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A New Avian Leukosis Virus-Based Packaging Cell Line That Uses Two Separate Transcomplementing Helper Genomes

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An avian leukosis virus-based packaging cell line was constructed from the genome of the Rous-associated virus type 1. The *gag*, *pol*, and *env* genes were separated on two different plasmids; the packaging signal and the 3′ long terminal repeat were removed. On a plasmid expressing the *gag* and *pol* genes, the *env* gene was replaced by the hygromycin resistance gene. The phleomycin resistance gene was inserted in the place of the *gag-pol* genes on a plasmid expressing the *env* gene. The plasmid containing the *gag*, *pol*, and Hygro^r genes was transfected into QT6 cells. Clones that produced high levels of p27^{gag} were transfected with the plasmid containing the Phleo^r and *env* genes. Clones that produced high levels of *env* protein (as measured by an interference assay) were tested for their ability to package Neo^R-expressing replication-defective vectors (TXN3′). One of the clones (Isolde) was able to transfer the Neo$^+$ phenotype to recipient cells at a titer of 10^5 resistance focus-forming units per ml. Titers of supernatants of cells infected with Rous-associated virus type 1 prior to transfection by Neo^r vectors were similar. Tests for recombination events that might result in intact helper virus showed no evidence for the generation of replication-competent virus. The use of selectable genes inserted next to the viral genes to generate high-producer packaging cell lines is discussed.

Livestock improvement as well as studies of cell lineage in the chicken embryo have both raised the need of generating helper-free replication-defective retroviruses at high titers (3, 14, 25–27). Recently, an avian leukosis virus (ALV)-based packaging cell line has been constructed in which the helper virus genome is not a transmissible replication-competent virus (30). The signal for packaging of viral RNA has been deleted. Thus, while it produces all the viral proteins necessary for virus replication and assembly, there is no release of wild-type helper virus by this packaging cell line. However, when the packaging line was transfected with a replication-defective vector containing an intact packaging signal required for its own packaging, replication-competent viruses could arise, probably by a recombination event between the helper and the vector genomes. Contaminating helper virus is a major problem in the use of retroviral vectors for introducing foreign genes in the chicken germ line because its proliferation induces leukosis (35). To circumvent this problem, we constructed a new ALV-based packaging cell line in which the helper genome was further disabled by deleting the 3′ long terminal repeat (LTR) (29). Two recombination events would then be necessary to produce replication-competent retrovirus. We showed that this packaging cell line did not produce any wild-type retrovirus. However, vector titers obtained from this packaging line were always 10 to 20 times lower than titers obtained with a coinfecting helper virus. High-titer replication-defective vectors are a major requirement for experiments whose goal is to infect the germ line because only a very small proportion of the cells is committed to the germ line in the newly laid egg (32).

We approached this problem by generating a new packaging cell line expressing the *gag*, *pol*, and *env* genes on two separate plasmids. In addition, the two packaging genomes were further disabled by the deletion of their packaging signal and their 3′ LTR. This strategy was shown to be very safe as far as production of replication-competent retrovirus is concerned (10, 12, 20). To get high levels of expression of the viral genes, a selectable gene (Hygro^r and Phleo^r genes) was inserted within the viral genome so that the selectable and the viral genes were expressed from the same transcription unit. A packaging cell line was generated, producing as many vector retrovirus as cells coinfected with helper virus. In addition, the three recombination events necessary to generate replication-competent helper virus could not be detected.

MATERIALS AND METHODS

Plasmids. Plasmids pRAV1, containing the permuted genome of Rous-associated virus type 1 (RAV-1), was obtained from J. M. Bishop (University of California, San Francisco). Plasmids pLG89 and pX343, containing the bacterial Hygro^r gene driven by the simian virus 40 early promoter, were obtained from H. Diggelman (ISREC, Lausanne, Switzerland). Plasmid pUT56, containing the bacterial Phleo^r gene, was obtained from G. Tiraby (Université Paul Sabatier, Toulouse, France). Plasmid pHF13 is a packaging mutant of RAV-1 (29). pTXN3′, pTXN5′, pXJ12, and pNL53 are avian erythroblastosis virus based vectors (see Fig. 2). pTXN3′, pTXN5′, and pXJ12 have been described elsewhere (1). pNL53 is a derivative of pTXN5′ described previously (1). It carries and expresses the Neo^r gene and the *Escherichia coli lacZ* gene, both under the control of the 5′ LTR. The *lacZ*

