0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(5):1946–1953

doi: 10.1210/en.2006-1542

Reporter Expression, Induced by a Growth Hormone Promoter-Driven Cre Recombinase (rGHp-Cre) Transgene, Questions the Developmental Relationship between Somatotropes and Lactotropes in the Adult Mouse Pituitary Gland

Raul M. Luque, Geraldine Amargo, Shinya Ishii, Corrinne Lobe, Roberta Franks, Hiro Kiyokawa, and Rhonda D. Kineman

Section of Endocrinology, Diabetes, and Metabolism, Department of Medicine (R.M.L., G.A., S.I., R.D.K.) and Transgenic Production Service (R.F.), Research Resources Center, University of Illinois at Chicago, Chicago, Illinois 60612; Research and Development Division (R.M.L., S.I., R.D.K.), Jesse Brown Veteran's Administration Medical Center, Chicago, Illinois 60612; Sunnybrook and Women's College Health Sciences Centre (C.L.), Toronto, Ontario, Canada M4N 3M5; and Department of Molecular Pharmacology and Biological Chemistry (H.K.), Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

This report describes the development and validation of the rGHp-Cre transgenic mouse that allows for selective Cre-mediated recombination of loxP-modified alleles in the GH-producing cells of the anterior pituitary. Initial screening of the rGHp-Cre parental line showed Cre mRNA was specifically expressed in the anterior pituitary gland of adult Cre+/− mice and cephalic extracts of e17 Cre+/− fetuses. Heterozygote rGHp-Cre transgenic mice were crossbred with Z/AP reporter mice to generate Cre+/−,Z/AP+/− offspring. In this model system, the GH promoter-driven, Cre-mediated recombination of the Z/AP reporter leads to human placental alkaline phosphatase (hPLAP) expression that serves to mark cells that currently produce GH, in addition to cells that would have differentiated from GH cells but currently do not express the GH gene. Double immunocytochemistry of adult male and female Cre+/−,Z/AP+/− pituitary cells revealed the majority (~99%) of GH-producing cells of the anterior pituitary also expressed hPLAP, whereas ACTH-, TSH-, and LH-producing cells were negative for hPLAP, confirming previous reports that corticotropes, thyrotropes, and gonadotropes develop independently of the somatotrope lineage. A small subset (~10%) of the prolactin-producing cells was positive for hPLAP, consistent with previous reports showing lactotropes can arise from somatotropes during pituitary development. However, the fact that 90% of prolactin-producing cells were negative for hPLAP suggests that the majority of lactotropes in the adult mouse pituitary gland develop independently of the somatotrope lineage. In addition to developmental studies, the rGHp-Cre transgenic mouse will provide a versatile tool to study the role of a variety of genes in somatotrope function and neoplastic transformation. (*Endocrinology* 148: 1946–1953, 2007)

CONVENTIONAL KNOCKOUT (NULL) mice generated by recombination-based gene targeting in embryonic stem cells has provided a wealth of information as to the requirement of specific genes for normal cellular development and differentiated function. However, genome-wide inactivation of genes critical for embryonic development can result in a lethal phenotype, making it impossible to study the role of these genes in adult tissues. Also, genome-wide inactivation of gene function may affect multiple tissues, confounding any detailed study of these genes in lineage-specific function. This latter concern is particularly relevant when examining the development and function of the GH-producing cells of the anterior pituitary gland (somatotropes) because developmental and functional control of this cell population is mediated by multiple inputs including: central signaling from peptides released from hypothalamic neurons, feedback of target organ products, and interactions between heterologous cell types within the pituitary gland itself (for review, see Refs. 1–9). Therefore, it is difficult to determine whether alterations in somatotrope development or function observed in conventional gene knockout mice are due to disruption of intrasomatotropic signaling or perturbations in central, local, and/or systemic inputs. To circumvent this problem, we have developed and validated a transgenic mouse line that expresses Cre recombinase within the anterior pituitary gland and selectively directs recombination of a loxP-modified reporter allele in the somatotrope population, as well as a small subset of lactotropes.

First Published Online February 8, 2007

Abbreviations: AP, Alkaline phosphatase; As, antisense; CMV, cytomegalovirus; FVB, FVB/NHsd; hGH, human GH; hPLAP, human placental alkaline phosphatase; no-RT, not reversed transcribed; PRL, prolactin; Sn, sense; TBS, Tris-buffered saline.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Materials and Methods

Animals

All experimental procedures were approved by the Animal Care and Use Committees of the University of Illinois at Chicago and the Jesse

Brown Veterans Administration Medical Center. C57BL/6J (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME), and FVB/NHsd (FVB) mice were purchased from Harlan (Indianapolis, IN). Mice were housed under standard conditions of light (12-h light, 12-h dark cycle; lights on at 0700 h) and temperature (22–24 C), with free access to standard rodent chow (LabDiet, St. Louis, MO; catalog no. 5008) and tap water.

Construct design

To construct the transgene (Fig. 1A), the rGHp-human GH (hGH) plasmid (AmpR+), originally reported by Behringer *et al.* (10) and provided by Dr. Richard Palmiter (University of Washington, Seattle, WA), was modified by placing a oligonucleotide linker, containing a *Mlu*I site, into a *Xho*I site located immediately downstream of the rGH promoter. The design of the linker preserved the *Xho*I site. The *Cre* recombinase gene construct was excised from the cytomegalovirus (CMV)-Cre plasmid [provided by L. Hennighhausen and K. Wagner, National Institutes of Health, Bethesda, MD (11)] using *Xho*I and *Mlu*I and inserted into the complementary site of the modified rGHp-hGH plasmid located within the first exon of the hGH gene, converting it to a 3′-untranslated region. The rGHp-Cre gene was excised using *Kpn*I and *Eco*RI, and the fragment was column purified and sequenced to confirm the structural genes were in the correct orientation and in-frame.

