Enhancer-Mediated High Level Expression of Mouse Pituitary Glycoprotein Hormone α-Subunit Transgene in Thyrotropes, Gonadotropes, and Developing Pituitary Gland

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The pituitary hormones LH, FSH, and TSH are heterodimers composed of a common α-subunit and unique β-subunits. We demonstrate that 4.6, 2.7, 1.49, or 0.48 kilobases (kb) mouse α-subunit 5′-flanking sequences are sufficient for transgene expression in both gonadotropes and thyrotropes but not in inappropriate pituitary cells. In contrast, transgenes with bovine or human α-subunit flanking sequences have been shown to confer reporter gene expression only to gonadotrope cells, suggesting that the elements regulating cell-specific expression may differ between species. Equal levels of reporter gene expression were conferred by 5.0 and 0.48 kb in transiently transfected thyrotrope tumor-derived cells. In contrast, in transgenic mice, high level expression was only obtained with 4.6 kb 5′-flanking sequences, indicating the presence of an enhancer element between 4.6 and 2.7 kb. The 4.6 kb of 5′-flanking sequences are sufficient for both hormonal and developmental regulation of transgene expression. Mice rendered hypothyroid by radiothyroidectomy had significantly higher levels of transgene expression than either hyperthyroid or euthyroid animals. The temporal and spatial pattern of transgene expression in Rathke's pouch paralleled that of the endogenous gene; the onset of transgene expression occurred by embryonic day 9.5. Low level expression of both the transgene and the endogenous α-subunit gene were detected in some unexpected peripheral sites, such as the embryonic extraocular and olfactory regions, suggesting that α-subunit may have a more diverse role in development than previously considered. (Molecular Endocrinology 8: 1420–1433, 1994)

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INTRODUCTION

During ontogeny, unique pituitary cell types differentiate in a sequential pattern and begin to synthesize and secrete specific hormones. Many of these circulating hormones play critical roles in the development of other organs. Three of these hormones are heterodimers composed of a common α-subunit and unique β-subunits: TSH, LH, and FSH.

TSH stimulates thyroid hormone production in the thyroid gland and plays an important role in thyroid folliculogenesis in the fetus. Several mouse mutants demonstrate the importance of TSH for thyroid development. Snell dwarf mice lack TSH and have small, disorganized thyroid glands in which folliculogenesis appears to have been initiated but has arrested early (1). The thyroid phenotype can be reversed by TSH replacement therapy (2). Poor thyroid follicle develop-

ment is also evident in *hyt* homozygotes (3), which carry a point mutation in the TSH receptor, producing unresponsiveness to TSH stimulation (4).

LH and FSH are critical for sexual maturation during puberty and during gonadal development. LH stimulation of testicular LH receptors in the fetal testis causes an increase in testosterone production. Fetal testosterone is important for male sexual differentiation (5). The stage at which the ovaries first respond to LH and FSH varies among species (6), but gonadotropins are necessary for development of ovarian follicles in the newborn rat (5).

In the anterior pituitary gland, transcription of the common α- and unique β-subunit genes occurs in thyrotropes that secrete TSH and gonadotropes that secrete LH and FSH. The appearance of the α-subunit precedes the β-subunits in both rat and human embryogenesis (7–9). The DNA sequences important for the onset of α- and β-subunit transcription have not been defined. However, sequences important for cell-specific expression of the α-subunit gene have been defined by gene transfer studies. Transient transfection experiments using cultured thyrotropic or gonadotropic-derived cells or thyrotropic tumor cells have demonstrated that separate DNA sequences within the proximal 0.5 kilobase (kb) of the human and mouse promoters are important for expression of α-subunit in gonadotropes and thyrotropes (10–13).

Transgenic mouse studies support the view that the cell specificity of α-subunit expression is regulated by separate elements and suggest the possibility that there may be different DNA sequence requirements for expression in cell culture and animals. Expression in gonadotropes but not thyrotropes was observed with both human and bovine α-subunit transgenes, suggesting that thyrotrope expression requires separate DNA sequences (14–16). Although cell culture studies defined separate thyrotropic and gonadotropic elements within 0.5 kb of the human α-subunit promoter, neither 1.6 (14) nor 1.8 kb (16) of the human 5′-flanking sequences conferred thyrotrope expression. Thus, additional DNA sequences must be required for expression of the human gene in the context of the intact animal.

Species-specific differences in expression of the α-subunit gene have also been noted. α-Subunit is expressed in the placentas of primates and horses but not in other animals (17, 18). Functional differences in promoter activity in transfection assays suggest that differences in nucleotide sequence rather than in trans-acting factors may account for the placental expression. For example, although placental-specific elements are located within the proximal 184 base pairs (bp) of both the human and equine α-subunit 5′-flanking regions, a similar fragment of the mouse gene is inactive in JEG3 human placental cells (19–22). Sequence differences between the human and bovine α-subunit promoters also account for differences in expression and cAMP regulation in placental cells (23). Expression in the pituitary also appears to be regulated by species-specific DNA sequences. In mouse αTSH cells, the profound reduction in basal activity observed when the mouse promoter was truncated from −507 to −424 bp was not evident when similar constructs of the human gene promoter were tested (11).

We used transgenic mice to identify the DNA sequence elements that regulate expression of the mouse α-subunit in adult and developing mice. We initiated these studies with the mouse α-subunit gene because species-specific differences in expression of the gene suggested that the homologous mouse genomic sequences would be more likely to be appropriately expressed. We present evidence that 4.6 kb of mouse α-subunit 5′-flanking region is not only sufficient for cell-specific and hormonally regulated expression in adult mice, but also contains the temporal cues for initiating expression during development. We identified an enhancer-like element between −4.6 and −2.7 kb that is essential for high level expression in transgenic mice but inactive in cell culture. However, as predicted from cell culture studies, 0.48 kb of promoter proximal sequences are sufficient for low-level cell-specific expression. Finally, we have identified novel sites of α-subunit expression in the developing embryo, suggesting the possibility of additional functional roles of this gene in ontogeny.

RESULTS

4.6 kb of Mouse α-Subunit 5′-Sequences Are Sufficient for High Level Expression in the Anterior Pituitary Gland of Transgenic Mice

A 4.6-kb fragment containing the mouse α-subunit promoter was joined within the 5′-untranslated region of exon 1 to a *lacZ* reporter gene containing a nuclear localization signal (Fig. 1). Eight transgenic mouse lines were generated with this construct, 4.6 mα-βgal, and screened for transgene expression by inspection of X-gal staining in pituitary frozen sections. Transgene expression was detected in seven of the eight lines. No background β-galactosidase staining was detected in frozen sections of any of the nontransgenic pituitaries. The level of transgene expression was quantitated in the six highest expressing lines by a fluorometric assay for β-galactosidase enzyme activity in extracts made from individual pituitary glands. The level of activity ranged from 200-fold over C57BL/6J background levels in transgenic line 8365 to 5-fold over background in line 8316 (Table 1). Notably, these levels of β-galactosidase expression were high enough to be readily detected in a 30-min assay rather than overnight assays (25). There was no correlation between the level of β-galactosidase activity and the copy number of the transgene, suggesting some influence of integration sites on expression.

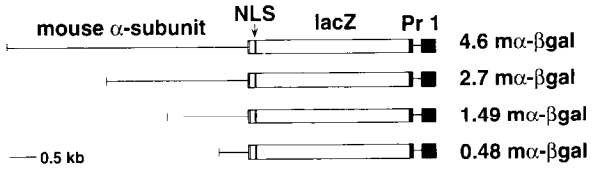

Fig. 1. Transgene Constructs

Four transgenes, 4.6 mα-βgal, 2.7 mα-βgal, 1.49 mα-βgal, and 0.48 mα-βgal, were isolated from either the 5.0 mα-βgal or 0.48 mα-βgal plasmid construct as described in *Materials and Methods*. The mouse α-subunit sequences (mα) span from −4.6, −2.7, −1.49, or −0.48 kb upstream of the transcription start site to +43 bp of 5′-untranslated sequences in exon 1. In all cases, the mouse α-subunit promoter is joined to a modified form of the *E. coli* β-galactosidase (*lac Z*) gene. The modified *lac Z* gene (*open box*) includes a translation initiation site and the SV40 T-antigen nuclear localization signal (NLS). An intron, 3′-untranslated region, and polyadenylation signal are provided by the mouse protamine-1 gene (Pr-1, *shaded*) (59).

