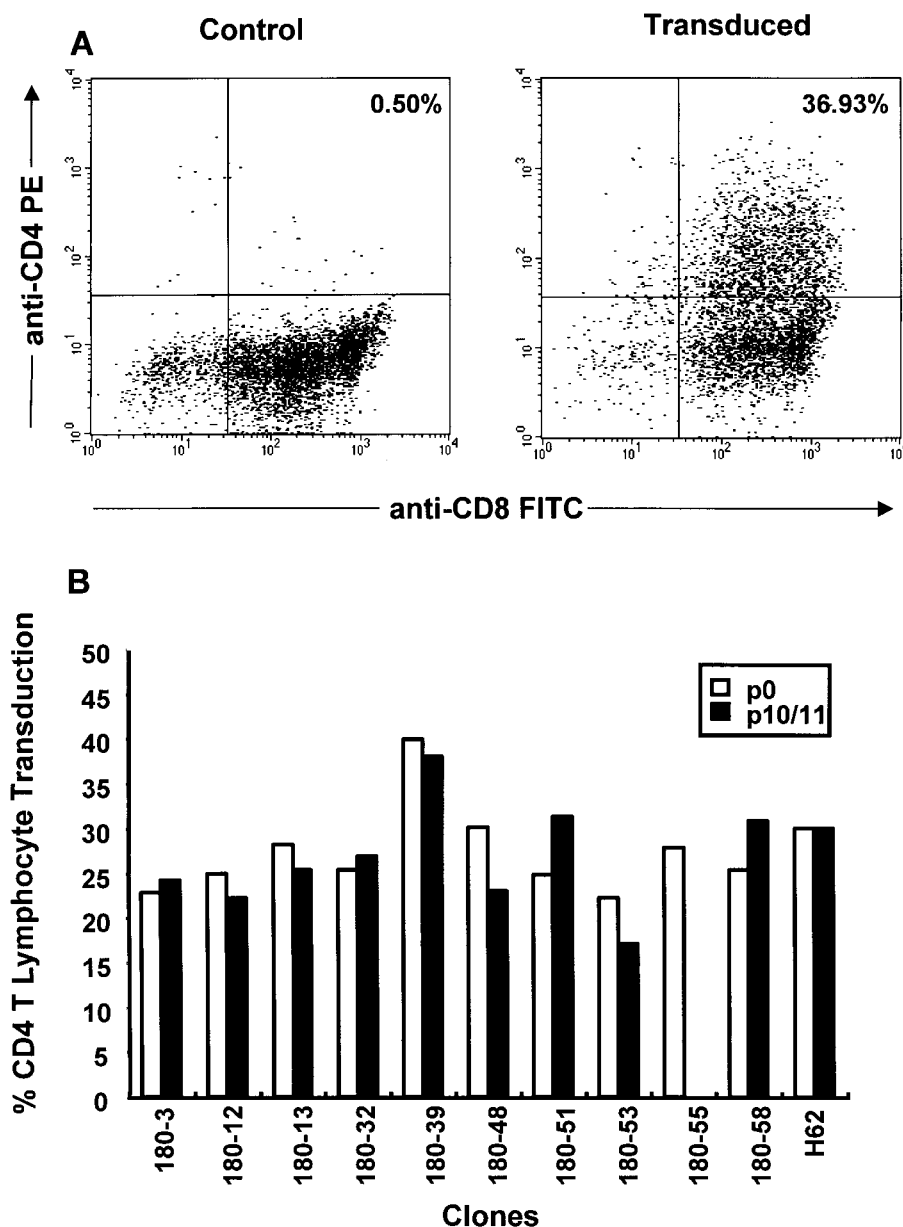


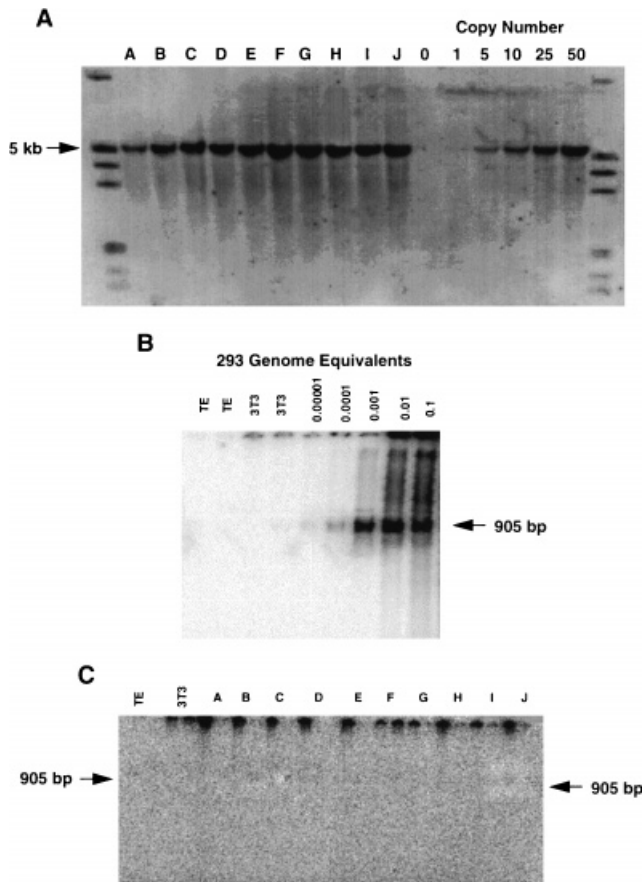
Figure 2. CD4 $\zeta$  transduction of human T lymphocytes by stable producers. The ten producer clones generated from Puzikat2 that yielded the greatest T lymphocyte transduction were evaluated for stability over 10–11 passages. Supernatants were collected and used to transduce primary human CD8 $^{+}$  T lymphocytes. (A) Two days post transduction T lymphocytes were evaluated for CD4 surface expression by FACS analysis using PE-labeled anti-CD4 monoclonal antibody. Non-transduced T lymphocytes were included as a FACS control. (B) T lymphocyte transduction by the ten most efficient clones at passages 0 and 11 expressed as % CD4 positive cells. H62 is a CD4 $\zeta$  producer generated in the single-genome packaging clone Puzikat1 used for comparison

demonstrated the presence of HERV-H RNA in 293 cells, yet no transmission of HERV-H sequences was detected.

### Clinical Scale-up

Having demonstrated that our 293-based amphotropic packaging lines yield producer clones of sufficient titer to efficiently transduce chimeric immune receptor genes into primary human T lymphocytes at laboratory scale, we investigated whether we could produce RCR-free vector at large scale (>50 l) for the clinic. Puzikat2 was serially infected with xenotropic CC49 $\zeta$  supernatants generated from a transient cotransfection of tsA54 cells