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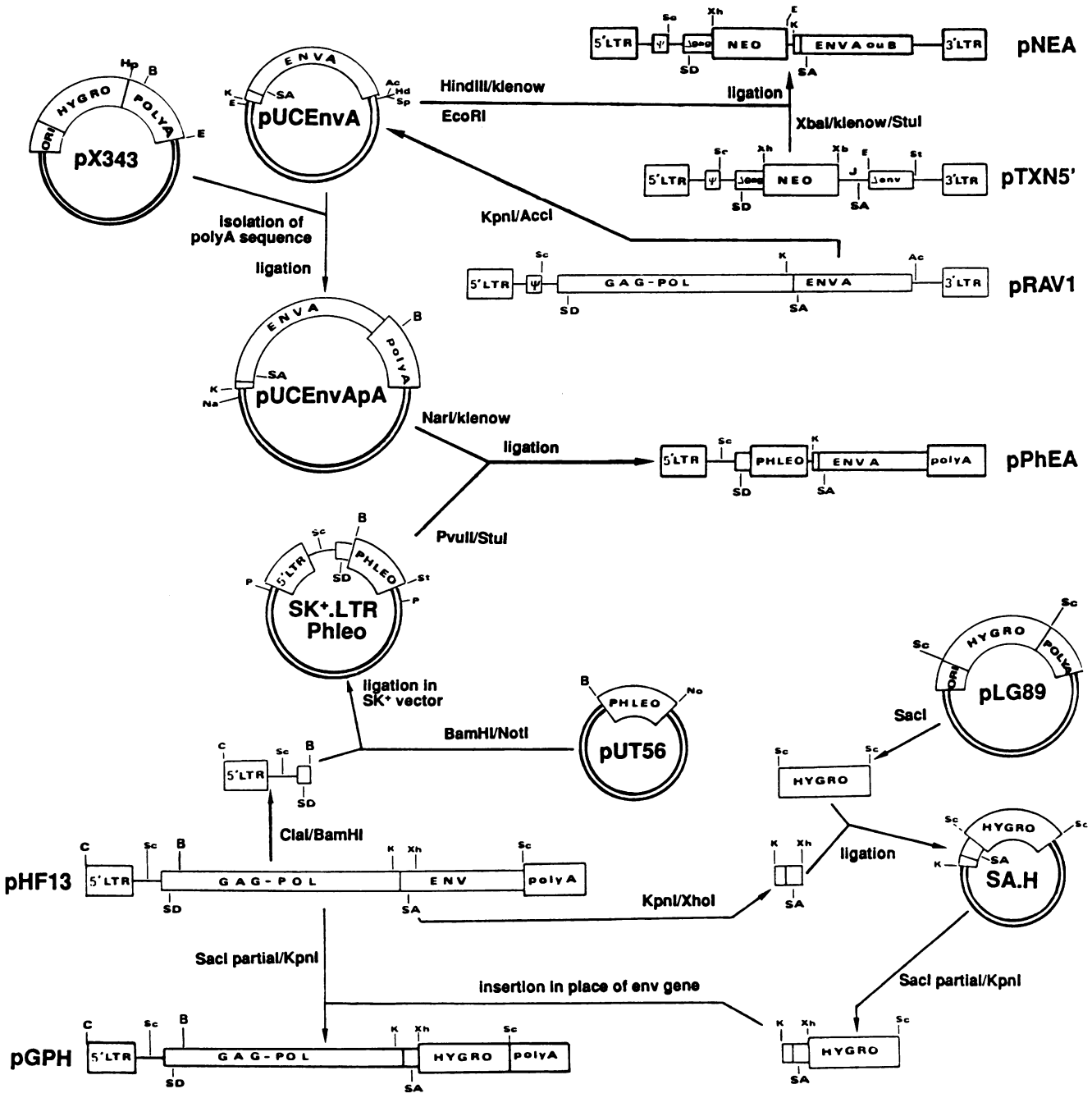

FIG. 1. Schematic diagram of plasmid construction. The doubled line represent procaryotic DNA elements; the small open boxes represent retroviral genes; the large open boxes represent the LTRs, polyadenylation signals, and selectable genes; Ψ, packaging signal; SD and SA, splice donor and splice acceptor sites, respectively. Restriction enzymes: Ac, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Hd, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; Na, *Nar*I; No, *Not*I; P, *Pvu*II; Sc, *Sac*I; Sp, *Sph*I; St, *Stu*I; Xh, *Xho*I.

gene and the *gag* gene residue are in the same reading frame. Translation of the *lacZ* gene starts at the initiator codon of the δ*gag* gene after splicing of genomic RNA. δ*gag* indicates the 5′ end of the *gag* gene, which remains intact in our vectors. Similarly, δ*env* indicates the 3′ end of the *env* gene.

All plasmids were amplified in *E. coli* HB101 or DH5α strains.

Enzymes. Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, the large fragment of *E. coli* DNA polymerase I, and calf intestinal phosphatase were purchased from Boehringer Mannheim France SA.

Construction of vectors. Plasmid constructions are summarized in Fig. 1. A 1.2-kilobase (kb) *Sac*I fragment (isolated from pLG89) containing the Hygro^r gene was subcloned into the *Sac*I restriction site of the SK$^+$ polylinker (STRATAGENE). A 263-base-pair *Kpn*I-*Xho*I fragment containing the *env* splice acceptor site of RAV-1 was isolated from pHF13 (29). Plasmid SA-H was generated by inserting

this splice acceptor site upstream from the Hygro^r gene, which was inserted in the same reading frame as the original *env* coding sequence. Plasmid SA-H was then double digested with *Kpn*I and *Sac*I. A 1.5-kb fragment containing the splice acceptor site and the Hygro^r gene was inserted between the *Kpn*I and *Sac*I restriction sites of pHF13 in the place of the *env* gene to generate the packaging mutant pGPH.

