Development of High-Titer Retroviral Producer Cell Lines by Using Cre-Mediated Recombination

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Retroviral gene transfer is widely used in experimental and human gene therapy applications. We have devised a novel method of generating high-titer retroviral producer cell lines based on the P1 bacteriophage recombinase system Cre-loxP. Incorporation of loxP sites flanking a Neo<sup>r</sup>-SVTK cassette in the proviral DNA allows excision of these selectable markers through expression of Cre recombinase after production of a high-titer producer cell line. The resultant producer line contains a single loxP site flanked by the viral long terminal repeats. Retransfection of this line with the Cre expression vector and a plasmid containing a gene of interest flanked by loxP sites allows insertional recombination of the gene into the favorable preexisting site in the genome and the generation of a new line with a titer equivalent to that of the parental producer cell line. The efficiency of the process is sufficient to allow the generation of multiple new producer lines without the addition of antibiotic resistance genes. We have successfully generated retroviral vectors carrying different genes by using this approach and discuss the potential applications of this method in gene therapy.

Retrovirus-mediated gene transfer is a frequently utilized method to study the expression of foreign gene sequences in mammalian cells (19). More recently, it has become the most widely used vector system in gene therapy protocols initiated to cure various diseases in humans (21). In addition to inborn errors of metabolism and other monogenic disorders, retrovirus-based gene therapy trials have been expanded to incorporate treatment for cancer and AIDS (7, 18). The majority of these trials utilize vectors derived from Moloney murine leukemia virus (Mo-MuLV) (21). Structurally, this vector is dependent on two elements, the trans-complementing genes (gag, pol, and env) and the viral cis-acting sequences (U3, R, U5; the primer-binding site, polypurine track, and encapsidation and dimerization signals) (33). Many of the initial difficulties facing this vector system have been overcome. Safety issues concerning generation of wild-type virus in vector preparations have largely been circumvented with the design of new packaging systems which require multiple recombination events for breakthrough to occur (8, 9, 17, 20, 32). Similarly, progress has been made on the restrictions of cell tropism with the advent of pseudotyped vectors and other strategies to expand the range of cells expressing the amphotropic receptor (4, 15, 36, 37).

Despite these advances, a number of problems inherent to retroviral vector production and usage still remain. These problems include: (i) the need for high-titer viral stocks for efficient target cell transduction which often necessitates screening of large numbers of clones (21); (ii) the genomic integration of regulatory elements in the viral long terminal repeats (LTRs) which can transactivate cellular genes, increasing the risk of oncogenesis (30, 32); (iii) down-regulated expression of the gene of interest by viral regulatory sequences or the selectable marker used in producer line generation (1, 5, 16); and (iv) interference with the metabolism of the recipient cells or stimulation of an unwanted host immune response by selectable marker genes (31, 34).

This study details a unifying strategy used in the generation of retroviral producer cell lines which circumvents some of these problems. It is based on the incorporation of bacteriophage P1 recombination system in the design of retroviral vectors. Bacteriophage P1 encodes a site-specific recombination system comprising the phage-encoded recombinase Cre and a site on the phage genome, loxP, where recombination takes place (2, 12, 26). The loxP site consists of two 13-bp inverted repeats, binding sites for the Cre protein, and an 8-bp asymmetric core region in which recombination occurs and which is responsible for the directionality of the site (12, 27). Recombination between two directly orientated loxP sites excising the DNA as a circular molecule can be mediated by Cre recombinase. Targeted insertion of genetic material into a single loxP site in the genome is also possible with cotransfection of a Cre protein expression vector and a circular piece of DNA containing a single loxP site (27). We have utilized this system to modify producer cell lines constructed with retroviral vectors containing loxP sites. We demonstrate with this approach that we can remove selectable markers and reinsert genes of interest while maintaining production of functional retrovirus.

