Codon-Improved Cre Recombinase (iCre) Expression in the Mouse

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Summary: By applying the mammalian codon usage to Cre recombinase, we improved Cre expression, as determined by immunoblot and functional analysis, in three different mammalian cell lines. The improved Cre (iCre) gene was also designed to reduce the high CpG content of the prokaryotic coding sequence, thereby reducing the chances of epigenetic silencing in mammals. Transgenic iCre expressing mice were obtained with good frequency, and in these mice loxP-mediated DNA recombination was observed in all cells expressing iCre. Moreover, iCre fused to two estrogen receptor hormone binding domains for temporal control of Cre activity could also be expressed in transgenic mice. However, Cre induction after administration of tamoxifen yielded only low Cre activity. Thus, whereas efficient activation of Cre fusion proteins in the brain needs further improvements, our studies indicate that iCre should facilitate genetic experiments in the mouse. genesis 32:19–26, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

The bacteriophage P1-derived Cre recombinase (Cre) is widely used to introduce specific gene deletions in selected cell populations of genetically modified mice (reviewed by Nagy, 2000). Despite a growing list of successful applications, recent experience indicates that several parameters can be critical for Cre-mediated recombination efficiency at a given allele between two loxP-flanked target sites. These parameters include (i) the distance between the two recombination target sites (Ringrose et al., 1999); (ii) genomic position effects that may differ according to where the loxP sites are placed in the genome and in which cell type recombination is desired (Vooijs et al., 2001); and (iii) the level of Cre expression in the target cell population. Here we aimed to enhance the level of Cre recombinase expression in mammalian systems. Because Cre is derived from a prokaryotic source, its codon usage is not optimal for eukaryotes, and it also contains an undesirably high frequency of CpG dinucleotides—65 CpG dinucleotides in the Cre gene—which can lead to epigenetic silencing during mammalian development (Cohen-Tannoudji et al., 2000). Furthermore, expression levels are particularly important to applications of regulated recombinases, either by regulated expression (Kuhn et al., 1995) or by regulated activity using fusion proteins of Cre with ligand binding domains of steroid receptors (LBD; Logie and Stewart, 1995). Therefore, we decided to optimize the codon usage of the Cre gene for use in mammals (Haas et al., 1996). The altered version, termed iCre (improved Cre), was assembled from oligonucleotides (Stemmer et al., 1995) to introduce silent base mutations corresponding to human codon-usage preferences, minimize CpG content, eliminate putative cryptic splice sites (splice site prediction programs: www.cbs.dtu.dk/services/NetGene2/ and www.fruitfly.org/seq_tools/splice.html) and alter the stop codon. We also included an optimal Kozak consensus sequence (Kozak, 1997), and an additional valine codon in front of the simian virus 40 large T nuclear localization signal (T-NLS, Fig. 1) for potentially increased N-end rule stability (Varshavsky, 1997).

In a first comparison, equal amounts of iCre and the prokaryotic Cre (Gu et al., 1994) expression vectors—both carried N-terminal the large T-NLS—were each transiently transfected together with a lacZ expression plasmid in human embryonic kidney 293 (HEK293) cells. From individual transfections the beta-galactosidase (β-gal) activity was measured and showed transfection efficiency variations of about 0.5% (n = 9). When the amount of Cre protein in the cell extracts was determined in immunoblots, normalized for β-gal.
FIG. 1. Nucleotide sequence comparison of improved Cre recombinase (iCre) and phage P1 Cre recombinase (Cre). Sequence differences are boxed. Start and stop codons are in gray boxes. Both DNAs encode a nuclear localization sequence, codons of which are underlined. Important restriction sites are indicated.
of iCre was 1.58 ± 0.15 (mean ± SEM; P < 0.05; n = 6) fold higher than that of Cre expressed from the same vector (Fig. 2a). Increased iCre expression was confirmed in an enzymatic test (Fig. 2b). Protein extracts of transfected cells were incubated with a substrate for Cre in the form of linearized DNA containing two loxP sites and releasing a 2,895 bp fragment upon Cre recombination. In agreement with the enhanced expression levels of iCre (Fig. 2a), DNA recombination was 1.83 ± 0.18 fold higher (P < 0.01; n = 2; β-gal normalized) by extracts containing iCre than Cre (Fig. 2b). Transfection experiments with CV1/lacZ indicator cells (Ludwig et al., 1994) yielded a similar result (Fig. 2c). In this assay, Cre activity removed the floxed stop insert and activated β-gal expression. Again, iCre had 1.68 ± 0.02 (P < 0.01; n = 2) fold higher activity than Cre (Fig. 2c). In this experiment an expression plasmid coding for alkaline phosphatase (AP) was cotransfected. The number of AP-positive cells was used to normalize for transfection efficiency.