Table 1. The 4.6 mα-βgal Transgene Confers High Level Expression in Adult Pituitary Glands

Transgenic Line	β-gal Activity in Pituitary Extracts[a] (nmol 4MU/pituitary)	Transgene Copy Number[b]
8365	73	10–50
8690	53	10–50
8612	40	50
8364	31	50
8588	10	10
8316	2	1–5
8346	nd[c]	10–50
8691	nd[c]	10–50
C57BL/6	0.4	0

[a] The amount (nanomoles) of the fluorescent product 4-methylumbelliferone (4MU) generated by cleavage of the substrate 4-methylumbelliferyl-β-D-galactoside was quantitated in homogenates prepared from individual pituitary glands and averaged. Three to eight individuals were assayed from the five highest expressing lines.

[b] The copy number was estimated by comparison with standards on Southern blots (see *Materials and Methods*).

[c] Not determined. X-gal-stained frozen sections revealed very few lightly stained cells in line 8346 and no expression in line 8691.

Transgene Expression Is Specific for Anterior Pituitary Thyrotropes and Gonadotropes

Transgene expression was confined to the anterior lobe of the pituitary gland in all six expressing lines (Fig. 2A). No expression was detected in the intermediate or posterior pituitary lobes. In line 8365, cell counts revealed that approximately 10–12% of the anterior pituitary cells expressed the transgene. The cell specificity of transgene expression was examined in lines 8364, 8365, and 8588 using a costaining technique that takes advantage of the nuclear localization of the β-galactosidase reporter gene and the localization of stored hormones in the cytoplasm. Whole pituitaries were incubated with X-gal, embedded, sectioned, and immunostained for the anterior pituitary hormones. Cells expressing the transgene and either LH or TSH were identified by blue-staining nuclei and immunostained cytoplasms. In line 8365, the transgene was expressed in 90% of LH-positive cells and 80% of TSH-positive cells (Fig. 2, B and C). None of the cells staining for PRL, ACTH, or GH exhibited transgene expression (Fig. 2, D, E, and F). We cannot exclude the possibility that the transgene is expressed in a few somatotropes. Coexpression of α-subunit and GH has been observed in human pituitary tumors and in normal human pituitary cells (26–29). Gonadotrope- and thyrotrope-specific transgene expression was also demonstrated in lines 8364 and 8588 (data not shown).

Transgene Expression Responds to Changes in Thyroid Status

We tested whether the 4.6 mα-βgal transgene was regulated by thyroid hormone. Groups of mice from the highest expressing transgenic line (8365) were placed into three groups (n = 6). One group served as a euthyroid control and had mean T_4 serum levels of 3.2 ± 0.3 μg/dl and mean T_3 serum levels of 23 ± 4.6 ng/dl. A second group was given L-T_4 in their drinking water for 28 days (hyperthyroid). This resulted in mean serum T_4 and T_3 levels of 18 ± 1.9 μg/dl and 363 ± 70 ng/dl, respectively. The third group was radiothyroidectomized 167 days before the experiment to produce a hypothyroid state. The mean serum T_4 levels were below the detectability of the assay (<1 μg/dl) while the mean T_3 levels were 8.3 ± 1.8 ng/dl. The α-subunit promoter activity was assessed in anterior pituitary gland homogenates using the chemiluminescent β-galactosidase assay. When pituitary extracts from nontransgenic mice were assayed, no detectable β-galactosidase activity could be measured. As shown in Fig. 3, a high level of promoter activity (118 ± 11 ng/pituitary) was exhibited by the pituitaries from the control euthyroid group. Activity from the hypothyroid animals increased 230% (270 ± 21 ng/pituitary) above the control group. Hyperthyroid animals exhibited a 43% decrease in activity (68 ± 9 ng/pituitary) relative to the control group. There was a statistically significant difference between the groups ($P < 0.001$) using the Kruskal-Wallis one-way analysis of variance on ranks. All pairwise multiple comparisons between groups were statistically significant ($P < 0.05$) using the Student Newman-Keuls method. Thus, the transgene is subject to regulation in response to the thyroidal state.

Low Levels of Transgene Expression Are Found in Some Adult Peripheral Tissues

A subset of the high-expressing transgenic lines was selected for further analysis. Extracts of kidney, heart, lung, liver, gonads, and brain were prepared from at least two individuals from lines 8365 and 8364. Expres-

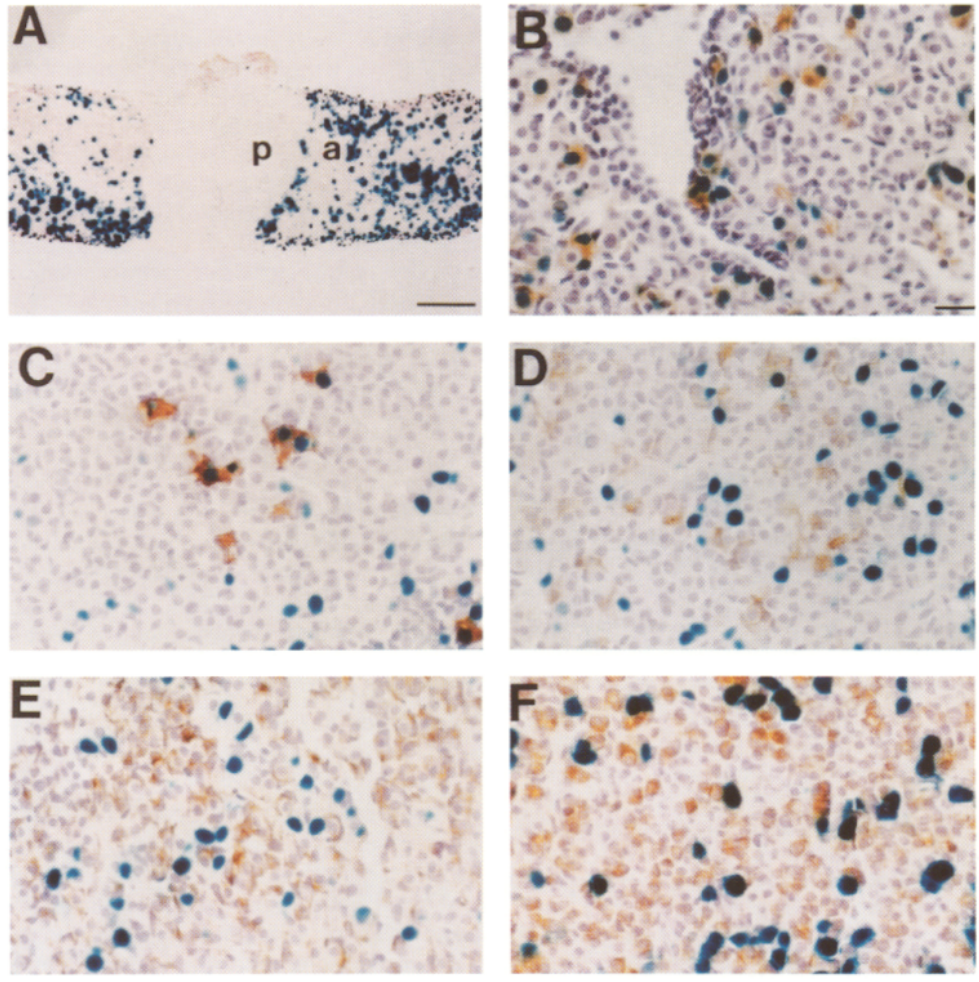

Fig. 2. Colocalization of 4.6 mα-βgal Transgene Expression and TSH and LH Immunoreactivity in Adult Anterior Pituitary Glands

A pituitary frozen section from transgenic line 8612 was stained for β-galactosidase expression and counterstained with neutral red. High level β-galactosidase expression is detected by a blue precipitate found only in the nuclei of the anterior lobe (a) but not in the posterior (p) and intermediate lobes (panel A). Pituitaries from transgenic line 8365 were stained for β-galactosidase expression and embedded in paraffin, and sections were immunostained with antibodies against LH (panel B), TSH (C), ACTH (D), PRL (E), and GH (F). Transgene expression is evident only in cells immunostained for TSH and LH. *Magnification bars* shown in A and B represent 200 and 25 μm, respectively. Magnification was constant for B-F.

sion was quantitated as nanomoles of the fluorescent product 4-methylumbelliferone generated per gram of tissue/h. In homogenates, the background level of β-galactosidase activity is characteristic of the tissue and can be significant. Enzyme activity levels that were less than 2-fold over background were not considered significant. Whereas pituitary expression in lines 8365 and 8364 was 200- to 100-fold over background, potentially significant peripheral tissue expression was noted only in the brain (5-fold) in line 8365 and the ovaries/oviducts in lines 8365 (14-fold) and 8364 (4-fold). No significant expression was noted in the kidney, heart, lung, liver, or testis of either line (data not shown).

