RAPID COMMUNICATION

kat: A High-Efficiency Retroviral Transduction System for Primary Human T Lymphocytes

By Mitchell H. Finer, Thomas J. Dull, Lu Qin, Deborah Farson, and Margo R. Roberts

We describe a novel retroviral packaging system in which high titer amphotropic retrovirus was produced without the need to generate stable producer clones. kat expression vectors, which produce high levels of retroviral vector transcripts and retroviral packaging functions, were transduced into 293 cells followed by virus harvest 48 hours posttransfection. Viral titers as high as 3.8 proviral copies/cell/mL of frozen supernatant in 3T3 cells were obtained, 10- to 50-fold greater than transient viral titers reported using 3T3-based retroviral packaging lines. Cocultivation of primary human CD8+ T lymphocytes after transient transfection of 293 cells with kat plasmids resulted in transduction efficiencies of 10% to 40%, 5- to 10-fold greater compared to cocultivation with a high titer PA317 producer clone and significantly greater than previously reported results for transduction of primary human T lymphocytes with retroviral vectors. Virus produced using the kat system was shown to be free of detectable replication competent retrovirus by an extended provirus mobilization assay, demonstrating that this system is as safe as currently available stable packaging lines. The kat virus production system should be of general use for the rapid production of high titer viral supernatants, as well as for high-efficiency transduction hematopoietic cell types refractory to retroviral transduction.

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RETROVIRUS VECTORS have become the primary tool for gene delivery in human gene therapy applications.1 Their ability to deliver an unarranged, single-copy gene into a broad range of human somatic cells in primary culture makes them well suited for this purpose. Identification and subsequent deletion of the packaging signals (ψ) for murine retroviruses enabled the development of cell lines that supply the proteins necessary for production of infectious virions, but were unable to package their own viral genomic mRNA.2 Although early murine retroviral packaging lines were highly prone to copackaging and transfer of the ψ genome,3 or generation of replication-competent retrovirus (RCR),4 second-generation packaging lines that have dramatically reduced ability to generate RCR have been constructed.5-8

Even though the currently available packaging lines have yielded producer clones of sufficient titer to transduce human cells for gene therapy applications and have led to the initiation of several human gene therapy clinical trials,1 these lines are deficient in two areas. First, design of the appropriate retroviral vectors for a particular application requires the construction and testing of several vector configurations. It was necessary for Belmont et al9 to construct stable producer lines from 16 retroviral vectors to identify the vector that yielded the greatest titer and the greatest ADA expression level for use in hematopoietic stem cell transduction. A packaging system that would enable rapid, high-titer virus production without the need to construct stable producer lines would be highly advantageous, because it would eliminate of the laborious and time-consuming process of identifying high-titer producer clones for each of several constructs. Second, the transduction efficiency of primary cultures of mammalian somatic cells with high-titer amphotropic retrovirus varies considerably and is often reduced compared with NIH 3T3 cells.10-11 Purified human tumor infiltrating lymphocytes (TILs) and human peripheral blood-derived CD4+ and CD8+ T lymphocytes have been infected to levels of only 6% to 9%,12-14 and primate hematopoietic stem cells have only been infected to ≤1%.15,16 Although populations that are highly enriched for transduced cells can be obtained by selection in G418, selectable marker expression has been shown to have deleterious effects on long-term gene expression in vivo.17

This report describes a novel retrovirus packaging system, kat, that enables rapid production of recombinant amphotropic retrovirus vectors and eliminates the need for the generation of stable producer lines. Viral supernatants produced using this method yielded titers as high as 3.8 proviral copies/cell/mL of frozen supernatant, assayed on NIH 3T3 cells. In addition, transduction efficiencies obtained by cocultivation of kat virus-producing cells with primary human CD8+ T lymphocytes resulted in transduction efficiencies of 10% to 40%, significantly greater than previously reported. The kat system will greatly accelerate the evaluation of retroviral constructs directly in the appropriate primary cells without the need for stable producer clones. In addition, this approach will potentially lead to greater infectivity of cell types previously described as refractory to retroviral transduction.