To establish whether the improved expression properties of iCre could confer improved expression onto a Cre-LBD fusion protein, and also to test expression from stably integrated transgenes, plasmids encoding Cre-ABD (androgen receptor binding domain) or iCre-ABD were constructed (Fig. 3a) and stably integrated into mouse embryonic stem (ES) cells. The N-terminal NLS was removed from Cre and iCre for this experiment because its presence increases background Cre recombination by Cre-LBD fusion proteins before activation by ligands (Angrand et al., unpublished results). After selection for stable integration of the Cre-ABD plasmids, pools of primary colonies were taken for immunoblot analysis (Fig. 3b). In two experiments, iCre-ABD expression levels were 2.5 ± 0.5 (P < 0.05) greater than Cre-ABD expression levels. The ES cells used for this experiment were of the Ex3 line that carries a stably integrated loxP/lacZ recombination reporter in which β-gal reports recombination (Kellendonk et al., 1996). Immediately after electroporation, 10⁻⁷ M mibolerone was added to plates so that iCre-ABD or Cre-ABD would be induced while stable, G418 resistant colonies arose. The plates were then stained for β-gal expression in situ. Of 158 G418-resistant colonies on the Cre-ABD plate, 52 (32.9%) showed β-gal expression, whereas on the iCre-ABD a 1.8-fold increase of colonies (146/155, 94.2%) were positive for β-gal despite its high basal activity (basal activity without inducing ligand was 0.2% for Cre-ABD and 34.6% for iCre-ABD). This result supports the immunoblot analysis and indicates that iCre can improve expression levels of Cre-LBD but increase the basal activity of the ABD fusion protein.

Based upon the improved properties of iCre in cell culture experiments, we verified functionality in transgenic mice and expressed iCre via a mouse gonadotropin-releasing hormone (GnRH) promoter fragment. In previous studies using this fragment (Spergel et al., 2001) or similar ones (Pape et al., 1999), GnRH pro-
motor activity in the mouse brain was observed in most GnRH immunopositive neurons and in a second population of cells about 18 times larger than the GnRH expressing population. When we used this GnRH promoter fragment to drive nuclear iCre expression, we obtained four mouse lines exhibiting high expression, three with low expression, and two with no iCre expression. In all four high-expressing lines, cell nuclei of GnRH neurons and neurons of the second population were immunopositive for iCre (Fig. 4c, e, f). Double labeling with GnRH and Cre antisera confirmed cellular colocalization of cytoplasmic GnRH and nuclear Cre in 89/110065% (n/110055 mice) of GnRH neurons (Fig. 4e).

Cre-activated β-gal expression as determined by X-Gal staining was observed in offspring of transgenic (TgGnRH-iCre) mice bred with a Cre indicator mouse line (GTRosa26; Soriano, 1999). In double positive (GTRosa26/TgGnRH-iCre) mice, Cre activity was detected in 97 ± 0.5% (n = 2 mice) of GnRH neurons and also in the second population (Fig. 4d, g). Thus, in the first test in mice, iCre presented good operational properties. Neurons in other areas, mainly of the limbic system, also exhibited Cre activity. This widespread iCre activity seen in GTRosa26/TgGnRH-iCre mice may reflect in part a developmental regulation of the GnRH promoter (Skynner et al., 1999). In the adult GTRosa26/TgGnRH-iCre mouse, Cre protein is only detected in GnRH neurons and in neurons of the second population. Thus, later in development, the activity of the GnRH promoter might be more restricted.

