A novel technique for temporally regulated cell type-specific Cre expression and recombination in the pituitary gonadotroph

Karuna Naik1, Isaiah Pittman IV1, Andrew Wolfe2, Ryan S Miller3, Sally Radovick3 and Fredric E Wondisford3,4

Departments of 1Medicine, 2Pediatrics, University of Chicago, Chicago, Illinois 60637, USA
Departments of 3Pediatrics, 4Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, USA

(Requests for offprints should be addressed to F E Wondisford; Email: fwondisford@jhmi.edu)

Abstract

Inducing tissue-specific genetic alterations under temporal control allows for the analysis of gene function in particular cell types at specified points in time. We have generated a system for tetracycline-controlled expression of Cre recombinase in mice using the unique CreTeR vector. The gonadotroph-specific bovine α-subunit (Bα) promoter fragment was subcloned into the CreTeR vector, creating a technique for highly regulated expression of Cre recombinase exclusively in pituitary gonadotrophs. Control of Cre recombinase in the CreTeR vector was demonstrated in LjT2 pituitary cell lines, where Cre protein was detected in cells treated with doxycycline, but not in untreated cells. In transgenic mice, Cre was expressed in pituitary gonadotrophs of mice treated with doxycycline, but not in non-pituitary tissues or in transgenic mice not treated with doxycycline. We demonstrated Cre expression in the gonadotroph by immunostaining showing co-localization of Cre recombinase with the β-subunit of LH (LH-β). Furthermore, by crossing Bα/CreTeR with R26R mice, we were able to demonstrate functional recombination within pituitary gonadotrophs, detected by lacZ expression. The Bα/CreTeR mice described here can be used to study the function of virtually any gene in the gonadotroph; in particular, this will be useful in studying genes which may have distinct roles in development and in the adult.

Journal of Molecular Endocrinology (2006) 37, 63–69

Introduction

Targeted gene disruption is a powerful tool to study gene function in vivo. Homologous recombination of genes in embryonic stem cells was the first system used for deletion of targeted genes. However, this process results in gene mutation in all tissues of the body. The Cre/loxP system is a tool that allows researchers to study the effects of a gene deletion in a particular tissue or cell type. This system utilizes the site-specific recombinase Cre from bacteriophage P1, which recognizes consensus loxP target sequences and excises any sequence placed between two loxP sites of the same relative orientation (Sauer & Henderson 1988, Müller, 1999, Nagy 2000). By crossing a transgenic mouse expressing Cre under a cell-specific promoter with a mouse containing a gene flanked by loxP sites, one can obtain an animal with a cell-specific knockout of the particular gene of interest. This technique is especially useful when a generalized knockout of a gene produces embryonic lethality or when there are multiple tissue effects of deleting the targeted gene (Tsien et al. 1996, Lewandoski et al. 1997, Yu et al. 1998).

In order to study the effects of gene deletion in the pituitary, we have generated transgenic mice that express Cre recombinase only in pituitary gonadotrophs. Previously, the Cre/loxP system has been used to successfully target gene disruption to multiple cell types of the anterior pituitary. Luteinizing hormone (LH), follicle-stimulating hormone, and thyrotropin-stimulating hormone (TSH) are heterodimeric glycoprotein hormones that share a common α-subunit, but contain unique β-subunits that confer their biological function. We and others have used the common α-subunit promoter to direct expression of Cre recombinase to the anterior pituitary of mice (Kendall et al. 1995, Cushman et al. 2000). This results in the targeted deletion of genes in both gonadotrophs and thyrotrophs. As gene deletions in the thyrotrph may complicate the physiological assessment of transgenic animals, we sought to utilize a construct that would specifically target only the gonadotrophs in the anterior pituitary.