MATERIALS AND METHODS

Construction of the retroviral packaging plasmids pkat. Plasmids encoding the functions required in trans for the packaging of retroviral vector transcripts were constructed in pkat expression vectors, pkat1 was constructed by insertion of the following elements, cloned by polymerase chain reaction (PCR) into pSKII (Stratagene, La Jolla, CA): the enhancer-promoter-first exon and splice donor of the human cytoomegalovirus (hCMV) immediate-early region,18 the splice acceptor of human α-globin gene exon 2, and the SV40 early region polyadenylation site and SV40 origin of replication.20 The fragment encoding the hCMV enhancer-promoter of pkat1 was removed and replaced with a PCR-generated fragment encoding the Moloney murine sarcoma virus (MMSV) U321 to generate pkat4. pkat2 was derived from pkat1 by replacement of the hCMV enhancer-promoter with a modified Moloney
murine leukemia virus (MMLV) U3 in which the enhancer fragment of the MMLV U3 had been deleted (pΔHB; gift of Paul Robbins, University of Pittsburgh, Pittsburgh, PA) and replaced with the PCR-generated C4 fragment of the hCMV IE enhancer,\(^\text{19}\)

The single genome pkat2ampac retroviral packaging plasmid (Fig 1, 1a1), similar to the \(\psi^-\) plasmid pAM3,\(^2\) contained the gag-pol genes from ecotropic MMLV and the envelope gene from the 4070A amphotropic MLV\(^3\) and was constructed by insertion of the \(\psi^-\) 3.813-bp Sac I/Sal I fragment of MMLV\(^4\) and the 4.140-bp Sal I-Nhe I fragment of pCRIPamgag-2 (gift of Olivier Danos, Institute
Pasteur, Paris, France), into pkat2. pkat1.gag/pol/ATG (Fig 1, la2) was constructed by addition of an Nhe I linker to the Sca I site of the A1I I-Sca I fragment of MMLV (+644 to +580), followed by ligation with an oligonucleotide that encoded nucleotides +621 to +644 (A1I I) of MMLV between the Neo I and Nhe I sites of pkat1. pkat1 lamin/ATG (Fig 1, la3) was constructed by ligation of the 325-bp HpaI-A1I I fragment and the 1.7-kb A1I I/Nhe I fragment from the 4070A env gene with a synthetic heptamer encoding the ATG the of MLV V4070A env gene to nucleotide +7 between the Neo I and Nhe I sites of pkat1.

Construction of rkat retroviral vectors. kat retroviral vectors (rkat) that produce high steady-state levels of packagable transcripts in 293 cells were based on a neo version of pZIPneoSVX.23 pZen.4 Viral gag sequences up to the Nal I site at nucleotide 1038 of MMLV22 and a polynucleotide linked downstream of the splice acceptor encoding EcoRI and Ava I cloning sites were added and the Xho I to Cla I fragment was deleted from pZIPneoSVX to yield rkat 1.27 The 5' LTR U3 region was replaced by a precise fusion of the hCMV enhancer-promoter to the MMLV R region to generate rkat 2 (Fig 1, l1b). rkat3 (Fig 1, l1b) was constructed by ligation of the HindIII to EcoRI fragment encoding the complete retroviral genome, of rkat 2, as well as the 3' flanking genomic DNA present downstream of the 3' LTR, into pkat1. rkat4 (Fig 1, l1b3) was constructed by replacement of the MMLV U3 region in the 5' LTR with the MMSV U3 from pkat4. cDNA expression cassettes were cloned downstream of the viral splice acceptor in the site normally occupied by the neo cassette in pZIPneoSVX. The retroviral vectors rkat2F3 and rkat2F15 encode chimeric receptors containing either the extracellular domain of human CD4 (F3) or a single-chain antibody against gp41 of HIV (F15), respectively, fused to the cytoplasmic domain of ß chain of the T-cell receptor (M.R.R., unpublished data; Dan Capon and Doug Smith, personal communication, January 1993).

