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Transcript Abundance in Mouse Pituitaries with Altered Growth Hormone Expression Quantified by Reverse Transcriptase Polymerase Chain Reaction Implicates Transcription Factor Zn-16 in Gene Regulation In Vivo

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The correlation of growth hormone (GH) mRNA abundance and expression of specific transcription factors was studied in pituitaries of panhypopituitary (Ames df/df **and Snell** dw^J/dw^J **dwarf), isolated GH-deficient (**lit/lit**), and GH-overproducing (growth hormone–releasing hormone [GHRH] transgenic) mice compared with normal littermates. A fluorescence-based reverse transcriptase polymerase chain reaction assay was developed for seven target mRNAs: GH, prolactin (PRL), proopiomelanocortin (POMC), α-subunit of the glycoprotein hormones (αSU), Pit-1, Prop-1, and Zn-16. Amplification parameters for each of these primer pairs were determined in order to calculate initial mRNA transcript number. The reproducibility of the assay was found to be ±10% for either Pit-1 or Zn-16 mRNAs measured in characterized murine GHFT1-5 somatotroph precursor cells. The cell extracts also showed an increased abundance of both Zn-16 and Pit-1 mRNAs when compared with whole pituitary extracts. Measurement of copy number in normal pituitaries showed that for every 10^6 GH or PRL mRNAs, there were 3×10^5 POMC, 4×10^4 αSU, 2×10^3 Pit-1, and only 70 Zn-16 or Prop-1 transcripts. Transcript abundance in GH-altered mice as a percentage of copy number per normal gland showed that POMC was significantly reduced in** dw^J/dw^J **($p < 0.01$) and** df/df **($p < 0.05$) mice. αSU mRNA was reduced in** df/df **($p < 0.05$),** dw^J/dw^J **($p < 0.05$), and** lit/lit **($p < 0.05$) mice, but not in GHRH-excess mice. PRL mRNA was not detected in dwarf mice, reduced to 52% of normal in** lit/lit **($p < 0.05$), and unchanged in GHRH-excess animals. GH mRNA was not detected in dwarf mice, reduced to 1.3% in** lit/lit **($p < 0.005$), and increased to 242% in GHRH-excess mice ($p < 0.05$). Pit-1 mRNA was not detected in dwarf mice, was 2.9% of normal in** lit/lit **($p < 0.005$) mice, and increased to 200% in GHRH-excess mice ($p < 0.05$). Prop-1 was not present in dwarf mice, was decreased to 1.4% in** lit/lit **($p < 0.01$), and increased to 223% in GHRH-excess mice ($p < 0.05$). Zn-16 abundance in** df/df **mice was significantly reduced ($p < 0.05$) to 4.8% of normals, to 6.3% of normals in** dw^J/dw^J **($p < 0.005$), to 6.1% of normals in** lit/lit **($p < 0.005$) mice, and significantly elevated in GHRH-excess mice to 197% ($p < 0.05$). Altered pituitary mRNA abundance was found for several products not previously measured, or thought not to be affected by these mutations. Correlation of GH mRNA abundance with transcription factor copy number showed a significant correlation for Pit-1, Prop-1, and Zn-16. These quantitative analyses provide the first in vivo evidence that Zn-16 mRNA abundance correlates with GH expression.**

Key Words: Somatotroph; gene expression; neuroendocrine regulation.

Introduction

Spontaneous and transgenic mutations in mice that alter growth hormone (GH) expression have been used to study GH effects and pituitary differentiation, as well as to identify similar human mutations *(1–5)*. Comparison of expression among the mutant models, which range in GH from undetectable to greater than normal, of related hormones and regulatory factors could provide information about transcriptional regulation. These models include Snell *(6,7)* and Ames *(8)* dwarf mice with combined pituitary hormone (GH, prolactin [PRL] and thyroid-stimulating hormone) deficiency, the little mouse *(9)* with isolated GH deficiency, and transgenic human GH-releasing hormone (GHRH)-expressing mice with increased endogenous GH *(10)*. The spontaneous mutations have now been characterized. The Snell dwarf mouse was found to have a mutation in the pituitary-specific transcription factor, Pit-1, as either an amino acid substitution (gene symbol **dw**) or an independently arising

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large-scale rearrangement (Snell Jackson, gene symbol **dw^J**) *(11)*. The Ames dwarf mouse (gene symbol **df**) was found to have a mutation affecting a transcription factor named Prophet of Pit-1 (Prop- 1), which is necessary for the initiation of Pit-1 expression *(12)*. The little mouse (gene symbol **lit**) has a mutated GHRH receptor (GHRH-R) that prevents proper signaling from the hypothalamus to activate somatotroph lineage expansion and GH production *(13–15)*. Mutations in the same genes have been found in patients with similar endocrine profiles: Pit-1 *(16,17)*, Prop-1 *(18)*, and GHRH-R *(19)*.

