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Negative Selection with the *Diphtheria toxin A fragment* Gene Improves Frequency of Cre-Mediated Cassette Exchange in ES Cells

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The Cre-*lox* system is an important tool for genetic manipulation in embryonic stem cells. We previously reported that the cassette exchange strategy using the mutant *lox*66/71 and *lox*2272 combination showed high recombination efficiency and stability. However, the efficiency was strongly affected by the position of chromosomal target *lox* sites. To enrich successful cassette exchange events, even in clones showing lower recombination efficiency, we have improved exchange vector. The *Diphtheria toxin A fragment* gene was placed in the un-exchanged region for negative selection and the *puromycin N-acetyltransferase* gene, instead of the *neomycin phosphotransferase* gene, was used for positive selection. By reducing random integration, the frequency of successful cassette exchange increased up to 2–4 fold. Furthermore, by adding the third *lox* site to induce intrarmolecular recombination, the recombination efficiency of cassette exchange itself was improved, and the frequency increased to maximum 5 fold, in which the percentage of exchanged clones reached to 50–70%. This strategy should be useful for other recombinase-mediated cassette exchanges.

Key words: cassette exchange, Cre recombinase, *Diphtheria toxin A fragment* (*DT-A*) gene, embryonic stem (ES) cells, site-directed recombination.

Abbreviations: bsr, blasticidin S resistant; DT-A, diphtheria toxin A fragment; ES, embryonic stem; neo, neomycin phosphotransferase; NLSLacZ, *lacZ* gene fused with the nuclear localizing signal, pA, polyadenylation; pac, puromycin *N*-acetyltransferase; Pgk, phosphoglycerate kinase-1; RMCE, Recombinase Mediated Cassette Exchange; tk, thymidine kinase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

The Cre-mediated site-specific targeting system is a powerful tool for genome engineering in mammals, especially in mouse embryonic stem (ES) cells, because it allows precise and repeated knock-ins of any DNA to target *lox* sites introduced by gene targeting or gene trapping. (*1–3*) However, intermolecular recombination between wild-type *lox*P sites, *i.e.*, integrative recombination, is inefficient due to re-excision through intramolecular recombination (*4*).

In order to perform Cre-mediated insertion or replacement, two kinds of mutant *lox* sites have been developed. One is a pair of *lox* sites with a 5 bp mutation in the left or right end of the *lox* sequence, such as *lox*66/71 (*5*, *6*). Recombination between a chromosomally located *lox*71 site and a *lox*66 site on a targeting plasmid results in site-specific integration of the plasmid producing a double mutant *lox* site at both ends and a wild-type *lox*P site. Since the binding affinity of the double *lox* mutant site for Cre recombinase is reduced, the integrated plasmid is stably retained. The other mutant site is a heterospecific *lox* site that has mutation(s) in the central 8 bp spacer region (*7–9*). The recombination using heterospecific *lox* sites is termed Recombinase Mediated Cassette Exchange (RMCE) (*10*), in which the recombination does not occur between two *lox* sites differing in the spacer region whereas *lox* sites with identical spacer regions can be recombined efficiently. Until now, *lox*511 (*11*), *lox*2272 (*12*) and *lox*5171 (*13*) have been successfully used in ES cells.

Recently, we have shown that the combination of *lox* 66/71 and the heterospecific *lox*2272 site gave high recombination efficiency and stability even with Cre recombinase (*14*), and developed an exchangeable gene trap vector carrying *lox*71, *lox*P and *lox*2272 (*3*). Using the trap vector, we can initially carry out random insertional mutagenesis in ES cells, and then replace the reporter gene in the trap vector with any gene of interest to be expressed under the control of the trapped promoter through RMCE.

In RMCE, a targeting plasmid carrying an integrated floxed cassette is co-electroporated with a Cre-expression vector into cells in their circular forms to reduce random integration. However, random integration of targeting plasmid also occurs at a considerable frequency, probably due to nicks in plasmid DNA strands. In our previous study, the percentage of random integrants was over 50% even in the clone that showed the highest RMCE frequency, indicating that random integration is more efficient than RMCE. The most effective method to eliminate random integrants is negative selection using the *thymidine kinase* (*tk*) gene of the Herpes simplex virus (*15*). In this method, the *tk* gene is placed on a chromosomal target construct between two heterospecific *lox* sites, and recombined clones, where the *tk* gene should be removed by

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replacement with other DNA on the targeting vector, are selected with ganciclovir. Several groups reported that almost all clones obtained after electroporation had targeted replacement (*12*, *16*, *17*). This *tk* negative selection is very useful because it is not necessary to have any positive marker on targeting vectors. On the other hand, the necessity of placing the *tk* gene in a chromosomal target construct is quite inconvenient for gene trapping, since it is preferable that trap vectors should consist of minimum elements to avoid unexpected effect(s) caused by introduction of vector elements. Actually, male sterility caused by ectopic expression of the *tk* gene in testis has been reported (*18*).

