Cre-Mediated Recombination in the Pituitary Gland

Lisa J. Cushman,[1] **Heather L. Burrows,**[2] **Audrey F. Seasholtz,**[3] **Mark Lewandoski,**[4,†]
Nicholas Muzyczka,[5] **and Sally A. Camper**[1,2]*

[1]Department of Human Genetics, University of Michigan, Ann Arbor, Michigan
[2]Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, Michigan
[3]Mental Health Research Institute and Department of Biochemistry, University of Michigan, Ann Arbor, Michigan
[4]Department of Developmental Biology, University of California, San Francisco, California
[5]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida

Summary: Organ-specific expression of a cre recombinase transgene allows for the analysis of gene function in a particular tissue or cell type. Using a 4.6 kb promoter from the mouse glycoprotein hormone α-subunit (*αGSU* or *Cga*) gene, we have generated and characterized a line of transgenic mice that express cre recombinase in the anterior and intermediate lobes of the pituitary gland. Utilizing a cre-responsive reporter transgene, αGSU-cre transgene expression was detected in the pituitary primordium and in all five cell types of the adult anterior pituitary. αGSU-cre transgene activity was also detected in the cardiac and skeletal muscle. Little or no activity was evident in the gonads, adrenal glands, brain, ventromedial hypothalamus, or kidneys. The αGSU-cre transgenic mice characterized here will be a valuable tool for examining gene function in the pituitary gland. genesis 28:167–174, 2000.   © 2000 Wiley-Liss, Inc.

Key words: cre recombinase; pituitary; αGSU; transgenic mouse; muscle

Gene targeting using the traditional method of homologous recombination in embryonic stem cells leads to the deletion of the targeted gene in all tissues of the body (Muller, 1999). For genes essential for the development of the animal, this can result in embryonic or perinatal lethality, limiting the utility of the engineered mouse to reveal gene function. With the advent of the cre/*loxP* system in transgenic mice, it is now possible to examine the effects of gene deletion in a particular cell type or tissue (Orban *et al.*, 1992; Tsien *et al.*, 1996; Lewandoski *et al.*, 1997; Ray *et al.*, 1998) or at a specific developmental stage (Yu *et al.*, 1998; Utomo *et al.*, 1999).

Several genes that are expressed in the murine anterior pituitary are critical for the survival and proper development of the animal (Burrows *et al.*, 1999). Deletion of these genes results in early lethality when targeted using conventional methods, making it difficult to isolate their roles in the process of pituitary gland organogenesis. For example, homozygous mutants for the orphan nuclear receptor steroidogenic factor-1 (*SF-1/Nr5a1*) die by postnatal day 8 due to adrenal insuffi-

ciency (Ingraham *et al.*, 1994; Luo *et al.*, 1994). While the essential role of *SF-1* in the development of the adrenal glands and gonads is clear from analysis of SF-1-deficient mice, the early lethality of homozygotes precludes a complete analysis of its role in pituitary gland function. Pituitary-specific targeting of *SF-1* will allow the function of this gene in pituitary gland organogenesis to be examined without complications from its critical role in other tissues.

In order to study the pituitary-specific effects of gene deletion, we have generated transgenic mice that express cre recombinase in the pituitary gland. These transgenic mice express cre under the control of a 4.6 kb mouse *αGSU* promoter (Fig. 1). The *αGSU* (*Cga*) gene encodes the α-subunit of the heterodimeric glycoprotein hormones thyroid-stimulating hormone (TSH), follicle stimulating hormone (FSH), and luteinizing hormone (LH), which are made and secreted by the anterior lobe of the pituitary gland. These hormones are comprised of the same α-subunit and a unique β-subunit that dictates the biological function of each. *αGSU* expression is one of the earliest molecular markers of the pituitary primordium (Japon *et al.*, 1994). Transcripts are initially detected throughout Rathke's pouch and later become restricted to the developing anterior lobe. Although *αGSU* expression is fairly specific to the pituitary gland, some *αGSU* expression can be detected in the olfactory epithelium and in the developing extraocular muscles (Kendall *et al.*, 1994). The 4.6 kb *αGSU* promoter used in the αGSU-cre transgene has been previously shown to drive high levels of transgene expression in the pituitary primordium beginning at embryonic day 9.5 (e9.5). In adults, this promoter confers high levels of hormonally
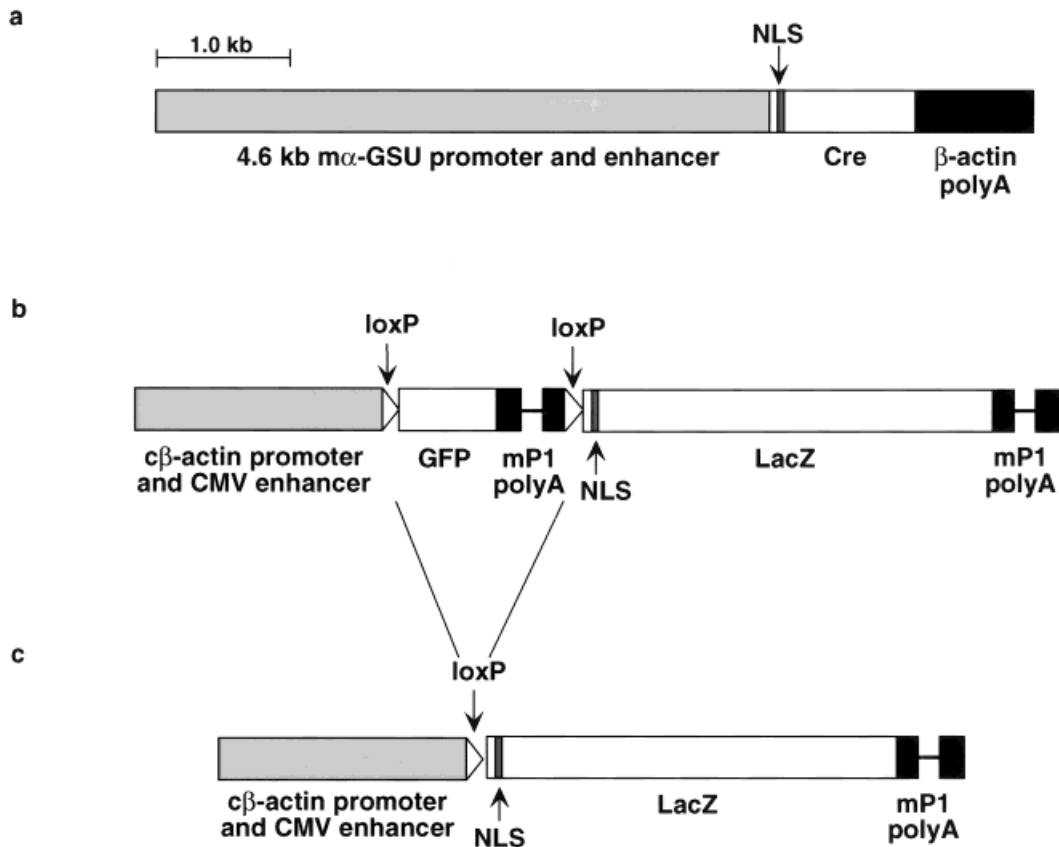
FIG. 1. Schematic diagrams of the transgene constructs. (**a**) The αGSU-cre transgene was created from the 4.6 kb mouse α*GSU* promoter, a fragment containing the gene for cre recombinase and a nuclear localization signal (NLS), and a β-*actin* polyA signal. (**b**) The reporter transgene contains the chicken β-*actin* promoter and CMV enhancer, a floxed GFP cassette, and a *LacZ* fragment containing a nuclear localization signal. A mouse *protamine-1* (m*P1*) polyA signal and intron follows both the GFP and *LacZ* sequences. In mice that carry only the reporter transgene, the presence of the m*P1* polyA signal and the GFP translation termination sequence will prevent expression of *LacZ*. (**c**) A recombined allele is present in the pituitaries of reporter, αGSU-cre doubly transgenic animals. In this recombined allele, the GFP sequence is excised, leaving a single *loxP* site followed by the *LacZ* reporter gene. Since the m*P1* polyA signal and the GFP translation termination sequence have been removed, *LacZ* expression can now be driven from the chicken β-*actin* promoter and CMV enhancer.