Studies of pituitary hormone expression in these models have been reviewed by Phillips et al. *(20)*, and later by Phelps *(21)*. These reviews point out differences from normal not expected from the known properties of the mutant gene products, such as reduced gonadotropins in **df/df** mice *(22)*. While thyrotrophs are absent in the dwarf pituitary *(23,24)*, gonadotrophs might be either normal or reduced in activity; measuring expression of the common α-subunit of the glycoprotein hormones (αSU) might address this area of question. Adrenocorticotroph hormone (ACTH) is present in dwarf pituitary *(25,26)*, and proopiomelanocortin (POMC) expression should be unaffected by any of the mutations so that abundance of POMC mRNA could serve as a functional control in comparisons. Further, although circulating levels of many hormones have been assayed, pituitary content is often unknown. For example, the PRL content of **lit/lit** pituitaries has not been determined, although lactotrophs might be affected by the loss of precursor somatolactotrophs *(27, 28)*. Thus, it would be useful to have a more nearly complete panel of results from the GH-altered mice to examine the extent of altered expression in the pituitary, especially for correlation with the functions of the mutant gene products in cell type development and maintenance *(1)*.

It was hypothesized that changes in GH expression would correlate with changes in mRNA copy number of transcription factors that regulate GH. Because large differences in mRNA abundance would be predicted when comparing normal and GH-altered pituitaries, a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed to provide the needed range of sensitivity. The reproducibility of the RT-PCR assay was tested first with cloned templates, then with mRNA from murine GHFT1-5 cells, a somatotroph precursor cell line previously characterized for hormone mRNA abundance *(29)*, as well as having detectable mRNAs for Pit-1 *(29)* and Zn-16 *(30)*. Then, copy number was quantified for seven mRNA targets in pituitaries from individual Ames **df/df** dwarf, Snell **dw^J/dw^J** dwarf, little **lit/lit**, and GHRH-excess giant mice: GH; PRL; POMC; αSU; and transcription factors Pit-1, Prop-1 and Zn-16.

Whether Zn-16 and GH mRNA abundance were correlated was a focus of the present study. Zn-16 is a bipartite transcription factor with multiple zinc fingers *(30)* that has been shown in vitro to act with Pit-1 on the GH promoter by binding to a recognition element that is conserved in mammals *(31)*. However, there is no information about the in vivo role of Zn-16. Sequencing mouse Zn-16 showed that the predicted amino acid sequence is ≈90% similar to that of rat and provided the information necessary to design primers for RT-PCR assay in pituitary *(30)*. It was hypothesized that Zn-16 mRNA abundance would correlate with GH mRNA transcripts in this panel of mouse models, as a test of the physiologic relevance of Zn-16 in GH transcriptional control.

Table 1
Pit-1 and Zn-16 mRNA Abundance
in Normal Pituitary Tissue and GHFT1-5 Cell Extracts[a]

		Copy no./μg total RNA	
RNA source	Trial	Pit-1 mRNA	Zn-16 mRNA
Pituitary	1	640	53
	2	571	35
	3	423	27
	4	408	25
	5	390	28
mean ± SEM		486 ± 50	34 ± 5
GHFT1-5	1	638	66
	2	495	52
	3	629	64
	4	798	83
	5	545	56
mean ± SEM		621 ± 52*	64 ± 5**

[a]Quantitative fluorescence-based RT-PCR assay of both Pit-1 and Zn-16 mRNA was performed as described in Materials and Methods. Total RNA was extracted from five separate samples of pituitary or cells, and then independent reverse transcriptions and amplifications for either Pit-1 or Zn-16 were performed. Values shown are initial copy numbers of each target divided by the amount of total RNA in the sample measured spectrophotometrically. Significant differences between GHFT1-5 and pituitary samples are indicated as follows: *$p < 0.05$; **$p < 0.01$.

