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Expression of Pituitary Hormones in the Pax8–/– Mouse Model of Congenital Hypothyroidism

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Signaling mechanisms in pituitary morphogenesis as well as pituitary cell fate determination during early embryonic development are relatively well characterized. In contrast, the cues that determine the progression of the various anterior pituitary cell types during postnatal periods are poorly defined. Pax8−/− mice, which are born without a thyroid gland, were used to study the influence of thyroid hormones on the expression of pituitary hormones during early postnatal life. Serum pituitary hormones were determined by RIAs, and the pituitaries were analyzed by Northern blotting, *in situ* hybridization histochemistry, and immunocytochemistry. In 21-d-old Pax8−/− mice, the cellular composition of the anterior pituitary was dramatically distorted. Thyrotropes exhibited hypertrophy and hyperplasia, the number of detectable somatotropes was drastically reduced, and lactotropes were almost undetectable. Expression of LH and FSH was also reduced, but ACTH and proopiomelanocortin expression was not significantly different. Serum pituitary hormone levels were changed correspondingly. T_4 replacement therapy for variable time periods normalized TSH and GH mRNA expression within 3 d but not prolactin expression, not even when T_4 was administered for 6 d in combination with estradiol. These findings reveal the importance of thyroid hormones in developing the appropriate proportions of anterior pituitary cell types, especially with regard to lactotropes. (*Endocrinology* 145: 1276–1283, 2004)

CONGENITAL HYPOTHYROIDISM (CH) is a relatively common disorder occurring once in approximately 3600 live births (1). If not treated immediately after birth by thyroid hormone replacement therapy, severe forms of thyroid hormone deficiency leads to the syndrome of cretinism, a disorder that is characterized by growth retardation, metabolic disturbances, and severe neurological deficits (for review, see Refs. 2 and 3).

CH mainly results from thyroid dysgenesis and in many cases even from thyroid agenesis (1). Most of these cases appear sporadically with the consequence that the hypothyroid fetus is born by a euthyroid mother. This condition perfectly matches with the situation of athyroid Pax8−/− mice, which are born by euthyroid Pax8+/− dams. Deletion of the Pax8 gene in mice results in thyroid agenesis with the consequence that the athyroid Pax8−/− mice die within the first few weeks of life (4). When treated with thyroxine, however, Pax8−/− pups survive weaning and develop properly without overt deficits, indicating that the deletion of the paired box-transcription factor Pax8 specifically affects the development of the thyroid gland. Other structures expressing Pax8, such as the developing kidney or spinal cord (5), are clearly not affected, a phenomenon that is most likely explained by the redundant function of other Pax genes (6).

As an animal model, Pax8−/− mice are ideally suited to study the consequence of CH for the development of the adenohypophysis during critical periods of late embryonic development and early postnatal life. This is of special interest because this master gland of the endocrine system is a major component for the maintenance of homeostasis, metabolism, reproduction, growth, and lactation in all higher life forms. Homeostasis within the anterior pituitary (AP) is controlled by hypothalamic factors, intrapituitary communication systems, and peripheral hormones via feedback regulatory mechanisms. As a consequence, changes in different physiological, pathological, and developmental states considerably influence the synthesis and secretion of pituitary hormones from distinct cell types as well as the overall pituitary cell composition. This is clinically evident in various forms of pituitary hormone hypersecretion and hyperplasia caused by the loss of negative feedback regulatory mechanisms. Lactotrope hyperplasia also occurs naturally during pregnancy and lactation, a process that is linked to increased blood levels of estrogens (7, 8).

Developmentally, the progression of the various cell types in the AP still continues after birth. This is most evident for the generation of lactotropes whose cell numbers rapidly increase during postnatal life, slightly preceded by gonadotropes (9, 10). Whereas the organogenesis and morphogenesis of the AP during early embryonic development has been extensively studied by genetic analysis (11), our knowledge about the extra- and intracellular cues that induce the developmental patterns during late embryonic stages and early postnatal life is still very limited. The role of thyroid hormones during these phases has not been ultimately defined,

Abbreviations: AP, Anterior pituitary; BW, body weight; CH, congential hypothyroidism; α-GSU, α-glycoprotein subunit; ISH, *in situ* hybridization histochemistry; P3, postnatal d 3; POMC, proopiomelanocortin; RT, room temperature.

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and therefore, we were interested in studying the expression of pituitary hormones in the athyroid Pax8 mutant mice during early postnatal life.

Materials and Methods

Experimental animals

Animals were maintained according to the guidelines of the Animal Welfare Committee of the Medizinische Hochschule Hannover (Hannover, Germany). Standard laboratory chow and tap water were provided *ad libitum*. A temperature of 21 C and alternating 12-h light, 12-h dark cycles were controlled automatically. Pax8+/− male and female mice were used for mating. After birth, litter size was successively reduced to four pups to increase the survival of the mutants. Experiments were performed with young animals kept with their mothers. Wild-type controls were littermates of Pax8 mutants. Groups of six animals were injected sc with T_4 [20 ng/g body weight (BW)] from P3–P20, P15–P20, or P18–P20. In addition, two groups of six animals were treated from P18–P20 with estradiol injected sc, one group with 5 ng/g BW and the other group with 100 ng/g BW. Animals were decapitated, and tissues were removed quickly, frozen in liquid nitrogen, and stored at −80 C until further processing. Trunk blood was collected, and serum, obtained by centrifugation, was stored at −80 C.