regulated expression to at least 80% of gonadotropes and 90% of thyrotropes. Low levels of expression can be detected in the expected nonpituitary sites, such as the extraocular muscles, as well as in occasional ectopic sites (Kendall *et al.*, 1994; Burrows *et al.*, 1996).

We report here the characterization of an αGSU-cre transgenic line that will be useful for gene targeting experiments in the pituitary gland. In this line of mice, cre recombinase is highly expressed in the anterior pituitary and will delete a floxed gene in the majority of anterior pituitary cells.

To examine the level and specificity of αGSU-cre transgene (Fig. 1A) expression, mice carrying a cre-responsive reporter transgene were also generated. The reporter transgene consists of the coding sequence for green fluorescent protein (GFP) flanked by *loxP* sites and followed by the β-galactosidase gene. This transgene is driven by the chicken β-*actin* promoter and a CMV enhancer element (Fig. 1B). Transgenes under the control of this promoter should be widely expressed from a very early point in gestation onward (Niwa *et al.*, 1991;

Sands *et al.*, 1993; Ikawa *et al.*, 1995). When hemizygous αGSU-cre mice are mated with hemizygous reporter mice, one quarter of the progeny will be doubly transgenic. In these mice, any cell that expresses α*GSU* will produce cre. The cre will then catalyze recombination between the two *loxP* sites in the reporter transgene leading to excision of GFP and expression of β-galactosidase (Fig. 1C). Because the excision is a permanent and heritable DNA change, the descendants of these cre-expressing cells will also express β-galactosidase, even if they no longer express cre. Thus, αGSU-cre transgene expression can be characterized by analyzing β-galactosidase expression in doubly transgenic mice. The highest expressing reporter line (J7 or TgN(flox-lacZ)[J7Sac]) was selected for use in cre transgene analysis. The transgene in this line is present as a single copy.

Six αGSU-cre transgenic lines were generated by pronuclear injection. The level of transgene expression in each of these lines was examined by mating αGSU-cre mice with J7 reporter animals. The pituitary glands from adult J7, αGSU-cre doubly transgenic progeny were re-

moved and stained with X-gal. Four of the six lines (S2, S3, S5, and S6) had a high level of β-galactosidase expression in the anterior pituitary, which indicates the cre recombinase is both highly expressed and active in each of these lines.

The cell specificity of cre transgene expression in the anterior pituitary was analyzed in the S3 and S6 αGSU-cre lines. Because the β-galactosidase enzymatic assay is not suitable for analysis of pituitary cell specificity due to release of the X-gal precipitate from the cells, double immunostaining was performed using an antibody against β-galactosidase followed by antibodies against each of the pituitary hormone markers. In both lines S3 and S6, the αGSU-cre transgene is active in all five cell types of the anterior pituitary (Fig. 2A), with nearly equivalent penetrance. β-galactosidase expression was also analyzed in J7,CMV-cre doubly transgenic mice (Fig. 2B) In these animals, β-galactosidase expression was also detected in all five anterior pituitary cell types. The labeling of all five pituitary cell types in J7 reporter, αGSU-cre double transgenics is expected as the endogenous αGSU gene and transgenes with the 4.6 kb promoter are expressed throughout Rathke's pouch in early development (Kendall et al., 1994). Because the change that occurs in cre-expressing cells is permanent and heritable, labeling Rathke's pouch in its entirety would lead to the labeling the cells of both the anterior and intermediate lobes of the adult pituitary gland.