Generation of rGHp-Cre transgenic mice

Purified DNA was microinjected into pronuclear stage FVB zygotes, which were transferred into pseudopregnant recipients. The offspring were initially screened for the transgene incorporation by PCR of genomic DNA obtained from tail snips at weaning using primers that amplify Cre [sense (Sn), CGTACTGACGGTGGGAGAAT and antisense (As), CCCGGCAAAACAGGTAGTTA primers yielding a 166-bp product; GenBank accession no. X03453]. Genomic DNA from Cre-positive mice was further evaluated by Southern blot to confirm the PCR genotyping and estimate transgene copy number. Specifically, purified genomic DNA or rGHp-Cre plasmid DNA (as a standard) was digested using *Bam*HI, and samples were size separated on a 0.8% TAE agarose gel and capillary transferred to Immobilon-Ny+ Transfer Membranes (Millipore Corp., Bedford, MA). Membranes were exposed to UV light to cross-link DNA and subsequently incubated with a radiolabeled probe prepared by random hexamer labeling (HexaLabel Plus DNA Labeling Kit, MRI Fermentas, Hanover, MD) of the *Xho*I/*Mlu*I fragment from the CMV-Cre plasmid (see Fig. 1, A and B). Membranes were washed and exposed to a PhosphorImager screen, and band intensity was evaluated by image analysis software (Molecular Dynamics, Sunnyvale, CA). Germline transmission was verified by cross-breeding rGHp-Cre-positive founders with wild-type B6 mice. To maintain the rGHp-Cre transgenic line, all subsequent backcrosses were performed using pure B6 mice.

Verification of pituitary-specific Cre expression and activity

RNA isolation and RT-PCR. Tissues were obtained from 8- to 10-wk-old mice and processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA), with deoxyribonuclease treatment. The amount of RNA recovered was determined using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1 μg) was reverse transcribed in a 20-μl volume using random hexamer primers, with enzyme and buffers supplied in the cDNA First Strand Synthesis kit (MRI Fermentas). cDNA was treated with ribonuclease H, and duplicate aliquots (1 μl) were amplified by standard PCR (2× Master mix PCR reagent, MRI Fermentas) or quantitative real-time PCR (Brilliant SYBR green QPCR Master Mix, Stratagene) using the same primer set as used for genotyping. Samples not reversed transcribed (no-RT) were used to control for genomic contamination and PCR reactions run without cDNA samples were used to assess reagent contamination. For quantitative assessment of Cre mRNA levels, tissue sample cDNA was run against a synthetic Cre cDNA standard to estimate copy number. Development and validation of the quantitative real-time RT-PCR technique has been described previously (12), with the exception that the annealing temperature for the Cre primer set is 63 C.

To determine whether Cre recombinase was expressed early in the developing pituitary, adult rGHp,Cre+/− females mice were caged with

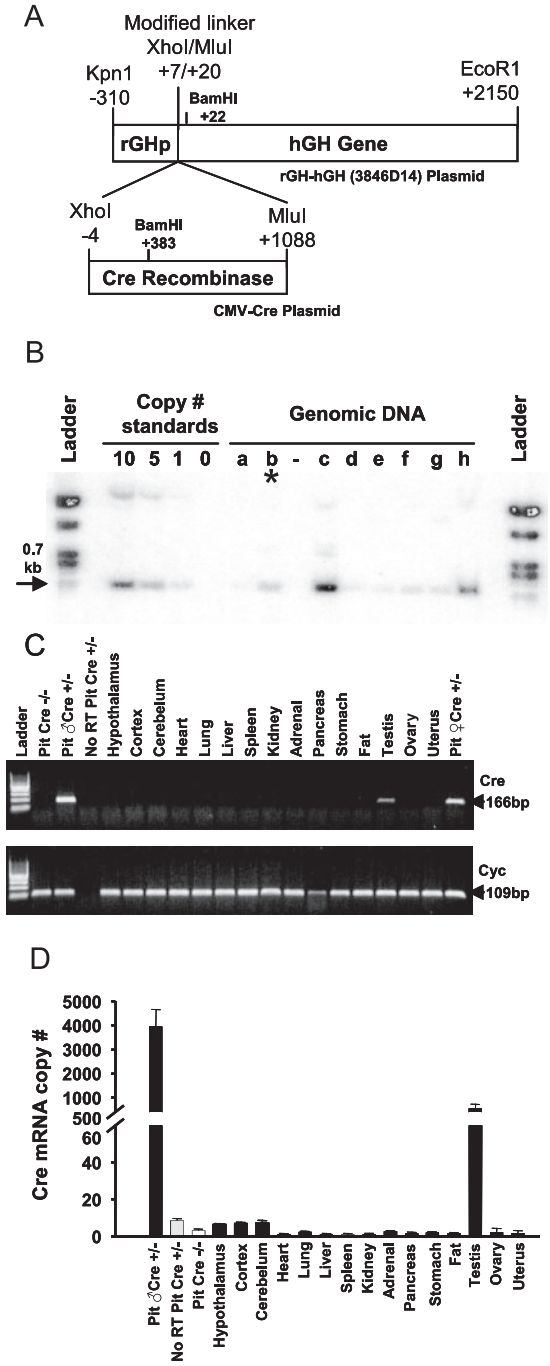

FIG. 1. Design and validation of the rGHp-Cre transgenic mouse. A, Construct design of the rGHp-Cre transgene, with the relevant positions of the structural genes and the key cloning sites, where insertion of Cre downstream of the rGHp converts the hGH gene to a 3′-untranslated region. B, Southern blot confirming PCR genotyping of rGHp-Cre founders (a–h) and estimation of transgene copy number. *Arrow*, Expected size (0.7 kb) of the genomic *Bam*HI fragment containing the Cre transgene, which hybridizes with a radiolabeled *Xho*I/*Mlu*I fragment from the CMV-Cre plasmid. *Asterisk*, rGHp-Cre transgenic mouse line (b), described in detail in this report. C, Representative agarose gel of Cre and cyclophilin (Cyc; used as housekeeping gene) RT-PCR products from various tissues obtained from rGHp-Cre (Cre+/−) transgenic mice. Negative controls consisted of cDNA from a pituitary of a wild-type (Cre−/−) mouse and cDNA from a pituitary of a Cre+/− mouse that was not reverse transcribed (No RT). D, Analysis of Cre mRNA copy number by quantitative real-time RT-PCR in tissue samples from rGHp-Cre mice. Values shown are the mean ± SEM of n = 3–13 mice.

wild-type breeding males and checked daily for a vaginal plug. The morning a plug was found was considered as d 0 postcoital. Pregnant females were killed 17 d postcoital, and fetuses were removed and rinsed in PBS. A hind limb was used to extract genomic DNA for genotype analysis, and the head was taken for RNA extraction. Total RNA (5 μg) was reversed transcribed, and 1 μl was used as a template to amplify the Cre, GH, and prolactin (PRL) transcripts by quantitative real-time RT-PCR, as described above. The following primers were used to amplify mouse PRL: Sn, GGCCATCTTGGAGAAGTGTG and As, ACAGATTGGCAGAGGCTGAA, yielding a 140-bp product (GenBank accession no. NM_011164; annealing, 61 C). Primer sequences used to amplify mouse GH have been previously reported (12).