It has been shown that the (−313 to +48) fragment of the α-subunit promoter directs expression in gonadotrophs, but not in thyrotrophs. Hamernik et al. (1992) demonstrated the presence of cis-regulatory elements of the promoter involved in signal transduction in gonadotropin-releasing hormone-responsive cells, but not in thyrotrophs. Kendall et al. (1991) demonstrated that α-subunit expression in gonadotrophs versus thyrotrphs is regulated by discrete
regulatory elements in the α-subunit promoter. Transgenic animals expressing the diphtheria toxin A chain under control of the (−313 to +48) bp α-subunit promoter had total ablation of gonadotrophs (demonstrated by immunohistochemical staining for LH, reproductive failure, and lack of gonadal differentiation) and intact thyrotrophs, as demonstrated by normal T4 levels, normal thyroid gland, and staining for TSH. We subcloned the gonadotroph-specific fragment of the bovine α-subunit promoter (Bz) region (−313 to +48 bp) into the CreTeR vector to generate mice expressing Cre recombinase only in pituitary gonadotrophs.

In certain circumstances, it would also be advantageous to have temporal regulation of Cre recombinase expression. This would be useful when deletion of the gene of interest in the embryo results in failure of the affected tissues to develop or develop normally. By combining the Cre/loxP system with a tetracycline-mediated system of regulation of gene expression, Cre-mediated gene recombination can be turned on at any time in the development of an organism (Utomo et al. 1999). A cell- or tissue-specific promoter can be subcloned into the vector, allowing application of this system to cell- and/or tissue-specific expression of Cre recombinase. To disrupt the genes in a temporal fashion, we utilized a transgenic system (CreTeR) containing the Cre expression vector, the tetracycline operator (TetO), and tetracycline repressor (TetR) within the same vector. In the absence of doxycycline, TetR is bound to TetO, blocking the expression of Cre recombinase. In the presence of doxycycline, TetR is released from TetO by a conformational change induced by the binding of doxycycline to the TetR and Cre expression is activated (Postle et al. 1984, Yao et al. 1998).

The ROSA26 mouse strain (R26R) was developed as a reporter line for monitoring Cre expression. In this strain, a stop codon is flanked by loxP sites such that following induction of Cre recombinase, the stop codon is excised and β-galactosidase is expressed in tissues where Cre is expressed (Holzmann & Johnson 1983, MacGregor et al. 1995, Soriano 1999). In order to assess functionality of the CreTeR vector and tissue specificity conferred by the Bz promoter, we crossed Bz/CreTeR mice with R26R mice and demonstrated β-galactosidase expression specifically in the pituitary gonadotroph after exposure to doxycycline.

Others have used the Bz promoter to target Cre expression to the gonadotroph. However, there are no reports of temporal regulation of Cre expression in these animals. We report the generation of transgenic mice that express Cre recombinase in a gonadotroph-specific, and temporally regulated manner using a unique CreTeR vector.

Materials and methods

Bovine α/CreTeR transgene construction
To construct the CreTeR transgene, a 1·7 kb fragment containing the Cre coding region and a nuclear localization signal was subcloned into the HindIII site downstream of the TetO in pcDNA4/TO (Invitrogen). A cytomegalovirus-TetR (CMV-TetR) cassette from pcDNA6/TO (Invitrogen) was then cloned downstream of Cre generating the CreTeR vector. A (−313 bp to +48 BglII fragment of the proximal Bz region was subcloned into the CreTeR vector generating Bz/CreTeR.

Bovine α/CreTeR transgenic mice
A purified Sap1 fragment of the Bz/CreTeR construct was injected into fertilized oocytes derived from a CD1 mouse to generate transgenic lines. Transgenic mice were identified using PCR and Southern blot analysis. Hybridization was performed with a 1·0 kb BamHI probe containing the Cre coding region. The 1·0 kb BamHI fragment was labeled with 3²PdCTP using DNA labeling beads (Amersham Biosciences). Genotyping was performed on DNA isolated from the tails of 3-week-old mice. Transgenic Bz/CreTeR mice were identified by PCR analysis using the primers (5’ primer 5’gatctggtatataggaagttga3’ and 3’ primer 5’catctcctttgctggaac3’). Three founder lines were identified and characterized.

R26R/Bz/CreTeR bitransgenic mice
To evaluate for functional Cre recombinase expression, Bz/CreTeR mice were crossed with R26R mice (Jackson Laboratories, Bar Harbor, ME, USA). R26R mice were genotyped using primers to amplify the transgene (~300 bp band; 5’ primer 5’gccaagaggtttgcttcatca3’ and 3’ primer 5’aaatctgtgttttatgtatt3’) or wild-type sequence (~550 bp band; 5’ primer 5’ggagaggggagaggt3’ and 3’ primer 5’aaatctgtgttttatgtatt3’). Bitransgenic mice were treated with 2 mg/ml doxycycline hydrochloride (Sigma) added to the drinking water for 7 days. Tissue was then harvested for X-gal (Roche Diagnostics) staining per manufacturer’s instructions.

