VIROLOGY 218, 290–295 (1996)
ARTICLE NO. 0194

SHORT COMMUNICATION

A Novel Human Amphotropic Packaging Cell Line: High Titer, Complement Resistance, and Improved Safety

RICHARD J. RIGG, JINGYI CHEN, JONATHAN S. DANDO, SEAN P. FORESTELL, IVAN PLAVEC, and ERNST BÖHNLEIN[1]

SyStemix, 3155 Porter Drive, Palo Alto, California 94304

Received December 20, 1995; accepted February 8, 1996

Successful retroviral-mediated gene therapy will depend on safe, efficient packaging cell lines for vector particle production. Existing packaging lines for murine leukemia virus (MLV)-based vectors are predominantly derived from NIH/3T3 cells which carry endogenous MLV sequences that could participate in recombination to form replication-competent retrovirus (RCR). To identify cells devoid of such sequences, we screened genomic DNA from eight cell lines. DNA from the human 293 cell line did not cross-hybridize with MLV sequences, and these cells were able to secrete Gag particles after transfection. We derived a stable amphotropic packaging cell line (called ProPak-A) in 293 cells in which the Gag-Pol and Env (packaging) functions are expressed separately from a heterologous (non-MLV) promoter, to maximally reduce homology between packaging and vector sequences. ProPak-A-based producer cells are efficient, yielding higher stable titers than PA317-based producers. In addition, a vector that consistently gave rise to RCR in PA317 cells never resulted in detectable RCR in ProPak-A-based producer cultures. We have also shown that ProPak-A-packaged particles are not inactivated by human serum. Thus, the packaging cells we describe are as efficient and safer than the amphotropic packaging cells most commonly used in clinical gene therapy work and are also more appropriate for *in vivo* gene delivery.

The application of retroviral vector-mediated human gene therapy demands greater safety margins than experimental gene transfer. The majority of cell lines currently available for stable production of retroviral vector supernatants are derived from NIH/3T3 cells (*6, 7, 21–24, 28*). Although packaging constructs have been designed such that three recombination events are required for replication-competent retrovirus (RCR) generation (*6, 7, 21–23, 28*), the NIH/3T3 cells from which these lines were derived express endogenous murine leukemia virus (MLV) sequences (*15*) that can be packaged (*36*) and participate in recombination to form RCR (*4, 42*), particularly in mass culture during large-scale clinical vector production.

Table 1 summarizes the steps taken to generate and characterize the ProPak-A packaging cells. We initiated our work by screening cell lines for endogenous MLV sequences. Genomic DNA from a variety of cell lines was analyzed by Southern blot hybridization (*35*) using probes from the MLV long terminal repeat (LTR) or *gag-pol* sequences (Table 2). We screened the following cell lines: 293 cells that are used to produce adenoviral vectors for clinical gene therapy applications (*3*) and retroviral vectors (*31, 12*), Vero and MRC-5 cells in which vaccines are produced (*44*), and CHO cells which are used to produce recombinant proteins (*18*). *Mus dunni* tail fibroblasts were also included since these cells are reportedly free of endogenous MLV sequences (*20*). Genomic DNA from NIH/3T3 cells, the basis for the majority of existing packaging cell lines, hybridized very strongly with both MLV-specific probes at low or high stringency (Fig. 1; Table 2), consistent with a previous report (*20*). Strong hybridization was also seen with CHO-K1 cell DNA. In contrast, neither probe cross-hybridized with genomic DNA from 293 (Fig. 1) or MRC-5 cells, even at low stringency (Table 2). In addition, no cross-hybridization was seen with genomic DNA from *M. dunni,* MDCK (Fig. 1), Vero, or fox lung cells at high stringency (Table 2). Fox lung and MRC-5 were discounted due to poor growth and limited cell division potential, respectively, which would preclude subcloning of stably transfected cells. Thus, 293, MDCK, *M. dunni,* and Vero cells were identified as candidate cell lines in which to derive packaging cell lines.

To decrease the probability of RCR formation, we constructed separate expression plasmids for *gag-pol* and *env,* as previously described (*6, 7, 21–23, 28*). In contrast to existing packaging constructs, however, we included only the minimum genetic information required to encode Gag-Pol and Env proteins. Figure 2 details the construction of Gag-Pol and Env expression plasmids.

[1] To whom correspondence and reprint requests should be addressed.

