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A Safe Packaging Line for Gene Transfer: Separating Viral Genes on Two Different Plasmids

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A retrovirus packaging cell line was constructed by using portions of the Moloney murine leukemia virus in which the *gag*, *pol*, and *env* genes of the helper virus were separated onto two different plasmids and in which the ψ packaging signal and 3′ long terminal repeat were removed. The plasmid containing the *gag* and *pol* genes and the plasmid containing the *env* gene were cotransfected into NIH 3T3 cells. Clones that produced high levels of reverse transcriptase and *env* protein were tested for their ability to package the replication-defective retrovirus vectors Δneo and N2. One of the *gag-pol* and *env* clones (GP+E-86) was able to transfer G418 resistance to recipient cells at a titer of as high as 1.7×10^5 when it was used to package Δneo and as high as 4×10^6 when it was used to package N2. Supernatants of clones transfected with the intact parent *gag-pol-env* plasmid 3P0 had comparable titers (as high as 6.5×10^4 with Δneo; as high as 1.7×10^5 with N2). Tests for recombination events that might result in intact retrovirus showed no evidence for the generation of replication-competent virus. These results suggest that *gag*, *pol*, and *env*, when present on different plasmids, may provide an efficient and safe packaging line for use in retroviral gene transfer.

The cloning, transfer, and expression of human globin genes into erythroid cells in culture and in mice has raised the possibility of autotransplantation of bone marrow cells with a normal β-globin gene as an approach to therapy of sickle cell anemia and β-thalassemia in humans (5). Retroviral vectors are the most efficient means of transferring genes into cells. This high efficiency is a requirement for experiments whose goal is human globin gene therapy since only a limited number of bone marrow stem cells can be obtained, and as many cells as possible must acquire and express the transferred genes to ensure repopulation of sufficient bone marrow elements to produce normal amounts of hemoglobin. Another major prerequisite in these experiments is safety (1). The major danger of the use of retroviral vectors is the possibility that replication-competent viruses could form and that the proliferation of those viruses would lead to multiple integrations into the genome. Those integrations could result in activation of potentially harmful genes such as oncogenes (21, 24) and could lead to other harmful consequences of their proliferation. To avoid these complications, packaging lines have been constructed in which the retroviral sequences in the helper virus are not by themselves transmissible, replication-competent viruses (4, 15, 18, 23, 25). In these defective viral constructs, the signal for packaging of viral RNA (ψ sequence) has been deleted. Thus, while the required *gag*, *pol*, and *env* genes of the retrovirus are intact, there is no release of wild-type helper virus by these packaging lines. However, when the ψ packaging lines are transfected with replication-defective retroviral vectors containing an intact ψ sequence required for their own packaging, wild-type retrovirus can arise, presumably by recombination events (15). For example, high-titer amphotropic retroviral stocks generated by the transfer of a defective neomycin-containing retrovirus into the amphotropic packaging cell line PA12 (containing the ψ deletion) have been shown to produce infectious amphotropic helper virus (11, 16). To circumvent this problem, additional mutations have been made in the defective virus of newer helper cell lines (2, 16). These have included deletions in the 3′ long terminal repeat (LTR) of the helper virus component, and additional deletions of portions of the 5′ LTR as well. One of these defective amphotropic constructs was used to produce a retroviral packaging line, PA317, that has recently been reported to eliminate wild-type retrovirus production after retroviral transfection. However, by use of this packaging line, two recombinational events could still produce intact retrovirus. Cell lines containing both 3′ and 5′ LTR deletions and the packaging mutation were also constructed but were not useful because of the relatively low titers obtained with these constructs.

We approached this problem by attempting to create a packaging cell line by using helper virus DNA in which the *gag* and *pol* genes were on one plasmid while the *env* gene was on another. In addition, the two plasmids had deletions of the ψ packaging sequence and the 3′ LTRs. A stable and efficient packaging line was created by using these two plasmids, and as predicted, the three recombinational events necessary to lead to the production of intact retrovirus could not be detected. Thus, this approach satisfies the requirement for human gene therapy that the use of retroviral vectors must involve, as close as possible, completely safe retroviral packaging lines and must permit efficient gene transfer into recipient cells.