RESULTS

Growth and transfection of established cell lines. 293 cells (ATCC CRL1573) and tsa201 cells26 were grown in DMEM (JRH Biosciences), 1 g/L glucose, 10% donor calf serum (Tissue Culture Biologics), and split 1:10 every 3 days. Cells, 1 × 10^6 per 10-cm plate, were plated 48 hours before transfection. PA317 cells were grown in DMEM (JRH Biosciences, Lenexa, KS), 4.5 g/L glucose, 10% fetal bovine serum (Tissue Culture Biologics), and split 1:20 every 3 days. Cells, 5 × 10^6 per 10-cm plate, were 24 hours before transfection. AM12 cells were grown in DMEM (JRH Biosciences), 4.5 g/L glucose, 10% newborn calf serum (Sigma, St Louis, MO), and split 1:10 every 3 days. Cells, 1 × 10^6 per 10-cm plate, were 24 hours before transfection. Ten micrograms of each retroviral vector, in the absence or presence of a packaging plasmid, was transfected as described above. Transduction efficiency was measured by flow cytometry at days 3, 7, 17, 21, and 28 posttransfection.29

Viral transduction and analysis of transduction. All 3T3 transductions were performed with viral supernatants that had been filtered through a 0.45-μm filter at the time of harvest, frozen at −70°C, and thawed before transfection. 3T3 cells, 1 × 10^6 per 10-cm plate, were infected in 5 mL of complete media containing viral supernatant and 8 μg/mL polybrene (Sigma). Polybrene-free media was added 24 hours after transduction, followed 24 hours later by flow cytometry to assay surface expression of CD4 (F3) or Fv (F15). Cells were removed from plates by addition of 1 mL of 40 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 150 mmol/L NaCl (CAT 1), pipetted into a single-cell suspension, and washed twice in phosphate-buffered saline/5% fetal bovine serum (PBS/FBS). The pellet was resuspended in 100 μL of PBS/FBS, 10 % of the appropriate dilution of fluorescein isothiocyanate (FITC)-conjugated OKT4A or mouse-anti-human Fv (Ortho Scientific, Raritan, NJ) was added and incubated for 40 minutes at 4°C. The cells were diluted to 1 mL with PBS/FBS, washed twice in PBS/FBS, resuspended in 1% formaldehyde, and analyzed by flow cytometry.

Growth and transduction of primary human CD8+ T lymphocytes. Human CD8+ T lymphocytes were purified from peripheral blood lymphocytes (PBL) and grown as described by Morecki et al.29 293 cells were plated and transfected as described above. Twenty-four hours posttransfection, the media were removed, replaced with T-cell growth media,33 and 2 to 4 hours later, the transfected 293 cells were cocultivated with 0.5 × 10^6 purified CD8+ T lymphocytes. Twenty-four hours after plating the 293 cells for the initial transfection, a second set of 293 cells was plated and transfected as described above. T lymphocytes were removed from the first cocultivation after 24 hours and transferred to the second transfected plate for cocultivation for an additional 24 hours. T lymphocytes were harvested, grown in the absence of purification, and sampled for transduction efficiency by flow cytometry at days 3, 7, 17, 21, and 28 posttransfection as described above.

293 cells produce high-titer retrovirus after transient transfection. Cocultivation of an ecotropic producer together with an amphotropic packaging line has been used to substantially increase viral titer of retroviral producer lines, due to increased copies of the viral vector genome resulting in increased steady-state levels of packagable transcripts.28 The drawback to the "ping-pong" approach was rapid formation of RCR, caused by the recombination between ψ− and viral vector or endogenous retroviral genomes during multiple rounds of viral replication.33 Our approach was to increase viral titer by construction of viral vectors and packaging-function plasmids that would produce high steady-state levels of packagable transcripts and packaging functions when introduced into the appropriate cell line. The human 293 cell line28 was an ideal candidate producer cell line because of the ability of the adenovirus 5 E1A and E1B gene products to trans-activate transfected genes.31 Even though the expression of 125 and 135 E1A gene products substantially inhibits transcription from retroviral long terminal repeats,32 constructs containing the human cytomegalovirus immediate early region (hCMV IE) enhancer-promoter were shown to be super-activated in the presence of these gene products.33 Therefore, kat plasmid expression vectors encoding retroviral packaging functions, as well as retrovirus vector plasmids were constructed in hCMV enhancer containing expression plasmids.

