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Genetics

Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges

(retroviral vectors/packaging cell lines/gene transfer)

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ABSTRACT **We have constructed a set of packaging cell lines useful for the generation of helper-free recombinant retroviruses with amphotropic and ecotropic host ranges. To eliminate the problems of transfer of packaging functions and helper virus formation encountered with the previously available packaging systems, two mutant Moloney murine leukemia virus-derived proviral genomes carrying complementary mutations in the *gag–pol* or *env* regions were sequentially introduced into NIH 3T3 cells by cotransformation. Both genomes contained a deletion of the ψ sequence necessary for the efficient encapsidation of retroviral genomes into virus particles and additional alterations at the 3′ end of the provirus. We show that the resulting packaging cell lines ψCRIP and ψCRE can be used to isolate clones that stably produce high titers (10^6 colony-forming units/ml) of recombinant retroviruses with amphotropic and ecotropic host ranges, respectively. More importantly, we demonstrate that viral producers derived from the packaging cell lines do not transfer the packaging functions, or yield helper virus, even under conditions where existing packaging cell lines can be shown to yield transfer of packaging functions and/or helper virus. These properties of the ψCRIP and ψCRE packaging lines make them particularly valuable reagents for *in vivo* gene transfer studies aimed at cell lineage analysis and the development of human gene replacement therapies.**

Most often, the initial step in the generation of recombinant retrovirus for mammalian gene transfer studies is the introduction of a suitable proviral DNA vector into fibroblastic cells that produce the necessary viral proteins for encapsidation of the desired recombinant RNA. Since, for most gene transfer applications, the generation of pure stocks of recombinant virus free of replication-competent helper virus is desirable, there has been considerable interest in the development of cell lines that produce the necessary viral gene products for encapsidation yet do not themselves yield detectable helper virus or transfer of viral genes (1–6). In the first generation of such "helper-free" packaging cell lines, expression of the necessary viral proteins was achieved through the stable introduction of a mutant Moloney murine leukemia virus (Mo-MuLV) proviral genome containing a 350-base-pair (bp) deletion of the ψ sequence (2), a sequence required for efficient encapsidation of the Mo-MuLV genome. Although such a cell line, termed ψ-2 (2), and its derivative, ψ-AM (4), have been successfully used by many investigators, we and others have shown that virus-producing cell lines derived from ψ-2 and ψ-AM produce low levels of virus containing the ψ^- genome and, therefore, are able to transfer the mutant proviral genome to recipient cells, albeit at low efficiency (ref. 5 and see below). In addition, in a minority of cases, the encapsidation of the ψ^- genome appears to lead to the generation of wild-type virus through recombinational events involving a copackaged recombinant genome carrying the ψ sequence (4–9).

Others have sought to eliminate these problems by introducing additional alterations into the ψ^- genome (5). Although in these latter packaging systems the chances of transferring the packaging functions or generating wild-type virus are significantly reduced, the formal possibility of the events occurring remains, since the additional mutations could still be corrected by recombinational events involving a ψ^+ vector genome. Indeed, the presence of helper virus has been reported in virus-producing cell lines derived from such a packaging cell (ref. 6 and see below).

In the hopes of practically eliminating the problem of packaging-function transfer and generation of helper virus, we have generated packaging cell lines useful for the generation of virus with amphotropic or ecotropic host ranges by sequentially introducing into mouse fibroblasts two modified Mo-MuLV genomes bearing complementary mutations in the viral functions necessary for encapsidation. Here we describe the construction of such cell lines and characterize their efficiency and safety in comparison to other existing cell lines.

MATERIALS AND METHODS

Cell Lines and Plasmids. NIH 3T3 cells were obtained from G. Cooper (Dana–Farber Cancer Institute) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. Drug-resistance selections in transfected or infected NIH 3T3 cells were done in the following concentrations of compounds: G418 (GIBCO) at 1 mg/ml, hygromycin (Calbiochem) at 0.2 mg/ml, and histidinol (Sigma) at 0.5 mM.