To confirm that the Cre mRNA was capable of being translated into an active protein that could excise loxP-flanked DNA, heterozygote rGHp-Cre mice (Cre+/−) were crossbred to heterozygote Z/AP mice (13). Z/AP mice express human placental alkaline phosphatase (hPLAP) upon Cre/loxP-mediated excision of the reporter construct. Genomic DNA obtained from offspring from Cre+/− × Z/AP+/− mice was screened by standard PCR for Cre (as described above) and hPLAP (Sn, TGGACGGGAAGAATCTGGTG and As, CATGACGTGCGCTATGAAGGT primers yielding a 704-bp product; GenBank accession no. NM_001632; annealing, 63 C) transgenes. Whole tissues or tissue slices were taken from Cre+,Z/AP+ and Z/AP+ (as negative controls), fixed, heated to 75 C [to inactivate endogenous heat-sensitive alkaline phosphatases (APs)], and processed for AP activity using BM Purple AP Substrate (Roche Diagnostics, Mannheim, Germany), as previously described (13). In addition, some pituitaries from Cre+,Z/AP+ and Z/AP+ mice were fixed in 10% formalin, paraffin embedded, sectioned (5 μm), and processed for AP activity. To verify that the AP activity was the result of hPLAP expression, cDNA samples from pituitaries, testis, and epididymis of Cre+,Z/AP+ and Z/AP+ male mice were evaluated for hPLAP mRNA levels using quantitative real-time RT-PCR (Sn, GAAACGGTCCAGGCTATGTG and As, ATGACGTGCGCTATGAAGGT primers yielding a 205-bp product; GenBank accession no. NM_001632; annealing, 62 C). Finally, anterior pituitaries from Cre+, Z/AP+ and Z/AP+ male and female mice were enzymatically dispersed into single cells as previously described (14), plated on tissue culture slides, fixed, and processed for AP activity or double immunostaining for hPLAP and each pituitary hormone (see below).

Immunocolocalization of the Cre recombinase reporter (hPLAP) and pituitary hormones.

Dispersed pituitary cells (50,000 cells/50 μl α-MEM, Invitrogen, Grand Island, NY) were plated on poly-L-lysine-coated slides (catalog no. 22247; Polysciences, Inc., Warrington, PA). After a 1-h incubation at 37 C, cells were fixed in 4% paraformaldehyde/10 mM PBS for 15 min at room temperature. After fixation, cells were rinsed three times (5 min each) in 20 mM Tris-buffered saline (TBS). Slides were blocked (2 h) with a solution containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; catalog no. 017-000-121) and 1× Power block (BioGenex, San Ramon, CA; catalog no. HK085-5K), diluted in TBS-0.05% Triton X-100. Cells were incubated (2 h, room temperature) with a primary antibody for hPLAP (1:1000; sheep polyclonal PLAP; American Research Products, Belmont, MA; catalog no. 13-2355) together with a rabbit polyclonal antibody from Dr. Albert Parlow (National Hormone and Peptide Program, Torrance, CA), directed against mouse GH (mGH, 1:10,000; lot no. AFP5672099Rb), mouse PRL (1:10,000; lot no. AFP131078Rb), human ACTH (1:10,000; lot no. AFP632803), rat TSH β-subunit (1:5000; lot no. AFP1274789), or rat LH β-subunit (1:5000; lot no. AFP571292393R), diluted in TBS-0.05% Triton X-100. In addition, select slides were incubated simultaneously with antibodies to GH, PRL, and hPLAP. Cells were then washed sequentially with TBS-0.05% Triton X-100 and TBS only, then incubated for 1 h with fluorescent-labeled secondary antibodies diluted 1:200 in TBS (fluorescein isothiocyanate-conjugated AffiniPure Donkey Anti-Sheep IgG and Rhodamine Red-X-conjugated AffiniPure Donkey Anti-Rabbit IgG; Jackson ImmunoResearch Laboratories, catalog nos. 713-095-147 and 711-295-152, respectively). Finally, cells were washed in TBS and mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA; catalog no. H-1200). The secondary antibody incubation and all subsequent steps were performed in low light. The fluorescent signal was visualized using a Zeiss AxioPlan 2 fluorescence microscope with Axio Vision 4.3 software (Carl Zeiss GmbH, Jena, Germany). Controls consisted of Cre+,Z/AP+ pituitary cell preparations processed as described above with the exception of excluding each or both primary antibodies to hPLAP and pituitary hormones. Also, pituitary cell preparations from Z/AP+ mice were processed using the primary antibody to hPLAP, confirming negligible background staining in the absence of the Cre transgene.

Results and Discussion

Generation of rGHp-Cre transgenic mice

To target Cre recombinase expression to GH-producing cells of the anterior pituitary gland, we used 310 bp 5′ of the initiation codon of the rat GH gene (rGHp). This promoter was chosen because this sequence was previously shown in mice to: 1) selectively express the hGH reporter transgene in GH-producing cells of the anterior pituitary gland (10); 2) direct the expression of a diphtheria toxin transgene, resulting in the destruction of the developing somatotrope lineage, GH deficiency, and a dwarf phenotype (10); and 3) direct the expression of a cholera toxin transgene, resulting in constitutive activation of the cAMP signal transduction pathway within the somatotrope, leading to hyperplasia and tumor formation (15). Although the promoter used for the generation of these transgenic mice is of rat origin, the rat sequence is 91% identical with the mouse sequence, and the critical response elements are conserved (16, 17).

Of the embryos injected with the rGHp-Cre construct, 36 mice were born, and eight were positive for the rGHp-Cre transgene, where anterior pituitaries from two lines expressed Cre mRNA as assessed by standard RT-PCR. The following narrative is focused on the rGHp-Cre line that was most effective in mediating recombination of the Z/AP reporter construct in the GH-producing cells of the anterior pituitary. This particular line was shown to carry 3 copies of the rGHp-Cre transgene, as determined by Southern analysis (Fig. 1B, *asterisk*). The growth pattern (published as supplemental Fig. S1 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), physical attributes, and behavior of the rGHp-Cre transgenic mice were identical with their wild-type littermates, suggesting insertion and/or expression of the rGHp-Cre transgene did not perturb normal function.