Southern blotting

Genotypes were determined on tail lysates by Southern blot analysis. The isolated DNA was digested with *Pst*1, size separated in a 1% Tris-borate EDTA buffer (90 mM Tris, 90 mM borate, and 1 mM EDTA, pH 8.4) agarose gel and then capillary transferred to a nylon membrane (Hybond N, Amersham, Freiburg, Germany). The membranes were hybridized with probes specific for the neomycin gene and the replaced exon 4 of the Pax8 gene (4) and then washed twice in 20 mM NaP_i (pH 7.2)/1% SDS at 60 C and once at 65 C. The signals were analyzed using a phosphoimager (Fujix BAS 1000; Fuji Photo Film Co., Düsseldorf, Germany).

Northern blotting

For each experimental group, 10 pituitaries were pooled and homogenized in lysis buffer. Polyadenylated RNA was prepared by using magnetic oligo (deoxythymidine) Dynabeads (Deutsche Dynal, Hamburg, Germany) as suggested by the supplier. Samples were size fractionated by electrophoresis in a denaturing formaldehyde/agarose gel, capillary transferred to a nylon membrane (Hybond XL; Amersham, Freiburg, Germany) and cross-linked by UV irradiation. Hybridization was performed under high-stringency conditions (42 C; 16 h in 50% formamide, 0.5% SDS, 100 μg/ml salmon sperm DNA, 0.9 M NaCl, 12 mM EDTA, and 0.09 M sodium phosphate, pH 7.4) with 100 ng of the labeled cDNA fragments that were generated by random prime labeling of template DNA with [α-^{32}P]dCTP. The membranes were washed to a final stringency of 0.2× SSPE (30 mM NaCl, 2 mM Na_2HPO_4, and 0.2 mM EDTA, pH 7.4) and 0.3% SDS at 59 C for 30 min and exposed to x-ray film BIOMAX MS (Kodak, Rochester, NY). The signals were quantified by phosphorimaging. After stripping the membranes, cyclophilin mRNA was similarly determined to confirm the integrity and uniformity of RNA loading (12).

In situ hybridization

After the animals were decapitated, pituitaries were removed rapidly, embedded in Tissue-Tek medium (Sakura Finetek, Torrance, CA), and frozen on dry ice. Sections (16 μm) were cut on a cryostat (Leica, Bentheim, Germany), thaw mounted on silane-treated slides, and stored at −80 C until further processing.

In situ hybridization histochemistry was carried out as described previously (13). Briefly, frozen sections were fixed in a 4% phosphate-buffered paraformaldehyde solution (pH 7.4) for 1 h at room temperature (RT), rinsed with PBS, and treated with 0.4% phosphate-buffered Triton X-100 solution for 10 min. After washing with PBS and water, tissue sections were incubated in 0.1 M triethanolamine (pH 8) containing 0.25% (vol/vol) acetic anhydride for 10 min. After acetylation, sections were rinsed several times with PBS, dehydrated by successive washing with increasing ethanol concentrations, and air dried.

Digoxigenin-labeled probes were generated from cDNA subclones in pGEM-plasmids (Promega, Mannheim, Germany) with a DIG RNA labeling kit (Boehringer, Mannheim, Germany). *In vitro* transcription was carried out according to standard protocols. Probes were generated from cDNA fragments corresponding to nucleotides (nt) 190–445 (accession no. M10902) of TSH, nt 248–445 (accession no. U62779) of GH, nt 1–879 (accession no. M36804) of FSH, nt 1566–1749 (accession no. J00769) of prolactin, nt 56–526 (accession no. J00759) of proopiomelanocortin (POMC), nt 31–488 (accession no. NM_012858) of LH, and nt 11–355 (accession no. AH003532) of α-glycoprotein subunit (α-GSU).

The digoxigenin-labeled probes were diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris/HCl (pH 7.4), 1× Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA, 1 mM EDTA, and 10 mM dithiotreitol. After applying the hybridization mix, sections were coverslipped and incubated in a humid chamber at 50 C for 16 h. After hybridization, coverslips were removed in 2× standard sodium citrate (SSC; 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). The sections were then treated with RNase A (20 μg/ml) and RNase T_1 (1 U/ml) at 37 C for 30 min. Successive washes followed at RT in 1×, 0.5×, and 0.2× SSC for 20 min each and in 0.2× SSC at 60 C for 1 h. Sections were rinsed with B1 (100 mM Tris, 150 mM NaCl, pH 7.5) and then incubated for 2 h in blocking solution provided by the manufacturer of the kit. After incubation overnight with antidigoxigenin antibody conjugated with alkaline phosphatase (1:500 dilution; Boehringer), the tissue sections were washed with B1. Staining proceeded for 2–16 h in substrate solution containing nitroblue tetrazolium chloride (340 μg/ml; Biomol, Hamburg, Germany), X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate, 175 μg/ml; Biomol), 100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$ (pH 9.0).