Consequently, we expressed iCre flanked by two estrogen receptor-binding domains (ER, Zhang et al., 1996; Indra et al., 1999) via the GnRH promoter to achieve temporal regulation of iCre activity. Again we obtained high (n = 3) and low (n = 2) expressing lines (TgGnRH-ERiCreER). In all lines, tamoxifen induced nuclear accumulation of the Cre immunostain from its hsp70-mediated cytoplasmic localization. In tamoxifen-injected offspring of TgGnRH-ERiCreER mice containing the lacZ gene of GTRosa26 we detected loxP-directed recombination at the lacZ locus of the indicator gene by PCR of total brain DNA (data not shown). However, β-gal expression in GTRosa26/TgGnRH-ERiCreER mice could not be detected indicating that the activity of iCre recombine is too low. Hormone-binding domains flanking Cre reduce the recombination efficiency in vitro (Kellendonk et al., 1996), and it may be further reduced in a living organism. In addition, the chromatin structure may hinder the accessibility of the loxP elements in the mouse genome, thereby reducing the effectiveness of ERiCreER to act on these loxP sites. As a result, recombination events at the Cre indicator gene in GTRosa26/TgGnRH-ERiCreER may occur in only a few cells not detectable by X-Gal staining.

In summary, in our mammalian cell culture studies, iCre was expressed better than prokaryotic Cre and also better than the humanized Cre described by Koresawa et al. (2000). In our transgenic mouse model TgGnRH-iCreER iCre was expressed in four out of nine founders at high levels and with high activity. Thus, adapting codon usage elevated Cre expression, as was the case with GFP from jellyfish (Zolotukhin et al., 1996) and with the lac repres-
sor (Cronin et al., 2001). We conclude that iCre, when expressed in select tissues and cell populations (Casanova et al., 2001), is well suited for studying gene function in the mouse.

MATERIALS AND METHODS

Synthesis of the Improved Cre

To synthesize the iCre gene (Fig. 1, Genbank accession no. AY056050), the Cre DNA sequence of pMC-Cre (Gu et al., 1994) was modified. Twenty-four 70-mer oligonucleotides encoding the iCre sequence were assembled by PCR (Stemmer et al., 1995). Fragments of the expected length (1.091 kb) were isolated, reamplified, and sequenced with primers C0 (5'-H11032-GAGGAAGCTTGTC-CACCATGGTGC-3') and R0 (5'-H11032-CGCTCCGTCGACT-CAGTTTCAGTC-3').

Plasmids

The iCre gene was cloned into a prokaryotic expression vector composed of pBAD (Invitrogen, Groningen, The Netherlands) and part of pPKM-6 (Reiss et al., 1984) via HindIII and SalI and introduced into DH5α cells containing the Cre indicator plasmid pSVpaX1 (Buchholz...
Out of 31 recombinant clones, two harbored the expected sequence. Plasmid pL29mGnRH.iCre was constructed by substituting the GFP fragment in the plasmid pL29mGnRH.hGFP2 (Spergel et al., 1999) by iCre via HindIII and SalI. Plasmid pL29mGnRH.ERiCreER was constructed as described above except that the GFP-SV40 sequence was substituted by the ERiCreER-hghpolyA (polyadenylation signal of the human growth hormone) sequence via a 5'/-blunt and 3'/-BssHII ligation. Plasmid pBKC-iCre-ABD was constructed by substituting Cre with the 5'/-altered iCre fragment via HindIII and XhoI in pBKC-Cre-ABD.

**Transfection and Staining Assays**

HEK293 cells were transfected with plasmid pRK (Schall et al., 1990) containing the iCre or Cre recombinase gene (5 µg) and cotransfected with pCMV-lacZ (5 µg) to monitor transfection efficiency by β-gal (Miller et al., 1992). The pH vectors (Kellendonk et al., 1996) encoding iCre and Cre (2 µg) were transfected into CV1 cells carrying a lacZ indicator plasmid (Ludwig et al., 1994) and cotransfected with pHD-AP (1 µg) for controlling transfection efficiency by Fast Red staining (Roche, Mannheim, Germany). β-gal activity was detected with X-Gal solution (Kellendonk et al., 1996). Cre activity of 1 µg Cre expression plasmid is given as the number of blue cells/number of AP-positive cells. Ex3 ES cells (5 × 10^5) were electroporated (240V, 500 µF) with 20 µg Cre-ABD or iCre-ABD plasmids, linearized by ApaL1. Selection (G418, 200 µg/ml) was initiated 1 day after plating.

