genesis 32:19–26 (2002)
DOI 10.1002/gene.10023

TECHNOLOGY REPORT

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

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Received 24 September 2001; Accepted 20 November 2001

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.

Key words: iCre recombinase; loxP; GnRH promoter; LBD; ER

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, the amount

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Grant sponsors: Deutsche Forschungsgemeinschaft and the Volkswagen Foundation.

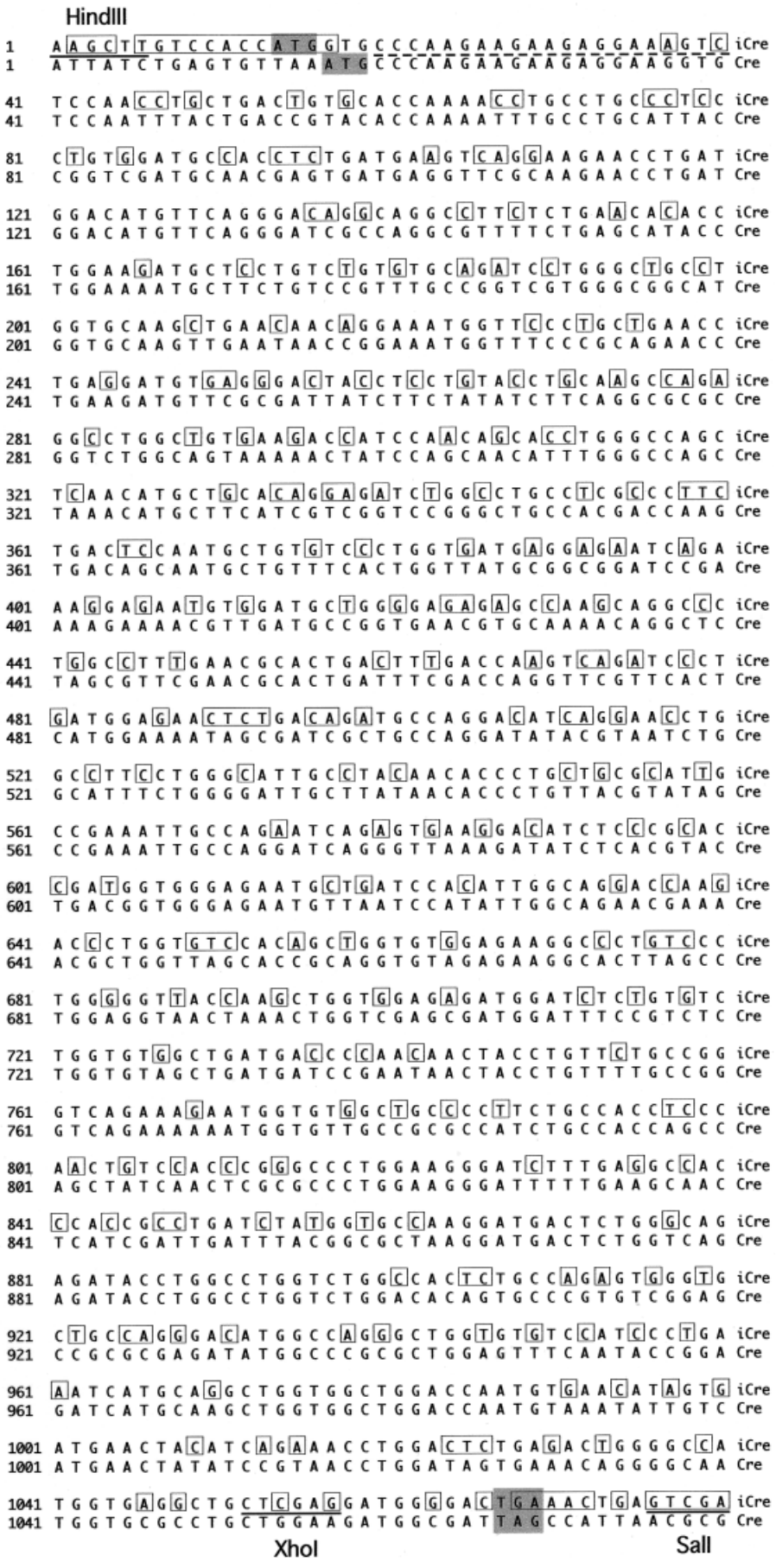

FIG. 1. Nucleotide sequence comparison of improved Cre recombinase (iCre) and phage P1 Cre recombinase (Cre). Sequence differences are boxed. Start and stop codons are in gray boxes. Both DNAs encode a nuclear localization sequence, codons of which are underlined. Important restriction sites are indicated.

