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Pax6 Is Implicated in Murine Pituitary Endocrine Function

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***Pax6*, an evolutionarily conserved transcription factor, is expressed in the murine and zebrafish embryonic pituitary, but its role in pituitary development and endocrine function has not been described. To study the role of *Pax6* in vivo , we examined *Pax6* mutant mouse (*Sey^{Neu}*) pituitaries. Mice homozygous for the *Sey^{Neu}* mutation die at birth; therefore, we examined peptide hormone expression by the differentiated pituitary cell types as well as developmental marker expression in the intermediate and anterior lobes of the embryonic pituitary. GH- and PRL-immunopositive cells appear severely decreased in an outbred ICR background at embryonic d 17.5, although mRNA expression of these peptide hormones is present, as is expression of other pituitary markers. This suggests that pituitary cell types are able to differentiate in mutant embryos. To identify the cellular or physiologic mechanism responsible for less GH- and PRL-immunoreactivity in *Pax6* mutant mice, we tested serum levels of GH and PRL. *Pax6* homozygous mutant mice have GH serum levels one fifth that of controls at embryonic d 17.5, and one-third that of controls at postnatal d 0. PRL serum levels, which are very low during embryonic and neonatal stages, were below assay detection limits in both the wild-type and mutant groups. Taken together, these data suggest that *Pax6* is not essential for pituitary differentiation, but rather functions to establish appropriate neonatal homeostatic levels of GH and PRL, possibly through regulation of translational or secretory mechanisms.**

Key Words: *Pax6*; pituitary; *Sey^{Neu}*.

Introduction

The *Small eye* mouse mutant was first descibed over thirty years ago *(1)*. The small eye mutation (*Sey* or *Sey^{Neu}*) was later found to reside in the *Pax6* gene *(2)*. *Pax6* is a transcription factor renowned as an evolutionarily conserved master regulator of eye development. *Pax6* contains two DNA binding domains, a paired domain and a paired-class homeodomain, as well as a carboxy-terminal activation domain *(3)*. Mice homozygous for the *Pax6* mutation lack of eyes and nasal placodes *(4)*, display impaired neural crest cell migration *(5,6)* fail to differentiate glucagon-producing α-cells of the pancreas *(7)*, and die shortly after birth. The *Sey^{Neu}* phenotypes result from a point mutation in the *Pax6* gene that abolishes splicing, leading to truncation of the protein before the activation domain, due to the inclusion of an intronic stop codon, thus, functionally inactivating *Pax6 (2)*. Here we utilize the *Sey^{Neu}* mutant to examine the role of *Pax6* in pituitary development and function.

It is well established that the pituitary serves a critical role in the maintenance of homeostasis in the organism. The differentiation of the five anterior pituitary cell types and the regulation and secretion of their respective polypeptide hormones is highly dependent upon signals from all endocrine organs, in addition to paracrine and autocrine signals from within the pituitary itself *(8)*. Determining how these signals are processed and integrated by the pituitary to establish and maintain homeostasis is critical for understanding pituitary development and function. The conserved expression of *Pax6* in the mouse and zebrafish pituitary *(9,10)* suggests that this transcription factor may serve as an important pituitary effector molecule.

Pax6 is expressed in the mouse embryonic pituitary as well as in tissues predicted by the mutant phenotypes: developing eye and nasal epithelium, forebrain, hind brain, neural tube, and pancreas *(2,9,11)*. The anterior pituitary is derived from Rathke's pouch, an invagination in the oral ectoderm, which is initiated at mouse embryonic d 8.5 (E8.5) *(12)*. *Pax6* is detectable at E11.5 in the developing pouch *(9)* as dorsal regions press into and extend the neuroectoderm of the diencephalon, and as ventral regions are separating from the oral ectoderm. *Pax6* mRNA levels peak at E12.5, concomitant with the thickening of Rathke's pouch and further separation from the oral ectoderm *(12)*. E12.5 to E14.5 is marked by rapid proliferation of the ventral wall of Rathke's pouch to form the primordium of the anterior lobe *(13)*. During this time, *Pax6* mRNA levels

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decline until at E15.5 the transcript is detectable only in the epithelium lining the remaining lumen of Rathke's pouch *(9)*. Levels of *Pax6* mRNA remain just above background levels in this epithelial lining at E18.5 *(9)*. The cytodifferentiation of the five major anterior pituitary cell types, defined by the production of their respective polypeptide hormones, occurs in a defined temporal order between E12.5 and E18 within distinct regions of the pituitary *(14)*.

