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 insufficiency (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
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 expression (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 αGSU 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(loxp-lacz)^y/sac) 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 tissue specific expression of the S3 αGSU-cre line is not due to limited potential of the J7 reporter transgene. The tissue specificity of the S5 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 activation 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 endogenous 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 Sall and Asp718. This fragment was subcloned into the ClaI site downstream of the αGSU promoter (-5,000 to +43) in pGEM7Zf+ (Kendall et al.,
**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. Immunohistochemical 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.
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 (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 GTG CCA-3') (Zolotukhin et al., 1996). These primers insert a loxP sequence and a PstI site 5' and BglII

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.
and HindIII 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 PstI and HindIII and then subcloned into the poly linker of pGEM4Z (p4Z: 2.7, Brett Spear, University of Kentucky). A 0.5 kb fragment containing a mouse protamine 1 (mP1 or Prm1) intron and polyadenylation signal was isolated from pnlacF (Kendall et al., 1994) and placed downstream of the GFP sequence using HindIII sites present in both clones, the BglII site placed 3′ to GFP during amplification, and the BamHI site present at the 3′ end of the mP1 clone. A 3.6 kb fragment containing the β-galactosidase (β-gal) gene and the mP1 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 BglII site and a loxP sequence 5′ of the β-gal gene. The fragment was digested with BglII and subcloned into the BglII site downstream of the loxP-GFP-mP1 sequence, generating a loxP-GFP-loxP-β-gal fragment. This 4.8 kb loxP-GFP-loxP-β-gal cassette was excised by PstI 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 SalI and XhoI. This was then subcloned into the SalI site in the poly linker of pGEM3Z (Promega Corp., Madison, WI) to produce pCAG3Z. The loxP-GFP-loxP-β-gal cassette was then subcloned into the PstI site of pCAG3Z. The identity of CAG-GFP-β-gal clone was confirmed by sequence analysis at the various

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.
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 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 MgCl₂, 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 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 αGUS-cre; J7,S5 αGUS-cre; J7,S5 αGUS-cre; and J7,S6 αGUS-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 NaH2PO4 (pH 7.3), 2mM MgCl2, 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 K4Fe(CN)6·3H2O in wash buffer). Tissues were rinsed in wash buffer and then postfixed in buffer 4% paraformaldehyde for at least 6 h at 4°C. Samples were dehydrated in wash buffer and then postfixed in buffer 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 αGUS-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,S5 αGUS-cre; and J7,S6 αGUS-cre doubly transgenic animals, fixed in buffer 4% paraformaldehyde for 6 h, dehydrated, and then embedded in paraffin. Following paraffin sectioning, immunostaining was performed using polyclonal antisera 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% NiCl2, and 0.06% H2O2 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, AFP111S), rat LHβ (1:1000, AFP22258790POLLB), 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.

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LITERATURE CITED