Plasmids pA5 and pR21 (10), containing mutated Mo-MuLV proviral sequences, were kindly provided by S. Goff (Columbia University). The mutant pA5 carries an 8-bp *Sac* II linker inserted at position 623 of the Mo-MuLV genome and pR21 has two 10-bp *Eco*RI linkers at position 5987. The cloned 4070A amphotropic genome pL1 was obtained from A. Oliff (Merck), and pSV2Hm was from P. Berg (Standford University). All plasmids were grown in *Escherichia coli* MC1061.

Nucleic Acids Procedures and Enzymatic Assays. DNA constructions, isolation of genomic DNA, and blot analysis were performed by standard procedures (11). High-specific-activity ^{32}P-labeled DNA probes were synthesized by using the random-priming method (12). The method of Goff *et al.* (13) was used to assay for the presence of reverse transcriptase activity in the culture medium of exponentially growing cells. Staining for β-galactosidase activity in intact cells was done according to Sanes *et al.* (14).

Transfections, Infections, and Determination of Viral Titers. Transfection of calcium phosphate/DNA coprecipitates (15) and infection (16) of NIH 3T3 were done as described.

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus; cfu, colony-forming unit; LTR, long terminal repeat; SV40, simian virus 40.

For the determination of viral titers, producers were grown to subconfluence (5×10^6 cells on a 10-cm dish), 10 ml of fresh medium was added, and virus was harvested 16 hr later. Viral stocks were filtered through a 0.45-μm membrane and 2 ml of a dilution was applied to 5×10^5 NIH 3T3 cells on a 10-cm dish for 2.5 hr. The infected cells were grown for 48 hr. At this point, cells were lysed and their DNA was prepared for Southern blot analysis (11), or, when the retroviral vector contained a selectable marker, infected cells were split at a ratio of 1:10 in selective medium. The number of resistant colonies obtained, divided by two, was the titer in colony-forming units (cfu)/ml of the diluted stock used for the infection.

Mobilization Assay for the Production of Helper Virus and the Transfer of Packaging Functions. The test cell line 116 was isolated after infection of NIH 3T3 by a recombinant retrovirus carrying the *hisD* selectable marker (MSVhisD; S. Hartman and R.C.M., unpublished results). The cell line contains a single copy of the replication defective provirus (J. Morgan, personal communication). Cells to be tested were grown to subconfluence and fresh medium was added. After 16 hr, medium was harvested and filtered through a 0.45-μm filter. Two milliliters of this medium was applied to a dish containing 5×10^5 116 cells. After 48 hr, the 116 cells were split at a ratio of 1:20 and allowed to grow for another 48 hr where the culture medium was changed and virus was harvested 16 hr later. The presence of *hisD* virus released by the 116 cells was then assayed by applying 2 ml of filtered culture medium to NIH 3T3. Infected cells were selected in medium without histidine but containing 0.5 mM histidinol.

RESULTS

General Approach. As described above, the determining events in the transfer of viral genes and the generation of wild-type virus by existing packaging cell lines appear to be the inefficient, but detectable encapsidation of ψ^- genomes into virus particles, and subsequent recombination between copackaged ψ^- genomes and ψ^+ vector genomes during the process of reverse transcription. As shown in Fig. 1, the ψ^- genome utilized in the construction of ψ-2 and ψ-AM cells can be encapsidated into virus particles at low efficiency and transferred to recipient cells without any recombinational events (17, 18) (referred to as "transfer" in Fig. 1). Surprisingly, the generation of wild-type virus by ψ-2-derived cells (referred to as "helper formation" in Fig. 1) is not frequent, although the retention of specific viral sequences in the vector genome can increase the likelihood of obtaining wild-type virus through one recombinational event (5, 8, 9). Although the additional alterations in the 3′ long terminal repeat (LTR) of ψ^- genomes, employed in the generation of the PA317 cell line by Miller and Buttimore (5), would not be expected to affect the encapsidation of the resulting ψ^- genomes (19), efficient transfer of the ψ^- genomes to cells would require a recombinational event. Accordingly, transfer of the viral genes to cells should be dramatically reduced in comparison to ψ-2 and ψ-AM cells. In addition, the generation of wild-type virus from such cells would require two recombinational events (Fig. 1).