The tissue specificity of the αGSU-cre transgene was assessed by X-gal staining a variety of adult tissues from J7, αGSU-cre doubly transgenic animals. In animals that have both the J7 reporter and the S3 αGSU-cre transgenes, high levels of β-galactosidase expression were detected in the skeletal muscle and in specific regions of the heart (Fig. 3A). β-galactosidase expression in the posterior lobe of the pituitary, lungs, kidneys, brain, adrenal glands, and gonads is low to absent. These results, which were consistent for the S3, S5, and S6 αGSU-cre lines (data not shown), drastically contrast those obtained in the J7,S2 αGSU-cre doubly transgenic mice. In J7,S2 αGSU-cre animals, high levels of β-galactosidase expression are seen in every tissue examined, including the pituitary gland, heart, skeletal muscle, lungs, gonads, and kidneys (Fig. 3B). This reveals that the restricted tissue specificity of the S3 αGSU-cre line is not due to limited potential of the J7 reporter transgene. The tissue specificity of the S3 αGSU-cre transgene was analyzed at the single cell level in sections of the X-gal stained tissues. High levels of expression in the heart and skeletal muscle were confirmed with this analysis (Fig. 4). β-galactosidase expression in the lung, adrenal glands, ovary, and brain is sparse and scattered, while essentially no expression is detected in the kidney. The nonpituitary β-galactosidase expression seen with the S3, S5, and S6 αGSU-cre transgenes most likely results from the transgenes being expressed for a brief period early in development. It is clear from the reporter gene analysis of the αGSU-cre transgenes that this method is much more sensitive at detecting ectopic promoter ac-

tivation than a reporter gene linked directly to the αGSU promoter.

To assess the developmental expression of the S3 αGSU-cre transgene, J7,S3 doubly transgenic embryos were stained with X-gal. Previous experiments with αGSU-LacZ transgenic mice suggest that transgenes driven by the 4.6 kb αGSU promoter should be active in the developing pituitary by e9.5 (Kendall et al., 1994; Burrows et al., 1996). Dissection of Rathke's pouch in J7,S3 double transgenics reveals that expression of the αGSU-cre transgene is robust at e12.5 (Fig. 5A, inset). In addition, β-galactosidase expression in the developing pituitary gland has a very specific pattern that is characteristic of endongenous αGSU-expressing cells. At this developmental timepoint, transgene expression is also evident in the somites and neural tube (Fig. 5A), consistent with the results obtained from the αGSU-LacZ transgene (Fig. 5B). Limited β-galactosidase expression is evident in J7,CMV-cre doubly transgenic embryos at e11.5 (data not shown), but by e12.5, J7 reporter transgene expression is evident throughout the embryo as well as in the developing pituitary gland (Fig. 5C). This result indicates that the J7 reporter transgene has limited value for assessing cre transgene expression prior to e12.5. Analysis of a control embryo that carries the J7 reporter transgene and no cre transgene demonstrates that J7 reporter animals do not exhibit leaky expression of β-galactosidase (Fig. 5D).

The usefulness of this αGSU-cre transgenic line [TgN(Cga-cre)[S3Sac]] in gene targeting experiments will be dependent on the gene to be deleted. The αGSU-cre transgenic line should be effective in targeting genes whose expression is not essential for the development or function of skeletal muscle and/or heart. For example, this line was effective in generating a pituitary-specific deletion of the SF-1 gene (K. Parker, University of Texas Southwestern Medical Center, personal communication). Crossing mice homozygous for a floxed SF-1 gene with S3 αGSU-cre transgenic animals results in a complete absence of SF-1 expression in the pituitary gland. In contrast to the SF-1-deficient mice generated by traditional gene targeting methods (Ingraham et al., 1994; Luo et al., 1994), the development of the adrenal glands, gonads, and ventromedial hypothalamus was unaffected in this pituitary-specific knockout of SF-1. In summary, we have generated and characterized a line of transgenic mice that express cre recombinase in the pituitary gland. These αGSU-cre transgenic mice will be a valuable tool for the analysis of gene function in the anterior pituitary.

