



## VIRAL TRANSFER TECHNOLOGY

## BRIEF COMMUNICATION

# Plat-E: an efficient and stable system for transient packaging of retroviruses

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A potent retrovirus packaging cell line named Platinum-E (Plat-E) was generated based on the 293T cell line. Plat-E is superior to existing packaging cell lines regarding efficiency, stability and safety. The novel packaging constructs utilized in establishment of Plat-E ensure high and stable expression of viral structural proteins. Conventional packaging constructs made use of the promoter of MuLV-LTR for expression of viral structural genes *gag-pol* and *env*, while our packaging constructs utilized the EF1 $\alpha$  promoter, which is 100-fold more potent than the MuLV-LTR in 293T cells in combination with the Kozak's consensus sequence upstream of the initiation codon resulting in high expression

of virus structural proteins in Plat-E cells. To maintain the high titers of retroviruses under drug selection pressure, we inserted the IRES (internal ribosome entry site) sequence between the gene encoding *gag-pol* or *env*, and the gene encoding a selectable marker in the packaging constructs. Plat-E cells can stably produce retroviruses with an average titer of  $1 \times 10^7$ /ml for at least 4 months. In addition, as we used only the coding sequences of viral structural genes to avoid inclusion of unnecessary retrovirus sequences in the packaging constructs, the probability of generating the replication competent retroviruses (RCR) by recombination can virtually be ruled out. Gene Therapy (2000) 7, 1063–1066.

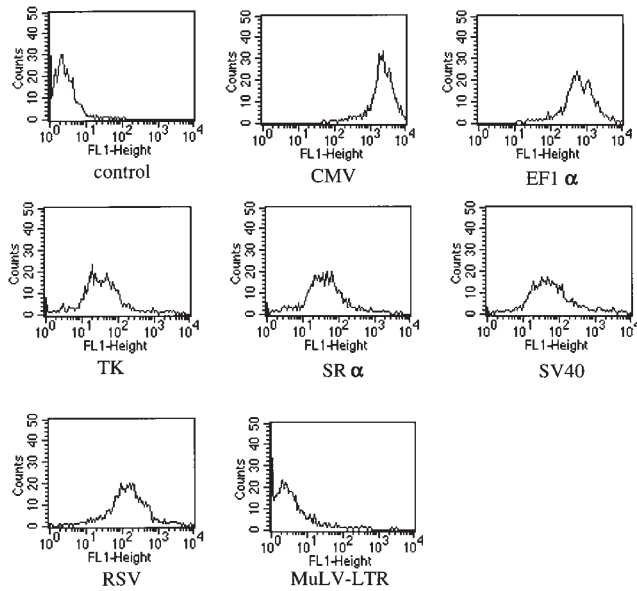
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Retroviral vectors and packaging cells are important tools for gene transfer applications. Introduction of retroviral vectors containing the gene of interest into suitable packaging cells enables production of infectious retroviruses, and these particles can infect target cells and stably transmit the gene of interest into chromosomes. In conventional strategies, stable high producers of a retrovirus vector harboring a gene of interest were established by transducing the retrovirus construct into NIH3T3-based packaging cells such as PA317,<sup>1</sup> and 2–3 months were usually needed to acquire high producers. Pear *et al*<sup>2</sup> developed a unique packaging system by which high titer retroviruses can be obtained in 3 days by transient transfection. The expression of viral structural genes was driven by the MuLV LTR in Bosc23 cells. For transient transfection, the combination of Bosc23 cells and the pMX-neo vector<sup>3</sup> produced  $1-3 \times 10^6$ /ml viruses, assessed based on the number of neomycin resistant colonies of the infected NIH3T3 cells (data not shown). Since Bosc23 cells carry the large T antigen, we attempted to increase titers of the retroviruses by introducing the SV40 origin to the pMX vector for amplification of the vector. However, this proved unfeasible (data not shown), suggesting that the limiting factor was the expression level of the viral structural proteins in the packaging cells. Bosc23 was obtained by cotransfection of the plasmid encoding *gag-pol* together with the plasmid encoding the hygromycin-resistant gene and the plasmid encoding *env*

together with the plasmid encoding another selection gene GPT (guanine phosphoribosyl transferase), one after the other. Therefore, expression of selectable markers did not guarantee the expression of *gag-pol* or *env* genes, which may account for the instability of the cells in producing high-titer retroviruses.