Tissue specificity of rGHp-Cre expression and activity

Use of standard (Fig. 1C) and quantitative real-time (Fig. 1D) RT-PCR to screen a variety of tissues of rGHp-Cre mice revealed Cre mRNA was strongly expressed in the pituitary, with lower level Cre expression in the testis, whereas all other tissues were negative compared with no RT controls. Cre recombinase expression was also detected in cephalic extracts of e17 Cre+/− fetuses (Fig. 2), a time during mouse pituitary development at which the somatotrope population is rapidly expanding, whereas very few lactotropes are present (18, 19), as reflected by high levels of GH and low levels of PRL mRNA detected in these same extracts.

To determine whether Cre mRNA was capable of being translated into an active protein that could excise loxP flanked genomic DNA, Cre+/− mice were crossbred to Z/AP+/− mice. As previously described (13), Z/AP mice act as a double reporter where a loxP-flanked lacZ reporter

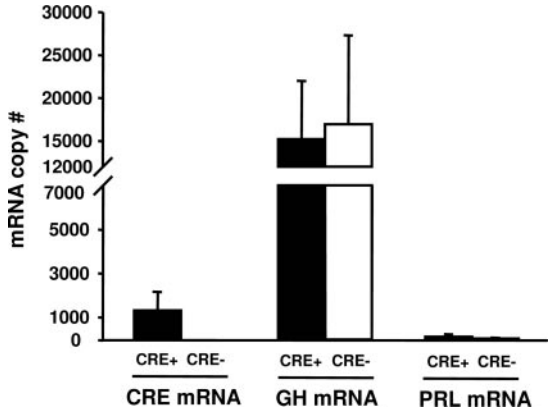

FIG. 2. Cre recombinase, GH, and PRL mRNA copy number in cephalic extracts from embryonic d 17 rGHp-Cre-positive and -negative fetuses, as assessed by quantitative real-time RT-PCR. Values shown are the mean ± SEM of n = 4–7 fetuses.

gene is expressed in the absence of Cre under the control of a CMV promoter. In the presence of Cre, the lacZ gene is excised, allowing for CMV-mediated hPLAP expression. Tissues were taken from Cre+/−,Z/AP+/− mice and Z/AP+/− mice (as negative controls) and processed for heat-stable AP activity using a substrate that stained cells blue. As shown in Fig. 3A, the anterior lobe of the pituitary from a Cre+/−,Z/AP+/− mouse showed strong AP activity (*dark blue*), whereas the intermediate and posterior lobes, as well as the whole pituitary from a Z/AP+/− mouse, were negative. The proportion of anterior pituitary cells of Cre+/−, Z/AP+/− mice that displayed AP activity ranged from 30–50%, depending on the gender of the mouse (for example see Fig. 3, B and C), where the proportion was consistent with the proportion of GH cells previously reported by our laboratory and others (1, 20, 21). All tissues from Cre+/−,Z/AP+/− mice that proved to be negative for Cre mRNA by RT-PCR (Fig. 1, C and D) were also negative for AP activity (published as supplemental Fig. S2 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). However, AP activity (*i.e.* blue staining) was observed in isolated areas in cross-sections of the testis, with strong staining in the epididymis of control mice (wild-type or Z/AP+/−), as well as Cre+/−,Z/AP+/− mice (Fig. 3D), consistent with previous

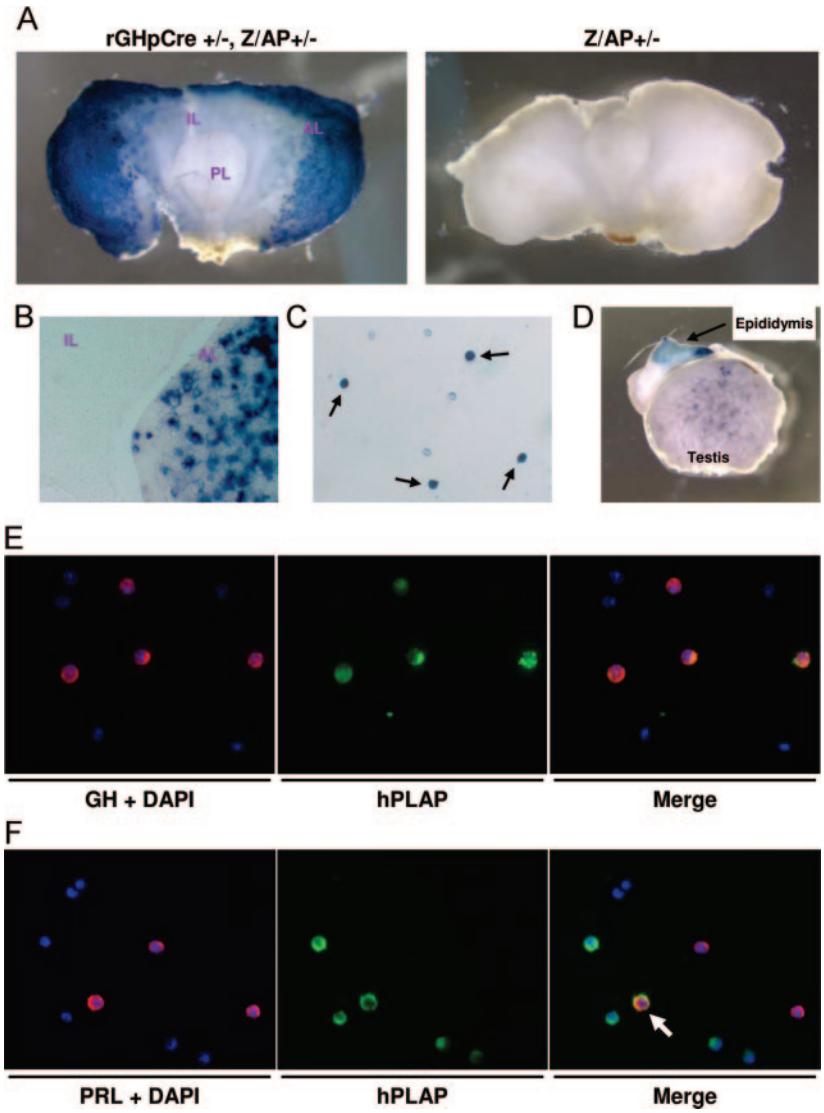

FIG. 3. A, Pituitaries of rGHpCre+/−,Z/AP+/− mice and Z/AP+/− mice (negative controls) stained for heat-stable, AP activity (*blue*). AL, Anterior lobe; PL, posterior lobe; IL, intermediate lobe. B, Paraffin-embedded cross-section (5 μm). C, Dispersed pituitary cells from rGHpCre+/−,Z/AP+/− mice processed for heat-stable AP activity (*arrows* indicate positive cells). D, Bisected testis and epididymis of a rGHpCre+/−,Z/AP+/− mouse processed for AP activity, demonstrating scattered activity (*blue* staining) within the testis and intense staining in the epididymis. A similar staining pattern was observed in wild-type and Z/AP+/− mice (data not shown). E and F, Dispersed pituitary cells from rGHpCre+/−,Z/AP+/− mice immunostained using antibodies directed against mouse (E) GH or (F) PRL (*red*) combined with an antibody directed against hPLAP (*green*). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, *blue*). Images were obtained and processed using a Zeiss AxioPlan 2 fluorescence microscope with Axio Vision 4.3 software. *Arrow* (*white*) indicates a cell that is immunopositive for both PRL and hPLAP.