In this study, we aimed to increase recombination frequency by improving exchange vectors, not chromosomal target constructs. We used the *Diphtheria toxin A fragment* (*DT-A*) gene (*19*) for negative selection to reduce random integrants, changed the *neomycin phosphotransferase* (*neo*) gene into the *puromycin N-acetyltransferase* (*pac*) gene, and added a *lox*66 site to induce intra-molecular recombination within the exchange vector. With the improved exchange vector, the frequency of exchanged clones was increased to 2–5 folds.

MATERIALS AND METHODS

Plasmids—Plasmids p66-2272 and pCAGGS-Cre were described previously (*14*, *20*). The plasmid containing the MC1 promoter–DT-A gene was kindly provided by Dr. S. Aizawa (*19*). However, The original MC1-DT-A cassette had no polyadenylation (pA) signal and the MC1 promoter of the cassette carried only one copy of the enhancer sequence. The MC1-DT-A-pA cassette used in this study was constructed by replacing the original promoter by the MC1 promoter from MC1-neo-pA cassette (STRATAGENE, La Jolla, USA) and by adding the pA signal of the rabbit β-grobin gene. The pMC1-DTA plasmid was constructed by inserting the MC1-DT-A-pA cassette into p66-2272. The pPGK-DTA and pPac-DTA were constructed by replacing the MC1-neo-pA cassette into the Pgk-neo-pA and Pgk-pac-pA cassette, respectively. The pPac-DTA-66 was constructed by inserting a *lox*66 fragment into pPac-DTA. The sequences of all *lox* sites in these plasmids were confirmed by DNA sequencing.

Cell Culture and Electroporation—ES cell culture and establishment of cell lines carrying a single copy of target *lox* sites was described previously (*14*). For RMCE, the cells (3–6 $\times$ 10^6 cells/0.8 ml in PBS) were electroporated at 400 V and 125 μF, and plated into two 10 cm plates. G418 selection was started after 24 h of electroporation at 200 μg/ml for 7 days. For puromycin selection, cells were fed 2 μg/ml of puromycin containing medium for 24 h $\times$ 2 times on day 1 and 4 after electroporation. On day 8, colonies were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) or picked and expanded for DNA analysis.

A series of 5 exchange vectors with a cell line was performed on the same day, and each series of electroporation was repeated at least four times on independent days.

PCR Analysis—Genomic DNA (0.05–0.1 μg) was subjected to 32 cycles of amplification (each cycle consisted of 1 min at 94°C, 1.5 min at 58°C and 1.5 min at 72°C) using AmpliTaq polymerase (Perkin-Elmer). Primer sequences are as follows; AG2, 5′-CTGCTAACCATGTTCATGCC-3′; LZUS3, 5′-GCGCATCGTAACCGTGCAT-3′; bsr-2, 5′-GCAGAAATCGGAGGAAGAAG-3′; bsr-3, 5′-CAACTCCCTACACATACCAC-3′; FRT-S, 5′-GCTTCAAAAGCGCTCTGAAG-3′; DTA1, 5′-TACCACGGGACTAAACCTGG-3′; DTA2, 5′-CGCTTAACGCTTTCGCCTGT-3′.

Statistical Analyses—The recombination efficiencies and relative number of blue or white colonies were evaluated by non-repeated measures ANOVA. Where a significant difference ($p < 0.05$) was identified, the differences were analyzed further with SNK tests for multiple comparisons.