0042-6822/96 $18.00

TABLE 1

ProPak-A Derivation Scheme

Objective	Result
1. Identify cells lacking endogenous MLV-like sequences (candidate cell lines)	See Table 2; 293, *Mus dunni* TF, Vero, and MDCK candidate cells.
2. Prepare minimal *gag-pol* ORF by pcr; subclone into expression plasmids	pCMV-gp (Fig. 2)
3. Prepare minimal amphotropic *env* ORF by pcr; subclone into expression plasmid	pCMV*Ea (Fig. 2)
4. Stably transfect *gag-pol* into candidate lines and screen for Gag secretion	293 cells secrete Gag; chosen as base cell line
5. Stably transfect pCMV*Ea into 293 cells; isolate and characterize clones	293.Env clones
6. Stably transfect pCMV-gp into 293.Env cells; isolate and characterize clones	ProPak-A clones
7. Determine titer: end point, transduction efficiency	Table 3, Fig. 3
8. Determine sensitivity to human complement	Fig. 4

To further identify the optimal cell line, sandwich ELISA assays were developed to detect Gag and Env proteins in transfected cells and supernatants. Plates were coated with hybridoma culture supernatants from either 83A25 (*11*) for Env or R187 (ATCC CRL 1912) for Gag. Captured proteins were detected with 79S-834 and 77S-227 antisera (Quality Biotech, Camden, NJ), respectively, and horseradish peroxidase-conjugated anti-species antibodies and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (Pierce, Rockford, IL). The ability to produce vector particles was assessed by transfection of a *gag-pol* expression plasmid into candidate cell lines and selection of drug-resistant pools. Only the supernatants from *gag-pol*-transfected 293 or *M. dunni* cells contained Gag protein. In sharp contrast, no Gag was secreted by transfected Vero or MDCK cells, although Gag was present in the cell lysates (data not shown). This lack of Gag secretion is puzzling since Vero-based producer cells can be established with the replication-competent 4070A virus (I. Plavec, unpublished results), but parallels a report that HIV-1 Gag expressed in Vero cells also remains intracellular (*9*). The extended MLV Gag protein sequence present in the replication-competent 4070A virus, but not in the Gag-Pol expression construct used here, may be required for secretion of Gag particles from Vero or MDCK cells. We discounted Vero and MDCK cells as candidate cell lines and elected to derive packaging cells based on human 293 cells because of the high transient transfection efficiency (*31, 12*).

TABLE 2

Screening of Cell Lines for Cross-Hybridization to MLV LTR or *gag/pol* Sequences

	Endogenous MLV sequences (hybridization)			
Hybridization probe:	LTR		*gag/pol*	
Wash stringency:	Low	High	Low	High
Cell lines tested				
293 (ATCC CRL 1573)	−	−	−	−
MDCK (ATCC CCL 34)	±	−	±	−
Mus dunni tail fibroblasts	±	−	±	−
Vero (ATCC CCL 81)	−	−	±	−
FoLu (ATCC CCL 168)	−	−	±	−
MRC-5 (ATCC CCL 171)	−	−	−	−
NIH/3T3 (ATCC CRL 1658)	++	++	++	++
CHO-K1 (ATC 61)	++	++	++	++

Note. Hybridization signal strength: −, none; ±, weak; ++, strong. Probes: LTR (positions relative to cap site of genomic RNA), nucleotides −232 (*Eco*RV) to 563 (*Pst*I of 5′ leader sequence); *gag/pol,* nucleotides 739 (*Pst*I in *gag*) to 3705 (*Sal*I in *pol*). Stringency (65°): Low, 500 m*M* Na^+; High, 50 m*M* Na^+. ATCC, American Type Culture Collection.

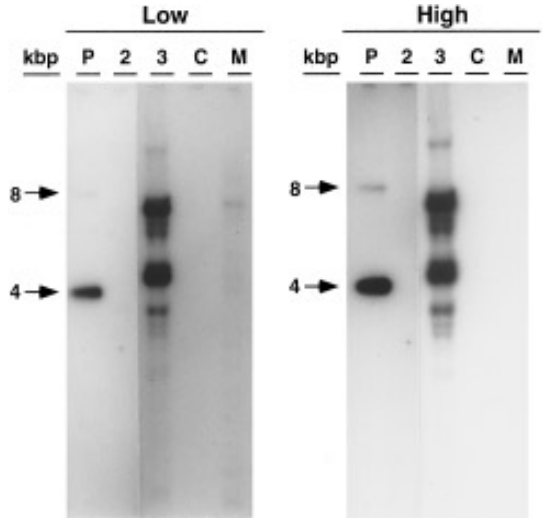

FIG. 1. Example of Southern blot analysis of cellular DNA for cross-hybridization with the MLV *gag/pol* probe after washing at low or high stringency (see Table 2). P, pVH2 DNA digested with *Eco*RI and *Sal*I. Genomic DNA from 2, 293; 3, NIH/3T3; C, MDCK; or M, *M. dunni* tail fibroblasts.