MATERIALS AND METHODS

Helper virus genomic construction. By using the Moloney murine leukemia virus (Mo-MULV) present in the plasmid 3P0 as the starting material (Fig. 1), two constructions were made: (i) p*gag-pol*gpt, containing the *gag* and *pol* genes of Mo-MULV; and (ii) p*env*, containing the *env* gene from this virus (Fig. 1).

p*gag-pol*gpt. p*gag-pol*gpt (Fig. 2) was constructed by using the plasmid pSV2gpt (20) as the source of simian virus 40 sequences and the *gpt* gene as the selectable marker. The plasmid 3P0 contains Mo-MULV proviral DNA with a 134-base-pair deletion of the ψ packaging signal, from *Ball* at

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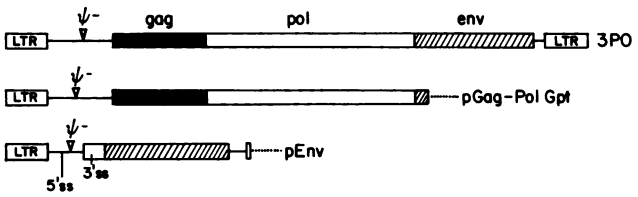

FIG. 1. Comparison of viral sequences contained in parent plasmid 3P0 and constructs p*gag-pol*gpt and p*env*. Mo-MULV LTRs and the ψ deletion (ψ⁻) are indicated. Symbols and abbreviations: solid regions, *gag* sequences; open regions, *pol* sequences; hatched regions, *env* sequences; solid lines, nontranslated viral sequences; 5′ ss, 5′ splice donor; 3′ ss, 3′ splice acceptor.

position 660 to *Xma*III at position 794 (L. Lobel and S. Goff, personal communication). 3P0 DNA was digested with *Sca*I and *Nae*I, both of which left blunt ends; and a 7.9-kilobase (kb) fragment containing the 5′ LTR and the *gag* and *pol* genes was isolated from a 1.7% agarose gel by electroelution. Plasmid pSV2gpt was digested at its unique *Bam*HI site, and its protruding 5′ ends were filled in by using the Klenow fragment of DNA polymerase and all four deoxynucleoside triphosphates. The 7.9-kb *gag-pol* fragment was then ligated to the blunt-ended 5.1-kb pSV2gpt vector, and positive colonies were isolated by using colony filter hybridization (10) and probing with a nick-translated 2.54-kb *Bgl*II fragment from 3P0 (*gag-pol* probe). DNAs from individual colonies were then tested for the correct orientation of the *gag-pol* insert by digesting them with *Eco*RI. The resulting 13.4-kb plasmid was named p*gag-pol*gpt (Fig. 2).

p*env*. Plasmid 3P0 was digested with *Bgl*II and *Nhe*I (Fig. 2). The 2.4-kb *env* fragment at positions 5858 to 8297

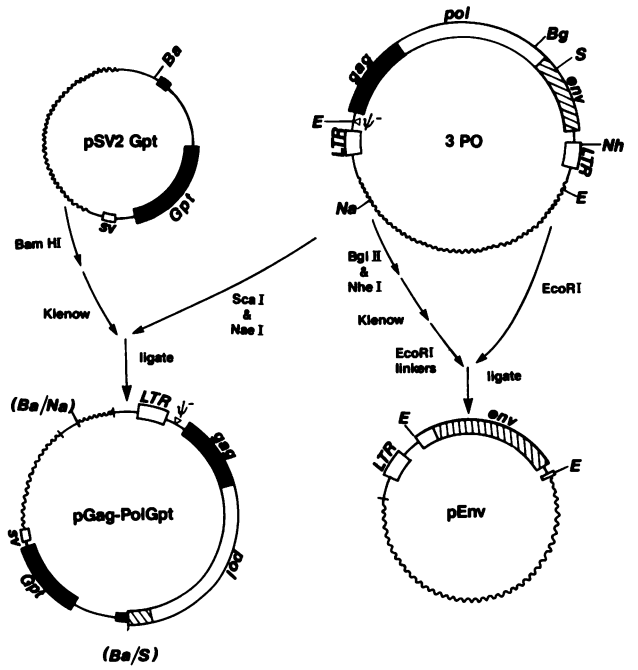

FIG. 2. Schematic diagram showing construction of plasmids p*gag-pol*gpt and p*env*. Symbols and abbreviations: wavy lines, pBR322 sequences; thin lines, plasmid sequences; small solid box, simian virus 40 poly(A) sequence; SV, simian virus 40 origin of replication; ψ⁻, deletion of ψ packaging sequences; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Na, *Nae*I; Nh, *Nhe*I; S, *Sca*I.

