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Pituitary-Specific Expression and Pit-1 Regulation of the Rat Growth Hormone-Releasing Hormone Receptor Gene

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The GHRH receptor is expressed in the somatotroph cell of the anterior pituitary, where it functions to mediate GHRH-stimulated GH release. To study pituitary and somatotroph cell-specific expression of this gene, a transgenic mouse model and complementary cell culture experiments were developed. The activity of the 1.6-kb proximal rat GHRH receptor promoter was examined *in vivo* by generating transgenic mice with the promoter directing expression of a luciferase reporter. The promoter directs tissue-specific expression; luciferase is highly expressed in the pituitary but absent from 14 other tissues. Immunocytochemistry experiments show that transgene expression is targeted to GH-expressing somatotroph cells. The transgene is 5-fold more highly expressed in males than females, and there is an increase in transgene expression leading up to the onset of puberty. The 1.6-kb promoter was further examined in cell culture experiments, which revealed that the promoter is selectively activated in pituitary cells and that promoter-reporter expression in nonpituitary cells can be enhanced by the pituitary-specific transcription factor Pit-1. EMSAs identified 10 short regions that specifically bind Pit-1 with highly variable relative affinities. The highest affinity site was previously identified and is required for Pit-1 activation of the promoter. Four additional sites contribute to Pit-1 regulation of the promoter and are important to achieving full activation of the gene. The results show that the 1.6-kb promoter is sufficient to direct tissue- and cell-specific expression *in vivo* and is regulated by Pit-1. (*Molecular Endocrinology* 21: 1969–1983, 2007)

THE ANTERIOR PITUITARY gland comprises five cell types that are defined by the hormones they secrete. Development of the anterior pituitary is characterized by a series of inductive signals from the neural ectoderm of the ventral diencephalon (1–5) that stimulates the growth and differentiation of the anterior pituitary primordium, Rathke's pouch, around embryonic d 8.5 in the mouse (6–8). Once established, Rathke's pouch creates an ectodermal boundary that allows subsequent signaling gradients (9), which stimulate the expression of transcription factors in overlapping patterns (9–14), leading to the differentiation of the various pituitary cell types, which are specified by embryonic d 17.5 (15). An opposing ventrodorsal gradient of BMP2 and FGF8 (9, 16) is required to differentiate the ventral cell phenotypes from the dorsal cell phenotypes, and reciprocal gradients of Pit-1 and GATA-2 are involved in the differentiation of the four most caudal cell lineages, gonadotrophs, thyrotrophs, lactotrophs, and somatotrophs (14).

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Abbreviations: DDT, Dithiothreitol; HDB, HEPES dissociation buffer; TBE, Tris-borate, EDTA.

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The pituitary somatotroph cell, which secretes GH, integrates signals from the hypothalamus to regulate GH release. In vertebrates GH promotes proliferation (17–21), differentiation (22–26), and metabolism (27–30) of target tissues throughout the body. GHRH, a hypothalamic peptide hormone, is the primary stimulus for GH synthesis (31, 32) and secretion (32–34) and acts by binding to its receptor on the surface of pituitary somatotroph cells. The GHRH receptor is a seven-transmembrane G protein-coupled receptor that is closely related to the receptors for the related peptide hormones, vasoactive intestinal polypeptide, secretin, and glucagon (35).

Pituitary somatotroph cells and lactotroph cells, which secrete prolactin, differentiate from a common precursor cell. Terminally differentiated somatotroph cells express the GHRH receptor and respond to GHRH stimulation (36). Therefore, discovery of how the GHRH receptor gene is regulated is expected to lead to a better understanding of what factors are involved in the differentiation of the somatotroph cell lineage from the four other cell types of the anterior pituitary gland. The GHRH receptor gene is known to be regulated by several factors, including glucocorticoids (37–41), thyroid hormone (41), retinoic acid (41), GHRH (36, 42), and the pituitary-specific transcription factor Pit-1 (38, 43–45), but the mechanism of regulation has not been elucidated for most of these factors.

The transcription factors Prop-1 (11, 12) and Pit-1 are required for the differentiation of the thyrotroph (46), lactotroph (47), and somatotroph (48) cell lineages from a common pituitary precursor. Pit-1 is a pituitary-specific POU homeodomain transcription factor (49, 50) that is involved in the transcriptional activation of the GH gene in somatotroph cells and of the prolactin gene in lactotroph cells (51–53). Previous work from our laboratory and others has shown that Pit-1 also leads to activation of the GHRH receptor promoter (38, 43, 44). Further indicative of the important role Pit-1 plays in stimulating GHRH receptor expression, a mutation in a human GHRH receptor Pit-1 binding site results in decreased expression of the receptor in a patient exhibiting GH deficiency (45). Pit-1 has recently been shown to be required for glucocorticoid regulation of the rat (r) GHRH receptor (54), suggesting that cooperation between Pit-1 and other factors may lead to differential cell-specific gene regulation.

Mutations in the GHRH receptor in human (55–58) and in animal models (59–61) lead to dwarfism and pituitary hypoplasia due to lack of somatotrophs, indicating an integral role for the receptor in regulation of appropriate GH levels. Given the importance of the GHRH receptor in mediating the neuroendocrine pathway regulating GH levels, understanding regulation of the GHRH receptor gene itself is important.

The current study seeks to determine what promoter elements are required to direct tissue- and cell-specific expression of the GHRH receptor gene. Using transgenic mice and cell culture systems, we show that the 1.6-kb promoter is sufficient to direct tissue- and cell-specific expression *in vivo* and that the cell specificity is mimicked *in vitro*. In further examination of elements within the 1.6-kb promoter, we mapped 10 Pit-1 binding sites and show that five of these contribute to Pit-1-regulated expression of the GHRH receptor. Of these, the greatest contribution to Pit-1-activated expression of the gene is achieved by two well-conserved, high-affinity sites, −888 to −857 and +129 to +164.

RESULTS

Pituitary-Specific Expression of the Luciferase Transgene

Two independent lines of transgenic mice were generated in which the 1.6-kb GHRH receptor promoter directs a luciferase reporter. Luciferase assays were performed on pituitaries taken from normal littermate and transgenic mice for each line. Luciferase expression is significant in transgenic pituitaries in each line (Fig. 1A). Transgenic mice in line 2 show significantly higher transgene expression than those in line 1 (Fig. 1A). Due to the robust transgene expression level in the pituitary of transgenic mice in line 2, this line was used for further analyses and data from line 2 are reported here.

To determine whether the 1.6-kb promoter is sufficient to direct tissue-specific expression of the GHRH receptor, multiple endocrine and nonendocrine tissues from both normal littermate and transgenic mice were examined for luciferase expression. In addition to pituitary the tissues tested include medial-basal hypothalamus, cerebellum, pancreas, cerebral cortex, testes/ovary, heart, lung, adrenal gland, kidney, spleen, muscle, skin, thymus, and liver. Only in the pituitary gland were relative light units significantly higher for the transgenic sample compared with the normal littermate sample (Fig. 1B).

Transgene Expression Is Targeted to Somatotroph Cells

To determine whether the transgene is restricted to somatotroph cells, immunocytochemistry was performed on dispersed pituitary cells from transgenic and normal littermate mice. In control samples where the primary antibodies are omitted, no fluorescence is observed (Fig. 2, A–H). An antibody to GH was used to localize somatotroph cells. A luciferase antibody was used to examine transgene localization. The GH antibody shows that somatotroph cells in both normal littermate (Fig. 2J) and transgenic (Fig. 2N) pituitaries occur at equivalent numbers. As expected, the luciferase antibody shows luciferase expression in pituitary cells of only the transgenic mice (Fig. 2O).

Overlays of the channels detecting GH and luciferase antibodies reveal that the two antibodies colocalize in the transgenic pituitary (Fig. 2P), indicating that the luciferase transgene is expressed in somatotroph cells of the anterior pituitary gland. In immunocytochemistry experiments, all somatotroph cells express the luciferase transgene ($n > 1000$ cells). In fewer than 5% of cells expressing the transgene, GH is not detected ($n > 1000$ cells). This small number of cells is not likely significant enough to represent an entire population of an additional pituitary cell type. Thus, we conclude that transgene expression is somatotroph cell specific.

Transgene Expression Is Sexually Dimorphic and Regulated during Postnatal Development

Transgene expression was examined in males compared with females to determine whether sexual dimorphism in transgene expression occurs. Transgene expression levels are strikingly higher in transgenic pituitaries from males than from females, with a difference of more than 5-fold (Fig. 3).

To determine whether the 1.6-kb promoter regulates changes in receptor expression during postnatal development and aging, luciferase assays were performed on pituitaries from transgenic animals ranging from d 1 postnatal through 1 yr of age (Fig. 4). Similar to reports on expression of the endogenous GHRH