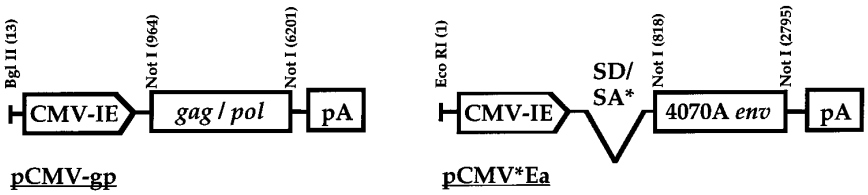

FIG. 2. Diagram of expression plasmid (Gag-Pol and Env) structure showing the positions of selected restriction sites in parentheses. First, the structural gene sequences were amplified by polymerase chain reaction (PCR) using the primers shown to obtain the open reading frames (ORFs) from the initiation to the termination codons of the *gag*/*pol* or *env* genes flanked by *Not*I restriction sites, and the fragments were subcloned into pBluescript (SK^+) (Stratagene, La Jolla, CA). Oligonucleotide primers (Genosys Biotechnologies, Woodlands, TX) corresponding to the N-terminus of the genes also placed the AUG in the ideal context for translation (*19*), and those corresponding to the C-terminus encoded a second in-frame stop codon. The integrity of PCR products was verified by DNA sequencing. *gag*/*pol:* The ORF was amplified from the plasmid pVH-2, which carries the infectious Moloney MLV sequence (*27*) using the primer pair 5′AAAAAAAAGCGGCCGCGCCGCCACCATGGGCCAGACTGTTACCAC3′ and 5′AAAAAAAAGCGGCCGCTCAttaGGGGGCCTCGCGGG3′. The underlined ATG is that of p15Gag (bases 621 to 623 (*37*)) and the codon in lowercase corresponds to the *pol* stop codon (bases 5835 to 5837 (*37*)). The expression plasmid pCMV-gp, with the human cytomegalovirus immediate early (CMV-IE) promoter, was constructed by inserting the *gag-pol* fragment into the pcDNA3 plasmid (Invitrogen, San Diego, CA) from which the neomycin resistance expression cassette (*Dra*III to *Bsm*I) had been deleted. Plasmids carrying the *gag-pol* ORF were propagated at 30° to prevent recombination (*16*). *env:* A contiguous amphotropic envelope sequence was constructed from p4070A (*30*) and amplified using the PCR primers 5′TAATCTACGCGGCCGCCACCATGGCGCGTTCAACGCTC3′ and 5′AATGTGATGCGGCCGCtcaTGGCTCGTACTCTATGG3′. The underlined ATG corresponds to bases 37 to 39, and the stop codon (lowercase), bases 1998 to 2000 (*30*). The CMV promoter-Env expression plasmid pCMV*Ea was created by insertion of the *env* ORF in place of the β-galactosidase gene of pCMVβ (Clontech, Palo Alto, CA) modified by mutation of an extraneous ATG in the SV40 intron to ACG (SD/SA*).

To derive packaging cells, we first introduced the pCMV*Ea plasmid (Env) into 293 cells by cotransfection (Profection kit, Promega, Madison, WI) with the pHA58 plasmid (*33*) conferring resistance to hygromycin B (250 μg/ml; Boehringer, Indianapolis, IN). Stably selected populations were stained with anti-Env antibody (83A25), and individual Env-positive cells were isolated by automatic cell deposition on a FACStar Plus (Becton Dickinson, San Jose, CA). Three clones with the highest fluorescence intensity were further characterized. All three yielded equivalent titers upon transient cotransfection with *gag-pol* and vector plasmids (data not shown).