In spite of the additional mutations in the proviral genomes used in the generation of PA317 cells, we (see below) and others (6) have detected the transfer of viral genes and the generation of wild-type virus in virus-producing cells derived from PA317. To further reduce the possibility of these events, we chose to construct separate proviral genomes that contained deletions of the ψ sequences and 3′ LTR and encoded either the *gag–pol* gene or the *env* gene (Fig. 2). Rather than attempt to express the specific subgenomic coding sequences by using heterologous expression vectors, we chose to retain "genomic-like" structures to ensure high levels of expression. Accordingly, well-characterized mutations in the *gag–pol* or *env* gene were transferred into ψ^- constructs to generate *gag–pol*$^+$ *env*$^-$ and *gag–pol*$^-$ *env*$^+$ constructs. To generate packaging cell lines with ecotropic host range, proviral constructs containing the *env* gene from the Mo-MuLV genome were used. To generate cells with amphotropic host range, the *env* coding sequences from the 4070A virus genome (20) were introduced in place of the Mo-MuLV-derived *env* sequences.

The resulting constructs, shown in Fig. 2, were then introduced sequentially into NIH 3T3 cells through two

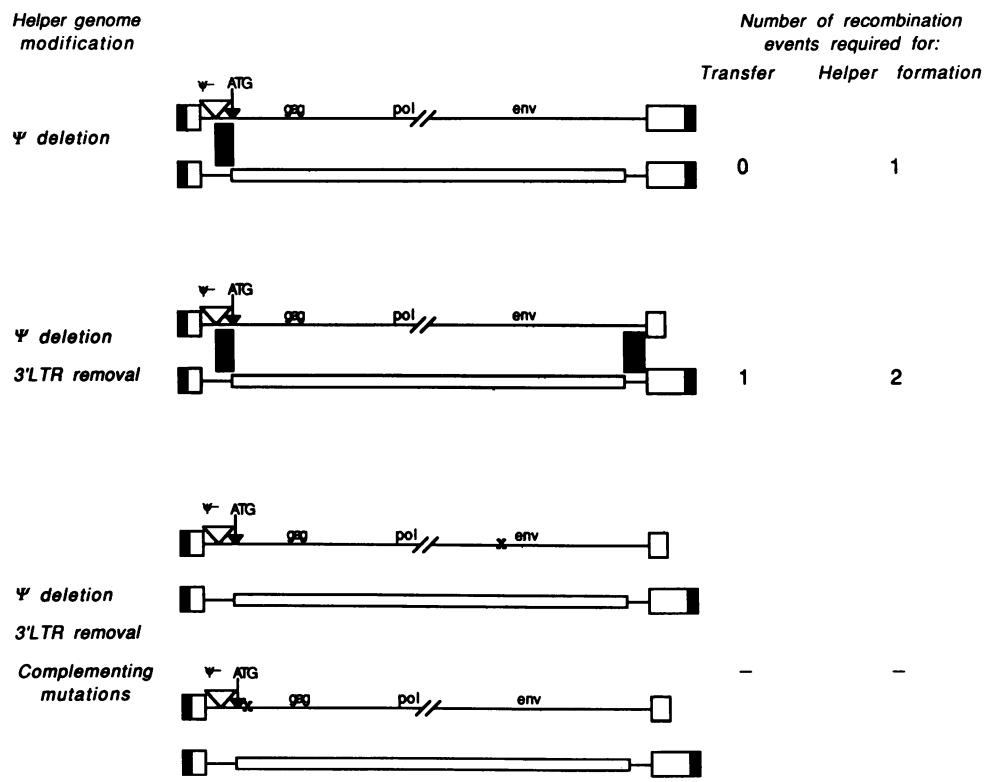

FIG. 1. Generation of helper virus by recombination in heterozygous virions. Three cases of copackaged RNA genomes (the helper virus genome carrying the *gag–pol* and *env* genes and the defective recombinant whose transduced insert is depicted as an open box) are presented. Solid and open boxes at the extremities represent the R-U5 and U3-R terminal sequences. Each case differs by the nature of the helper genome modification, listed on the left. The open box at the 3′ end represents the simian virus 40 (SV40) polyadenylylation signals and frameshift mutations are indicated by an x. The shaded areas indicate homologous regions on the genomes, through which, if recombination occurs, helper virus can be formed. Transfer refers to transmission of the ψ^- genome to recipient cells. Helper formation refers to the generation of wild-type replication-competent virus.