**Protein Preparation and Immunoblot Assays**

Protein from transfected HEK293 cells was prepared 24 h after transfection and immunoblots (10% separating and 4% stacking SDS-polyacrylamide gels) were performed. For immunoblotting of Ex3 ES cells, Ex3 cells were harvested by trypsinization. 2 × 10^6 cells were pelleted; resuspended in 1 ml 250 mM Tris-HCl, pH 7.5; supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT); and frozen in a dry ice/ethanol bath, thawed in a 37°C bath twice. NaCl was added to 400 mM, freeze/thaw cycle repeated twice and centrifugated at 1,600×g for 4 min (4°C). Protein in supernatant was precipitated with 10% ice-cold trichloroacetic acid (TCA) overnight (4°C) and centrifugated at 16,000×g for 10 min (4°C). Precipitate was washed twice in ice-cold acetone, dried and separated by 10% SDS-polyacrylamide (5% stacking zone) for immunoblot-
**In vitro Recombination Assay**

Equal amounts of protein extracts from each transfection were incubated with equal amounts of AffIII-linearized 4.995 bp plasmid pLoxPNeo-1 (Nagy et al., 1998) as described by Lee and Saito (1998). Recombination events were analyzed on a 1% agarose gel, scanned, quantified with Image Gauge 3.0, and normalized for transfection efficiency.

**Generation of Transgenic Mice**

Transgenic founders Tg^GnRH-iCre^ and Tg^GnRH-ERiCreER^ were generated by pronucleus injection of linearized (AvrII/NsiI) and purified pL29mGnRH.humCre and pL29mGnRH.ERiCreER minigenes (Spergel et al., 1999). Tg^GnRH-iCre^ and Tg^GnRH-ERiCreER^ mice were selected by PCR analysis of tail DNA (Spergel et al., 1999) with primers lac7 (5' -CTTCTCCACACCAGCTGTGGA-3') and iCre32 (5' -GCCTCCAGTACAGC-3'), which amplified a 414 bp DNA fragment or with iCre1 (5' -GACAGGCAGGCCTTCTCTGAA-3') and iCre2 (5' -CTTCTCCACACCAGCTGTGGA-3'), which amplified a 522 bp DNA fragment. GTRosa26/Tg^GnRH-iCre^ and GTRosa26/Tg^GnRH-ERiCreER^ double transgenic mice were identified by PCR analysis of tail DNA using two independent rounds of PCR, one for Tg^GnRH-iCre^ and Tg^GnRH-ERiCreER^, respectively, and another for the lacZ indicator gene with primers lac7 (5' -CCCATTACGGT-CAATCCGCACG-3') and lac8 (5' -GCCCTCACTAGC-GCGGCTG-3'), which amplified a 407 bp DNA fragment.

**Analysis of Cre Expression and Activity in Transgenic Mice**

Immunohistochemistry for iCre and GnRH was performed as described (Spergel et al., 1999). Cre antiserum (1:3,000, BAbCO, Richmond, CA) was used as primary antibody, horseradish peroxidase-coupled goat anti-rabbit IgG (1:600, Vector, Burlingame, CA) as secondary antibody, diaminobenzidine (DAB) as chromogen, and Eukitt (O. Kindler, Freiburg, Germany) as mounting medium. Costaining for GnRH was performed by repeating the staining procedure except that a GnRH antibody, the staining procedure except that a GnRH antibody, the staining procedure except that a GnRH antibody, was substituted for the Cre antibody, and an SG chromogen (Vector) was used instead of DAB. The staining for β-gal activity in GTRosa26/Tg^GnRH-iCre^ and GTRosa26/Tg^GnRH-ERiCreER^ mice on vibratome sections (Spergel et al., 1999) was performed as for the CVI cells. To induce Cre in Tg^GnRH-ERiCreER^ mice, i.p. injections of 2.5 mg/day tamoxifen diluted in sunflower oil (both from Sigma, Deisenhofen, Germany) were given for 5 days. Tg^GnRH-ERiCreER^ mice were analyzed for Cre expression at day 6 after the first injection. Activity of β-gal was assessed by X-Gal staining at the 10th and 42nd day after the first injection in GTRosa26/Tg^GnRH-ERiCreER^ mice.

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