

TECHNOLOGY REPORT

Efficient FLP Recombination in Mouse ES Cells and Oocytes

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Summary: We report an improved vector, pCAGGS-FLPe, for transient expression of the enhanced FLP recombinase in mouse ES cells and oocytes. In standard transfection experiments, about 6% of total ES colonies showed FLP recombination, albeit with mosaicism within each colony. After microinjection of pCAGGS-FLPe into oocytes, about one-third of heterozygotic mice born showed complete FLP recombination. Thus pCAGGS-FLPe presents two practical options for removal of FRT cassettes in mice. *genesis* 31:6–10, 2001. © 2001 Wiley-Liss, Inc.

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Site-specific recombination adds a unique dimension to genetic engineering. The two most widely used site-specific recombinases (SSRs) in genetic engineering are FLP recombinase from the yeast 2-micron episome and Cre recombinase from the coliphage P1. Both are tyrosine recombinases and share similar, but not identical, reaction mechanisms (Van Duyne, 2001; Chen *et al.*, 2000; Tribble *et al.*, 2000; Ringrose *et al.*, 1998). It is not yet clear how their differences influence efficiencies in genetic engineering applications, however, both enzymes have been successfully applied in flies, plants, and somatic cells in culture. For example, the first application of site-specific recombination in genetic engineering of higher eukaryotes involved FLP recombinase in flies (Golic and Lindquist, 1988; Golic, 1991), whereas success in mammalian cells and mice was first achieved with Cre (Sauer and Henderson, 1989; Lakso *et al.*, 1992).

A comparative biochemical analysis of Cre and FLP revealed that they have temperature optima concordant with their original hosts. Cre enzyme activity has an *in vitro* temperature optimum of 37–39°C, whereas FLP's is 25–30°C and activity is significantly reduced at 37°C (Buchholz *et al.*, 1996). The thermostability of FLP with respect to Cre correlates to enhanced sensitivity of FLP to thermal denaturation of the folded protein (Buchholz *et al.*, 1998). In accordance with this, many applications

of Cre in mice have been successful, however, only two with FLP succeeded with apparent moderate efficiencies (Dymecki, 1996; Vooijs *et al.*, 1998). Hence the preference for use of Cre in mice has emerged.

Although Cre is a remarkable aid for genetic engineering in the mouse, a variety of genetic engineering exercises rely upon, or could benefit from, the use of two SSRs. Most prominently, conditional strategies to engineer the mouse genome rely upon the good properties of Cre in mice, however, the removal of the selectable marker used to generate the conditional allele is best accomplished by use of a second SSR. Given two useful SSRs, other applications of two recombination events in engineering strategies can also be developed (Meyers *et al.*, 1998). To acquire a second recombinase with good properties in the mouse, we previously used molecular evolution to improve the thermostability of FLP recombinase and derived FLPe (Buchholz *et al.*, 1998). To validate the improved performance of FLPe at 37°C in that work, we subcloned FLP and FLPe into several expression vectors and tested their ability for recombination in ES cells. Whereas the FLPe vectors were consistently 4- to 10-fold better than their FLP counterparts, the best FLPe vector tested, pOG-FLPe, showed recombination in only 0.5–1% of total ES colonies. Subsequently, we have subcloned FLPe into a variety of different expression vectors in search of improved performance in ES cells. Here we report the best outcome. Expression of FLPe from a pCAGGS (Araki *et al.*, 1997) vector derivative, termed pCAGGS-FLPe (Fig. 1), delivers a 5- to 10-fold improvement over pOG-FLPe in the ES cell assay. Additionally, we report that pCAGGS-FLPe also works efficiently when microinjected into oocytes harbouring an FRT-flanked cassette. Both applications advance the utility of FLP recombination for