Immunohistochemistry

After animals were decapitated, the pituitaries were removed and immersed overnight in Bouin Hollande fixative. After dehydration in a graded series of 2-propanol solutions for several days, tissues were embedded in Paraplast Plus (Merck, Darmstadt, Germany). Serial sections of 5-μm thickness were cut on a rotating microtome (Leica, Bentheim, Germany) and mounted on adhesive slides. Polyclonal antisera directed against GH (NIDDK-anti-rGH-IC-1), TSH-β (NIDDK-anti-rβTSH-IC-1), and prolactin (NIDDK-anti-rPRL-IC-5) were used at a dilution of 1:30,000. The antisera against FSH-β (NIDDK-anti-rβFSH-IC-1) and ACTH (rabbit-anti-ACTH R3–3, kindly donated by E. Weber and K. Voigt, University of Marburg, Marburg, Germany) were diluted 1:10,000; the antisera against LH-β (NIDDK-anti-rβLH-IC-2) and α-GSU (NIDDK-anti-rα Subunit-IC) were used at a dilution of 1:20,000.

Immunohistochemistry was performed as described (14) with some modifications. After deparaffinization, endogenous peroxidase activity was blocked with 0.5% perhydrol in methanol for 30 min. Nonspecific binding sites were blocked with 5% BSA in 50 mM PBS followed by an avidin/biotin-blocking step (avidin/biotin-blocking kit, Vector Laboratories, Burlingame, CA). Sections were incubated with primary antibodies overnight at 16 C followed by 2 h at 37 C. After several washes in distilled water followed by rinsing in 50 mM PBS, species-specific biotinylated secondary antibodies (1:200; Dianova, Hamburg, Germany) were applied for 45 min at 37 C. After another series of washes, sections were incubated for 30 min with the ABC reagents (Vectastain ABC Kit; Vector) followed by a nickel-enhanced diaminobenzidine reaction (0.125 μg/ml diaminobenzidine and 0.75 μg/ml ammonium nickel sulfate) for 10 min at RT. For negative controls, primary antibodies were omitted. Sections were analyzed and photographed using an Olympus AX70 microscope equipped with an Olympus DP-50 camera.

RIA

Mouse serum pituitary hormone levels were determined by A.F.P. using the highly sensitive double-antibody method described recently (15). For these assays, the reagents provided by A.F.P and the National Institute of Diabetes and Digestive and Kidney Diseases's National Hormone and Pituitary Program were used. For the TSH assay, highly purified rat TSH (AFP11542B) was used as iodinated ligand, guinea pig antimouse TSH (AFP98991) as primary antibody, and mouse TSH

(AFP51718MP) as reference preparation. For the α-GSU assay, rat LH-α (AFP4403A) was used for iodination and anti-LH-α (AFP66P9986) as antiserum. FSH was measured with rat FSH (AFP12828B) as iodinated ligand, guinea pig antimouse FSH (AFP1760191), and mouse FSH (AFP5308D) as reference preparation. For prolactin, mouse prolactin (AFP10777D) was used for iodination, rabbit antimouse PRL (AFP 131078) as antiserum, and mouse PRL (AFP6476C) as reference preparation.

Results

After mating male and female Pax8+/− mice, Pax8−/− mice were born with the expected Mendelian frequency, but they were severely growth retarded (4). Pax8−/− mice that survived until P21 reached a BW of 5–7 g, whereas wild-type and Pax8+/− littermates grew to approximately 12 g at that age (16). Despite the difference in BW, the weights of the pituitaries were not different (0.87 ± 0.09 mg for Pax8−/− *vs.* 0.80 ± 0.08 mg for wild-type mice). It is interesting to note that the pituitaries of Pax8−/− mice exhibited a glassy/translucent and not an opalescent appearance like those of wild-type and heterozygote littermates. Heterozygote and wild-type animals were indistinguishable in all parameters tested. For the experiments described here, only male animals were used.

Northern blotting

PolyA+-enriched RNA was prepared from the pituitaries from 21-d-old animals and subjected to Northern blot analysis (Fig. 1). Hormone mRNA abundance was determined by densitometry, and cyclophilin expression was used to adjust the hormone message for differences in loading. As expected, TSH-β mRNA levels were found to be dramatically increased in Pax8 mutant mice compared with the wild-type littermates. In addition, α-GSU mRNA levels were increased in Pax 8−/− mice, albeit considerably less pronounced. In comparison, transcript levels of FSH-β were considerably reduced, whereas those of the LH-β subunit were decreased only moderately. POMC mRNA levels were similar in both groups of animals. As expected, the GH transcript levels were strongly down-regulated in the mutant mice compared with control animals. Surprisingly, prolactin mRNA expression was almost abolished in Pax8−/− mice, whereas a strong signal was detected in the wild-type littermates.

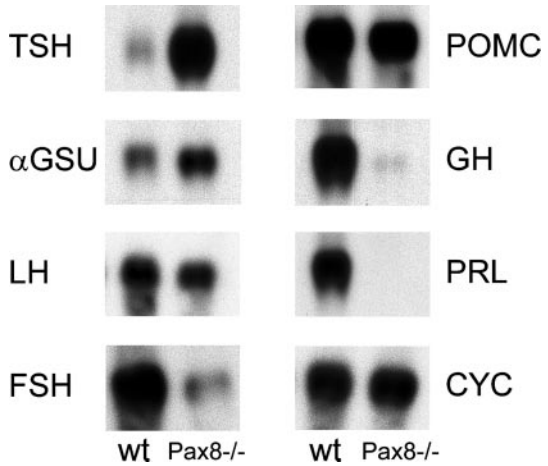

FIG. 1. mRNA expression of pituitary hormones of Pax8−/− and wild-type mice at P21. PolyA-enriched RNA from pituitaries of 21-d-old control animals and Pax8−/− mice was prepared and subjected to Northern blot analysis as described in *Materials and Methods*. After hybridization with radioactively labeled cDNA fragments for the different hormones, membranes were exposed to x-ray films. Equivalency of loading and transfer to membranes were monitored by hybridization with a cyclophilin (CYC) probe. PRL, Prolactin.