RESULTS

Experimental Design—The strategy to assess the RMCE frequencies used previously (*14*) is outlined in Fig. 1A. The chromosomal target is the CAG promoter-*lox*71-*Blasticidin S resistant* (*bsr*) gene-pA-*lox*511-FRT-*lox*2272. Exchange vectors contain *lox*66-the promoter-less *lacZ* gene fused with the nuclear localizing signal (*NLSlacZ*)-a selection marker gene-*lox*2272. ES cell lines carrying the chromosomal target are co-electroporated with the exchange vector and Cre-expression vector in their circular forms, and then selected with appropriate drug according to the selection marker gene. The *cre* gene on the expression vector is transiently expressed, and Cre protein mediates site-specific recombination between the chromosomal *lox*71 and *lox*66 on the targeting plasmid, and the chromosomal *lox*2272 and *lox*2272 on the targeting plasmid, resulting in cassette exchange of the *bsr* gene with the *NLSlacZ*-selection marker cassette. Since there is no negative selection marker on the chromosomal target construct, both random integrants and site-specific recombinants become drug resistant, but only the colonies where RMCE had occurred are stained blue with X-gal, since the *NLSLacZ* gene is inserted downstream of the CAG promoter. The percentage of blue colonies represents the frequency of RMCE. When only the exchange vector was electroporated, no blue colonies appeared (data not shown), indicating that gene trapping events hardly occur with electroporation using the exchange vectors in circular forms.

We used four ES cell lines, 71-5F2-7, 71-5F2-10, 71-5F2-23 and 71-5F2-26 carrying a single copy of the chromosomal target construct. The cell lines 71-5F2-10 and 71-5F2-23 were used in our previous study (*14*), and showed lower and higher recombination frequency, respectively. We added two cell lines, 71-5F2-7 and 71-5F2-26, which showed lower recombination frequency, to examine enrichment effects of exchange vectors on several different "inactive" positions. Since the original 71-5F2-7, 71-5F2-10 and 71-5F2-26 clones were contaminated with wild-type ES cells at a considerable percentage (data not shown), all four lines were re-cloned through limiting dilution and Blasticidin S selection. Table 1 shows the RMCE frequencies and the number of blue colonies in each re-cloned line electroporated with pCAGGS-Cre and p66-2272, which contains only the MC1-*neo*-pA cassette as a positive selection marker (Fig. 1B, top) and gave the best frequency in the previous study. Since the conditions of electroporation were optimized for Cre-mediated recombination, the frequency in 71-5F2-23 increased to 35.3% from the frequency obtained in the previous study, 26.3%. In 71-5F2-23, the number of blue colonies was 3–4 times higher than in the