reports of an endogenous heat-stable AP in M phase spermatocytes and in seminal plasma (22, 23). Therefore, as an alternative strategy to evaluate whether Cre-mediated recombination does occur in the testis, we used quantitative real-time RT-PCR to compare the absolute levels of hPLAP and Cre mRNA in the pituitary, testis, and epididymis of Cre+/−,Z/AP+/− and Z/AP+/− mice of a mixed genetic background (ICR × B6 × FVB). Low expression of hPLAP mRNA was detected in tissues of Z/AP+/− mice (Fig. 4A), whereas hPLAP mRNA was not detected in no-RT samples or in samples obtained from wild-type controls (data not shown), indicating that the hPLAP signal was not due to genomic contamination and that the primers used are specific for the human transgene and did not amplify an endogenous AP transcript. These results indicate that expression of the hPLAP transgene can occur at low levels, independent of the Cre transgene. In the testis, hPLAP mRNA levels in Cre+/−,Z/AP+/− mice did not significantly differ from Z/AP+/− mice (Fig. 4A), indicating that low level of expression of Cre mRNA in the testis does not translate into additional activation of the hPLAP transgene above background levels. It should also be noted that the level of Cre expression in the testis of Cre+/−,Z/AP+/− mice (ICR × B6 × FVB background; Fig. 4B) was significantly less than observed in our screening of the parent rGHp-Cre line (F9 generation of FVB × B6 backcross; Fig. 1D), suggesting that genetic background may influence the nonspecific expression of the transgene in the testis. Nonetheless, the pituitaries of Cre+/−,Z/AP+/− mice expressed high levels of both hPLAP and Cre mRNA (Fig. 4, A and B, respectively), consistent with the strong AP activity as shown in Fig. 3, A–C. Taken together these results demonstrate that rGHp-Cre transgenic mice represent a model system that allows for anterior pituitary-specific recombination of loxP-modified alleles.

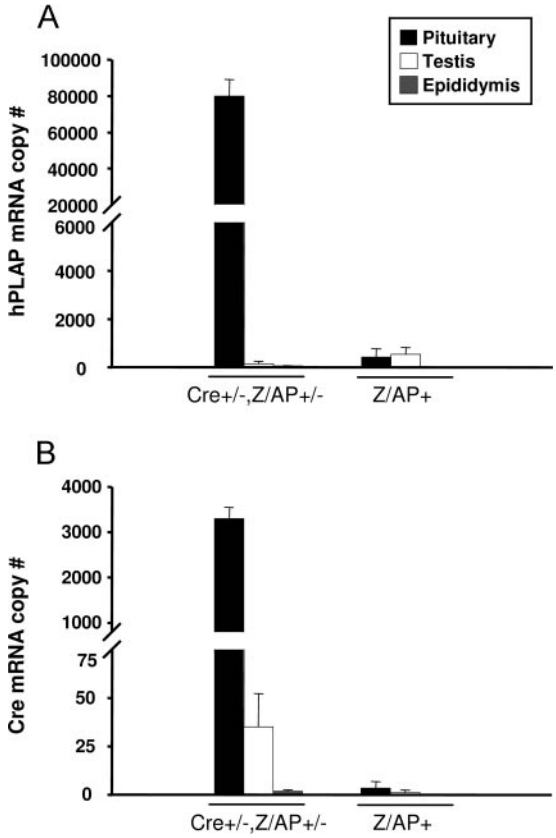

FIG. 4. hPLAP (A) and Cre recombinase (B) mRNA copy numbers in pituitary, testis, and epididymis of Cre+/−,Z/AP+/− and Z/AP+/− male mice, as assessed by quantitative real-time RT-PCR. Values shown represent the mean ± SEM of three mice per group.

rGHp-Cre-mediated recombination in anterior pituitary cell types

To determine which anterior pituitary cell types undergo Cre-mediated recombination, we performed double immunocytochemistry for hPLAP and each pituitary hormone (GH, PRL, ACTH, TSH, and LH) on pituitary cell preparations from Cre+/−,Z/AP+/− mice. rGHp-Cre mediated recombination of the Z/AP transgene did not occur in corticotropes, thyrotropes, or gonadotropes, in that all cells that were immunopositive for ACTH, TSH, or LH were negative for hPLAP (Fig. 5 and supplemental Fig. S3 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). However, the majority of GH-positive cells (Fig. 3E, shown in *red*) also stained positive for hPLAP (shown in *green*), where the relative proportions of somatotropes (Fig. 5) were greater in male mice (46.8 ± 2.4%), compared with female mice (29.7 ± 1.3%), consistent with previous reports (24–26). However, it should be noted that a small percentage of GH-positive cells (males, 0.8 ± 0.2%; females, 1.2 ± 0.6% of all pituitary cells) were clearly negative for hPLAP (*solid arrow*; supplemental Fig. S4 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Although we cannot completely exclude the possibility that this small subset of somatotropes is due to the inability of the rGH promoter used to target all somatotropes, it is also possible that these somatotropes express hPLAP at levels well below the detection of the assay system, or select somatotropes can inactivate the Cre or hPLAP transgene. It should be noted that Behringer *et al.* (10) also reported that a small subset of GH cells (~1%) in the rGH-DT-A transgenic mouse pituitary escaped rGHp-mediated diphtheria toxin destruction. Because these somatotropes were found in clusters, the authors hypothesized their survival may be due to clonal inactivation of the transgene, possibly by hypermethylation.