Based on the expression of *Pax6* in the embryonic pituitary during the morphological origination of this gland and the differentiation of its cell types, we hypothesized that *Pax6* plays a role in pituitary ontogeny and function. The conserved tissue expression of *Pax6* in the mouse and zebrafish tissues, including the pituitary *(9,10)* also suggests the possibility that *Pax6* performs a conserved pituitary function. The present study examines the putative role of *Pax6* in mammalian pituitary ontogeny and the establishment of homeostatic levels of pituitary hormones.

Results

Immunohistochemical Analysis of Pituitary Hormones in Sey^{Neu}/Sey^{Neu} Pituitaries

Because mice homozygous for the Sey^{Neu} mutation die at birth, we examined peptide hormone protein expression of the differentiated pituitary cell types in the embryonic pituitary at E17.5. Specifically, immunoreactivity of ACTH, GH, PRL, and TSHβ subunit antibodies were studied; we were unable to obtain effective antiserum against the β subunits of LH or FSH. For all immunohistochemical analyses, homozygous *Pax6* mutants are compared to homozygous wild-type animals. Although the Sey^{Neu}/Sey^{Neu} pituitaries displayed no hypocellularity and appeared morphologically normal, a striking deficiency of GH- and PRL-immunopositive cells was observed in the mutant pituitaries ($n = 2$) in an outbred ICR background (Fig. 1). Occasional GH-immunopositive cells were observed in these mutants, but PRL-immunopositive cells were never found.

In the mixed CB6/ICR background, GH-, PRL-, ACTH- and TSHβ-immunoreactivity was qualitatively lower in the Sey^{Neu} mutant pituitaries compared to wild-type controls as judged by intensity of chromagen reaction ($n = 4$; Fig. 1). Qualitative observations were made based on criteria described in Materials and Methods. This observation is supported quantitatively by RIA analysis to follow.

Analysis of Pituitary Developmental Marker and Peptide Hormone mRNAs in Sey^{Neu}/Sey^{Neu} Embryos by In Situ Hybridization

Expression of *Pax6* in the embryonic pituitary suggests that it may function in pituitary ontogeny. To determine if the deficiency of GH- and PRL-immunoreactivity in Sey^{Neu}/Sey^{Neu} embryos resulted from ontogenic or transcriptional failure, we analyzed the expression of pituitary developmental marker mRNAs and pituitary hormone mRNAs, respectively. We looked at the developmental markers Prop-1 and Pit-1 in the ICR background because this line completely lacks immunoreactivity for both PRL and GH.

Because Prop-1, which contains a paired-class homeodomain, and Pit-1, a POU-homeodomain protein, are both required for thyrotrope/somatotrope/lactotrope development *(15–17)*, we utilized these genes as early markers for the differentiation of these lineages. Prop-1 activates Pit-1 expression and the temporal expression of Prop-1 is closely followed by *Pax6* expression. We observed no differences in Prop-1 mRNA expression in the Sey^{Neu}/Sey^{Neu} pituitaries ($n = 3$) compared to the wild-type at E12.5 (Fig. 2), using criteria described in Materials and Methods. Conversely, Pit-1 mRNA signal appears lower in the mutant pituitary at E15.5 ($n = 3$) and higher in the mutant pituitary at E17.5 (+/+, $n = 2$ and Sey^{Neu}/Sey^{Neu}, $n = 3$; Fig. 2).