A 1.9-kb *Kpn*I-*Acc*I fragment containing the splice acceptor site and the *env* gene was isolated from plasmid pRAV1 (subgroup A). Plasmid pUCEnvA was generated by subcloning this fragment in the mp19 polylinker of pUC19 vector (BIOLABS). Plasmid pUCEnvApA was generated by inserting a 0.9-kb *Hpa*I-*Eco*RI fragment containing the polyadenylation signal from simian virus 40 (isolated from pX343) in pUCEnvA. A 0.9-kb *Cla*I-*Bam*HI fragment was isolated from pHF13 DNA and contains the 5′ LTR, the leader sequence minus its packaging signal (29), as well as the splice donor site located at the beginning of the *gag* gene. A 0.6-kb *Bam*HI-*Not*I fragment containing the Phleo^r gene was isolated from plasmid pUT56. Both fragments (0.9 and 0.6 kb) were coligated into the *Cla*I and *Not*I sites of the SK$^+$ vector polylinker. This gave rise to the SK$^+$-LTR-PHLEO plasmid in which the Phleo^r gene and the δ*gag* gene are in the same reading frame. A 1.5-kb *Pvu*II-*Stu*I fragment containing the 5′ LTR, the leader region, and the δ*gag*-Phleo^r fusion gene was isolated from SK$^+$-LTR-PHLEO and was inserted in plasmid pUCEnvApA upstream from the *env* gene. The resulting plasmid was called pPhEA.

A 1.9-kb *Eco*RI-*Hin*dIII-filled fragment containing the *env* gene (and its splice acceptor site) was isolated from pUCEnvA and inserted into pTXN5′ previously deleted of its J region (which contains the splice acceptor site of avian erythroblastosis virus) and of AEV-δ*env* residue (Fig. 2). The resulting retroviral vector was called pNEA.

Cells and media. The QT6 cell line was obtained from C. Moscovici (Gainesville, Fla.), (24). Chicken embryo fibroblasts (CEFs) were prepared from C/O SPAFAS 10-day-old chicken embryos and grown as described previously (13). QT6 and CEF cells were grown in F10 medium supplemented with 5% calf serum and 1% chicken serum. Medium and sera were purchased from Flow Laboratories. G418 (GIBCO-BRL), hygromycin B (Boehringer-Mannheim), and phleomycin (Cayla) were dissolved in phosphate-buffered saline (PBS) and sterilized by filtration through 0.22-μm-pore-size membranes.

DNA transfection. Plasmids DNAs were transfected into QT6 cells as described by Kawaï and Nishizaka (17). Transfected cells were selected in medium containing 200 μg of G418 per ml, 30 to 80 μg of hygromycin B per ml, or 10 to 50 μg of phleomycin per ml. Medium containing the antibiotics was replaced every 3 days for 7 to 15 days. Resistant colonies were isolated by using cloning cylinders.

Detection of viral proteins. Production of viral proteins was analyzed from culture supernatants and cell lysates by an enzyme-linked immunosorbent assay (ELISA) with an anti-p27^{gag} (7), as described previously (29).

Virus assay. Fresh media (4 ml) were added on confluent producer cells plated on 100-mm dishes, and 6 to 8 h later, virus samples were collected and then filtrated through 0.22-μm-pore-size membranes to remove cells and debris and stored at −80°C.

Titers of vectors expressing the Neo^r gene as a selectable marker were determined by induction of G418 resistance to fresh QT6 cells. QT6 cells were infected with diluted virus suspensions for 6 h. Culture medium was then replaced by selective medium containing 200 μg of G418 per ml. This medium was replaced every 3 days, and resulting Neo$^+$ colonies were scored 10 days later.

Recombinant vectors carrying the bacterial *lacZ* gene were titrated on QT6 cells by cytochemical staining. QT6 cells were plated in 60-mm dishes, at a density of 5×10^5 cells per plate. The next day, QT6 cells were infected with diluted virus suspensions for 6 h. Three days later, they were fixed in 4% paraformaldehyde and washed in PBS. Fixed cells were incubated overnight at 37°C in a solution containing 5 mM potassium hexacyanoferrate III [$K_3Fe(CN)_6 \cdot 3H_2O$], 5 mM potassium hexacyanoferrate II [$K_4Fe(CN)_6 \cdot 3H_2O$], 2 mM $MgCl_2$, 3% dimethyl sulfoxide, and 1 mg of X-Gal (5-bromo-4-chloro-indolyl-β-D-galactoside) per ml. Blue-stained clones of β-galactosidase producer cells were scored.

For assay of replication-competent virus, CEFs were seeded at 10^6 cells per 60-mm dish. One day later, virus samples were added. The medium was replaced every 3 days thereafter, and cells were passaged for 2 weeks to allow replication-competent virus to spread. Culture supernatants from these cells were then assayed by ELISA for the p27^{gag} protein.

Helper virus was also titrated by in situ detection of p27^{gag} producer cells. QT6 cells were infected with virus suspensions and overlaid with agar 12 h later. When cells were confluent, they were washed in PBS, fixed in 4% paraformaldehyde, and washed again in PBS containing 0.25% Triton X-100. Polyclonal rabbit anti-p27^{gag} (Life Science, Inc., St. Petersburg, Fla.) was added (45 min with 3% bovine serum albumin) followed by an alkaline phosphatase-conjugated goat anti-rabbit serum (Biosys, Compiègne, France) (45 min). Alkaline phosphatase activity was revealed with substrate kit III (Vector Laboratories, Inc., Burlingame, Calif.).

