J. Biochem. 127, 367–372 (2000)

Synthesis of a New Cre Recombinase Gene Based on Optimal Codon Usage for Mammalian Systems[1]

Yukie Koresawa,* Shuji Miyagawa,*,2 Masahito Ikawa,† Katsuyoshi Matsunami,* Mako Yamada,* Ryota Shirakura,* and Masaru Okabe†

**Division of Organ Transplantation, Biomedical Research Center, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; and †Genome Information Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871*

Received November 5, 1999; accepted December 2, 1999

The origin of the Cre recombinase gene is bacteriophage P1, and thus the codon usages are different from in mammals. In order to adapt this codon usage for mammals, we synthesized a "mammalian Cre recombinase gene" and examined its expression in Chinese hamster ovarian tumor (CHO) cells. Significant increases in protein production as well as mRNA levels were observed. When the recombination efficiency was compared using CHO cell transfectants having a cDNA containing *loxP* sites, the "mammalian Cre recombinase gene" recombined the *loxP* sites much more efficiently than the wild-type Cre recombinase gene.

Key words: codon usage, Cre recombinase, *loxP*.

Organ-specific gene targeting has great potential in the field of transplantation, especially in the area of clinical xenotransplantation (*1*). For example, as a strategy for overcoming -Galα(1,3)Gal (*2*), which plays a major role in the antigenicity on swine to human xenotransplantation, the knockout (KO) of α1,3 galactosyltransferase (α1,3GT) (*3*) has been of considerable interest. α1,3GT KO mice have been reported, but these mice develop cataracts at 4–6 weeks of age (*4*, *5*). In addition, the KO of this gene might well have other harmful effects on swine. Therefore, a system which can provide partial KO of this gene in swine would be highly desirable and useful (*6*).

The bacteriophage P1 site–specific recombination system (*7*, *8*), which consists of two components, a *loxP* site, at which recombination occurs, and a recombinase protein, Cre (*9*), has been commonly employed in a number of studies on a conditional homologous recombination system. However, the efficiency of recombination *via* the Cre-*loxP* system leaves considerable room for improvement. The insufficient expression of wild-type Cre proteins limits the efficacy of recombination of the *loxP* sites, making it difficult to ultimately develop tools for organ- or cell-specific expression (*10–12*).

In this study, in order to determine the efficiency of recombination, we used a strategy involving the activation of silent enhanced-green fluorescent protein (EGFP) (*13–15*) by Cre recombinase.

MATERIALS AND METHODS

Cell Cultures—Chinese hamster ovarian tumor (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Ham's F12 medium, which also contained 10% fetal bovine serum, L-glutamine and kanamycin/amphotericin. Cultures were maintained under a 5% CO_2/95% air atmosphere at 37°C.

Construction of the Mammalian Cre Recombinase Gene—The strategy used for construction of the completely modified Cre recombinase gene was based on the overlap extension PCR method involving long oligonucleotides as the starting materials (*16*). The codon usage for expression in mammals was adopted from a previous report (*17*). The actual codons used here for each amino acid are summarized in Table I. The sequences of the synthetic Cre recombinase gene (sCre) were verified with an ABI 310 autosequencer (Perkin-Elmer). The cDNA of the wild-type Cre recombinase gene (wtCre) was a gift from Dr. Rajewsky (University of Cologne, Germany) (*18*).

The cDNA of the wild-type and synthetic Cre recombinases with a Kozak sequence and a nuclear localization signal was subcloned into the pCXN site (*19*); a β-actin promoter and a cytomegalovirus enhancer, and the pMC1 site; a thymidine kinase (TK) promoter and a polyoma enhancer.

Expression of cDNAs—The cDNAs of pCXN-Cre (50 μg) were introduced into CHO cells by electroporation under the following conditions: 25 microfarads (μF), 200 Ω, and 2.0 kV. Transfected CHO cells were maintained in Ham's F12 complete medium up to day 5. The expression of plasmids was confirmed by Western blotting and Northern blotting, as described below.