MATERIALS AND METHODS

Transgene Construction

To generate the αGSU-cre transgene (Fig. 1A), a 2.1 kb fragment containing a nuclear localization signal (NLS), the bacteriophage P1 gene cre recombinase, and the β-actin polyadenylation signal was excised from pML78 by digestion with SalI and Asp718. This fragment was subcloned into the ClaI site downstream of the αGSU promoter (-5,000 to +43) in pGEM7Zf+ (Kendall et al.,
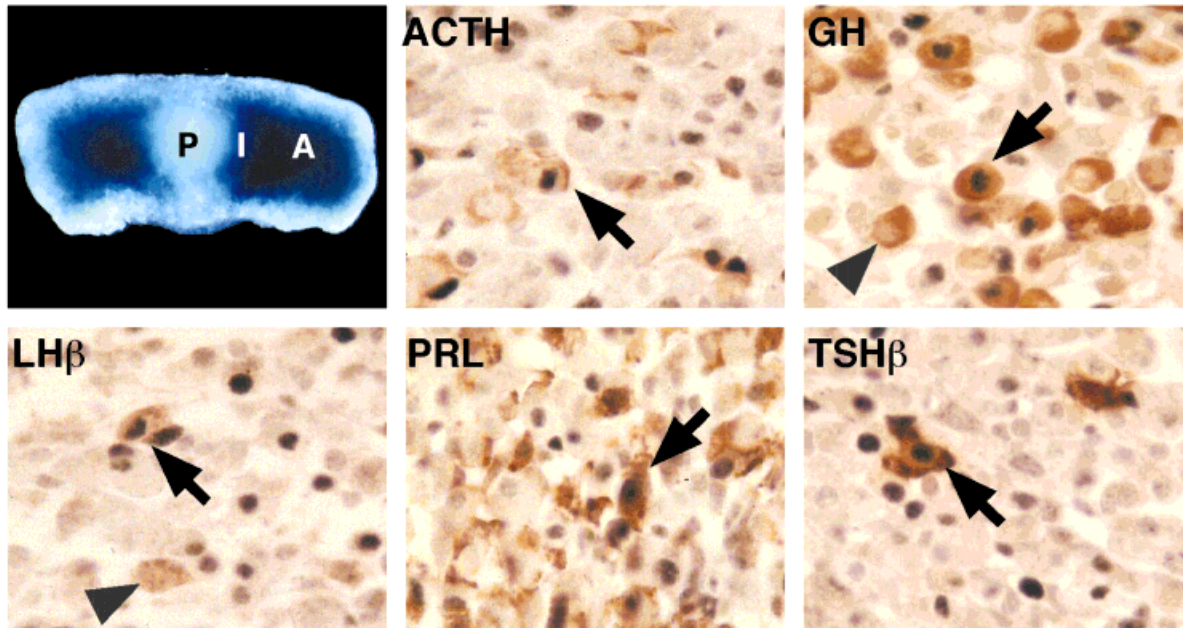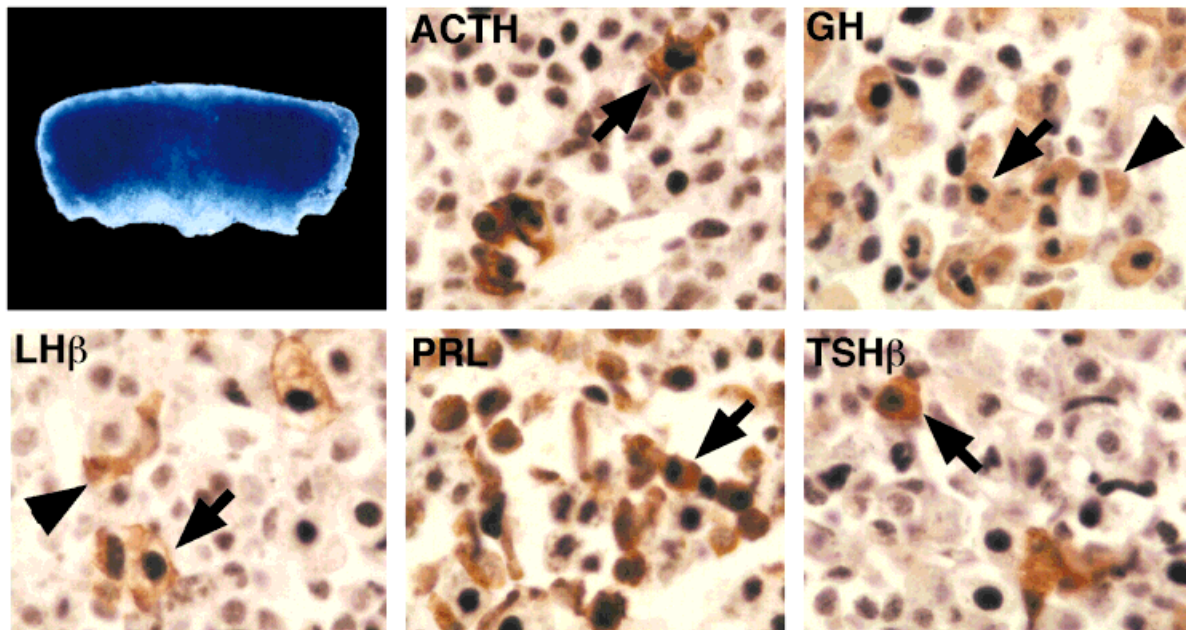
a   J7 Reporter,S3 αGSU-cre