A similar packaging cell line Phoenix-E<sup>4</sup> has also been developed. In Phoenix-E cells, the plasmids encoding the *gag-pol* and *env* genes were cotransfected with selection markers, which did not warrant the stable expression of the *gag-pol* and *env* genes in the selection drug, hygromycin and diphtheria toxin, respectively. There were several improvements in the Phoenix-E cells when compared with Bosc23 cells. First, the RSV and CMV promoters, which are much stronger than MuLV-LTR in 293T cells, were used to express the *gag-pol* and *env* genes, respectively. Second, the internal ribosome entry site (IRES)<sup>5</sup> sequence was used to express *gag-pol* and a cell surface marker CD8 simultaneously which enables sorting of high expressers of the *gag-pol* gene. However, one needs to sort cells from time to time to maintain the expression levels of the *gag-pol* gene.

To design a packaging cell line which can stably produce retroviruses with high titer, we searched for a strong promoter to drive expression of viral structural proteins in 293T cells using the FACS-GAL assay.<sup>6</sup> Among seven promoters tested, the EF1 $\alpha$  and CMV promoters induced high expression of *lacZ* (Figure 1). The activities driven by these promoters were 100-fold higher than those by LTR utilized in Bosc23 cells, and even exceeded those by SV40 and SR $\alpha$  promoters, which enable amplification of vectors in 293T cells expressing



**Figure 1** Activities of various promoters in 293T cells. The activities of the seven promoters, SV40, SR $\alpha$ , EF1 $\alpha$ , RSV, TK, MuLV LTR and CMV were evaluated by expression of lacZ under the control of each promoter in 293T<sup>r</sup> cells by the FACS-GAL assay as described.<sup>6</sup> Briefly, cells ( $1 \times 10^6$ ) transfected with each promoter construct were suspended in 50  $\mu$ l of phosphate buffered saline (PBS), then incubated for 5 min at 37°C. FDG (fluorescein di- $\beta$ -D-galactopyranoside; Molecular Probes, Eugene, OR, USA) was dissolved in distilled water, warmed at 37°C and 50  $\mu$ l of 2 mM FDG solution was added to 50  $\mu$ l of cell suspension. After 1 min of incubation at 37°C, 1 ml of PBS was added followed by incubation on ice for 2 h. To stop the reaction, 20  $\mu$ l of 50 mM PETG (phenylethyl- $\beta$ -D-thiogalactoside; Sigma, St Louis, MO, USA) was added, and the preparation was placed on ice until being subjected to FACS analysis.

the SV40 large T antigen. Because we thought that the promoters of housekeeping genes were more suitable than the viral promoters for driving stable gene expression in mammalian cells, we used the EF1 $\alpha$  promoter to express the viral structural proteins in 293T cells (Figure 2). In addition, IRES was inserted between the gag-pol or env gene and the selection marker in the packaging constructs described here. Therefore, expression of the selection marker is a direct reflection of gag-pol or env expression in the same cells.