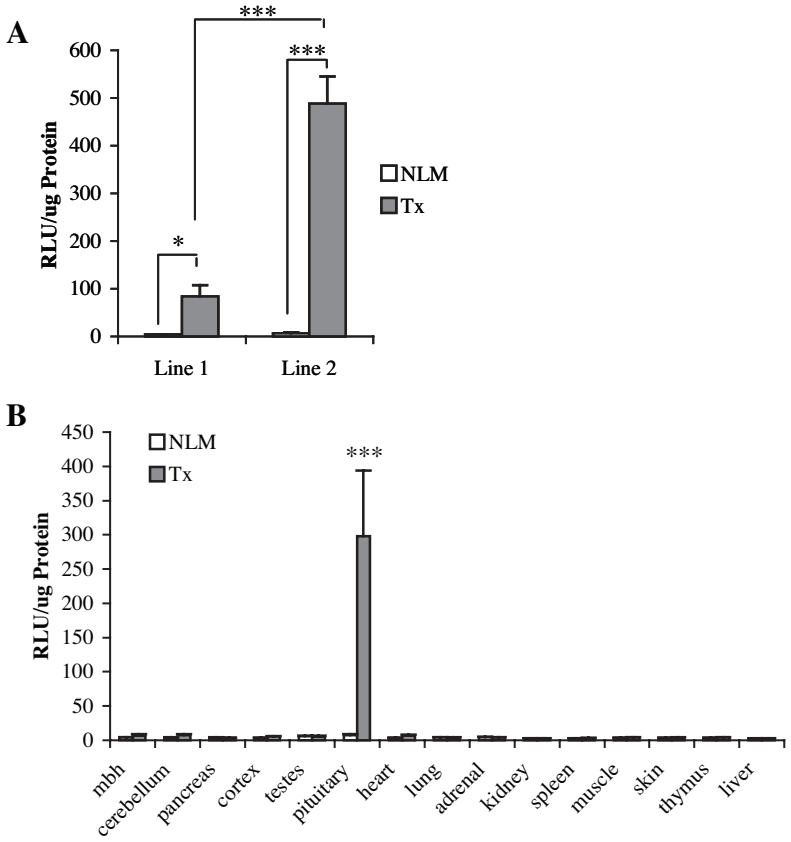


Fig. 1. Luciferase Transgene Expression in Two Independent Lines

A, Transgene expression in normal littermate (NLM) and transgenic (Tx) male mice from two lines detected by luciferase assay performed on homogenized pituitary tissue and normalized to protein. B, Luciferase assay of 15 homogenized tissues from normal littermate and transgenic males, normalized to protein (mbh: medial-basal hypothalamus). Statistical analysis was performed using a two-way ANOVA (*, $P < 0.05$; ***, $P < 0.001$; $n \geq 5$ for each group). RLU, Relative light units.

receptor (62), there is a significant increase in transgene expression in the time leading up to puberty (30–35 d in the male mouse) (Fig. 4). In contrast to the endogenous receptor, transgene expression levels do not consistently decrease as the animal ages (Fig. 4).

Cell-Specific and Pit-1-Activated Expression of the rGHRH Receptor Promoter in Cell Culture

Having established the 1.6-kb promoter as sufficient *in vivo* to direct tissue- and cell-specific expression, we further examined regulation of this promoter in cell culture experiments. A rGHRH receptor promoter-luciferase reporter construct (rGHRH receptor −1673) is not expressed in a nonpituitary cell line, HeLa T4 cells, but is highly expressed in a GH-secreting pituitary cell line, GH3 cells (Fig. 5). In nonpituitary HeLa cells, coexpression of Pit-1 leads to significant activation of the rGHRH receptor −1673 construct. Because Pit-1 is expressed in GH3 cells, Pit-1 coexpression in transfections of these cells leads to little or no further activation of the GHRH receptor promoter reporter (Fig. 5). Pit-1-activated levels of this promoter in HeLa cells are 8-fold lower than basal expression levels in GH3 cells, indicating that factors in addition to Pit-1 are required for full activation of the promoter (Fig. 5). Given the ability of Pit-1 to stimulate expression of the promoter in a nonpituitary cell line, we focused on identifying functional binding sites for this transcription factor, which appears to be a critical determinant of GHRH receptor gene expression.

Pit-1 Binding to the 1.6-kb rGHRH Receptor Promoter

To determine whether Pit-1 binds to the rGHRH receptor promoter and where it binds, EMSAs were performed using overlapping probes spanning the proximal promoter. A Western blot shows Pit-1 expression in nuclear extracts from cells transfected with the Pit-1 construct, which were used in the binding reactions, and no expression in nuclear extracts from mock-transfected cells, which were used as a negative control (Fig. 6A). DNA probes are shown schematically in Fig. 6C. In the rat, four transcriptional start sites for the GHRH receptor gene have been identified (43); these are depicted as *arrows* in the *schematic* (Fig. 6C). We refer to the

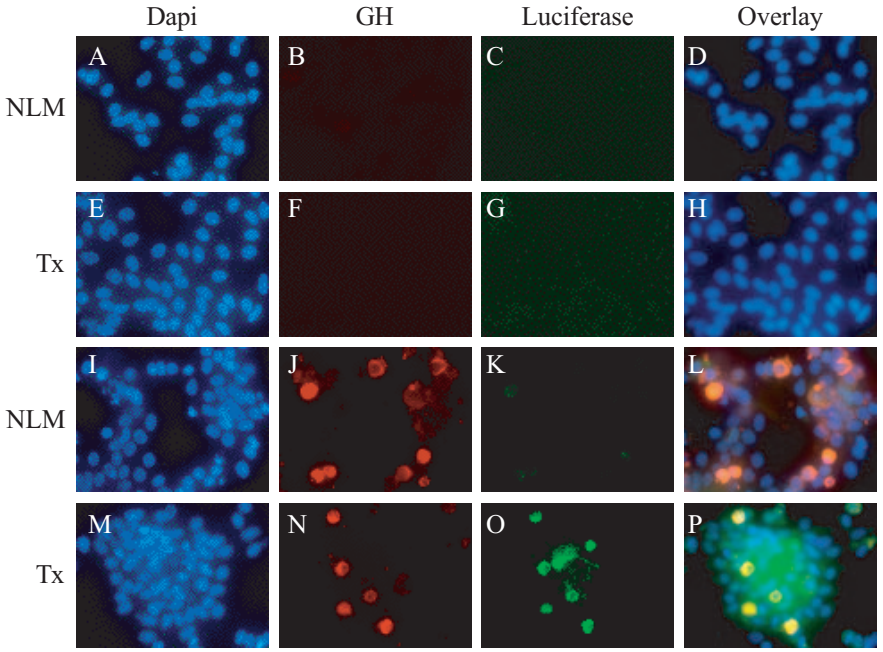


Fig. 2. Cell Specificity of Transgene Expression

Pituitaries from normal littermate (A–D; I–L) and transgenic (E–H; M–P) male mice, age 21–45 d, were necropsied and cells were dispersed. Immunocytochemistry was performed using primary antibodies to GH and luciferase. Additional samples were processed without primary antibody incubation (normal littermate, A–D; transgenic, E–H). A 4′,6-diamidino-2-phenylindole (Dapi) stain identifies cell nuclei (A, E, I, and M). GH-positive cells are visualized on the Texas Red channel (B, F, J, and N), and luciferase-positive cells are visualized on the fluorescein isothiocyanate channel (C, G, K, and O). *Overlays* show where colocalization of GH and luciferase occurs (D, H, L, and P). Data are representative of at least three independent experiments with n ≥ 6 animals per condition in each experiment. NLM, Normal littermate; Tx, transgenic.

5′-most of these start sites as "+1." Only the 3′-most probe was negative for Pit-1 binding (Fig. 6B). Pit-1 binds to each of the other seven probes, as nuclear extracts from cells overexpressing Pit-1 shift the mobility of the promoter probes (Fig. 6B, lanes 3). An unlabeled oligonucleotide composed of a consensus Pit-1 binding site competes away Pit-1 binding (Fig. 6B, lanes 4), but an unlabeled oligonucleotide with a mutated Pit-1 binding site has no effect on binding (Fig. 6B, lanes 5). Incubation of the nuclear extracts with an antibody to Pit-1 supershifts the Pit-1 binding band (Fig. 6B, lanes 6),

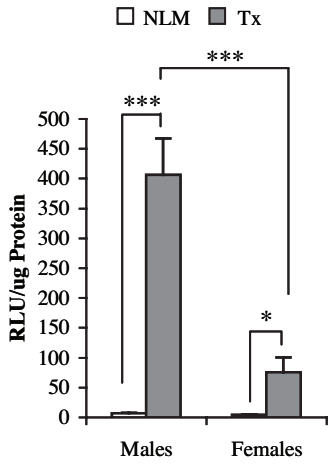


Fig. 3. Sexual Dimorphism of Transgene Expression

Pituitaries from male and female normal littermate and transgenic mice were necropsied and homogenized to perform luciferase assays, normalized to protein. Statistical analysis was performed using a two-way ANOVA (*, $P < 0.05$; ***, $P < 0.001$; n ≥ 7). NLM, Normal littermate; RLU, relative light units; Tx, transgenic.

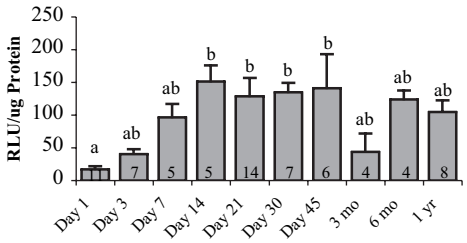


Fig. 4. Transgene Expression during Postnatal Development and Aging

Pituitaries from male transgenic mice age 1 d postnatal through 1 yr were necropsied and homogenized to perform luciferase assays, normalized to protein. Statistical analysis was performed using a one-way ANOVA [data points with *different letters* are significantly different from one another ($P < 0.05$); n ≥ 4 for each age group and is indicated at the *bottom of each bar*]. RLU, Relative light units.

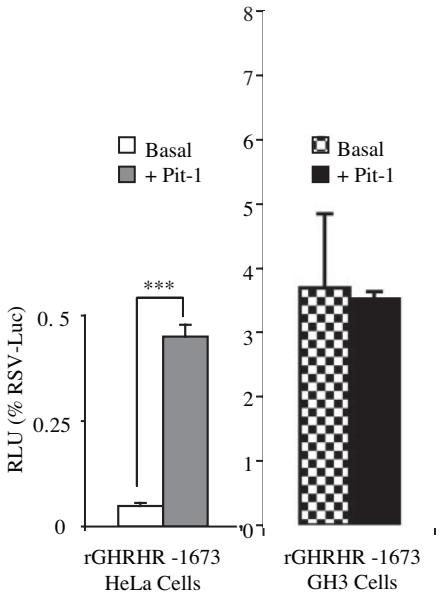


Fig. 5. Expression of rGHRH Receptor Promoter Luciferase Reporter in Pituitary and Nonpituitary Cell Lines

The full −1673 rGHRH receptor promoter-luciferase construct was transfected into nonpituitary HeLa cells and pituitary GH3 cells alone or with cotransfected Pit-1 to examine relative expression levels. Data are plotted as relative light units per microgram of protein, as a percentage of Rous sarcoma virus-luciferase values, to normalize for variability in transfection efficiencies of the cell lines. Data represent the average of at least three independent experiments. *Error bars* represent the SEM. Statistical analysis was performed using a Student's *t* test. (***, $P < 0.001$). RLU, Relative light units.

whereas normal serum does not affect binding (Fig. 6B, lanes 7). Pit-1 binding to the −1673 to −1323 region is less definitive than for the other six regions, because normal serum, in addition to the Pit-1 antibody, supershifted the Pit-1 band, although this band has a distinct mobility from the Pit-1 antibody shift.