In situ hybridization histochemistry

Changes in pituitary hormone mRNA expression observed by Northern blot analysis were reflected by dramatic changes in cellular distribution pattern and signal intensities as analyzed by *in situ* hybridization histochemistry (ISH) (Fig. 2). Compared with control animals, not only the intensity of the TSH-β mRNA signals was dramatically increased in the mutant animals but also the number of cells expressing TSH-β transcripts. In Pax8−/− mice, hyperplasia and increased signal intensity was also observed for the α-GSU mRNA-expressing cells albeit more moderately. In contrast, the FSH-β transcript levels and the number of FSH-β mRNA-expressing cells were considerably reduced in Pax8−/− mice, whereas the expression of LH-β transcripts was only moderately affected. The POMC mRNA expression patterns were not significantly different. In both groups of animals, very strong hybridization signals were found in the intermediate lobe, whereas modest and scattered signals could be detected in the APs. GH mRNA was highly expressed in wild-type animals but not in Pax8−/− mice where the expression levels and numbers of detectable somatotropes were considerably reduced. Most striking were the differences in the prolactin mRNA expression patterns. In the pituitaries of wild-type animals, prolactin transcripts were highly abundant in numerous lactotropes, but in mutant mice only few, faintly labeled lactotropes could be detected.

Immunohistochemical analysis

Information as to the protein expression patterns, especially important for hormone-secreting cells, was obtained by immunohistochemistry (Fig. 3). Staining with TSH-β-specific antibodies revealed a dramatic increase in both the number and intensity of immunoreactive cells in tissue sections of Pax8−/−-derived pituitaries. Many of the thyrotropes were located in dense clusters and had an increased cell size suggesting both hypertrophy and hyperplasia of thyrotropes in the mutants. Similar changes in the immunostaining pattern, although less pronounced, could be observed after incubating Pax8−/− pituitary sections with antibodies against α-GSU. Staining intensity and the number of immunopositive LH-β and FSH-β cells were decreased in mutants compared with control animals, whereas in both groups, staining patterns of corticotrophic cells were similar. As expected, the number of GH-immunopositive cells was greatly reduced in the pituitaries of Pax8−/− mice. Moreover, in these mutant mice, only few, faintly stained lactotrope cells were identified in contrast to the high number of prolactin-immunopositive cells in control animals. Thus, changes in the cellular protein expression pattern of pituitary hormones in Pax8−/− mice nicely paralleled changes in hormone mRNA levels as revealed by ISH and Northern blot analysis.

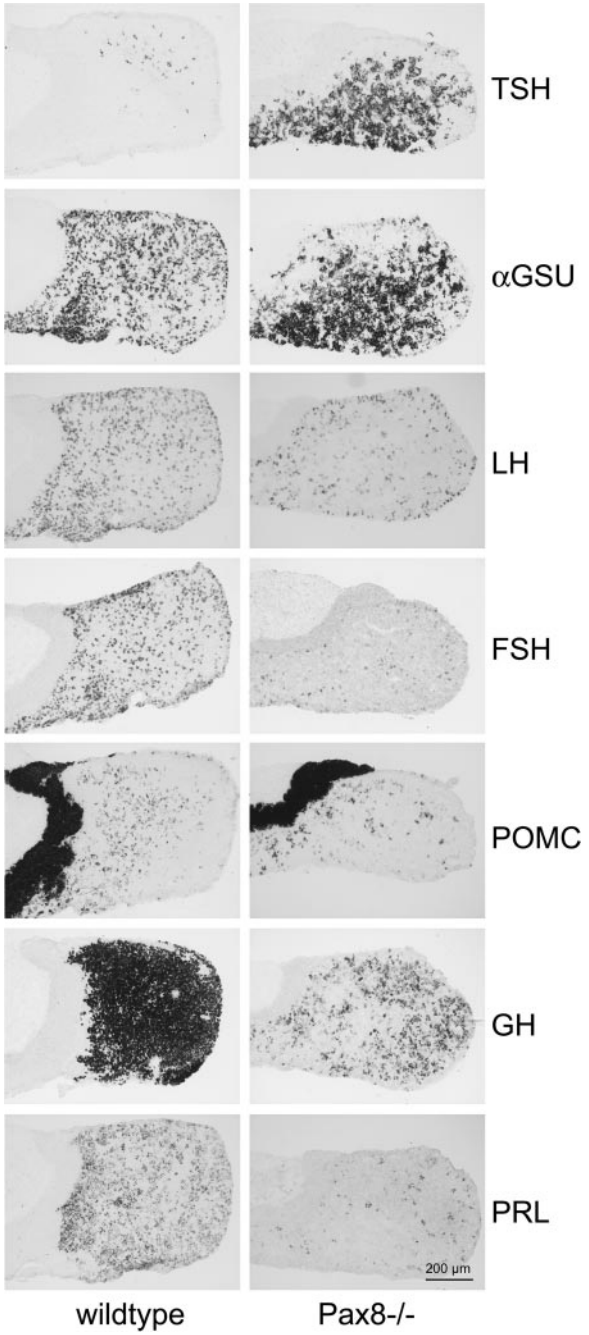

FIG. 2. Differential mRNA expression of pituitary hormones in 21-d-old wild-type and Pax8−/− mice as analyzed by ISH. Pituitaries were isolated from wild-type and Pax8−/− mice. As described in *Materials and Methods*, 16-μm-thick sections were hybridized with digoxigenin-labeled cRNA probes for TSH, FSH, LH, α-GSU, prolactin (PRL), GH, and POMC.