FIG. 3. Replication-defective retroviral vector Δneo. Symbols and abbreviations: ψ, packaging sequence; wavy line, pBR322 sequences; solid box, simian virus 40 promoter and origin of replication.

containing the 3′ acceptor splice site was isolated by electroelution from a 1.2% agarose gel. The ends were filled in with the Klenow fragment of DNA polymerase and all four deoxynucleoside triphosphates, and *Eco*RI linkers were ligated to both ends. The 5′ LTR and 5′ donor splice site were prepared by digesting 3P0 DNA with *Eco*RI and isolating the 6.2-kb fragment by electroelution from a 1% agarose gel. The 6.2-kb fragment was treated with phosphatase and then ligated to the 2.4-kb *env* fragment. Positive transformants were isolated by using the colony filter hybridization technique, probed with a labeled 1.3-kb *Hpa*I fragment from 3P0 (*env* probe). DNAs from positive colonies were then tested for the correct orientation of the *env* insert by digesting them with either *Xba*I or *Sca*I. The resulting 8.6-kb plasmid was named p*env* (Fig. 2).

Electroporation and cell analysis. NIH 3T3 cells were transfected with p*gag-pol*gpt or the p*env* plasmid by electroporation (22). For each experiment, 10^7 cells were collected by centrifugation and suspended in 0.5 ml of sterile, 1× phosphate-buffered saline. The cells were then mixed with 10 μg of nonselectable plasmid DNA and 5 μg of selectable plasmid DNA or with 5 μg of selectable plasmid DNA alone. The cell-DNA suspension was loaded into a 0.5-ml electroporation chamber (model ZA1000; Prototype Design Services, Madison, Wis.) and a bank of capacitors (effective capacity, 14 μF), charged to 500 to 1,000 V, and discharged via an electronic switch through the solution. The cells were then suspended in 100 ml of Dulbecco modified Eagle medium, supplemented with 10% newborn calf serum–penicillin (100 μg/ml)–streptomycin (100 μg/ml)–amphotericin B (0.25 μg/ml), and plated in four 24-well plates. Selective medium was added 48 to 72 h after the electroporation.

The plasmids p*gag-pol*gpt and p*env* were coelectroporated into NIH 3T3 cells; as a control, 3P0 and pSV2gpt were also coelectroporated into NIH 3T3 cells. Cells were selected for the presence of the *gpt* gene with medium containing 15 μg of hypoxanthine per ml, 250 μg of xanthine per ml, and 25 μg of mycophenolic acid (MA) per ml (HXM medium). Clones selected with HXM medium were then analyzed for reverse transcriptase (RT) production as described previously (8). Positive controls for RT activity were ψ2 cell (15) supernatants and supernatants from wild-type Mo-MULV clone 4 cells. Negative controls for RT activity were untransfected NIH 3T3 supernatants and RT cocktail alone.

Packaging lines were transfected with the retroviral vector plasmids Δneo and N2 by electroporation of 10^7 NIH 3T3 cells with 5 μg of plasmid DNA. Both plasmids contain a neomycin resistance (*neo*^{r}) gene; eucaryotic cells expressing the gene were selected with the antibiotic G418 (800 μg/ml). Δneo is a 6.6-kb replication-defective retroviral plasmid in which the *neo*^{r} gene is flanked by intact LTRs and has 5′ *gag* sequences, including an intact ψ sequence (Fig. 3) (14a); N2 has been described previously (12).

Analysis of viral proteins. The presence and expression of p*env* was analyzed by metabolic labeling and immunoprecipitation of gPr80*env*, the *env* protein, with *env* antiserum as follows. Clones of confluent cells (on 10-cm-diameter plates) were starved for 20 min in Dulbecco modified Eagle