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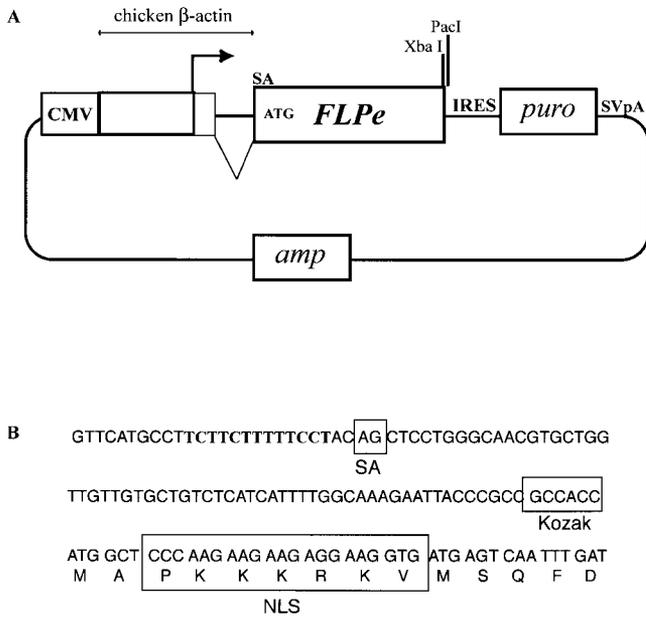


FIG. 1. Details of pCAGGS-FLPe. **(A)** Schematic presentation of the pCAGGS-FLPe plasmid. FLP-e is expressed from the chicken β -actin promoter (arrow) with the CMV-IE enhancer upstream. The chicken β -actin sequences include the first intron and start of the second exon. FLPe was positioned in the second exon. Included downstream are the EMCV IRES fused to the puromycin resistance gene, followed by the SV40 polyadenylation signal. **(B)** Sequence at the 5' end of the FLP-e coding region including the splice acceptor (SA), a Kozak sequence, the nuclear localisation signal from SV40 large-T antigen (NLS), and the first 5 amino acids of FLP-e recombinase, including the FLP-e-specific replacement of proline at amino acid 2 with serine.

engineering the mouse genome and provide complementary alternatives to the recently described FLPe deleter mice (Rodriguez *et al.*, 2000; Farley *et al.*, 2000).

In addition to the FLPe coding region, pCAGGS-FLPe was also constructed to (a) improve the Kozak consensus sequence upstream of FLPe and (b) fuse a large-T consensus nuclear localisation signal (nls) to the N-terminus of FLPe. Furthermore, the pCAGGS vector used contained an IRES puromycin resistance gene cassette (Fig. 1).

Using the same ES cell assay used previously to evaluate FLP recombination by induction of lacZ expression (Buchholz *et al.*, 1998), approximately $6\% \pm 0.5\%$ (4 experiments) of total colonies plated after electroporation with pCAGGS-FLPe showed FLP recombination (Fig. 2). As in our previous experiments with pOG-FLP and pOG-FLPe, we again observed mosaicism in the ES cell colonies with only rare colonies showing complete FLP recombination. In anticipation of this problem, we used a pCAGGS derivative that included an IRES-puromycin resistance gene cassette so that the transient puromycin selection technique to enhance Cre recombinase efficiencies (Taniguchi *et al.*, 1998) could be explored. However, in tests with a different allele in which a 6.2-kb FRT cassette was excised by recombination, we were

