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^r-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 the 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 Lox6 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^r 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 *SpeI* sites and also verified by sequencing. A *NotI/SalI* fragment of pG1NaSVTK containing the Neo^r gene was then subcloned into the *NotI/XhoI* 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%

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FIG. 1. Schematic representation of proviral integrants obtained with Cre-mediated recombination. The structure and response to selection with G418 or ganciclovir are shown for the parental provirus (Lox6), the provirus after Cre-mediated excisional recombination of Neo^r and TK genes (Lox6Cre1), and the provirus after Cre-mediated insertional recombination of a gene of interest (Lox6Cre1GeneX). The positions of the LTRs, *loxP* sites, and selectable marker genes are indicated. Resistance to selection is denoted by R, and sensitivity is indicated by S. SV40 P, simian virus 40 promoter.

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 (Pharmacia Biotech Inc.). The oligonucleotides used were as follows: *loxP*, sense, 5' CGGGATCCATAACTTCGTATAGCAT ACATTATACGAAGTTATAG 3'; and *loxP*, antisense, 5' CTATAACTTCGT ATAATGTATGCTATACGAAGTTATGGATCCCG 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 2× 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 extracted once with phenol-chloroform and precipitated with ethanol. The RNA pellets were dissolved in 0.25 ml of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and loaded onto a membrane using a slot blot apparatus (Schleicher and Schuell). The membrane was removed, rinsed in 10× 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 probing the membrane with a randomly primed ³²P-labeled restriction fragment from the Neo^r gene at 60°C for 12 to 16 h. After the membrane was washed at 65°C in 2× SSC for 30 min and then in 0.5× SSC for 30 min, the membrane was dried and imaged by autoradiography.

Biological viral titers were obtained by functional assay of Neo^r CFU (CFU per milliliter). NIH 3T3 cells were seeded at 5 × 10⁵ cells per 100-mm-diameter tissue culture plate and incubated for 24 h. Virus-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 *NheI*, 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^r); (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 polyurine 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^r 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

FIG. 2. Molecular analysis of the Lox6 producer cell line. (A) Southern blot analysis of the Lox6 proviruses in GP+E86 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 32 P-labeled Neo^r probe. SV40 P, simian virus 40 promoter. (B) Integration site analysis of Lox6 provirus in GP+E86 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 32 P-labeled Neo^r 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 32 P-labeled Neo^r probe.

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 transfected with supernatant from the Lox6 producer cell line. As shown in Fig. 2C, G418-resistant NIH 3T3 clones all demonstrated the presence of an unarranged 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^r 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^r 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 10^6 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^r colonies emerged, indicating that the excisional recombination was complete, removing both Neo^r and TK genes (data not shown). This finding was confirmed by genomic Southern analysis with a probe for Neo^r (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

FIG. 3. Cre-mediated excision of the Neo^r 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 (10^6) 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 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+E86 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 32 P-labeled Neo^r probe. Genomic DNA from Lox6 (lane 1) served as the control.

FIG. 4. Cre-mediated insertion of the Neo^r gene into the Lox6Cre1 producer cell line. (A) Insertional recombination of Neo^r into the Lox6Cre1 producer cell line is Cre dependent. Lox6Cre1 cells (10^6) were cotransfected with pEGFP-C3, pMC-Cre expression vector, and a plasmid containing loxP sites flanking the Neo^r gene (Neo^Rlox) in a ratio of 1:10:10. Cotransfection of the Neo^Rlox 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 GP+E86 cell line. Genomic DNAs from seven clones described above were digested with *Nhe*I, fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a ³²P-labeled Neo^r probe.

mammalian cells (27). We sought to utilize this observation to generate a new producer cell line based on Lox6Cre1. Lox6Cre1 cells (10^6) were cotransfected with pEGFP-C3, pMC-Cre expression vector, and a plasmid containing loxP sites flanking the Neo^r gene (Neo^Rlox) in a ratio of 1:10:10. Cotransfection of the Neo^Rlox plasmid with pEGFP-C3 and pUC19 served as a control. FACS analysis revealed that the transfection efficiencies were comparable in both experiments. After FACS analysis for green fluorescent protein (GFP) fluorescence, both transfections were selected in G418-containing media. In the cells transfected with the Cre expression vector, an initial excisional recombination event would occur in the Neo^r plasmid, creating circular DNA containing a single loxP site and the Neo^r gene. Subsequent Cre-mediated recombinational insertion would juxtapose the Neo^r 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 three different experiments. In contrast, in the absence of Cre, only eight clones were obtained in each experiment. These clones presumably represent random insertion of the Neo^r gene adjacent to a genomic regulatory sequence ca-

pable 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^r 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 Neo^Rlox 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.