RESULTS

Helper and vector genomes. The *gag-pol-env* packaging genome of ALVs was separated on two plasmids, one expressing the *gag* and *pol* genes (plasmid pGPH) and another one expressing the A-subgroup *env* gene (plasmid pPhEA). Details of the constructions are given in Materials and Methods. The pGPH genome (Fig. 2) is a derivative of the pRAV1 genome in which the *env* gene has been replaced by the Hygro^r gene, which confers resistance to hygromycin B. Furthermore, the packaging signal has been deleted, and the 3′ LTR was removed and replaced by the polyanenylation sequence from the herpes simplex thymidine kinase gene. In plasmid pGPH, transcription from the 5′ LTR gave rise to two kinds of mRNAs: a genomic RNA from which the *gag-pol* gene was translated and a subgenomic RNA processed by splicing of the genomic mRNA, leading to Hygro^r gene translation as a δ*gag*-δ*env*-Hygro^r fusion protein. The pPhEA genome (Fig. 2) is also a derivative of pRAV1 in which the *gag* and *pol* genes have been removed and replaced by the Phleo^r gene, which confers resistance to phleomycin. This plasmid was also mutated in the packaging signal and in the 3′ LTR. pPhEA should direct transcriptions of genomic mRNA leading to Phleo^r gene expression and of subgenomic mRNA leading to gp85^{env} and gp37^{env} production. Retroviral vector pNEA (Fig. 2) was generated by inserting the A-subgroup *env* gene into pTXN5′. The retroviral vector pNEA, carrying two genes, expresses the Neo^r gene from the genomic mRNA and the *env* gene from the subgenomic mRNA.

Testing of the pGPH packaging mutant. QT6 cells were

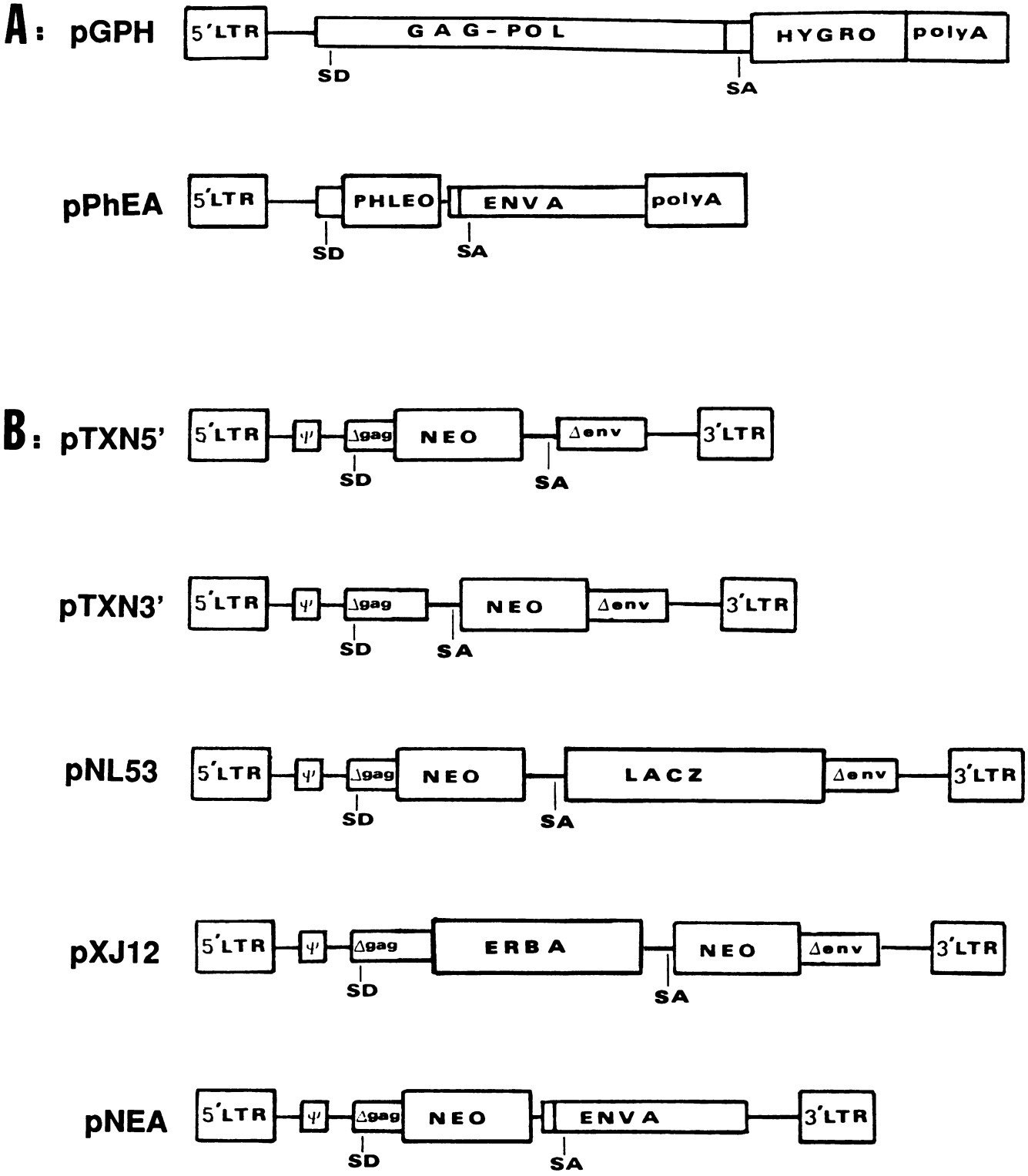

FIG. 2. Structure of packaging mutants pGPH and pPhEA (A) and of retroviral vectors pTXN5', pTXN3', pNL53, pXJ12, and pNEA (B). The small open boxes represent retroviral genes, and the large open boxes represent the LTRs, polyadenylation signals, and selectable genes. SD and SA, splice donor and splice acceptor sites, respectively; Ψ, packaging signal.