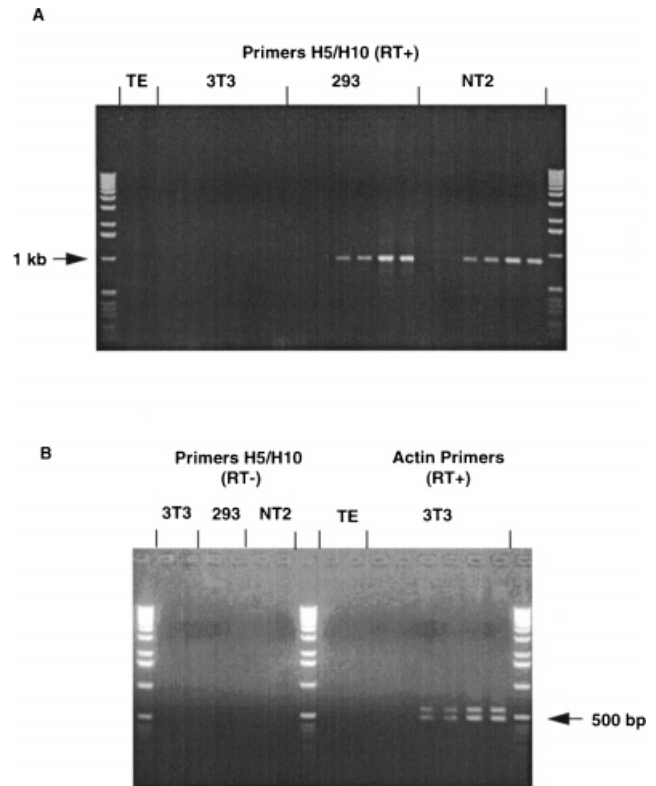
with *pkat2gagpol*, *pkat2xenoenv*, and *rkat43.2CC49 $\zeta$* . JC1-L1, a CC49 $\zeta$  producer clone which was isolated from the population by dilution cloning had titers of  $1\text{--}2 \times 10^7$  TU/ml on NIH-3T3 cells by FACS analysis. Analysis by Southern blot demonstrated that clone JC1-L1 had 10–11 copies of CC49 $\zeta$  (data not shown). At laboratory scale, this clone yielded stable virus production over 14 passages and was RCR-free by PG4 S+L- assay (data not shown). JC1-L1 was expanded to  $90 \times 850$  cm $^2$  roller bottles and 18 l of supernatant was harvested at 24 h intervals for three consecutive days. Five production runs were carried out for a total of 270 l of retroviral supernatant. In each production run 5% of the third



**Figure 3.** HERV-H analysis of NIH-3T3 cells serially transduced with CD4 $\zeta$  viral supernatants. (A) NIH-3T3 were serially infected a total of ten times. Genomic DNA was isolated from each transduced cell line (A–J) and from naive NIH-3T3 cells and digested with XbaI. Naive NIH-3T3 genomic DNA was also mixed with XbaI/NdeI digested *rkat43.2CD4 $\zeta$*  plasmid as a copy number reconstruction (0–50 copies). Hybridization was performed with a human CD4 probe that detected a 5 kb fragment. (B) PCR standard curve. 293 DNA mixed into NIH-3T3 DNA equivalent to 10<sup>4</sup> cells/reaction. Mixtures were analyzed by PCR with primers H5/H10 as described in Methods. (C) DNA equivalent to 10<sup>4</sup> NIH-3T3 cells/reaction from each serial infection (A–J) was analyzed by PCR in duplicate with primers H5/H10. Naive NIH-3T3 DNA and TE were included as PCR negative controls

harvest, 900 ml, and 10<sup>8</sup> post-production cells were assayed for RCR by PG4 S+L– assays and determined to be RCR-free. The NIH-3T3 titers of the large-scale lots averaged 5 × 10<sup>6</sup> TU/ml (Table 2).

The ability of these vector preparations to transduce human primary T lymphocytes at manufacturing scale was assessed by transduction of T lymphocytes from 13 patients with metastatic colorectal cancer. Patients were



**Figure 4.** HERV-H RNA analysis. Reverse transcriptase was used to generate cDNA from total cellular RNA, and PCR was then run to detect the presence of HERV-H sequences. (A) 0, 0.25 and 1.0  $\mu$ g of NIH-3T3, 293 and NT2 RNAs were analyzed with primers H5/H10 in duplicate. (B) Duplicate RT(-) controls on 1.0  $\mu$ g each of NIH-3T3, 293 and NT2 RNAs demonstrated the absence of DNA in the RNA preparations. 0, 0.25 and 1.0  $\mu$ g of NIH-3T3 RNA were analyzed with  $\beta$ -actin primers in duplicate to demonstrate the integrity of the RNA preparation. TE negative control lanes for each primer pair are indicated. All molecular weight markers are 1kb ladder