Next, the pCMV-gp construct was stably transfected into one of the three 293.Env clones by cotransfection with the plasmid pSV2pac (*43*). Puromycin-resistant (1 μg/ml; Sigma, St. Louis, MO) clones were grown to confluence, medium was exchanged, and supernatants were analyzed for Gag and Env content by ELISA. Clones were identified (16 of 37) that secreted high levels of Gag and Env antigens (data not shown). Of these, six clones produced virus in transient transfections at titers within two- to threefold of Oz 2 cells (Table 3A), the amphotropic equivalent of BOSC 23 cells (*31*). Transient titers reflect the efficiency of transient transfection, and the titers obtained with ProPak-A cells are lower than those achieved with Oz 2 cells, possibly because Oz 2 is based on a 293T cell clone selected for high transient transfection efficiency (*31*).

For gene therapy applications, it is necessary to generate large volumes of characterized supernatants, which cannot be easily prepared by transient transfection. Therefore, we were more interested in determining the stable end-point titers and the transduction efficiencies (*13*). End-point titers were determined for supernatants from producer cell clones which had been transduced with the LMTNL vector (*10*), in which an internal thymidine kinase promoter (T in vector name) drives the neomycin phosphotransferase gene (N). End-point titers from ProPak-A-based producer cells were marginally higher than those for our best PA317-based producer clone (Table 3). In addition, the titers from ProPak-A.LMTNL producer pools were stable when passaged for 3 months in the absence of drug selection (data not shown).

While end-point titers are broadly used, transduction efficiency is a better measure of gene transfer potency (*13*). However, the assay is laborious with vectors encoding drug resistance genes. We therefore prepared PA317- or ProPak-A-based producer cell populations carrying a vector (LLySN) derived from the LXSN vector (*26*) by insertion of the Lyt2 surface marker gene (*39*). Surface expression of the Lyt2 antigen allows simple, quantitative determination of transduction efficiency by FACS (*34*). Higher transduction efficiencies were achieved with supernatants from two independently derived ProPak-A. LLySN populations than with supernatants from three PA317.LLySN pools (Fig. 3). Higher transduction efficiencies of NIH/3T3 cells and other cells (data not shown) were achieved with ProPak-A supernatants, even though supernatants from PA317 or ProPak-A cells had similar end-point titers (Table 3C). We have previously postulated that PA317-packaged vector contains a viral inhibitor of transduction, possibly envelope protein (*13*), and it may be that ProPak-A supernatants contain less inhibitor.

One of our major concerns was to evaluate the safety of the ProPak-A cells. In previous work (S. Forestell, I. Plavec, and G. Veres, unpublished) we found that the vector BC140revM10 (*1*) reproducibly gave rise to RCR in PA317 cells. BC140revM10 carries the extended packag-

TABLE 3

Comparison of End-Point Titers from Transiently Transfected ProPak-A Cell Clones (A) or Stable Producer Cell Clones (B) or Pools (C)

(A) Transient titers — MFG lacZ vector

Cell line or clone	End-point titer (cfu $\times 10^{-5}$/ml)
Oz2	13.8 ± 0.3
ProPak-A.12	8.8 ± 0.8
ProPak-A.31	8.0 ± 0.5
ProPak-A.6	7.5 ± 0.0
ProPak-A.27	6.8 ± 1.2
ProPak-A.21	6.0 ± 1.0
ProPak-A.5	6.0 ± 2.0

(B) Stable titers — LMTNL vector

Producer clone	End-point titer (G418^r cfu $\times 10^{-6}$/ml)
PA317.LMTNL	1.7 ± 0.7
ProPak-A.6.LMTNL.6	2.1 ± 0.3
ProPak-A.6.LMTNL.7	2.2 ± 0.8

(C) Stable titers — LLySN Vector

Producer pools	End-point titer (G418^r cfu $\times 10^{-6}$/ml)
PA317.LLySN 1	1.5 ± 0.3
PA317.LLySN 2	1.4 ± 0.4
PA317.LLySN 3	0.9 ± 0.1
ProPak-A.6. LLySN 1	1.9 ± 0.5
ProPak-A.6. LLySN 2	1.6 ± 0.3

Note. Supernatants were collected after 16 hr at 32°, and end-point titers determined on NIH/3T3 cells (*13*). cfu, colony-forming units. (A) Cells were seeded at 2×10^5 cells/cm^2 in six-well plates and transfected 16 hr later with 2.5 μg DNA/well MFG-lacZ (*8*) in the presence of 25 μM chloroquine (*31*). Titers are the average and range for duplicate transfections. Oz 2 cells are also called Bing cells. (B and C) Supernatants were harvested from confluent cultures of producer cell clones (B) or pools (C) in T-75 flasks. (C) End-point titers for supernatants for which transduction efficiency determinations are shown (Fig. 3). The average and range for triplicate samples are given.