b   J7 Reporter,CMV-cre

FIG. 2. The S3 αGSU-cre transgene is expressed in all five cell types of the anterior pituitary. (**a**) An X-gal-stained pituitary gland from an adult J7 reporter, S3 αGSU-cre doubly transgenic animal reveals high levels of the S3 αGSU-cre transgene activity in the anterior and intermediate lobes of the pituitary gland, with little or no staining in the posterior lobe. The pituitary cell specificity of this transgene was assessed by immunohistochemical analysis with a β-galactosidase antibody and antibodies specific for each pituitary hormone. In a pituitary from a J7,S3 double transgenic, the β-galactosidase protein (black nuclear stain) colocalizes with each of the five of the anterior pituitary hormone markers (brown cytoplasmic stain) used: ACTH, GH, LHβ, PRL, and TSHβ. (**b**) An X-gal stained pituitary from a J7,CMV-cre double transgenic demonstrates the activity of the J7 reporter transgene in all three lobes of the pituitary gland. Immunohis-tochemical analysis reveals that the J7 reporter transgene is expressed in all five anterior pituitary cell types, as expected. Arrows indicate cells that are positive for both the β-galactosidase and the hormone marker. Arrowheads point to cells that are hormone-positive but β-galactosidase-negative. A, anterior lobe; I, intermediate lobe; P, posterior lobe.
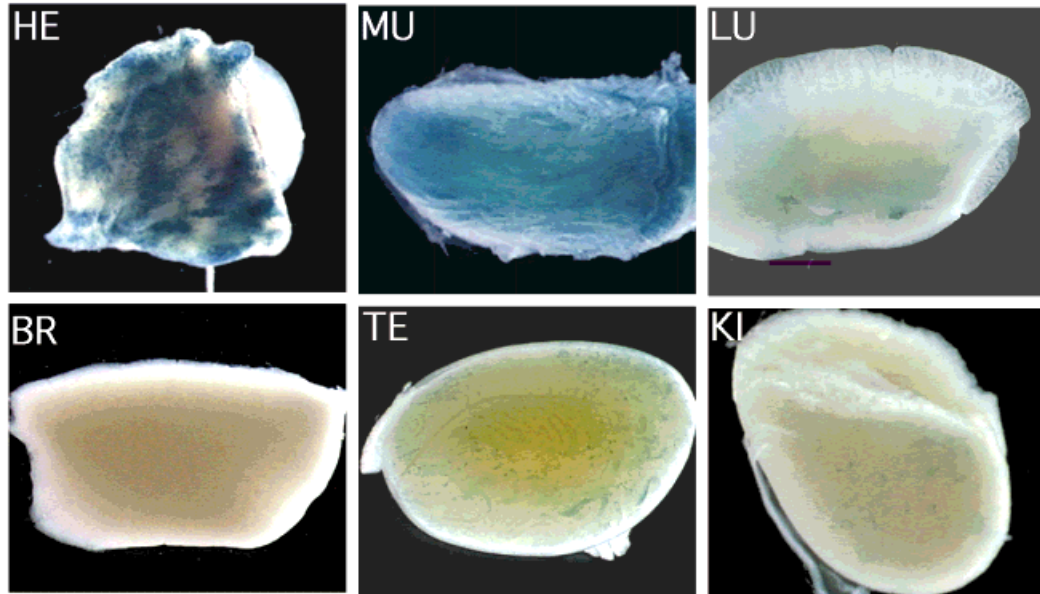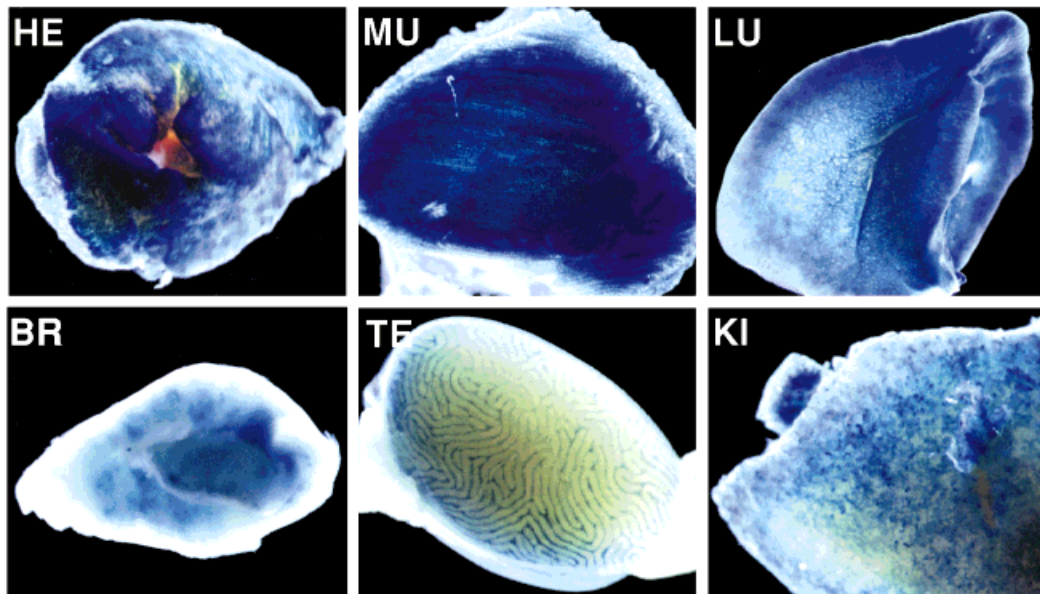
a J7 Reporter, S3 αGSU-cre

b J7 Reporter, S2 αGSU-cre

FIG. 3. S3 αGSU-cre transgene expression is specific and consistent. (**a**) X-gal stained tissues from J7,S3 αGSU-cre doubly transgenic mice reveals that the S3 αGSU-cre transgene is highly expressed in heart (HE) and skeletal muscle (MU). Lower levels of expression are detected in the lung (LU), brain (BR), testis (TE), and kidney (KI). (**b**) These results drastically contrast those found with a ubiquitously expressed transgene (S2 αGSU-cre). Robust X-gal staining was detected in all tissues examined from J7,S2 αGSU-cre animals, demonstrating that the apparent tissue specificity of the S3 αGSU-cre line is not due to limited expression of the J7 reporter transgene.

1994). Prior to cloning, both the cre-containing fragment and pGEM7Zf+ were incubated with Klenow to generate blunt ends. The orientation of the insert and identity of this clone was confirmed by restriction mapping and partial DNA sequencing.