Pit-1 Specifically Binds 10 Sites in the 1.6-kb rGHRH Receptor Promoter

To determine short DNA regions that bind Pit-1 specifically, sequential sets of overlapping smaller oligonucleotide probes were generated to each region mapped in Fig. 6 and used in EMSAs. In a previous report, four putative Pit-1 binding sites in the 1.6-kb promoter were identified computationally (43). The binding experiments show that, in fact, 10 short regions of the 1.6-kb rGHRH receptor promoter bind Pit-1 specifically (Fig. 7, lanes 3). The binding of Pit-1 to these probes is competed by an unlabeled Pit-1 site oligonucleotide (Fig. 7, lanes 4), not competed by a mutated Pit-1 site oligonucleotide (Fig. 7, lanes 5), supershifted by incubation with an antibody to Pit-1 (Fig. 7, lanes 6), and unaffected by incubation with normal rabbit serum (Fig. 7, lanes 7). Not surprisingly, several of these promoter probes also bind nuclear proteins from mock-transfected cells (Fig. 7, lanes 2). For most of these, the band representing binding of other (non-Pit-1) proteins migrates at a different size than Pit-1. For others (−348 to −313 and −81 to −46) the band migrates at a similar size, but the band intensity is much greater in the Pit-1 nuclear extract lane. Pit-1-specific binding was determined by competition with a cold Pit-1 consensus oligonucleotide (but not with a mutated version of it) and supershift with Pit-1 antibody. No defined binding sites were detectable within the −1673 to −1323 region, possibly indicative of the existence of multiple weak-affinity sites within this region, or lack of specificity of binding, as discussed for Fig. 6.

When key nucleotides are mutated from A/T to G (Table 1), Pit-1 binding to these sequences is completely abolished (Fig. 7, Mut panels). Because no strong consensus Pit-1 binding site exists, it was not possible to predetermine a single target for effective mutation in these probes. Instead, several nucleotides were mutated within the presumed core sequence of each Pit-1 probe (Table 1).

The rGHRH Receptor Promoter Pit-1 Binding Sites Exhibit Varying Affinities for Pit-1

The 10 Pit-1 binding sites were examined more closely to determine which site or sites might be important to Pit-1 activation of the GHRH receptor gene. Because the observation was made in initial experiments that different sites seem to bind Pit-1 with different affinities, we examined relative binding affinities of the sites. EMSAs were performed by incubating increasing amounts of Pit-1 nuclear extracts, ranging from 0 μM to 2000 μM, with each of the labeled probes (Fig. 8, A and B). Average relative affinities were compared with the affinity of a known Pit-1 binding sequence (63), which is referred to as the Pit-1 consensus sequence (Fig. 8). Relative affinities for the 10 sites are highly variable, with EC_{50} values ranging from 300 μM for the +129 to +164 site to greater than 2000 μM for six of the sites (Fig. 8C). Affinity ranks are listed for comparison (Fig. 8C).

Conservation of Pit-1 Binding Sequences across Multiple Species

To examine evolutionary conservation of the Pit-1 binding sequences, the rat promoter sequence was aligned to the genomic sequences of other species (data not shown). As might be expected, there was strong homology between rat and mouse that was less robust in other species (human, chimpanzee, dog, and rhesus monkey). A percent conservation and conservation raking was calculated for each of the sites, and the ranks are shown (Fig. 8C). There is some correlation between relative affinities and amount of conservation of the sequences. For example, the most highly conserved sequence, +129 to +164, is the site with the highest relative affinity

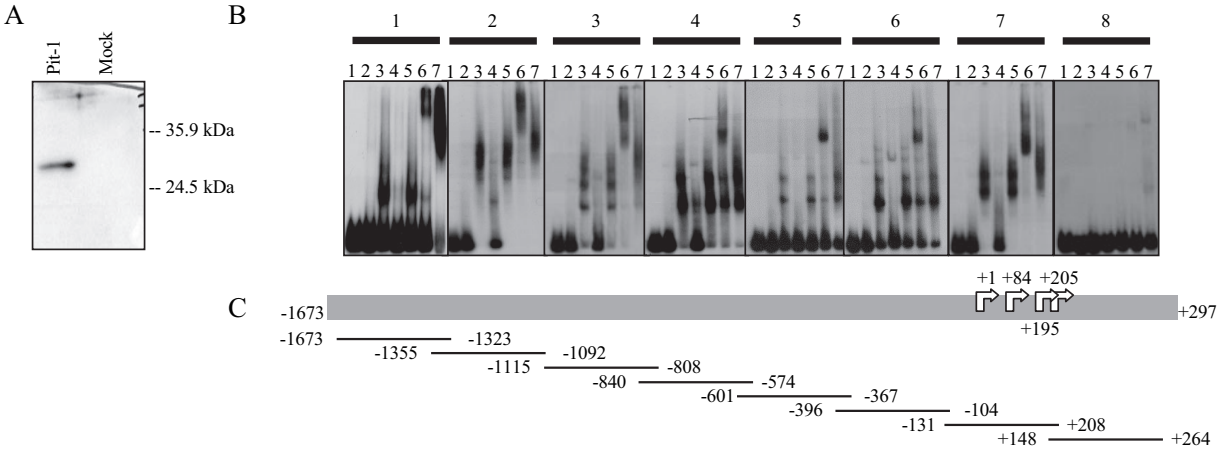


Fig. 6. Expression of Pit-1 in Nuclear Extracts and Pit-1 Binding across the 1.6-kb rGHRH Receptor Promoter

A, Nuclear extracts from either Pit-1 overexpressing HeLa T4 cells (lane 1) or mock-transfected HeLa T4 cells (lane 2) were separated by SDS-PAGE on a 10% gel and transferred to nitrocellulose. The membrane was incubated with an antibody to Pit-1, and antibody-antigen complexes were detected by chemiluminescence. B, EMSAs were run by incubating nuclear extracts with radiolabeled DNA probes spanning the promoter. Lane 1 in each panel is probe alone. Lane 2 is probe incubated with nuclear extracts from mock-transfected cells. Lanes 3–7 are probes incubated with nuclear extracts from Pit-1-overexpressing cells. Lane 4 includes an unlabeled oligonucleotide including a Pit-1 consensus binding sequence. Lane 5 includes an unlabeled mutated Pit-1 oligonucleotide binding sequence. Lane 6 includes an antibody to Pit-1, and lane 7 includes normal rabbit serum. C, A schematic represents the overlapping DNA promoter probes and the four transcriptional start sites in the rGHRH receptor promoter. Data are representative of at least three independent experiments.

for Pit-1, and the least well-conserved sequence, −348 to −313, is among the lowest affinity sites (Fig. 8C).

Two human GHRH receptor promoter Pit-1 binding sites have been identified (44). The more 5′ of these human sites, which contributes most to Pit-1 activation of the human GHRH receptor, is highly conserved and corresponds to the high-affinity +129 to +164 rat site, indicating a possible functional importance for this site in regulating the rGHRH receptor gene.

Functional Analysis of Pit-1 Binding Sites

Because Pit-1 does not further activate expression of the 1.6-kb rGHRH receptor construct in GH3 cells, we performed experiments in HeLa cells to examine the contribution of Pit-1 to regulation of the receptor gene and compared these data to basal reporter expression in GH3 cells.

In HeLa cells, Pit-1 significantly activates a series of 5′-deletion constructs up to the rGHRH receptor −131 construct, which still contains the high-affinity, highly conserved +129 to +164 site (Fig. 9A). When this site is removed, in the rGHRH receptor +148 construct, Pit-1 activation is lost (Fig. 9A). Because basal expression of the 5′-deletion constructs varied, we also examined fold increase in reporter expression with Pit-1 cotransfection for each of the promoter deletion constructs. This analysis shows that Pit-1 significantly increases promoter activity for each construct except the shortest, which lacks Pit-1 binding sites (Fig. 9B). Pit-1 coexpression has the greatest effect on the full-length −1673 construct, suggesting that multiple binding sites are necessary for full Pit-1 responsiveness of this gene (Fig. 9B).

To further examine the individual contributions of the identified Pit-1 binding sites, functional analysis of the Pit-1 binding site mutations was performed. Five of the Pit-1 binding sites exhibit significantly reduced Pit-1-activated expression when mutated (Fig. 9C). The most dramatic loss of Pit-1 activation is observed when either the −888 to −857 or +129 to +164 site is mutated. Because changes in basal expression were apparent in these experiments, fold increase in reporter expression with Pit-1 coexpression was examined for these mutation constructs. The fold increase is significant for each of the mutations except the +129 to +164 site (Fig. 9D), supporting that Pit-1 binding to this site is essential for activating the rGHRH receptor promoter.

GHRH receptor promoter deletion and mutation constructs were also examined in pituitary GH3 cells. Interestingly, the pattern of basal reporter expression for the promoter 5′-deletion constructs follows the pattern of Pit-1-activated reporter expression seen in HeLa cells (Fig. 10A). Mutation of seven of the 10 Pit-1 binding sites results in significantly reduced basal expression in GH3 cells (Fig. 10B). These sites include those that exhibit reduced Pit-1-activated expression when mutated in HeLa cells, in addition to two others. As in HeLa cells, no activation of the reporter occurs when Pit-1 is unable to bind the critical +129 to +164 site (Fig. 10B).