Differentiation of corticotropes, thyrotropes, lactotropes, somatotropes, and gonadotropes in the *Pax6* mutant pitutitary was demonstrated by mRNA expression of POMC, TSHβ, PRL, GH, and LHβ, respectivley, in both the ICR and CB6/ICR lines. PRL mRNA levels were too low for reliable *in situ* hybridization analysis. All peptide hormone mRNAs were generally seen to be expressed at lower levels in the *Pax6* mutant pituitaries (Fig. 2). We examined POMC and TSHβ mRNAs in mutant and control pituitaries at E12.5 ($n = 3$), E15.5 ($n = 3$), and E17.5 (+/+, $n = 2$ and Sey^{Neu}/Sey^{Neu}, $n = 4$) (E15.5. shown in Fig. 2). At E15.5, POMC and TSHβ appeared lower in the mutants, reflecting the protein deficiencies observed by immunohistochemistry. Distribution of expression of these two markers was normal: POMC mRNA was appropriately expressed in the intermediate lobe of the *Pax6*-mutant pituitary and TSHβ mRNA was expressed in the anterior lobe as well as in the rostral tip of the mutant pituitary. GH and LHβ mRNA levels also appeared lower in mutant pituitaries compared to controls at E17.5 ($n = 4$, Fig. 2). In contrast to immunohistochemical data, no differences in the peptide hormone mRNAs were observed between the ICR and CB6/ICR lines at E17.5. Despite the less robust levels of the pituitary hormone mRNA expression in mutatnt pituitaries, the hormone mRNA expression was spatially normal.

RIA Analysis of Serum GH and PRL

To gain a quantitative measure of pituitary function in the *Pax6* mutant mice, we employed RIA analysis of serum GH, PRL and TSHβ levels. Because the outbred ICR background was lost to rederivation, RIA analysis was carried out only in the mixed CB6/ICR line. At E17.5, serum levels of GH in control animals (homozygous wild-type and heterozygous) is approximately fivefold higher than levels observed in the Sey^{Neu}/Sey^{Neu} animals (Table 1). This difference is statistically significant. The difference is less severe at P0, although still statistically significant, with GH levels in control animals testing about three times higher than Sey^{Neu}/Sey^{Neu} animals. PRL levels in both control and mutant embryos were too low to assess differences at E17.5,