lymphapheresed and their T lymphocytes were stimulated with anti-CD3 and anti-CD28 coated beads, followed five days later by centrifugation in CC49 $\zeta$  retroviral supernatants. For seven of the patient samples this transduction was repeated at day seven. The T lymphocytes were expanded for a total of 14 days and frozen down for subsequent use in the clinical trial. T lymphocytes were analyzed for CC49 $\zeta$  gene transfer and gene expression by FACS and PCR (Figure 5). PCR analysis of six sets of T lymphocytes receiving two transductions had an average of 0.5 CC49 $\zeta$  proviral copies per cell, compared to 0.27 copies per cell in the six sets receiving only a single transduction. In each case the copy numbers determined by FACS were lower than those determined by PCR,

**Table 2.** Production of PUZ*kat2* retrovirus encoding CC49 $\bullet$  for clinical use

CC49 $\bullet$ production lots	volume collected	3T3 titers × 10 <sup>6</sup> TU/ml	sample volume	Producer cells tested	RCR test results
E3020	3 × 18 l	5.11	900 ml	10 <sup>8</sup>	negative
E3022	3 × 18 l	6.92	900 ml	10 <sup>8</sup>	negative
E3027	3 × 18 l	6.68	900 ml	10 <sup>8</sup>	negative
E3028	3 × 18 l	2.32	900 ml	10 <sup>8</sup>	negative
E3031	3 × 18 l	4.99	900 ml	10 <sup>8</sup>	negative
Totals	270 l	5.20	4.5 l	5 × 10 <sup>8</sup>	all negative