MATERIALS AND METHODS

DNA construction and cell culture. The Loxh retroviral vector was derived from pG1NaSVTK, a Mo-MuLV-based vector. Oligonucleotides encoding minimal loxP sites were subcloned into a blunted NotI site 5′ of the Neo<sup>r</sup> gene and into a blunted ClaI site 3′ of the thymidine kinase (TK) gene in PG1NaSVTK. The integrity and direction of the oligonucleotides were confirmed by DNA sequencing using the chain termination method (Sequenase; U.S. Biochemical Corp.). Constructs containing selectable markers flanked by loxP sites were derived from the plasmid PSL1190 (Pharmacia Biotech Inc.). Oligonucleotides encoding the minimal loxP sites were subcloned into blunted EcoRI and SphI sites and also verified by sequencing. A NotI/Sall fragment of pG1NaSVTK containing the Neo<sup>r</sup> gene was then subcloned into the NotI/Xhol sites in PSL1190LoxP to give PSL1190LoxPNeo.

The Cre expression vector pMC-Cre was a kind gift of K. Rajewsky. The green fluorescence protein expression vector pEGFP-C3 was obtained from Clontech. NIH 3T3 cells and all producer cell lines were grown at 37°C with 5% CO₂, 95%
air in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated calf serum, 4.5 mg of glucose per ml, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

**Oligonucleotide synthesis.** Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380B using phosphoramidite chemistry and purified on Sephadex G-25 columns (Whatman). The oligonucleotides used were as follows: `loxP` sense, 5′ CCGGATCCATAACTTCGTATAGCAT ACATTATACGAAGTTATAG 3′; and `loxP` antisense, 5′ CTATAACCTCGTATAATGTATGCTATACGAAGTTATGGATCCCG 3′.

**Cell transfection, transinfection, selection, and viral titer determination.** To generate the Lox6 producer cell line, plasmid DNA was prepared by the Qiagen (Chatsworth, Calif.) procedure and transfected into the amphotropic packaging cell line PA317 by calcium phosphate precipitation using standard conditions. Viral supernatant from these cells was harvested, filtered through 0.45-μm-pore-size filters (Millipore Inc.), and used to transfect the ecotropic GP+E86 packaging cell line in the presence of 6 μg of Polybrene (Sigma, St. Louis, Mo.) per ml. Selection was imposed by the addition of G418 (500 μg/ml, active). An estimate of viral titer was obtained by RNA slot blot analysis. The viral particles in 1 ml of culture medium were precipitated by adding 0.5 ml of PEG solution (30% PEG 8000, 1.5 M NaCl). After 30 min on ice, the samples were centrifuged for 5 min at 4°C. The pellets were resuspended in 2 ml of VTR buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 20 mM vanadyl ribonucleoside complex, 100 μg of yeast tRNA per ml) and then lysed by adding 0.2 ml of 20% SDS, 0.6 M NaCl, 20 mM EDTA, 20 mM Tris-HCl; pH 7.4. The viral RNA was extracted once with phenol-chloroform and precipitated with ethanol. The RNA pellets were dissolved in 0.25 ml of 20X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then lysed by adding 0.2 ml of 2X lysis buffer (1% sodium dodecyl sulfate [SDS], 0.6 M NaCl, 20 mM EDTA, 20 mM Tris-HCl; pH 7.4). The viral RNA was then denatured and immobilized on a nylon membrane using a slot blot apparatus (Schleicher and Schuell). After 4 h of hybridization in 10X SSC, air dried, baked at 80°C for 2 h under vacuum, and prehybridized in the hybridization solution (10% dextran sulfate, 1% SDS, 1 M NaCl) at 60°C for 2 h. Hybridization was performed by incubating the membrane with a radiolabeled restriction fragment from the Neo gene at 60°C for 2 h. The membrane was washed at 65°C in 2X SSC for 30 min and then in 0.5X SSC for 30 min, the membrane was dried and imaged by autoradiography.