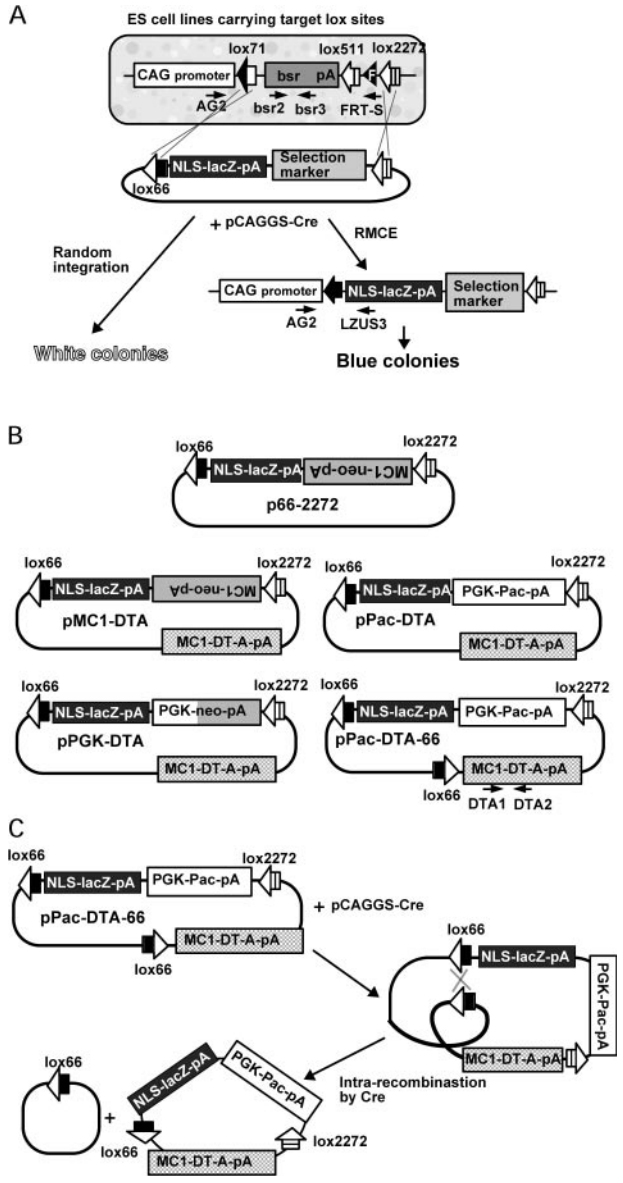

Fig. 1. **(A) Experimental strategy for comparison of frequency of RMCE.** ES cell lines carrying a single copy of the CAG-*lox*71-bsr-pA-*lox*511-FRT-*lox*2272 fragment were established. The cell lines were co-electroporated with the Cre expression vector and the targeting plasmids carrying the promoter-less *NLS-lacZ* gene. Through RMCE, the *NLSlacZ* gene is joined to the CAG promoter, resulting in positive staining with X-gal. Since the targeting plasmids contain a selection marker gene, random integrants can also appear. However, colonies with random integration are not stained because there is no promoter for the *NLSlacZ* gene. The percentage of blue colonies represents the frequency of RMCE. Positions of PCR primers used in Table 2 are shown as small arrows with the name of the primer. **(B) Targeting vectors used in this study. (C) Predicted recombination intermediate of pPac-DTA-66.** Since pPac-DTA-66 carries two *lox*66 sites, intra-molecular recombination should occur first to divide into two circular molecules. The molecule including *lox*66 and *lox*2272 becomes a substrate of RMCE.

other three clones, indicating high recombination efficiency probably due to open chromosomal configuration around the target *lox* sites. The goal of this study is to improve targeting vectors to enrich blue colonies in all cell lines, including the "lower" recombination-frequency clones.

Table 1. **Recombination frequency with p66-2272 targeting vector in clones used in this study.**

Cell line	Number of Blue colonies ± SD	Recombination Frequency (%) ± SD
71-5F2-7	121 ± 29.6	20.2 ± 5.89
71-5F2-10	92.0 ± 34.6	16.5 ± 2.67
71-5F2-23	311 ± 166	35.3 ± 7.89
71-5F2-26	67.0 ± 17.8	11.6 ± 2.44

$N = 4$ in each clone.

Exchange Vectors—Exchange vectors used in this study are shown in Fig. 1B. In pMC1-DTA, we added the MC1 promoter-*DT-A* gene-pA cassette to p66-2272 in the un-integrated region by RMCE (outside of two *lox* sites) for negative selection to reduce random integrants. The choice of selection marker gene is also important for colony formation efficiency. In pPGK-DTA, the mouse *phosphoglycerate kinase-1* (*Pgk*) promoter–*neo*-pA cassette, which shows higher colony efficiency than MC1-neo, is used, and in pPac-DTA, the *Pgk* promoter–*pac*-pA cassette, which is known to that puromycin sensitive cells are killed quite quickly and the drug selection completes within 24–48 h, is used. In addition, we constructed a pPac-DTA-66 plasmid, in which an additional *lox*66 site was placed between the DT-A cassette and plasmid vector sequence. After electroporation with Cre expression plasmid, intra-molecular recombination between *lox*66 sites should take place first, resulting in two circular molecules as shown Fig. 1C. By minimizing the size of the targeting DNA molecule, we expected to reduce random integration.

Recombination Frequencies—Percentages of blue colonies, *i.e.*, RMCE frequencies, in the 4 lines are shown in Fig. 2A. The pPac-DTA-66 plasmid (vector no. 5) gave the highest RMCE frequency in all lines with statistical significant differences. Even in the 'lower' recombination-frequency clone 71-5F2-26, the frequency exceeded 50%, and in the "high" recombination-frequency clone 71-5F2-23 line, it reached to 75%. In order to evaluate the enrichment effect, relative numbers of blue or white colonies against the number of blue colonies obtained with p66-2272 were calculated and are shown in Fig. 2B. When the DT-A cassette was added to p66-2272 (pMC1-DTA), the number of white colonies was significantly reduced to almost half (vector no. 2), indicating that negative selection of the DT-A was effective. However, since the number of blue colonies was also slightly reduced, the percentages of blue colonies did not change significantly, but were higher than in p66-2272. With the use of the PGK-neo-pA, both the number of blue and white colonies increased, but no significant changes of frequency compared to pMC1-DTA observed (vector no. 3).With the use of PGK-pac-pA, the RMCE frequency increased to 30–50% (vector no. 4). Interestingly, with pPac-DTA-66 plasmid, the numbers of blue colonies (targeted integration) were significantly increased in all lines, whereas the numbers of white colonies (random integration) were unchanged (vector no. 5). This indicates that smaller DNA molecule can access more easily to chromosomal target *lox* sites.