To construct the CAG-GFP-β-gal reporter transgene, a 718 bp *loxP*-GFP fragment was generated by PCR using HPLC-purified oligonucleotides to amplify the human codon optimized GFP contained in PBS-GFP$_h$ (5′-AAA ACT GCA GAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATC CAC CAT GAG CAA GGG CGA G-3′ and 5′-CCC CCC AAG CTT GGG AGA TCT TCA CTT GTA CAG CTC GTC CCA-3′) (Zolotukhin *et al.*, 1996). These primers insert a *loxP* sequence and a *Pst*I site 5′ and *Bgl*II
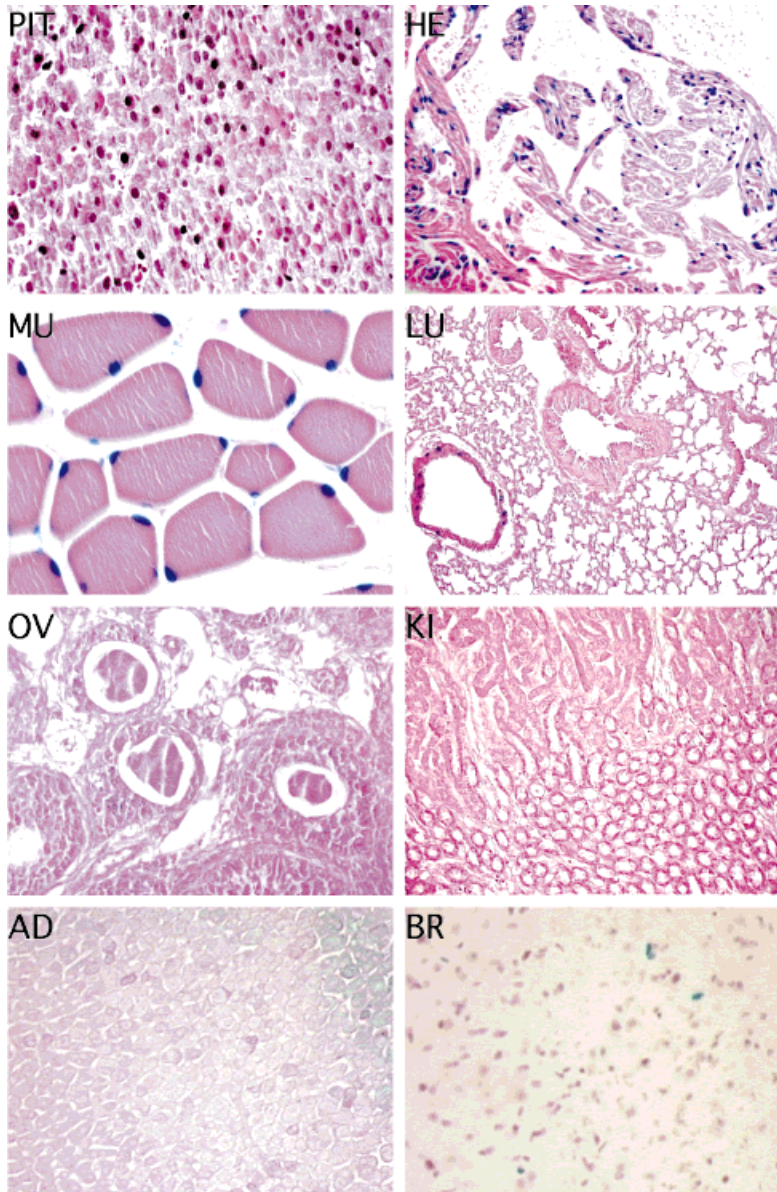
FIG. 4. The S3 αGSU-cre transgene is expressed in the adult pituitary gland and other select tissues. Analysis of β-galactosidase expression in a J7 reporter, S3 αGSU-cre doubly transgenic adult indicates that the S3 αGSU-cre transgene is highly active in the anterior pituitary (PIT) of double transgenics. High levels of αGSU-cre transgene expression are also evident in the heart (HE) and skeletal muscle (MU). Cre transgene activity in the lung (LU), ovary (OV), kidney (KI), adrenal gland (AD), and brain (BR) is low to absent. Analysis of transgene expression in the pituitary gland was assessed by utilizing a β-galactosidase antibody. Expression in all other tissues was examined by X-gal staining.

and *Hind*III sites 3′ of the GFP sequence. Pwo high fidelity DNA polymerase (Boehringer Mannheim, Indianapolis, IN) was used in the amplification to decrease the possibility of sequence errors. This fragment was digested with *Pst*I and *Hind*III and then subcloned into the polylinker of pGEM4Z (p4Z: 2.7, Brett Spear, University of Kentucky). A 0.5 kb fragment containing a mouse protamine 1 (m*P1* or *Prm1*) intron and polyadenylation signal was isolated from pnlacF (Kendall *et al.*, 1994) and placed downstream of the GFP sequence using *Hind*III sites present in both clones, the *Bgl*II site placed 3′ to GFP during amplification, and the *Bam*HI site present at the 3′ end of the m*P1* clone. A 3.6 kb fragment containing the β-galactosidase (β-gal) gene and the m*P1* intron and polyadenylation signal was generated by PCR using HPLC-purified oligonucleotides from pnlacF (5′-GGA GAT CTA TTA CTT CGT ATA GCA TAC ATT ATA CGA