Peripheral tissue expression was examined further in transgenic lines 8365, 8690, and 8364 using an X-gal histochemical assay on frozen sections. The nuclear localization of the reporter gene facilitated distinguishing transgene expression from background β-galactosidase activity in the cytoplasm. A few scattered blue nuclei were noted in the kidneys and brains of all three lines and in the ovaries and oviducts from two of two tested lines (8365 and 8364). In 20-μm frozen sections of kidney, expression was localized exclusively in the artery walls near the vascular pole of glomeruli. Four-micrometer frozen sections confirmed the localization to a subset of vascular wall cells in the afferent arterioles of glomeruli, possibly in granular cells, the major source of endocrine renin production (Fig. 4A). In the brain, expressing cells were located predominately in the arcuate hypothalamic nucleus and the cortex of all three lines and in the lateral septal nucleus of the highest expressing lines 8365 and 8690 (Fig. 4B). The level of transgene expression in the kidney and in these regions of the brain was much lower than the transgene expres-

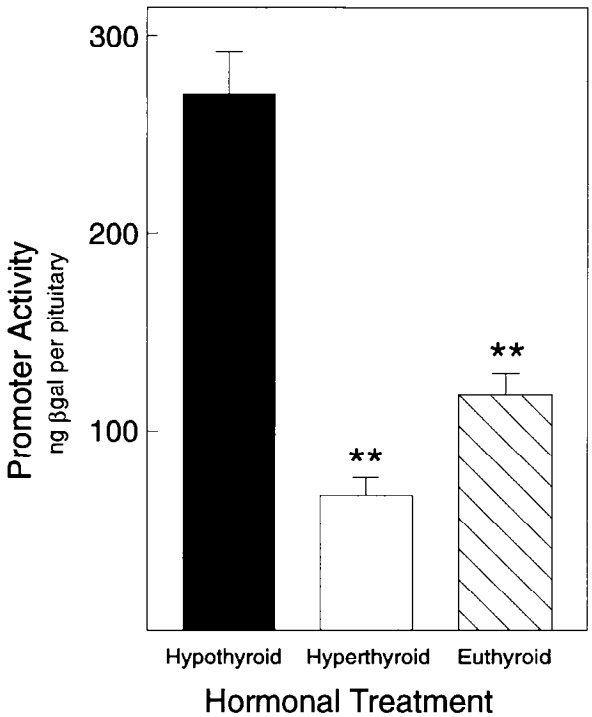

Fig. 3. Expression of the 4.6 mα-βgal Transgene Is Increased in Response to Hypothyroidism and Decreased in Hyperthyroid Mice

Groups of adult transgenic mice (n = 6) were radiothyroidectomized (hypothyroid, *black bar*) or treated with L-T_4 (hyperthyroid, *shaded bar*) as described in *Materials and Methods*. A control group was untreated (euthyroid, *striped bar*). Thyroidal status was confirmed by measurement of serum T_4 and T_3 levels. Anterior pituitaries were harvested and cell extracts were assayed for β-galactosidase activity and are expressed as nanograms of β-galactosidase per pituitary. *Error bars* represent the SEM. There was a statistically significant difference (**) between the groups ($P < 0.05$) for all pairwise multiple comparisons using the Student-Newman-Keuls method.

sion in the pituitary gland (Fig. 4C). No transgene expression was detected in heart, lung, liver, and testis.

Expression of the endogenous α-subunit gene in the brain, kidney, and ovary was examined by *in situ* hybridization. As expected, endogenous α-subunit mRNA transcripts were readily detected in the anterior pituitary after 3 days exposure of emulsion-dipped slides (data not shown). Expression evident in the *pars tuberalis* of the pituitary also served as a positive control (Fig. 4D), but no significant endogenous gene expression was detected in the brain, ovary/oviduct, or kidney after 3 weeks exposure (data not shown).

α-Subunit expression, if present in these peripheral tissues, is below the limit of detection by our *in situ* hybridization assay. Nevertheless, transgene expression in the granular cells of the kidney provides a marker for purification of these cells (30).

Appropriate Developmental Onset of Transgene Expression in Rathke's Pouch

The α-subunit mRNA is first detected in the developing rat embryo in the hypophyseal placode which becomes Rathke's pouch (8, 9). Embryos were collected from transgenic lines 8365 and 8364 at embryonic day 8.5, 9.5, and 12.5 and examined for evidence of transgene expression. No expression was detected in the region of the developing pituitary in whole embroyonic day 8.5 (e 85) embryos stained with X-gal (data not shown). Blue-staining cells were evident in essentially all of the cells of Rathke's pouch at e 9.5 (Fig. 5A). No background β-galactosidase activity was detected in C57BL/6J control littermate embryos. The morphology of Rathke's pouch in e 9.5 mouse embryos is comparable to the stage when α-subunit is first detected in rat embryos, e 11 (8, 9, 31). This suggests that the transgene is activated between e 8.5 and e 9.5, as expected. At e 12.5, expression in the developing pituitary was much stronger. Blue-staining was detectable throughout Rathke's pouch, but the majority of staining appeared to be restricted to the ventral and anterior portion, in the rostral tip (Fig. 5, B and C).

Identification of Novel Regions of Endogenous α-Subunit Gene Expression in Developing Mice

In addition to transgene expression in the developing pituitary gland, low levels of transgene expression were detected in several other regions at each embryonic stage examined. In transgenic lines 8365 and 8364, expression was detected at e 9.5 in the trigeminal area in the region of condensing mesenchyme (Fig. 5A) and, at much lower levels, in the umbilical region. At e 12.5, low levels of expression were detectable in the condensing mesenchyme in the region of forming extraocular muscle (Fig. 5, B and E), the first branchial pouch, a portion of the trigeminal area, the medial wall of the vestibular or cochlear apparatus, the marginal zone of the spinal cord, the connective tissue core of the genital tubercle, and pancreatic primordium (Table 2). In line 8364, transgene expression was also detected in cells present in the mesenchyme near the olfactory epithelium, likely to be neurons migrating from this region to the hypothalamus (data not shown). Transgene expression patterns were reproducible within a line and identical in lines 8364 and 8365 except for some minor differences such as the expression in the olfactory region. At e 12.5, expression in Rathke's pouch was significantly greater than expression in any of these peripheral regions.

To compare endogenous expression with transgene expression at e 12.5, *in situ* hybridization was performed on embryo sections from C57BL/6J and transgenic line 8364. Endogenous α-subunit mRNA was detected in the condensing mesenchyme in the region of forming extraocular muscle (Fig. 5F) and in cells present in the mesenchyme near the olfactory epithelium (Fig 5D). These are the same regions in which transgene expression was evident. As noted for the transgene, the level of expression in these peripheral regions was much lower than the level of expression in the developing pituitary.