Results

Pit-1 and Zn-16 Expression in Murine Presomatotroph GHFT1-5 Cells

To test the reproducibility of the quantitative RT-PCR assay (see Materials and Methods) on cells in which transcription was expected to be uniform, mRNAs present in the GHFT1-5 cell line were quantified. In agreement with previous analysis *(29)*, GH, POMC, αSU, and PRL transcripts were not detectable in GHFT1-5 cell extracts (data not shown). Pit-1 and Zn-16 were detectable, and the abundance of each was determined in five separate assays of independently prepared samples from GHFT1-5 cells (Table 1). The results show that in independent trials of the assays, there were 621 ± 52 Pit-1 and 64 ± 5 Zn-16 mRNAs/μg of total RNA in the GHFT1-5 cells, so that, even with separate

Table 2
mRNA Copy Number in GH-Altered and Normal Mouse Pituitaries[a]

	Target mRNA						
Mouse type	POMC	αSU	PRL	GH	Pit-1	PROP-1	Zn-16
Normal **DF/?**	311,667	57,900	1,131,667	1,378,000	1501	52	21
	±75,614	±33,051	±238,787	±534,029	±448	±5	±1
df/df	57,600	1919	7	2	0	0	1
	±1971*	±546*	±4†	±2†	±0†	±0†	±1*
Normal **DWJ/?**	428,200	24,954	1,790,400	1,639,800	2761	48	16
	±76,741	±9,707	±92,001	±198,049	±329	±15	±5
dw^J/dw^J	47,620	1450	14	23	0	0	1
	±4535**	±687*	±9†	±23†	±0†	±0†	±0†
Normal **LIT/?**	336,000	23,860	716,000	578,600	1435	74	147
	±41,378	±2328	±57,890	±127,162	±184	±31	±28
lit/lit	144,675	13,388	374,025	7383	42	1	9
	±47,563	±3703*	±50,002*	±2742†	±12†	±1**	±1†
Nontransgenic	189,660	75,940	1,504,200	896,600	2131	108	109
	±44,767	±25,604	±642,685	±229,301	±770	±44	±35
GHRH giant	128,167	77,683	2,378,333	2,173,500	4258	241	215
	±17,130	±18,229	±120,538	±354,639*	±1,168*	±44*	±46*

[a] Values are the average copy number ± SEM for each mRNA in all four types of GH-altered mice and the four normal littermates from 1 μL of RNA extract used for quantitative RT-PCR as described in Materials and Methods. Statistical differences determined by student's *t*-test are indicated as follows: *$p < 0.05$; **$p < 0.01$; †$p < 0.005$. Numbers of animals for each group were as follows: normal **DF** ($n = 4$); **df/df** ($n = 4$); normal **dw^J** ($n = 5$); **dw^J/dw^J** ($n = 4$); normal **LIT** ($n = 4$); **lit/lit** ($n = 5$); nontransgenic ($n = 6$); GHRH-transgenic giant ($n = 5$).

extractions, variance was low (SEM ≤10%). Under the same protocol, normal mouse pituitaries showed significantly lower numbers of transcripts for both Pit-1 (486 ± 50; $p < 0.05$) and Zn-16 (34 ± 5; $p < 0.01$). Using GHFT1-5 cells or whole pituitary extracts, assay reproducibility as indicated by the SEM for these repeated trials was 9.3–11.5% for Pit-1 and 9.3–14.7% for Zn-16.

Transcript Comparisons in GH-Altered Mouse Pituitaries

Efficiency calculated for each primer pair was used to determine the relative number of transcripts present/μL of total RNA extracted from whole pituitaries from individual mice. From these data, the transcript copy number of each of the seven mRNAs in normal and GHaltered pituitaries of each strain was calculated (Table 2). In the four types of normal mice, it was found that for every 10^6 copies of GH mRNA detected, there were 10^6 PRL, 3×10^5 POMC, 4×10^4 αSU, 2×10^3 Pit-1, and 70 transcripts each for Zn-16 and Prop-1. These relative abundance values did not differ significantly among the four strains of normals.