Cell culture and transfection studies
LβT2 pituitary cell lines were transiently transfected with the Bz/CreTeR vector using Lipofectamine Plus reagent (Invitrogen) or Fugene 6 (Roche Diagnostics) as per manufacturer’s instructions. Cells were allowed to recover in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were treated with PBS or 2 μg/ml doxycycline hydrochloride.
for 24 h following transfection. Whole cell extract was prepared with RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 40 μl/ml Protease Inhibitor Cocktail; Roche Molecular Biochemicals) for Western blot analysis.

**Western blot analysis**

Animals were anesthetized; pituitaries were dissected and frozen immediately. Whole cell extracts were prepared as described above. Proteins were resolved by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). Membranes were first probed with a rabbit polyclonal anti-Cre antibody (Covance Research Products, Denver, PA, USA) or mouse monoclonal anti-actin (Chemicon, Temecula, CA, USA) followed by an appropriate secondary antibody (Amersham). Bands were visualized using the ECL-Plus Western Blotting System (Amersham).

**Immunohistochemistry**

Pituitaries were removed and fixed in 4% paraformaldehyde followed by 30% sucrose. Frozen 5 μm sections were stained using monoclonal anti-Cre antibody (Covance Research Products, Denver, PA, USA) or mouse monoclonal anti-actin (Chemicon, Temecula, CA, USA) followed by an appropriate secondary antibody (Amersham). The X-gal staining kit (Roche Diagnostics) was used according to manufacturer’s directions to detect β-galactosidase.

**Doxycycline treatment**

For systemic administration, doxycycline hydrochloride (Sigma) was dissolved in the drinking water to a concentration of 2 mg/ml. Mice that received doxycycline were treated for a period of 7 days. All experiments were done in mice aged 6–10 weeks. Animals were housed in standard cages with a 12 h light:12 h darkness cycle and provided with water and standard chow *ad libitum*. Animal care and experimental procedures were approved by the institutional Animal Care and Use Committee.

**Results**

**Cre expression in LβT2 pituitary cell line in the presence of doxycycline**

The Bz/CreTeR construct (Fig. 1A) was transiently transfected into LβT2 cells in order to assess expression of Cre recombinase in the presence or absence of doxycycline in a gonadotroph-derived cell line. Transiently transfected LβT2 cells were treated with doxycycline and whole cell extracts were harvested for western blot analysis (Fig. 1B). Maximal levels of Cre protein were detected at 24 h in transfected cells treated with doxycycline (other time-points not shown). Transfected cells not treated with doxycycline were negative for Cre protein, as were untransfected cells.

**Cell- and time-specific expression of Cre in Bz/CreTeR mice**

The Bz/CreTeR construct was injected into oocytes as a linearized purified fragment (Fig. 2A). Founder mice were identified by Southern blot analysis and PCR (Fig. 2B). From a total of 37 mice, three founders with different sites of integration were identified and propagated.

**Cre expression in adult F1 Bz/CreTeR mice**

Animals were treated with doxycycline for a period of 7 days. Immunoblotting for Cre protein was then performed on whole cell extract of pituitaries from doxycycline-treated and untreated mice. Three founder lines were studied. One line was found to be leaky, as Cre recombinase was expressed in the pituitary in the absence of doxycycline (data not shown). In another line, Cre recombinase was expressed weakly in response to doxycycline (data not shown). Therefore, these two founder lines were not propagated. Offspring from the third founder line demonstrated highly inducible pituitary Cre recombinase expression with doxycycline treatment and no detectable Cre recombinase expression in untreated mice (Fig. 2C). In this line, Cre recombinase expression was also found to be pituitary-specific; protein was detected in the pituitary, but not in the heart, kidney, liver, or spleen of transgenic animals treated with doxycycline (Fig. 2D). The presence of the transgene had no effect on fertility rates or viability of offspring, and there was no decrease in expected frequency of the transgene in offspring.

