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Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, *pac*: a transient gene-integration marker for ES cells

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ABSTRACT

Gene targeting in embryonic stem (ES) cells is a powerful tool for generating mice carrying specifically designed mutations in the germline. Puromycin can completely kill ES cells within 24 to 48 h whereas G418 and hygromycin cannot. We have, therefore, proposed that the puromycin N-acetyltransferase (*pac*) gene, may be utilized as a transient gene-integration marker. Using a circular expression vector of *cre* and *pac* genes, Cre-mediated mutant cells were effectively enriched by pulse treatment of puromycin without stable integration of their genes. We have thus demonstrated the first application of *pac* as a transient gene-integration marker for ES cells.

Puromycin can effectively kill ES cells within a short period (2 days) and at a low dose, independent of G418 selection. The low dose of puromycin, which effectively eliminates wild-type ES cells, has little influence on the feeder cells from mouse embryonic fibroblasts, enabling the use of wild-type feeder cells for puromycin selection. The puromycin selected ES cells effectively colonized into germ cells in chimeric mice (1). Because the time course profile for puromycin to kill ES cells is different from that of other drugs such as G418 and hygromycin, the puromycin-*pac* gene system may be utilized not only in producing stable integrants, but also for the selection of transient transgene integrants.

To evaluate selectional efficiency for transient *pac* gene integrants, we used the Cre-mediated *loxP* recombination system, as no gene markers remain in the chromosome of transient gene integrants. Transient expression of the *cre* gene is employed for the acquisition of Cre-*loxP*-mediated recombinants without other chromosomal DNA damages. This system promotes gene disruption between two *loxP* sites through the transient expression of Cre recombinase. Using this method for ES cells allows us to produce various specifically designed mutant mice (2,3). To enrich for Cre-*loxP*-mediated recombinants in ES cells, Gu *et al*. used supercoiled Cre-encoding plasmid to transiently express the Cre enzyme, followed by selection with Gancyclovir (GANC) to eliminate cells containing the stable herpes simplex virus-thymidine kinase (*HSV-tk*) gene, which was located within the flanking DNA region between the *loxP* sites (4).

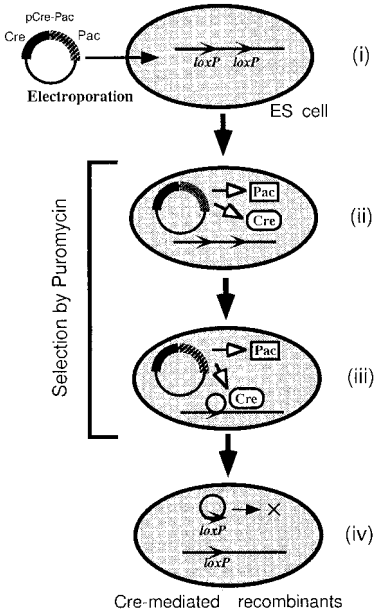

Figure 1. Recombination using pCre-Pac plasmid. Strategy for recombination using pCre-Pac plasmid. (plasmid) Closed circular pCre-Pac DNA is transfected by electroporation into ES cells containing two *loxP* sites. (ii) Transfected pCre-Pac DNA expressing Cre and Pac products. (iii) Cre protein recognizes the *loxP* sites and recombines between these sites. ES cells are selected by puromycin at (ii) and (iii) periods. (iv) Cre-mediated recombination between *loxP* sites is complete in ES cells. The chromosome containing a single *loxP* site and excised circular DNA containing a single *loxP* site are lost. The 1.7 kb *Sal*I fragment from pPGKPuro was ligated into the 4.3 kb *Xho*I–*Sal*I fragment from pPGKPuro was ligated into the 4.3 kb *Xho*I–*Sal*I fragment from pMC1-Cre (5) to yield pCre-Pac.

The experimental strategy is outlined in Figure 1. To test this, we constructed a circular pCre-Pac plasmid which coincidentally expressed both the *pac* and *cre* genes in ES cells, and obtained a gene-targeted cell line; clone-121, containing three *loxP* sites at the

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Table 1. Frequency of recombination by pCre-Pac plasmid

Experiment	Treatment time course of puromycin after electroporation (hrs)	Total clones ($\times 10^3$)	Number of clones analyzed	Number of single genotype clones	Locus types of targeted allele (%)				Rate of Cre-mediated recombination (%)
					type-1[a]	type-2[a]	type-3[a]	no recombination	
1	12 → 72	0.12	24	19	11 (58)	0	1 (5.3)	7 (37)	12/19 (63)
	24 → 72	0.35	24	13	7 (54)	0	0	6 (46)	7/13 (54)
2	6 → 30	0.60	32	32	2 (6.3)	1 (3.1)	0	29 (91)	3/32 (9.4)
	6 → 42	0.55	26	25	6 (24)	0	0	19 (76)	6/25 (24)
	6 → 54	0.14	26	24	2 (8.3)	2 (8.3)	1 (4.2)	19 (79)	5/24 (21)
	no puromycin	15	---	---	---	---	---	---	---
3	6 → 30	5.5	---	---	---	---	---	---	---
	6 → 42	4.0	---	---	---	---	---	---	---
	6 → 54	1.0	65	51	22 (43)	1 (2.0)	1 (2.0)	27 (53)	24/51 (47)
	no puromycin	48	---	---	---	---	---	---	---

