Genomic targeting with purified Cre recombinase

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ABSTRACT

Purified Cre recombinase protein introduced directly into cultured mammalian cells by lipofection catalyzes both site-specific chromosomal integration of a co-transfected *lox* targeting vector and precise excision of genomic DNA flanked by directly repeated *lox* sites. This procedure eliminates the need to transfect *cre* expression plasmids to activate recombination at *lox* sites. We used this simplified procedure to investigate the effect on targeting efficiency of both *lox* vector design and chromosomal position of the *lox* target. We show that such chromosomal position effects can exert at least a 50-fold per *lox* target difference in targeting efficiency in a human osteosarcoma cell line.

INTRODUCTION

The 38 kDa Cre recombinase from bacteriophage P1 catalyzes site specific recombination at *loxP* sites in both prokaryotic (1) and eukaryotic cells (2, 3). In addition to Cre, a number of other site specific recombinases have been shown to be active in heterologous eukaryotic cells (4–7). These recombinases catalyze defined recombination events at pre-engineered sites in the chromosome and, hence, can be of considerable utility in manipulating the *tansgene* in vivo. For example, a gene flanked by directly repeated 34 bp *loxP* sites and incorporated into the genome by standard gene transfer procedures is efficiently evicted from the chromosome by subsequent expression of the Cre recombinase (8). Cre catalyzes conservative site specific recombination at the *loxP* sites to excise the intervening gene, leaving a single *loxP* site behind in the chromosome. Conversely, prior placement of a *lox* target in the chromosome allows subsequent targeting of that site by the Cre recombinase (9). Because Cre directs single copy site-specific integration of a *lox* targeting vector, isogenic cell lines can be quickly generated to more easily analyze transcriptional regulation or DNA recombination at a particular chromosomal site.

To select for site specific integrants we have used *lox* fusion vectors that reconstruct a functional, selectable gene after the desired recombinational event (Fig. 1). For example, the chromosomal target can be a promoterless, ATG-less *lox-neo* translational fusion (10) that generates a functional *lox-neo* fusion gene upon targeted integration of an appropriately designed *lox* targeting vector (Fig. 1A). Alternatively, a simple promoter

fusion strategy (Fig. 1B) can be used in which a promoterless *tk* gene on the targeting vector is fused to a chromosomally placed promoter by Cre-mediated recombination at *loxP* sites located 5' to *tk* and 3' to the promoter, respectively (9).

For the integration reaction it is important to temporally limit Cre mediated recombination. Integration of the *lox* targeting vectors requires only transient presence of the Cre recombinase: continuous presence of Cre results in a second round of recombination that removes the targeting vector from the chromosome. Temporally limited expression of the *cre* gene can be achieved either by placing *cre* under the transcriptional control of a tightly regulated promoter or by relying on transient expression from a *cre* expression vector co-transfected with the *lox* targeting vector. We show here that Cre recombinase activity can be provided by direct introduction of the purified Cre recombinase protein into a variety of cultured cells. Direct introduction of purified Cre recombinase obviates the need for a co-transfecting *cre* expression vector and thus eliminates the possibility of unwanted incorporation of the *cre* gene into the genome.

MATERIALS AND METHODS

Cell culture

The thymidine kinase (*tk*⁻) human osteosarcoma cell line 143B, obtained from the American Type Culture Collection, and derivatives were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Tk⁺ derivatives were selected in HAT medium (11). We have observed no spontaneous Tk⁺ derivatives of 143B after examination of more than 1 × 10⁻⁹ cells. The *lox* target cell line 14-1-2 (10), a CHO cell derivative, and the *lox*² excision assay cell line 12HG-1 (8), a derivative of the LMtk⁻ cell line (12), were cultured in alpha medium and DMEM, respectively, as previously described.