Although the majority of the hPLAP-immunopositive cells in Cre+/−,Z/AP+/− pituitaries were somatotropes (Fig. 5), a small percentage of the total pituitary cell population was clearly hPLAP positive but GH negative (males, 3.2 ± 0.4%; females, 4.9 ± 1.9%; see supplemental Fig. S4, *open arrow*). Given that these GH-negative/hPLAP-positive cells could not be accounted for by corticotropes, thyrotropes, and gonadotropes, coupled with the fact that their proportions were similar to the proportion of cells that expressed both hPLAP and PRL (Fig. 3F) in males (3.2 ± 0.3%; Fig. 5, *top*) and females (5.1 ± 0.5%; Fig. 5, *bottom*), suggest that the majority of these cells may represent a subset of the lactotrope population. To directly test this hypothesis, additional slides from the same pituitary cell preparations were also incubated with both GH and PRL antibodies (to label all acidophils) and the hPLAP antibody (to label cells that have undergone recombination). As shown in Fig. 6A, the majority of hPLAP-

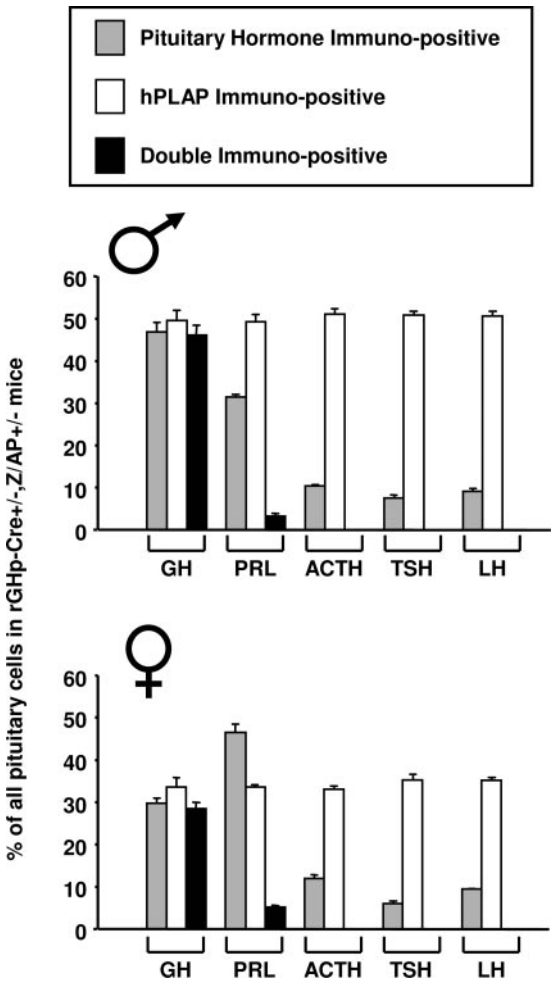

FIG. 5. Relative proportions of GH-, PRL-, ACTH-, TSH-, and LH-immunopositive cells (*gray*), hPLAP-immunopositive cells (*white*), and those cells that were immunopositive for both hPLAP and a pituitary hormone (*black*) in dispersed pituitaries of Cre+/−,Z/AP+/− mice. Immunocytochemistry for each pituitary hormone, combined with immunostaining for the hPLAP transgene, was performed on separate slides prepared from a single pituitary. The data shown represent the mean ± SEM of three to four male and female pituitary cell preparations, where the proportions shown were derived from counting approximately 1000 cells/slide for each pituitary hormone tested.

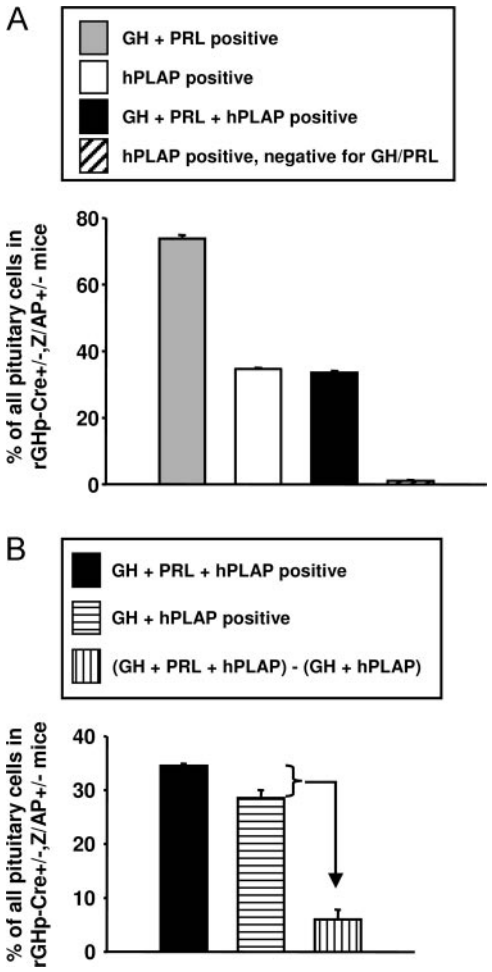

FIG. 6. A, Relative proportions of all GH- and PRL-immunopositive cells (*gray bar*), hPLAP-immunopositive cells (*white*), immunopositive cells for hPLAP, GH, and PRL (*black bar*), and those cells that were immunopositive for hPLAP but negative for GH/PRL (*diagonal-lined bar*) in dispersed pituitary cell preparations from Cre+/−,Z/AP+/− female mice. Immunocytochemistry was performed using primary antibodies for GH, PRL, and hPLAP on the same slide. B, Relative proportions of immunopositive cells for hPLAP, GH, and PRL (*black bar*, repeated from A), compared with the proportion of cells immunopositive for GH and hPLAP (*horizontal-lined bar*, as determined from different slides of the same pituitary preparations processed at the same time). The difference between these values (*vertical-lined bar*) represents lactotropes that have undergone Cre-mediated recombination. The data shown represent the mean ± SEM of three separate pituitary cell preparations, where the proportions shown were derived from counting approximately 1000 cells/slide.

immunopositive cells in female pituitary cell preparations (*white bar*) are indeed acidophils (GH or PRL immunopositive, *black bar*). Given the relative proportion of acidophils that express hPLAP (Fig. 6B, 34.5 ± 0.4%; *black bar*) is greater than ($P < 0.01$) the proportion of somatotropes that express hPLAP (28.5 ± 1.5%; determined from slides incubated with antibodies to GH and hPLAP, Fig. 6B, *horizontal-lined bar*), we can conclude that the difference (6 ± 1.8%, *vertical-lined bar*) represents lactotropes that have undergone Cre-mediated recombination. Also of interest is the fact that the proportion of acidophils in females (74%, Fig. 6A, *gray bar*) is less than the sum of the proportions of GH (30%) and PRL (47%) cells shown in Fig. 5, indicating that there is a functional overlap of these cell populations (3%), supporting the existence of a pituitary cell type that can simultaneously produce GH and PRL. These dual hormone-producing cells are commonly referred to as mammosomatotropes and have been reported to comprise from 2.5–10% of the anterior pituitary cell population, depending on the species, gender, and physiologic status of the tissue donor (26–29). However, it should be noted that the approach used in the current report does not allow us to determine which of the acidophils that have undergone Cre-mediated recombination (*i.e.* are hPLAP positive) are active mammosomatotropes.