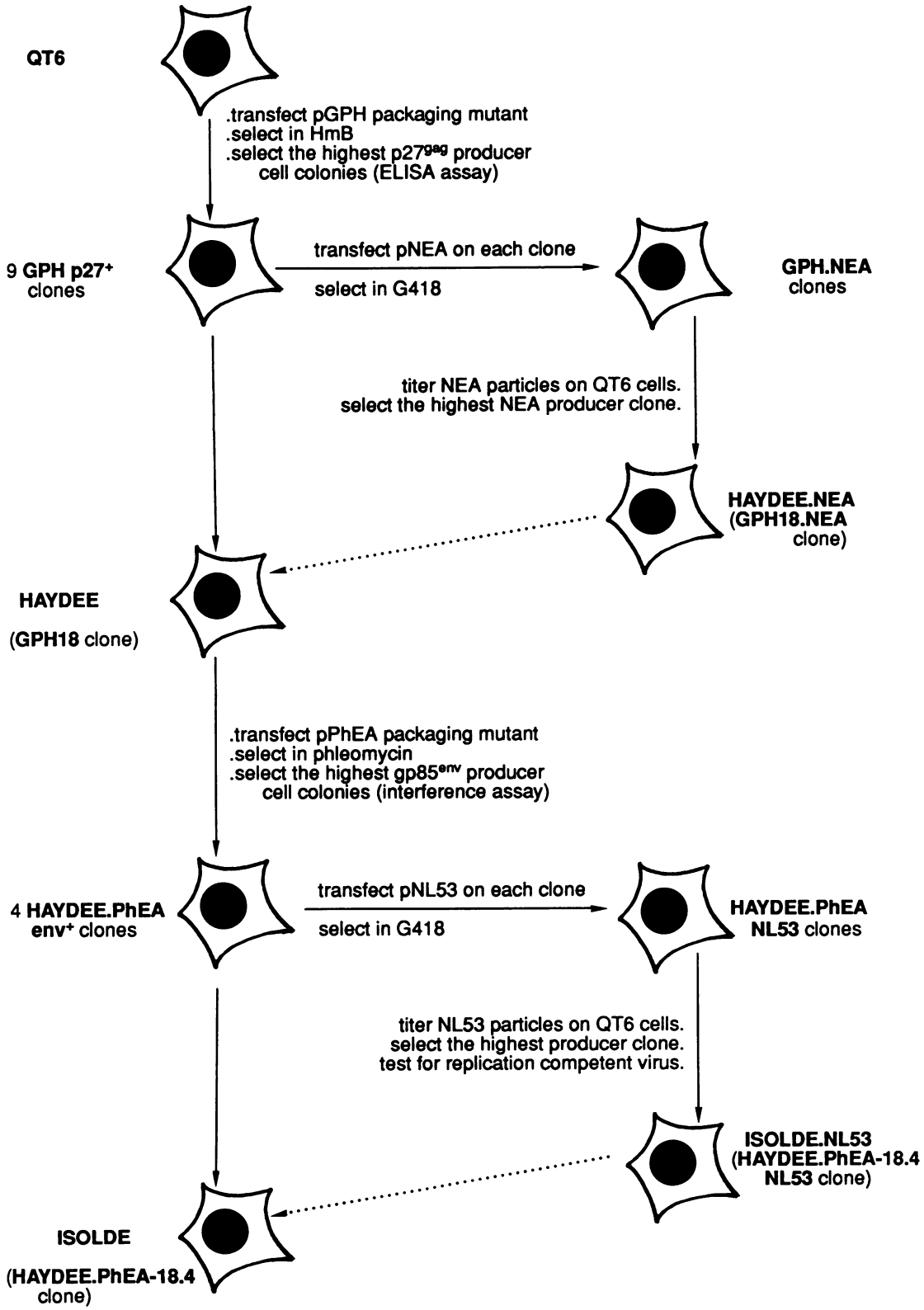

FIG. 3. Diagram of the strategy used for generating ALV-based packaging cell lines with two separate helper genomes carrying selectable markers.

TABLE 1. $p27^{gag}$ production from GPH clones and transcomplementing activity as tested by their ability to package the NEA retroviral vector

Cell or clone	$p27^{gag}$ extra (OD_{405})[a]	$p27^{gag}$ intra (OD_{405})[b]	NEA titer (RFU/ml)[c]
GPH clones			
1	0.160	ND	ND
2	0.720	ND	ND
3	0.800	ND	ND
4	0.770	ND	ND
5	0.400	ND	ND
6	0.200	ND	ND
7	0.35	ND	ND
8	0.59	ND	ND
9	0.46	ND	ND
10	0.50	ND	ND
11	0.80	ND	ND
12	0.17	ND	ND
13	1.06	ND	ND
13	1.060	0.91	ND
15	0.96	0.83	ND
16	1.06	ND	80
17	1.29	0.960	3.2×10^3
18	1.072	0.90	1.5×10^5
19	1.25	0.95	0
20	1.22	0.95	0
21	0.97	0.86	3
22	1.22	ND	0
23	0.95	0.91	1
QT6	0.23	0.200	0
RAV1-QT6	1.3	1.1	ND

[a] $p27^{gag}$ level in GPH clone supernatants ($p27^{gag}$ extra) (100 μl) was determined by ELISA. OD_{405}, Optical density at 405 nm.

[b] $p27^{gag}$ level in GPH clone crude cell lysates ($p27^{gag}$ intra) was determined by ELISA. Cell proteins (3 μg) were diluted 1/500. ND, Not determined.