Biological viral titers were obtained by functional assay of Neo CFU (CFU per milliliter). NIH 3T3 cells were seeded at 5 × 10^4 cells per 100-mm-diameter tissue culture plate and incubated for 24 h. Cells-containing culture medium was diluted in 10-fold decrements in medium containing 6 μg of Polybrene per ml and added to plates, which were incubated for 24 h. The medium was then replaced with standard medium containing G418. After 14 days, plates were stained with crystal violet and colonies were counted to provide an estimate of viral titer.

**Genomic Southern analysis.** Genomic Southern analysis was performed as previously described (25). Experiments designed to assess proviral integrity utilized SphI, which cuts in both viral LTRs. For integration site analysis, an enzyme cutting once in the provirus (EcoRI) was employed.

**RESULTS**

**Strategy and vector design.** A retroviral vector, Lox6 containing two directly orientated `loxP` sites was designed (Fig. 1). This vector was based on G1NaSVTK and contained the following from 5′ to 3′: (i) the Mo-MuLV LTR with packaging signal; (ii) an 18-bp `loxP` site derived from the P1 bacteriophage; (iii) the neomycin resistance gene (Neo); (iv) the simian virus 40 promoter driving the TK gene; (v) a second `loxP` site; and (vi) a second Mo-MuLV LTR preceded by its purine track. The configuration of this vector would allow selection of transduced cell lines with the neomycin analog, G418. We predicted that following transient transfection of Cre recombinase expressing constructs in these lines, the DNA segment flanked by the `loxP` sites would be excised. The resultant clones (Lox6Cre1) would contain a proviral integrant consisting of the two viral LTRs and a single residual `loxP` site (Fig. 1). As a result, these cell lines would be sensitive to the effects of G418 and resistant to ganciclovir, allowing selection with the antiviral agent. We postulated that subsequent transfection of a Lox6Cre1 line with the Cre expression vector and a circular piece of DNA containing a gene of interest and a directly orientated `loxP` site would result in insertional recombination of the gene of interest into the single `loxP` site of the proviral integrant (Fig. 1). This strategy should therefore yield a new retroviral producer cell line with the same titer as that of the parental Lox6 line.

**Generation and characterization of the Lox6 producer cell line.** The Lox6 retroviral vector was transfected into the amphotropic packaging cell line, PA317. Culture medium containing retroviral particles generated by this line was used to transfect the ecotropic packaging cell line GP+E86 and individual clones isolated with G418 selection. The content of vector RNA in the culture media of 40 clones was determined by RNA slot blot analysis with a Neo probe, and 7 clones with the highest apparent titer were selected for subsequent experiments. Genomic Southern analysis of these clones confirmed the integrity of the proviral integrant (Fig. 2A). A single line with an intact provirus, Lox6 (clone 4) was selected for all
subsequent experiments. Integration site genomic Southern analysis of Lox6 revealed that the line contained a single retroviral insertion (Fig. 2B). As recombination has been observed in retroviral vectors containing repetitive sequences, we evaluated proviral integrity in NIH 3T3 cells transinfected with supernatant from the Lox6 producer cell line. As shown in Fig. 2C, G418-resistant NIH 3T3 clones all demonstrated the presence of an unrearranged proviral integrant in genomic Southern analysis. This finding established that the Lox6 retrovirus was not prone to intrinsic rearrangement.