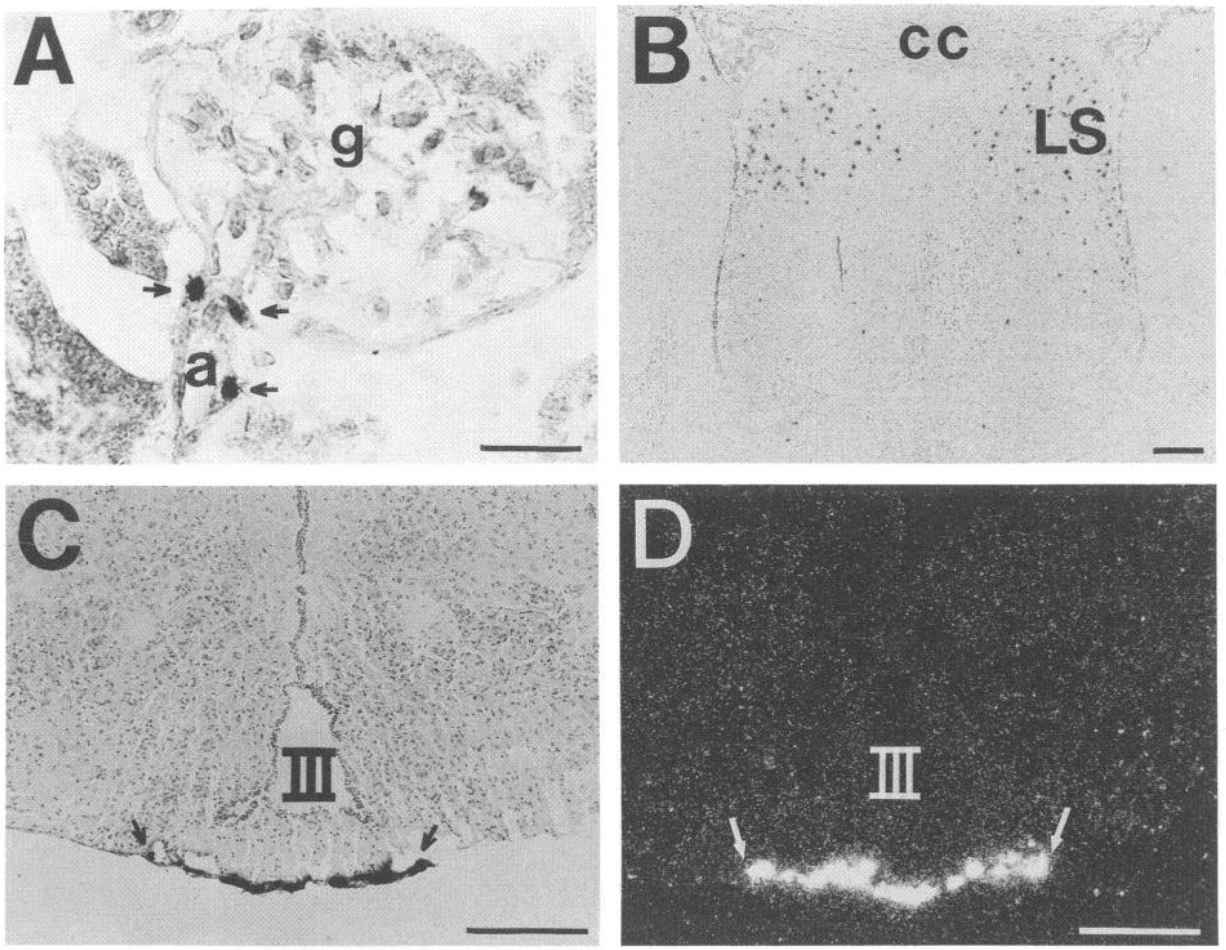

Fig. 4. The 4.6 mα-βgal Transgene Is Expressed at Much Lower Levels in Adult Peripheral Tissues Than in the Anterior Pituitary Gland

Frozen brain and kidney sections from transgenic line 8365 were stained for β-galactosidase activity by X-gal histochemistry and counterstained with 0.5% neutral red. In the kidney (panel A), expression was restricted to the vasculature in close proximity to the vascular pole of glomeruli (g). Here a longitudinal section through an afferent arteriole (a) demonstrates three positive vascular wall cells, possibly granular cells (*arrows*). Transgene expression is also evident in the lateral septal nucleus of the brain (LS) which lies below the corpus callosum (cc) (panel B). Transgene expression in the pituitary stalk (pars tuberalis, *arrows*), located just below the median eminence of the hypothalamus and the third ventricle (III), is evident in a frozen brain section of line 8365 after X-gal histochemistry (panel C). Expression of the endogenous α-subunit gene was detected in the pars tuberalis (*arrows*) by *in situ* hybridization histochemical analysis of an adjacent brain section with a mouse α-subunit cDNA antisense riboprobe (panel D, darkfield). No endogenous mouse α-subunit transcripts were found in other areas of the brain. The *magnification bars* represent 20 μm (panel A) and 250 μm (panels B-D).

DNA Sequences Between −4.6 and −2.7 kb Are Necessary for High Level Expression of α-Subunit in Whole Animals

Sequences within the 480 bp proximal to the mouse α-subunit transcription start site are sufficient for expression in thyrotrope- and gonadotrope-derived cell lines (11–13). Lower levels of expression in somatotrope cell lines suggest that 480 bp is sufficient for cell specificity (12). To determine whether 480 bp can confer cell-specific expression in whole animals, 480 bp of the mouse α-subunit 5′-flank were joined within exon 1 to the nuclear-localized *lacZ* reporter gene (0.48 mα-βgal, Fig. 1). The integrity of the construct was confirmed by transient transfection into αTSH cells. Both the 5.0 mα-βgal and 0.48 mα-βgal constructs gave high levels of expression when compared to the promoterless reporter construct. No significant difference was noted in promoter activity between the two constructs (Fig. 6).

Eleven transgenic founders were generated with the 0.48 mα-βgal construct. The fluorometric assay was used to quantify β-galactosidase activity in extracts prepared from a portion of the anterior lobe from these founders. For analysis of cell specificity, the remainder of the pituitary was incubated in X-gal and embedded in paraffin, and sections were immunostained. In order

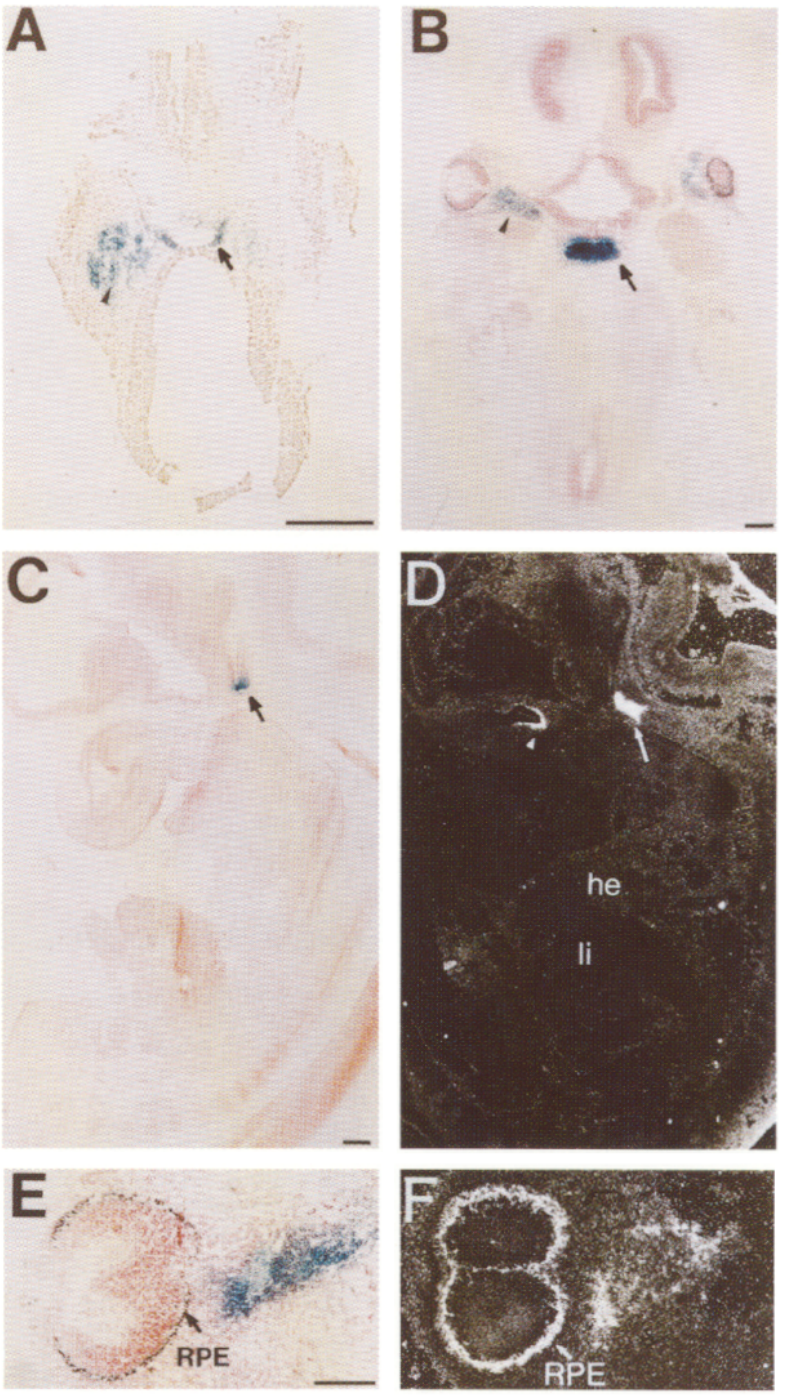

Fig. 5. Appropriate Expression of the 4.6 mα-βgal Transgene in Rathke's Pouch and Novel Embryonic Tissues