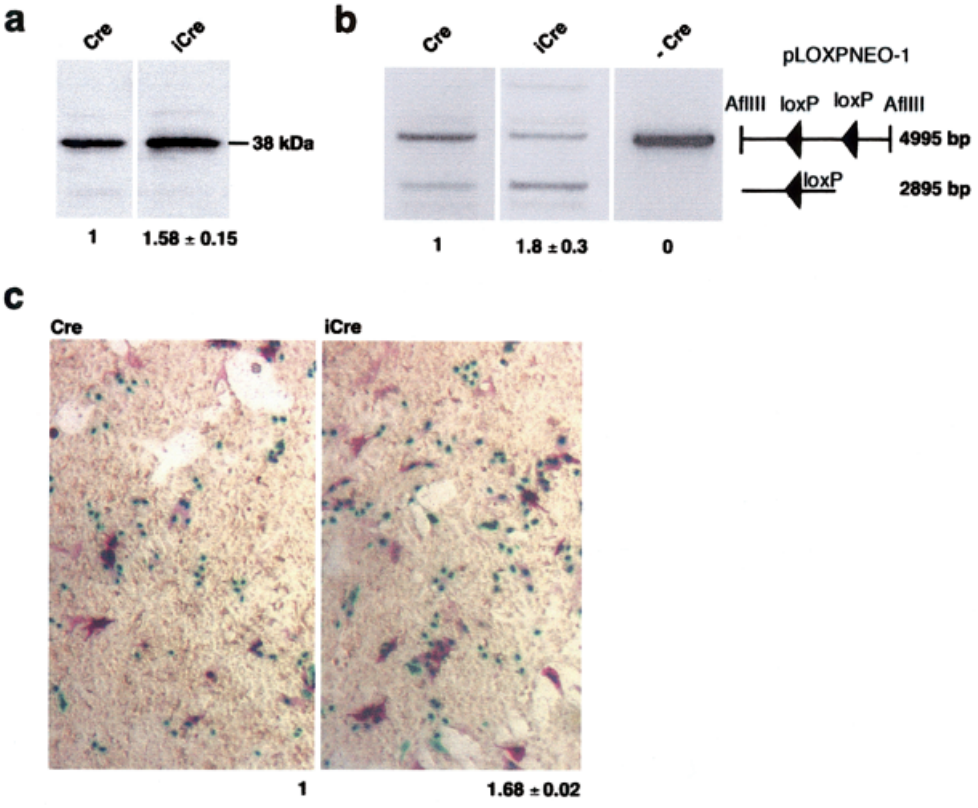

FIG. 2. Comparison of iCre and Cre expression in HEK293 and CV1/lacZ indicator cells. (**a**) Cre-immunoblot of protein extracts from transfected HEK293 cells. Quantitative evaluations of six experiments are given below the panels. (**b**) Agarose gel showing recombination of pLoxPNeo-1 induced by Cre in vitro. A 2,895 bp linear fragment was released after Cre acted on the *loxP* elements; the remaining plasmid circularized. Quantitative evaluations for two independent experiments are given below the panels. (**c**) Recombination in transfected CV1/lacZ indicator cells. The ratio of blue (recombination-positive) to red (alkaline phosphatase-positive) cell number gives the recombination efficiency shown below the panels.