DISCUSSION

The primary goal of this study was to expand our understanding of pituitary- and somatotroph cell-

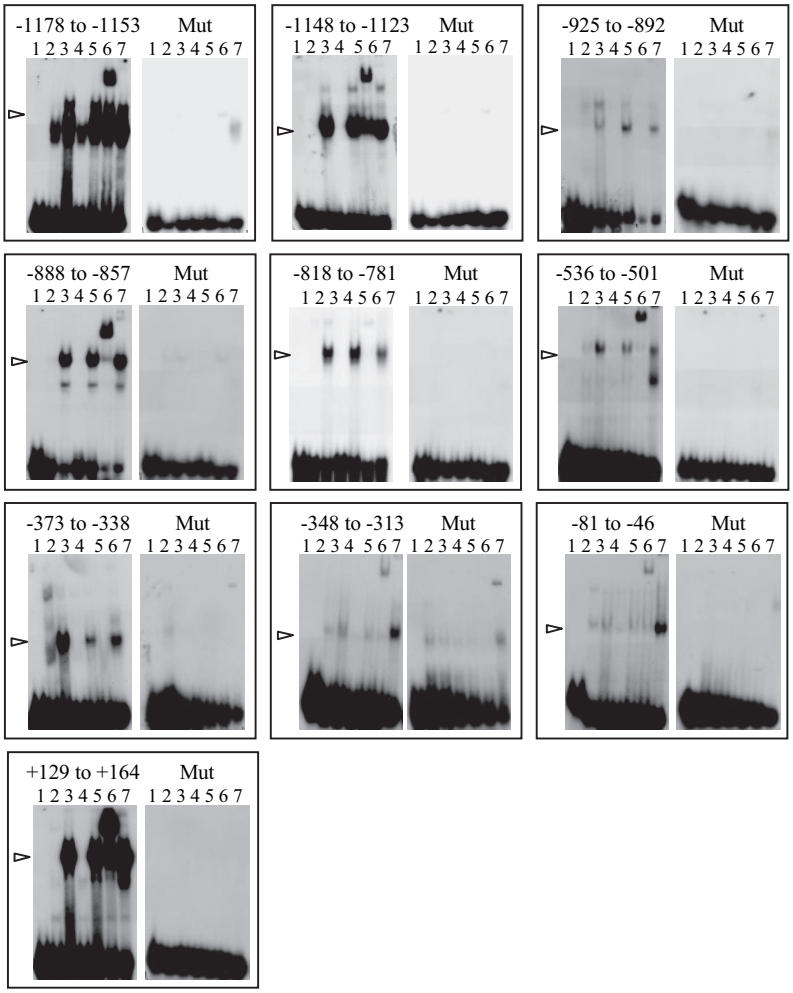


Fig. 7. Identification of 10 Pit-1 Binding Sites in the 1.6-kb rGHRH Receptor Promoter

EMSAs were performed using nuclear extracts from Pit-1-overexpressing HeLa T4 cells and radiolabeled oligonucleotide probes. Shown are the 10 short probes that specifically bind Pit-1. Lane 1 in each panel is probe alone. Lane 2 is probe incubated with nuclear extracts from mock-transfected cells. Lanes 3–7 are probes incubated with nuclear extracts from cells overexpressing Pit-1. Lane 4 includes an unlabeled oligonucleotide including a Pit-1 consensus binding sequence. Lane 5 includes an unlabeled mutated Pit-1 oligonucleotide binding sequence. Lane 6 includes an antibody to Pit-1, and lane 7 includes normal rabbit serum. An *arrow* marks the Pit-1 band. The second gel in each panel is an identical experiment with a mutated probe. Data are representative of at least three independent experiments. Mut, Mutation.

specific expression of the GHRH receptor gene. Examination of transcriptional regulation of this gene, which is expressed early in terminally differentiated somatotroph cells, will lead to a better understanding of the events leading to cell specification of the somatotroph lineage. The approach we took was to characterize regulation of the rGHRH receptor promoter *in vivo*, in an attempt to define elements required to direct tissue- and cell-specific expression, and to further study regulation of the defined promoter in a cell culture system.

Previous cell culture experiments have shown that the 1.6-kb rGHRH receptor promoter was able to direct expression of reporter constructs in a pituitary cell line (43). To determine whether this part of the promoter was sufficient *in vivo* to direct tissue- and cell-specific expression of the receptor, transgenic mice were generated. The transgene contains the −1673 to +289 fragment cloned upstream of a luciferase reporter. The data show that the transgene is specifically expressed in the pituitary, is targeted to somatotroph cells, and is expressed in a sexually dimorphic and age-dependent manner.

Just as endogenous GHRH receptor expression is largely restricted to the pituitary (35), transgene expression is specific to the pituitary in transgenic mice, suggesting that tissue specificity of expression is driven by elements within the 1.6-kb promoter. Interestingly, no transgene expression is observed in the kidney, where an alternative GHRH receptor mRNA is expressed in the rat (35). Studies in our group (Zheng, J., and K. Mayo, unpublished) suggest that this mRNA is transcribed from a different promoter. A report has suggested that endogenous GHRH receptor expres-

Table 1. rGHRH Receptor Promoter Pit-1 Binding Sequences and Mutations

Probe Location	Probe Sequence and Mutated Nucleotides
−1178 to −1153	tattgattattaataccactaatgc ** ** *
−1148 to −1123	ggagatgaagcatgactaatcacgt ** *
−925 to −892	tgatgtcaaatgattcccagatgcagatgtggt ** **
−888 to −857	ggaagctttccacattcatgacacaaattc ** *
−818 to −781	tctgtaagtggatacggtcattccgtggt *** **
−536 to −501	cagaaactttgcctaacgcatgtgagttgggggcc ** *
−373 to −338	ggagccacacacatgttgaccaagtgctttaccac ** * *
−348 to −313	gctttaccactgagcgacactcccagccctgcatg ** * *
−81 to −46	ctgaaaacaatgggaaaacatactaagtggaacag * * **
+129 to +164	CTGTTCAATATTCAGCTGGGTGTCCCTCCTGTTG ** **

Each Pit-1 binding sequence and promoter location is shown, along with the mutations that affect binding, which were also examined in functional experiments. The nucleotides marked with *asterisks* were mutated to guanines for binding and functional assays.

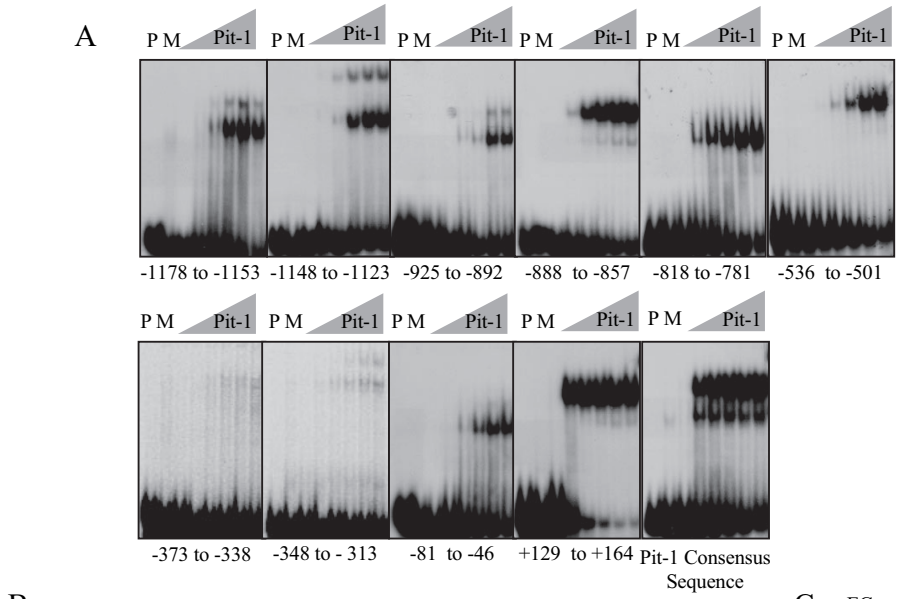
sion is restricted to somatotroph cells of the anterior pituitary gland (64) in experiments in which cell type was morphologically determined. Although a small percentage of nonsomatotroph cells express the GHRH receptor promoter transgene, all somatotroph cells, defined by GH-positive immunocytochemistry, express the transgene. The results support the finding that endogenous GHRH receptor expression is somatotroph specific and suggest that the cell-specific expression is determined by elements in the 1.6-kb proximal promoter.

Conflicting data have been reported on whether GHRH receptor expression is dependent upon sex. Some reports suggest that males express the receptor mRNA at much higher levels than females (36, 65). Other studies have reported no difference between receptor expression in males and females (66, 67). The explanation for the conflict in the literature is not clear. One possibility is that female cycle status should be taken into account, considering that the GHRH receptor is known to be regulated by estrogen (38, 68). Expression of the GHRH receptor promoter transgene is consistently higher in males than in age-matched females, with an approximate 5-fold difference. The data indicate that sexual dimorphism of GHRH receptor expression is controlled by elements in the 1.6-kb receptor promoter. Sexually dimorphic GHRH receptor gene expression may contribute to the differing pulsatile pattern of GH release in males and females and the resulting average size difference between the sexes.

A previous study in the rat using ribonuclease protection assays has shown that expression of the endogenous GHRH receptor transcript is regulated during early postnatal development and aging. Endogenous GHRH receptor expression is high in newborns and declines until puberty, when expression increases, before again declining throughout aging (62). Subsequent reports have further substantiated that GHRH receptor mRNA expression dramatically decreases in the aged animal (66, 67). In the transgenic mouse, the transgene expression pattern during postnatal development shows an increase in expression as the animals approach puberty, although transgene levels do not steadily decrease during aging, as endogenous expression levels do. The data suggest that the regulatory elements for the pubertal surge in GHRH receptor expression are contained within the 1.6-kb promoter, but other upstream elements might be involved in the decreased expression during the aging of the animal. Alternatively, regulation of GHRH receptor protein levels may differ from mRNA regulation, possibly explaining the differences reported here. Another possibility is that this aspect of regulation of the GHRH receptor gene differs between rat and mouse species.

The data show that 1.6 kb of the rGHRH receptor promoter is sufficient to target tissue- and cell-specific expression of the receptor *in vivo*. In addition, the data suggest that the increase in GHRH receptor expression coincident with the pubertal increase in GH levels and sex-dependent expression of the receptor are regulated by elements within this 1.6-kb proximal promoter. Having established this part of the promoter as sufficient *in vivo* for appropriate expression, we further examined its regulation in cell culture experiments.