[a]Type-1, -2 and -3 represent recombination types null, conditional and knocked-in *lac*Z, respectively.

mouse *semaphorin III/D* locus (5). The enrichment of Cre-mediated recombinants at the *loxP* sites by transient puromycin selection was determined by electroporating the circular pCre-Pac plasmid into clone-121; thereafter we altered the time-course of puromycin selection. In experiments, we transiently administered 1 μg/ml puromycin during 24 or 48 h at several time-courses (Table 1). To separate a single type of recombinant, we subcultured the transformants on new feeder cell layers after pulse selection by puromycin. After six days with normal ES cell medium, we analyzed the Cre-*loxP*-mediated recombinants by PCR and Southern blot analyses. Since clone-121 contained three tandem *loxP* sites which was located at the 5′ non-coding region of the first exon and both edges of *lacZ-neo* cassette at the first intron of the *semaphorin III/D* locus (5), three types of site-directed recombinants were generated: type-1, -2 or -3 deletions resulted, respectively, in the following phenotypes: null, the deletion of both the first exon and the *lacZ-neo* cassette; conditional, the deletion of the external *lacZ-neo* cassette; or 'knocked-in' *lacZ* gene, the deletion of the first exon. These deletion types were firstly detectable by PCR analyses (data not shown). Cre-*loxP* mediated recombinants were from 9.4 to 63% of the surviving single-genotype cells after puromycin selection at various time courses (Table 1). At the time course of 6–54 h in experiment 2 or 3, we could obtain all three type recombinants at 39% (29/75). Among these 29 recombinants, 24, 3 and 2 were identified as type-1, -2 and -3, respectively. Since 1.4 and 10×10^2 clones arose by pulse selection with puromycin from 6 to 54 h in contrast to 1.5 and 4.8×10^4 clones without the puromycin, the pulse selection of puromycin enriched transient-transgene integrants 50–100-fold. In previous reports on the selection of Cre-mediated recombinants by the *HSV-tk* gene and GANC, ~20% of the GANC-selected colonies resulted from the site-directed recombination (4). This evidence indicates that our Cre-*loxP* recombination system is a more efficient method for the enrichment of recombinants in comparison to other drugs. However, 83% (24/29) of the site-directed recombinants were type-1 at the 6–54 h time course. This type-1 distortion suggests that the activity of Cre with this puromycin-*pac* system may be too high to limit site-directed recombination at a single event. To efficiently enrich for type-2 or -3 recombinants, an attempt should be made to use a weaker promoter of *cre* gene or less activity of Cre enzyme.

After the three types of site-directed recombinants were confirmed by Southern blot analyses (data not shown), we chose every one clone for each type of recombinants; 121-12-22 (type-1), 121-48-55 (type-2) and 121-48-26 (type-3), and produced chimeric mice. Neither the *pac* nor *cre* gene was detectable by PCR and Southern blot analyses (data not shown). Ten of each ES cell-line were microinjected into an 8-cell stage CD-1 mouse embryo (6). The microinjected embryos were cultured in M16 medium overnight to blastocyst stage. Seven blastocysts were transplanted into each uterus of recipient CD-1 mice. Chimeric mice were identified by eye and coat colors and germline transmission was confirmed by coat color of offspring with crossing with CD-1 females. One chimeric male from parental clone-121 was obtained with an agouti coat color of >80%, which exhibited a germline-differentiating potency when mated with albino CD-1 females (5). In contrast, 8, 4 and 5 chimeric males obtained by microinjection with type-1, -2 and -3 recombinants, respectively, into CD-1 embryos had >80% agouti hair color. By crossing these chimeric mice with CD-1 females, 3, 1 and 2, respectively, produced ES-cell derived offspring. All types of Cre-mediated recombinants thus maintained the germline-differentiating potency. Cre-mediated recombinants possessed similar or even greater potency in comparison to the parental clone-121 cell to differentiate each tissue and germ cell in chimeric mice. These results indicated that neither pulse selection of puromycin nor Cre and/or Pac transient expression influenced the stability of germline-differentiating potency in ES cell culture.

Instead of this *pac* gene, GFP gene can also be used for selecting Cre recombinants (7). Nevertheless these results establish the applicability of the puromycin-*pac* gene system as a novel selection system for transient extragene integrants, and demonstrate a highly efficient and facile method for screening Cre-mediated site-directed recombinants of ES cells with strong germline differentiating potency. This method is based on the transient integration of a circular pCre-Pac plasmid, which allows selection of transient expression of Cre recombinase by short puromycin exposure. These results indicate that transient Pac expression allows us to select and enrich the transient gene integrants with puromycin. Thus, this is a first selection marker for transient gene integration.

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