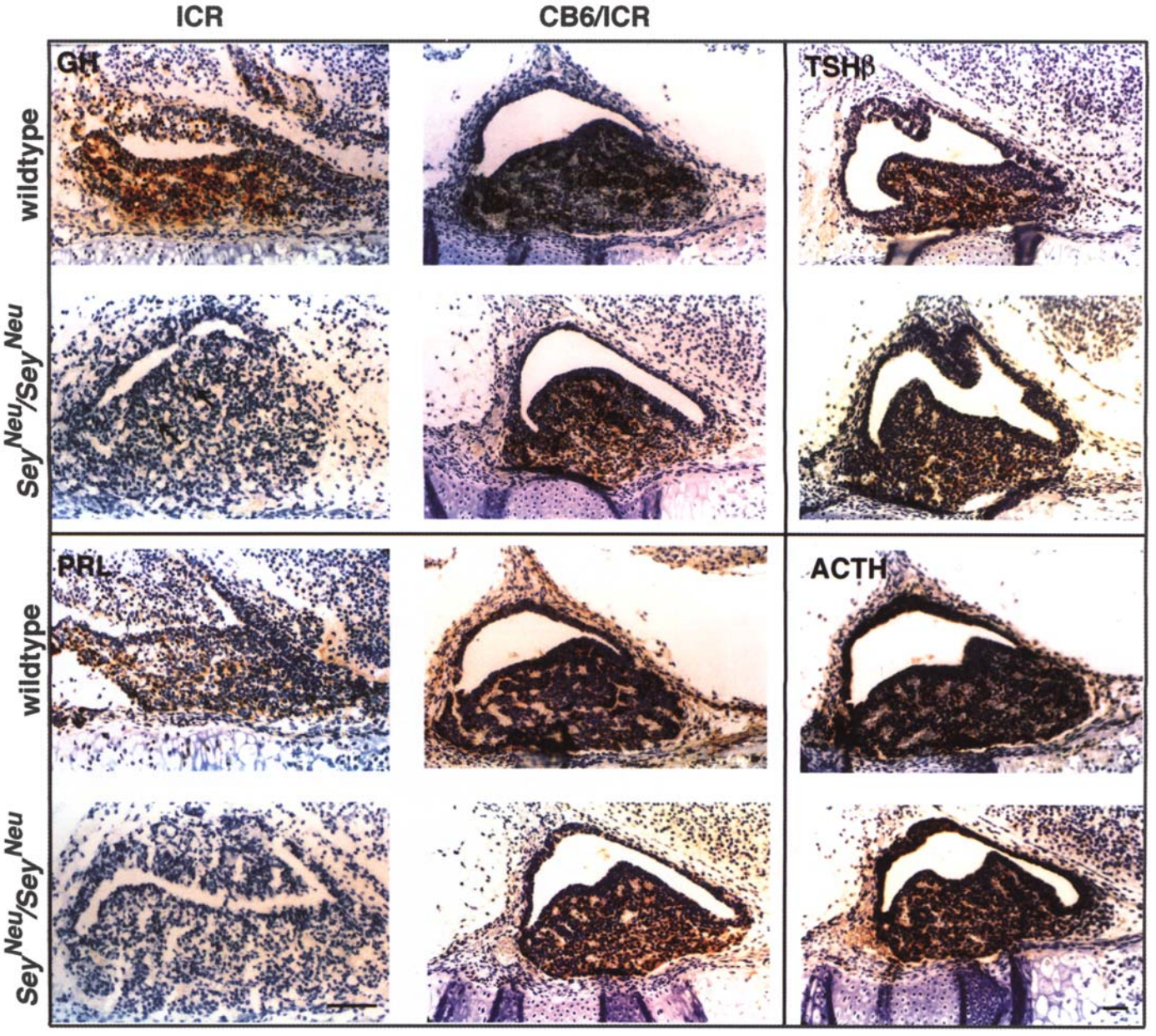

Fig. 1. Immunohistochemical detection of GH, PRL, TSHβ, and ACTH in sagittal sections of wild-type and *Pax6* mutant E17.5 pituitaries. GH- and PRL-immunopositive cells are present in the wild-type pituitaries of ICR (7 μm frozen sections) and CB6/ICR mice (5 μm paraffin sections). Immunopositive staining of these hormones is severely decreased in the Sey^{Neu}/Sey^{Neu} pituitaries in the ICR background, but not in the CB6/ICR background. Although a few GH-immunoreactive cells can be observed in the ICR Sey^{Neu} mutant pituitary (arrows), no PRL-immunoreactive cells are seen. TSHβ- and ACTH- immunopositive cells are present in both wild-type and mutant pituitaries of the CB6/ICR embryos, but immunoreactivity for these peptides is slightly reduced in the mutants. Bars, 100 μm.

and adequate amounts of serum could not be obtained to assess PRL leves at P0. Levels of TSHβ were nearly two-fold higher in the wild-type serum compared to Sey^{Neu}/Sey^{Neu} serum, but this difference is not statistically significant. The confidence intervals for this hormone are very broad because a crude extract of mouse pituitary gland is used as the antigen for iodination and the cold standard.

Discussion

Our data indicate that *Pax6* is required for normal pituitary endocrine function, which appears generally decreased in the *Pax6* mutant pituitary as demonstrated by lower peptide hormone protein and mRNA levels. Markedly decreased levels of serum GH in late prenatal Sey^{Neu}/Sey^{Neu} animals indicates that *Pax6* functions to establish appropriate neonatal serum levels of GH. Analysis of pituitary marker mRNAs, however, including GH mRNA, indicates that serum GH deficiency in the *Pax6* mutants does not result from failure of the pituitary to differentiate normally.

The Sey^{Neu} pituitary phenotype may result secondarily from hypothalamic defects. *Pax6* is known to be expressed