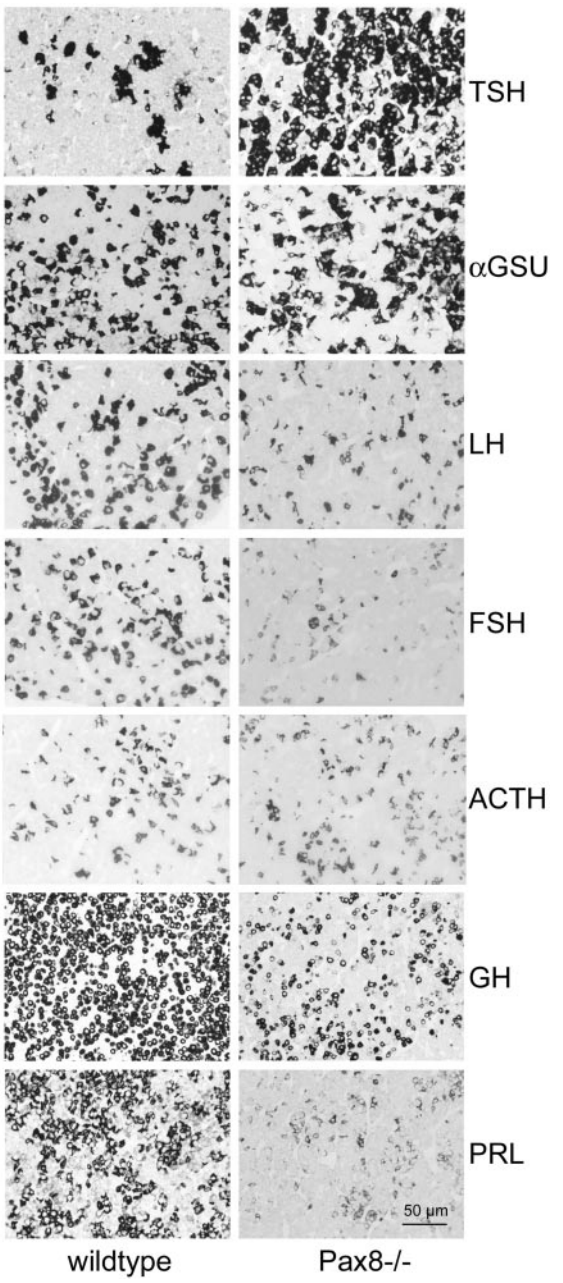

FIG. 3. Protein expression of pituitary hormones in 21-d-old wild-type and Pax8−/− mice as analyzed by immunohistochemistry. Pituitaries were isolated, immersed overnight in Bouin Hollande fixative, embedded in paraffin, and sectioned as described in *Materials and Methods*. Immunohistochemistry was performed with primary antibodies for TSH, FSH, LH, α-GSU, prolactin (PRL), GH, and POMC.

Analysis of pituitary hormones in serum

Serum levels of some pituitary hormones were measured by RIAs. As expected, there were no significant differences between wild-type and heterozygote animals (Table 1). In the serum of Pax8−/− mice, however, we detected exorbitantly high levels of TSH, approximately 130 times higher than in control animals. In addition, serum content of α-GSU was elevated by a factor of 10 in Pax8−/− animals. As expected from the immunocytochemical data, significant amounts of FSH and prolactin were present in the serum of wild-type and heterozygote animals, whereas in Pax8−/− mice, serum levels of both FSH and prolactin were below the detection limits of the assays. Unfortunately, reliable results could not be obtained for GH because in young animals, GH levels are known to be very low and difficult to measure due to the pulsatile secretion of this hormone.

TABLE 1. Pituitary hormone levels in the serum of 21-d-old wild-type, Pax8+/−, and Pax8−/− littermates

	Pax8 genotype		
	+/+	+/−	−/−
TSH	162 ± 27	141.7 ± 28.1	19652 ± 3316
α-GSU	1.6 ± 0.5	2.3 ± 0.5	23.2 ± 5.9
FSH	21.2 ± 4.7	19.8 ± 7.5	ND
Prolactin	6.8 ± 2.2	6.5 ± 3.3	ND

Concentrations are given in nanograms per milliliter serum. Values represent mean ± SD of at least five assays with serum pools of two to five animals. ND, Not detectable.

Effect of thyroid hormone replacement and estradiol treatment

As revealed by ISH, the pituitary hormone mRNA expression patterns normalized when Pax8−/− mice received daily injections of T_4 (20 ng/g BW) from P3 through P20 (Fig. 4). This was also true for LH and FSH (data not shown). Compared with control animals, expression of TSH mRNA was reduced in the T_4-treated mutant mice due to the slightly elevated thyroid hormone levels (105 ± 3 nmol T_4/liter and 1.12 ± 0.05 nmol T_3/liter in treated Pax8−/− mice *vs.* 79 ± 13 nmol T_4/liter and 0.98 ± 0.16 nmol T_3/liter in wild-type animals).