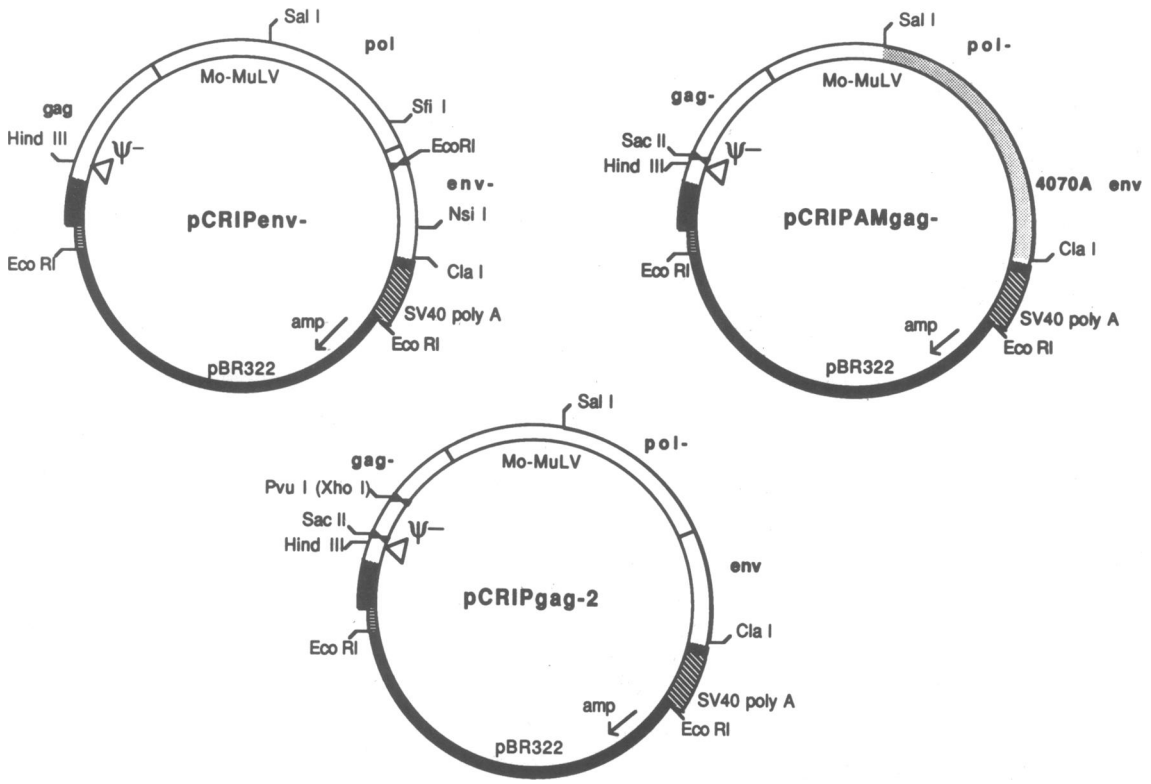

FIG. 2. Structure of the CRIP plasmids. A parental plasmid, pCRIP (data not shown), was first constructed by replacing most of the 3′ LTR in the pMOVψ^- by the SV40 early polyadenylylation region. For this, the *Cla* I–*Eco*RI fragment of pMOVψ^- encompassing the 3′ end of the *env* gene, the 3′ LTR, and mouse cellular sequences was replaced by two fragments: a *Cla* I–*Sal* I fragment taken from pZipNeoSVX(enh$^-$) (16) that contained a *Sal* I linker at the original *Pvu* II site (position 7934) and a *Hpa* I–*Eco*RI fragment from the SV40 genome (positions 2666–1782) with a *Xho* I linker at its *Hpa* I extremity. The pCRIPenv$^-$ plasmid was obtained by exchanging the *Sfi* I–*Nsi* I fragment (positions 5382–7054) with the equivalent segment from the env$^-$ mutant genome pR21 (ref. 10). To obtain pCRIPAMgag$^-$, a fragment from *Pst* I (position 563, *Hin*dIII linkered) to *Sal* I (position 3705) of the gag–pol$^-$ mutant pA5 was ligated, in a pCRIP backbone, to the *Sal* I–*Cla* I fragment of the 4070A amphotropic Mo-MuLV cloned genome (20). The pCRIPgag$^-$2 plasmid contained the same *Pst* I–*Sal* I fragment from pA5 introduced in the pCRIP backbone. The ecotropic envelope gene was kept and a second mutation in the *gag* sequences was introduced by cutting with *Xho* I (position 1560), filling in the extremities with the Klenow fragment of *E. coli* DNA polymerase I and religating. Mutations in the coding sequences are shown as ⧗.

rounds of transfection and coselection with different dominant-acting selectable markers. This protocol was chosen to optimize the stable expression of each construct and to prevent recombination between the complementary plasmid DNAs that might occur at the DNA level if they were cotransfected together (21).

Isolation of Packaging Cell Lines. The starting material for all of the constructions shown in Fig. 2 was the pMOVψ^- DNA (2). In each construction, a majority of the 3′ LTR was removed and replaced with a DNA segment containing the SV40 early polyadenylylation site (see legend to Fig. 2 for the precise boundaries of the constructions). The pCRIPenv$^-$ plasmid DNA was generated by exchanging a fragment spanning the 5′ half of the *env* gene