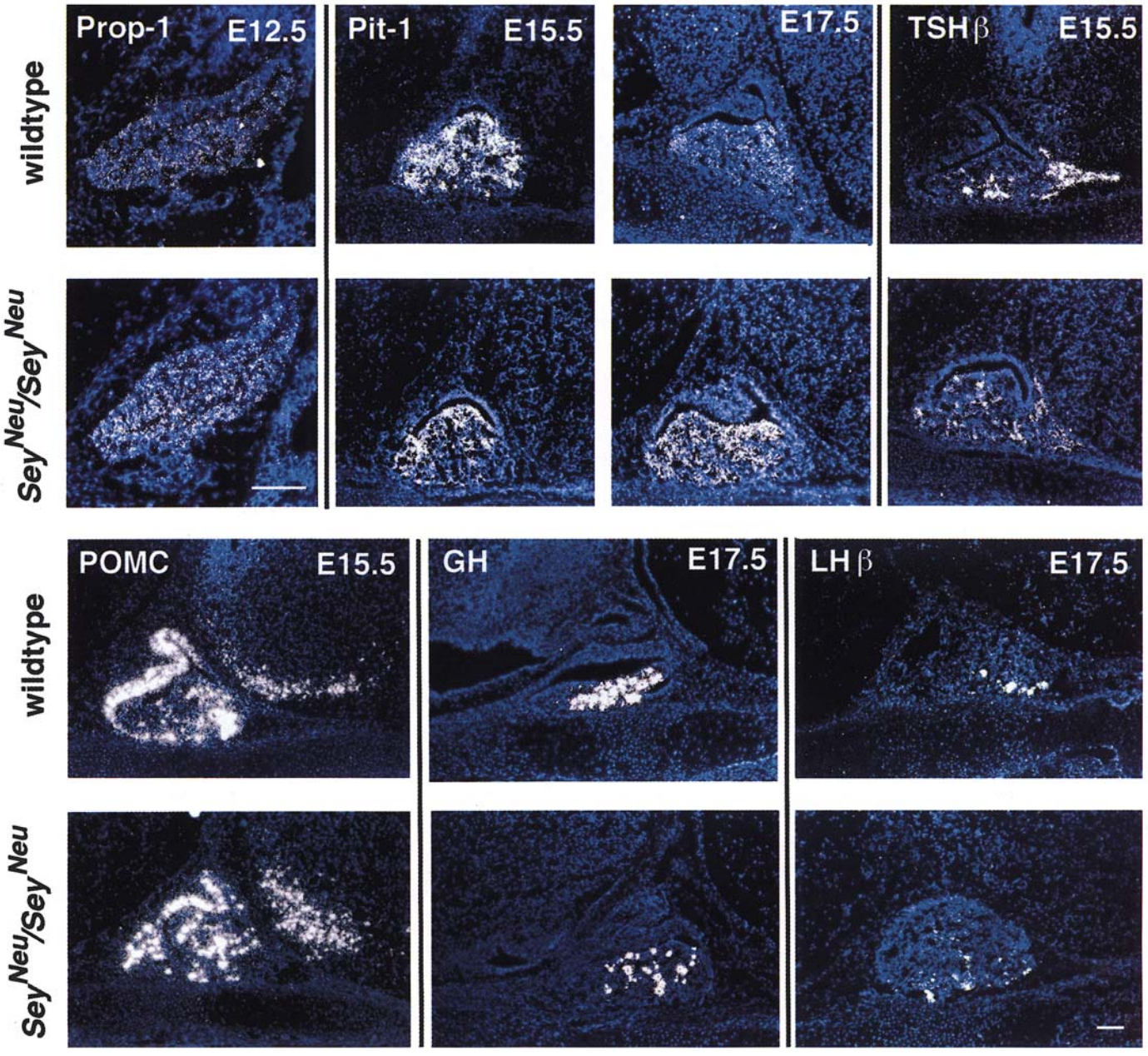

Fig. 2. *In situ* hybridization of antisense riboprobes of pituitary markers Prop-1, Pit-1, TSHβ, POMC, GH, and LHβ in sagittal sections of wild-type and *Pax6* mutant pituitaries. Prop-1 mRNA expression is equivilent in both wild-type and mutant pituitaries. Representative sections of Rathke's pouch are shown at E12.5 in the ICR background. Represenative sections of Pit-1 mRNA expression in the ICR background at E15.5, show that Pit-1 signal is lower in the *Pax6* mutant pituitary. Representative sections at E17.5, however, show that Pit-1 expression in the ICR background is higher in the mutants (5 μm frozen sections). TSHβ and POMC are expressed in control and mutant pituitaries, but signal is reduced is mutant pituitaries (7 μm frozen sections in the ICR background). GH and LHβ mRNA expression at E17.5 is reduced in Sey^{Neu}/Sey^{Neu} anterior lobes compared to wild-type controls at E17.5 in both ICR and CB6/ICR backgrounds (7 μm frozen sections). Bars, 100 μm.