**Cre recombinase expression in the Lox6 line excises the Neo’ and TK genes.** To derive a “master producer cell line” into which genes of interest could be inserted, it was first necessary to remove the Neo’ and TK genes from Lox6 by excisional recombination. The bacteriophage recombinase Cre has been shown to promote recombination between directionally orientated **loxP** sites. The intervening DNA is excised as a circular molecular with a single **loxP** site, leaving a single **loxP** site in the host genome. We transfected 10⁶ Lox6 cells with the green fluorescence protein expression vector pEGFP-C3 and a vector containing the bacteriophage recombinase Cre driven by a synthetic herpes simplex virus TK promoter and enhancer (pMC-Cre) in a ratio of 1:10 (11). Cells transfected with pEGFP-C3 and pUC19 served as a control. Transfected cells were analyzed by fluorescence-activated cell sorting (FACS) after 48 h, and cells fluorescent at a wavelength of 488 nm were sorted and selected in ganciclovir, G418, or both agents for 14 days. As seen in Fig. 3A, Cre-transfected Lox6 cells produced equivalent numbers of colonies on the G418 and ganciclovir plates, indicating that the efficiency of excisional recombination was comparable to the previously reported figure of 50%. No colonies were observed in the presence of both selection agents (Fig. 3A) or in the pUC19-transfected Lox6 cells selected with ganciclovir. As expected, pUC19-transfected cells grew to confluence in G418 plates. To ensure that excisional recombination had removed all intervening sequence between the **loxP** sites in the proviral integrant, Cre-transfected clones resistant to ganciclovir were expanded and placed under G418 selection. No Neo’ colonies emerged, indicating that the excisional recombination was complete, removing both Neo’ and TK genes (data not shown). This finding was confirmed by genomic Southern analysis with a probe for Neo’ (Fig. 3B). A single clone (Lox6Cre1) in which recombination had been achieved was selected for further study.

**Insertional recombination of a gene of interest into the Lox6Cre1 producer cell line.** Cre-mediated insertional recombination of circular DNA containing a **loxP** site into a genomic site containing a second **loxP** site has been demonstrated in

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**FIG. 2.** Molecular analysis of the Lox6 producer cell line. (A) Southern blot analysis of the Lox6 proviruses in GP + E66 cell lines. Genomic DNAs from seven different Lox6 clones (lanes 1 to 7) digested with NheI were fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled Neo’ probe. SV40 P, simian virus 40 promoter. (B) Integration site analysis of Lox6 provirus in the GP + E66 cell line. Genomic DNA from the clone in lane 4 of panel A digested with EcoRI was fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled Neo’ probe (lane 1). Lox6 DNA digested with NheI served as the control (lane 2). (C) Southern blot analysis of the Lox6 provirus in NIH 3T3 cells. Genomic DNAs from seven different clones (lanes 1 to 7) digested with NheI were fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled Neo’ probe.

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**FIG. 3.** Cre-mediated excision of the Neo’ and TK genes from the Lox6 producer cell line. (A) Selection of Lox6 producer cell lines in G418 and ganciclovir after exposure to Cre recombinase. Lox6 cells (10⁶) were transfected with the green fluorescence protein expression vector pEGFP-C3 and a Cre expression vector, pMC-Cre, in a ratio of 1:10. Transfected cells were analyzed by FACS after 48 h, and cells fluorescent at a wavelength of 488 nm were sorted and selected with G418, ganciclovir, or both agents for 14 days. Clones in which the proviral genome remains intact are G418 resistant, whereas clones that have undergone Cre-mediated excision become resistant to ganciclovir. All of the cells remain sensitive to a combination of G418 and ganciclovir as predicted. The plates were then stained with crystal violet, and colonies were counted. (B) Southern blot analysis of the Lox6Cre1 provirus in a GP + E66 cell line. Genomic DNA from a ganciclovir-resistant clone digested with NheI was fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled Neo’ probe. Genomic DNA from Lox6 (lane 1) served as the control.
excisional recombination event would occur in the Neor plasmid.

Analysis for green fluorescent protein (GFP) fluorescence, efficiencies were comparable in both experiments. After FACS analysis of Lox6Cre1 cells transfected with pMC-Cre ex- pression vector, a plasmid containing loxP sites flanking the Neo gene (NeoRlox) in a ratio of 1:10. Cotransfection of the NeoRlox plasmid with pEGFP-C3 and pUC19 served as a control. After FACS analysis for GFP fluorescence, equal numbers of cells from both transfections were selected in G418-containing media for 14 days. The plates were then stained with crystal violet, and colonies were counted. + Cre, with Cre; – Cre, without Cre. (B) Southern blot analysis of Lox6Cre1Neo provirus in a GP1 cell line. Genomic DNAs from seven clones described above were digested with NheI, fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled probe. as seen in Table 1, the efficiency of recombination in cells transfected with pMC-Cre was approximately 1 in 30.