293 cells were assayed for their ability to produce retrovirus by cotransfection of pkat packaging and rkat retroviral vector plasmids, followed by virus harvest 48 hours posttransfection and analysis of virus production by transduction of 3T3 cells with viral supernatants. Cotransfection of pkat2ampac (Fig 1, la1) and rkat2F3 (Fig 1, l1b) into 293 cells yielded viral supernatants whose transduction efficiency was 48% CD4+ 3T3 cells/mL frozen supernatant (Table 1). Virus production was highly reproducible in four transfection experiments and was dependent on cotransfection of a packaging function plasmid and a retroviral construct. Supernatants obtained after transfection of 293 cells with rkat2F3 in the absence of pkat2ampac, or transfection of a CD4+ expression vector (pkat1F3) in the presence or absence of pkat2ampac, resulted in a 3T3 transduction frequency of ≤1% (Table 1).
aging plasmids into 293 cells, in duplicate, followed by addition of fresh filtration through a 0.2-pm filter and flash freezing on dry ice. Genomic DNA from infected 3T3 cells was digested with EcoRV and electrophoresed on an agarose gel, followed Southern blotting to a 32P-labeled 605-bp probe. Supernatants from mock-transfected 293 cells (lane 1), 293 cells transfected with rkat2F3 (lanes 2 and 3), 293 cells cotransfected with pkat2ampac and rkat2F3 (lanes 4 and 5), transfected with pkat1F3 (lanes 6 and 7), or cotransfected with pkat2ampac and pkat1F3 (lanes 8 and 9). Lanes 10, 11, 12, and 13: rkat1F3 plasmid DNA corresponding to three copies per cell of integrated provirus. Lane 14, EcoRV digested rkat2F3 plasmid DNA corresponding to three copies per cell of transfected plasmid.

Table 1. Virus Production After Transient Transfection of 293, tsa201, PA317, and AM12 Cells*

<table>
<thead>
<tr>
<th>Retroviral Construct</th>
<th>Packaging Vector</th>
<th>Packaging Cell Line</th>
<th>3T3 Transduction Efficiency/mL</th>
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</thead>
<tbody>
<tr>
<td>rkat2F3</td>
<td>pkat2ampac</td>
<td>293</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td>rkat2F3</td>
<td>pkat1 gagpol ATG</td>
<td>293</td>
<td>26.25 ± 0.1</td>
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<tr>
<td>rkat2F3</td>
<td>pkat1 amenv ATG</td>
<td>293</td>
<td>≤1</td>
</tr>
<tr>
<td>pkat1F3</td>
<td>—</td>
<td>293</td>
<td>≤1</td>
</tr>
<tr>
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<td>293</td>
<td>35.3 ± 2.255</td>
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<td>pkat2ampac</td>
<td>tsa201</td>
<td>37.8 ± 5.775</td>
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<tr>
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<td>293</td>
<td>69.75 ± 0.35</td>
</tr>
<tr>
<td>rkat2F3</td>
<td>—</td>
<td>PA317</td>
<td>1.0</td>
</tr>
<tr>
<td>rkat2F3</td>
<td>—</td>
<td>AM12</td>
<td>≤1</td>
</tr>
</tbody>
</table>

* rkat retroviral constructs were transfected with or without pkat packaging plasmids into 293 cells, in duplicate, followed by addition of fresh media 24 hours posttransfection. Virus was harvested 24 hours later by filtration through a 0.2-μm filter and flash freezing on dry ice. ** Transfected in quadruplicate. t Percent transduction, expressed as percent CD4+ (F3) or F4 (F15) 3T3 cells. 106 3T3 cells were infected with 1 mL flash-frozen and thawed supernatant, followed by flow cytometric analysis for surface expression 48 hours later. § 100 μL of supernatant was used in duplicate infections.