in the embryonic hypothalamus *(9)*, a region of the brain that regulates the production and secretion of the pituitary peptide hormones. It has been shown that factors originating in the hypothalamus are important for differentiation and proliferation of the various pituitary cell types *(18)*. For example, hypothalamically produced growth hormone releasing factor (GHRF) acts on pituitary somatotropes, in conjunction with the pituitary transcription factor Pit-1, to facilitate the differentiation and proliferation of this cell type. Thyrotropes are also known to be dependent on the synergistic action of Pit-1 and the hypothalamic factor thyrotropin releasing hormone (TRH) *(18)*. Although it is not clear if the pituitary phenotype seen in the Sey^{Neu} mutants results from a requirement for functional *Pax6* in the pituitary or in the hypothalamus, it is clear that *Pax6* is required for appropriate function of the hypothalamic–pituitary axis.

Table 1

RIA Analysis of GH, TSHβ, and PRL

Age	Genotype	mGH (ng/mL)[a]	95% Confidence intervals	mPRL (ng/mL)[b]	mTSHβ[c] (ng/mL)	95% Confidence intervals
E17.5–18	+/+ and +/Sey^{Neu}	188	160–221	<1	252	173–361
E17.5–18	Sey^{Neu}/Sey^{Neu}	37	32–42	<1	141	95–204
P0	+/+ and +/Sey^{Neu}	191	163–225	ND[d]	ND	ND
P0	Sey^{Neu}/Sey^{Neu}	60	52–69	ND	ND	ND

GH and TSHβ serum levels along with 95% confidence intervals and PRL serum levels are shown for control (wild-type and heterozygous Sey^{Neu}) and Sey^{Neu}/Sey^{Neu} mutant animals at ages E17.5–18 and P0 in the ICR background. GH levels in control serum are about five times that of serum from mutant animals at E17.5–18 and about three times higher at P0.

[a]GH serum levels are expressed as nanogram-equivalents of mouse GH AFP10783B per mL of serum. PRL serum levels were found to be too low at E17.5 to quantitate, less than 1 nanogram equivalent per mL of serum.

[b]PRL serum levels are expressed as nanogram-equivalents of mouse PRL AFP6476C per mL of serum. TSHβ levels in control serum are about twice as high as that measured from mutant animals, but this is not statistically significant.

[c]TSHβ serum levels are expressed as nanogram-equivalents of mouse TSHβ AFP5171.8MP.

[d]ND = not determined.

The background-dependent variation in GH and PRL deficiency in Sey^{Neu} pituitaries underscores the importance of examining more than one line of mice. This analysis gives us a broader and more complete picture of how *Pax6* may act in the pituitary. Examination of the CB6/ICR line enabled us to consider the possibility that *Pax6* is not required for translation of GH, but rather interacts with other factors to promote normal transcriptional levels. The differences in protein levels seen in the two backgrounds may be a result of strain-specific modifier genes or may simply result from the differences in gestation time. Quinn et al. *(19)* have shown that variable penetrance of supernumerary upper incisor teeth and nasal capsule-derived cartilaginous "spurs" in mice homozygous for a *Pax6* mutation is dependent on genetic background.

Alternatively, the phenotypic difference observed between the ICR and CB6/ICR backgounds may be the result of differences in gestation time. The ICR outbred mice are born between 18.5 to 19 d post-coitum, while the mixed CB6/ICR mice are born between 17.5 to 18 d post-coitum; therefore, observations made at E17.5 may not represent equivalent stages of development. Because the deficiency in GH serum levels becomes less severe in the older P0 as compared to the E17.5 animals, homeostatic conditions appear to be delayed. The striking deficiency of GH- and PRL-immunopositive cells in the ICR mutant embryos may, therefore, be explained by a translational delay which is no longer apparent by immunohistochemical analysis in the mutant CB6/ICR embryos. However, it is difficult to speculate on how gestation time may have a bearing on actual stage of development. We can conclude that *Pax6* deficiency effects GH protein levels to some degree in both backgrounds.