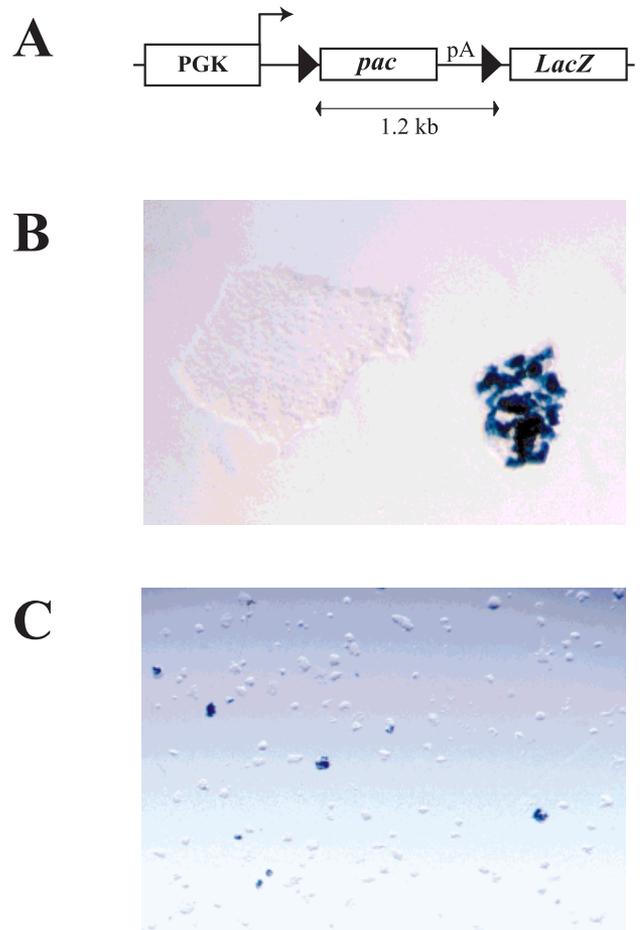


FIG. 2. FLPe deletion in ES cells. **(A)** Schematic presentation of the pPGKpaZ11 recombinase reporter (Buchholz *et al.*, 1996). The puromycin resistance gene (*pac*) and SV40 late polyadenylation signal (pA) lie between two FRT sites (depicted as solid triangles), followed by the β -galactosidase gene (*LacZ*). Expression is driven by the phosphoglyceratekinase (PGK) promoter. Before recombination, the cells are resistant to puromycin selection and do not express lacZ. Upon Cre recombination, the 1.2-kb *puro* pA fragment is deleted so that the cell will now express lacZ. Thus the recombination event can be monitored in situ by X-Gal staining. **(B)** X-Gal-stained single colonies 5 days after electroporation with pCAGGS-FLP-e. Approximately 70% of the cells in the positive colony have undergone recombination.

unable to identify conditions under which transient puromycin selection enhanced relative FLP recombination efficiencies. Rather we found that recombination efficiencies and mosaicism remained at about 4% across a broad range of diminishing numbers of surviving cells (results not shown). In contrast to Taniguchi *et al.*, we consistently achieve 10% or greater Cre recombination in equivalent experiments with Cre expression plasmids without transient puromycin selection, with little mosaicism. Hence we find that the transient puromycin selection procedure is of no benefit for enhancement of either Cre or FLP recombination products.

Nevertheless, pCAGGS-FLPe is the best option to date for FLP recombination in ES cells in culture. Since mo-