The high transduction efficiency observed by flow cytometry was confirmed by Southern blotting of integrated proviral DNA from infected 3T3 cells. High molecular weight genomic DNA from infected 3T3 cells was digested with EcoRV and the resulting Southern blots were probed with a 605-bp fragment encoding the transmembrane and cytoplasmic domains of the t antigen gene. EcoRV digestion of the transfected plasmid rkat2F3, which contains the CMV IE enhancer-promoter in the U3 LTR, yielded a 4.2-kb band (Fig 2, lane 14). After viral transduction and integration, duplication of the MMLV U3 region originally present only in the 3' LTR would result in a proviral EcoRV fragment of 3.6 kb (Fig 2, lanes 10 through 13), equivalent in size to a plasmid that contained two identical MMLV LTRs. A 3.6-kb band, identical to the band present in the rkat1F3 plasmid control lanes (which contains both 5' and 3' MMLV LTRs), and indicative of integrated provirus (Fig 2, lanes 4 and 5), was only observed following 3T3 cell transduction with supernatants from 293 cells cotransfected with rkat2F3 and pkat2ampac. Quantitative analysis of Southern blots by scanning densitometry revealed a transduction efficiency of 0.5 proviral copies/cell/mL of frozen viral supernatant, consistent with the results obtained by flow cytometry.

To further increase viral titer, vectors designed to replicate in SV40 T antigen-expressing cells were constructed. The construct rkat3F3 contained the entire rkat2F3 retroviral backbone and 700 bp of DNA downstream of the 3' LTR, followed by the SV40 polyadenylation signal and the SV40 origin of replication cloned into pkat1 (Fig 1, lane 2). rkat3F3 and pkat2ampac were cotransfected into tsa201 cells, a derivative of the 293 line which express the SV40 T antigen and enables replication of SV40 origin-containing plasmids,26 yielding supernatants having a transduction efficiency of 37.8% per 100 μL frozen supernatant (Table 1). Although 293 cells do not enable replication of SV40 origin containing plasmids, the titer obtained after cotransfection of rkat3F3 and pkat2ampac into these cells was 35% 3T3 transduction per 100 μL of supernatant, similar to the titer in tsa201 cells. Even in the absence of plasmid replication, the rkat3F3 vector yielded a titer equivalent to 3.4 proviral copies/cell/mL of frozen viral supernatant, sevenfold greater than rkat2F3.

The effect of cDNA insert on viral titer was examined by construction of rkat2F15, which contained the anti-gp41 (F15) chimeric receptor insert. Supernatants from 293 cells cotransfected with rkat2F15 and pkat2ampac had a transient titer of 70%, a 1.5-fold greater titer compared with rkat2F3. Southern blot analysis of DNA from rkat2F15-infect 3T3 cells probed with the t probe confirmed the flow cytometry titer for this construct (data not shown). The size of the EcoRV fragment observed on Southern blots for both
constructs was 0.6 kb less than the transfected plasmid, which showed that although both the F3 and F15 constructs encoded a hybrid 5' LTR, these constructs were able to be transcribed and packaged in 293 cells, as well as undergoing normal reverse transcription and integration upon 3T3 transduction to yield a provirus of the expected size.

Separation of the packaging functions into two genomes dramatically decreases the potential for the formation of RCR. 6-8 The expression vectors pkat1 gagpolATG and pka- tlamenvATG (Fig 1, la2 and 1a3), which encode the ecotropic gag-pol and 4070A amphotropic env cDNAs, respectively, were constructed in the hCMV IE based expression vector pkat1. The titer after cotransfection of the split genome packaging plasmids together with rkat2F3 into 293 cells was 27% per mL, a twofold decrease compared with the single genome constructs.

The transient viral titers produced by cotransfection of the kat packaging and retroviral constructs into 293 cells were compared with transient viral titers produced by transfection of the kat retroviral constructs into the murine amphotropic packaging lines PA317 3 and AM12.7 Transfection of rkat2F3 into PA317 cells yielded transient titers on 3T3 cells of 1% per mL frozen supernatant, in contrast to the 48% per mL frozen supernatant transient titer after cotransfection of rkat2F3 and pkat2amapc into 293 cells. The transient titer of supernatants from AM12 cells transfected with rkat2F3 was undetectable (Table 1).