TSH and GH expression patterns also normalized after short-term treatment of Pax8−/− mice with the same T_4 dose, starting either at P15 or P18. Prolactin mRNA expression, however, was not affected by treatment with T_4 for 3 d and only slightly increased when the mutant mice received daily injections of T_4 from P15 onward (data not shown).

Because prolactin expression is stimulated by estradiol (17–19), Pax8−/− mice were also treated with this steroid hormone. Injection of 5 ng estradiol/g BW for 3 d (P18-P20) had no effect on prolactin transcript levels (data not shown). When Pax8−/− mice were treated for 3 d with a pharmacological dose of estradiol (100 ng/g BW), the prolactin expression levels seemed to be slightly increased, but the number of prolactin mRNA-expressing cells still remained extremely low. In another set of experiments, Pax8−/− mice were treated with 20 ng T_4/g BW starting at P15 and subsequently with 100 ng estradiol/g BW from P18 onward. Prolactin mRNA expression on d 21 was comparable with that of Pax8−/− mice treated for 6 d with T_4 only. The number of prolactin-expressing cells still remained very low (data not shown).

Expression of pituitary hormones at early postnatal stages

Although the circulating thyroid hormone levels have been shown to be very low during the first postnatal days (16), TSH expression levels in the pituitary of the 3-d-old athyroid Pax8−/− mice were already significantly upregulated (Fig. 5). Immunocytochemically, signs of thyrotrope hypertrophy and hyperplasia were already evident at this early stage of postnatal life, indicating that the negative feedback regulatory system is firmly established. In contrast, GH expression was not yet significantly altered. As is already known (10, 11), the prolactin-expressing cell type appears late in postnatal development, mainly during the second postnatal week. Accordingly, only a few lactotropes could be detected in the 3-d-old animals of both groups (data not shown). At P9, progression of thyrotrope hypertrophy was noticed in the Pax8−/− mutants (Fig. 5). Interestingly, a high number of GH-immunopositive cells could still be detected in the pituitaries of the athyroid Pax8 mutants. At this developmental stage, an increasing number of prolactin-expressing cells were detected in the wild-type animals but not in the Pax8−/− mice (data not shown).

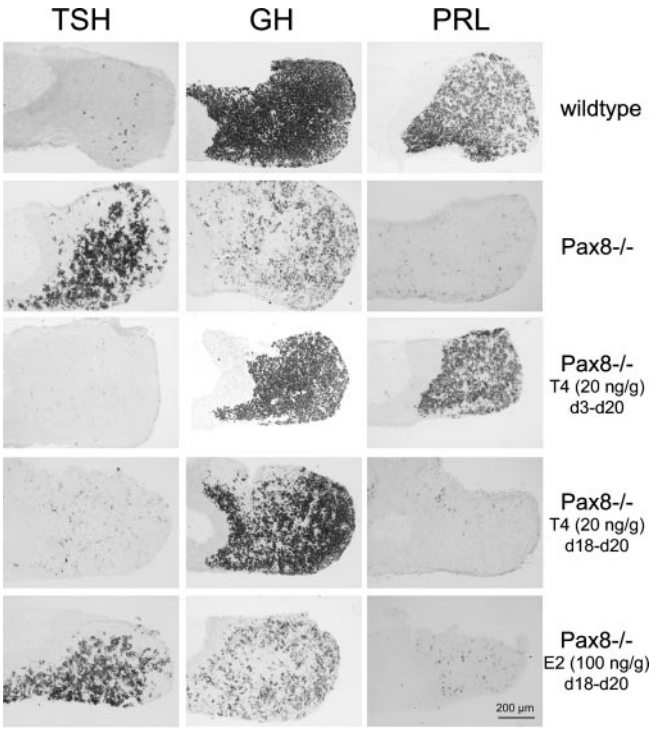

FIG. 4. Analysis of TSH, GH, and prolactin (PRL) mRNA expression by ISH in pituitaries of 21-d-old male Pax8−/− mice after treatment with T_4 and/or estradiol (E2) for different time periods. Pax8−/− mice were injected with T_4 (20 ng/g BW) from P3–P20 or P18–P20 and with E2 (100 ng/g BW) from P18–P20, respectively. The animals were killed 24 h after the last injection. Pituitaries from wild-type animals and from Pax8−/− mice treated either with hormones or not were subjected to ISH with digoxigenin-labeled cRNA probes for TSH, GH, and PRL as described in *Materials and Methods*.

Discussion

The Pax8−/− mouse can be used as an ideal animal model to study the effects of thyroid hormone deficiency on pituitary cell type development because Pax8−/− mice are completely athyroid and not only hypothyroid to variable degrees as in experimentally manipulated hypothyroid animals. Moreover, neither in these animals nor in their mothers is it necessary to manipulate the thyroid hormone levels by goitrogens, surgery, and/or radiothyroidectomy, means that are known to cause adverse side effects for the newborns and/or the mothers.