ing sequence, including the ATG of the *gag* ORF. The LMTNL vector (*10*), in contrast, lacks part of the 5′ untranslated region and contains no *gag* sequences and is therefore less likely to recombine and form RCR. We introduced the BC140revM10 or LMTNL vectors into PA317 or ProPak-A cells and tested culture supernatants for RCR (*14, 32*) at weekly intervals. The PA317/BC140revM10 combination (transfected or transduced) gave rise to RCR detectable by direct inoculation of culture supernatant onto PG4 cells at 4 weeks (Table 4). Cultures were maintained for 4 more weeks and also tested by coculture of producer cells with *M. dunni* cells to amplify any RCR in the culture, followed by S+L− assay on PG4 cells. Even by this stringent assay for RCR, the ProPak-A-based producer pools were all free of RCR (Table 4).

Recently, interest has arisen in the *in vivo* application of retroviral gene transfer by direct administration of vector particles to human beings. In addition, targeting of particles bearing hybrid ligand-ecotropic envelope glycoproteins specific for cell surface molecules has been reported (*5, 17, 38*). A prerequisite is that the particles are not inactivated by human serum. Therefore, we analyzed the susceptibility of ProPak-A- or PA317-packaged vector particles to inactivation by human serum. In addition, we analyzed ecotropic supernatants packaged in either PE501 cells (NIH/3T3-based; *26*) or 293 cells. Vector particles with either envelope generated from 293 cells were resistant to human serum, while supernatants packaged in NIH/3T3 cells were inactivated by incubation with human serum (Fig. 4). Takeuchi *et al.* (*41*) concluded that resistance of vector particles to human serum was determined by both the host cell type and the viral envelope. Our data suggest that packaging of amphotropic and ecotropic vectors in 293-based cells is sufficient to confer resistance to human complement, and studies to understand this phenomenon are ongoing.

The ProPak-A cells described in this study are safer than existing packaging cell lines because they carry the

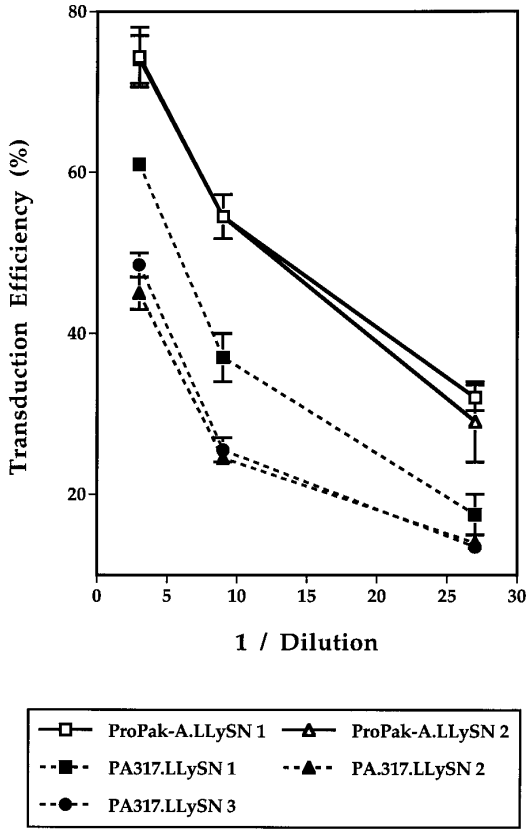

FIG. 3. Transduction efficiencies achieved with Lyt2-encoding (LLySN) vector supernatants from PA317 or ProPak-A-based producer cells were quantitated as the proportion (%) of NIH/3T3 cells that stained (FACScan, Becton Dickinson) with anti-Lyt2 antibody (Pharmingen, San Diego, CA) 2 days after inoculation with the dilutions of vector supernatant shown. Supernatants were prepared from confluent producer cell cultures after 12 hr at 32°.

