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A simple analysis system for the estimation of recombination efficiency using fluorescence-activated cell sorting

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Abstract

A simple and accurate analysis system for the estimation of recombination efficiency *in vivo* using fluorescence-activated cell sorting (FACS) was designed and was subsequently used to compare the efficiency of recombination related to different spacer mutants. F_3 and F_5 mutant sequences were used for Flpe-mediated cassette exchange, and *m2* and *lox*2272 mutant sequences were used for Cre-mediated cassette exchange due to their high incompatibility with wild-type sequences. The incompatibilities with wild-type were almost the same between mutant sequences. However, the recombination efficiencies were different. F_3 and *m2* could mediate more efficient recombination than F_5 and *lox*2272, respectively. These results are consistent with the fact that the sequence of spacer region affects not only the reactivity upon wild-type sequence but also the recombination efficiency. It was also confirmed that the recombination process was mediated in a site-specific manner through PCR analysis using different sizes of exchange cassettes. In this experiment, the feasibility of the FACS analysis system for the estimation of recombination efficiency was verified. This system should be readily applicable for estimating recombination efficiency of other various mutant candidates, which will contribute to more precise and efficient site-specific recombination.

Keywords: Site-specific recombinase; Recombinase-mediated cassette exchange; Incompatibility; Recombination efficiency; FACS

1. Introduction

The random integration of foreign genes into a chromosome during typical transfection leads to unpredictable gene expression. Actually, most transgenes are repressed in the chromosome in a position-dependent manner (Festenstein et al., 1996). The importance of the chromosomal site of integration is more prominent during long term culture. Transgene expression can be silenced if the gene is integrated near the heterochromatic part of the chromosome (Bell and Felsenfeld, 1999).

To circumvent this position effect, the use of site-specific recombinases has been applied. Cre from bacteriophage P1 (Hoess et al., 1982), Flp from *Saccharomyces cerevisiae* (McLeod et al., 1986), and

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its thermostable variant, Flpe (Buchholz et al., 1998) are widely used recombinases. Targeted integration of transgenes into predefined chromosomal loci by these site-specific recombinases can lead to more predictable and elevated transgene expression. For example, a hot-spot on a chromosome, which allows for high-level expression from a single or only a few gene copies, can be targeted reproducibly for high-level expression of any transgene by site-specific recombination. With this approach, the tedious and time-consuming cell line development process, which occurs with traditional random integration, can be improved.

Among various recombination strategies, it is believed that recombinase-mediated cassette exchange (RMCE) is one of the most promising strategies for the targeted integration of transgenes. RMCE guarantees effective recombination and excludes unnecessary vector sequences of prokaryotic origin which can inhibit transgene expression by methylation-dependent inactivation (Baer and Bode, 2001).

For stable integration of a transgene by the RMCE strategy, mutant recognition sequences, which are incompatible with wild-type sequence, are indispensable, as in general recombination strategies. For the past decade, much work concerning mutant sequence has been performed. The induction of mutation in inverted repeat regions of a wild-type recognition sequence increased the recombination efficiency by decreasing a reverse reaction or an excision (Albert et al., 1995). However, the binding affinity of DNA against recombinase can be impaired in this system. Knowledge concerning the role of nucleotide sequences within the *FRT* spacer region and the *lox*P spacer region led to the use of a mutant with a mutation in its spacer region (Umlauf and Cox, 1988; Lee and Jayaram, 1995; Lee and Saito, 1998). This promoted the recombination process by decreased reactivity upon wild-type recognition sequence and intact binding affinity against recombinase. Among various mutant sequences, it seems that *lox*2272 (Lee and Saito, 1998) and *m2* (Langer et al., 2002) in the Cre/*lox*P system, F_3 and F_5 (Schlake and Bode, 1994) in the Flpe/*FRT* system have the lowest reactivity upon a wild-type sequence (Branda and Dymecki, 2004).

Despite much work for the identification of novel mutants, studies on the efficiency of recombination related to these mutants *in vivo* were insufficient. Therefore, it will be meaningful to investigate whether these mutant sequences can contribute to effective site-specific recombination and to compare the efficiency of the recombination related to different mutants. A similar approach was performed by Kolot et al. on the coliphage HK022 integrase system (Kolot et al., 2003). However, we have focused on the recombination events mediated by the RMCE strategy, which is one of the most powerful tools for gene targeting, and applied the most widely used recombinases (Cre and Flpe). Here, we present a simple and accurate analysis system for the estimation of recombination efficiency *in vivo* using FACS.

2. Materials and methods

2.1. Plasmid construction

2.1.1. pbleo family vectors

pIRESbleo-MCS was obtained by slight modification of pIRESbleo (BD Biosciences, Franklin Lakes, NJ). EGFP coding region was obtained from pIRES2-EGFP (BD Biosciences) by PCR amplification. The primers used for this PCR reaction were 5′-GAATTCATGGTGAGCAAGGGCGAGGA-3′ and 5′-GAATTCGATCTAGAGTCGCGGCCGCT-3′. The EGFP coding region was cloned into the *Eco*RI site of the pIRESbleo-MCS vector, resulting in pbleo-EGFP. The EGFP gene can then be expressed by the CMV promoter which pre-exists in pIRESbleo-MCS.

pbleo-EGFP-*lox*P and pbleo-EGFP-*FRT* were prepared by inserting synthesized *lox*P and *FRT* fragments between *Not*I and *Sac*II sites of pbleo-EGFP. Subsequent ligation of *Hin*dIII–*Bam*HI fragments of various mutants and wild-type sequences resulted in pbleo-*lox*P-EGFP-*lox*P, pbleo-*m2*-EGFP-*lox*P, pbleo-*lox*2272-EGFP-*lox*P, pbleo-*FRT*-EGFP-*FRT*, pbleo-F_3-EGFP-*FRT*, and pbleo-F_5-EGFP-*FRT*, respectively. These re-iterated constructs were referred to as pbleo family vectors (Fig. 1), which were used to investigate the incompatibilities of various spacer mutants. Incompatibility is defined as the ability of a recognition sequence to discriminate against recombination with another recognition sequence (Langer et al., 2002). A high level of incompatibility with wild-type sequence is an important factor to consider when identifying mutants for efficient recombination. Each vector has an EGFP gene for monitoring the level of green