Insertional recombination generates a new virus with a titer equivalent to that of Lox6Cre1. To determine whether recombinational insertion recreated an active retrovirus, slot blot analysis of Cre-dependent neomycin-resistant clones was performed. As shown in Fig. 5A, all positive clones demonstrated the presence of viral RNA in culture supernatants. To formally determine the biological titer of these viruses, an assay on NIH 3T3 cells was performed. Various dilutions of retroviral supernatants from the parental Lox6 producer cell line and Lox6Cre1Neo clone 1 (Lox6Cre1Neo1) were added to NIH 3T3 cells which were then selected in G418. As shown in Fig. 5B, the titer of the virus produced by the recombined Lox6Cre1Neo clone was comparable to that of the original line. A repeat of this experiment utilizing supernatants from four additional Lox6Cre1Neo clones yielded similar results (Table 2). The growth characteristics of these five clones were also comparable to those of the parental Lox6 line. Genomic Southern analysis of Neor NIH 3T3 clones obtained with Lox6Cre1Neo1 demonstrated a proviral integrant of the predicted size, indicating that the new producer cell line generates a stable retrovirus (Fig. 5C).

The Lox6Cre1 line functions as a master producer cell line. To determine whether Lox6Cre1 could function as a master producer cell line, we examined recombinational insertion with a second gene flanked by loxP sites. For this purpose, we utilized the puromycin resistance gene (PuroR). We cotransfected a PuroRlox construct with the Cre expression vector into the Lox6Cre1 cell line and selected clones in puromycin. Several hundred clones were obtained, and genomic Southern analysis of positive clones demonstrated a band of the predicted size for a proviral integrant containing the Puro gene (Fig. 6A). Viral titering by RNA slot blot revealed that three of the four selected clones had comparable titers. A fourth clone containing, creating circular DNA containing a single loxP site and the Neo gene. Subsequent Cre-mediated recombinational insertion would juxtapose the Neo gene and the viral LTR, allowing gene expression. As seen in Fig. 4A, in the presence of Cre, more than 550 neomycin-resistant colonies were observed in each of these different experiments. In contrast, in the absence of Cre, only eight colonies were obtained in each experiment. These clones presumably represent random insertion of the Neo gene adjacent to a genomic regulatory sequence capable of driving expression. Genomic Southern analysis of seven neomycin-resistant clones obtained with Cre expression (Lox6Cre1Neo1 to Lox6Cre1Neo7) revealed a predicted band of 1.1 kb with a restriction enzyme cutting at the 5′ end of the Neo gene and in the 3′ LTR (Fig. 4B). In contrast, non-Cre-dependent clones showed random integration events (data not shown). To evaluate the efficiency of insertional recombination, the NeoRlox plasmid was again cotransfected into Lox6Cre1 cells with pMC-Cre and pEGFP-C3 and fluorescent cells were sorted and plated in serial dilutions in standard medium in the presence and absence of G418. Colonies were counted at 14 days, and the ratios of colonies on the two plates were compared. as seen in Table 1, the efficiency of recombination in cells transfected with pMC-Cre was approximately 1 in 30.

mammalian cells (27). We sought to utilize this observation to generate a new producer cell line based on Lox6Cre1. Lox6Cre1 cells (10⁶) were cotransfected with pEGFP-C3, pMC-Cre expression vector, and a plasmid containing loxP sites flanking the Neo gene (NeoRlox) in a ratio of 1:10. Cotransfection of the NeoRlox plasmid with pEGFP-C3 and pUC19 served as a control. After FACS analysis for GFP fluorescence, equal numbers of cells from both transfections were selected in G418-containing media for 14 days. The plates were then stained with crystal violet, and colonies were counted. + Cre, with Cre; – Cre, without Cre. (B) Southern blot analysis of Lox6Cre1Neo provirus in a GP1 cell line. Genomic DNAs from seven clones described above were digested with NheI, fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a 32P-labeled probe.