Plasmids and molecular biology

All plasmids were constructed and prepared by standard techniques (13). The *loxP*-containing plasmid pBS64 (14), the SV40 promoter-*loxP-neo* plasmid pBS74 (8), the *loxP-tk* targeting vector pBS153 (9), and the *lox* translational fusion targeting vectors pBS226 (10) and pSF23 (15) have been described previously. Plasmid pSF23 is identical to pBS226 except that the

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CMV promoter has been replaced by the 250 bp herpes simplex virus type I *tk* promoter. A cDNA expression cassette (consisting of the CMV promoter, a multiple cloning site, and the SV40 polyadenylation signal) was obtained from A. Colberg-Poley (Children's National Medical Center) and inserted 5' to the *tk* promoter of pSF23, in either the same or opposite orientation with respect to the *tk* promoter, to give pBS261 and pBS266, respectively. The CMV-*lacZ* expression plasmid p324 was a gift from A. Colberg-Poley (Children's National Medical Center).

The number of *transgenes* inserted into the genome after gene transfer of pBS74 into the 143B cell line was determined by quantitative Southern analysis (16), using a *transgene* probe and an endogenous genomic probe to normalize for gel loading variability. Briefly, 5 μ g genomic DNA from candidate cell lines was digested both with EcoRI and with BamHI, electrophoresed on a 0.8% agarose gel and then blotted to GeneScreen Plus (DuPont Co.). Hybridization was with a *neo* probe to detect pBS74 and with the human angiotensin Type I receptor gene (a gift from Martha Corjay, DuPont Merck) as the internal control. In addition a titration series of linearized pBS74 corresponding to 1–64 copies per genome were electrophoresed and blotted in parallel with the genomic samples. The copy number of the *transgene* in these cells was determined by densitometry of autoradiograms from both the EcoRI and the BamHI digests.

Gene transfer

A *loxP* target was incorporated into the genome of 143B cells by microinjection of pBS74 and selecting for resistance to 400 μ g/ml G418. Single cells were microinjected with the Zeiss-Eppendorf AIS Automated Injection System (17). Plasmid pBS74 contains the SV40 promoter *neo* cassette with a single *loxP* site inserted between the promoter and the *neo* gene as shown in Fig. 1B. Plasmid pBS74 was linearized by digestion with EcoRI and then approximately 120 individual cells on a glass slide were each injected with a volume of 1 μ l containing about 1000 linearized plasmid molecules. Cells were trypsinized 4 hr after injection and replated in 10 cm dishes. Two days after injection cells were selected for resistance to 400 μ g/ml G418. Twelve G418 resistant transformants were observed 21 days after microinjection. These were picked and expanded for further analysis.

Lipofection of DNA and protein into cultured cells was with TransfectACE™ (Life Technologies, Inc.), a 1:2.5 (w/w) formulation of dimethyl dioctadecylammonium bromide and dioleoyl phosphatidylethanolamine, and was performed essentially as suggested by the manufacturer, with the following modifications. Cells were seeded in a six well tissue culture plate and allowed to reach ~80% confluency. DNA and Cre protein (NEN—DuPont Co.) were mixed with Opti-MEM (Life Technologies, Inc.), giving a final volume of 100 μ l, and incubated at 0°C for 5 min. In a separate aliquot, 12 μ l TransfectACE was added to 88 μ l Opti-MEM. The two aliquots were combined and incubated 10 min at room temperature. The volume was brought to 1 ml by addition of Opti-MEM and the mixture then added to a single well of cells that had been previously washed twice with Opti-MEM. Cells were incubated for 5 hr at 37°C, 1ml of α^- medium + 20% dialyzed fetal bovine serum was added and incubation was continued overnight. The next day cells were trypsinized and replated. Selection for stable transformants was imposed 48 hr after the lipofection procedure.