As demonstrated, the cellular composition of Pax8−/− mouse pituitaries is completely distorted compared with that of their wild-type littermates. All hormone-producing cells except the corticotropes were significantly affected. Pax8−/− mice survive and their pituitary cellular makeup normalizes if thyroid hormone treatment is initiated early during postnatal life, thus clearly demonstrating that the

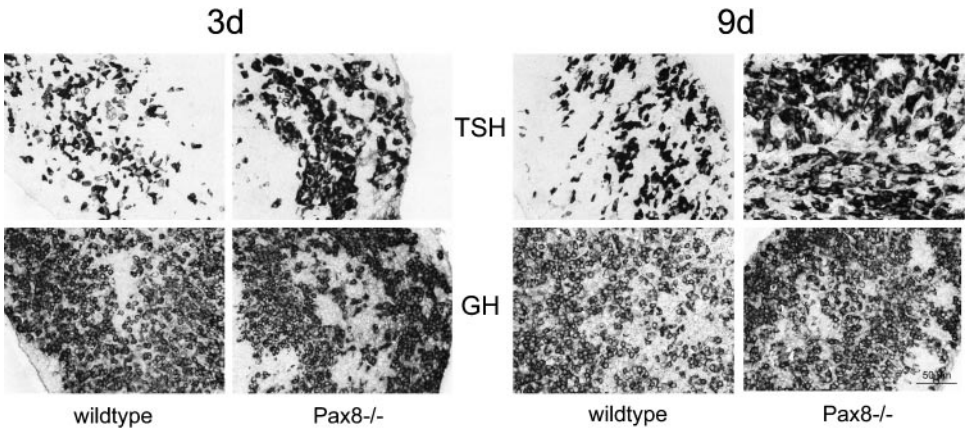

FIG. 5. Expression of TSH and GH in 3-d-old and 9-d-old wild-type and Pax8−/− mice as analyzed by immunohistochemistry. Pituitaries were isolated and processed as described in Fig. 3.

defects observed are caused solely by the athyroidism and not by the disruption of the Pax8 gene itself.

Thyrotrope development under athyroid conditions

TSH-β and α-GSU mRNA levels as well as the expression of both proteins was extremely up-regulated in the pituitaries of Pax8−/− compared with control mice. Moreover, extensive thyrotrope hypertrophy and hyperplasia was observed in the 21-d-old mutants. In fact, hypertrophic thyrotropes were already evident in the 3-d-old athyroid animals. Therefore, the exorbitantly high serum TSH levels were not surprising and actually could be expected because, at least in thyrotropes, transcription of the TSH-β gene and the α-GSU gene is directly regulated by T_3 (20–22) via thyroid hormone receptors. In fact, negative T_3-responsive elements have been identified in the gene promoters of TSH-β and of α-GSU as well (23, 24).

With adult rats rendered hypothyroid by treatment with goitrogens, similar although less pronounced effects have been observed previously (25, 26). Our results also fit well with data obtained by analyzing the pituitaries of various mouse mutants with congenital hypothyroidism. For example, severe hypertrophy of thyrotropes and high serum TSH levels were also observed in the pituitaries of adult hyt/hyt mice (27), a mouse mutant in which the thyroid hormone levels are drastically reduced (5- to 10-fold) due to a hypoplastic thyroid gland that is caused by an inactivating point mutation in the TSH receptor gene (28; for review, see Ref. 29). In α-GSU−/− mice, generated by targeted disruption of the α-GSU gene, the lack of functional TSH also leads to severe hypothyroidism and consequently to thyrotrope hypertrophy with increasing age (30).

In contrast to the hyt/hyt and the α-GSU mutant mice, distinct thyrotrope hypertrophy is already evident in 3-d-old Pax8/– mice. This difference is most likely explained by the fact that after birth Pax8−/− mice are completely athyroid, whereas in the hyt/hyt and the α-GSU−/− mice, severe hypothyroidism develops with age. As an additional consequence of this, untreated Pax8−/− mice die at the latest during the fourth week after birth (4) whereas the other mutants are viable and reach adult life.

Somatotrope development

The somatotropes are normally the most abundant cell type in the AP. In the Pax8−/− mouse, however, GH transcription was strongly reduced and the detection of GH mRNA-expressing cells and GH-immunopositive cells was greatly restricted. This effect was actually expected as previous studies with adult rats rendered hypothyroid by propylthiouracil treatment or surgery (26, 31) and the analysis of congenitally hypothyroid mice [*e.g.* the hyt/hyt (27) and the α-GSU−/− mouse (30)] clearly demonstrated that GH expression is strongly influenced by the thyroid status of the animals. Furthermore, thyroid hormones in rodents are known to be directly and positively involved in GH gene transcription (32–35).

Lactotrope development

The role played by thyroid hormones in the regulation of prolactin synthesis and secretion remains uncertain. In man, the association between hypothyroidism and hyperprolactinemia is well recognized (36, 37) and suggests an inhibitory role of thyroid hormone in the regulation of prolactin release. Alternatively, this phenomenon is explained by the increase of hypothalamic TRH and the increased number of TRH receptors on pituitary target sites. In the intact adult rat, hypothyroidism is associated with reduced pituitary prolactin synthesis and prolactin mRNA accumulation (31, 38), changes that are reversed by thyroid hormone replacement. The results from *in vitro* studies using primary monolayer pituitary cell cultures or pituitary cell lines are contradictory, ranging from inhibitory to stimulatory effects of thyroid hormone on prolactin synthesis (39–41). Contradictory results were also obtained by studies on the thyroid hormone-regulated transcription of the rat prolactin gene using different prolactin-synthesizing tumor cell lines (40, 42, 43).

Compared with the wild-type littermates, we observed in Pax8−/− mice a dramatic decrease in prolactin transcription as well as in protein expression. Only few prolactin-immunopositive cells could be detected in the 21-d-old mutant mice, whereas at this stage of development numerous prolactin-positive cells were readily detected in control animals.