TABLE 4

Assay for Presence of RCR in Cultures Carrying the BC140revM10 or LMTNL Vectors

Packaging cell line	Transfected with	Transduced with	Supernatant RCR (wk)	Coculture RCR (wk 8)
ProPak-A	pBC140revM10	N/A	(>8)	Negative
ProPak-A	pLMTNL	N/A	(>8)	Negative
ProPak-A	N/A	M(G).BC140revM10	(>8)	Negative
ProPak-A	N/A	M(G).LMTNL	(>8)	Negative
PA317	pBC140revM10	N/A	4	Not tested
PA317	pLMTNL	N/A	(>8)	Negative
PA317	N/A	M(G).BC140revM10	4	Positive
PA317	N/A	M(G).LMTNL	(>8)	Negative

Note. RCR detected by S+L- assay on PG4 cells (ATCC CRL 2032) by inoculation with supernatant from producer cell cultures, or after three passages of coculture with *Mus dunni* cells. N/A, not applicable. (>8), no RCR detected 8 weeks after G418-resistant pools established. M(G)., transient MLV(VSV-G) pseudotype (*45*) used as inoculum.

minimum MLV sequences. The 293 cells in which we established the ProPak-A line are devoid of sequences that cross-hybridize with MLV and therefore no endogenous sequences could recombine to form RCR, in contrast to the situation in producer cells derived from NIH/3T3 cells (*7, 21, 22, 28*). In addition to separating *gag-pol* and *env* genes on different expression plasmids (*7, 21, 22, 28*), the probability of recombination was minimized by including only the ORFs and no flanking sequences for the MLV *gag-pol* and *env* genes. Also, in contrast to previous studies using the MLV-LTR promoter (*7, 21, 22, 28, 31*), we expressed the structural proteins from the human CMV promoter, reducing the MLV-derived sequences present in the packaging cells to a minimum. NIH/3T3-based packaging cells have been described using a heterologous promoter (*2, 40*). However, low stable titers were achieved (*2*) unless the episomal plasmid copy number was amplified (*40*), probably because the metallothionein promoter that was used is weaker than the CMV promoter. In practice, the ProPak-