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-

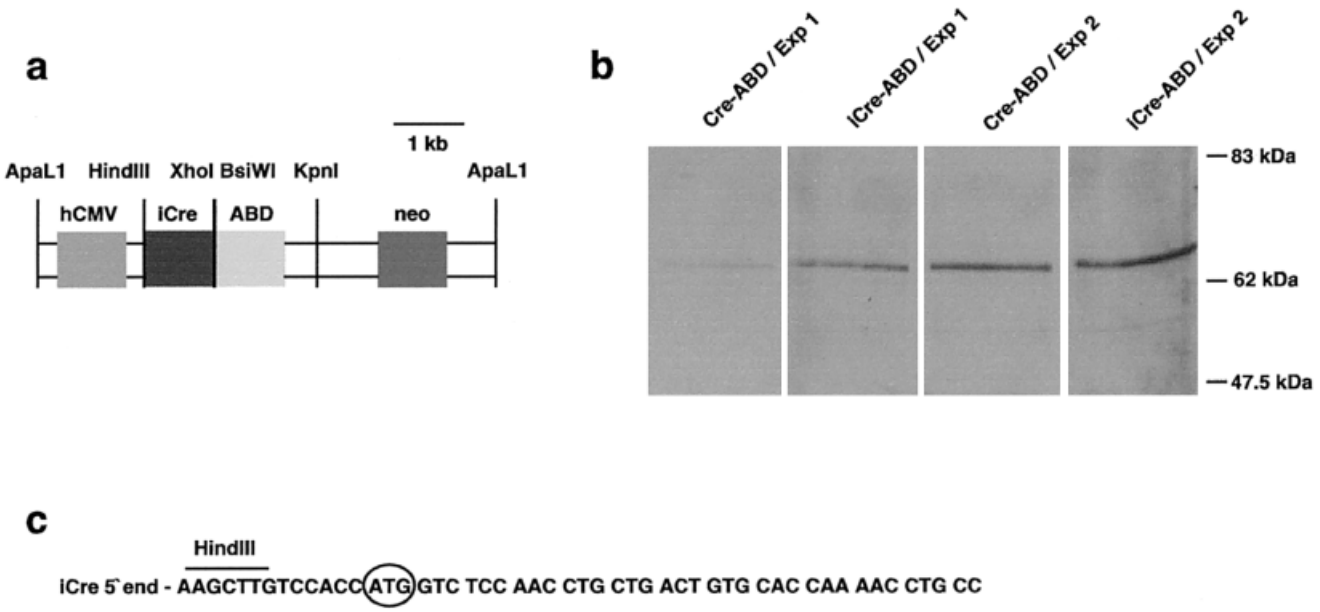

FIG. 3. Analysis of stably expressed iCre in mouse ES cells. (**a**) Diagram of the linearized plasmid pBKC-iCre-ABD (6,711 bp) used for stable integration. hCMV (late enhancer/promoter cassette of human CMV); neo (kanamycin/neomycin resistence gene). (**b**) Immunoblot analysis (two experiments) from total cell extracts of Ex3 ES cells. The two experiments differed in the presence (Exp 1) or absence (Exp 2) of 1 g/ml puromycin co-selection with G418. (**c**) The sequence of the 5′-end of the iCre-ABD and Cre-ABD coding regions. Both iCre-ABD and Cre-ABD genes carry this sequence at their 5′-ends. Translational start codon is encircled.

moter 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 ± 5% (n = 5 mice) of GnRH neurons (Fig. 4e).

Cre-activated β-gal expression as determined by X-Gal staining was observed in offspring of transgenic ($Tg^{GnRH\text{-}iCre}$) mice bred with a Cre indicator mouse line (*GTRosa26*; Soriano, 1999). In double positive (*GTRosa26*/$Tg^{GnRH\text{-}iCre}$) 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*/$Tg^{GnRH\text{-}iCre}$ mice may reflect in part a developmental regulation of the GnRH promoter (Skynner *et al.*, 1999). In the adult *GTRosa26*/$Tg^{GnRH\text{-}iCre}$ 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 ($Tg^{GnRH\text{-}ERiCreER}$). In all lines, tamoxifen induced nuclear accumulation of the Cre immunostain (Fig. 5b, c, d), indicating that the estrogen receptor antagonist released the iCre fusion protein from its hsp70-mediated cytoplasmic localization. In tamoxifen-injected offspring of $Tg^{GnRH\text{-}ERiCreER}$ mice containing the lacZ gene of *GTRosa26* mice 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*/$Tg^{GnRH\text{-}ERiCreER}$ mice could not be detected indicating that the activity of iCre recombinase 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*/$Tg^{GnRH\text{-}ERiCreER}$ 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 $Tg^{GnRH\text{-}iCre}$ 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-