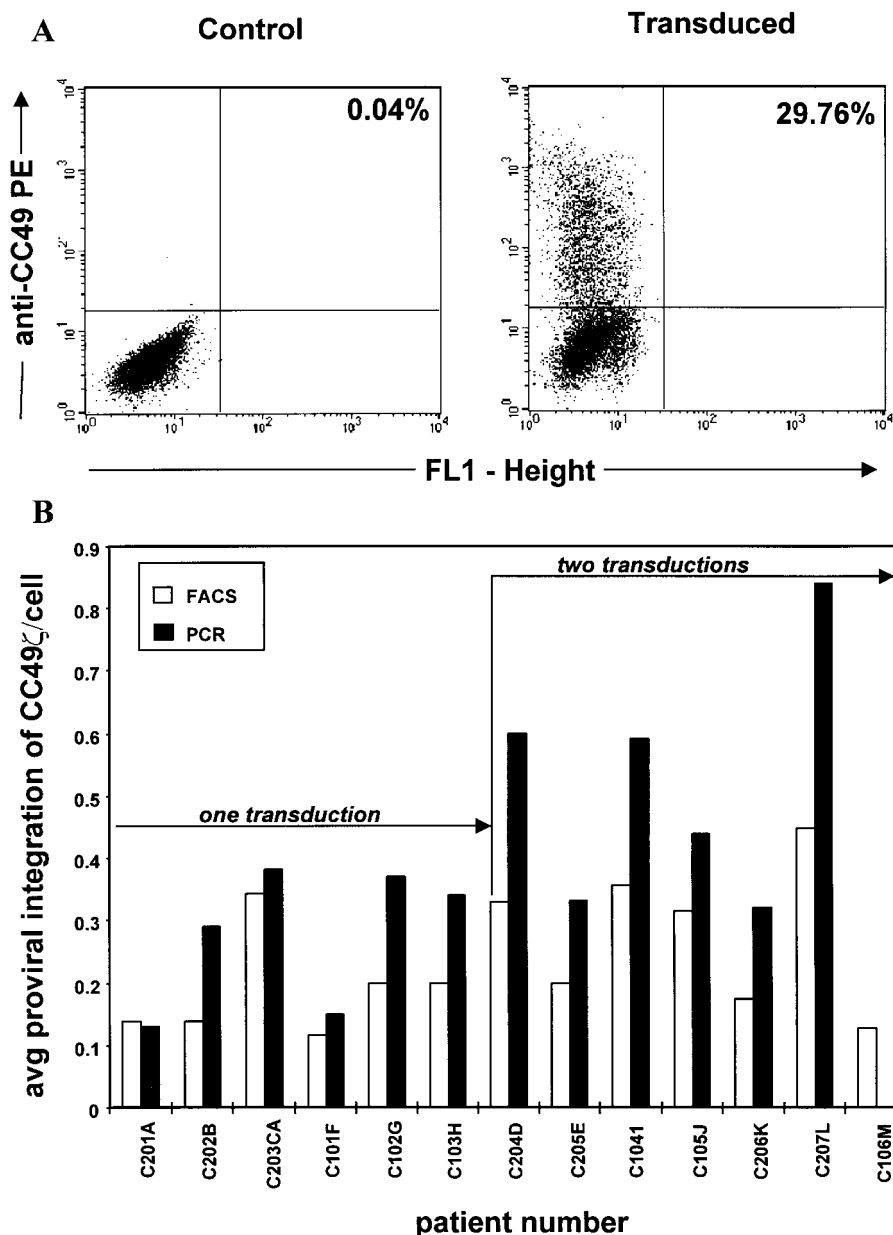


Figure 5. High efficiency T lymphocyte gene transfer in clinical protocol with PUZikat2 producers. (A) FACS profile of patient #C203CA T cell transduction with CC49 $\zeta$  retroviral supernatants. (B) Thirteen patients were lymphapheresed, their T lymphocytes isolated, followed by gene transfer with PUZikat2 CC49 $\zeta$  supernatants. T lymphocytes were evaluated for CC49 $\zeta$  surface expression by FACS and for gene integration by PCR. Average proviral copy number per transduced T cell was determined by assuming the frequency of integrated vector genomes follows a Poisson distribution as described in Methods

which would be expected since not all integration events lead to gene expression and the FACS assays do not differentiate between cells containing greater than one proviral integration event. At day 14, 5% of the supernatant and  $10^8$  cells were assayed by culture on *M. dunnii* followed by S+L- assay on PG4 cells. Each set of transduced T lymphocytes was determined to be RCR-free.

### Retroviral envelope impacts T lymphocyte transduction

In preparation for future clinical trials we examined the infectivity of different envelopes on the transduction of

human CD8<sup>+</sup> T lymphocytes. We defined infectivity as the average proviral copy number in  $10^6$  T lymphocytes transduced with 1 ml of supernatant, divided by particles per ml of supernatant. CD4 $\zeta$  producer populations were made by serial transduction of each of the three packaging clones PUZikat2, STRAk2 and ALLIk2 with transient CD4 $\zeta$ s supernatants of appropriately pseudotyped retrovirus. Producer clones were isolated and characterized for titer, stability and RCR as described above. Murine producer clones from single-genome amphotropic packaging line PA317, and human producer clones from our 293-based single-genome packaging line, PUZikat1, were used for comparison. Retroviral supernatants were collected from several clones of each

Table 3. Retroviral envelope and T lymphocyte transduction

Producer Clone #	Envelope	% CD4 $\zeta$ + T cells	Average proviral copy # per cell <sup>1</sup>	Particles ( $\times 10^8$ )/ml <sup>2</sup>	Infectivity ( $\times 10^{-4}$ ) <sup>3</sup>
3t3 1-genome	ampho	13.66	0.1468	7.632	1.92
293 1-genome	ampho	22.41	0.2537	8.839	2.87
#39	ampho	27.17	0.3170	16.183	1.96
#48	ampho	25.22	0.2906	12.547	2.32
#17	xeno	45.77	0.6119	8.948	6.84
#18	xeno	39.27	0.4987	7.621	6.54
#37	xeno	44.40	0.5870	7.745	7.58
#49	10A1	34.42	0.4219	2.617	16.10
#71	10A1	41.53	0.5367	2.696	11.90

<sup>1</sup>Average proviral copy number per transduced T lymphocytes assumes the frequency of integrated vector genome follows a Poisson distribution as described in Methods.