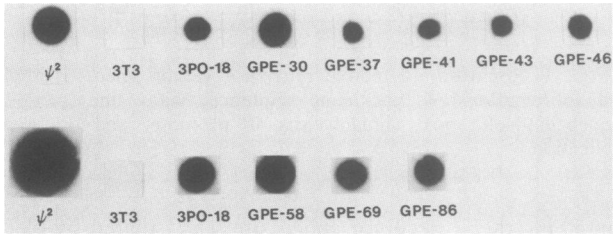

FIG. 4. RT assays of supernatants from clones of cells transfected with 3P0 or cotransfected with p*gag-pol*gpt and p*env*. Individual clones resistant to MA were isolated, and the supernatant fluid was assayed for RT on an exogenous template (8). Results are shown from two different experiments (top and bottom lines). Included in each experiment are positive (ψ^2 supernatant) and negative (NIH 3T3 supernatant) controls.

medium minus methionine, and 150 μCi of [^{35}S]methionine (Amersham Corp. Arlington Heights, Ill.) was added for 40 min. The cells were lysed in 1% Triton X-100–0.5% deoxycholate–0.1% sodium dodecyl sulfate–10 mM sodium phosphate (pH 7.5)–0.1 M NaCl; the cell lysate was spun down in a TI50 or TI80 rotor at 35,000 × *g* for 3 h at 4°C. The supernatant was incubated with normal goat serum, and nonspecifically bound proteins were precipitated with pansorbin (staph A protein; Calbiochem-Behring, La Jolla, Calif.). The remaining supernatant was incubated with *env* antiserum (795-771; National Cancer Institute, Rockville, Md.) overnight, and the immunoprecipitates were collected with pansorbin. The labeled proteins were analyzed by electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide gel (13), followed by fluorography.

Virus production. Titers of CFU were determined by infection of NIH 3T3 cells with dilutions of the viral harvest as follows. NIH 3T3 cells (5×10^5) were seeded in a 6-cm-diameter petri dish. Eighteen hours later the viral harvest supernatants from clones of semiconfluent cells were filtered through 0.45-μm-pore-size filters (Millipore Corp., Bedford, Mass.), and 1 ml was applied to the cells. Polybrene (8 μg/ml; dextran) was added to the supernatants to enhance the titer. After 2 h at 37°C, 4 ml of medium was added to the cells; 48 h later the cells were trypsinized and plated onto a 10-cm-diameter plate in medium containing 800 μg of G418 per ml; 10 to 14 days later clones were counted.

RESULTS

Generation of the packaging line. To generate cell lines expressing *gag-pol* and *env* regions from different plasmids, NIH 3T3 cells were cotransfected with p*gag-pol*gpt and p*env* DNAs. Recipient cells were then selected with medium containing MA. Eighty-six MA-resistant (GP+E) clones were isolated, and their supernatants were tested for RT. Twenty-seven clones were found to produce a high level of RT (Fig. 4). In a separate electroporation, 3P0 DNA, containing an intact set of *gag*, *pol*, and *env* genes, was coelectroporated with pSV2gpt into NIH 3T3 cells. Of 16 MA-resistant clones obtained from this electroporation and tested, supernatants from 3 were high in RT (Fig. 4). The RT levels of the high RT-producing GP+E clones were equal to those of the high RT-producing 3P0 clones.

Twenty-one of the high RT-producing GP+E clones were then analyzed for *env* protein expression by metabolic labeling, followed by immunoprecipitation with *env* antiserum. A total of 11 of the clones were positive for gPr80*env*; 3 clones produced a strong signal, 4 produced a medium signal, and 4 produced a weak signal (Fig. 5).

Ability of cell lines to package retroviral vectors. To test the packaging ability of cell lines containing a fragmented retroviral genome, five GP+E clones that expressed high levels of RT and medium to high levels of *env* were transfected with Δneo DNA, and recipient cells were selected with G418 as described above. A number of G418-resistant clones (GP+E+Δneo) were collected from each transfected GP+E cell line.

To determine viral titers, supernatants from GP+E+Δneo clones were filtered, and a 1-ml portion of undiluted or diluted supernatant was used to infect NIH 3T3 cells as described above. The titers of the GP+E+Δneo clones ranged from 1×10^2 to 1.7×10^5 CFU/ml (Table 1). Supernatants from G418-resistant clones assayed for Δneo virus titers in the *gag-pol-env* packaging clone 3P0-18 pro-