Packaging constructs pEnv-IRES-puro<sup>r</sup> and pGag-pol-IRES-bs<sup>r</sup>, which were constructed as described above, were sequentially transfected into 293T cells and 50 subclones resistant to both puromycin and blasticidin were isolated. Among 50 subclones, clone 1 named Platinum-E (Plat-E) produced the retrovirus which had the highest infection efficiency and was used for further analysis. The titer of the retroviruses was about  $1 \times 10^7$ /ml when tested on NIH3T3 cells, using serially diluted virus supernatants of Plat-E cells transfected with pMX-lacZ (data not shown). We next compared early passages of Plat-E cells with those of Bosc23 cells and Phoenix-E cells with regards to long-term stability to produce high-titer retroviruses by transient transfection (Figure 3). Culture conditions of the three packaging cell lines were as follows: Bosc23 cells were grown in DMEM with 10% fetal bovine serum containing the GPT selection reagents as indicated by the manufacturer (Specialty Media, Lavallette, NJ, USA). Phoenix-E cells were sorted by FACS for expression of CD8 and maintained in DMEM with 10%

pGag-pol-IRES-bs<sup>r</sup>



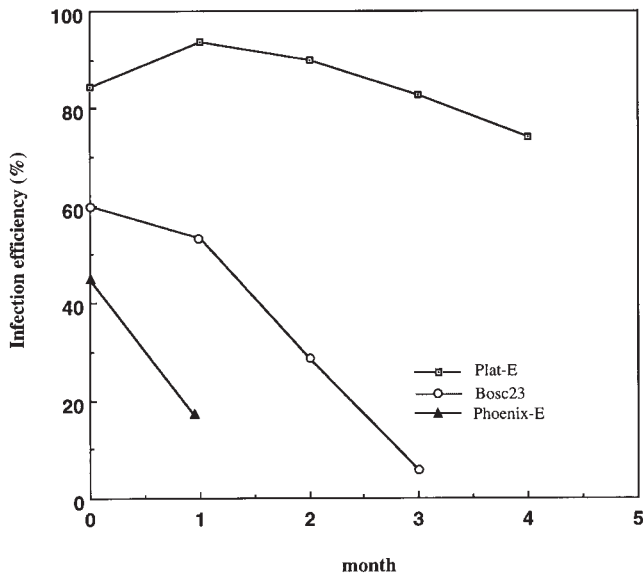
pEnv-IRES-puro<sup>r</sup>



**Figure 2** Schematic diagrams of packaging constructs. The packaging constructs used for development of Plat-E are shown. The fragment carrying the selectable marker, the blasticidin resistant gene (bs<sup>r</sup>) or the puromycin resistant gene (puro<sup>r</sup>), was obtained by PCR using a pair of oligonucleotides (for bs<sup>r</sup>: 5'-AAAACATTTAACATTTCTCAACAAG-3', 5'-ACGCGTGCAGTAAATTTTCGGGTATATTGAGTG-3', for puro<sup>r</sup>: 5'-ACCGAGTACAAGCCCACG-3', 5'-ACGCAGATCTTCAGGCACCGGCTTG-3'), and were inserted into the NcoI and Sall site (for bs<sup>r</sup>), or in the NcoI and BglII site (for puro<sup>r</sup>) of pMX-IRES-EGFP.