RESULTS

Protein mediated targeting

Using the *lox-neo* fusion system (Fig. 1A) purified Cre protein was introduced with the pBS226 targeting vector (Fig. 2) by means of a lipofection procedure. As demonstrated previously, G418 resistance is diagnostic of Cre-mediated site-specific recombination in this system (10). Titration of Cre with a constant amount of *lox* targeting vector (1 μ g) showed optimal targeting (highest frequency of G418 resistance) with 8.4 μ g of Cre, and

Figure 1. Cre-mediated integrative recombination. A. Targeted integration using a chromosomal *lox-neo* fusion target. Upon site-specific recombination of the promoter-ATG-*lox* targeting vector with the chromosomal target (having an inactive *neo* gene), a functional *lox-neo* fusion gene is produced to confer G418 resistance on the cell. B. Targeting with a simple promoter *lox* fusion vector. The chromosomal target is the SV40 promoter-*lox-neo* fragment from pBS74. The targeting vector, pBS153, has a promoterless *tk* gene preceded by a *loxP* site. Cre-mediated recombination at the *lox* sites places the *tk* gene under the control of the SV40 promoter and confers HAT resistance. Note that in this binary system the defective structural gene can be either placed in the chromosome (A) or in the targeting vector (B). In both cases Cre-mediated integration results in a *lox*² product that is itself a substrate for excisive recombination.

Figure 2. *lox-neo* fusion targeting vectors. The *lox* site is represented by the open white arrow and is preceded either by an in-frame ATG codon and the CMV promoter (pBS226) or by the *tk* promoter (pBS261 and pBS266). Unique restriction sites in the polylinker of the CMV expression cassette are indicated for pBS261 and pBS266.

little or no targeting with less than 3 μ g Cre (Fig. 3). Higher amounts of Cre reduced targeting efficiency.

Lipofection procedures require that an optimal amount of DNA (ratio of DNA to lipid) be used in the lipofection procedure. We first determined that 1 μ g DNA was optimal for stable (illegitimate) transformation with a SV40 neo vector in these cells (data not shown). Because increasing amounts of targeting vector result in increased targeting when co-electroporated with a Cre expression vector (9), we examined targeting efficiency in the lipofection procedure as a function of the amount of targeting vector, holding the amount of Cre protein constant at the optimized level (Fig. 4).

Between 1 and 3 μ g of DNA gave optimal targeting efficiency with the pBS226 targeting vector. For comparison, two other lox targeting vectors were also examined in this assay: pBS261 and pBS266 (Fig. 2). These two vectors are similar to pBS226, but use the weak HSV-1 tk promoter to drive the lox - neo fusion gene after integration instead of the strong CMV promoter. In

Figure 3. Targeting dependence on Cre recombinase. Cell line 14-1-2 was lipofected with 1 μ g pBS226 and the indicated amount of Cre recombinase, as described in Materials and Methods. Targeted integration results in a G418 resistant colony. The results shown are the average of two experiments. In a parallel experiment to determine the frequency of illegitimate recombination under these conditions, cells were lipofected with 1 μ g of pBS74, which has a functional neo gene, to give an average of 791 G418 resistant colonies.

Figure 4. Targeting dependence on the targeting vector. Cell line 14-1-2 was lipofected with 8.4 μ g Cre and the indicated amount of each lox targeting vector. The results shown are the average of two experiments.

addition, both contain a CMV promoter-driven cDNA expression module, but in opposite orientations. Both pBS261 and pBS266 are also proficient in targeting and showed optimal targeting at 1 μ g DNA, although at a substantially lower frequency than that seen with pBS226.

Excision

The Cre recombinase catalyzes precise removal of a *transgene* from the genome (18). This activity can be used both to eliminate a gene's activity and to activate an adjacent gene. For example, we have previously described (8) a mouse cell line, 12HG-1, in which expression of the *neo* gene is attenuated by insertion of the yeast *LEU2* gene flanked by directly repeated *lox* sites between the SV40 promoter and the *neo* structural gene. Cre-mediated recombination excises the *LEU2* gene to regenerate a functional *neo* expression unit, thus conferring high level G418 resistance.