We showed that the cell specificity of expression driven by the 1.6-kb promoter is mimicked in cell culture. Because coexpression of Pit-1 led to activation of this promoter reporter construct in nonpituitary



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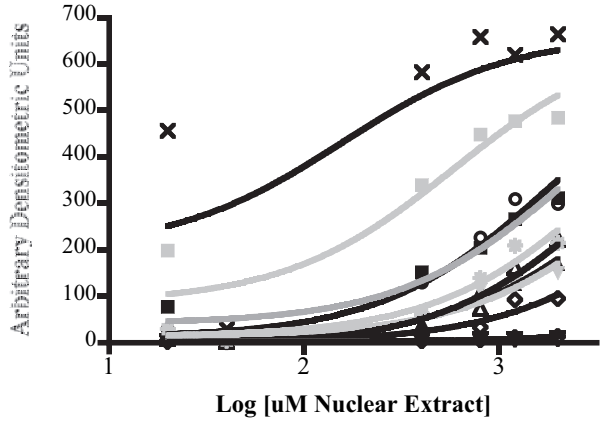

C

	EC_{50} (μM N.E.)	Affinity Rank	% Sequence Conservation	Conservation Rank
Pit-1 Consensus	400	-	-	-
-1178 to -1153	>2000	6th	32.6	8th
-1148 to -1123	>2000	7th	32	9th
-925 to -892	>2000	8th	42.9	6th
-888 to -857	800	2nd	55.4	3rd
-818 to -781	1200	3rd	55	4th
-536 to -501	>2000	5th	44.8	5th
-373 to -338	>2000	10th	35.7	7th
-348 to -313	>2000	9th	27.6	10th
-81 to -46	1600	4th	68	2nd
+129 to +164	300	1st	78.7	1st

Fig. 8. Varying Relative Affinities for Pit-1 among the 10 Binding Sites in the rGHRH Receptor Promoter

Relative affinities were deduced from EMSAs using increasing amounts of Pit-1 nuclear extracts. A, In each panel, the first lane, marked "P," is probe alone. The second lane, marked "M," is probe incubated with mock-transfected nuclear extract. The last six lanes are probe incubated with increasing amounts of nuclear extract from cells overexpressing Pit-1 in the following order: 1 μg (40 μM), 5 μg (200 μM), 10 μg (400 μM), 20 μg (800 μM), 30 μg (1200 μM), and 50 μg (2000 μM). Shown is a representative of three independent experiments. B, Arbitrary densitometric units were calculated for each probe using ImageQuant software. Data are representative of three independent experiments. C, Relative affinities of Pit-1 binding sites were determined by calculation of the EC_{50} values for each probe, using the GraphPad Prism program (N.E., nuclear extract). A relative affinity rank is also provided, with the rank of first having the highest affinity and the rank of tenth having the lowest. Conservation of Pit-1 binding probe sequences was determined across multiple species, and the degree of conservation was translated to a rank, shown here. The rank of first is most highly conserved, and tenth is least highly conserved.

HeLa cells, we focused on Pit-1 regulation of the 1.6-kb rGHRH receptor promoter with a goal of identifying functional Pit-1 binding sites.

We were able to effectively narrow down specific Pit-1 binding sites to ten 25- to 35-bp regions. Only two of the 10 sites correspond to computationally predicted Pit-1 binding sites (43), illuminating the lack of consensus for Pit-1 binding sequences. To determine whether a single or few sites contributes most to Pit-1 activation of the rGHRH receptor promoter, we examined relative binding affinities and sequence conservation to zero in on candidate regions. Combined with functional analysis of promoter 5′-deletion and Pit-1 binding mutation constructs, the data revealed that multiple binding regions contribute to Pit-1 activation of the rGHRH receptor promoter. Of particular importance are two well-conserved sites with high relative affinities for Pit-1, −888 to −857 and +129 to +164.

The required +129 to +164 site has previously been identified as a Pit-1 binding site that synergizes with glucocorticoid receptor to activate transcription of the

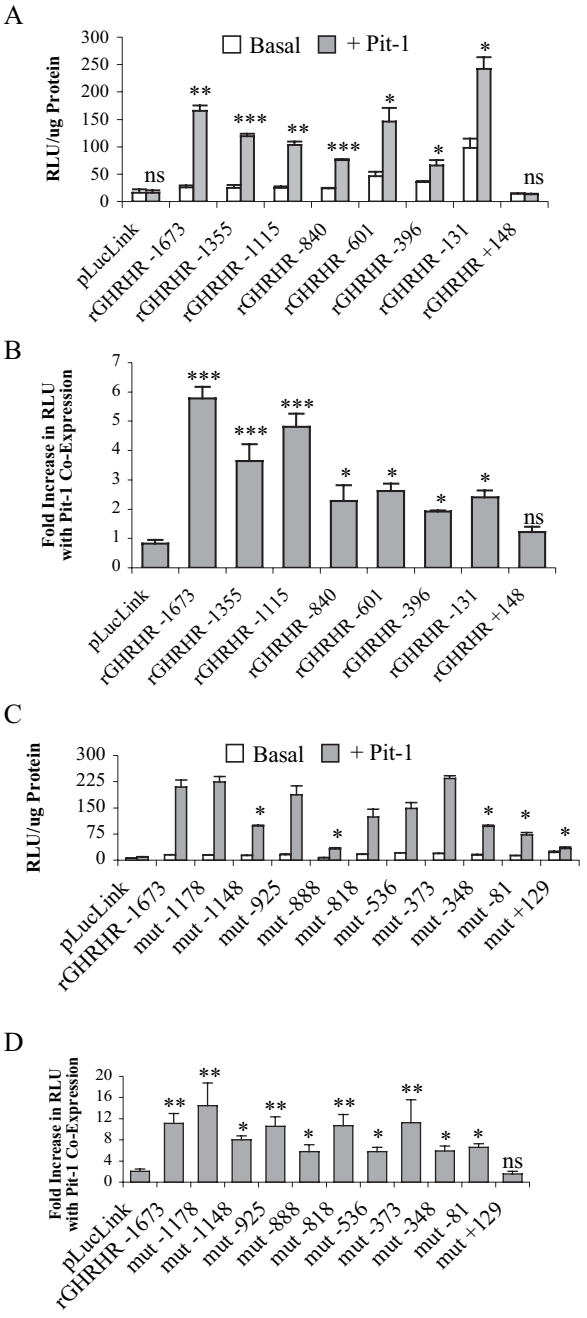

Fig. 9. Pit-1 Activation of rGHRH Receptor Promoter Luciferase Reporter Deletion and Mutation Constructs

A, HeLa T4 cells were transfected with 5′-deletion rGHRH receptor promoter constructs alone or with a cotransfected Pit-1 expression construct and analyzed for luciferase expression. Data are representative of at least three independent experiments performed in triplicate. *Error bars* represent the SEM. Statistical analysis was performed using a Student's *t* test (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). B, The fold increase in reporter activity with Pit-1 coexpression is plotted for each of the 5′-promoter-deletion constructs. Data represent the average of at least three independent experiments. *Error bars* represent the SEM. Statistical analysis was performed using a one-way ANOVA (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$). C, Individual mutations of the 10 Pit-1 binding sites were examined by transfection and luciferase assay in HeLa cells. Data are representative of at least three independent experiments performed in triplicate. *Error bars* represent the SEM. Statistical analysis was performed using a Student's *t* test (ns, $P > 0.05$; *, $P < 0.05$). D, The fold increase in reporter activity with Pit-1 coexpression is plotted for each of the 5′-promoter deletion constructs. Data represent the average of at least three independent experiments. *Error bars* represent the SEM. Statistical analysis was performed using a one-way ANOVA (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$). Mut, Mutation; ns, nonsignificant; RLU, relative light units.

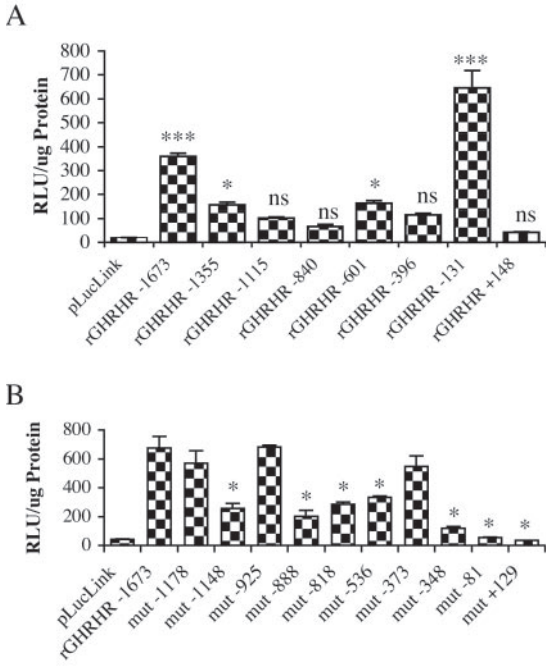

Fig. 10. Expression of rGHRH Receptor Promoter Luciferase Reporter Deletion and Mutation Constructs in Pituitary Cells

A, GH3 cells were transfected with 5′-deletion rGHRH receptor promoter constructs and analyzed for luciferase expression. Data are representative of at least three independent experiments performed in triplicate. *Error bars* represent the SEM. Statistical analysis was performed using a one-way ANOVA (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$). B, Mutation of the +129 to +164 Pit-1 binding site was examined by transfection and luciferase assay in GH3 cells. Data are representative of six independent experiments performed in triplicate. *Error bars* represent the SEM. Statistical analysis was performed using Student's *t* test (ns, $P > 0.05$; *, $P < 0.05$). mut, Mutation; ns, nonsignificant.

rGHRH receptor promoter (54). In addition, this site corresponds to one of the human sites identified by Iguchi *et al.* (44), which contributes most to Pit-1 activation of the human GHRH receptor promoter. Interestingly, a mutation in a different human Pit-1 site, which corresponds to a region in the rat that does not bind Pit-1, has been discovered in a human familial isolated GH deficiency type IB case (45). Our data

confirm that the +129 to +164 site is essential to Pit-1 activation of the GHRH receptor gene. Furthermore, we have identified four additional functional Pit-1 binding sites that contribute to activation of the rat promoter. Of these, the −888 to −857 site contributes most to Pit-1 activation. Interestingly, there is a predicted glucocorticoid receptor binding site within this region, suggesting that Pit-1 might synergize with GR at this site, as well.