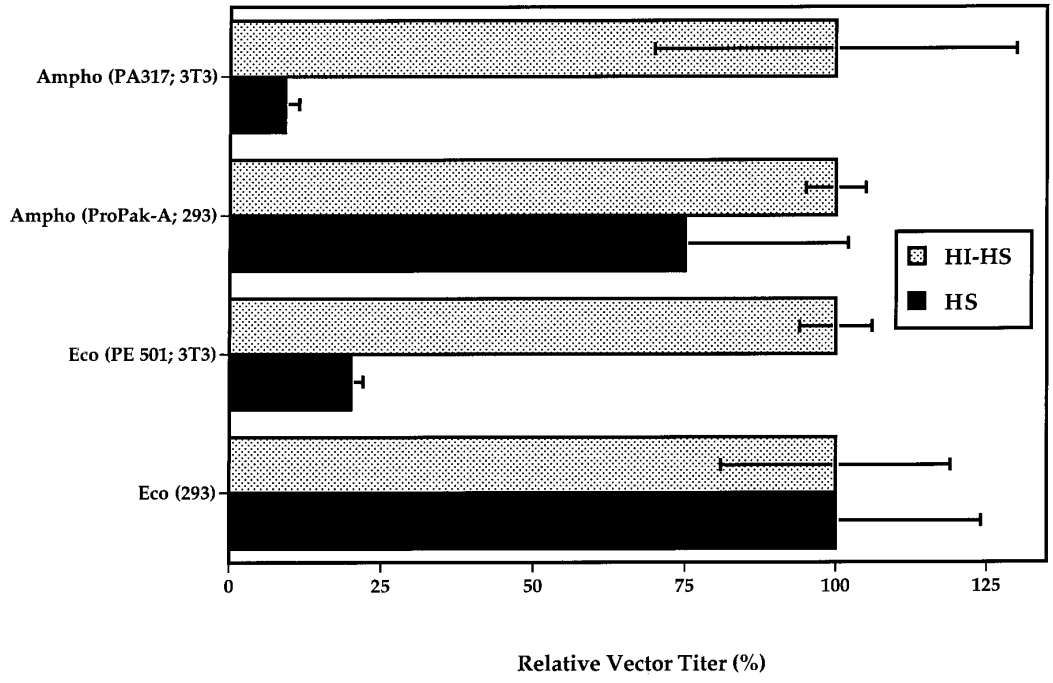

FIG. 4. Resistance to human serum of vector particles packaged in either murine or human cells. LacZ-encoding vector supernatants were prepared from stable producer cells (PA317; PE501), by transient transfection of vector into packaging cells (ProPak-A), or by cotransfection of packaging and vector constructs into 293 cells (293). Supernatants were mixed with an equal volume of a pool of human serum from five healthy donors and incubated for 1 hr at 37°, and the residual titer was determined on NIH/3T3 cells (*41*). The serum was either untreated (HS) or had been heat-inactivated for 30 min at 56° (HI-HS). The human serum pool had a hemolytic titer (CH_{50}; EZ Complement Assay, Diamedix, Miami, FL) of 66 to 137 before, and <8 after heat-inactivation. End-point titers (cfu $\times 10^{-5}$/ml) of supernatants treated with heat-inactivated serum (100%) were PA317, 5.0; ProPak-A, 1.0; PE501, 1.4; and 293, 1.1. The bars indicate the range for duplicate samples.

A cells did not give rise to RCR with a vector carrying the extended packaging sequence which consistently yielded RCR in PA317 cells, the packaging cells most commonly used to prepare retroviral vectors for gene therapy applications (*29*).

As well as being safer, the titers and transduction efficiencies obtained with stable ProPak-A-based producers are higher than those achieved with PA317-based producers. ProPak-A cells are not resistant to G418, and therefore producers can be selected with vectors that encode the commonly used neomycin phosphotransferase gene, unlike Oz 2 cells, which were derived from G418-resistant 293T cells (*31*). Thus, ProPak-A is a safe, efficient, and convenient packaging cell for the preparation of stable producer cell lines and supernatants for gene therapy. We are currently optimizing conditions for the production of high-quality supernatants from ProPak-A-based producers and deriving a similar packaging cell line with the xenotropic envelope. This complementary line will allow preparation of amphotropic or xenotropic supernatants from transduced producers to facilitate derivation of high-titer producer cell lines with tropisms for distinct receptors on human cells.

ACKNOWLEDGMENTS

We thank J.-L. Darlix for the pVH2 plasmid; R. Friedrich for plasmid p4070A, and M. Lander for *Mus dunni* tail fibroblast cells. D. Adams and P. Lekas assisted with cell sorting. E. Gilboa provided advice, and U. Junker, W. Kerr, and G. Veres critically reviewed the manuscript.