<sup>8</sup> The fragments containing the IRES sequence and either of bs<sup>r</sup> and puro<sup>r</sup> were excised from the vector by NotI and Sall for bs<sup>r</sup>, or NotI and BglII for puro<sup>r</sup>, respectively. The viral structural genes, gag-pol and env were amplified by PCR, using the MoMuLV genome as a template, and the oligonucleotide primers were used as followed. Each primer contains either the EcoRI site or the NotI site (underlined) and the 5' primers also contain a Kozak's consensus sequence GCCGCCACC located upstream of the initiation codon. gag-pol: 5'-CGAATTCGCCGCCACCATGGGCCAGACTGTTACCACTCCCTTAA-3'; 5'-TACGCGGCCGCTCTGAGCATCAGAA GAA-3'; env: 5'-cGAATTCGCCGCCACCATGGCGCGTTCAACGCTCTCAAAA-3'; 5'-TACGCGGCCGCTATGGCTCGTACTCTAT-3'. The resulting PCR fragments were digested with the EcoRI and the NotI fragment. Finally, the fragment containing the viral structural genes, and the fragment containing the IRES sequence and the selection marker were inserted downstream of the EF1 $\alpha$  promoter in the pCHO vector, a derivative of pEF-BOS.<sup>9</sup> For construction of the pGag-pol-IRES-bs<sup>r</sup>, pCHO was digested with BamHI, and converted to a blunt end by Klenow reaction, and then ligated with Sall linker (Stratagene, La Jolla, CA, USA). The EcoRI-NotI fragment of gag-pol, and the NotI-Sall fragment of IRES-bs<sup>r</sup> were inserted into the EcoRI and the Sall site of pCHO by triple ligation. To construct pEnv-IRES-puro<sup>r</sup>, pCHO was digested with EcoRI and BamHI, and the EcoRI-NotI fragment of env, and the NotI-Sall fragment of IRES-puro<sup>r</sup> were inserted into the EcoRI and the BamHI sites of pCHO. Packaging constructs pEnv-IRES-puro<sup>r</sup> and pGag-pol-IRES-bs<sup>r</sup> were sequentially transfected into 293T cells using Fugene (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. One day after transfection with pEnv-IRES-puro<sup>r</sup>, 293T cells were selected in DMEM containing 1  $\mu$ g/ml puromycin. The selected cells were then transfected with the pGag-pol-IRES-bs<sup>r</sup> vector, and subcloned in the presence of puromycin and blasticidin (10  $\mu$ g/ml). The selected clones were tested for their potential to produce retroviruses. EF1 $\alpha$ , EF1 $\alpha$  promoter; IRES, internal ribosome entry site; bs<sup>r</sup>, blasticidin resistant gene; puro<sup>r</sup>, puromycin resistant gene.