Western Blotting—Cell lysates of CHO cell transfectants and naive cells were quantified by the BCA method

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and the Program for the Promotion of Basic Research Activities for Innovative Biosciences.

[2] To whom correspondence should be addressed. Tel: +81-6-6879-3062, Fax: +81-6-6879-3069, E-mail: miyagawa@orgtrp.med.osaka-u.ac.jp

Abbreviations: CHO, Chinese hamster ovarian tumor; KO, knockout; α1,3GT, α1,3 galactosyl transferase; EGFP, enhanced-green fluorescent protein; sCre, synthetic Cre recombinase gene; wtCre, wild-type Cre recombinase gene.

Fig. 1. **Characteristics of the synthetic Cre recombinase gene.** Nucleotide and deduced amino acid sequences of sCre induding a Kozak sequence (-----) and a nuclear localization signal (—). Differences between wtCre used in this study and sCre are indicated above the nucleotide sequence of the latter.

(Pierce), and 20 μg aliquots of the obtained proteins were subjected to 10% SDS/PAGE unde reducing conditions by the method of Laemmli (*20*), and then transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in 5% skim milk in tris-buffered saline/0.05% Tween 20 (TBST) for 1 h at 25°C and then incubated in 1% bovine serum albumin (BSA)/0.5% skim milk/TBST with rabbit anti–mouse Cre polyclonal antibodies (Novagen) for 1 h at 25°C. After washing, the blots were incubated with horseradish peroxidase–conjugated secondary antibodies and the signal was developed using an ECL detection system (Amersham).

Northern Blotting—Total RNA was isolated from transfectants and naive cells with TORIZOL (GIBCO BRL), and then separated by electrophoresis (25 μg /lane). The probe used for hybridization was the PCR product that was generated from a portion of the poly A tail (167 bp) of pCXN, using the primers; 5′-ACTCCTCAGGTGCAGGCTGC-3′ and 5′-GCAATGAAAATAAATTTCCTTTATTAG-3′, since this probe should hybridize equally well with both mRNAs, and then labeled with the ECL detection system (Amersham). Hybridization signals were evaluated with FAST SCAN (Molecular Dynamics).

Construction of pCXN-YK1—A plasmid, pCXN-YK1, was constructed from pCXN for the expression of site-specific recombination. First, the cDNAs of EGFP and CD59 (*21*, *22*) were inserted into the cloning sites of pCXN and pCX, respectively. Next, pCX-LCL was constructed from pCX-CD59. The *loxP* site and the *loxP* site with the *Eag*I site were inserted into the *Eag*I and *Hin*dIII sites of pCX-CD59, respectively, using linkers. Finally, the *Eag*I fragment of established pCX-LCL was ligated into the *Eag*I site of pCXN-EGFP to give pCXN-YK1.

pCXN-YK1 was introduced into CHO cells by electroporation, and then the transfected CHO cells were maintained in the complete medium for several days, and then transferred to the complete medium containing 1.0 mg/ml

TABLE I. **Codon usage in each gene of Cre (actual number of codons).**[a]