In order to analyze integration patterns, 71-5F2-7 and 71-5F2-10 colonies were picked from cells electroporated

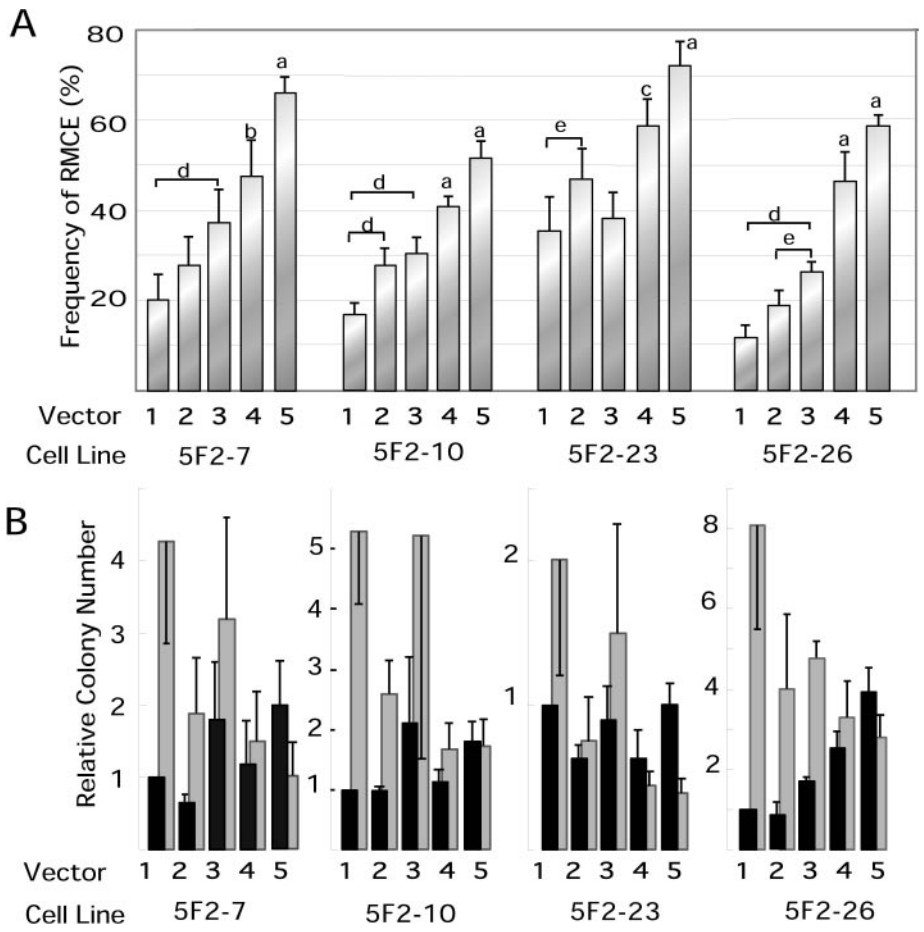

Fig. 2. **(A) Frequency of RMCE.** Twenty micrograms of each replacement plasmid and the Cre-expressing vector were co-electroporated, and after drug selection for 7 days, colonies were stained with X-gal, and the percentage of positive colonies was scored as the frequency of RMCE. Numbers under the graph indicate the targeting vector used in each electroporation. 1, p66-2272; 2, pMC1-DTA; 3, pPGK-DTA; 4, pPac-DTA, 5, pPac-DTA-66. The means ± SD of at least four independent electroporations are represented. All cell lines show significant differences at $P < 0.01$ by ANOVA analysis. Statistical significances among vectors were further analyzed by the SNK test. a, $P < 0.01$ compared to all other vectors; b, $P < 0.01$ compared to vector 1, 2 and 5 and $P < 0.05$ compared to vector 3; c, $P < 0.01$ compared to vector 1, 3 and 5 and $P < 0.05$ compared to vector 2; d, $P < 0.01$ compared to the indicated vector; e, $P < 0.05$ compared to the indicated vector. **(B) Relative Blue (solid bars) or white (gray bars) colony numbers.** The number of blue colonies obtained with p66-2272 arbitrarily set at 1.