The current study identifies the 1.6-kb promoter as sufficient for tissue- and cell-specific expression of the rGHRH receptor gene *in vivo*. Further analysis of the promoter shows that cell specificity of expression is maintained in cell culture. Pit-1 is an important activator of the GHRH receptor promoter, and an in-depth characterization of Pit-1 regulation of this gene reveals at least 10 short regions that specifically bind Pit-1. The identification of several Pit-1 binding sites within the promoter of a single gene is not unexpected. Multiple binding sites for Pit-1 have been identified in the promoters of other known Pit-1 targets (48, 63, 69, 70), as well, which seems to be indicative of the importance of this factor in regulating cell-specific genes in the thyrotroph, lactotroph, and somatotroph lineages.

A report on estrogen regulation of the prolactin promoter shows a requirement for multiple Pit-1 binding sites to facilitate estrogen responsiveness (70), which confers cell-specific expression of this gene. It is likely that the Pit-1 binding sites identified in the rGHRH receptor promoter similarly synergize with cell-specific factors to facilitate somatotroph-specific expression of the gene. Future work will focus on examining the factors that work in addition to Pit-1, or in synergy with it, to regulate the cell-specific expression of the GHRH receptor gene.

MATERIALS AND METHODS

Generation and Maintenance of Transgenic Mice

Transgenic mice were generated by the Case Western Reserve University (CWRU) transgenic facility according to the standard technique described in Ref. 71 and following protocols approved by the CWRU institutional animal care and use committee. The transgene was created by cloning the 1.6-kb GHRH receptor proximal promoter into the pA3 luciferase vector. *Nco*I digestion linearized the transgene, which was purified and diluted in T10E1 buffer. Transgene DNA was microinjected into the pronucleus of zygotes at 2–4 ng/μl. Injected embryos were surgically transferred to the oviducts of pseudopregnant recipient females. Animals used to generate founders were F_1 hybrids of C57/Bl6 × SJL. Two founder male mice were identified and used to establish two separate transgenic lines. The transgenic lines were maintained by crossing transgenic offspring to C57/Bl6 mice. Genotyping was performed on genomic DNA extracted from tail biopsies by PCR using primers to amplify the luciferase reporter (5′-GATTCTAAAACGGATTACCAG-3′ and 5′-GTGTTGTAACAATATCGATTC-3′). Mice were housed and bred in a controlled barrier facility at Northwestern University's Center of Comparative Medicine. Temperature, humidity, and photoperiod (12-h light, 12-h dark) were kept constant. Mice were fed a phytoestrogen-free diet (Harlan Teklad Global 2019 or Harlan Teklad Breeder diet 2919). All animal protocols were approved by the animal care and use committees of CWRU (Cleveland, OH) and Northwestern University (Evanston, IL), and animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Luciferase Assays on Pituitary Protein Extracts

Pituitary glands were removed after euthanasia by CO_2 and decapitation. Pituitaries were placed in 100 μl 1× Reporter Lysis Buffer (Promega Corp., Madison, WI) and homogenized using a hand-held motorized grinder. Samples were stored on ice for 15 min, cleared by centrifugation, and frozen at −80 C for at least 1 h to facilitate efficient cell lysis. Thawed samples were recentrifuged, and 25 μl of the supernatant was used in a luciferase assay by injecting 100 μl of Luciferase Assay Reagent (Promega Corp.) with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Protein assays were performed using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA). Relative light units were normalized to protein concentration for each sample. Statistical analysis was performed using GraphPad PRISM 4.0 (GraphPad Software, Inc., San Diego, CA).

Pituitary Dispersions

Pituitaries were removed after euthanasia by CO_2 and decapitation. Tissues were immediately transferred to Wash/Collection Media [3 g/liter BSA; 25 mM HEPES (pH 7.4), in low-glucose DMEM (Invitrogen Life Technologies, Inc., Gaithersburg, MD), with penicillin/streptomycin and fungizone] on ice. Pituitaries were quartered with a scalpel on a siliconized glass slide and washed twice with wash/collection media. Pituitary quarters were rinsed in HEPES Dissociation Buffer (HDB) [1× Hanks' Balanced Salt Solution (Invitrogen Life Technologies, Inc., Gaithersburg, MD) and 25 mM HEPES, pH 7.4] in a 15-ml conical tube. HDB was removed, and pituitaries were dissociated in 5 ml Collagenase II solution [0.4% Collagenase, type II (Invitrogen Life Technologies, Inc.)] 0.4% BSA Fraction V (Sigma, St. Louis, MO), 0.2% glucose, 80 U/ml DNase II (Sigma) in HDB; filter-sterilized aliquots were stored at −20 C before use in a 15-ml conical tube by shaking at 100 rpm in a 37 C water bath for 2 h. During the last half-hour of dispersion, the cells were triturated with a siliconized Pasteur pipette. Dispersed cells were centrifuged at 1000 rpm, and the supernatant was discarded. Freshly prepared 1× pancreatin (Sigma) (2.5 mg/ml in HDB) was added to the cells, and the cells were incubated at 37 C, 100 rpm for an additional 7 min. Charcoal-stripped fetal bovine serum (2 ml) (Invitrogen Life Technologies, Inc.) was added to stop the action of pancreatin, and cells were centrifuged at 1000 rpm for 10 min. Cells were washed twice with 420 μl culture media (low-glucose DMEM with 10% horse serum and 2.5% fetal bovine serum with penicillin/streptomycin and fungizone). Cells were resuspended in culture media, filtered through nylon mesh, and viable cells were counted on a hemocytometer using Trypan Blue staining. Cells were plated on collagen I-coated eight-well chamber slides (BD Biosciences, San Jose, CA) at approximately 1.5×10^5 cells per well.

Immunocytochemistry

Immunocytochemistry was performed on dispersed pituitary cells after culturing for 16–24 h at 37 C in culture media. Cells were fixed in 1% paraformaldehyde for 30 min at 4 C and permeabilized in 0.02% Triton X-100 for 5 min at room temperature, and blocking was performed by incubation in 6% BSA for 30 min at room temperature. Primary antibodies were diluted in 1% BSA in 1× PBS. Mouse antiluciferase (Zymed

Laboratories, South San Francisco, CA; Invitrogen) was used at 30 μg/ml, and guinea pig antirat GH (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) was used at a 1:50 dilution. Primary antibody incubations were performed at 4 C for 16 h. A two-step secondary antibody incubation was performed using the Alexa Fluor Signal Amplification Kit (Molecular Probes, Invitrogen). For the first step, cells were incubated for 30 min at room temperature in a 1:200 dilution of rabbit antimouse conjugated to Alexa Fluor 488 and a 1:200 dilution of goat antiguinea pig conjugated to Alexa Fluor 546 (Molecular Probes, Eugene, OR) in 1% BSA in 1× PBS. To amplify the signal of the luciferase primary antibody, a second step was performed. Cells were incubated for 30 min at room temperature in a 1:200 dilution of goat antirabbit conjugated to Alexa Fluor 488 and a 1:200 dilution of goat antiguinea pig conjugated to Alexa Fluor 546. Vectashield mounting media with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) was applied to the samples and the slides were coverslipped. Fluorescence was examined using a Leica DM5000B fluorescence microscope and OpenLab 4.0 software (Improvision, Lexington, MA). Images were captured at ×400.

Vaccinia Transfection System

HeLa T4 cells, maintained in DMEM with 4.5 g/liter glucose and L-glutamine (Mediatech, Inc., Herndon, VA) and 5% fetal bovine serum (Mediatech, Inc.), were transfected with constructs using the vaccinia virus-T7 polymerase expression system (obtained under license from Dr. Bernard Moss, National Institutes of Health, Bethesda, MD), as described elsewhere (72). For transfection, the cells were incubated with vaccinia at a multiplicity of infection of 10 in PBS/0.1% BSA for 30 min. Cells were transfected either with a Pit-1 expression construct (a gift from Dr. Holly A. Ingraham, University of California at San Francisco) or with empty vector. Plasmid DNAs to be transfected were incubated with liposomes (73, 74) at 5 μg lipid/μg DNA in OptiMEM media (Life Technologies, Inc., Gaithersburg, MD) for 15–20 min at room temperature. After infection, the virus was aspirated, and the DNA/transfectAce was added. Cells were transfected for 6 h at 37 C in 5% CO_2 and allowed to recover overnight in DMEM.

Extraction of Nuclear Proteins

After transfection, nuclear extracts were collected, as described previously (75). HeLa cells were washed twice with cold 1× PBS and scrape collected in 1 ml 1× PBS with protease inhibitors (1 μg/ml aprotinin, leupeptin, antipain, and pepstatin) in microcentrifuge tubes. Cells were pelleted by spinning for 5 min at 500 × *g* at 4 C. The cell pellet was resuspended in 5× volume of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride with 1 μg/ml aprotinin, leupeptin, antipain and pepstatin] and incubated on ice for 15 min, followed by homogenization by 10 strokes with a glass-glass Dounce homogenizer. Samples were spun at 1,500 × *g* for 2 min at 4 C. The nuclear pellet was resuspended in two thirds volume Buffer C (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.2 mM EDTA; 25% glycerol; 0.5 mM DTT; 0.5 mM phenylmethylsulfonylfluoride with 1 μg/ml aprotinin, leupeptin, antipain, and pepstatin) while constantly stirring with a micro stir bar on ice. After stirring for 30 min, samples were spun at 13,000 × *g* for 10 min. The supernatant was aliquoted and stored at −80 C. Protein concentration was determined by Bradford Protein Assay using Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Nuclear extracts (10 μg) were boiled for 5 min in sample buffer and size separated by SDS-PAGE on a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane (BA-85; Schleicher & Schuell, Keene, NH) by electrophoresis. The membrane was blocked with 3% nonfat dry milk in PBS (blocking buffer) for 20 min at room temperature with shaking, followed by incubation with a 1:100 dilution of Pit-1 antibody (PRB-230C; Covance Laboratories, Inc., Berkeley, CA) in blocking buffer overnight at 4 C with shaking. The blot was washed twice with water and incubated with a 1:3000 dilution of secondary antibody [goat-antirabbit conjugated to horseradish peroxidase (Promega)] in blocking buffer for 90 min. The blot was washed twice with water, then in PBS/0.05% Tween for 5 min, and finally washed in five changes of water. The antibody-antigen complexes were detected by enhanced chemiluminescence (ECL-Plus kit, Amersham, Little Chalfont, Buckinghamshire, UK).