Codon	AA	wt	s	Codon	AA	wt	s	Codon	AA	wt	s	Codon	AA	wt	s
TTT	Phe	5	—	TCT	Ser	5	—	TAT	Tyr	4	—	TGT	Cys	—	—
TTC	Phe	3	8	TCC	Ser	4	—	TAC	Tyr	3	7	TGC	Cys	4	4
TTA	Leu	4	—	TCA	Ser	5	—	TAA	Stop	—	—	TGA	Stop	—	—
TTG	Leu	4	—	TCG	Ser	1	—	TAG	Stop	1	1	TGG	Trp	7	7
CTT	Leu	5	—	CCT	Pro	2	—	CAT	His	5	—	CGT	Arg	8	—
CTC	Leu	1	—	CCC	Pro	1	9	CAC	His	2	7	CGC	Arg	9	34
CTA	Leu	4	—	CCA	Pro	3	—	CAA	Gln	3	—	CGA	Arg	5	—
CTG	Leu	18	36	CCG	Pro	3	—	CAG	Gln	9	12	CGG*	Arg	6	1
ATT	Ile	7	—	ACT	Thr	6	—	ATT	Asn	10	—	AGT	Ser	4	—
ATC	Ile	7	17	ACC	Thr	6	16	AAC	Asn	8	18	AGC	Ser	5	24
ATA	Ile	3	—	ACA	Thr	1	—	AAA	Lys	9	—	AGA	Arg	4	—
ATG	Met	12	12	ACG	Thr	3	—	AAG	Lys	4	13	AGG	Arg	3	—
GTT	Val	11	—	GCT	Ala	7	—	GAT	Asp	17	—	GGT	Gly	8	—
GTC	Val	6	—	GCC	Ala	10	34	GAC	Asp	5	22	GGC	Gly	5	22
GTA	Val	6	—	GCA	Ala	11	—	GAA	Glu	11	—	GGA	Gly	3	—
GTG	Val	1	24	GCG	Ala	6	—	GAG	Glu	5	16	GGG	Gly	6	—

[a]The actual codons used for each amino acid in sCre were the most prevalent ones in mammals, according to a previous report. In particular, a CGG-codon was used for Arg in one place of sCre for the sake of convenience in the construction. A.A., amino acid; wt, wtCre; s, sCre.

G418 (GIBCO/BRL) for selection (*23*). A stable CHO cell clone with pCXN-YK1 was then established. The expression of plasmids was confirmed by flow cytometry as described below.

Detection of Cre Recombinase Activity—Closed circular pCXN or pMC1-Cre was transfected by electroporation into the CHO cell line which contained pCXN-YK1, and the rate of switching from CD59 to EGFP was determined by flow cytometry.

Flow Cytometry—CHO cell transfectants with pCXN-YK1 were treated with anti-CD59 mAb (5H8; gift from Dr. M. Tomita, Showa University, Tokyo) at 4°C for 1 h, washed, and then incubated with 0.25 μg of phycoerythrin (PE)-conjugated anti–mouse IgG (Southern Biotechnology Associates) as a second antibody for 1 h at 4°C. The stained cells were determined with a FACS Calibur flow cytometer (Becton Dickinson). The green fluorescence of the CHO cell line was also determined using a FACS Calibur.

Statistics—Data are presented as means ± SEM. The Student's *t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Construction of the Synthetic Cre Recombinase Gene—The coding region of sCre with an extremely high G+C content was divided into five segments to allow the synthesis of the gene using oligonucleotides which could be easily constructed (Fig. 1). The actual codons used here for each amino acid were the most prevalent ones in mammals. In particular, a CGG-codon was used for Arg in one place for the sake of convenience in the construction. Overall 64% of the codons of the coding sequence were replaced by more suitable codons (Table I).

Western Blot and Northern Blot Analysis—pCXN-wtCre and pCXN-sCre were transfected into CHO cells. Screening of the transfected CHO cells was performed by determining the protein and mRNA levels in cell extracts from day 1 to day 5. The measurements indicated that pCXN-sCre provided the CHO cells with 3–10 times higher Cre protein levels than pCXN-wtCre did. Next, Cre mRNA accumulation in CHO cells containing pCXN-wtCre or pCXN-sCre was detected by Northern blot analysis. Twenty-five micrograms of total RNA was loaded per lane. The data showed that the increase in the Cre protein levels, after transfection, was partially matched by a concomitant increase in the mRNA levels (Fig. 2).