Table 2. **Genomic DNA analysis of isolated subclones by PCR.**

Cell line	Total no. of clones analyzed	X-Gal staining (%)	PCR pimers			
			AG2/LZUS3	bsr2/bsr3	AG2/FRT-AS	DTA1/DTA2
71-5F2-7	18	Blue 8 (44)	8	0	ND	0
		White 10 (56)	0	5	5	2
71-5F2-10	23	Blue 14 (61)	14	0	ND	0
		White 9 (39)	0	6	3	0

Subclones obtained after coelectroporation with pPac-DTA-66 and pCAGGS-Cre were picked and expanded for genomic DNA preparation and PCR analysis. Part of each clone was stained with X-gal, and the number of blue or white clones is represented. PCR analyses were performed with the indicated primers (see Fig. 1), and among the each blue or white clones, the number of clones showed a band of expected size is represented. ND, not done.

with pPac-DTA-66 and pCAGGS-Cre, genomic DNAs were prepared, and PCR analysis was performed. As shown in Fig. 1A, the 5′-junction of the recombination can be amplified using the primers AG2 and LZUS3, and all blue clones gave a band of the expected size (Table 2, data of electrophoresis not shown). Then, the presence of the *bsr* gene, which should be removed through RMCE, was examined with the primers bsr-1 and bsr-2. All blue clones were negative for the *bsr* gene detecting PCR, however, only 11 clones out of 19 white clones retained the bsr gene, unexpectedly. Since it is reported that *lox*511 can be recombined with *lox*P or *lox*71 (*9*), PCR analysis with AG2 and FRT-AS primers was performed to detect recombination between *lox*71 and *lox*511. The 8 clones that did not carry the *bsr* gene exhibited a 201-bp band. We cloned the product into the T-vector and confirmed that the sequence corresponded to the recombination product *lox*71 and *lox*511. The spacer sequence of the *lox* site in the recombined product was the wild-type sequence (data not shown). The *DT-A* gene was detected in only 2 clones by PCR, indicating successful negative selection. Since the amplified region was the inside of the ORF, the promoter or pA signal might be deleted in these 2 clones.

DISCUSSION

We have shown here that the combined usage of negative selection of the *DT-A* gene, positive selection of the *Pac* gene and the third *lox* site for intra-molecular recombination efficiently enriched RMCE events. The enrichment

effect was more apparent in the 'lower' recombination-frequency clones, and we could obtain a frequency of 50% and more, which is high enough for the practical use of RMCE.

We could enrich RMCE event through two strategies, one is the use of the *DT-A* negative selection marker gene and the other is the change of positive selection marker gene. The usefulness of the *DT-A* gene which reduce random integration has been already reported in gene targeting (*21*), however, enrichment effect in gene targeting frequency by changing selection marker has not been clearly observed. In comparing two plasmids having the same promoter, pPac-DTA (Pgk-Pac) and pPGK-DTA (Pgk-neo), the number of white colonies was reduced to almost half, probably due to the difference of colony formation efficiency of the *pac* and *neo* gene. On the other hand, in comparison between pMC1-DTA (MC1-neo) and pPac-DTA (Pgk-pac), there is no statistical difference in number of white colonies or blue colonies, except of blue colonies in 71-5F2-26, nevertheless, the RMCE frequency showed statistical difference in the all lines used in this experiment. Why the use of the Pgk-*pac* cassete resulted in higher RMCE frequency? We speculate that the difference of time course in G418 and puromycin selection might be the cause of the enrichment effect. Puromycin kills almost all sensitive cells within 24hrs, whereas G418 takes 2–3 days. The quick elimination of non-transfected cells might result in reduction of spontaneously drug-resistant colonies.

In addition to reduction of random integration, we could increase recombination efficiency itself by adding the third *lox* site to induce intra-molecular recombination, which results in 33% of size reduction of targeting DNA molecule by removal of the plasmid vector sequence. Interestingly, the numbers of blue colonies with pPac-DTA-lox66 plasmid were 1.5–1.7 times higher than those with pPac-DTA plasmid, thus, the RMCE efficiencies increase approximately in inverse proportion with the size of targeting DNA molecule. This indicates that the probability of encounter of chromosomal *lox* site and targeting plasmid depends on physical mobility of DNA molecule. In the higher-recombination efficiency clone 71-5F2-23, the chromosomal configuration around *lox* sites is considered to be open, therefore, they always show high efficiency even when the molecular weight of targeting vector is relatively large. On the other hand, in the lower-recombination efficiency clone 71-5F2-26, the chromosomal configuration around *lox* sites is considered to be close, therefore, reduction of the molecular weight of targeting DNA is quite effective to increase RMCE frequency by improving accessibility to chromosomal target *lox* sites. Our results predict that RMCE using large molecular weight plasmid, *ex.* bacterial artificial chromosome, may show low frequency.