In the current model system, the GH promoter-driven, Cre-mediated recombination of the Z/AP reporter leads to hPLAP expression, driven by the constitutive CMV promoter, which serves to mark cells that produce GH, in ad-

dition to cells that would have differentiated from GH cells but currently do not express the GH gene. Therefore, the hPLAP reporter serves as a permanent marker of GH ancestry and provides a unique advantage over other systems in which reporter expression is directly driven by the GH promoter and is dependent on continued expression of the endogenous GH gene. Our results indicate that approximately 10% of the lactotrope population, in adult male and female mice, are derived from a somatotrope lineage. This small percentage is somewhat surprising in light of the fact that the pituitaries of rGH-DT-A transgenic mice, which used the same rGH promoter sequence as the current study to target diphtheria toxin-mediated destruction of GH-producing cells, are severely depleted of lactotropes (10). A similar depletion of the somatotrope and lactotrope populations was observed in rGHp-thymidine kinase transgenic mice, treated with (1-(2-deoxy-2-fluoro-β-D-arabino furanosyl) 5-iodouracil) to kill dividing cells (30). These observations, coupled with the fact that the development of PRL-secreting cells in the rat and cow (28, 31) follows the appearance of GH-secreting cells and that these early lactotropes also secrete GH, has led to the conclusion that the majority of lactotropes arise from GH-producing cells during pituitary development. However, the differences in our current results, compared with the findings of others, might be reconciled by comparing the phenotype of the transgenic models used. In the current system, Cre-mediated recombination of the Z/AP reporter construct and subsequent expression of hPLAP do not perturb normal somatotrope development and function, as indicated by the fact that the body weights, pituitary cell number, percent GH-immunopositive cells, and GH mRNA levels did not differ between Cre+/−,Z/AP+/− mice and their Cre+/− or wild-type littermates (supplemental Table S1 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). However, targeted destruction of the majority of GH cells in rGH-DT-A or rGHp-TK mice resulted in a dramatic reduction in circulating GH and IGF-I and a dwarf phenotype (10, 30). Several pieces of evidence suggest that IGF-I is required for optimal expansion of the lactotrope population. First, pituitaries of adult IGF-I knockout mice are approximately half the size of those of IGF-I intact controls and the proportion of lactotropes is reduced by 50%, whereas the proportion of somatotropes is maintained (32). The number of PRL-immunopositive cells are also reduced in GH receptor knockout mice, which have circulating IGF-I levels one tenth of wild-type controls (21). Finally, IGF-I promotes proliferation of lactotropes in primary rat pituitary cultures as measured by 3[H]-thymidine incorporation (33). Therefore, we suggest that the reduction of lactotropes in the pituitaries of rGH-DT-A and rGHp-TK mice is secondary to the reduction in IGF-I input. Additional support for the parallel development of the somatotrope and lactotrope populations in the mouse pituitary gland comes from the observations of Japon et al. (19), who used in situ hybridization to track the development of the major pituitary cell populations from embryonic d 9.5 to postnatal d 1. They observed that both PRL and GH cells were first detectable at e15.5. However, PRL cells were localized to the ventromedial area of the developing pituitary gland, whereas GH cells were located more laterally. Of note is the fact that this differential distribution was maintained as the GH cell population dramatically expanded with advancing age. These observations were interpreted to indicate that at least some lactotropes and somatotropes arise from distinct groups of committed cells, consistent with our current findings.

Summary

This report describes the development and validation of the rGHp-Cre transgenic mouse that will allow for selective Cre-mediated recombination of loxP-modified alleles in the GH-producing cells of the anterior pituitary. The rGHp-Cre mouse represents a highly versatile experimental tool because these mice can be crossbred with existing mouse strains that carry conditional alleles resulting in excision, inversion, or translocation of loxP-flanked sequences that would inactivate or activate the gene of interest, depending on the specific design of the loxP-modified allele. To date, over 100 strains of mice expressing loxP-modified alleles have been reported, and more are being developed (as discussed in a special issue of *Genesis*, vol. 32, issue 2, 2002, "Conditional Alleles in Mice"; http://www.wiley.com/legacy/products/subject/life/anatomy/genesis_mice.html). Therefore, the rGHp-Cre transgenic mouse provides a unique opportunity to answer questions regarding the specific role of genes in somatotrope development, function, and neoplastic transformation.

Acknowledgments

We thank Drs. Richard Palmiter (University of Washington, Seattle, WA), Hugo Vankelecom (University of Leuven, K.U. Leuven, Belgium), Mary Gillam (Northwestern University, Feinberg School of Medicine, Chicago, IL), and Jennifer Schmidt (University of Illinois at Chicago, Chicago, IL) for helpful comments. We also thank Dr. Douglas Feinstein and Paul Polak (University of Illinois at Chicago, Chicago, Illinois) for help and advice regarding fluorescence microscopy.

Received November 17, 2006. Accepted January 26, 2007.

Address all correspondence and requests for reprints to: Rhonda D. Kineman, Ph.D., Jesse Brown Veterans Administration Medical Center, Research and Development Division, Mail Post 151, West Side, Suite 6215, 820 South Damen Avenue, Chicago, Illinois 60612. E-mail: Kineman@uic.edu.

This work was supported by the Secretaria de Universidades, Investigación y Tecnología de la Junta de Andalucia (to R.M.L.), by a grant from the University of Illinois at Chicago Campus Research Board, and by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases Grant 30677 (to R.D.K.).

The authors have nothing to disclose.