<sup>2</sup>The particles per ml of supernatant as determined by quantitative reverse transcriptase assay.

<sup>3</sup>Infectivity is defined as the average proviral copy number in  $10^6$  T lymphocytes transduced with 1 ml of supernatant, divided by particles per ml of supernatant.

envelope and used to transduce human CD8+ T lymphocytes. Infectivity was determined as a ratio of the average proviral copy number per cell (derived from CD4 expression by FACS) divided by the number of virus particles in the retroviral supernatants (determined by quantitative RT assay).

Table 3 shows the analysis of two to three clones from each of the human two-genome amphotropic, xenotropic and 10A1-tropic producer clones, as well as the clones from single-genome murine or human producers. When comparing the murine (3T3) and human (293) amphotropic producers it is interesting to note that while the T lymphocyte transduction is consistently two-fold higher in the 293 producers, the particle numbers are also twice as high, thus resulting in comparable infectivity rates for all of the amphotropic producers. In contrast we found that differences in the infectivity of supernatants from the different amphotropic, xenotropic and 10A1 clones were dependent upon the envelope. Xenotropic clone infectivity was consistently three-fold higher than amphotropic clone infectivity, and the 10A1 clones were twice as infective as the xenotropic clones. Therefore the 10A1 clones are the most potent and potentially most useful in future gene therapy protocols

## Discussion

The use of MMLV retroviral vectors in large scale immunotherapy clinical trials requires the development of efficient, high-titer, safe packaging and producer cell lines. The first packaging cell lines  $\psi$ 2 and PA317 were developed in mouse NIH-3T3 cell lines with single plasmid configurations. Despite the deletion of the  $\psi$  packaging signal in  $\psi$ 2, and the further deletion of *cis*-acting sequences in PA317, the generation of replication competent retrovirus was a problem in producer clones derived from these packaging lines [9]. The next generation of packaging clones, AM12, cre and crip, while going a step further and splitting the viral genome between two plasmids, still retained portions of *cis*-acting sequences. These packaging cell lines were prone to RCR production in scale-up [12,38], and additionally the

transduction of T lymphocytes was poor, only 6 to 9% [2,10,11].

In the past several years new retroviral packaging systems have been described by ourselves and others [12–16], which utilize split-genome plasmids in human cells such as 293 and HT1080. These systems have been shown to produce virus titers of  $10^6$ – $10^7$ TU/ml which is sufficient for gene transfer. Pear *et al.* described the creation of the BOSC cell line in 293T cells, wherein they serially transfected the pCRIPenv- and pCRIPgag-2 plasmids [13]. However these two plasmids are identical with the exception of mutations in the *env* and *gagpol* regions respectively (Figure 1B). They also share overlap with the MFG transfer vector [39]: therefore it is theoretically possible to generate RCR through multiple crossover events. The development of a safe packaging line was taken a step further by Cosset *et al.* who utilized *gagpol* and *env* expression plasmids that lessened viral sequences. While a small volume of viral supernatant was tested for RCR and found to be RCR-free, they reported that partial recombinant retroviruses expressing either MMLV *gagpol* or *env* proteins could be detected. This is not surprising given the significant homology between the two packaging plasmids, as well as with the transfer plasmid and portions of the 5' LTR that were retained in their HT1080 clone FLY [12] (Figure 1B). Stuhlmann and Berg using MMLV constructs previously demonstrated that homology in co-packaged retrovirus consistently generated RCR [40]. This raises the possibility for the emergence of RCR with the larger cell volumes required for clinical production.