with the homologous fragment from an *in vitro*-generated mutant of Mo-MuLV containing two *Eco*RI linkers inserted in tandem (20-bp insert) at position 5987 of the Mo-MuLV genome (10). In pCRIPAMgag$^-$, the region surrounding the start of the *gag–pol* gene was replaced by a homologous fragment from the mutant pA5, which contains an 8-bp *Sac* II linker at position 623 after the initiation codon for *gag* translation (J. Colicelli, personal communication). In addition, the *Sal* I–*Cla* I fragment spanning the 3′ end of the *pol* gene and most of the *env* gene was replaced by the corresponding fragment from the cloned 4070A amphotropic virus genome (20). The third construction, pCRIPgag$^-$2, contains the same basic structure as the pCRIPAMgag$^-$, except that the ecotropic *env* sequences were retained and an additional mutation at the *Xho* I site at position 1560 was introduced (see legend to Fig. 2). This latter mutation was designed to further prevent the possibility of recombinational events that could lead to the emergence of helper virus.

Pairs of complementary constructs (pCRIPenv$^-$ and pCRIPAMgag$^-$ or pCRIPenv$^-$ and pCRIPgag$^-$2) were sequentially introduced into NIH 3T3 cells by DNA-mediated cotransformation. In the first round of transfection, the pCRIPenv$^-$ plasmid was cotransfected with the plasmid pSVHm, which confers resistance to hygromycin B (22). Stable transformants were selected in the presence of hygromycin B (200 μg/ml). Sixteen clones were isolated and individually grown, and the presence of reverse transcriptase activity in the culture medium was assayed. The parental NIH 3T3 line was used as a negative control in the assay and positive controls included ψ-2 and ψ-AM cells. Thirteen clones were found to release reverse transcriptase activity, among which 9 showed significantly higher levels (data not shown). Two of these positive clones (env$^-$1 and env$^-$15) were subsequently used: env$^-$1 was initially chosen to derive the amphotropic ψCRIP packaging line as described below, and env$^-$15 was later selected as the parental clone for the ecotropic ψCRE line, since it showed a 2-fold higher level of Mo-MuLV-specific transcripts in an RNA gel blot analysis (data not shown).