Frozen transverse sections through the cephalic region of e 9.5 (line 8365, panel A) and e 12.5 embryos (line 8364, panel B) were stained for β-galactosidase expression by X-gal histochemistry and counterstained with 0.5% neutral red. Expression was detected in Rathke's pouch at both stages (*arrows*), in the trigeminal nucleus at e 9.5 (*arrowhead*) and in condensing mesenchyme in the region of forming extraocular muscles at e 12.5 (*arrowhead*). X-gal histochemical staining of a sagittal section of an e 12.5 embryo from transgenic line 8364 reveals expression throughout Rathke's pouch, with predominant expression in the ventral anterior portion of the rostral tip (*arrow*, Panel C). *In situ* hybridization with an α-subunit cDNA antisense riboprobe was carried out in sagittal sections of a C57BL/6J embryo, e 12.5, and photographed in darkfield (panel D). Endogenous α-subunit expression was detected in Rathke's pouch (*arrow*) and in cells present in the mesenchyme region near the olfactory epithelium (*arrowhead*). These cells are likely to be neurons migrating from this region to the hypothalamus. For orientation, heart (he) and liver (li) are indicated. At higher magnification, expression of the transgene in line 8364 at e 12.5 is evident by the *blue* precipitate associated with the condensing mesenchyme in the region of forming extraocular muscles at the level of the optic fissure (panel E). The pigmented epithelial cells of the retina contain melanin granules that appear black in brightfield and white in darkfield (RPE). Expression of the endogenous α-subunit gene in the same region was confirmed in an adjacent section by *in situ* hybridization

Table 2. Embryonic Expression in Transgenic Lines 8365 and 8364

Region	Level of Expression[a]	
	e 9.5	e 12.5
Rathke's pouch	++	++++++
Trigeminal nucleus	++	+
Umbilical region	+	−
Condensing mesenchyme in the extraocular muscle region	−	+++
First branchial arch	−	+
Medial wall of the vestibular apparatus	−	+
Olfactory region	−	+[b]
Marginal zone of the spinal cord	−	++
Genital tubercle	−	+
Pancreatic primordium	−	+

[a] The intensity of β-galactosidase staining in frozen sections was evaluated subjectively. Weak staining is scored as + and intense staining as ++++++.

[b] Expression was observed in transgenic line 8364 but not in line 8365.

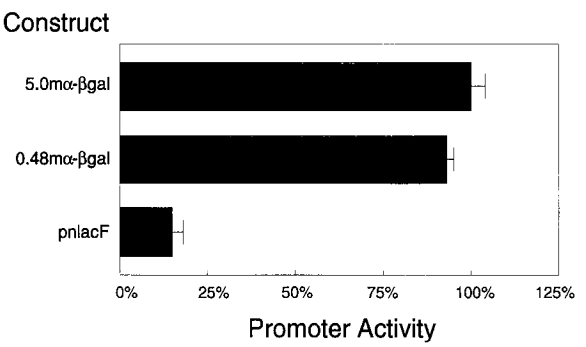

Fig. 6. Constructs with 5 kb or 480 bp of the Mouse α-Subunit 5′-Flanking Sequences Exhibit Equivalent Promoter Activity in αTSH Cells

αTSH cells (2–3 × 10^6) were transiently transfected in quadruplicate with 20 μg of a β-galactosidase expression vector containing either 5 kb or 480 bp of the α-subunit 5′-flanking regions or no promoter (pnlacF, *vertical axis*). One microgram of the luciferase expression vector $pA_3RSVLUC$ was cotransfected. After 24 h, cells were harvested and β-galactosidase and luciferase activities were measured as described in *Materials and Methods*. The results of four transfections are presented as the mean ± SEM, normalized to the percent activity relative to the 5.0 mα-βgal construct.

for staining to be visible, the concentration of X-gal was 10-fold higher than that used to stain pituitaries from mice with the 4.6 mα-βgal construct. In four founder animals tested, transgene expression was localized to cells expressing LH or TSH (Fig. 7) and was not present in cells expressing PRL, GH, or ACTH. In general, the 0.48 mα-βgal mice had fewer X-gal-stained gonadotrope and thyrotrope cells than the 4.6 mα-βgal mice, and the staining was lighter. The level of β-galactosidase activity in the 0.48 mα-βgal transgenic mice was compared to the activity measured in extracts prepared the same day from the 4.6 mα-βgal transgenic line 8365 (Fig. 8). Pituitaries from C57BL/6J mice served as negative controls. All 11 0.48 mα-βgal founders exhibited less than 10% of the expression levels observed in line 8365. In contrast, only three of the eight 4.6 mα-βgal transgenic lines had β-galactosidase levels in the low range. It is clear that although these constructs confer equivalent levels of expression in transient transfection, they have dramatically different activities in transgenic mice. No transgene expression was detected in X-gal-stained frozen sections of kidney, liver, heart, lung, or testis (data not shown).

In order to determine the location of enhancer sequences present in the 4.6 kb and missing in 480 bp, transgenes with 2.7 kb and 1.49 kb of the mouse α-subunit 5′-flank were generated (Fig. 1). Eight founders containing the 2.7 mα-βgal transgene and 10 founders containing the 1.49 mα-βgal transgene were analyzed by fluorometric assay and immunostaining as described for the 0.48 mα-βgal transgenic mice. The majority of these founders also expressed the transgene at a level below 10% that observed in line 8365 (Fig. 8). Cell-specific expression in both gonadotropes and thyrotropes was confirmed in at least two founder animals for each construct (data not shown).

Application of the one-way analysis of variance test confirmed a statistically significant effect of the 5′-flanking region on transgene expression ($P < 0.0007$). Expression of the transgenes with 2.7, 1.49, and 0.48 kb of 5′-flanking DNA did not differ significantly ($P < 0.60$). Pairwise multiple comparisons between groups using the Student Newman-Keuls method demonstrated that expression of the 4.6 mα-βgal transgene was statistically different from each of the others ($P < 0.05$)). Thus, sequences necessary for high level expression in both gonadotropes and thyrotropes of transgenic mice lie between −4.6 and −2.7 kb. This region is likely to contain an enhancer, although we have not demonstrated position- and orientation-independent function.

DISCUSSION

This is the first *in vivo* demonstration of α-subunit transgene expression in both pituitary thyrotrope and gonadotrope cells. Most 4.6 mα-βgal transgenic lines exhibited a high level of β-galactosidase reporter gene expression, and the transgene was expressed in 80–90% of the cells immunostained with LH and TSH.

with an α-subunit cDNA antisense riboprobe (panel F, darkfield). *Magnification bars* represent 200 μm (panels A and E) and 300 μm (panels B and C). The magnification of panels D and F are identical to panels C and E, respectively.

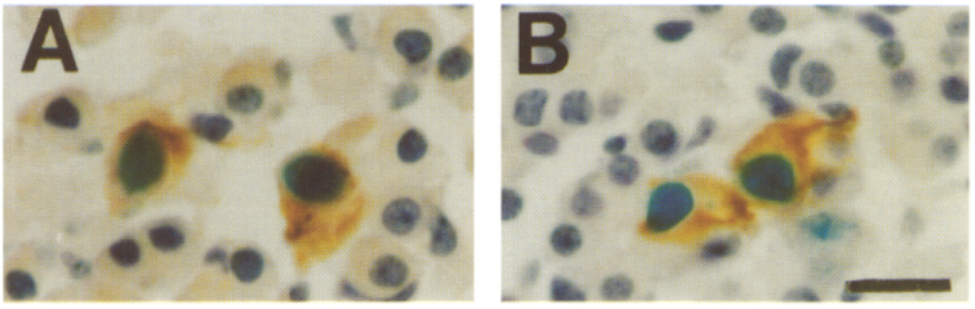

Fig. 7. Expression of the 0.48 mα-βgal Transgene Colocalizes with LH and TSH Immunoreactivity

The pituitary gland from a transgenic founder animal carrying the 0.48 mα-βgal construct was stained for β-galactosidase expression and embedded in paraffin, and sections were immunostained with antibodies against pituitary hormones. Transgene expression was evident only in cells immunostained for LH (panel A) or TSH (panel B). The magnification in panels A and B is the same, and the *magnification bar* represents 15 μm (panel B).