Insertional recombination generates a new virus with a titer equivalent to that of Lox6. 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 Neo^r 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 (Puro^r). We cotransfected a Puro^Rlox 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^r gene (Fig. 6A). Viral titration by RNA slot blot revealed that three of the four selected clones had comparable titers. A fourth clone

TABLE 1. Efficiency of insertional recombination into the Lox6Cre1 producer cell line^a

Expt. no.	No. of colonies		Ratio (-G418/+G418)
	-G418	+G418	
1	60	4	15:1
2	110	3	36:1
3	230	3	76:1
4	180	4	45:1
5	350	7	50:1
6	600	16	37:1
7	1,050	56	18:1
Total	2,580	93	27:1

^a The Neo^Rlox plasmid was cotransfected into Lox6Cre1 cells with pMC-Cre and pEGFP-C3, and fluorescent cells were sorted and plated in serial dilutions in standard media in the presence (+) and absence (-) of G418. Colonies were counted at 14 days, and the ratios of colonies on the two plates were compared.

FIG. 5. Lox6Cre1Neo producer cell lines have titers comparable with that of the parental line, Lox6. (A) Slot blot analysis of Lox6Cre1Neo producer cell lines. Viral RNAs from 1-ml portions of culture supernatants from Lox6Cre1Neo1 to Lox6Cre1Neo7 (rows 1 to 7) and Lox6 (row 8) were loaded onto a nylon membrane as detailed in Materials and Methods and hybridized to a ³²P-labeled Neo^r probe. (B) Viral titer analysis on NIH 3T3 cells of Lox6Cre1Neo producer cell lines. NIH 3T3 cells (5×10^5) were incubated with viral supernatant diluted in 10-fold decrements from Lox6Cre1Neo1 and Lox6 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. (C) Southern blot analysis of the Lox6Cre1Neo1 provirus in NIH 3T3 cells. Genomic DNAs from two different NIH 3T3 clones (lanes 2 and 3) digested with *NheI* were fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a ³²P-labeled Neo^r probe. Lox6 genomic DNA digested with *NheI* served as the control (lane 1).

had 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^r allowed selection of a high-titer (10^6 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^a

Producer cell line	Titer (CFU/ml)
Lox6.....	4×10^6
Lox6Cre1Neo1.....	2×10^7
Lox6Cre1Neo2.....	2×10^6
Lox6Cre1Neo3.....	4×10^6
Lox6Cre1Neo4.....	2×10^6
Lox6Cre1Neo5.....	4×10^6

^a Viral titer analysis on NIH 3T3 cells of producer cell lines was performed as detailed in the legend to Fig. 5B.

FIG. 6. Cre-mediated insertion of the Puro^R gene into Lox6Cre1 producer cell line. (A) Southern blot analysis of Lox6Cre1puro provirus in a GP+E86 cell line. Four puromycin-resistant clones were digested with *Nhe*I, fractionated on an agarose gel, blotted onto a nylon filter, and hybridized to a ³²P-labeled Neo^r probe. (B) Determination of titers of Lox6Cre1puro producer cell lines by RNA slot blot analysis. Viral RNAs from 1-ml portions of culture supernatants from the four Lox6Cre1puro clones in panel A (rows 1, 2, 3, and 5) were loaded onto a nylon membrane as detailed in Materials and Methods and hybridized to a ³²P-labeled Puro^r probe. Supernatant from a non-Cre-dependent puromycin-resistant clone served as a control (row 4).

prove the recombination efficiency (6). To this end, we are currently developing a Lox6Cre1 producer cell line containing a stable integrant of the Cre recombinase gene driven off a tetracycline-inducible promoter which will allow high levels of Cre expression prior to transfection with a gene of interest (10). This approach has been utilized successfully with a metallothionein promoter in mammalian cells (28). A second factor influencing the efficiency in our system is that the DNA used for transfection into the Lox6Cre1 line is a plasmid containing two *loxP* sites flanking the gene of interest. Hence, two recombination events are required, an initial excisional recombination in the plasmid generating the circular piece of DNA with a single *loxP* site and the gene of interest and the subsequent insertional recombination of this DNA into the genomic site. In experiments in which we evaluated excisional and insertional recombination in a single step by transfecting the Lox6 line with Puro^Rlox 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 Neo^r 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 Puro^r clones (Fig. 6B). However, our observation that one of our Puro^r 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 unpackageable 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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