Because pituitary α-GSU expression in Pax8−/− mice is up-regulated and the serum α-GSU levels are increased 10-fold, this observation supports the idea that for the differ-

entiation and proliferation of lactotropes a critical factor is missing that is not the free α-subunit as suggested (44–46). Together with the profound reduction in the number of lactotropes observed in the congenitally hypothyroid hyt/hyt and α-GSU mutant mice, these data rather indicate that thyroid hormones play a most important role in lactotrope proliferation or differentiation.

Effect of thyroid hormone replacement and estradiol treatment on cellular composition

Treatment of neonatal Pax8−/− mice with T_4 from P3–P20 completely normalized the cellular makeup of the pituitaries at the age of 21 d, indicating that the ontogeny of pituitary cells was not severely impaired by the hypothyroid conditions during the perinatal period. In adult α-GSU−/− mice, Stahl *et al.* (47) also observed that TSH-β and GH expression returned to normal after injecting thyroid hormone for 40 d. However, T_4 treatment of α-GSU−/− mice for only 3 d was not effective. Therefore, these authors concluded that the increase in GH cells after long-term T_4 treatment is attributable to the differentiation of precursor cells and is not due to enhanced GH production or storage in differentiated cells already present but inactive.

Furthermore, genetic analysis of Snell and Jackson dwarf mice (48; for review, see Refs. 49 and 50) established and other lines of evidence supported the concept that thyrotropes, somatotropes, and lactotropes derive from a common precursor that requires the POU-domain transcription factor Pit1 for the terminal differentiation of these three cell types (48). Therefore, Stahl *et al.* (47) also hypothesized that in α-GSU−/− mice the recruitment of thyrotropes from a common precursor pool depletes the pool available for the generation of somatotropes and lactotropes.

Both conclusions are not supported by our results. In Pax8−/− mice, thyroid hormone replacement for 3 d (from P18–P20) was sufficient to down-regulate the transcription of TSH-β and to increase GH mRNA expression to levels comparable to those of control mice. Our data clearly demonstrate that in the pituitaries of 21-d-old athyroid Pax8−/− mice, fully differentiated GH cells are present, but they are rather inactive and therefore difficult to detect. The apparent discrepancy in the two studies can easily be explained by the fact that we analyzed GH transcription by ISH, whereas Stahl *et al.* (47) used immunocytochemistry to follow the posttranscriptional and therefore delayed synthesis of GH protein.

The distribution pattern of GH mRNA-expressing cells in pituitaries of 21-d-old Pax8−/− mice treated with T_4 for 3 d also indicates that the differentiation of GH cells continues throughout the postnatal period analyzed. Interestingly, in 3-d- and also in 9-d-old Pax8−/− mice, GH expression comparable to that of control animals could be detected even by immunocytochemistry, indicating that GH synthesis at early postnatal age is not yet stringently regulated by thyroid hormones.

Based on the ontogenic expression patterns and elegant transgene ablation studies (51, 52) using toxins that are expressed under the control of the GH promoter and prolactin promoter, respectively, it is generally assumed that most lactotropes derive postmitotically from somatotropes via an intermediate cell type, the somatomammotropes, that produce prolactin and GH (53; for review, see Ref. 54). Although fully differentiated somatotropes are obviously present in the Pax8−/− mice, prolactin transcription in these mutants remained extremely low after T_4 treatment for 3 d and increased only slightly after thyroid hormone replacement for 6 d. Because in Pax8−/− mutants compared with control animals LH as well as FSH expression is significantly reduced and estradiol is known to act as a potent protagonist of lactotrope development, differentiation and prolactin production (at least at later stages of development although less likely during perinatal periods), Pax8−/− mice were also treated with estradiol. As expected, at low concentrations (5 ng/g BW) estradiol was completely ineffective (not shown), presumably because the bioavailability of circulating estradiol is blocked by α-fetoprotein, an estrogen-binding protein present in the plasma during early postnatal life (55, 56). When pharmacological doses of estradiol (100 ng/g BW) were injected, expression of prolactin mRNA seemed to be slightly increased but not the number of lactotrophic cells. Thus, estradiol seems not to be the crucial factor for lactotrope differentiation at this developmental stage. In line with this interpretation, only slightly reduced lactotrope cell numbers were found in the hpg/hpg mutants (47). These mice cannot produce GnRH, which consequently leads to hypogonadism and extremely low levels of circulating gonadal steroids. Correspondingly, in hypogonadal mice generated by transgene ablation of gonadotropes, the numbers of lactotropes are also not drastically reduced (57, 58). Furthermore, only a modest decrease in lactotrope cell density has been observed in estradiol receptor-α-deficient mice (59), indicating that estradiol receptor-α is not required for specification of the lactotrope cell phenotype as the somatotrope-lactotrope lineage progresses.

Based on the data presently available, we hypothesize that during early postnatal life differentiation of somatotropes into lactotropes requires a permissive factor that is regulated directly or indirectly by thyroid hormones. A wealth of evidence supports the notion that pituitary differentiation is influenced by intrapituitary chemical mediators acting via auto- or paracrine mechanisms as well as by cell-to-cell communication systems. So far, however, the factor(s) that are crucial for the differentiation of somatotropes into lactotropes have not been identified, and the mechanisms of T_4 action in this process warrant additional investigation.

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