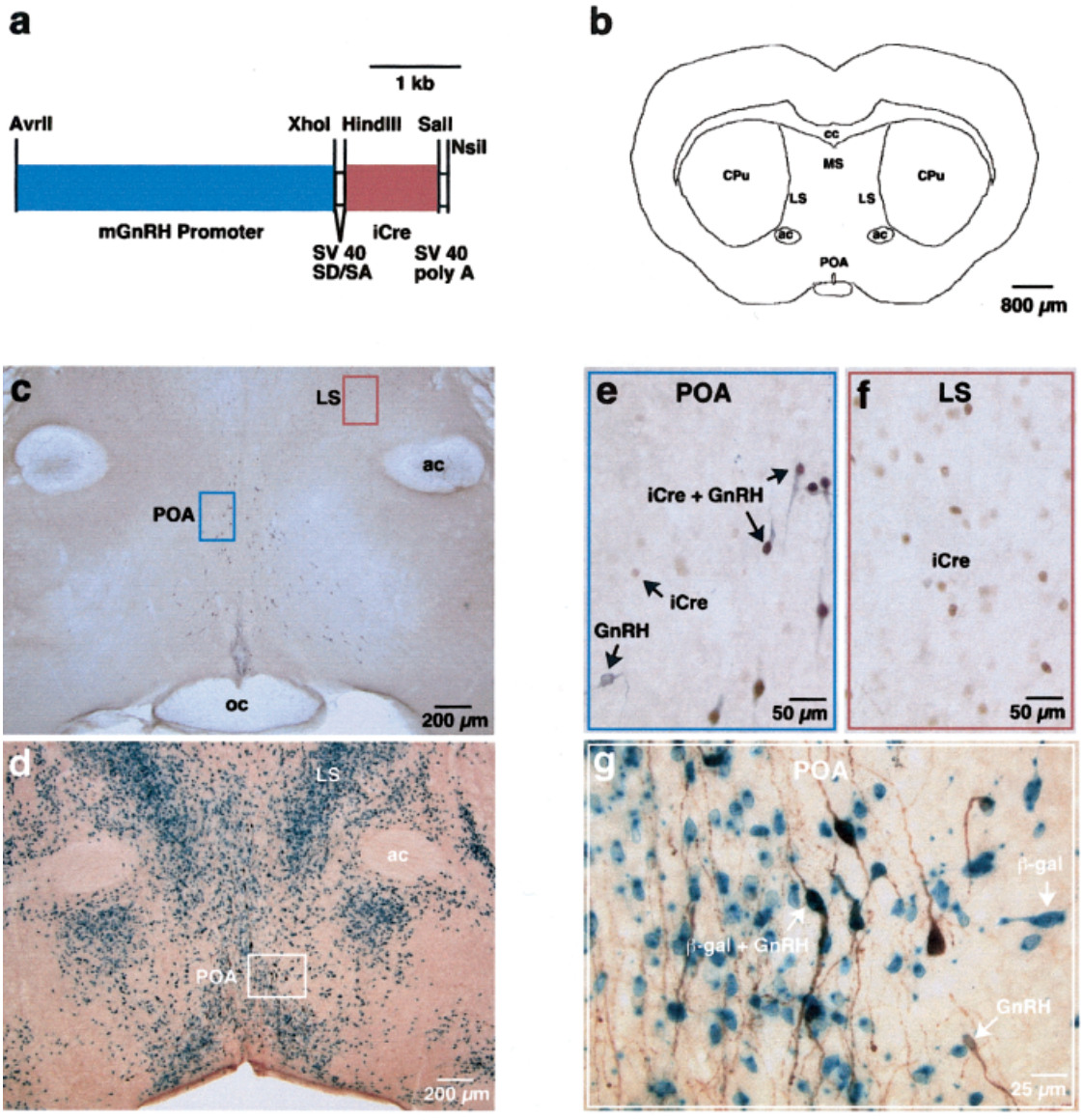

FIG. 4. GnRH–iCre construct and its expression in transgenic mice. (**a**) GnRH-iCre construct (5,028 bp) used to generate $Tg^{GnRH\text{-}iCre}$ mice. (**b**) Schematic diagram of a mouse brain slice. cc, corpus callosum; CPu, caudate putamen; MS, medial septal nucleus; LS, lateral septal nucleus; ac, anterior commissure; POA, preoptic area. (**c**) Expression and colocalization of iCre and GnRH in a coronal section. oc, optic chiasm. (**d**) Immunopositive GnRH neurons and β-gal expressing cells in *GTRosa26*/$Tg^{GnRH\text{-}iCre}$ mice. (**e, f, g**) Higher magnification showing GnRH- (blue) and iCre-positive (brown) cells in the POA (e), iCre-positive cells in the LS (f), β-gal and GnRH expression in the POA (g).

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′-GAGGAAGCTTGTCCACCATGGTGC-3′) and R0 (5′-CGCTCCGTCGACTCAGTTTCAGTC-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