We tested the ability of purified Cre protein to activate G418 resistance in 12HG-1 cells by excisive Cre-mediated recombination using the lipofection procedure. There is a low spontaneous frequency of G418 resistance with this cell line (Table I) and, as we had observed previously, this is probably due to occasional amplification of the *neo* gene (8). When cells are lipofected with Cre protein there is about a 40 fold stimulation of G418 resistance from excisive recombination so that about 10% of all treated cells become G418 resistant. This is approximately the same fraction of cells that express β -galactosidase after lipofection with CMV-*lacZ* DNA. Lipofection of pure Cre protein alone gives high levels of excisive recombination in these cells (Exp. 2, Table 1). Co-lipofection of Cre with either *loxP*-containing DNA (pBS64) or non-*loxP* DNA (p324) did not facilitate uptake of Cre by cells.

Effect of *lox* target copy number and position on integrative recombination

Next we examined the effect of position and copy number of the *lox* targets in the chromosome on the efficiency of Cre-mediated targeting using the targeting scheme shown in Fig. 1B. The tk^- human osteosarcoma cell line 143B was microinjected with plasmid pBS74 containing the SV40 promoter-*loxP*-*neo* target sequence and independent G418 resistant colonies were selected. Southern analysis showed that transformants had

Table I. Cre-mediated excision.

	Cre*	DNA [†]	% G418 ^R CFU [‡]
Exp. 1	—	—	0.25
	+	p324 (<i>loxP</i> [−])	14
	+	pBS64 (<i>loxP</i> ⁺)	14
Exp. 2	+	—	8.8
	+	p324 (<i>loxP</i> [−])	8.8
	+	pBS64 (<i>loxP</i> ⁺)	9.4

* 8.4 μ g Cre protein with Transfectace was added per well of a 6 well culture dish as described in Materials and Methods.

[†] 1.0 μ g of the indicated DNA was used.

[‡] CFU, colony forming unit. A series of aliquots was plated in duplicate either in the presence or absence of 800 μ g/ml G418 to obtain the ratio of drug resistant CFU to total CFU. The data presented are the average of two experiments. Approximately 10–15% of the treated cells took up DNA as determined by an in situ assay for β -galactosidase activity using the CMV-*lacZ* plasmid p324 (24).

between 2 and 60 copies of the *loxP* target, mostly integrated as tandem arrays. Representative cell lines are shown in Fig. 5. The copy number of the *loxP* target in eight of these cell lines was established by quantitative Southern analysis and the targeting efficiency was then determined for all eight by selecting for Tk^+ using the *loxP-tk* targeting vector containing a promoterless HSV-1 *tk* gene.

Three of these cell lines showed a high frequency of targeting, three showed a greater than 10 fold decreased targeting efficiency and two were intermediate (Table II). These results indicate that targeting efficiency is much more dependent on chromosomal position than on the copy number of *loxP*. The cell line showing the highest targeting efficiency, 143-3, is one with a lower copy number of the *loxP* target. Conversely, cell line 143-2 has the highest copy number of *loxP* targets but shows an intermediate frequency of targeting. The ratio of Tk^+ transformants to chromosomal *loxP* copy number varies from 0 to 15 and indicates that there is no simple relation between copy number and targeting efficiency. Thus, Cre-mediated targeting is subject to significant position effects. In particular, certain chromosomal loci are more accessible to site specific recombination than are others.

Figure 5. Representative cell lines containing the pBS74 *lox* target. DNA's (5 μ g each) from several G418 resistant pBS74 transformants of 143B cells were digested with EcoRI and then analyzed by Southern analysis of using a *neo* probe. A titration series of EcoRI digested pBS74 was electrophoresed in parallel on the same gel. Also shown is the same Southern blot reprobed with the human angiotensin AT1 receptor gene to normalize for variation in DNA/gel loading.