In the second series of transfections, either the pCRIPAMgag$^-$ or the pCRIPgag$^-$2 plasmid was introduced into the env$^-$ cells, along with the plasmid pSV2gpt, which contains the bacterial xanthine-guanine phosphoribosyltransferase gene as a dominant selectable marker (23). Clones isolated in

guanine phosphoribosyltransferase-selective medium were expanded and tested for their ability to package the BAG retroviral vector (Fig. 3 and ref. 24) into infectious particles. For this analysis, each clone to be tested was infected by a helper-free stock of BAG virus (ecotropic for the CRIP clones and amphotropic for the CRE clones), and populations of 50–100 G418-resistant colonies were derived from each infection and grown in mass cultures. Virus production from each population was then checked by using culture supernatant to transfer G418-resistance and β-galactosidase activity to NIH 3T3 cells. The packaging capacity, as measured by G418 or β-galactosidase titer of producer populations, varied within a 100-fold range (data not shown). As a control, NIH 3T3 or the env$^-$15 cells were shown to be negative in the packaging assay. Clone CRIP14 was selected as having the highest packaging capacity; when compared to ψ-AM and PA317 in the same assay, its packaging activity was shown to be 3.5-fold higher and equivalent, respectively. Clone CRE25 was chosen as the ecotropic packaging line and it was observed to be less active by a factor of 3–5 than ψ-2 cells.

Producer Clones Derived from ψCRIP and ψCRE. The titering of populations of BAG virus-producing cells served to select the best packaging clones but was not an accurate estimation of their performance. To obtain more quantitative data about the titers that could be generated with the ψCRIP and the ψCRE lines, the two cell lines were stably transfected with the vector HSGneo (see Fig. 3). Colonies were selected in the presence of G418, individually picked, and expanded. Supernatant from these cultures (16 producers for each of the packaging lines) was used to transfer G418 resistance to NIH 3T3 cells. Titers of the best producers were found to be 1.3 × 10^6 cfu/ml and 0.7 × 10^6 cfu/ml for ψCRIP and ψCRE, respectively. The best ψ-2 producer tested in parallel had a titer of 1.7 × 10^6 cfu/ml. The amphotropic host range of the ψCRIP-derived viruses was shown by the ability of the viruses to efficiently infect a wide range of mammalian cells of human, dog, rat, rabbit, and monkey origin (data not shown).

Characterization of the Packaging Lines for Transfer of Packaging Functions, Helper-Virus Formation, and Stability. To determine the extent to which recombinant virus generated from ψCRIP- or ψCRE-derived cell lines was helper-free, we employed a mobilization assay, in which the cell line 116, containing a single copy of the MSVhisD recombinant provirus, is challenged by a supernatant from virus-producing cell lines or infected cells and subsequently tested for release of the recombinant virus it harbors. Infection of the 116 cell line with culture supernatants harvested from ψ-2 cells or from ψ-2-derived producer cells led to the recovery of 2 × 10^3 *his* cfu/ml. Since these ψ-2 and ψ-2 producer cell lines remained negative in assays for replication-competent virus, the mobilization assay was likely detecting transfer of the ψ$^-$ genome. Culture supernatants from ψCRIP, ψCRE, PA317 (5), and all producer clones tested were not able to mobilize the MSVhisD provirus (data not shown).