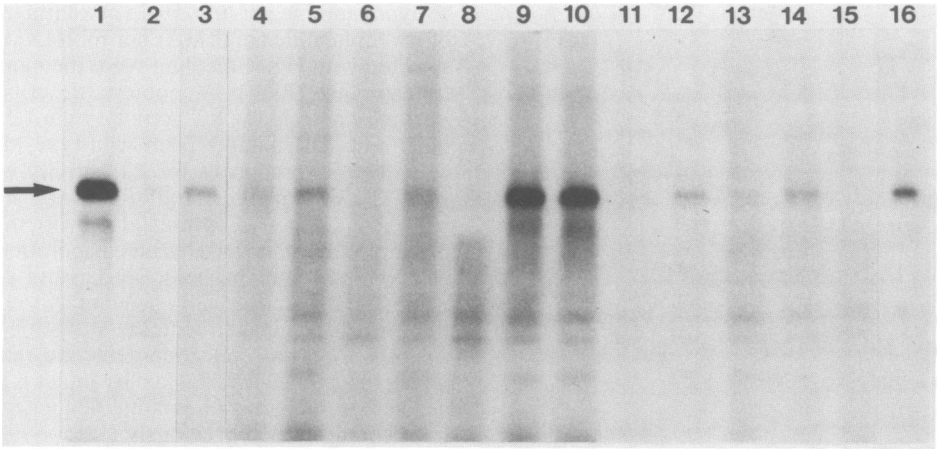

FIG. 5. Analysis of viral *env* protein synthesis in transfected NIH 3T3 cells. Plasmids p*gag-pol*gpt and p*env* were cotransfected into NIH 3T3 cells. Individual clones resistant to MA and which expressed high levels of RT were labeled with [^{35}S]methionine. The labeled proteins were analyzed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography, as described in the text. Lane 1, Proteins from 3P0-18 cells; lanes 2 to 16, proteins from GP+E clones 5, 21, 30, 37, 38, 41, 42, 43, 46, 56, 58, 66, 69, 75, and 86, respectively. The position of protein gPr80^{env} is indicated by the arrow.

TABLE 1. Virus production from packaging cells containing retroviral vectors

Packaging cell line	Vector	Clone	Titer (CFU/ml)	Secondary titer (CFU/ml)[a]	Secondary RT[b]
GP+E-86	Δneo	1	1.25×10^5	0	–
		8	3×10^4	0	–
		11	9×10^4	0	–
		17	6.5×10^4	0	–
		21	1.7×10^5	0	–
ψ2	Δneo	4	4.6×10^4		
		6	5.4×10^4		
3P0-18	Δneo	1	2.2×10^4	0	
		2	1.1×10^4		
		3	5.7×10^3		
		4	8×10^2		
		5	6.7×10^3	0	
		7	6.5×10^4	0	
GP+E-86	N2	3	1.23×10^6		
		7	3.5×10^6		
		8	2.6×10^6		
		9	3×10^6		
		11	1.33×10^6		
		12	4×10^6		
		13	3.6×10^6		
		17	2.5×10^6		
		20	1.34×10^6		
		22	3×10^6		
3P0-18	N2	1	3.75×10^4		
		2	1.85×10^4		
		3	4×10^4		
		6	10^5		
		9	6×10^4		
		10	10^5		
		11	1.7×10^5		

[a] Supernatants from pools of G418-resistant Δneo virus-infected NIH 3T3 cells were analyzed for the Δneo virus titer.

[b] Supernatants from pools of G418-resistant Δneo virus-infected NIH 3T3 cells were assayed for RT as described in the text.

duced titers from 8×10^2 to 6.5×10^4 CFU/ml. The titer of Δneo released from ψ2 cells was 4.6×10^4 to 5.4×10^4 CFU/ml. The GP+E-86 packaging line produced consistently higher titers than the other four packaging lines, and was therefore used in subsequent experiments.

GP+E-86 cells were also tested for their ability to package the retroviral vector N2. N2 DNA was electroporated into GP+E-86 cells, and 22 G418-resistant clones (GP+E+N2) were isolated. N2 viral titers from GP+E+N2 clones ranged from 5.3×10^3 to 4×10^6 CFU/ml, with 17 of 22 (77%) clones generating titers of $>10^5$ CFU/ml. Titers from clones of 3P0−18 cells transfected with N2 ranged from $<1 \times 10^2$ to 1.7×10^5 CFU/ml, with 5 of 11 (45%) generating titers of $>1 \times 10^5$ CFU/ml.