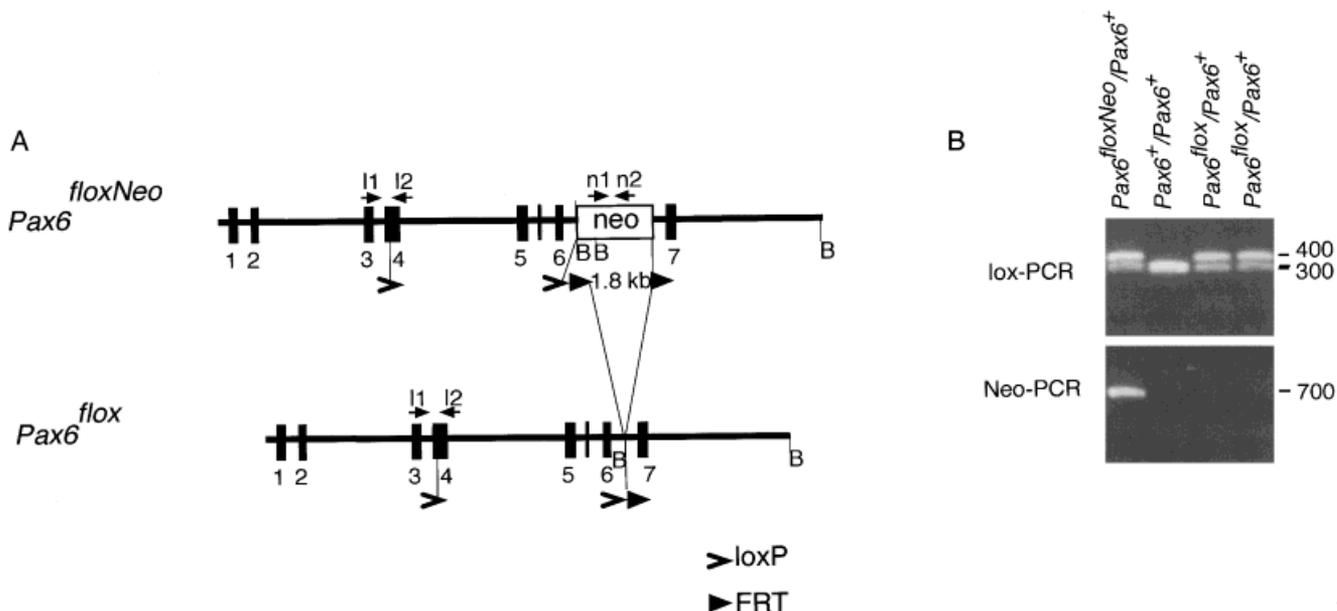


FIG. 3. FLPe deletion in oocytes. **(A)** Map of the $Pax6^{floxNeo}$ (before FLPe recombination) and $Pax6^{flox}$ (after FLPe recombination) alleles. The closed boxes mark $Pax6$ exons. The PGK-Neo cassette (Neo) flanked with FRT sequences (Meyers *et al.*, 1998) lies in intron between exon 6 and 7. Following recombination, the selection cassette was deleted, giving rise to the $Pax6^{flox}$ allele. Positions of PCR primers (arrows) to identify the loxP insertion (l1, l2), the selection cassette (n1, n2), and the BamHI (B) restriction sites employed to verify the recombination by Southern are shown. **(B)** PCR analyses to identify the alleles present in four mice born after injection of pCAGGS-FLPe into candidate oocytes. Upper panel: using oligos l1 and l2 that flank the upstream loxP site, the loxP insertion and wild-type alleles are distinguished (lox-PCR., 400 bp; wild type 300 bp). Lower panel: using oligos n1 and n2, the presence of the neomycin cassette is identified by PCR as a 700-bp band (Neo-PCR).

saicism is likely, we recommend that either (a) cells be passaged after an initial culture period of 2 to 3 days and replated at low density to obtain completely recombined colonies (see Protocol 1; Dymecki, 2000) or (b) screening strategies for FLP recombination in unpassaged primary colonies should be designed to evaluate colonies for the ratio of recombined to unrecombined cells. Thereby colonies with high ratios can be chosen for introduction into mice to increase the likelihood that complete, FLP recombined, F1 progeny can be acquired.

Previously, microinjection of a pCAGGS-Cre plasmid into oocytes has been shown to deliver good efficiencies of Cre recombination (Sunaga *et al.*, 1997). We explored this way for deletion of FRT-flanked cassettes by microinjection of pCAGGS-FLPe into oocytes. Fertilized eggs were harvested from mating between $Pax6^{floxNeo}/Pax6^+$ males (Ashery-Padan *et al.*, 2000) and wild-type FVB females. The $Pax6^{floxNeo}$ allele includes a 1.8-kb neomycin cassette flanked by FRT sites. FLP recombination was assessed by PCR analysis (Fig. 3) and confirmed by Southern blotting (data not shown). Germ-line transmission of the FLP recombined $Pax6^{flox}$ allele was tested in the offsprings of three $Pax6^{flox}$ mice. In all of the tested $Pax6^{flox}/Pax6^+$ progeny, the selection cassette could not be detected by PCR analysis (Table 1). Furthermore, pCAGGS-FLPe sequences could not be detected by PCR, indicating that the plasmid had not integrated into the genome. Hence, as with pCAGGS-Cre (Sunaga *et al.*, 1997), pCAGGS-FLPe can induce efficient recombination

Table 1
Summary of Oocyte Microinjection Experiments
with pCAGGS-FLPe

Experiments	1	2	3	Total
Total mice born following oocyte injection of pCAGGS-FLPe	4	14	21	39
Total mice born with targeted $Pax6$ allele	2	8	9	19
$Pax6^{floxNeo}/Pax6^-$ (not FLPe recombined)	1	7	4	12
$Pax6^{flox}/Pax6^+$ (FLPe recombined)	1	1	5	7
% FLPe recombined	50%	12%	55%	37%

in zygotes, allowing efficient deletion of the selection cassette from all cells without integration of foreign sequences into the genome.