[c] GPH clones were transfected with DNA from the NEA retroviral vector (Fig. 2). Titers are expressed as numbers of Neo^+ colonies induced by infection of QT6 cells with 1 ml of viral stock. RFU, Resistance focus-forming units.

transfected with pGPH plasmid DNA and selected with hygromycin B (Fig. 3). Hygromycin B-resistant ($Hygro^+$) cell colonies were isolated and grown individually. A total of 23 $Hygro^+$ clones were screened by an ELISA for production of $p27^{gag}$ protein both in culture supernatant and in crude cell lysates (Table 1). Some GPH ($Hygro^+$) clones were found to produce as much $p27^{gag}$ as RAV1-infected QT6 did. A positive correlation was found between levels of $p27^{gag}$ in culture supernatants and in cell lysates. Therefore, it was most likely that any variations in the level of $p27^{gag}$ between clones resulted from genuine variations in the activity of the proviruses and was not simply due to the inaccuracy of the measurement. It must be pointed out that all GPH clones were cultured in medium containing hygromycin B. Hygromycin B was omitted for collecting supernatants for $p27^{gag}$ assay only.

To determine whether these GPH $Hygro^+$ cell lines were providing all the proteins encoded by the *gag* and *pol* genes, nine of them were transfected with pNEA plasmid DNA (Fig. 3). pNEA is a packaging-competent vector which carries and expresses both Neo^r and *env* genes. Therefore, it could complement GPH cells for production of infectious viral particles by providing both *env* proteins and genomic RNAs. Transfected cells were selected with G418 (200 μg/ml) and polyclonal cultures (more than 100 Neo^+ colonies) were grown for each GPH cell line. Supernatants from these cultures were harvested and titrated by infecting fresh QT6 cells that were then selected with G418. Results reported in Table 1 show that clones GPH 17 and 18 produced NEA vector at high titers. The seven other clones produced NEA vector at low or undetectable levels. Hence, two GPH ($Hygro^+$) cell lines were found to complement the NEA vector for production of infectious vector particles, which provided evidence that both produce all *gag* and *pol* proteins. Because it produced vector at a very high titer (more than 10^5 resistance focus-forming units per ml) after transfection by pNEA, GPH clone 18 was chosen to generate a complete packaging cell line. From now on, clone GPH 18 will be referred to as the Haydee cell line.

TABLE 2. $gp85^{env}$ production from Haydee-PhEA clones tested by an interference assay and transcomplementing activity tested by their ability to package the NL53 retroviral vector

Haydee-PhEA packaging cell line	No. of clones of β-gal^+ cells (interference assay)[a]	Relative titer (*lacZ* CFU/ml)[b]
18.1	20	2×10^2
18.2	8	1×10^3
18.3	5	1×10^3
18.4	0	6×10^3
18.5	1	ND
18.6	62	ND
18.7	2	ND
18.8	3	ND
18.9	10^4	ND
18.10	10^2	ND
18.11	3	ND
QT6	$>10^4$	0
QT6-RAV1	0	5×10^3

[a] Packaging cell lines were superinfected with 5×10^4 NL53 particles (Fig. 2). Titers are expressed as *lacZ* CFU per 5×10^4 particles.

[b] Packaging cell lines were transfected with DNA from the NL53 vector (Fig. 2). Titers are expressed as *lacZ* CFU per milliliter of supernatants.

Testing of the pPhEA packaging mutant. The Haydee cell line expressing *gag* and *pol* genes was transfected with plasmid pPhEA (Fig. 3). pPhEA is a packaging-deficient mutant (Fig. 2) which carries and expresses the *env* gene as well as the $Phleo^r$ gene conferring resistance to phleomycin. Transfected cells were selected with phleomycin, and 11 colonies (called Haydee-PhEA) were isolated and grown individually. As mentioned previously for GPH clones, all Haydee-PhEA ($Phleo^+$) clones were cultured in medium containing both hygromycin B and phleomycin. Antibiotics were omitted before the $gp85^{env}$ assay only. Production of *env* proteins from these 11 Haydee-PhEA ($Phleo^+$) clones was tested by using an interference assay. Cells expressing the *env* gene at a high level should become resistant to superinfection by an exogenous virus of the same subgroup (11, 36). Such an assay was carried out by infecting each $Phleo^+$ cell lines (2×10^5 cells) with 5×10^4 CFU of retroviral vector NL53 pseudotyped with RAV1 (Fig. 2). NL53 carries and expresses the bacterial *lacZ* gene. Superinfected cells were detected by cytochemical staining to reveal production of β-galactosidase. Noninfected QT6 as well as RAV1-infected QT6 were also infected in the same conditions, as controls. Results are reported in Table 2. No β-galactosidase-positive cells were detected following superinfection of QT6-RAV1 with NL53, which demonstrates that RAV1-infected QT6 had become fully resistant to superinfection. By contrast, more than 10^4 *lacZ* CFU/ml were observed following infection of QT6 with NL53. Haydee-PhEA cell lines superinfected with NL53 displayed very

TABLE 3. Titers obtained with various retroviral vectors transfected into Isolde packaging cell line or into RAV1-infected QT6

Transfected retroviral vector[a]	Titer of virus produced by the transfected cells	
	Isolde[b]	RAV1-infected QT6[c]
NL53	5×10^{4d}	5×10^4
TXN3′	1×10^5	1.5×10^5
XJ12	8×10^4	9×10^4

[a] Structure of retroviral vectors is depicted in Fig. 2.
[b] Vector titers following transfection of Isolde packaging cell line with NL53, TXN3′, and XJ12. Supernatants were collected from a pool of Neo$^+$ clones. Titers are expressed as number of Neo$^+$ colonies per milliliter of supernatants.
[c] Vector titers following transfection of RAV1-infected QT6 with NL53, TXN3′, and XJ12. Titers are expressed as number of Neo$^+$ colonies per milliliter of supernatants.
[d] Titer of NL53 vector is 8 times higher than in the experiment reported in Table 2, because conditions used for harvesting the virus were improved.

unequal sensitivity to superinfection. One of them, Haydee-PhEA-18.9, was found to be as sensitive as QT6 to superinfection, whereas Haydee-PhEA-18.4 was found to be as resistant as RAV1-infected QT6. The other cell lines displayed intermediate status.