Analysis for recombinant infectious retrovirus. As a preliminary test for infectious retrovirus, supernatants from five high-titer GP+E+Δneo clones were used to infect NIH 3T3 cells. The infected NIH 3T3 cells were selected with G418 and allowed to develop into a confluent layer of G418-resistant clones. Supernatants from these plates (secondary GP+E+Δneo supernatants) were then used to infect fresh NIH 3T3 cells. These NIH 3T3 cells were again selected with G418, resulting in no surviving G418-resistant cells. These same supernatants also tested negative for RT. These results indicate that there is no viral rescue of Δneo from the initial NIH 3T3 cells infected with GP+E+Δneo primary supernatants.

In a more stringent test for infectious retrovirus, NIH 3T3 cells were infected with supernatants from high-titer GP+E+Δneo clones. The infected NIH 3T3 cells were passaged continuously for 1 month without G418 selection. This treatment would have allowed the spread of a rare recombinant wild-type virus throughout the population of cells and, therefore, the spread of infectious Δneo particles into originally noninfected NIH 3T3 cells (that would have been killed off had the cells been exposed to G418). After 1 month in culture, supernatants were tested for Δneo production by infecting fresh NIH 3T3 cells and testing them for G418 resistance. As in the previous experiment, no G418-resistant cells were obtained.

In a third test for the safety of the GP+E-86 packaging line, we designed an experiment that could detect a transfer of packaging function. NIH 3T3 cells were electroporated with N2 DNA, and pools of G418-resistant clones (NIH 3T3-N2 pools) were collected. Supernatant medium from GP+E-86 cells was used to infect the NIH 3T3-N2 pools. If the NIH 3T3-N2 pools became infected with the wild-type virus secreted from GP+E-86 cells, resulting in a transfer of packaging function, these cells would begin to secrete N2 virus. The supernatant from the infected NIH 3T3-N2 pools was harvested and used to infect NIH 3T3 cells. These NIH 3T3 cells were then assayed for the presence of N2 virus by exposing the cells to G418. Using this type of assay, we were unable to detect G418-resistant colony production; therefore, GP+E-86 cells are unable to transfer the packaging function, as demonstrated by an inability to rescue N2 virus from NIH 3T3 cells.

DISCUSSION

A safe packaging line essentially incapable of permitting recombinational events that can result in an intact infectious retroviral genome is a requirement for the use of retroviral vectors in human gene therapy. While recently used vector systems are relatively safe, their design is flawed by the potential for two recombination events resulting in the generation of infectious retrovirus. In the experiments described here, we separated portions of the helper virus genome so that the generation of an intact retroviral genome would be extraordinarily unlikely, if not impossible. We separated the *gag* and *pol* genes on one plasmid and the *env* gene on another plasmid. In addition, we kept the packaging (ψ) mutation in both of these plasmids and eliminated the 3′ LTR, thus, further diminishing the opportunity for recombination. We demonstrated that the fragmented helper virus genome, when introduced into NIH 3T3 cells, appears to produce titers of retroviral particles comparable to those of the intact Mo-MULV helper from which the components were derived. Analysis of NIH 3T3 cells transfected with these fragmented genomes has been simplified by the availability of a sensitive RT assay (8) to detect *gag-pol* expression and a sensitive *env* assay to measure the expression of this important gene.

We obtained an optimal packaging line (GP+E-86) that produces high *gag-pol* and *env* protein levels, as demonstrated by the RT assay and immunoprecipitation with *env* antiserum. GP+E-86 cells (which contain *gag* and *pol* on one plasmid and *env* on another plasmid), on transfection with the retroviral vectors Δneo and N2, released titers that were comparable to those released by 3P0-18 and ψ2 cells

(which contain an intact *gag-pol-env* plasmid). The titers that were obtained were comparable to those reported by other investigators who used defective retrovirus (2, 4, 5, 15, 16, 18, 23, 25) and were high enough for use in gene transfer experiments in animals (3, 6, 7, 9, 11, 14, 17, 26, 27).

Thus far, we have found no evidence for recombinational events occurring when *gag-pol* on one plasmid and *env* on another are used in plasmids that also contain ψ mutations and deletions of 3′ LTRs. Cells that were electroporated with these packaging plasmids and then with vector plasmids did not appear to produce the three recombination events needed for the generation of wild-type virus. Thus, this packaging line may be especially safe for use in experiments whose goal is human gene therapy. We are currently preparing a similar amphotropic packaging line for this purpose.

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