In order to create a packaging system with an improved safety profile we first described the use of minimal expression plasmids in the 293T *kat* transient system [16], in which the plasmids contained only the coding sequence for *gagpol* or the appropriate *env* under the control of a heterologous promoter, with an intron and polyadenylation site. Recognizing that this transient *kat* system presented the combination of safety and high retroviral expression that could be utilized in the creation of RCR-free, stable, high titer packaging cell lines for clinical trials, ourselves and others [14,15] developed plasmid-based packaging and producer clones. Here we

have taken it a step further and demonstrated that PUZ $ikat2$  producer clones can be safely scaled up to lot sizes of 54 l, and these lots are currently in use for gene transfer into CD4 $+$ /CD8 $+$  T lymphocytes for immunotherapy of colorectal carcinoma [41].

It has been reported that MMLV-based retroviral vectors can transmit endogenous murine VL30 sequences at efficiencies equivalent to the retroviral vectors themselves [8,42,43]. While the use of human cell lines should further reduce the opportunity for RCR production due to the absence of MMLV-packagable retrotransposons which are found in murine cells [38], we nevertheless wished to explore the potential transmission of human endogenous retroviral elements (HERVs) in our 293 *kat* packaging cells. We chose to evaluate the transfer of the HERV-H family because of its  $10^3$  copies per diploid genome and its RNA expression in 293 cells. Utilizing our sensitive PCR assays we were able to demonstrate that the transmission frequency to NIH-3T3 target cells was less than 1 HERV-H sequence per  $5 \times 10^5$  proviral integrations. Furthermore, if transfer were to occur at a level below the detection of this assay, expression of HERV-H encoded proteins would be highly unlikely due to the abundance of stop codons in all three reading frames. Likewise, any hybrid viral sequences resulting from recombination between HERV-H sequences and MMLV vector or packaging genes would be expected to contain multiple stop codons in all reading frames at an average of one every 150 nucleotides [44]. Our analysis is in agreement with a previous study, in which retroviral particles themselves were examined by RT-PCR for the inclusion of HERVs. Patience *et al.* concluded that viral particles produced by the HT1080 based producer line FLYA4L3 contained no detectable amount of the HERVs (HERV-K, HuRT, type C, and RTVL-H) studied [38].

For the current CC49 $\zeta$  clinical trial 270 l of retroviral supernatant were produced. The clinical supernatants were determined to be RCR-free in assays run on 5% of each final harvest, and  $5 \times 10^8$  of the final production cells. Additionally, RCR assays were conducted on the transduced patient cells and culture fluids at the time of cryopreservation (14 days of expansion), and all were shown to be RCR-free. Based on theoretical considerations and the data presented here, we believe these *kat* cell lines represent the most advanced design with respect to biosafety for a retroviral producer used in clinical trials to date.

The field of T lymphocyte transduction has also made significant advances. A number of other groups have also reported transduction of human T lymphocytes in the 10–80% range [45–47]. However, these transductions required numerous manipulations such as alterations in phosphate levels, temperature shifts, centrifugation, multiple infections and binding to fibronectin. Minimal *ex vivo* culture and rapid turn around for T lymphocyte immunotherapeutic protocols is necessary in order to maintain T lymphocyte function [19,48]. Minimal cell processing requires retroviral supernatants of the highest titer and greatest potency. In the current CC49 $\zeta$  clinical

trial, the supernatant titer was sufficiently high ( $5 \times 10^6$  TU/ml on NIH-3T3 cells) that only one or two transductions with centrifugation was necessary on patient T lymphocytes to get efficient gene transfer in the absence of additional manipulations such as phosphate depletion or temperature shifts. This significantly reduced the amount of time that the cells needed to be expanded in culture in order to deliver a potentially therapeutic dose of modified cells.

Our studies with retroviral vectors pseudotyped with xenotropic or 10A1 envelopes demonstrated that these cell lines (ALL $ikat2$  and STRA $kat2$ , respectively) delivered genes to target T lymphocytes more efficiently on a per particle basis than the amphotropic packaging clone used in the current clinical trial, with STRA $kat2$  being the most potent. The mechanisms involved in the increased potency of these clones are currently under investigation. Our results suggest that STRA $kat2$  will be a highly attractive candidate retroviral packaging line for use in future T lymphocyte immunotherapy protocols.

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