Hence, the Haydee-PhEA-18.4 cell line was found to display the highest level of interference. For this reason, it is most likely that this cell line displayed the highest level of expression of the *env* gene as well.

Introduction of retroviral vectors in Haydee-PhEA cell lines. The ability of Haydee-PhEA clones to package replication-defective vectors was tested by transfecting various Haydee-PhEA cell lines with the NL53 vectors (Fig. 3). Haydee-PhEA-18.4 was chosen because it displayed the highest level of expression of the *env* gene. Clones 18.1, 18.2, and 18.3, with lower levels of expression of *env* gene, were also retained. Transfected cells were selected with G418. A pool of more than 100 Neo$^+$ colonies were grown for each Haydee-PhEA cell line tested. Culture supernatants were collected and viruses were titrated by infecting fresh QT6 and scoring *lacZ* CFU per milliliter after cytochemical staining. Results are reported in Table 2. Clone 18.1, which displayed the lowest level of expression of the *env* gene according to the interference assay, also displayed the lowest virus titer in culture supernatant. Clone 18.4, which displayed the highest level of expression of the *env* gene, conversely also displayed the highest virus titer in culture supernatant. Therefore, packaging cell line Haydee-PhEA clone 18.4 was selected for further studies. From now on, it will be referred to as the Isolde cell line.

The Isolde cell line was transfected with various retroviral vectors. All vectors tested carried the Neo^r gene as a selectable gene (Fig. 2). RAV1-infected QT6 cells were also transfected with the same retroviral vectors as a control. Transfected cells were selected in G418. Neo$^+$ clones from Isolde cells were then cultured in medium containing hygromycin B (for selecting cells expressing the pGPH plasmid), phleomycin (for selecting cells expressing the pPHEA plasmid), and G418 (for selecting cells expressing the vector). The three antibiotics were omitted from the culture medium only when supernatants were collected for virus production. Virus was harvested and titrated by infecting fresh QT6. Results are reported in Table 3. For every retroviral vector tested, the Isolde helper cell line produced as many vector particles as QT6-RAV1. Titers ranged from 5×10^4 to 1×10^5 G418 CFU/ml. Differences in titers might have resulted solely from difference in the structure of the vectors.

Tests for release of replication-competent viruses. As a preliminary test for release of replication-competent viruses from packaging cell lines, culture supernatants from either NL53-, TXN3′-, or XJ12-vector-producer cell lines were used to infect CEFs, which were then passaged in culture for 2 weeks. All these CEF cultures remained p27^{gag} negative (as demonstrated by ELISA), and their supernatants were unable to transmit G418 resistance to normal CEFs.

Production of replication competent virus was further tested by in situ detection of p27^{gag} producing cells in QT6 cultures infected with each of the helper-free vector virus stocks. No p27^{gag}-positive cells were detected even when 5 ml of helper-free virus stock was used to infect fresh QT6. These data provide evidence that the Isolde packaging cell line does not release any helper virus.

DISCUSSION

Characteristics of the packaging mutants tested. A new ALV-based vector packaging cell line was designed in which several alterations of the wild-type assistant proviral genome were introduced. In avian and murine retroviruses, a region located between the 5′ LTR and the initiator codon of the *gag* gene is responsible for efficient packaging of viral RNAs into virions (16, 18, 19, 28, 33). The deletion of 52 nucleotides encompassing this sequence in the packaging mutant pHF13 had been previously reported (29). However, it has been shown for both murine (8, 19, 22) and avian (30) viruses that this deletion was not sufficient to fully abolish the generation of replication-competent viruses. Such viruses might arise from recombination between the overlapping sequences of packaging-competent vector genome and packaging-defective helper genome. To circumvent these problems, the helper genome was further crippled. Efficient packaging cell lines were designed by replacing the 3′ LTR with an heterologous polyadenylation sequence. This additional mutation was shown to be efficient at eliminating the formation of replication-competent viruses (10, 20, 21, 29).

To further reduce the probability of generating replication-competent viruses via intermolecular recombination, the viral genes were expressed from two separate plasmids, one expressing the *gag* and *pol* genes and the other one expressing the *env* gene. In both plasmids, the packaging sequence was deleted and the 3′ LTR was replaced by the polyadenylation sequence from heterologous viruses. This approach had already been reported (4, 10, 12, 20, 34). This alteration was shown to fully abolish the generation of replication-competent viruses (10, 12, 20).