Altered Pit-1 expression levels in the Sey^{Neu}/Sey^{Neu} pituitary further supports a developmental delay mechanism. Pit-1 expression normally peaks at E14.5, gradually decreases, and then remains at low levels even in the adult *(15)*. Lower Pit-1 expression at E15.5 in the Sey^{Neu}/Sey^{Neu} pituitary followed by continued high levels of expression may suggest that this marker is peaking late and remaining elevated longer. *Pax6* is among the growing number of homeobox-containing transcription factors found in the pituitary along with Pit-1 *(16,17)*, Prop-1 *(15)*, P-Lim *(20)*, Rpx *(21,22)*, and Msx1 *(23)*. Many of these factors interact with each other, leading to a complex transcriptional regulatory hierarchy controlling pituitary development and function *(15)*. *Pax6* appears to be part of this transcriptional network in the pituitary, affecting protein and mRNA levels of the polypeptide hormones.

The integration of signals in the pituitary gland to maintain homeostasis is thought to be established in the neonatal period. For example, neonatal thyroidectomy causes a significant decrease in PRL serum levels suggesting neonatal establishment of thyroid hormone dependent prolactin *(24)*. *Pax6* is expressed prior to, and at, neonatal ages making it a candidate for establishment of homeostasis; consequently, lack of embryonic expression of *Pax6* may be responsible for delay or failure of the establishment of neonatal GH homeostasis. The role of *Pax6* as a modulator of homeostasis is further supported by the expression of *Pax6* in the adult mouse pituitary as determined by RNase protection analysis in our laboratory and the preliminary finding of *Pax6*-immunopositive cells in anterior and intermediate lobes of the adult rat pituitary (data not shown). Targeted disruption of *Pax6* specifically in the pituitary will be critical for elucidating the role of *Pax6* in the postnatal mouse.

Our data show that GH is decreased in the pituitary of ICR mice, and is decreased in the serum of perinatal CB6/ICR mice. The similarity of this phenotype, decrease in GH, suggests that *Pax6* is acting in a similar capacity in both genetic backgrounds to establish appropriate neonatal

levels of GH. The critical differences between these phenotypes and the ultimate ramifications on homeostasis can only be understood by examination of a conditional or tissue-specific *Pax6* mutation in the postnatal mouse.

Materials and Methods

Animals and Tissue Preparation

All animals were maintained according to NIH guidelines. Experiments described were completed in two different background strains of mice carrying the Sey^{Neu} mutation: an outbred albino ICR background and/or a CB6/ICR background. Analysis in the ICR background is limited because this strain was lost to rederivation. Rederived animals, termed CB6/ICR, resulted from crossing an ICR outbred male to a CB6 (C57Black6 X BalbC) female.

All histological observations were made in embryos from at least two different litters (unless otherwise noted). Each antiserum and *in situ* probe was tested on three to six sections, representing both medial and lateral regions of the pituitary. To obtain a comprehensive view of immunohistochemistry and *in situ* hybridization results, each section was photographed. Qualitative observations reported are based on the visual comparisons of every photograph. Comparisons were only made between sections from the same experiment to standardize for variations between experiments such as probe specific activity in *in situ* hybridization and and chromagen reaction efficiency in immunohistochemistry.

For *in situ* hybridization, embryos examined at E12.5, E13.5, and E15.5 were on the outbred albino ICR background. All *in situ* hybridization analyses of E17.5 embryos were completed in both the mixed CB6/ICR background and the outbred ICR background (n includes at least one animal from each background), except for analysis of Pit-1 mRNA which was completed only in the ICR background.

For examination of *Pax6* mutants, matings were set up between heterozygous Sey^{Neu} mice. Noon of the day the plug was observed was counted as embryonic d 0.5 (E0.5). Embryos were surgically removed at E12.5, E15.5, and E17.5 and washed in ice cold phosphate buffered saline (PBS, pH 7.2) before removal of yolk sac to prevent contamination of PCR genotyping by maternal or sibling blood. Instruments used to remove and handle the yolk sac were washed in ethanol and then PBS between each embryo, again to prevent crosscontamination of DNA. Embryos were fixed in 4% paraformaldehyde in PBS for 24 h, transferred to 10% sucrose in PBS overnight, embedded in O.C.T. compound (Tissue-Tek, Torrance, CA), and 5–7 μm frozen sections were cut on a cryostat. Alternatively, fixed tissue was put into 70% EtOH and then paraffin embedded. Paraffin sections were cut on a microtome at 7 μm.