References

1. **Yeung CM, Chan CB, Leung PS, Cheng C** 2006 Cells of the anterior pituitary. Int J Biochem Cell Biol 38:1441–1449
2. **Rosenfeld MG, Briata P, Dasen J, Gleiberman AS, Kioussi C, Lin C, O'Connell SM, Ryan A, Szeto DP, Treier M** 2000 Multistep signaling and transcriptional requirements for pituitary organogenesis in vivo. Rec Prog Horm Res 55:1–13
3. **Cooke NE, Liebhaber SA** 1999 Regulation of growth hormone gene expression. In: Kostyo JL, Goodman HM, eds. Hormonal control of growth. New York: Oxford University Press; 163–185
4. **Wehrenberg WB, Giustina A** 1999 Feedback regulation of growth hormone secretion. In: Kostyo J, Goodman HM, eds. Hormonal control of growth. Vol V. New York: Oxford University Press; 299–327
5. **Chowen JA, Frago LM, Argente J** 2004 The regulation of GH secretion by sex steroids. Eur J Endocrinol 151:U95–U100
6. **Maccario M, Tassone F, Grottoli S, Rossetto R, Gauna C, Ghigo E** 2002

Neuroendocrine and metabolic determinants of the adaptation of GH/IGF-I axis to obesity. Ann Endocrinol (Paris) 63:140–144
7. **Scacchi M, Ida Pincelli A, Cavagnini F** 2003 Nutritional status in the neuroendocrine control of growth hormone secretion: the model of anorexia nervosa. Front Neuroendocrinol 24:200–224
8. **Weltman A, Wideman L, Weltman JY, Veldhuis JD** 2003 Neuroendocrine control of GH release during acute aerobic exercise. J Endocrinol Invest 26: 843–850
9. **Vankelecom H, Denef C** 1997 Paracrine communication in the anterior pituitary as studied in reaggregate cell cultures. Microsc Res Tech 39:150–156
10. **Behringer RR, Mathews LS, Palmiter RD, Brinster RL** 1988 Dwarf mice produced by genetic ablation of growth hormone-expressing cells. Genes Dev 2:453–461
11. **Wagner K, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li M, Furth PA, Hennighausen L** 1997 Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 25:4323–4330
12. **Luque RM, Kineman RD** 2006 Impact of obesity on the growth hormone axis: evidence for a direct inhibitory effect of hyperinsulinemia on pituitary function. Endocrinology 147:2754–2763
13. **Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A** 1999 Z/AP, a double reporter for cre-mediated recombination. Dev Biol 208:281–292
14. **Aleppo G, Moskal SF2, DeGrandis PA, Kineman RD, Frohman LA** 1997 Homologous down-regulation of growth hormone-releasing hormone receptor messenger ribonucleic acid levels. Endocrinology 138:1058–1065
15. **Burton FH, Hasel KW, Bloom FE, Sutcliffe JG** 1991 Pituitary hyperplasia and gigantism in mice caused by a cholera toxin transgene. Nature 350:74–77
16. **Das P, Meyer L, Seyfert H-M, Brockmann G, Schwerin M** 1996 Structure of the growth hormone-encoding gene and its promoter in mice. Gene 169:209–213
17. **Copp RP, Samuels HH** 1989 Identification of an adenosine 3′,5′-monophosphate (cAMP)- responsive region in the rat growth hormone gene: evidence for independent and synergistic effects of cAMP and thyroid hormone on gene expression. Mol Endocrinol 3:790–796
18. **Dolle P, Castrillo J-L, Theill LE, Deerinck T, Ellisman M, Karin M** 1990 Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. Cell 60:809–820
19. **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
20. **Kineman RD, Aleppo G, Frohman LA** 1996 The tyrosine hydroxylase-human growth hormone (GH) transgenic mouse as a model of hypothalamic GH deficiency: growth retardation is the result of a selective reduction in somatotrope numbers despite normal somatotrope function. Endocrinology 137: 4630–4636
21. **Kineman RD, Teixeira LT, Amargo GV, Coschigamo KT, Kopchick JJ, Frohman LA** 2001 The effect of GHRH on somatotrope hyperplasia and tumor formation in the presence and absence of GH signaling. Endocrinology 142: 3764–3773
22. **Narisawa S, Hofmann M-C, Ziomek CA, Millan JL** 1992 Embryonic alkaline phosphatase is expressed at M-phase in the spermatogenic lineage of the mouse. Development 116:159–165
23. **Tang Y** 1998 Galactosyltransferase, pyrophosphatase and phosphatase activities in luminal plasma of the cauda epididymidis and in the rete testis fluid of some mammals. J Reprod Fertil 114:277–285
24. **Ho KY, Leong DA, Sinha YN, Johnson ML, Evans WS, Thorner MO** 1986 Sex-related differences in GH secretion in rat using reverse hemolytic plaque assay. Am J Physiol 250:E650–E654
25. **Hoeffler JP, Frawley LS** 1986 Capacity of individual somatotropes to release growth hormone varies according to sex: analysis by reverse hemolytic plaque assay. Endocrinology 119:1037–1041
26. **Villalobos C, Nunez L, Garcia-Sancho J** 2004 Phenotypic characterization of multi-functional somatotropes, mammotropes and gonadotropes of the mouse anterior pituitary. Pflugers Arch Eur J Physiol 449:257–264
27. **Frawley LS, Boockfor FR** 1991 Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. Endocr Rev 12:337–355
28. **Kineman RD, Faught WJ, Frawley LS** 1992 The ontogenic and functional relationships between growth hormone- and prolactin-releasing cells during the development of the bovine pituitary. J Endocrinol 134:91–96
29. **Porter TE, Hill JB, Wiles CD, Frawley LS** 1990 Is the mammosomatotrope a transitional cell for the functional interconversion of growth hormone- and prolactin-secreting cells? Suggestive evidence from virgin, gestating, and lactating rats. Endocrinology 127:2789–2794
30. **Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM** 1989 Transgenic mice with inducible dwarfism. Nature 339:538–541
31. **Hoeffler JP, Boockfor FR, Frawley LS** 1985 Ontogeny of prolactin cells in neonatal rats: initial prolactin secretors also release growth hormone. Endocrinology 117:187–195
32. **Stefaneanu L, Powell-Braxton L, Won W, Chandrashekar V, Bartke A** 1999 Somatotroph and lactotroph changes in the adenohypophyses of mice with disrupted insulin-like growth factor I gene. Endocrinology 140:3881–3889
33. **Oomizu S, Takeuchi S, Takahashi S** 1998 Stimulatory effect of insulin-like growth factor I on proliferation of mouse pituitary cells in serum-free culture. J Endocrinol 157:53–62

Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**