High-efficiency transduction of primary human CD8⁺ T lymphocytes. Using conventional retroviral producer lines, only low-efficiency transduction of primary human CD8⁺ T lymphocytes has been reported. 12-14 We compared the transduction efficiency of primary human CD8⁺ T lymphocytes with retrovirus produced transiently using the kat system to the transduction efficiency with retrovirus produced from a high titer, stable producer clone obtained by infection of PA317A with supernatants from an ecotropic rkat4F3 retroviral producer line. rkat4F3 (Fig 1, 1b3) is similar in structure to rkat2F3. However, the hybrid CMV IE-R-U5 5' LTR has been replaced with an MMSV 5' LTR, derived from the N2 vector, 20 to ensure high titers in murine packaging lines. Fifty clones were screened and one clone (RT40.39) was isolated whose titer was 35% 3T3 transduction for 100 μL of frozen supernatant, equivalent to transduction of 3.5 proviral copies/cell/mL. Primary human CD8⁺ T lymphocytes that were ≥96% CD3⁺, CD8⁺ and ≤0.5% CD4⁺, F₄ were isolated (Fig 3A). The transduction efficiency measured 3 days after a 48-hour cocultivation with the PA317 producer clone 40.39 was 8% CD4⁺ cells, determined by flow cytometry (Fig 3B). The fraction of transduced cells remained unchanged after growth for 28 days, showing stable transduction and expression. Transduction efficiency was dependent on the T-lymphocyte donor and varied between 1% and 8% (data not shown).

In comparison, the transduction efficiency of CD8⁺ T lymphocytes by cotransfection of pkat2amapc and rkat2F3 into 293 cells followed by 48-hour cocultivation resulted in a 36.4% transduction efficiency, measured 17 days posttransduction (Fig 3C). High-efficiency transduction was dependent on cotransfection of 293 cells with pkat2amapc and rkat2F3. Transfection of rkat2F3 in the absence of pkat2ampc (Fig 3D) and cotransfection of an F3 expression vector pkat1F3 with pkat2amapc (data not shown) or pkat1F3 alone (data not shown) yielded only background levels (1% to 2%) of CD4⁺ T lymphocytes.

The kat cocultivation strategy was also used to transduce rkat2F15 into primary CD8⁺ T lymphocytes, to verify that the high-efficiency transduction observed was not specific to the CD4⁺ construct. 293 cells cotransfected with rkat2F15 and pkat2amapc were cocultivated with primary CD8⁺ T lymphocytes. Flow cytometry 17 days postcocultivation showed 16% F₄ cells (Fig 3E), demonstrating that high-efficiency transduction could be obtained reproducibly using the kat transduction system. The efficiency of kat-mediated transduction for both constructs varied between 10% and 40% transduced T lymphocytes, depending on the T-lymphocyte donor. However, 5- to 10-fold greater transduction efficiencies with the kat system compared with stable PA317 producers were reproducibly observed in each experiment (data not shown).

Although kat-mediated transduction of CD8⁺ T lymphocytes was highly efficient, the CD4⁺ fusion construct displayed an unusual behavior. Flow cytometry 3 days after cocultivation of 293 cells cotransfected with rkat2F3 and pkat2amapc [rkat2F3 (+), Fig 4A] showed 75% CD4⁺ T lymphocytes. However, flow cytometry on day 7, day 12, day 17, and day 28 postcocultivation showed that the percentage of CD4⁺ T lymphocytes decreased to 38% over the subsequent 14 days and reached a steady-state level at day 17. The percent CD4⁺ T lymphocytes was unchanged for additional 11 days and remained unchanged 4 weeks later (data not shown), reflecting stable transduction. In contrast, the percent CD4⁺ T lymphocytes after cocultivation of 293 cells transfected with rkat2F3 in the absence of pkat2amapc [rkat2F3 (-), Fig 4A] or cotransfection a nonretroviral F3 expression vector with pkat2amapc [pkat1F3 (+), Fig 4A] decreased to background levels by day 12 postcocultivation. EcoRV digestion of DNA prepared from cells 28 days posttransduction, followed by Southern blotting and hybridization to the 605-bp probe, showed the 3.6-kb fragment characteristic of integrated provirus at a signal intensity corresponding to 0.3 to 0.4 proviral copies/cell, confirming the flow cytometry data at 17 and 28 days posttransduction (data not shown). This behavior was significantly less pronounced with the anti-gp41 5' construct (rkat2F15), where the controls decreased to background levels by day 7 (Fig 4B).