Genotype Analysis

Genotype analysis was performed on DNA isolated from the yolk sac. Genotype analysis was carried out using *Hax-5* and *G15* primers in PCR as described *(25)*. The PCR reactions were precipitated, resuspended in water, digested with HindII, and visualized by ethidium bromide on a 2% aragose gel.

Immunohistochemistry

Immunohistochemical localization was used to examine *Pax6* and the pituitary peptide hormones: GH, the β subunit of TSH, PRL, and ACTH. Antigens were detected using standard protocols as described *(26)*. Peptide hormone antisera were supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) through the National Hormone and Pituitary Program, NIH. Primary antisera generated against the peptide hormones included monkey anti-rat GH (NIDDK# AFP411S) and rabbit anti-rat TSHβ-IC-1 (NIDDK# AFP1274789) at a 1:1000 dilution, rabbit anti-rat PRL (NIDDK# APF425_10_91) and rabbit anti-human ACTH (NIDDK # AFP39013083) at a 1:500 dilution. Following incubation of slides with antisera, sections were treated with the appropriate biotinylated secondary antisera (goat anti-rabbit or anti-human) and an avidin-biotin-horseradish peroxidase (-HRP) complex (Vector, Labs, Burlingame, CA). Cells immunopositive for the peptide hormones were visualized with a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromagen reaction resulting in a brown precipitate. Tissue was counter stained with hematoxylin nuclear stain (Richard-Allen Scientific, Kalamazoo, MI), and all slides were dehydrated and cover-slipped in DPX mountant (BDH Scientific Supplies, Ft. Washington, PA).

In Situ Hybridization

Pituitary marker mRNAs were analyzed using standard *in situ* hybridization protocols *(27)*. The ^{33}P-labeled riboprobe specific for mouse Prop-1 is 800 bases in length from the ATG start site and the mouse Pit-1 probe is 1.2 kb from an EcoRI fragment. The ^{33}P-labeled *Pax6* riboprobe (provided by P. Gruss, Max-Planck Institute) consists of 250 bases covering an EcoRI/NheI fragment of the paired domain. Mouse POMC (provided by P. Sawchenko, The Salk Institute) (Sma I insert), mouse TSHβ (Pst I insert), LHβ, rat GH, and mouse PRL cDNAs made riboprobes that were 500, 427, 217, 190, and 242 bases long, respectively. Prop-1, Pit-1, TSHβ, LHβ, GH, and PRL cDNAs were provided by M. Rosenfeld, UCSD. RNase-treated sections were incubated with individual probes to determine background levels of signal.

Radioimmunoassays

Radioimmunoassays (RIAs) were performed to determine GH, PRL, and TSHβ serum levels in Pax6 mutant and wild-type animals. RIAs were carried out in the CB6/ICR

background. Blood samples were collected from mice at E17.5–18 (seven litters of pups) and P0 (three litters of pups). At least one homozygous mutant pup was in each litter. Because of the quantity of serum required for this assay, we pooled blood from each group. At each age, blood samples from homozygous mutant or heterozygous/homozygous wild-type, as judged by the presence of eyes and nose, were pooled. Samples were allowed to remain at room temperature for approx 1 h and then stored at 4°C overnight. The following morning, samples were centrifuged at 14,000g for 20 min and supernatant (serum) removed. Mouse serum RIAs for GH and PRL were performed in duplicate by double antibody RIA method using anti-rat GH (NIDDK-anti-rat GH-RIA-5 [AFP]) or anti-mouse PRL (NIDDK-anti-mPRL Rb#1 3-10-78) and utilizing highly purified mouse pituitary GH (AFP10783B) or PRL (AFP6476C) as antigens for iodination, the same materials were used for cold standard. RIA for TSHβ utilized crude extract of TSHβ (AFP5171.8MP) from mouse pituitary gland as antigen for iodination, the same materials for cold standard, and anti-rat TSHβ (NIDDK-anti-rat TSHβ GP#9 8-9-91). All immunoreagents were distributed by NIDDK's National Hormone and Pituitary Program. GH serum levels were expressed as nanogram-equivalents of AFP10783B per mL of serum, PRL serum levels as nanogram-equivalents of AFP6476C per mL of serum and TSHβ serum levels as nanogram-equivalents of AFP5171.8MP.

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