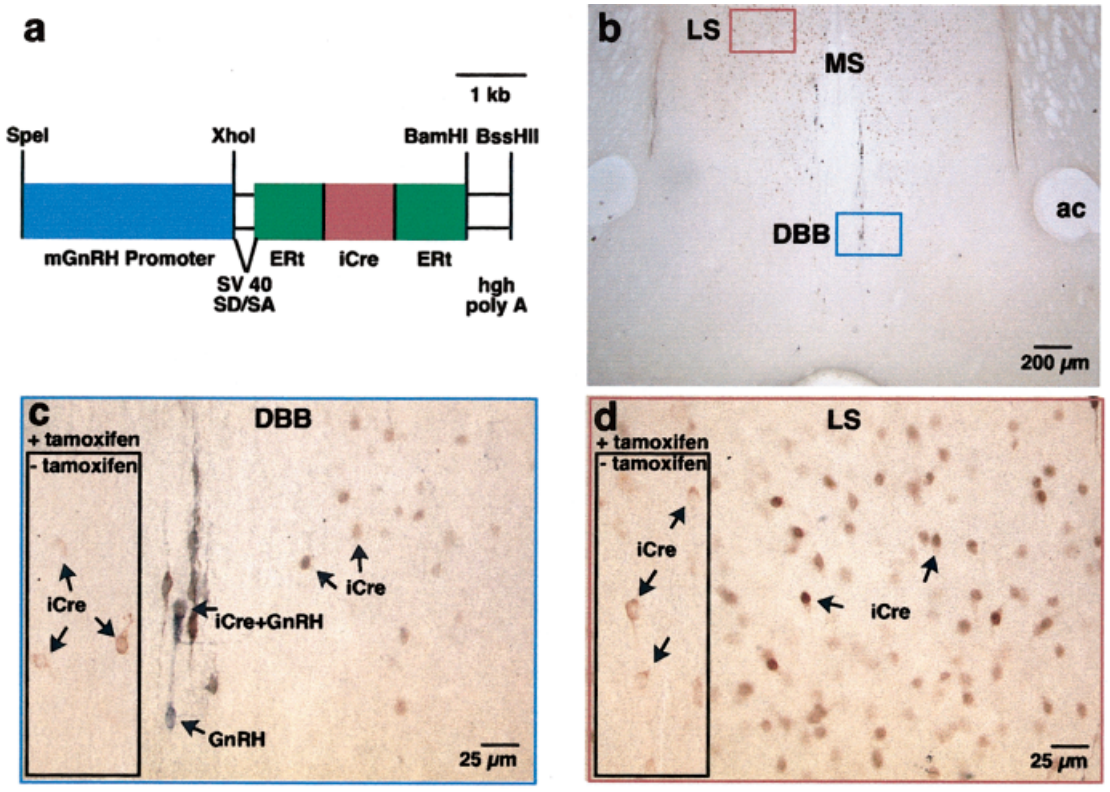

FIG. 5. GnRH-ERiCreER construct and its expression in transgenic mice. (**a**) GnRH-ERiCreER construct (7,350 bp) used to generate $Tg^{GnRH\text{-}ERiCreER}$ mice. (**b**) Expression and colocalization of iCre and GnRH in a coronal section of tamoxifen-treated $Tg^{GnRH\text{-}ERiCreER}$ mice. LS, lateral septal nucleus; MS, medial septal nucleus; DBB, diagonal band of Broca; ac, anterior commissure. (**c**) Higher magnification showing cells in the DBB that are iCre (brown) and GnRH (blue) immunopositive after tamoxifen treatment (inset in (c): iCre in the DBB without tamoxifen), (**d**) cells in the LS that are iCre immunopositive after tamoxifen treatment (inset in (d): iCre in the LS without tamoxifen).

et al., 1996). 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 pHD 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 (5×10^6) 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 1mM 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 centifugated at 1,600×g for 4 min (4°C). Protein in supernatant was precipitated with 10% ice-cold trichloracetic 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-

ing. Quantification was performed with Image Reader BAS1000 (Fuji) and Image Gauge 3.0.

In vitro Recombination Assay

Equal amounts of protein extracts from each transfection were incubated with equal amounts of AflIII-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 $Tg^{GnRH\text{-}iCre}$ and $Tg^{GnRH\text{-}ERiCreER}$ were generated by pronucleus injection of linearized (AvrII/NsiI) and purified pL29mGnRH.humCre and pL29mGnRH.ERiCreER minigenes (Spergel *et al.*, 1999). $Tg^{GnRH\text{-}iCre}$ and $Tg^{GnRH\text{-}ERiCreER}$ mice were selected by PCR analysis of tail DNA (Spergel *et al.