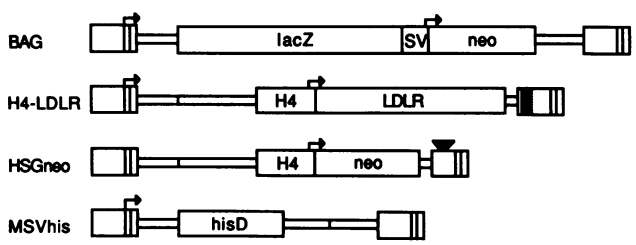

FIG. 3. Retroviral vectors used in the study. The BAG vector has been described (24). H4-LDLR (28) and HSGneo (B. C. Guild, M. H. Finer, D. E. Housman, and R.C.M., personal communication) share the same basic structure. Mo-MuLV sequences include the 5′ LTR, the 5′ noncoding region, and portion of the *gag* sequences up to the *Xho* I site at position 1560. The *gag* sequences were obtained from the pA5 mutant and the frameshift mutation is indicated as a vertical bar. The inserts are transcribed (arrows) by using an internal promoter from the human histone H4 gene. The 3′ retroviral sequences are from the *Cla* I site at position 7674 and include a deletion of the transcriptional enhancer in U3 (ref. 16, ▼) or, in the case of H4-LDLR, its replacement by the MPSV enhancer (cross-hatched area). MSVhisD was constructed by replacing the *gpt* gene in MSVgpt (2) by the *hisD* gene from *Salmonella typhimurium* (S. Hartman and R.C.M., unpublished results).

We then examined the stability of virus production and checked for the appearance of helper virus upon long-term cultivation of ψCRIP- and ψCRE-derived clones producing recombinant retrovirus. Cells were split at a ratio of 1:20 and grown for 3 days (3 or 4 doublings), at which point the culture medium was changed and virus was harvested 16 hr later. After this harvest, cells were split and the same cycle was repeated six times. Each collected culture supernatant was tested in the *his* mobilization assay and used to infect NIH 3T3 as described above. None of these virus stocks was able to transfer the packaging functions. Each population of infected NIH 3T3 cells contained a comparable number of copies of integrated recombinant provirus as revealed by Southern blot analysis. We did not detect any decrease in the viral titer or rearrangement of the transferred DNA (data not shown).

Unfortunately, the propagation of a virus-producing cell line in culture, even for long periods of time, may not reveal the generation of wild-type virus, since the envelope protein expressed on the surface of the cells would prevent their efficient infection with wild-type virus released from a rare virus-producing cell. To maximize our chances of observing recombination events due to reverse transcriptase-mediated recombination between packaging genomes and vector genomes, the following experiment was designed. A retroviral vector containing the 5′ *gag* sequences and the human low density lipoprotein receptor gene under the control of the H4 promoter (H4-LDLR, Fig. 3) was shuttled several times between amphotropic and ecotropic packaging lines and the appearance of helper virus was monitored in each infected cell population, by using the *his* mobilization assay. We started with two amphotropic viral stocks of equivalent titers, harvested from either a ψCRIP or a PA317 clone producing H4-LDLR and negative in the *his* assay. Two milliliters of each stock were then used to infect 5 × 10^5 ψCRE cells. A supernatant was collected 48 hr after infection, and ψCRIP or PA317 were infected again. The same cycle was repeated several times and seven viral stocks produced in each series of cross-infections (ψCRIP/ψCRE or PA317/ψCRE) were tested for their ability to mobilize the MSVhisD provirus (Table 1). In two separate experiments, all stocks from the ψCRIP/ψCRE series were negative. On the other hand, when PA317 was employed as the amphotropic packaging line, transfer of the packaging functions became apparent after the fifth (experiment 1) and fourth (experiment 2) round of

Table 1. Titers of culture supernatant from 116 cells infected by serial H4-LDLR viral stock

Viral stock, shuttle no.	Titer, cfu/ml			
	ψCRIP/ψCRE		PA317/ψCRE	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1 (ampho)	0	0	0	0
2 (eco)	0	0	0	0
3 (ampho)	0	0	0	0
4 (eco)	0	0	0	0
5 (ampho)	0	0	0	65
6 (eco)	0	0	325	5 × 10^4
7 (ampho)	0	0	475	5 × 10^4

ampho, Amphotropic; eco, ecotropic.