![FIG. 4. Cre-mediated insertion of the Neo gene into the Lox6Cre1 producer cell line.](image-url)

<table>
<thead>
<tr>
<th>No. of colonies</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>G418</td>
<td>+G418</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
</tr>
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<td>3</td>
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</tr>
<tr>
<td>6</td>
<td>600</td>
</tr>
<tr>
<td>7</td>
<td>1,050</td>
</tr>
</tbody>
</table>

Total: 2,580 colonies

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*Table 1. Efficiency of insertional recombination into the Lox6Cre1 producer cell line*
has a significantly reduced titer (Fig. 6B). An assay of the three high-titer clones with NIH 3T3 cells confirmed the production of biologically active virus.

**DISCUSSION**

This study reports a novel use of the P1 bacteriophage Cre-loxP recombinase system in the design and generation of retroviral producer cell lines. Incorporation of loxP sites into a parental producer cell line allows Cre-mediated removal of the selectable marker after selection of the highest-titer clones. The resultant cell line, Lox6Cre1, contains a single loxP site flanked by the viral LTRs and can function as a master producer cell line, allowing insertional recombination of genes of interest into an established genomic site. Once recombination occurs, the new producer lines generate stable retroviruses at a titer comparable to that of the parental cell line. The frequency of this recombination is such that new producer lines can be generated in the absence of a selectable marker in a shorter time frame than for conventional retrovirus production.

The choice of G1NaSVTK as our starting vector was influenced by the advantages inherent to the positive and negative selectable markers. The presence of Neo allowed selection of a high-titer (10⁶ CFU/ml) parental cell line, Lox6, in G418. Subsequent selection of lines in which Cre-mediated recombinational excision had occurred (Lox6Cre1) was achieved with ganciclovir with an efficiency of 50%, comparable to those reported for other genomic loci (27). Similar retroviral vectors have previously been utilized to modify target cell genomes after proviral integration with a reported excision efficiency of greater than 20% (3, 14).

The size of the oligonucleotides incorporated into the vector was critical, as an earlier retrovirus containing 53-bp lox sites flanked by restriction sites was unstable, demonstrating non-Cre-mediated excision of the lox sites and intervening DNA. Reduction of the loxP sites to the minimal 34-bp size was sufficient to alleviate this problem. This finding is in accord with previous studies which demonstrate that the frequency of deletion of direct sequence repeats in the non-LTR regions of retroviruses is proportional to the length of sequence (13).

Other investigators have circumvented this problem by cloning into the U3 region of the LTR which can tolerate up to 5 kb of extra sequence (23, 24). However, we initially elected not to use this approach in order to simplify the constructs necessary for subsequent insertional recombination.

Previous studies have shown that a circular piece of DNA containing a single loxP site can recombine into a host genome site also containing a single loxP site. The efficiency of this process is dramatically lower than excisional recombination in mammalian cells, occurring in only 1 in 10,000 clones (27). To improve the selection of insertional recombinants, we cotransfected a GFP expression plasmid with our Cre expression vector in a ratio of 1:10 and selected GFP-containing cells by FACS. Using this strategy, we were able to demonstrate insertional recombination in 1 of every 30 cells plated (Fig. 4C). This efficiency, although manageable in terms of obtaining producer cell lines without the need for selection, is less than optimal for the rapid generation of new producer lines. A previous study has demonstrated that expression of high levels of Cre enzyme very early in the transfection process may im-

**TABLE 2. Comparison of the biological titers of Lox6Cre1Neo producer cell lines and the parental Lox6 line**

<table>
<thead>
<tr>
<th>Producer cell line</th>
<th>Titer (CFU/ml)</th>
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<tbody>
<tr>
<td>Lox6</td>
<td>4 x 10⁶</td>
</tr>
<tr>
<td>Lox6Cre1Neo1</td>
<td>2 x 10⁷</td>
</tr>
<tr>
<td>Lox6Cre1Neo2</td>
<td>2 x 10⁷</td>
</tr>
<tr>
<td>Lox6Cre1Neo3</td>
<td>4 x 10⁶</td>
</tr>
<tr>
<td>Lox6Cre1Neo4</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>Lox6Cre1Neo5</td>
<td>4 x 10⁶</td>
</tr>
</tbody>
</table>