AGT TAT CCA CCA TGG GGC CCA AGA AG-3′ and the pUC18 primer 5′-GTA AAA CGA CGG CCA GT-3′). This introduces a *Bgl*II site and a *loxP* sequence 5′ of the β-gal gene. The fragment was digested with *Bgl*II and subcloned into the *Bgl*II site downstream of the *loxP*-GFP-m*P1* sequence, generating a *loxP*-GFP-*loxP*-β-gal fragment. This 4.8 kb *loxP*-GFP-*loxP*-β-gal cassette was excised by *Pst*I digestion. To generate CAG-*loxP*-GFP-*loxP*-β-gal, the CMV immediate early enhancer and the chicken *β-actin* (CAG) promoter were removed from pCAGGS (Jun-ichi Miyazaki, Tohoku University) (Niwa *et al.*, 1991) by digestion with *Sal*I and *Xho*I. This was then subcloned into the *Sal*I site in the polylinker of pGEM3Z (Promega Corp., Madison, WI) to produce pCAG3Z. The *loxP*-GFP-*loxP*-β-gal cassette was then subcloned into the *Pst*I site of pCAG3Z. The identity of CAG-GFP-β-gal clone was confirmed by sequence analysis at the various
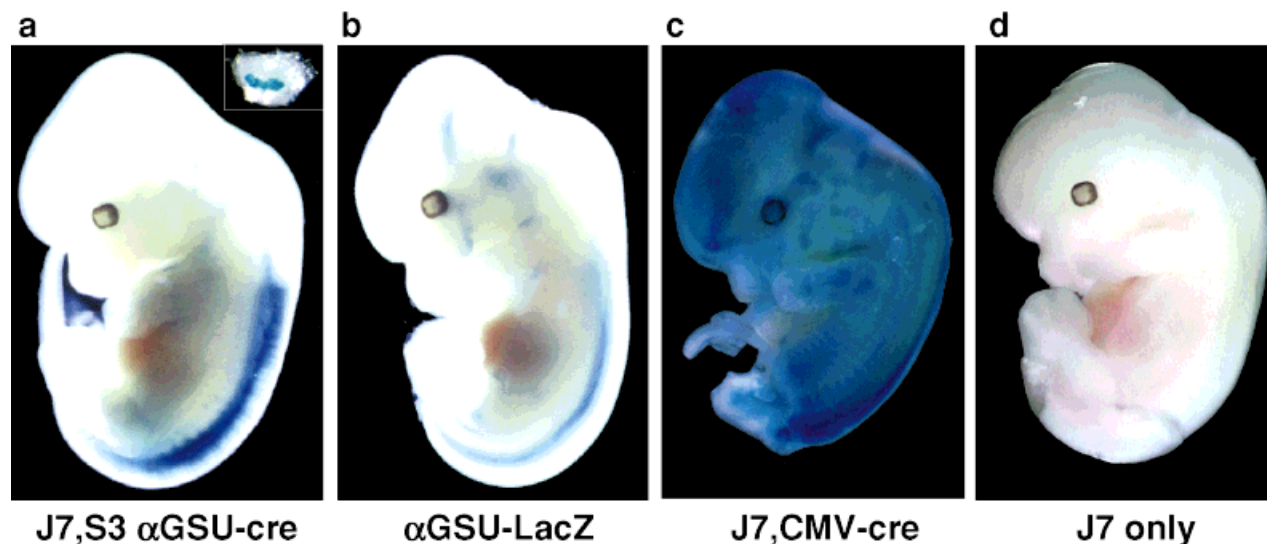
FIG. 5. The S3 αGSU-cre transgene is expressed in the developing pituitary gland by e12.5. (**a**) X-gal-stained J7 reporter, S3 αGSU-cre doubly transgenic embryos reveal that the S3 αGSU-cre transgene is active in the somites, neural tube, and pituitary gland at e12.5. The pattern of X-gal staining in the pituitary is characteristic of endogenous αGSU expression (inset). (**b**) At e12.5, the αGSU-cre transgene produces a staining pattern with the J7 reporter that, although weaker, is consistent with the expression of an αGSU-LacZ transgene at the same time. (**c**) An X-gal stained J7,CMV-cre doubly transgenic embryo indicates that β-galactosidase is being expressed throughout the embryo at e12.5. This indicates that the restricted pattern of expression seen in (**a**) is not limited by the J7 reporter transgene. (**d**) An X-gal stained embryo carrying only the J7 reporter transgene demonstrates that there is no leaky β-galactosidase expression in J7 transgenic animals.

stages of construction, as well as by restriction mapping of the completed plasmid.

Generation, Genotyping, and Breeding of Transgenic Mice

A 7.0 kb αGSU-cre fragment was generated by digestion of the αGSU-cre plasmid with *Kpn*I, which reduces the *αGSU* promoter to 4.6 kb. A 6.5 kb fragment containing the CAG-GFP-β-gal transgene was excised by digestion with *Kpn*I and *Hind*III. Both inserts were isolated by agarose gel electrophoresis and purified with the SpinBind DNA Recovery System (FMC Bioproducts, Rockland, ME). Purified DNA was microinjected into F2 zygotes from C57BL/6J X SJL parents at a concentration of approximately 2–3 ng/μl (Hogan *et al.*, 1994). Embryos at the two-cell stage were transferred to 0.5 postcoitum pseudopregnant CD-1 females. Genomic DNA was prepared from tail biopsies of all progeny born and then screened for the presence of the transgene injected. The αGSU-cre transgenics were identified by PCR using oligonucleotides that amplify a 150 bp product spanning the junction of the *αGSU* promoter and the cre coding sequence (5′-ACA TTG TTC CCC TCA GAT CG-3′ and 5′-CGG TAA TGC AGG CAA ATT TT-3′). Reactions were performed for 30 cycles of denaturation at 92°C for 15 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. CAG-GFP-β-gal transgenics were identified by amplification of a 364 bp product using β-galactosidase-specific primers (5′- TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA-3′ and 5′-ATG TGA GCG AGT AAC AAC CCG TCG GAT TCT-3′). Reactions proceeded for 30 cycles of

denaturation at 92°C for 15 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. All reactions were performed under standard conditions using approximately 100–200 ng of genomic DNA, 0.5 pmol/μl primers, 2.5 mM MgCl$_2$, and 0.02 units/μl Taq DNA polymerase per reaction.

Transgenic founders and their progeny were bred to C57BL/6J mice to establish and maintain lines. Six lines were generated with the αGSU-cre transgene (S1–S6) and seventeen with the CAG-GFP-β-gal transgene (J1–J17). Because GFP fluorescence in the reporter lines was too low for direct detection methods, transgene expression was assessed by breeding these mice to CMV-cre transgenic mice (R. Eisenman, Fred Hutchinson Cancer Research Center) and performing X-gal staining. Stronger fluorescence has been achieved in later generation GFP cassettes (Hadjantonakis *et al.*, 1998). Mice carrying the CMV-cre transgene were identified by PCR using primers that amplify a 268 bp fragment of the cre coding sequence (5′-GCA TAA CCA GTG AAA CAG CAT TGC TG-3′ and 5′-GGA CAT GTT CAG GGA TCG CCA GGC G-3′). Reactions were carried out for 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. To assess the level of αGSU-cre transgene expression, mice from all six lines were mated with mice from the highest expressing reporter line (J7).

(C57BL/6J X SJL/J) F1 mice (The Jackson Laboratory, Bar Harbor, ME) and CD-1 mice (Charles River, Wilmington, MA) were purchased and bred at the University of Michigan. All procedures using mice were approved by the University of Michigan Committee on Use and Care of

Animals. All experiments were conducted in accord with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Histology and Immunohistochemistry

To analyze transgene expression in adult tissues, J7,CMV-cre; J7,S2 αGSU-cre; J7,S3 αGSU-cre; J7,S5 αGSU-cre; and J7,S6 αGSU-cre doubly transgenic animals were sacrificed and whole tissues were removed. Specimens were fixed in 4% paraformaldehyde in phosphate buffered solution (pH 7.2) for 1 h at 4°C, washed in wash buffer (0.1M NaH_2PO_4 (pH 7.3), 2mM $MgCl_2$, 0.02% NP-40), and incubated at 37°C overnight in an X-gal stain solution (1mg/ml X-gal (Boehringer Mannheim, Indianapolis, IN), 5mM $K_3Fe(CN)_6$, and 5mM $K_4Fe(CN)_63H_20$ in wash buffer). Tissues were rinsed in wash buffer and then postfixed in buffered 4% paraformaldehyde for at least 6 h at 4°C. Samples were dehydrated and embedded in paraffin. Five-micron sections were prepared and counterstained with eosin (Sigma Diagnostics, St. Louis, MO).