To confirm that Cre recombinase is restricted to gonadotrophs in the pituitary, frozen sections of brain from untreated and doxycycline-treated mice were subjected to immunostaining with antibodies against Cre. Cell nuclei were stained with DAPI. Merged images demonstrated nuclear Cre expression in cells that express cytoplasmic LH, a marker for gonadotrophs (Fig. 3).

**Cre-mediated recombination in gonadotrophs**

R26R mice were crossed with Bz/CreTeR mice to evaluate whether the Cre recombinase expressed in the...
gonadotroph was functional. Bitransgenic mice have the Cre gene and a floxed stop codon upstream of the lacZ gene that will result in lacZ transcription when Cre recombinase is expressed. Bα/CreetR mice were bred to R26R mice and offspring were treated with doxycycline. Pituitaries were harvested and stained with X-gal for β-galactosidase. After evaluating multiple pituitary sections from untreated and doxycycline-treated animals, we found that 10% of the cells in either group stained positively for LH-β. In the untreated group, a few scattered weakly positive β-galactosidase cells were noted (<1 stained cell/section), and this staining pattern was not different from that found in non-transgenic, ROSA26 animals. We interpreted this staining, therefore, as leaky expression from the ROSA26 locus. In mice treated with doxycycline, β-galactosidase could be clearly detected in whole pituitary preparations (Fig. 4A) and histological sections (Fig. 4B). Staining for LH-β and β-galactosidase in doxycycline-treated animals demonstrated that all LH-β positive cells also stain for lacZ (Fig. 4C).

Figure 1 Schematic representation of the bovine α/CreTeR transgenic construct and evaluation of the construct in LβT2 pituitary cell line. (A) The Bα/CreTeR construct contains (1) the CMV-TetR cassette, which expresses high levels of the tetracycline repressor (TetR) under control of the human cytomegalovirus (CMV) promoter; (2) the Cre recombinase coding sequence (Cre) downstream of two tetracycline operator sites (TetO2) cloned within the CMV promoter. The TetO2 sequences serve as binding sites for TetR molecules, thus conferring tetracycline (and doxycycline) responsiveness for expression of Cre. In the absence of doxycycline, Cre expression is repressed by the binding of TetR to the TetO2 sequences. In the presence of doxycycline, repression is released by a conformational change, allowing Cre expression. (B) Western hybridization from untransfected LβT2 cells and LβT2 cells transiently transfected with the Bα/CreTeR and recovered in DMEM supplemented with 10% fetal bovine serum with or without 10 μg/ml doxycycline hydrochloride. Cre recombinase was maximally detected in transfected cells after treatment with doxycycline for 24 h, but not detected in untransfected cells or transfected cells not treated with doxycycline.
The Cre/loxP system is a powerful tool for in vivo-targeted gene disruption in mammalian models. In vivo recombination between loxP sites occurs with high efficiency if Cre is expressed at a sufficiently high level from a transgene. By placing Cre expression under the control of a cell type-specific promoter, one can target gene knockouts to specific cell types. Systems have also been developed that allow temporal regulation of Cre expression by subcloning a tetracycline-regulated element into the transgene. Thus, by combining the two approaches, we have developed an approach that allows us to knockout genes in a tissue-specific, temporally regulated fashion. This system is also easy to use in that doxycycline only has to be added to the drinking water in order to induce Cre expression.

The CreTeR vector can be used to target specific cell types by subcloning a tissue-specific promoter upstream of the TetO site. Tight regulation of Cre expression is observed using this vector both in vitro and in vivo. Cre transcription is silent in the absence of doxycycline, but in the presence of doxycycline, Cre is expressed in a tissue-specific manner. We used this vector to target pituitary gonadotrophs using a gonadotroph-specific fragment of the bovine glycoprotein α-subunit gene. We

![Diagram](image1)

**Figure 2** Genotyping and Cre recombinase expression (A) The transgenic construct showing a BamH1 region of the Cre coding region used a probe to detect the transgene. (B) Southern hybridization and PCR detection of Cre recombinase from tail DNA preparations of five representative transgenic lines. Lines 1–4 are negative for Cre while line 5 is one of the three positive founder lines identified. (C) Cre detection by immunoblot of pituitary tissue from untreated mice or mice treated with doxycycline hydrochloride (2 mg/ml added to drinking water for 7 days). Cre recombinase is detected in pituitaries of mice treated with doxycycline, but not in untreated mice. (D) Immunoblot of tissues in untreated (–) and doxycycline-treated mice demonstrates Cre is not expressed in heart, kidney, liver, and spleen.