DISCUSSION

We show here that active Cre recombinase can be directly introduced into mammalian cells using a lipofection procedure. The lipofected recombinase protein is proficient both for site-specific excision of DNA from the chromosome and for chromosomal targeted integration of a co-lipofected *lox* targeting vector. Our results are analogous to those of others who have previously shown the utility of directly introducing proteins into cells, for example, by electroporation, as has been shown for restriction enzymes (19), antibodies (20) and viral transactivator (21) proteins. In preliminary experiments, we've found that Cre protein can also be electroporated into cells to give site specific recombination (S. Fukushima and B. S., unpublished data).

Direct introduction of Cre recombinase into cells has a number of distinct advantages compared to using *cre* expression plasmids. First, use of purified protein rather than DNA eliminates the possibility of stable integration of the *cre* gene. For example, in the construction of targeted knockout mutations in embryonal stem cells it is often desirable to remove the selectable marker gene used to generate the knockout. This can be done by flanking the marker gene by directly repeated *loxP* sites in the targeting construct and later excising the marker gene in the resulting knockout cell line by transient expression of the *cre* gene (18). Use of purified Cre protein eliminates the need to check marker gene 'popout' colonies for presence of the *cre* gene. Second, because direct protein transfer should be possible with many different cell types, there is no need for individualized *cre* vectors having particular cell and tissue specific transcription and translation signals. Third, recombinase activity is temporally prescribed by the actual presence of functional Cre protein in the cell, thus averting problems for efficient integration of *lox* targeting vectors arising from long term persistence (and expression) of DNA, as occurs in certain cells, such as striatal muscle (22).

The efficiency of Cre-mediated genomic targeting depends in part on the *lox* targeting vector used. A higher frequency of site-specific integration occurs with the *lox-neo* fusion vector having the strong CMV major immediate early promoter driving expression of the fusion than with vectors using the *tk* promoter to drive expression. Moreover, proximity of the CMV promoter/enhancer to the *tk* promoter in these latter vectors increases targeting efficiency. This could be due to enhanced recovery of integrants from CMV enhanced expression of the *lox-neo* fusion, although selection at reduced concentrations of G418 does not result in increased targeting efficiency (data not shown).

Table II. Position effect of the chromosomal *loxP* target on targeting efficiency.

Recipient Cell Line	No. <i>loxP</i> Targets per Genome*	No. Tk^+ Transformants†	Ratio: Tk^+ /Target
143-1	3	9.5	3.2
143-2	58	19	0.33
143-3	4	59.5	15
143-6	37	40.5	1.1
143-7	24	46.5	1.9
143-8	7	5	0.71
143-11	2	0	0
143-12	21	4.5	0.21

* Determined by quantitative Southern analysis. See Materials and Methods.

† Average of two experiments, each using 1 μ g pBS153 and 8.4 μ g Cre per well of a 6 well plate, as described in Materials and Methods, and selecting for HAT resistance.

A second possibility is that the CMV enhancer potentiates site specific recombination with the chromosome, for example, by keeping the plasmid *lox* site accessible to Cre protein and to Cre-mediated synapsis with the chromosomal *lox* site. This may occur either by keeping the chromatin surrounding the *lox* site in an accessible configuration, or indirectly by transcription through the site. It has been suggested, for example, that transcription may stimulate homologous recombination in mammalian cells (23).

The efficiency of Cre-mediated genomic targeting is sensitive to chromosomal position effects. We observe a 50-fold difference in targeting efficiency per target *lox* site in human osteosarcoma cell lines engineered to have variously located *lox* targets. Previously we noted only marginal differences in targeting efficiency with four CHO cell lines each having a single copy *lox* target (10). This prior observation may be explained by the small number of CHO lines examined and by the inherent bias in choosing lines having only a single copy of the *lox* target and, hence, of the selected marker gene. The cause(s) of position effects on recombination and gene expression is not well understood. It will be interesting to see whether the position effect on Cre-mediated targeting correlates with position effects on transcription or on homologous recombination. Because Cre can direct integration of a series of *lox* integration vectors to the same target site, and thus construct isogenic cell lines, it should be possible to examine the effect of various DNA sequence motifs on these position effects.