The advantage of RMCE using our strategy is the minimum requirement of the chromosomal target structure, *i.e.*, only two heterospecific *lox* sites, and wide and easy application to gene targeting or gene trapping vectors. Thus, our enrichment strategy for RMCE will be a powerful tool in genetic manipulation in ES cells.

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REFERENCES

1. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**, 99–109
2. Branda, C.S. and Dymecki, S.M. (2004) Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev. Cell* **6**, 7–28
3. Taniwaki, T., Haruna, K., Nakamura, H., Sekimoto, T., Oike, Y., Imaizumi, T., Saito, F., Muta, M., Soejima, Y., Utoh, A., Nakagata, N., Araki, M., Yamamura, K., andAraki, K. (2005) Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-β*geo* cassette. *Dev. Growth Differ.* **47**, 163–72
4. Fukushige, S. and Sauer, B. (1992) Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **89**, 7905–9
5. Albert, H., Dale, E.C., Lee, E., and Ow, D.W. (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J.* **7**, 649–59
6. Araki, K., Araki, M., and Yamamura, K. (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.* **25**, 868–72
7. Hoess, R.H., Wierzbicki, A., and Abremski, K. (1986) The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**, 2287–300
8. Lee, G. and Saito, I. (1998) Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216**, 55–65
9. Langer, S.J., Ghafoori, A.P., Byrd, M., and Leinwand, L. (2002) A genetic screen identifies novel non-compatible loxP sites. *Nucleic Acids Res.* **30**, 3067–77
10. Bouhassira, E.E., Westerman, K., and Leboulch, P. (1997) Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood* **90**, 3332–44
11. Bethke, B. and Sauer, B. (1997) Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants. *Nucleic Acids Res.* **25**, 2828–34
12. Kolb, A.F. (2001) Selection-marker-free modification of the murine beta-casein gene using a lox2272 [correction of lox2722] site. *Anal. Biochem.* **290**, 260–71
13. Osipovich, A.B., Singh, A., and Ruley, H.E. (2005) Post-entrapment genome engineering: first exon size does not affect the expression of fusion transcripts generated by gene entrapment. *Genome Res.* **15**, 428–35
14. Araki, K., Araki, M., and Yamamura, K. (2002) Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic Acids Res.* **30**, e103
15. Seibler, J., Schubeler, D., Fiering, S., Groudine, M. and,Bode, J. (1998) DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* **37**, 6229–34
16. Soukharev, S., Miller, J.L., and Sauer, B. (1999) Segmental genomic replacement in embryonic stem cells by double lox targeting. *Nucleic Acids Res.* **27**, e21
17. Shmerling, D., Danzer, C.P., Mao, X., Boisclair, J., Haffner, M., Lemaistre, M., Schuler, V., Kaeslin, E., Korn, R., Burki, K., Ledermann, B., Kinzel, B., and Muller, M. (2005) Strong and ubiquitous expression of transgenes targeted into the beta-actin locus by Cre/lox cassette replacement. *Genesis* **42**, 229–35

18. al-Shawi, R., Burke, J., Wallace, H., Jones, C., Harrison, S., Buxton, D., Maley, S., Chandley, A. andBishop, J.O. (1991) The herpes simplex virus type 1 thymidine kinase is expressed in the testes of transgenic mice under the control of a cryptic promoter. *Mol. Cell. Biol.* **11**, 4207–16
19. Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y. andAizawa, S. (1993) A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. *Anal. Biochem.* **214**, 77–86
20. Araki, K., Imaizumi, T., Okuyama, K., Oike, Y., and Yamamura, K. (1997) Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. *J. Biochem.* **122**, 977–82
21. Yanagawa, Y., Kobayashi, T., Ohnishi, M., Tamura, S., Tsuzuki, T., Sanbo, M., Yagi, T., Tashiro, F., and Miyazaki, J. (1999) Enrichment and efficient screening of ES cells containing a targeted mutation: the use of DT-A gene with the polyadenylation signal as a negative selection maker. *Transgenic Res.* **8**, 215–21