**Discussion**

The Cre/loxP system is a powerful tool for in vivo-targeted gene disruption in mammalian models. In vivo recombination between loxP sites occurs with high efficiency if Cre is expressed at a sufficiently high level from a transgene. By placing Cre expression under the control of a cell type-specific promoter, one can target gene knockouts to specific cell types. Systems have also been developed that allow temporal regulation of Cre expression by subcloning a tetracycline-regulated element into the transgene. Thus, by combining the two approaches, we have developed an approach that allows us to knockout genes in a tissue-specific, temporally regulated fashion. This system is also easy to use in that doxycycline only has to be added to the drinking water in order to induce Cre expression.

The CreTeR vector can be used to target specific cell types by subcloning a tissue-specific promoter upstream of the TetO site. Tight regulation of Cre expression is observed using this vector both in vitro and in vivo. Cre transcription is silent in the absence of doxycycline, but in the presence of doxycycline, Cre is expressed in a tissue-specific manner. We used this vector to target pituitary gonadotrophs using a gonadotroph-specific fragment of the bovine glycoprotein α-subunit gene. We
Figure 4 Cre-mediated recombination in the gonadotroph. Bovine α/CreTeR mice were crossed with R26R mice for detection of a recombination event. In bitransgenic mice, Cre expression results in excision of a stop codon, thus allowing for β-galactosidase gene transcription. β-galactosidase is detected with X-gal staining. (A) X-gal staining of whole pituitary from F1 offspring demonstrates high levels of β-galactosidase expression in animals treated with doxycycline, but not in untreated mice. No other tissue tested demonstrated β-gal expression before or after doxycycline treatment (data not shown). (B) β-galactosidase is expressed in the ventral region of the pituitary in frozen sections of pituitaries from doxycycline-treated mice. (C) β-galactosidase is detected in cells that express the gonadotroph marker LH-β, indicating that in the pituitary, Cre is expressed only in gonadotrophs. Cells from untreated mice showed no β-galactosidase staining (lower left panel).
demonstrated that expression of Cre recombinase in the LβT2 gonadotroph-derived cell line can be tightly regulated by the presence or absence of doxycycline in the media.

We generated Bα/CreTeR mice and identified one founder line with tightly regulated Cre recombinase expression. We demonstrated that in vivo, Cre recombinase was strongly induced in the presence of doxycycline, and silent in untreated animals. Co-localization of LH with Cre indicates that Cre is expressed only in gonadotrophs, and not in other cell lines of the anterior pituitary. Furthermore, Cre is only expressed when doxycycline is present. Therefore, genes can be knocked out at any desired time-point in development.

By crossing Bα/CreTeR mice with R26R mice, we were able to demonstrate the functionality of the construct. In the presence of doxycycline, Cre recombinase excised the stop codon flanked by loxP sites in the pituitary of the bitransgenic mice, allowing the expression of β-galactosidase only in gonadotrophs.

By combining a tetracycline-responsive system with a tissue-specific promoter, we have generated an approach that allows for very precise expression of Cre recombinase. By crossing Bα/CreTeR mice with mice containing a floxed gene, one can study the effects of the loss of the gene at specific points in time. Recombination and deletion of the floxed gene would be limited to the gonadotroph and only after doxycycline treatment. This novel vector will be useful to study genes with different functions during development, and in the adult animal.

Acknowledgements

The LβT2 immortalized pituitary cell line was kindly provided by P Mellon.

Funding

This work was supported by research grants from the National Institutes of Health (U54 HD 58820 and T32 DK007751). There is no conflict of interest that would prejudice the impartiality of any of the authors of this manuscript.

References


Received in final form 5 May 2006
Accepted 30 May 2006
Made available online as an Accepted Preprint 5 June 2006