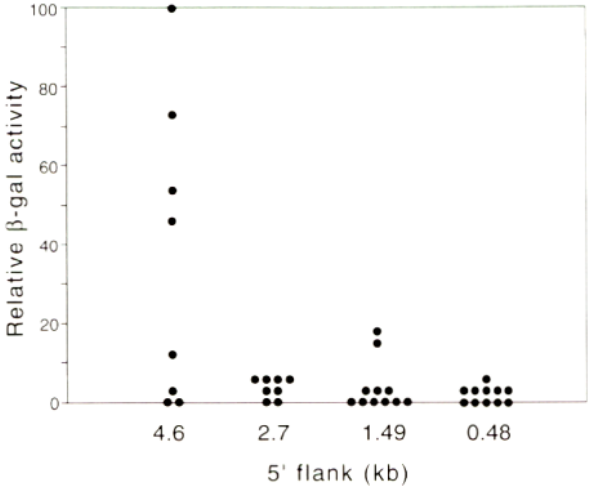

Fig. 8. Sequences Between −4.6 and −2.7 kb of the Mouse α-Subunit 5′-Flank Are Necessary for High Level Expression in Transgenic Mice

β-Galactosidase activity was measured in duplicate aliquots of individual pituitary gland homogenates by fluorometric assay and normalized to protein concentrations. The analysis was performed on transgenic founders carrying the 2.7 mα-βgal, 1.49 mα-βgal, and 0.48 mα-βgal transgenic constructs and transgenic progeny of 4.6 mα-βgal lines. In transgenic line 8365, the average β-galactosidase activity was 359 U enzyme per gram of protein. The background level of β-galactosidase activity in nontransgenic C57BL/6J pituitaries, 3.5 U/g, was subtracted from the readings for each transgenic pituitary, and values are shown as a percent of the activity of transgenic line 8365.

Based on transfection of αTSH cells and thyrotrope tumor cells, we predicted that 480 bp of 5′-flanking sequences would be sufficient to direct appropriate cell-specific expression in gonadotropes and thyrotropes (10–13). Despite the low level of expression obtained from transgenes truncated to −480 bp, thyrotrope- and gonadotrope- specific expression was retained, confirming the cell culture study. This finding contrasts with transgene experiments in which 1.6 and 1.8 kb of human α-subunit 5′-flank resulted in only gonadotrope expression (14, 16). Although a high degree of DNA sequence similarity exists between the mouse and human α-subunit 5′-flanking regions (>60% over approximately 500 bp), species differences may account for the lack of expression of the human promoter in the thyrotropes of transgenic mice. This idea is supported by other species-specific differences in α-subunit expression (11, 20, 23).

In transgenic mice a dramatic reduction in the level of reporter gene expression was observed with 480 bp compared to 4.6 kb of the mouse α-subunit 5′-flank. Deletion analysis in transgenic mice demonstrated that sequences between −4.6 kb and −2.7 kb are critical for high level expression in both gonadotropes and thyrotropes. In contrast, 5 kb and 480 bp yield identical levels of reporter expression in αTSH cells. Differences in the requirements for expression in cell lines and transgenic mice have been reported for other genes (32–34). There are several possible explanations for the inactivity of the enhancer element in the cell lines. Chromatin structure may be required for enhancer activity, necessitating the generation of stable transformants (35). Alternatively, the αTSH cells may lack one or more trans-acting factors that are critical for function of the enhancer sequences between −4.6 and −2.7 kb. The αTSH cell line and the transplantable tumor, MGH101A, from which it was derived, differ from the original thyrotrope tumor, MGH101, in that they do not express the TSH β-subunit gene, although they continue to express the α-subunit gene (36, 37). Thus, a change in transcription factor activity through altered gene expression or covalent modification could account for both the lack of α-subunit enhancer recognition and the lack of TSH β-subunit transcription in the cells. There are other examples of transformed cells with alterations in differentiated gene expression that may result from changes in transcription factor expression (38–40)

Expression of pituitary α-subunit is directly regulated at the transcription level by several hormones, including GnRH, TRH, and thyroid hormone. Distinct DNA se-

quences appear to mediate these responses. Many regulatory elements within 500 bp of the human, rat, or mouse α-subunit promoters have been identified in cell culture studies (12, 41–45). In transgenic mice both the bovine and human α-subunit promoters exhibit GnRH and estradiol responsiveness, but neither promoter exhibited thyroid hormone regulation (14). We demonstrated thyroid hormone regulation of the transgene containing 4.6 kb of mouse α-subunit 5′-flank. A 75% decrease in expression was observed in mice treated with T_4 compared to the hypothyroid state. This transgene response correlates with the changes seen in α-subunit mRNA levels in pituitaries (decrease of 40–50%) and in thyrotropic tumors (decrease of 85%) in similarly treated mice (46). This suggests that sequences within 4.6 kb of the mouse α-subunit promoter contain all of the *cis*-acting sequences necessary to confer the thyroid hormone response. This is consistent with transient transfection experiments in TtT-97 thyrotropic tumor cells in which we localized a thyroid-inhibitory response to a region between −62 and +43 bp of the mouse α-subunit promoter (12).

The developmental onset of transgene expression between e 8.5 and e 9.5 corresponds to the timing expected from previous studies of the ontogeny of α-subunit gene expression (8, 9). Transgene expression continued to follow the expected pattern; β-galactosidase levels increased and became more restricted to the ventral and anterior region of Rathke's pouch after e 9.5. The spatially restricted expression we observed corresponds with that reported for the endogenous α-subunit gene (8, 9, 47). This indicates that 4.6 kb of the mouse α-subunit 5′-flank also contains the *cis*-acting sequences necessary for developmental regulation. Thus, we have defined DNA sequences useful for targeting expression of other genes to the pituitary primordium.

Transcription of α-subunit during ontogeny has only been reported in Rathke's pouch (8, 9). Although we found the majority of transgene expression in Rathke's pouch, significant levels of expression were also present in other regions, including the extraocular and olfactory regions. Using *in situ* hybridization, we demonstrated that both of these areas of transgene expression correlate with regions of endogenous α-subunit gene expression. The reproducibility of low level transgene expression in other areas suggests that α-subunit may be expressed in these other regions at a level undetectable by the *in situ* hybridization assay.

If α-subunit transcripts are translated in Rathke's pouch or in the olfactory and extraocular regions, α-subunit may have a biological role in early development. In adult pituitary glands, the α-subunit is produced in excess of the β-subunits, and it is secreted as free α-subunit. Free bovine α-subunit has an additional O-linked oligosaccharide which prevents its association with β-subunits (48). Cell culture studies suggested that free α-subunit can act as an inducer of differentiation (49), although no α-subunit receptor has been identified. The presence of transcripts in the olfactory region and pituitary primordium is intriguing in light of the observation that GnRH cells develop from a group of progenitor cells in the olfactory placode at e 11 and migrate across the nasal septum into the forebrain and toward the hypothalamus (50–52). A role for α-subunit in differentiation or migration of brain, sensory, and/or pituitary gland cells will be tested directly by targeted disruption of the α-subunit gene by homologous recombination.

The expression of the α-subunit transgene in the brain was consistently observed in the cortex, lateral septal nucleus, and arcuate nucleus. The apparent lack of α-subunit mRNA transcripts in those regions may be attributable to insensitivity of the *in situ* hybridization assay relative to the β-galactosidase assay. The level of transgene expression in the brain was lower than that observed in the extraocular region of the developing embryo, where endogenous α-subunit transcripts were within the limit of detection. We cannot rule out the possibility that the transgene lacks DNA sequences that repress expression in the brain; however, the presence of immunoreactive LH, TSH, and α-subunit in the brain, and particularly the hypothalamus, has been reported (53–56). Other studies also were unable to detect α-subunit mRNA in brain and concluded that pituitary hormones might be transported into the brain (56). Our observations reopen the possibility that α-subunit is transcribed in the brain.