Supernatants harvested from infected CD8⁺ T lymphocytes 28 days post kat transduction were used to infect 3T3 lines that encoded a single copy of the BAG virus and screened for RCR by a provirus mobilization assay. 5,35 Supernatants harvested 48 hours posttransduction from the BAG-3T3 cells were used to infect naive 3T3 cells. X-gal positive 3T3 cells 48 hours posttransduction of naive 3T3 cells were absent, as well as G418-resistant clones after transduction of naive 3T3 cells and selection for 14 days in G418. The sensitivity of this assay was further enhanced by growth of the infected BAG-3T3 cells for an additional 3 weeks, followed by transduction of naive 3T3 cells with BAG-3T3 supernatants. Neither X-gal–stained cells nor G418-resistant clones were identified, which showed that
DISCUSSION

We describe a novel packaging system, designated kat, that results in rapid production of high-titer recombinant retrovirus, thereby eliminating the need to generate stable producer lines. In addition, this approach enables efficient transduction of primary human T lymphocytes. kat expression plasmids, designed to produce high steady-state levels of retroviral packaging functions and vector transcripts, were introduced into 293 cells by calcium phosphate cotransfection. Frozen viral supernatants harvested 48 hours posttransfection of retroviral constructs and single genome packaging constructs were capable of transduction of up to 3.8 proviral copies/cell/mL in NIH 3T3 cells. After one cycle of freezing at −70°C (D.F., unpublished data, April 1993), the titer of viral supernatants decreases approximately twofold. Therefore, titers as high as 7.5 proviral copies/cell/mL for fresh viral supernatants can be obtained using the kat system, at least twofold greater than those obtained using stable producer clones derived from ψCRIP, PA317, or AM12. Although transient titers greater than those reported here have been described for retroviral vectors containing the neo gene produced in PA317 or AM12, it would not be unusual for vectors containing the F3 (CD40 insert) to yield low transient titers when produced in murine packaging lines.

A transient retrovirus production system in which retrovirus was produced by cotransfection of packaging functions and a vector plasmid into COS cells was recently described, in which virus production was completely dependent on plasmid replication of both retrovirus vector and packaging functions. neo titers of $0.9 \times 10^5$ colony-forming units/mL of freshly harvested supernatants after COS cell transfection of packaging functions and viral vectors were reported, viral titers 5- to 50-fold less than those achieved with the kat system in the absence of plasmid replication.

**Fig 3.** Flow cytometry of transduced primary CD8+ T lymphocytes 28 days posttransduction. T lymphocytes were purified from PBMCs, transduced with the appropriate retroviral vector encoding the chimeric receptors F3 or F15, and analyzed for surface expression by flow cytometry with the appropriate antibodies. (A) Uninfected primary human CD8+ T lymphocytes stained with CD8 and CD3 antisera. (B) CD8+ T lymphocytes transduced by cocultivation with the PA317 producer and stained with CD4 and CD3 antisera. (C) CD8+ T lymphocytes transduced by cocultivation with 293 cells after pkat2ampac and rkat2F3 transfection, stained with CD4 and CD3 antisera. (D) CD8+ T lymphocytes transduced by cocultivation with 293 cells after rkat2F3 transfection, stained with CD4 and CD3 antisera. (E) CD8+ T lymphocytes transduced by cocultivation with 293 cells after pkat2ampac and rkat2F15 transfection, stained with Fc and CD3 antisera. The axes are labeled with the appropriate antibody and conjugated fluorophore.
HIGH-EFFICIENCY RETROVIRAL TRANSDUCTION