fetal bovine serum containing hygromycin (300  $\mu$ g/ml) and diphtheria toxin (1  $\mu$ g/ml) for 1 week, then cells were transferred to DMEM with 10% fetal bovine serum without hygromycin and diphtheria toxin. Plat-E cells were always maintained in DMEM with 10% fetal bovine serum containing blasticidin (10  $\mu$ g/ml) and puromycin (1  $\mu$ g/ml). On one hand, infection efficiency of retroviruses produced from Bosc23 was decreased within 3 months, and that of retroviruses produced from the Phoenix-E cells also decreased in time (Figure 3). On the other hand, Plat-E produced retroviruses with an infection efficiency greater than 75% with a titer of about  $1 \times 10^7$ /ml for at least 4 months under drug selection pressure.

To compare the expression level of gag-pol and env mRNA in Plat-E, Bosc23 and Phoenix-E packaging cell

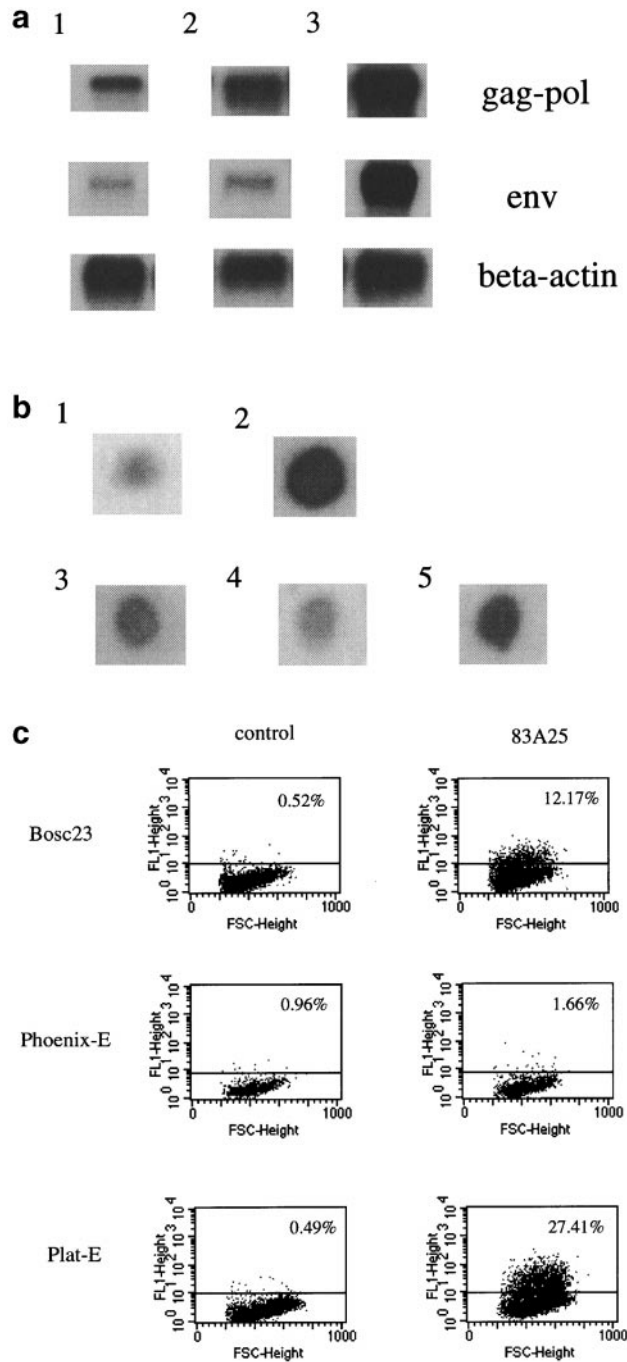


**Figure 3** Long-term stability of Plat-E in producing high titer retroviruses. The infection efficiencies of Ba/F3 cells using retroviruses derived from pMX-GFP produced by Plat-E, Bosc23 and Phoenix E were examined at the indicated times. pMX-GFP was constructed as follows. The GFP fragment was excised from the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) by EcoRI and NotI, and was inserted into the EcoRI-NotI site of the pMX vector.<sup>3</sup> Transfection and infection were performed as described<sup>10</sup> except that we used Fugene (Boehringer Mannheim) instead of LipofectAmine (Gibco-BRL, Rockville, MD, USA).

lines, Northern blot analysis was done using the cells cultured for 3 weeks. The expression levels of *gag-pol* and *env* mRNA was four-fold and 10-fold higher, respectively, in Plat-E cells than in the other packaging cell lines (Figure 4a). The RT activity in the cell lysate was also analyzed. Plat-E produced at least twice more RT activity when compared with Bosc23 and Phoenix-E cells (Figure 4b). In addition, the expression level of *env* protein was much greater than that of Bosc23 and Phoenix-E (Figure 4c) when evaluated by antibody staining raised against the *env* gene product.

As the retroviral structural genes were encoded on the two different plasmids, three recombination events are necessary to generate the replication competent retroviruses (RCR). In addition, the probability of recombination was minimized by using only the coding sequence of *gag-pol* and *env* genes isolated by PCR from MuLV genome in the packaging constructs. In fact, production of RCR was tested by the XC plaque assay,<sup>13</sup> and no RCR was detected from Plat-E cells after transfection of pMX-GFP. As for a positive control, a supernatant of MoMuLV-infected C3H2K cells (a gift from Dr Hoshino) was used after serial dilutions, and the viral titer of the wild-type MoMuLV produced from C3H2K cells was estimated as  $1 \times 10^4$ /ml.