EMSAs

DNA probes for EMSAs were generated either by PCR incorporating ^{32}P-dCTP (GE Healthcare, Piscataway, NJ) for probes longer than 100 bp or by end labeling annealed complementary oligonucleotides in a T4 kinase reaction using ^{32}P-γATP (MP Biomedicals, Solon, OH), for shorter probes. Labeled probes were gel purified on 5–12% acrylamide/TBE (Tris-borate, EDTA) gels and eluted from the gel in 0.5 M NH_4OAc and 1 mM EDTA. Probe eluates were spun through nylon wool, ethanol precipitated twice, and resuspended in 50 μl double distilled H_2O. Each probe (1 μl) was counted on a scintillation counter, and probes were diluted for use at 1.0×10^4 cpm per reaction. In each reaction, 5 μg nuclear extract was used, unless otherwise indicated. Nuclear extracts were incubated with 1× EMSA buffer (5× EMSA buffer: 5 mM DTT; 5 mM $MgCl_2$; 50 mM Tris, pH 7.5), 0.2 μg/μl polydeoxyinosinic deoxycytidylic acid double strand (Amersham Biosciences, Piscataway, NJ), and radiolabeled probe in a final volume of 10–15 μl for 20 min at room temperature. For supershift experiments, 2 μl Pit-1 antibody (200 μg/ml) (sc-16289; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with nuclear extract for 30 min on ice before the addition of the probes. TBE loading dye (30% glycerol/70% TBE with 16 mg/ml bromophenol blue and 16 mg/ml xylene cyanole) was added to each sample, and the samples were run on a 5% acrylamide/TBE gel at 4 C. Gels were exposed to Kodak X-OMAT AR film (Rochester, NY) overnight, and exposed to PhosphorImager screens (Molecular Dynamics, Inc., Sunnyvale, CA) for quantification using a STORM 860 phosphor imager (GE Healthcare) and ImageQuant 5.0 software (Molecular Dynamics).

Generation of 5′-Deletion and Mutation Constructs, Transient Transfections, and Luciferase Assays

rGHRH receptor promoter 5′-deletion constructs were generated by PCR using 5′-primers with a *Kpn*I site and a 3′-primer with either an *Nhe*I site (−1673, −1355, −1115) or a *Hin*dIII site (−840, −601, −396, −131) using the −1673 rGHRH receptor promoter construct as a template. Primers were generated by Integrated DNA Technologies (Coralville, IA). Primer sequences follow: −1673 5′, 5′-GCGGTACCCCATGGCCTC TGCATCAACTTCTG-3′; −1355 5′, 5′-GCGGTACCGGTTGTGGGAGCTCCCTACTGCAT-3′; −1115 5′, 5′-GCGGTAC CGGGTGTGGCAGCCTCTGTCCCCTTTTA-3′; −840 5′, 5′-GCGGTACCTGGCACCCCCC AGCACAACAGCTCTGTAA-3′; −601 5′, 5′-GCGGTACCAGCCCCAGAGATCCCTCTGAG AGTCCT-3′; −396 5′, 5′-GCGGTACCTGTGGTGCCAGGGATTTG AACCTGGAGCC-3′; −131 5′, 5′-GCGGTACCCGTGCAGGTATAGGAGGCCTCTCTGAG-3′; +148 5′, 5′-GCGGTA

CCTGTCCCTCCTGTTGGCCCTGCCTACACAA-3′; +264 *Nhe*I 3′, 5′-GGTGCTAGCCAAA GAGCCGTGGCCCTTC-CCTCCCA-3′ and +264 *Hin*dIII 3′, 5′-GGAAGCTTCCAAA-GAGCC GTGGCCCTTCCCTCCCA-3′. PCR products were digested with the appropriate enzymes and ligated into the pLuc IAV Link V.4 luciferase vector (a gift from Dr. Richard Day, University of Virginia). The −1673 luciferase clone was used as a template for generation of Pit-1 binding site mutation clones. Mutations are shown in Table 1 and were generated using the QuikChange (Stratagene, La Jolla, CA) protocol by PCR with complementary oligonucleotides incorporating the desired mutations. The parental DNA was digested by *Dpn*I for 1 h at 37 C, and the mutant DNA was transformed by electroporation into Top 10 competent cells.

Clones were sequence verified and used in transient transfections of GH3 and HeLa T4 cells. Cells were plated into 12-well plates and cotransfected while subconfluent with mutation or deletion constructs and either a Pit-1 expression vector (a gift from Dr. Holly Ingraham, University of California at San Francisco) or empty vector with 1 μg each plasmid (2 μg total DNA per well) in Opti-MEM containing lipofectamine 2000 (2 μl/μg DNA) (Invitrogen, Carlsbad, CA) for 4–6 h. Cells transfected with an Rous sarcoma virus-luciferase construct served as a positive control, to which individual transfection experiments were normalized. Transfected cells were allowed to recover overnight in complete media. Cells were washed with 1× PBS and lysed in 150 μl 1× Reporter Lysis buffer (Promega) for 20 min on ice. Cells were scrape collected, and lysis was completed by freezing at −80 C for at least 1 h. For the luciferase assay, 100 μl of each lysate was aliquoted into cuvettes, which were injected with luciferase assay substrate (Promega), and light emission was measured using a Monolight 2010 luminometer (Analytical Luminescense Laboratory, San Diego, CA). Relative light units were normalized to protein concentration, which was determined by Bradford Protein Assay using Bio-Rad dye reagent (Bio-Rad Laboratories).

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REFERENCES

1. Ferrand R 1972 [Experimental study of the factors in cytological differentiation of the adenohypophysis in the chick embryo]. Arch Biol (Liege) 83:297–371
2. Daikoku S, Chikamori M, Adachi T, Maki Y 1982 Effect of the basal diencephalon on the development of Rathke's pouch in rats: a study in combined organ cultures. Dev Biol 90:198–202
3. Watanabe YG 1982 Effects of brain and mesenchyme upon the cytogenesis of rat adenohypophysis in vitro. I. Differentiation of adrenocorticotropes. Cell Tissue Res 227:257–266
4. Kawamura K, Kikuyama S 1995 Induction from posterior hypothalamus is essential for the development of the pituitary proopiomelacortin (POMC) cells of the toad (*Bufo japonicus*). Cell Tissue Res 279:233–239
5. Takuma N, Sheng HZ, Furuta Y, Ward JM, Sharma K, Hogan BL, Pfaff SL, Westphal H, Kimura S, Mahon KA 1998 Formation of Rathke's pouch requires dual induction from the diencephalon. Development 125: 4835–4840
6. Takor TT, Pearse AG 1975 Neuroectodermal origin of avian hypothalamo-hypophyseal complex: the role of the ventral neural ridge. J Embryol Exp Morphol 34:311–325
7. elAmraoui A, Dubois PM 1993 Experimental evidence for the early commitment of the presumptive adenohypophysis. Neuroendocrinology 58:609–615
8. Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K 1994 The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. Dev Biol 164:409–419
9. Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP, Rosenfeld MG 1998 Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev 12:1691–1704
10. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW 1990 Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev 4:695–711
11. Gage PJ, Brinkmeier ML, Scarlett LM, Knapp LT, Camper SA, Mahon KA 1996 The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. Mol Endocrinol 10:1570–1581
12. Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, Gukovsky I, Carriere C, Ryan AK, Miller AP, Zuo L, Gleiberman AS, Andersen B, Beamer WG, Rosenfeld MG 1996 Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. Nature 384:327–333
13. Gage PJ, Camper SA 1997 Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. Hum Mol Genet 6:457–464
14. Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK, Rosenfeld MG 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. Cell 97:587–598
15. Japon MA, Rubinstein M, Low MJ 1994 In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem 42:1117–1125
16. Ericson J, Norlin S, Jessell TM, Edlund T 1998 Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 125: 1005–1015
17. Smith P 1930 The effect of hypophysectomy upon the involution of the thymus in the rat. Anat Rec 47:119–143