Fig 4. Time course of kat-mediated T-cell transduction. Retroviral or plasmid expression vectors encoding either F3 (CD4+) or F15 (anti-gp41) constructs were cotransfected with (+) or without (−) pkat2Fampac into 293 cells, followed 24 hours later by cocultivation with CD8+ T lymphocytes for 48 hours. T lymphocytes were purified and flow cytometry was performed at day 3, day 7, day 12, day 17, and day 28 postcocultivation. (A) F3 constructs. ○, rkat2F3 (+); □, rkat2F3 (−); △, pkat1F3 (+). (B) F15 constructs. ○, rkat2F15 (+); □, rkat2F15 (−); △, pkat1F15 (+).

Primary CD8+ T lymphocytes were transduced at efficiencies of 10% to 40% using the kat system and rkat2F3. These levels were 5- to 10-fold greater than a high-titer PA317 producer and significantly greater than previously reported.12-14 Transduction at these levels eliminates the need for the retroviral vector to encode the neo gene, which has adverse effects on gene expression in vivo.15 Based on a sevenfold increase in titer on 3T3 cells, significantly greater infectivity of primary T lymphocytes could be obtained using the rkat3F3 construct. The kat system could be useful for transduction of human or primate hematopoietic stem cells, which are currently difficult to transduce at high efficiencies.16,17 Although we do not currently understand the mechanism of high-efficiency kat transduction, during cocultivation, there is the potential for surface molecules on 293 cells and CD8+ T lymphocytes to make contact, thereby activating the T lymphocytes and rendering them more susceptible to retroviral transduction. For example, 293 cells express the LFA-3 molecule on their surface (Gib Otten, personal communication, May 1993) and the surface molecule CD2, the receptor for LFA-3, is found on CD8+ T lymphocytes.37 Interaction of these molecules, or others, may potentiate increased transduction of CD8+ T lymphocytes.

One unusual feature of kat-mediated transduction of CD8+ T lymphocytes was the initial high levels of CD4+ T lymphocytes observed even in the absence of retroviral construct or packaging functions, which decreased to background levels at 2 weeks posttransduction. This phenomenon was more pronounced with the CD4+ construct and could be caused by efficient passive antigen transfer during cocultivation,38 possibly facilitated in part by the high levels of class II major histocompatibility complex (MHC) molecules present on activated T lymphocytes and the interaction between CD4 and class II MHC molecules. We have monitored transduced T lymphocytes by flow cytometry for up to 2 months postcocultivation and have observed that the levels observed at day 17, after the initial decrease, were maintained. These results have been confirmed by Southern analysis, using a restriction digest that can differentiate between integrated provirus and transfected plasmid.

Recently, we have obtained preliminary data that showed kat-mediated transduction of mouse bone marrow isolated 2 days post 5-fluorouracil treatment, followed by injection into lethally irradiated recipients, resulted in high-efficiency transduction of day 14 spleen colonies (CFU-S). In addition, we have observed multilineage transduction and expression in lethally irradiated recipients examined 7 weeks posttransplant at a frequency identical to that obtained using a high-titer stable producer (K. Zsebo, K. Cooke, M. Finer, unpublished data, July 1993). In addition, human bone marrow-derived CD34+ are also efficiently transduced using the kat system (K. Zsebo, K. Cooke, T. Dull, M. Finer, unpublished data). These preliminary data, together with the human T-lymphocyte transduction data presented here, suggest that kat-mediated transduction of hematopoietic cell types will be highly useful in both basic research and potentially in gene therapy settings.

NOTE ADDED IN PROOF

Pear et al (Proc Natl Acad Sci USA 90:8392, 1993) have recently described production of high-titer retroviral supernatants after transient transfection of BOSC 23, a retroviral producer line derived from the tsa 201<sup>CC</sup> cell line.

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