infection. The amphotropic nature of the virus mobilized from the 116 cells with the viral stocks of the PA317/ψCRE series was demonstrated by showing that the virus efficiently infected CV1 (monkey) and the env$^-$15 cells, yet did not infect ψCRIP cells. Although we have not characterized the structure of the amphotropic proviral genome, it appears that the virus recovered from experiment 1 may be defective, since it does not spread, whereas the virus recombinant from experiment 2 is replication competent (data not shown).

DISCUSSION

We have designed retrovirus packaging cell lines in which complementary frameshift mutations were introduced in the retroviral genes encoding the packaging functions, in addition to the cis-acting alterations described (2, 5). Since these mutations cannot be rescued upon recombination with a replication-defective vector genome, the generation of helper virus in the ψCRIP and ψCRE packaging lines now requires a complex and unlikely chain of events. One of the mutated genomes would first have to regain a 3′ LTR and a ψ sequence by a double recombination with a vector genome, to be able, in a subsequent round of infection, to recombine with the complementary mutant genome and yield a wild-type genome. Alternatively, one could imagine a reversion of one of the mutations and then a double-recombination event. The probability of these outcomes is extremely low and we have shown here that when a high-titer viral stock was pseudotyped several times, alternatively by using ψCRIP or ψCRE to increase the probability of observing a reverse transcriptase-mediated recombination event, no transmissible functional helper genome was generated. In a parallel experiment where PA317 was used as the amphotropic packaging line, we observed the emergence of such a recombinant. Although we have not studied its structure, we assume that it was the product of at least one recombination event involving the retroviral vector and the ψ^- genome, presumably within the 88-base segment they share at their 3′ extremities (from the *Cla* I site at position 7674 to the *Rsa* I site at position 7762).

Earlier reports have described similar attempts to obtain stable packaging cell lines where the retroviral functions were carried on different plasmids (3, 6). However, the cell lines described in those reports were either unstable or functioned poorly. In addition, the authors cotransfected the two complementary constructs, thereby losing most of the advantage of having physically separated the viral genes, since recombination between transfected plasmids before they integrate is known to happen at a high frequency (21). Our strategy was to introduce the constructs into the recipient cell line by using two independent rounds of transfections. This way, we were able to first select clones expressing optimal levels of gag and pol proteins by measuring the levels of reverse transcriptase they released and then test for the production of a functional envelope in secondary transfectants by using a packaging assay. An interesting by-product of this procedure was the generation of an env$^-$ cell line that may prove useful for the generation of viral pseudotypes containing retroviral gag proteins and other nonretroviral envelope proteins.

High-titer viral stocks can be routinely obtained with producer clones isolated from ψCRIP or ψCRE. For instance, after transfection of the packaging lines with the HSGneo vector, which includes the 5′ portion of the *gag* sequences as well as the enhancer deletion, we isolated producer clones with titers of 10^6 cfu/ml. This is equivalent to what can be achieved with ψ-2. Such titers are high enough to ensure infection of the minor pool of pluripotent stem cells present in bone marrow (25–27). Indeed, we have observed efficient transduction of murine hematopoietic stem cells with ψCRIP and ψCRE producers.

So far, in our laboratory, high-titer viral producers have been derived from both ψCRIP and ψCRE with more than 15 constructs. Whenever tested in the *his* mobilization assay, the producers were negative for transfer of the packaging functions and, therefore, free of helper virus, even after carrying the cells in culture for a long period of time. For many gene transfer applications, the level of packaging function transfer and frequency of helper virus formation found with the previously developed cell lines may be inconsequential. However, the practical elimination of any transfer and/or recombination events potentially leading to the emergence of helper virus would appear to be important for studies of cell lineage and may prove to be extremely important in the establishment of safe and efficient conditions for somatic gene transfer in large animals and human gene replacement therapies.

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