Our transgenic mouse studies have confirmed that 480 bp of promoter proximal regions are sufficient for cell-specific expression of the α-subunit in thyrotropes and gonadotropes. However, we also localized an element between −4.6 and −2.7 kb that exhibits enhancer activity in both gonadotrope and thyrotrope cells *in vivo* but is inactive in cell culture studies. Sequences important for hormonal regulation and temporal specific expression were contained within 4.6 kb as well. These studies of transgene expression in development led to identification of novel regions of endogenous α-subunit gene expression in developing embryos. The 4.6-kb fragment described will provide an effective tool for direction of foreign gene expression in the anterior pituitary gland.

MATERIALS AND METHODS

Construction of the Mouse α-Subunit-β-Galactosidase Clones

A 3.2-kb *Hin*dIII to *Bam*HI fragment containing sequences from −5.0 kb to −1.7 kb of the mouse α-subunit gene was excised from a 8.5-kb *Hin*dIII to *Sal*I genomic clone (57), and a 1744-bp *Bam*HI to *Hin*dIII fragment containing genomic sequences from −1.7 kb to +43 bp was excised from a luciferase reporter clone (58). These two fragments were subcloned into the *Hin*dIII site of pGEM7Zf+ to generate a −5 kb to +43 bp *Hin*dIII to *Hin*dIII insert. This fragment was subsequently isolated, incubated with AMV reverse transcriptase (Promega, Madison, WI) and all four deoxynucleoside triphosphates to produce blunt ends, and subcloned into the *Sma*I site of the β-galactosidase expression vector pnlacF (kindly provided by

Jacques Peschon and Richard Palmiter). This vector contains the *Escherichia coli lac Z* gene modified to encode the SV40 T antigen nuclear localization signal at the amino terminus. Sequences from the mouse protamine-1 gene present at the 3′-end of the pnlacF vector provide 3′-untranslated sequences, an intron, and polyadenylation signal (59). Similarly, the −480 to +43 *Hin*dIII genomic fragment was cloned into the *Sma*I site of pnlacF. The integrity of the recombinant expression clones was verified by restriction mapping, and the clone junctions were confirmed by DNA sequence analysis.

The 5.0 mα-βgal clone and the 0.48 mα-βgal clone were digested with *Kpn*I and *Hin*dIII to remove plasmid sequences and produce the transgenes 4.6 mα-βgal and 0.48 mα-βgal. The 5.0 mα-βgal plasmid was also digested with *Xho*I and *Hin*dIII to generate the 2.7 mα-βgal transgene and with *Sph*I to generate the 1.49 mα-βgal transgene. The fragments were isolated by agarose gel electrophoresis and purified for microinjection with either the Nucleobond AX plasmid kit (The Nest Group, Inc., Southborough, MA) or with the FMC SpinBind DNA Recovery System for Agarose Gels (FMC BioProducts, Rockland, ME).

Generation and Breeding of Transgenic Mice

The purified inserts were microinjected into F2 hybrid zygotes from C57BL/6J × SJL/J parents at a total concentration of approximately 2–3 ng/μl. After overnight incubation, embryos at the two cell stage were transferred to day 0.5 postcoitum pseudopregnant CD-1 females. The 4.6 mα-βgal transgenic founders were mated to C57BL/6J mice to establish lines.

C57BL/6J and SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) and CD-1 mice (Charles River, Wilmington, MA) were bred at the University of Michigan. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accord with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Identification of Transgenic Mice

Genomic DNA prepared from tail biopsies was screened for the presence of the transgene by polymerase chain reaction (PCR) (60). A 30 bp sense oligo (5′ TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA 3′) and a 30-bp antisense oligo (5′ ATG TGA GCG AGT AAC AAC CCG TCG GAT TCT 3′) were used to amplify a 364-bp fragment of the β-galactosidase gene corresponding to nucleotides 15–379 (61). The PCR reactions were carried out under standard conditions using 100–200 ng genomic DNA, 0.5 pmol/μl primers, 2.5 mM $MgCl_2$, and 1.7 U Taq DNA polymerase per reaction (62). Reactions proceeded for 30 cycles of denaturation at 92 C for 30 sec, annealing at 60 C for 1.5 min, extension at 72 C for 2 min, and final extension at 72 C for 10 min.

To estimate transgene copy numbers and to confirm the integrity of the transgene, Southern blots were performed on transgenic mice identified by PCR. Mouse tail genomic DNA (10 μg) was digested overnight in the presence of 4 mM spermidine. The DNA fragments were separated by electrophoresis in an agarose gel, transferred to a nylon filter (BioRad, Richmond, CA), and attached by cross-linking with UV light. The pnlacF plasmid was linearized and radiolabeled by random priming for use as a probe. Hybridization was carried out at 65 C overnight in 7% sodium dodecyl sulfate, 0.5 M Na_2HPO_4, and 1 mM EDTA (pH 7.3). Filters were washed at 57 C to a final stringency of 0.1 × SSC, 0.1% sodium dodecyl sulfate (1 × SSC consists of 0.5 M NaCl, 0.015 M sodium citrate, pH 7.0). Copy number standards were prepared by mixing the appropriate amount of microinjection solution with genomic DNA from a nontransgenic mouse.

β-Galactosidase Fluorometric Assays

Transgene expression was quantitated in tissue extracts using a fluorometric assay for β-galactosidase. Individual pituitary glands from 4.6 mα-βgal transgenic mice were homogenized in 1 ml 250 mM Tris-Cl, pH 8, using Dounce homogenizers. Samples were freeze-thawed by incubation at 37 C and dry ice alternately for three cycles, and centrifuged for 10 min. Forty microliters of each supernatant were combined with 160 μl of a reaction mixture containing 25 mM Tris-Cl, pH 7.5, 125 mM NaCl, 2 mM $MgCl_2$, 12 mM 2-mercaptoethanol, and the substrate, 0.3 mM 4-methylumbelliferyl-β-D-galactoside (Sigma, St. Louis, MO). The reactions were incubated for 30 min at 37 C, stopped by addition of 50 μl 25% trichloroacetic acid, placed on ice, and centrifuged. Twenty or 40 μl of each supernatant were diluted in 2 ml of a pH 10.7 buffer containing 133 mM glycine and 83 mM Na_2CO_3. Fluorescence was measured using a TKO Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA). A standard curve was prepared using 50–175 nM of a methylumbelliferone standard diluted in the glycine carbonate reagent. Liver, heart, lung, testis, ovary, and brain homogenates were prepared and analyzed as described for the pituitaries except 10% tissue homogenates (grams per ml) were prepared using a Polytron (Brinkmann Instruments, Westbury, NY). Tissues from two mice from each transgenic line were analyzed, fluorometric readings were averaged, and background values were subtracted. Only measurements that were more than 2-fold greater than background were considered significant.

Modifications in the β-galactosidase fluorometric assay were made to facilitate quantification of transgene expression in founder mice. A section of the anterior lobe of the pituitary was excised using a scalpel and homogenized for up to 10 sec in 0.5 ml 250 mM Tris-Cl, pH 8, by sonication with cooling on ice. The remainder of the pituitary gland was processed for immunohistochemistry. Extracts were also prepared from progeny of line 8365 for comparison. Forty microliters of each homogenate were combined with 160 μl of a modified reaction cocktail containing 1.8 mM 4-methylumbelliferyl-β-D-galactoside. A standard curve was prepared using known amounts of β-galactosidase enzyme (5.0×10^{-3} to 5.0×10^{-5} U) (Sigma). Reactions were incubated for 4 h at 37 C, stopped by addition of 25% trichloroacetic acid on ice, and centrifuged. Forty microliters of each supernatant were diluted as above and fluorescence was measured with an American Instruments fluorometer (American Instruments Co., Inc., Silver Spring, MD) modified by D. Ballou and G. S. Ford (Department of Biological Chemistry, University of Michigan, Ann Arbor, MI). Reactions were performed in duplicate, fluorometric readings were averaged, and C57BL/6J background levels were subtracted.