Pattern of the Recombination Product—A closed circular plasmid containing wtCre or sCre was introduced into stable CHO cell transfectants with pCXN-YK1, by electroporation (clone 29 and clone 30, which contained one copy of pCXN-YK1; data not shown). The transient expression of the Cre recombinase gene lead to excision of the sequence located between two *loxP* sites. That is, as indicated in Fig. 3A, when Cre-mediated recombination between the *loxP* sites occurred in CHO cells with pCXN-YK1, YK1 containing a single *loxP* site and a circular DNA containing a single *loxP* site and cDNA of CD59 were generated. Consequently, EGFP is then ligated into a CAG promoter. Figure 3C shows the standard pattern on flow cytometric analysis of CD59 and EGFP in clone 29 cells before and after transfection of the pCXN-Cre gene.

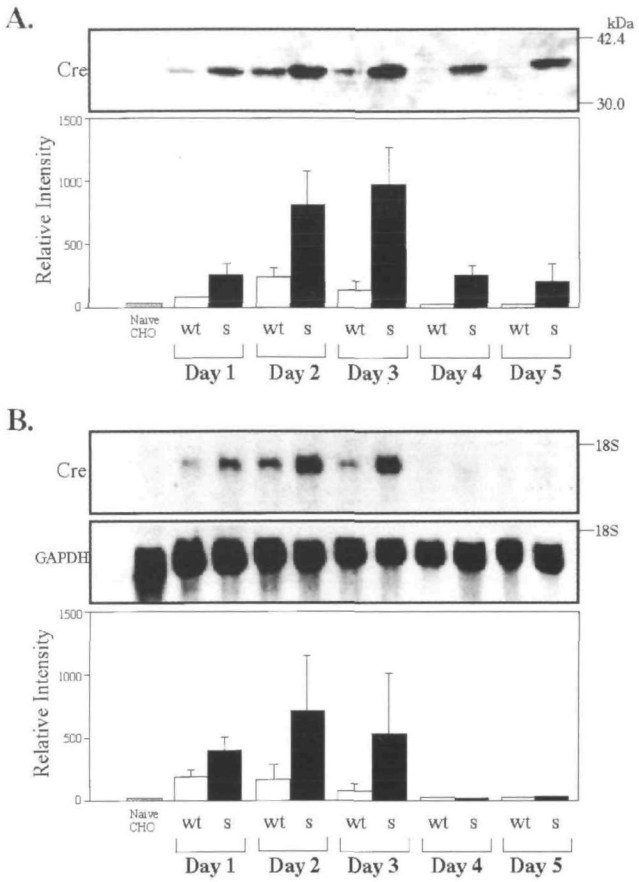

Fig. 2. **Expression of the Cre protein and mRNA in CHO cells temporarily transfected with the wild-type or synthetic Cre recombinase gene.** (A) A typical Western blot of the Cre protein in CHO cell lysates on days 1–5 after transfection. The results of quantitative analysis of blotted bands obtained in three independent experiments carried out with a FAST SCAN are shown in the lower panel, using arbitrary units. (B) Northern blot analysis of the Cre mRNA in CHO cells on days 1–5 after transfection. The results of quantitative analysis of blotted bands obtained in three independent experiments are shown in the lower panel. Each value is expressed as the mean ± SEM. [▨: naive CHO, □: wtCre (wt), ■: sCre (s)].

The Cre Recombinase Gene Transient Expression System—The efficiency of the intramolecular recombination of the two *loxP* sites was measured by flow cytometric analysis for five consecutive days, the results being summarized in Fig. 4. In the present experiment, the recombination increased significantly for the first 3 days and then reached a plateau. The maximum recombination frequency was not dependent on the promoter carrying the Cre recombinase gene, provided a sufficient amount of cDNA was transfected. However, it was demonstrated that *loxP* sites are much more efficiently recombined by sCre when transfected at a lower dose, especially in the case of the pMC1 promoter. For example, the recombination frequency with 20 μg of the transfected pMC1-sCre was nearly the same as that with 50 μg of pMC1-wtCre in clone 29, and much higher than in clone 30.