In conclusion, we report here a stable retrovirus packaging cell line Plat-E which has several advantages over the existing packaging cell lines. First, the EF1 $\alpha$  promoter in the packaging constructs, in combination with the Kozak's consensus sequence, allows production of retroviruses with a titer of  $1 \times 10^7$ /ml. Second, a bicistronic vector carrying the IRES sequence was used in the packaging constructs to ensure stable expression of the viral structural genes under the drug selection pressure, which



**Figure 4** Comparison of *gag-pol* and *env* expression in Bosc23, Phoenix-E and Plat-E. (a) Northern hybridization of *gag-pol* and *env*. Expression of *gag-pol* and *env* in Plat-E (3) was compared with that in Bosc23 (1) and Phoenix-E (2) by Northern hybridization. The probes used were the EcoRI-NotI fragment of pGag-pol-IRES-bs<sup>r</sup> and pEnv-IRES-puro<sup>r</sup>. (b) RT assay of cell lysate of Bosc23, Phoenix-E and Plat-E. Cell lysate of 293T cells was used as a negative control (1), 1 ng of HIV RT was mixed with the cell lysate of 293T cells (2), and was used as a positive control. RT activities derived from Bosc23 (3), Phoenix-E (4), Plat-E (5) were measured as described.<sup>11</sup> (c) Expression of *env* in Bosc23, Phoenix E and Plat-E cells. The expression of *env* was determined by cell surface fluorescence of Bosc23, Phoenix-E and Plat-E using the rat monoclonal antibody raised against the *env* proteins termed 83A25.<sup>12</sup> Staining procedure was performed as described<sup>12</sup> and then subjected to FACS analysis. As a control, these cells were stained only with the second antibody (FITC-conjugated goat anti-rat IgG as second antibody).

makes it possible to maintain the titer of retroviruses derived from the Plat-E cells by simply culturing the cells in the presence of selection drugs. Finally, to lessen the possibility of generation of RCR, the minimum virus sequences were used in the packaging constructs. Thus, Plat-E cells can stably produce helper-free retroviruses at high titers for a long time.

Using retroviruses produced by Plat-E cells, we can efficiently transfer genes to many different cells including cells in primary culture such as T cells and mast cells (data not shown). Recently, it has been reported that by introducing the coding region of the polyomavirus early gene into the packaging cell lines, the titers of recombinant retrovirus produced by these cell lines were 10–100 times higher than those produced by the parent cell line.<sup>14</sup> Introduction of the polyomavirus early region into Plat-E may lead to more efficient production of retroviruses with high titer. Plat-E is an ecotropic packaging cell line and generation of its amphotropic counterpart, the Plat-A cell line should prove useful in human gene therapy.

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### References

- 1 Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 1986; **6**: 2983–2902.
- 2 Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 1993; **90**: 8392–8396.
- 3 Onishi M *et al*. Application of retrovirus-mediated expression cloning. *Exp Hematol* 1996; **24**: 324–329.
- 4 <http://www.stanford.edu/group/nolan/>.
- 5 Gattas IR, Sanesm JR, Major JE. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryo. *Mol Cell Biol* 1991; **11**: 5848–5859.
- 6 Fiering SN *et al*. Improved FACS-Gal: flow cytometric analysis and sorting of viable eukaryotic cells expressing reporter gene constructs. *Cytometry* 1991; **12**: 291–301.
- 7 Dubridge RB, Tang P, Hsia HC. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol* 1987; **7**: 379–387.
- 8 Nosaka T *et al*. STAT5 as a molecular regulator of proliferation, differentiation, and apoptosis in hematopoietic cells. *EMBO J* 1999; **18**: 4754–4765.
- 9 Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 1990; **18**: 5332.
- 10 Kitamura T *et al*. Efficient screening of retroviral cDNA expression libraries. *Proc Natl Acad Sci USA* 1995; **92**: 9146–9150.
- 11 Mathias S *et al*. Reverse transcriptase encoded by a human transposable element. *Science* 1991; **254**: 1808–1810.
- 12 Evans LH *et al*. A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. *J Virol* 1990; **64**: 6176–6183.
- 13 Rowe WP, Pugh WE, Hartly JW. Plaque assay techniques for murine leukemia viruses. *Virology* 1970; **42**: 1136–1139.
- 14 Yoshimatsu T *et al*. Improvement of retroviral packaging cell lines by introducing the polyomavirus early region. *Hum Gene Ther* 1998; **9**: 161–172.