Here we have developed new options for removal of FRT-flanked sequences in the mouse genome, either in ES cells in culture or by microinjection of oocytes. The efficiencies of either approach permits predictable success and provide complementary paths for FLP recombination in mice in addition to that recently described using FLPe deleter mice (Rodriguez *et al.*, 2000; Farley *et al.*, 2000). The advantage of the approaches described here is that the genetic background is not changed despite the genomic manipulation.

The work again demonstrates that FLPe can play the sought-after role as a second SSR for use in mice. However, we also garnered an indication that FLPe is, operationally, less efficient than Cre. At this stage, we recom-

mend that SSR strategies in mice should pivot on the use of Cre with FLPe employed for secondary tasks, such as removal of operational sequences in the germ line. However, Cre and FLP differ in several mechanistic ways and the question of whether Cre is indeed more efficient than FLPe in mice remains to be settled by further experiments with FLPe. For example, the stability of their synaptic complexes differs by more than 100-fold, with Cre being more stable (Ringrose *et al.*, 1998). In vitro, using excision recombination substrates, this difference is reflected as a faster rate of Cre recombination to equilibrium (i.e., 50%, excised), and thereafter recombination does not proceed beyond 50% (Ringrose *et al.*, 1998). This probably reflects the stability of the Cre synaptic complex to hold all components and flick between forward (excision products) and backward (substrate) states. In contrast, FLP proceeds toward 100% excision in vitro (Ringrose *et al.*, 1998), which reflects the fact that excision is favored if the synaptic complex disassembles after recombination (Logie and Stewart, 1995). How these and other differences between Cre and FLP influence performance in mice and whether new SSRs will offer efficient alternatives (Thorpe and Smith, 1998; Thyagarajan *et al.*, 2001) are amongst several exciting issues that will be addressed in the near future.

MATERIALS AND METHODS

The eZ1 derivative of R1 ES cells, which contains a stably integrated copy of pPGKpaZ11, has been described (Kellendonk *et al.*, 1996). The cells were cultured, electroporated, and stained with X-Gal as described (Kellendonk *et al.*, 1996), except that 10 μ g of supercoiled pCAGGS-FLPe plasmid were used for a single electroporation of 5×10^6 cells in 500 μ l. Thereafter, cells were diluted 1,000-fold and aliquots plated.

For microinjection experiments, 10 ng/ μ l circular pCAAGS-FLPe plasmid, diluted in 1 mM Tris-HCl, pH 8, 0.1 mM EDTA, were microinjected into oocytes harvested from mating between Pax6^{loxNco}/Pax6⁺ males (Ashery-Padan *et al.*, 2000) and wild-type FVB females.

In (37%) of the Pax6^{loxNco}/Pax6⁺ progeny, the neomycin selection cassette was not detected by PCR analysis. Primers for identifying the loxP insertion were 11-5'-GCG GTT GAG TAG CTC AAT TCT A; 12-5'-AGT GGC TTG GAC TCC TCA AGA. Primers for identifying the neomycin gene were n1-5'-CCG GCC GCT TGG GTG GAG n2-5'-CGG TCC GCC ACA CCC AGC. The recombined allele was termed Pax6^{lox} and was confirmed by Southern blot analysis performed on genomic DNA extracted from the Pax6^{lox}/Pax6⁺ mice digested with BamHI and probed with probe B. Germ-line transmission of the Pax6^{lox} allele was tested in the next generation. In all of the tested Pax6^{lox}/Pax6⁺ progeny, the selection cassette could not be detected by PCR analysis. Furthermore, the pCAAGS-FLPe sequences could not be detected by PCR, indicating that the plasmid did not integrate to the genome.

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