β-Galactosidase activity was normalized for the amount of protein in each homogenate. The Bradford protein assay (63) was performed with the Coomassie brilliant blue dye reagent (BioRad) on 15–30 μl homogenate in microtiter plates as described (BioRad Laboratories, Hercules, CA). Absorbance of the samples and the BSA protein standard was measured at 630 nm using an EL311sx Auto Reader (Bio-TeK Instruments, Wihooski, VT).

Histology and Immunohistochemistry on Adult Pituitary Gland and Peripheral Tissues

Pituitaries and peripheral tissues were removed and frozen immediately in OCT (Miles Scientific, Elkhart, IN) on dry ice. Brains were frozen whole in 2-methyl butane at −35/−40 C for 30 sec and stored at −70 C. Twenty micrometer sections, and 4-μm sections of kidney, were cut in a cryostat (Bright Instrument Company, Huntingdon, England) and mounted on poly-L-lysine-coated slides.

Sections were fixed for 5 min in 0.2% glutaraldehyde, containing 0.1 M NaH_2PO_4 (pH 7.3), 5 mM EGTA, and 2 mM $MgCl_2$, washed three times in wash buffer [0.1 M NaH_2PO_4 (pH 7.3), 2 mM $MgCl_2$, 0.02% NP-40], and incubated at 37 C overnight in an X-Gal solution containing 1 mg/ml X-Gal (Boehringer Mannheim, Indianapolis, IN), 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6 \cdot {}^3H_20$ in wash buffer (above). The counterstain was 0.5% neutral red (Sigma).

The cell specificity of transgene expression was evaluated by X-Gal enzymatic assay followed by immunohistochemistry for individual pituitary hormones. Pituitaries were fixed for 1 h in 4% paraformaldehyde in pH 7.2 sodium phosphate buffer and incubated at room temperature overnight in an X-Gal solution as above, except that X-Gal was reduced to 0.1 mg/ml for pituitaries from the 4.6 mα-βgal transgenic lines. After 2–4 h postfixation in the buffered paraformaldehyde, the samples were embedded in paraffin and 3- to 4-μm sections were prepared. Immunostaining was performed with polyclonal antisera against rat pituitary PRL (1:2000, AFP-10505B), rat GH (1:1000, AFP411S), rat LHβ (1:2000, AFP22238790GP0LHB), rat TSHβ (1:1000 AFP1274789) (National Hormone and Pituitary Program, NIDDK, Bethesda, MD), and human ACTH (1:1000) (Dako, Santa Barbara, CA). Biotinylated secondary antibodies were used in conjunction with avidin and biotinylated peroxidase (Vectastain guinea pig, rabbit, and human kits, Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen. Normal serum was substituted for the primary antibody in negative controls. Sections were counterstained with Gill's triple strength hematoxylin (Fisher, Pittsburgh, PA). Cell counts were performed as previously described (15).

Thyroid Hormone Regulation

To assess transgene response to thyroid status, groups of transgenic mice from line 8365 (n = 6 per group) were rendered hypothyroid, hyperthyroid, or left untreated. Radiothyroidectomy by ip injection of 150 μCi $Na^{131}I$ (New England Nuclear Corp., Boston, MA) was performed 167 days before experimentation to produce hypothyroid mice. Another group of animals were administered L-T_4 (5 mg/liter in 0.75% EtOH) (Sigma) in their drinking water for 28 days (hyperthyroid), and one group served as an untreated control (euthyroid). Mice were anesthetized by methoxyflurane inhalation and killed by cervical dislocation. Trunk blood was collected and serum was assayed for T_4 and T_3 by RIA (64). Anterior pituitary glands were collected in microfuge tubes containing 250 μl cell lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol) and homogenized with a pestle. Extracts were prepared by subjecting tissue homogenates to three cycles of freezing and thawing followed by sonication for 2 × 10 sec with cooling on ice in between. After centrifugation for 5 min, an aliquot of the supernatant was removed and diluted 1:10 with water. β-Galactosidase activity was determined using a chemiluminescent reporter assay (Galacto-Light, Tropix, Inc., Bedford, MA) (65) under conditions in which endogenous enzyme activity is suppressed. Briefly, triplicate 10-μl aliquots of the diluted sample were added to luminometer cuvettes followed by 50 μl reaction buffer. Tubes were incubated at room temperature for 60 min followed by addition of 100 μl accelerator solution. After a 10-sec delay, light production was measured for 10 sec with a luminometer (model 2010, Analytical Bioluminescence Laboratory, San Diego, CA). A standard curve was produced by assaying known amounts of β-galactosidase (Sigma, 300 U/mg) from 1×10^{-6} U to 5×10^{-3} U.

Analysis of Transgene Expression in Developing Mice

Transgenic males were mated to C57BL/6J females. Midday after detection of a vaginal plug was considered embryonic day 0.5 (e 0.5). Whole e 9.5 embryos were fixed in 0.2% glutaraldehyde for 1 h and stained in X-Gal solution (1 mg/ml) overnight. Subsequently, the embryos were oriented in OCT compound, and frozen in hexane cooled in an acetone-dry ice slurry. Twenty-micrometer serial transverse and sagittal sections were cut using a cryostat, mounted onto poly-L-lysine-coated slides, and counterstained with 0.5% neutral red. The processing of e 12.5 embryos was similar except embryos were frozen in OCT compound in hexane before fixation. Sections were postfixed for 5 min in 0.2% glutaraldehyde and stained in an X-Gal solution (1 mg/ml) overnight at 37 C. Transgenic embryos were identified by PCR amplification of the β-galactosidase reporter gene in genomic DNA prepared from placentas.

In Situ Hybridization

In situ hybridization was performed as described (66, 67) except that fetal tissue sections were rinsed in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.3) after fixation and deproteinated in lower levels of proteinase K (0.1 μg/ml) (Boehringer Mannheim) for 5 min at 37 C. Adult tissues were deproteinized in 1 μg/ml proteinase K for 10 min at 37 C. Antisense and sense riboprobes were generated from a 460-bp Pst1 fragment of the mouse α-subunit cDNA cloned into the pGEM3Zf+ vector (Promega, Madison, WI) (68). The probes were labeled with [^{35}S]uridine triphosphate using T7 or SP6 polymerase. Slides were hybridized overnight in 75% formamide hybridization buffer (66) containing 1.5×10^6 cpm of riboprobe at 57 C in a humidified chamber. Posthybridization treatment was as described (66). The final wash was performed in 0.5 × SSC at 65 C (67). Sections were dehydrated and air dried, dipped in Kodak NTB-2 nuclear emulsion diluted 1:1 with water, exposed for 1 day to 3 weeks, developed, and counterstained with cresyl violet (Sigma).

Cell Culture, Transient Transfection, and Reporter Gene Assays

Suspension cultures of αTSH cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum as previously described (12). Cells were placed in medium containing 10% charcoal-stripped (T_3 depleted) fetal calf serum for 48 h before transfection, which was performed as previously described (69) with minor modifications. Briefly, 100 μl cell suspension containing 2×10^6 viable cells in DMEM was mixed with 20 μg of the test plasmid and 2 μg RSV-luciferase as an internal control in a sterile gene-pulse chamber and subjected to a controlled electrical field, then transferred to 60 mm^2 culture plates containing 4 ml DMEM supplemented with 10% charcoal-stripped fetal calf serum and placed in a 5% CO_2 incubator at 37 C. Each construct was transfected in quadruplicate. After incubation for 48 h, cells were harvested and lysed by three cycles of freezing and thawing, and both β-galactosidase and luciferase activity were determined in duplicate 10-μl aliquots. For measurement of β-galactosidase activity, a chemiluminescent assay was performed using a commercially available kit (Tropix, Bedford, MA, Galacto-Light). Light emitted was measured after a 2-sec delay and integrated for the next 5 sec using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). A standard curve was constructed using known amounts (1×10^{-6} to 1.5×10^{-4} U) of β-galactosidase (Boehringer Mannheim). For measurement of luciferase activity, light emitted in the presence of 15 mM ATP, 10 mM $MgSO_4$, and 1 mM luciferin was measured and integrated for the initial 10 sec using a Monolight 2010 luminometer. The units of β-galactosidase activity were corrected to the light units of luciferase activity and normalized to CMV promoter activity, which was transfected in parallel. The data are expressed as a percentage of activity for each construct relative to the 5.0 mα-βgal construct ± SEM (n = 4).

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