18. Bullough WS 1954 A study of the hormonal relations of epidermal mitotic activity in vitro. I. Technique. Exp Cell Res 7:176–185
19. Kember NF 1971 Growth hormone and cartilage cell division in hypophysectomized rats. Cell Tissue Kinet 4:193–199
20. Thorngren KG, Hansson LI 1974 Effect of withdrawal of growth hormone adminstration on longitudinal bone growth in the hypophysectomized rat. Acta Endocrinol (Copenh) 75:11–23
21. Isaksson OG, Lindahl A, Nilsson A, Isgaard J 1987 Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocr Rev 8:426–438
22. Reddi AH, Sullivan NE 1980 Matrix-induced endochondral bone differentiation: influence of hypophysectomy, growth hormone, and thyroid-stimulating hormone. Endocrinology 107:1291–1299
23. Morikawa M, Nixon T, Green H 1982 Growth hormone and the adipose conversion of 3T3 cells. Cell 29:783–789
24. Cobb MH, Robbins DJ, Boulton TG 1991 ERKs, extracellular signal-regulated MAP-2 kinases. Curr Opin Cell Biol 3:1025–1032
25. Campbell GS, Pang L, Miyasaka T, Saltiel AR, Carter-Su C 1992 Stimulation by growth hormone of MAP kinase activity in 3T3–F442A fibroblasts. J Biol Chem 267: 6074–6080
26. Winston LA, Bertics PJ 1992 Growth hormone stimulates the tyrosine phosphorylation of 42- and 45-kDa ERK-related proteins. J Biol Chem 267:4747–4751
27. Goodman HM 1965 Early and late effects of growth hormone on the metabolism of glucose in adipose tissue. Endocrinology 76:1134–1140
28. Goodman HM 1968 Growth hormone and the metabolism of carbohydrate and lipid in adipose tissue. Ann NY Acad Sci 148:419–440
29. Cheek DB, Graystone JE 1969 The action of insulin, growth hormone, and epinephrine on cell growth in liver, muscle, and brain of the hypophysectomized rat. Pediatr Res 3:77–88
30. Lindahl A, Isgaard J, Isaksson OG 1987 Growth hormone in vivo potentiates the stimulatory effect of insulin-like growth factor-1 in vitro on colony formation of epiphyseal chondrocytes isolated from hypophysectomized rats. Endocrinology 121:1070–1075
31. Barinaga M, Yamonoto G, Rivier C, Vale W, Evans R, Rosenfeld MG 1983 Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. Nature 306:84–85
32. Barinaga M, Bilezikjian LM, Vale WW, Rosenfeld MG, Evans RM 1985 Independent effects of growth hormone releasing factor on growth hormone release and gene transcription. Nature 314:279–281
33. Guillemin R, Brazeau P, Bohlen P, Esch F, Ling N, Wehrenberg WB 1982 Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585–587
34. Rivier J, Spiess J, Thorner M, Vale W 1982 Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature 300:276–278
35. Mayo KE 1992 Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone. Mol Endocrinol 6:1734–1744
36. Mayo KE, Miller T, DeAlmeida V, Godfrey P, Zheng J, Cunha SR 2000 Regulation of the pituitary somatotroph cell by GHRH and its receptor. Recent Prog Horm Res 55:237–266; discussion 266–267
37. Miller TL, Mayo KE 1997 Glucocorticoids regulate pituitary growth hormone-releasing hormone receptor messenger ribonucleic acid expression. Endocrinology 138: 2458–2465
38. Petersenn S, Rasch AC, Heyens M, Schulte HM 1998 Structure and regulation of the human growth hormone-releasing hormone receptor gene. Mol Endocrinol 12: 233–247
39. Gaylinn BD 1999 Molecular and cell biology of the growth hormone-releasing hormone receptor. Growth Horm IGF Res 9(Suppl A):37–44
40. Nogami H, Inoue K, Moriya H, Ishida A, Kobayashi S, Hisano S, Katayama M, Kawamura K 1999 Regulation of growth hormone-releasing hormone receptor messenger ribonucleic acid expression by glucocorticoids in MtT-S cells and in the pituitary gland of fetal rats. Endocrinology 140:2763–2770
41. Nogami H, Hiraoka Y, Matsubara M, Nonobe E, Harigaya T, Katayama M, Hemmi N, Kobayashi S, Mogi K, Aiso S, Kawamura K, Hisano S 2002 A composite hormone response element regulates transcription of the rat GHRH receptor gene. Endocrinology 143:1318–1326
42. Girard N, Boulanger L, Denis S, Gaudreau P 1999 Differential in vivo regulation of the pituitary growth hormone-releasing hormone (GHRH) receptor by GHRH in young and aged rats. Endocrinology 140:2836–2842
43. Miller TL, Godfrey PA, Dealmeida VI, Mayo KE 1999 The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signaling properties. Endocrinology 140:4152–4165
44. Iguchi G, Okimura Y, Takahashi T, Mizuno I, Fumoto M, Takahashi Y, Kaji H, Abe H, Chihara K 1999 Cloning and characterization of the 5′-flanking region of the human growth hormone-releasing hormone receptor gene. J Biol Chem 274:12108–12114
45. Salvatori R, Fan X, Mullis PE, Haile A, Levine MA 2002 Decreased expression of the GHRH receptor gene due to a mutation in a Pit-1 binding site. Mol Endocrinol 16: 450–458
46. Lin SC, Li S, Drolet DW, Rosenfeld MG 1994 Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. Development 120:515–522
47. Crenshaw III EB, Kalla K, Simmons DM, Swanson LW, Rosenfeld MG 1989 Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. Genes Dev 3:959–972
48. Lira SA, Kalla KA, Glass CK, Drolet DW, Rosenfeld MG 1993 Synergistic interactions between Pit-1 and other elements are required for effective somatotroph rat growth hormone gene expression in transgenic mice. Mol Endocrinol 7:694–701
49. Bodner M, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M 1988 The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55: 505–518
50. Ingraham HA, Chen RP, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG 1988 A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55: 519–529
51. Mangalam HJ, Albert VR, Ingraham HA, Kapiloff M, Wilson L, Nelson C, Elsholtz H, Rosenfeld MG 1989 A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. Genes Dev 3:946–958
52. Day RN, Koike S, Sakai M, Muramatsu M, Maurer RA 1990 Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol Endocrinol 4:1964–1971
53. Fox SR, Jong MT, Casanova J, Ye ZS, Stanley F, Samuels HH 1990 The homeodomain protein, Pit-1/GHF-1, is capable of binding to and activating cell-specific elements of both the growth hormone and prolactin gene promoters. Mol Endocrinol 4:1069–1080
54. Nogami H, Hiraoka Y, Ogasawara K, Aiso S, Hisano S 2005 The role of pit-1 in the regulation of the rat growth

hormone-releasing hormone receptor gene transcription by glucocorticoids. J Mol Endocrinol 35:477–488
55. Baumann G, Maheshwari H 1997 The Dwarfs of Sindh: severe growth hormone (GH) deficiency caused by a mutation in the GH-releasing hormone receptor gene. Acta Paediatr Suppl 423:33–38
56. Salvatori R, Hayashida CY, Aguiar-Oliveira MH, Phillips III JA, Souza AH, Gondo RG, Toledo SP, Conceicao MM, Prince M, Maheshwari HG, Baumann G, Levine MA 1999 Familial dwarfism due to a novel mutation of the growth hormone-releasing hormone receptor gene. J Clin Endocrinol Metab 84:917–923
57. Salvatori R, Fan X, Phillips 3rd JA, Espigares-Martin R, Martin De Lara I, Freeman KL, Plotnick L, Al-Ashwal A, Levine MA 2001 Three new mutations in the gene for the growth hormone (GH)-releasing hormone receptor in familial isolated GH deficiency type IB. J Clin Endocrinol Metab 86:273–279
58. Hayashida CY, Gondo RG, Ferrari C, Toledo SP, Salvatori R, Levine MA, Ezabella MC, Abelin N, Gianella-Neto D, Wajchenberg BL 2000 Familial growth hormone deficiency with mutated GHRH receptor gene: clinical and hormonal findings in homozygous and heterozygous individuals from Itabaianinha. Eur J Endocrinol 142: 557–563
59. Godfrey P, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE 1993 GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. Nat Genet 4:227–232
60. Lin SC, Lin CR, Gukovsky I, Lusis AJ, Sawchenko PE, Rosenfeld MG 1993 Molecular basis of the little mouse phenotype and implications for cell type-specific growth. Nature 364:208–213
61. Gaylinn BD, Dealmeida VI, Lyons Jr CE, Wu KC, Mayo KE, Thorner MO 1999 The mutant growth hormone-releasing hormone (GHRH) receptor of the little mouse does not bind GHRH. Endocrinology 140:5066–5074
62. Korytko AI, Zeitler P, Cuttler L 1996 Developmental regulation of pituitary growth hormone-releasing hormone receptor gene expression in the rat. Endocrinology 137: 1326–1331
63. Shewchuk BM, Asa SL, Cooke NE, Liebhaber SA 1999 Pit-1 binding sites at the somatotrope-specific DNase I hypersensitive sites I, II of the human growth hormone locus control region are essential for in vivo hGH-N gene activation. J Biol Chem 274:35725–35733
64. Morel G, Gallego R, Boulanger L, Pintos E, Garcia-Caballero T, Gaudreau P 1999 Restricted presence of the growth hormone-releasing hormone receptor to somatotropes in rat and human pituitaries. Neuroendocrinology 70:128–136
65. Ono M, Miki N, Murata Y, Osaki E, Tamitsu K, Ri T, Yamada M, Demura H 1995 Sexually dimorphic expression of pituitary growth hormone-releasing factor receptor in the rat. Biochem Biophys Res Commun 216: 1060–1066
66. Carmignac DF, Flavell DM, Robinson IC 1996 Pituitary growth hormone-releasing factor receptor expression in normal and dwarf rats. Neuroendocrinology 64:177–185
67. Kamegai J, Wakabayashi I, Kineman RD, Frohman LA 1999 Growth hormone-releasing hormone receptor (GHRH-R) and growth hormone secretagogue receptor (GHS-R) mRNA levels during postnatal development in male and female rats. J Neuroendocrinol 11:299–306
68. Yan M, Jones ME, Hernandez M, Liu D, Simpson ER, Chen C 2004 Functional modification of pituitary somatotropes in the aromatase knockout mouse and the effect of estrogen replacement. Endocrinology 145:604–612
69. Steinfelder HJ, Radovick S, Wondisford FE 1992 Hormonal regulation of the thyrotropin β-subunit gene by phosphorylation of the pituitary-specific transcription factor Pit-1. Proc Natl Acad Sci USA 89:5942–5945
70. Nowakowski BE, Maurer RA 1994 Multiple Pit-1-binding sites facilitate estrogen responsiveness of the prolactin gene. Mol Endocrinol 8:1742–1749
71. Bokar JA, Keri RA, Farmerie TA, Fenstermaker RA, Andersen B, Hamernik DL, Yun J, Wagner T, Nilson JH 1989 Expression of the glycoprotein hormone α-subunit gene in the placenta requires a functional cyclic AMP response element, whereas a different cis-acting element mediates pituitary-specific expression. Mol Cell Biol 9:5113–5122
72. Fuerst TR, Earl PL, Moss B 1987 Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol 7:2538–2544
73. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M 1987 Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84: 7413–7417
74. Campbell MJ 1995 Lipofection reagents prepared by a simple ethanol injection technique. Biotechniques 18: 1027–1032
75. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489

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