Use of selectable packaging mutants. As opposed to cotransfection of a conventional packaging mutant with a second plasmid carrying the selectable marker, the selectable gene was inserted inside the retroviral genome (Fig. 2). The Phleo^r gene, conferring resistance to phleomycin, was introduced in place of the *gag* and *pol* genes next to the *env* gene in plasmid pPhEA. Similarly, the Hygro^r gene, conferring resistance to hygromycin B, was inserted in place of the *env* gene in plasmid pGPH expressing also the *gag* and *pol* genes. Because the selectable gene and the viral genes were coexpressed from the same transcription unit, we thought this strategy would result in a more straightforward selection of clones expressing high levels of viral proteins. As a matter of fact, a packaging cell line producing 10^5 TXN3′ infectious particles (as many as produced by RAV-1/TXN3′-infected QT6 cells) was isolated from only 34 clones screened for

$p27^{gag}$ or $gp85^{env}$ production (23 Hygro^r GPH clones plus 11 Phleo^r Haydee-PhEA clones). By comparison, in our previous report (29), a packaging cell line (pHF-g) producing only 10^4 TXN3′ infectious particles was isolated from 126 clones screened for $p27^{gag}$ and/or virus production.

However, it might be pointed out that packaging cells had to be cultured in medium containing hygromycin, phleomycin, and G418 in order to maintain high virus production. Under these conditions, high titers were obtained after the cells were passaged in culture for several weeks. In contrast, in the absence of selective pressure, virus production dropped dramatically within few weeks. Antibiotics were omitted in medium used to harvest virus only.

It is theoretically possible that, by increasing the concentration of antibiotic in culture medium, resistant cell colonies with a higher level of expression of both the selectable gene and the viral genes might be isolated. This would allow a more straightforward isolation of higher-producer helper cell lines. However, it might also lead to isolation of clones containing altered proviral DNAs favoring expression of the selectable gene at the expense of the viral genes. It has been shown that some specific sequences located within the *gag-pol* region have a *cis*-acting effect on splicing by regulating the balance of genomic versus subgenomic RNAs (9, 15, 23, 31). Mutational events within such sequences in the pGPH packaging mutant (in which the Hygro^r gene is expressed from the subgenomic RNA) might artificially increase expression of the Hygro^r gene by increasing the amount of subgenomic RNAs at the expense of genomic RNAs.

Screening of putative packaging cell lines. Screening of GPH Hygro$^+$ cell colonies for production of *gag* and *pol* viral proteins was carried out in two steps (Fig. 3). First, production of viral proteins was measured by detecting $p27^{gag}$ in culture supernatant with a quantitative ELISA. Then, the nine highest $p27^{gag}$ producer clones were further screened by a complementation assay. They were transfected with a plasmid containing the genome of the retroviral vector pNEA expressing the *env* gene (Fig. 3). Then, production of infectious viral particles was analyzed. Surprisingly, no correlation between $p27^{gag}$ production and viral production was found. Some GPH clones with the highest level of $p27^{gag}$ production produced very few particles. Conversely, clone GPH18 (later called Haydee), which displayed the highest level of viral production, produced only an intermediate level of $p27^{gag}$. Since, for each GPH Hygro$^+$ clone, viral production was measured from a large polyclonal population of cells transfected with the retroviral vector expressing the *env* gene, one may be confident that any variation in viral production between GPH clones might not arise from limiting expression of *env* proteins. Rather, it seems very likely that the discrepancies between GPH clones for production of *env* vector particles might arise from limiting production of *gag* peptides other than $p27^{gag}$ and/or from limiting production of *pol* polypeptide. This highlights the fact that helper cell lines with the highest level of viral production might not be necessarily found among clones displaying the highest level of $p27^{gag}$ in culture supernatant. As opposed to $p27^{gag}$ production assay, the interference assay used to measure the level of expression of the *env* gene in Haydee-PhEA cell lines seemed to be correlated to the actual vector titer although the sample size is too small to provide definitive evidence for that. We attributed this difference to the fact that Haydee-PhEA cell clones were screened for *env* gene expression with a biologic assay. Unlike the ELISA, which just reveals the presence of $p27^{gag}$ epitopes, the test of interference demonstrates the functionality of the $gp85^{env}$ protein.

Use of two separate packaging mutants for generating helper-free hybrid viruses. The two-separate-plasmid strategy was adopted because in addition to preventing packaging cell lines from producing replication-competent viruses, it offered the possibility of generating a helper cell line expressing only the *gag* and *pol* genes. Such a defective packaging cell line would be an intermediate to generate genuine helper cell lines expressing the *env* genes of different subgroup specificities. Such a system would allow production of different pseudotypes of the same retroviral vector to transfer exogenous genes into various tissues expressing different retrovirus receptors (5, 6). On the other hand, coculture of packaging cell lines expressing different host range envelopes could be used to amplify a retroviral vector stock throught multiple rounds of infection by avoiding the barrier of interference (2). Each cell line would be resistant to superinfection by the virus it releases, but it would be sensitive to infection by viruses produced by the other packaging cell lines. Therefore it allow the retroviral vector to replicate and to amplify in the absence of a transmissible helper virus.

Finally, a *gag-pol* producer cell line also offers the possibility of generating hybrid viruses with *gag* and *pol* proteins from ALV and *env* proteins from heterologous viruses. Such hybrid viruses have been produced by coexpressing the *gag* and *pol* genes from the Moloney murine leukemia virus and the *env* gene from the gibbon ape leukemia virus in murine fibroblasts. Primate cells were found to be very sensitive to infection by this hybrid virus (37). This procedure might be of considerable interest for introducing genes into organisms for which no genetically engineered ecotropic retroviruses are available or for which amphotropic viruses are found to be very inefficient.

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