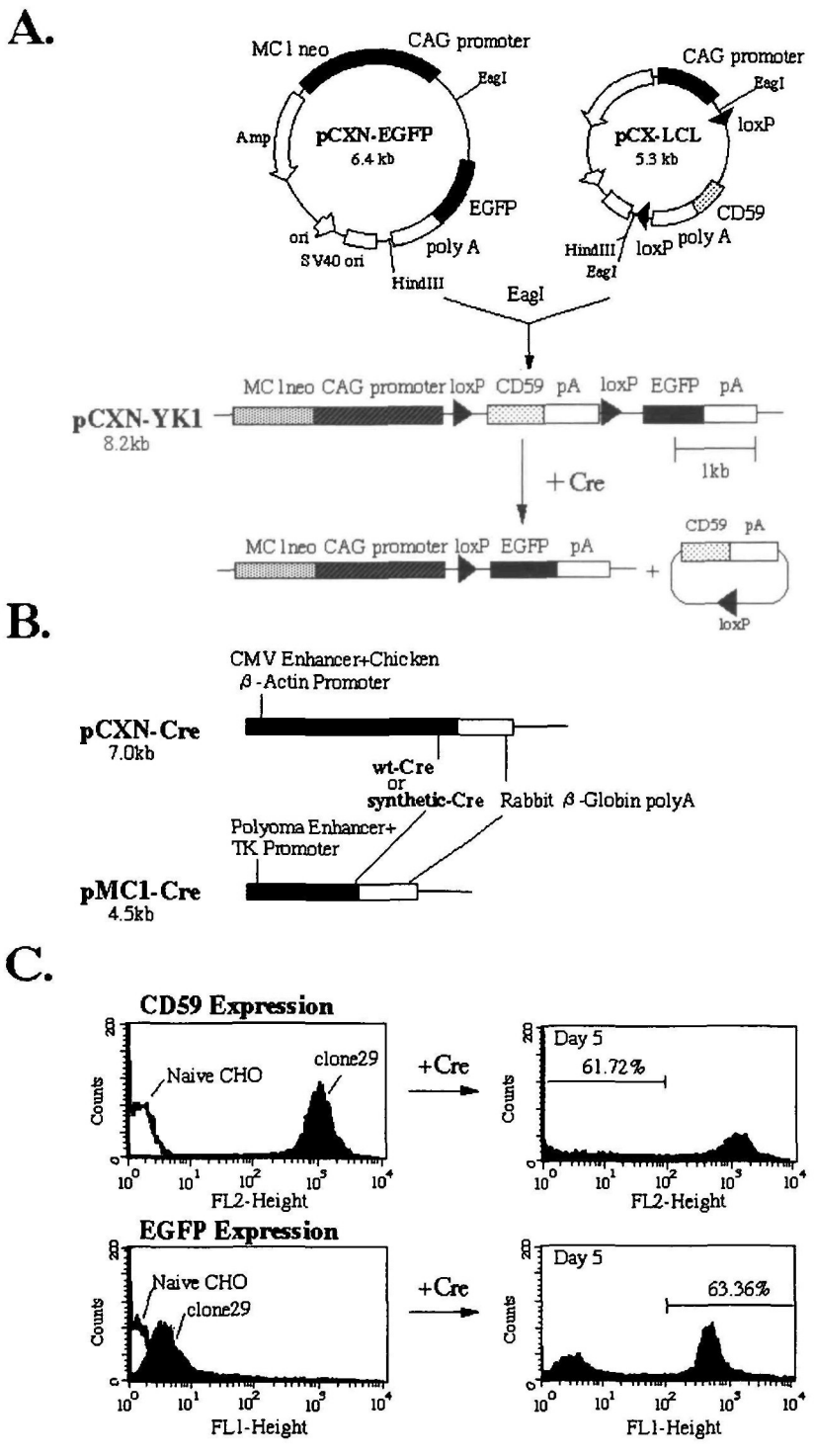

Fig. 3. **Experimental design.** (A) pCX-LCL was constructed from pCX-CD59. A *loxP* site and a *loxP* site with an *Eag*I site were inserted into the *Eag*I and *Hin*dIII sites of pCX-CD59, respectively, using linkers. The *Eag*I fragment of the established pCX-LCL was ligated into the *Eag*I site of pCXN-EGFP to obtain pCXN-YK1. A diagram of the pCXN-YK1 constructs used for the assay to assess the efficacy of wtCre or sCre is presented. When Cre-mediated recombination between the *loxP* sites occurred in CHO cells with pCXN-YK1, YK1 containing a single *loxP* site and a circular DNA containing a single *loxP* site and cDNA of CD59 were generated. Consequently, EGFP is then ligated into a CAG promoter. (B) Schematic diagrams of the constructs pCXN-Cre and pMC1-Cre. (C) FACS analysis of a CHO cell transfectant, clone 29, carrying pCXN-YK1. The histograms shown are representative ones, and the percent values shown were calculated from the percentages of unshifted or shifted cells. CD59 expression in clone 29 prior to and 5 days after transfection of pCXN-Cre is shown in the upper histograms. The percent value is for CD59 (-), recombinated cells. On the other hand, EGFP expression in clone 29 is shown prior to and 5 days after transfection of pCXN-Cre in the lower histograms. The percent of EGFP expression in the recombinated cells is shown. The percent of recombinated cells in the upper histogram nearly coincides with that in the lower histogram (FL1: 530 nm for FITC; FL2: 585 nm for PE).

DISCUSSION

A strategy for achieving high-level expression of the Cre protein was examined in this study. All codons substituted in the Cre recombinase gene were converted to ones which are more frequently used in mammals (as defined using the Genbank sequences available in 1990) (*17*).

To determine whether or not the high expression observed on Western blotting is produced by the synthetic sequence only at the translational stage, Northern blot analysis was carried out. In this area, some studies have revealed that the codon-optimized gene was correlated with the upregulated mRNA (*16*, *24*), while it has been concluded by others that the effect of the high expression was not due to transcriptional synthesis or stability (*25*, *26*). In our study, Northern blot analysis showed that the transcripts which encode sCre were more abundant than ones encoding