*, 1999) with primers GnRH51 (5′-GAAGTACTCAACCTACCAACG-GAAG-3′) and iCre32 (5′-CACAGACAGGAGCATCTTC-CAG-3′), which amplified a 414 bp DNA fragment or with EiCre1 (5′-GACAGGCAGGCCTTCTCTGAA-3′) and EiCre2 (5′-CTTCTCCACACCAGCTGTGGA-3′), which amplified a 522 bp DNA fragment. *GTRosa26*/$Tg^{GnRH\text{-}iCre}$ and *GTRosa26*/$Tg^{GnRH\text{-}ERiCreER}$ double transgenic mice were identified by PCR analysis of tail DNA using two independent rounds of PCR, one for $Tg^{GnRH\text{-}iCre}$ and $Tg^{GnRH\text{-}ERiCreER}$, respectively, and another for the lacZ indicator gene with primers lac7 (5′-CCCATTACGGT-CAATCCGCCG-3′) and lac8 (5′-GCCTCCAGTACAGC-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 (LR-1, 1:10,000) was substituted for the Cre antibody, and an SG chromogen (Vector) was used instead of DAB. Staining for β-gal activity in *GTRosa26*/$Tg^{GnRH\text{-}iCre}$ and *GTRosa26*/$Tg^{GnRH\text{-}ERiCreER}$ mice on vibratome sections (Spergel *et al.*, 1999) was performed as for the CV1 cells. To induce Cre in $Tg^{GnRH\text{-}ERiCreER}$ mice, i.p. injections of 2.5 mg/day tamoxifen diluted in sunflower oil (both from Sigma, Deisenhofen, Germany) were given for 5 days. $Tg^{GnRH\text{-}ERiCreER}$ 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*/$Tg^{GnRH\text{-}ERiCreER}$ mice.

ACKNOWLEDGMENTS

We thank A. Herold for making the GnRH-iCre construct, Dr. R. Benoit for GnRH antibody LR-1, F. Zimmermann for pronucleus injections, Dr. P. Soriano for lacZ reporter mice, Drs. Y. Gu and K. Rajewsky for Cre cDNA, and Dr. A. Nagy for pLoxPNeo-1. D. R. S. was a recipient of a Deutsche Forschungsgemeinschaft (DFG) long-term fellowship. D. J. S. was supported by a SFB grant from the DFG. Funded, in part, by a grant from the Volkswagen Foundation to R. S. and P. H. S. Experiments were in accordance with protocol 35-9185.81/35/97 approved by the Regierungspraesidium Karlsruhe, Germany.

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