*Viral titer analysis on NIH 3T3 cells of producer cell lines was performed as detailed in the legend to Fig. 5B.
recombination. The initial transfection may improve the efficiency of insertional recombination in a single step by transfecting the site and the gene of interest and the subsequent insertional recombination of this DNA into the genomic site. In experiments in which we evaluated exonsional recombination in a single step by transfecting the Lox6 line with PuroRlox and the Cre expression plasmid and selecting in ganciclovir and puromycin, we noted that the efficiency of generating Cre-mediated puromycin-resistant clones was at least fourfold less than with Lox6Cre1 as the starting producer cell line. The use of a circular piece of DNA containing a single loxP site and the gene of interest in the initial transfection may improve the efficiency of insertional recombination.

The comparable titers of the new NeoR producer cell lines and of the parental Lox6 line indicates that genomic integration site is one critical determinant of virus production (Fig. 5B). This was also true for the majority of our PuroR clones (Fig. 6B). However, our observation that one of our PuroR clones had a considerably lower titer suggests that clonal sublines of the parental packaging line can possess different titers, despite having identical integration sites. Nevertheless, the ability to direct genes of interest into a favorable genomic site avoids the need to screen large numbers of clones to obtain a high-titer line. The Lox6 line contains a single proviral integrant, and hence new producer lines derived from Lox6Cre1 are also single-integrant lines. This ensures homogeneity of the vector particles generated from these producer cell lines and avoids clones with multiple integrants, which often contain rearranged proviruses, leading to production of heterozygous vector particles and inefficient gene transfer (21).

Several investigators have incorporated the Cre-loxP recombinase system into the design of retroviral vectors (3, 6, 14, 24, 35). However, the role of recombinase in these systems has centered on manipulation of either the target cell genome or the proviral integrant after gene transfer, rather than modification of cell lines to facilitate retroviral production. In one approach designed to generate a self-inactivating vector (SIN), a loxP site is inserted within the U3 of the 3' LTR along with a gene of interest. Following the LTR-mediated loxP duplication, the LTRs can be recombined by the Cre enzyme. The resulting provirus in the host genome consists of a single LTR with viral enhancers deleted and a single copy of the gene of interest (6, 24). An additional strategy incorporating loxP sites into retroviral constructs has been used to generate vectors to study reversible immortalization of mammalian cells (35). In this system, Cre recombinase is utilized to excise an oncogene from the target cell genome after cell immortalization. Simple modifications of vector design would also allow production of these viruses in our system.

The modification of retroviral packaging cell lines we describe should also be applicable to other viral packaging systems, when available. In support of this are experiments in which a single loxP site was inserted into a pseudorabies viral vector to allow transfer of genes of interest into mammalian cells (29). Several parallels exist between these and our experiments, in particular the stability of the resulting recombinant vectors and the efficiency of recombinational insertion. However, the retroviral system offers advantages in its ability to generate recombinant vectors with reproducible titers and in the homogeneity of the viral progeny produced without the need for plaque purification. Recently, a different application for the Cre recombinase system in viral vectors has been described, in which loxP sites have been incorporated into the genome of helper viruses in adenoviral vector packaging cell lines (22). Exposure of these viruses to Cre excises the packaging signals of the helper virus, rendering it unpackagable without affecting replication. This approach could also be complementary to our approach, and we are currently exploring the use of loxP sites in adenoviral vectors.

The approach to retroviral producer cell line production described here resolves several of the difficulties of generating reproducibly high-titer retroviruses lacking a selectable marker. Used in combination with other modifications, it should result in improved vector performance with less effects on the expression of the gene of interest by viral regulatory sequences or the selectable marker used in producer line generation. In addition, it provides a means to generate high-titer SIN vectors, thus diminishing the potential risk of oncogenesis associated with retroviral insertion.

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