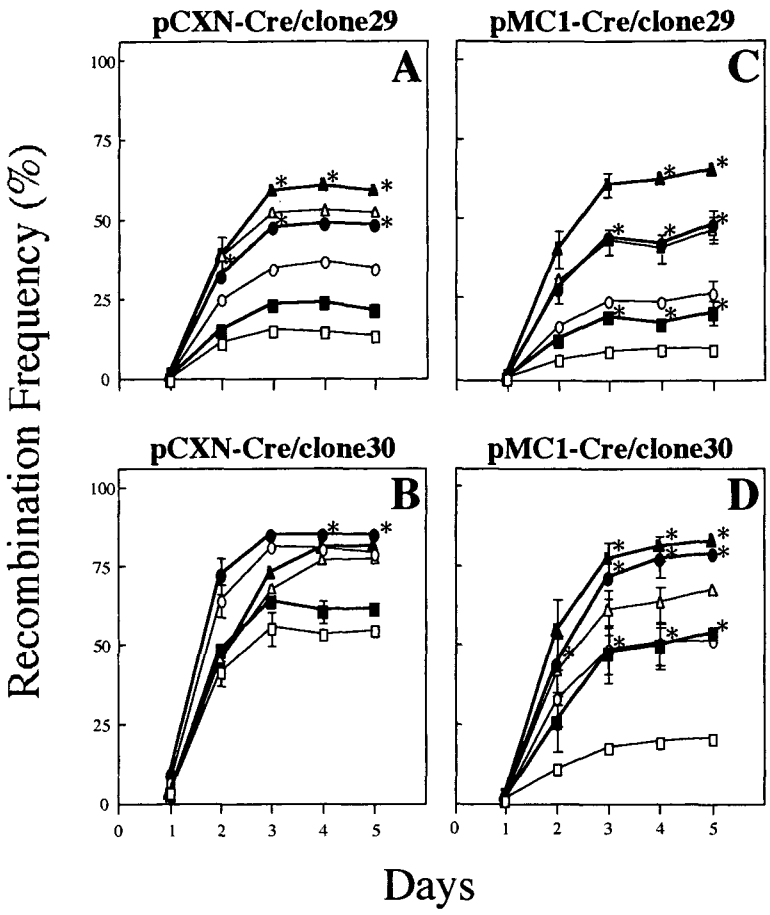

Fig. 4. **FACS analysis of the site-specific recombination effect of the wild-type or synthetic Cre recombinase gene with the pCXN or pMC1 promoter.** 5, 20, or 50 μg of pCXN (A and B), or pMC1 (C and D)-wtCre or sCre was transfected into CHO cells carrying pCXN-YK1, clone 29 (A and C) or clone 30 (B and D). The recombination frequency after day 3 was significantly higher in both clones with pMC1-sCre, compared to with pMC1-wtCre, whereas the differences in the recombination frequency were not always significant in the case of pCXN-Cre. For the recombination frequency, the percent EGFP expression in each clone was employed. Each value is expressed as the mean ± SEM for three to five independent experiments. $^*p < 0.05$ groups with sCre *versus* wtCre at the same dose (□: wtCre-5 μg, ■: sCre-5 μg, ○: wtCre-20 μg, ●: sCre-20 μg, △: wtCre-50 μg, ▲: sCre-50 μg).

wtCre. In addition, while the mRNA levels of wtCre reached its peak on day 1 and decreased on day 4, those of sCre were prolonged by one day. Similar changes were observed in the protein levels, indicating that the effect was not exclusively translational.

The choice of an expression system for the high level production of a recombinant protein depends on a variety of factors, including promoters, enhancers, transcriptional or translational terminators, and mRNA stability. In our case, the same promoter and poly A tail were used, and a cloning site of cDNA exists on exon 2 in pCXN. Therefore, both mRNAs are destined to decay *via* the same pathway, although the possibility exists that unknown exonucleases might rapidly degrade only the wild-type Cre mRNA. Codon optimization altered the translation, and may have influenced the half-life of Cre mRNA either through direct effects on mRNA stability determinants, or indirectly *via* an interplay between mRNA translation and degradation (*27, 28*).

In spite of the 3–10 times differences in the abundance of the protein between pCXN-wtCre and pCXN-sCre, as observed on Western blot analysis, the efficiency of recombination of the *loxP* sites in the CHO cells which encode pCXN-YK1 is not widely divergent. However, on the whole, sCre led to a significantly higher rate of recombination of the *loxP* sites, as shown by many of the data points in Fig. 4, especially when promoted by the pMC1 promoter, a relatively weak promoter (*12*). Although the maximum frequency of recombination of the *loxP* sites in both clones with pMC1-Cre is nearly the same as those with pCXN-Cre, the difference in vector size between pMC1 (3.5 kbp) and pCXN (6.0 kbp) may have an influence on this matter. In the case of organ-specific or cell-specific promoters, strong promoter activities are not always observed in the transgenic mouse. In addition, the copy number of the transgene is also variable. Therefore, the optimization of the codon usage of the Cre recombinase gene as to the mammalian system must be a critical factor for upregulation of the ratio of recombination of the *loxP* sites.

Regarding the relation between the amount of the transfected Cre recombinase gene and the recombination effect, a higher amount of the Cre recombinase gene does not always upregulate the recombination efficiency in the case of pCXN-Cre/clone 30 (Fig. 4B). Several reports have demonstrated the same phenomenon, the optimal amount of the transfected gene being noted in each case (*29, 30*).

Finally, the synthetic Cre recombinase gene showed a high recombination effect, suggesting that it will be a useful material for use in site-specific recombination not only *in vitro* but also *in vivo*.

The authors thank Dr. Milton S. Feather for editing the manuscript.

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