To assess transgene expression during development, timed pregnancies were generated by crossing J7 with CMV-cre mice and J7 with S3 αGSU-cre mice. Embryos were harvested at embryonic days 11.5, 12.5, and 14.5. Noon of the day the copulatory plug was detected was designated embryonic day 0.5. Following the removal of tails for genotyping (performed as described in the previous section), embryos were treated as described for the whole mount tissues, except that postfixation was done overnight.

The cell specificity of reporter transgene expression in the pituitary gland was evaluated by immunostaining for β-galactosidase followed by immunostaining for the various pituitary hormones. Pituitaries were collected from J7,CMV-cre; J7,S3 αGSU-cre; and J7,S6 αGSU-cre doubly transgenic animals, fixed in buffered 4% paraformaldehyde for 6 h, dehydrated, and then embedded in paraffin. Following paraffin sectioning, immunostaining was performed using polyclonal antiserum against rabbit β-galactosidase (1:500) (Eppendorf- 5 Prime, Inc., Boulder, CO). A biotinylated secondary antibody was used in conjunction with avidin and biotinylated peroxidase (Vectastain rabbit kit, Vector Laboratories, Burlingame, CA). The chromogenic substrate used was 0.0125% diaminobenzidine (DAB), 0.015% $NiCl_2$, and 0.06% H_2O_2 in phosphate buffered solution (pH 7.2), which produces a black precipitate. Immunostaining was then carried out with polyclonal antisera against rat PRL (1:2,000, AFP1050B), rat GH (1:1,000, AFP411S), rat LHβ (1:1000, AFP22238790GPOLHB), rat TSHβ (1:1,000, AFP1274789) (National Hormone and Pituitary Program, NIDDK, Bethesda, MD), and human ACTH (1:1,000, Dako, Santa Barbara, CA) (Kendall *et al.*, 1994). Biotinylated secondary antibodies were used in conjunction with avidin and biotinylated peroxidase (Vectastain guinea pig, rabbit, and human kits, Vector Laboratories, Burlingame, CA). Diaminobenzidine alone, which produces a brown precipitate, was used as the second chromogen.

LITERATURE CITED

Burrows HL, Birkmeier TS, Seasholtz AF, Camper SA. 1996. Targeted ablation of cells in the pituitary primordia of transgenic mice. Mol Endocrinol 10:1467–1477.

Burrows HL, Douglas KR, Seasholtz AF, Camper SA. 1999. Genealogy of the anterior pituitary gland: tracing a family tree. Trends Endocrinol Metab 10:343–352.

Hadjantonakis A, Gertsenstein M, Ikawa M, Okabe M, Nagy A. 1998. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev 76:79–90.

Hogan B, Beddington R, Costantini F, Lacey E. 1994. Manipulating the mouse embryo: a laboratory manual. Plainview, NY: Cold Spring Harbor Laboratory Press.

Ikawa M, Kominami K, Yoshimura W, Tanaka K, Nishimune Y, Okabe M. 1995. Green fluorescent protein as a marker in transgenic mice. Dev Growth Differ 37:455–459.

Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen W-H, Nachtigal MW, Abbud R, Nilson JH, Parker KL. 1994. The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. Genes Dev 8:2302–2312.

Japon MA, Rubinstein M, Low MJ. 1994. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem 42:1117–1125.

Kendall SK, Gordon DF, Birkmeier TS, Petrey D, Sarapura VD, O'Shea KS, Wood WM, Lloyd RV, Ridgway EC, Camper SA. 1994. Enhancer-mediated high level expression of mouse pituitary glycoprotein hormone α-subunit transgene in thyrotropes, gonadotropes, and developing pituitary gland. Mol Endocrinol 8:1420–1433.

Lewandoski M, Wassarman KM, Martin GR. 1997. *Zp-3*, a transgenic mouse line for the activation or inactivation of *loxP*-flanked target genes specifically in the female germ line. Curr Biol 7:148–151.

Luo X, Ikeda Y, Parker KL. 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77:481–490.

Muller U. 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech Dev 82:3–21.

Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.

Orban PC, Chui D, Marth JD. 1992. Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci 89:6861–6865.

Ray M, Fagan S, Moldovan S, DeMayo F, Bruhicardi F. 1998. A mouse model for beta cell-specific ablation of target gene(s) using the cre-loxP system. Biochem Biophys Res Comm 253:65–69.

Sands A, Hansen T, Demayo F, Stanley L, Xin L, Schwartz R. 1993. Cytoplasmic beta-actin promoter produces germ cell and preimplantation embryonic transgene expression. Mol Reprod Dev 34: 117–126.

Tsien J, Chen D, Gerber D, Tom C, Mercer E, Anderson D, Mayford M, Kandel E, Tonegawa S. 1996. Subregion- and cell type-restricted gene knockout in mouse brain. Cell 87:1317–1326.

Utomo AR, Nikitin AY, Lee W-H. 1999. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. Nat Biotechnol 17:1091–1096.

Yu RN, Ito M, Saunders TL, Camper SA, Jameson JL. 1998. Role of *Dax1* in gonadal development and gametogenesis. Nat Genetics 20:353–357.

Zolotukhin S, Potter M, Hauswirth W, Guy J, Muzyczka N. 1996. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J Virol 70:4646–4654.