REFERENCES

1. Bevec, D., Dobrovnik, M., Hauber, J., and Böhnlein, E., *Proc. Natl. Acad. Sci. USA* 89, 9870–9874 (1992).
2. Bosselman, R. A., Hsu, R.-Y., Bruszewski, J., Hu, S., Martin, F., and Nicolson, M., *Mol. Cell. Biol.* 7, 1797–1806 (1987).
3. Bramson, J. L., Graham, F. L., and Gauldie, J., *Curr. Opin. Biotechnol.* 6, 590–595 (1995).
4. Cosset, F. L., Girod, A., Flamant, F., Drynda, A., Ronforth, C., Valsesia, S., Molina, R.-M., Faure, C., Nigon, V.-M., and Verdier, G., *Virology* 193, 385–395 (1993).
5. Cosset, F. L., Morling, F. J., Takeuchi, Y., Weiss, R. A., Collins, M. K. L., and Russell, S. J., *J. Virol.* 69, 6314–6322 (1995).
6. Danos, O., *In* "Methods in Molecular Virology," Vol. 8, "Practical Molecular Virology: Viral Vectors for Gene Expression" (M. Collins, Ed.), pp. 17–27. Humana Press Inc., Clifton, NJ, 1991.
7. Danos, O., and Mulligan, R., *Proc. Natl. Acad. Sci. USA* 85, 6460–6464 (1988).
8. Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C., *Proc. Natl. Acad. Sci. USA* 90, 3539–3543 (1993).
9. Dru, A., Ludosky, M.-A., Cartaud, J., and Beaud, G., *AIDS Res. Hum. Retroviruses* 10, 383–390 (1994).
10. Escaich, S. E., Kalfoglou, C., Plavec, I., Kaushal, S., Mosca, J. D., and Böhnlein, E., *Hum. Gene Ther.* 6, 625–634 (1995).
11. Evans, L. H., Morrison, R. P., Malik, F. G., Portis, J., and Britt, W. J., *J. Virol.* 64, 6176–6183 (1990).
12. Finer, M. F., Dull, T. J., Qin, L., Farson, D., and Roberts, M. R., *Blood* 83, 43–50 (1994).
13. Forestell, S. P., Böhnlein, E., and Rigg, R. J., *Gene Ther.* 2, 723–730 (1995).
14. Haapala, D. K., Robey, W. G., Oroszlan, S. D., and Tsai, W. P., *J. Virol.* 53, 827–833 (1985).
15. Irving, J., Chang, L. W. S., and Castillo, F. J., *Bio/Technology* 11, 1042–1046 (1993).
16. Joshi, A., and Jeang, K.-T., *BioTechniques* 14, 883–886 (1993).
17. Kasahara, N., Dozy, A. M., and Kan, Y. W., *Science* 266, 1373–1376 (1994).
18. de Koning, W. J., Walsh, G. A., Wrynn, A. S., and Headon, D. R., *Bio/Technology* 12, 988–990 (1994).
19. Kozak, M., *J. Mol. Biol.* 196, 947–950 (1987).
20. Lander, M. R., and Chattopadhyay, S. K., *J. Virol.* 52, 695–698 (1984).
21. Markovitz, D., Goff, S., and Bank, A., *J. Virol.* 62, 1120–1124 (1988).
22. Markovitz, D., Goff, S., and Bank, A., *Virology* 167, 400–406 (1988).
23. Miller, A. D., *Hum. Gene Ther.* 1, 5–14 (1990).
24. Miller, A. D., and Buttimore, C., *Mol. Cell. Biol.* 6, 2895–2902 (1986).
25. Miller, A. D., Garcia, J. V., Suhr, N., Lynch, C. M., Wilson, C., and Eiden, M. V., *J. Virol.* 65, 2220–2224 (1991).
26. Miller, A. D., and Rosman, G. J., *BioTechniques* 7, 980–990 (1989).
27. Miller, A. D., and Verma, I. M., *J. Virol.* 49, 214–222 (1984).
28. Morgenstern, J. P., and Land, H., *Nucleic Acids Res.* 18, 3587–3596 (1990).
29. ORDA Reports. Recombinant DNA Advisory Committee Data Management Report, June 1994. *Hum. Gene Ther.* 5, 1295–1302 (1994).
30. Ott, D., Friedrich, R., and Rein, A., *J. Virol.* 64, 757–766 (1990).
31. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D., *Proc. Natl. Acad. Sci. USA* 90, 8392–8396 (1993).
32. Printz, M., Reynolds, J., Mento, S. J., Jolly, D., Kowal, K., and Sajjadi, N., *Gene Ther.* 2, 143–150 (1995).
33. Riele, H. t., Maandag, E. R., Clarke, A., Hooper, M., and Berns, A., *Nature* 348, 649–651 (1990).
34. Rigg, R. J., Dando, J. S., Escaich, S., Plavec, I., and Böhnlein, E., *J. Immunol. Methods* 188, 187–195 (1995).
35. Sambrook, J., Fritsch, E. F., and Maniatis, Y., "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
36. Scadden, D. T., Fuller, B., and Cunningham, J. M., *J. Virol.* 64, 424–427 (1990).
37. Shinnick, T. M., Lerner, R. A., and Sutcliffe, J. G., *Nature* 293, 543–548 (1981).
38. Somia, N. J., Zoppé, M., and Verma, I. M., *Proc. Natl. Acad. Sci. USA* 92, 7570–7574 (1995).
39. Tagawa, M., Nakauchi, H., Herzenberg, L. A., and Nolan, G. P., *Proc. Natl. Acad. Sci. USA* 83, 3422–3426 (1986).
40. Takahara, Y., Hamada, K., and Housman, D. E., *J. Virol.* 66, 3725–3732 (1992).
41. Takeuchi, Y., Cosset, F.-I., Lachmann, P. J., Okada, H., Weiss, R. A., and Collins, M. K. L., *J. Virol.* 68, 8001–8007 (1994).
42. Vanin, E. F., Kaloss, M., Broscius, C., and Nienhuis, A. W., *J. Virol.* 68, 4241–4250 (1994).
43. Vara, J. A., Portela, A., Ortín, J., and Jiménez, A., *Nucleic Acids Res.* 14, 4617–4624 (1986).
44. WHO, *In* "Technical Report Series No. 786." WHO, Geneva, 1989.
45. Yee, J.-K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C., and Friedmann, T., *Proc. Natl. Acad. Sci. USA* 91, 9564–9568 (1994).