The ability of lipofected Cre protein to catalyze both integrative and excisive site specific recombination in cultured cells has important practical implications. Because of the ease of the procedure, it can be used for transformation of a target cell line with a battery of targeting plasmids to allow rapid generation of a series of isogenic cell lines. Moreover, removal of a marker gene flanked by *lox* sites is simply accomplished by direct introduction of Cre protein, obviating the possibility of unwanted adventitious integration of a *cre* expression plasmid. Chromosomal manipulations with purified Cre recombinase should be of particular use in engineering both fertilized zygotes and embryonal stem cells for the production of transgenic mice (18, 25, 26).

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REFERENCES

1. Hoess, R.H. and Abremski, K. In Eckstein, F. and Lilley, D.M.J. (eds.), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin Heidelberg, Vol. 4, pp. 99–109.
2. Sauer, B. (1987) *Mol. Cell. Biol.*, **7**, 2087–2096.
3. Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5166–5170.
4. Golic, K.G. and Lindquist, S. (1989) *Cell*, **59**, 499–509.
5. O'Gorman, S., Fox, D.T. and Wahl, G.M. (1991) *Science*, **251**, 1351–1355.
6. Maeser, S. and Kahmann, R. (1991) *Mol. Gen. Genet.*, **230**, 170–176.
7. Onouchi, H., Yokoi, K., Machida, C., Matsuzaki, H., Oshima, Y., Matsuoka, K., Nakamura, K. and Machida, Y. (1992) *Nucleic Acids Res.*, **19**, 6373–6378.
8. Sauer, B. and Henderson, N. (1989) *Nucleic Acids Res.*, **17**, 147–161.
9. Sauer, B. and Henderson, N. (1990) *New Biologist*, **2**, 441–449.
10. Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7905–7909.
11. Szybalski, W., Szybalski, E.H. and Ragni, G. (1962) National Cancer Institute Monograph, **7**, National Cancer Institute, Bethesda, MD.
12. Wigler, M., Levy, D. and Perucho, M. (1981) *Cell*, **24**, 33–40.
13. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
14. Sauer, B., Whealy, M., Robbins, A. and Enquist, L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9108–9112.
15. Sauer, B., Baubonis, W., Fukushige, S. and Santomenna, L. (1992) *Methods: A Companion to Methods in Enzymol.*, **4**, 143–149.
16. Nakamura, N., Sugino, H., Takahara, K., Fukushige, S. and Matsubara, K. (1991) *Cytogenet. Cell Genet.*, **57**, 18–22.
17. Pepperkok, R., Zanetti, M., King, R., Delia, D., Ansorge, W., Philipson, L. and Schneider, C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6748–6752.
18. Sauer, B. (1993) *Methods Enzymol.*, **225**, in press.
19. Winegar, R.A., Phillips, J.W., Youngblom, J.H. and Morgan, W.F. (1989) *Mutation Res.*, **225**, 49–53.
20. Berglund, D.L. and Sarkey, J.R. (1991) *Cytometry*, **12**, 6467.
21. Kashanchi, F., Duvall, J.F. and Brady, J.N. (1992) *Nucleic Acids Res.*, **20**, 4673–4674.
22. Wolff, J.A., Ludtke, J.J., Acsadi, G., Williams, P. and Jani, A. (1992) *Human Molecular Genetics*, **1**, 363–369.
23. Nickoloff, J.A. (1992) *Mol. Cell. Biol.*, **12**, 5311–5318.
24. Sanes, J.R., Rubenstein, J.L.R. and Nicolas, J.-F. (1986) *EMBO J.*, **5**, 3133–3142.
25. Lakso, M., Sauer, B., Mosinger, Jr., B., Lee, E.J., Manning, R.W., Yu, S.-H., Mulder, K.W. and Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6232–6236.
26. Orban, P.C., Chui, D. and Marth, J.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6861–6865.