The results in Table 2 show that POMC abundance was reduced significantly in **dw^J/dw^J** ($p < 0.01$) and **df/df** ($p < 0.05$) mice compared with normal littermates; POMC abundance was not significantly different from normals in **lit/lit** and GHRH-excess mice. Abundance of αSU mRNA was reduced significantly in **df/df** ($p < 0.05$), **dw^J/dw^J** ($p < 0.05$), and **lit/lit** ($p < 0.05$) mice but was the same in GHRH-excess as in normal mice. PRL mRNA was not detected in **df/df** and **dw^J/dw^J** mice, was reduced significantly ($p < 0.05$) in **lit/lit** pituitary to 52% of normal, and was not different from normal in GHRH-excess pituitary. GH mRNA was not detected in **df/df** or **dw^J/dw^J** mice, reduced significantly ($p < 0.005$) in **lit/lit** to 1.3% of normal, and elevated significantly ($p < 0.05$) in GHRH-excess pituitary to 242% of normal. Pit-1 mRNA was not detected in **df/df** or **dw^J/dw^J** mice, was at 2.9% of normal ($p < 0.005$) in **lit/lit** mice, and was increased significantly to 200% in GHRH-excess pituitary ($p < 0.05$). Prop-1 was undetectable in **df/df** and **dw^J/dw^J** mice, was decreased significantly ($p < 0.01$) in **lit/lit** to 1.4% that of normal pituitaries, and increased significantly ($p < 0.05$) to 223% in GHRH-excess mice. Zn-16 abundance in **df/df** mice was reduced significantly ($p < 0.05$) to 4.8% of normals, to 6.3% of normals in **dw^J/dw^J** ($p < 0.005$) mice, to 6.1% of normals in **lit/lit** ($p < 0.005$) mice, and elevated significantly in GHRH-excess pituitary to 197% ($p < 0.05$).

Figure 1 compares mouse type on the *x*-axis and percentage of GH, Pit-1, Prop-1, and Zn-16 mRNA relative to normal littermates on the *y*-axis. Average copy numbers in all normal pituitaries within each strain were used to determine this relative percentage; the 100% level is indicated by a dotted line in Fig. 1. Regression analysis using copy numbers showed that there was no correlation of POMC mRNA with any of the transcription factors. There were significant correlations for Pit-1 vs GH ($p < 0.001$), Pit-1 vs PRL ($p < 0.001$), Prop-1 vs GH ($p < 0.001$), Prop-1 vs PRL ($p < 0.001$), Zn-16 vs GH ($p < 0.001$), and Zn-16 vs PRL ($p < 0.001$).

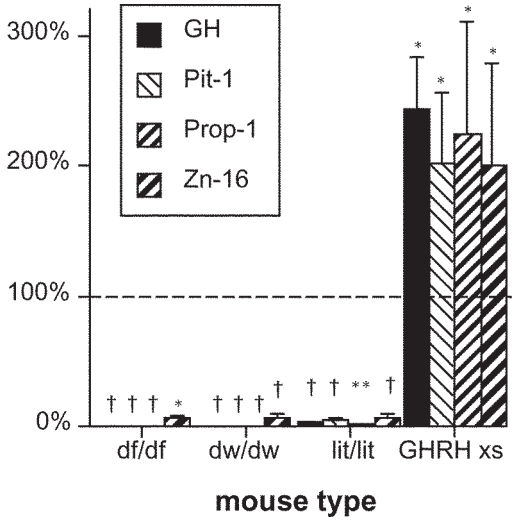

Fig. 1. Relative expression in Ames dwarf (**df/df**), Snell dwarf (**dw^J/dw^J**), little (**lit/lit**) and GHRH transgenic "giant" (**GHRH xs**) mice for GH, Pit-1, Prop-1, and Zn-16 mRNAs. Columns represent the mean percentages of normal expression in GH-altered mouse pituitary samples determined using RT-PCR assay. A line is drawn at 100%, indicating the average of the values for expression in normal littermates of that strain. Vertical bars denote SEM. Values that were significantly different from normal littermates by student's *t*-test are designated as follows: *$p < 0.05$; **$p < 0.01$; †$p < 0.005$.

Discussion

Quantitative RT-PCR-based assays have the advantages of speed, sensitivity, and selectivity for studies of gene expression *(32–37)*. A previous RT-PCR analysis of dwarf pituitaries simply determined the presence or absence of certain transcripts *(38)*. In the present study, quantitative detection of RT-PCR products combined with determined efficiencies of amplification for each primer set was used to calculate the initial copy number of mRNAs for four hormones and three transcription factors present in the anterior pituitary. Because the abundance of transcripts showed a wide range, from 70 copies of Zn-16 to >1,000,000 copies of GH or PRL mRNA, it was important to determine the reliability of measurement at quantity extremes. Repetitions with known amounts of input transcripts ranging from 1 to 1000 copies produced from cloned templates varied between 7.5 and 10.5%, similar to previously reported RT-PCR assays *(32–37)*. The variation was not different for mRNAs with different lengths as well as for different initial abundance.

Reproducibility was also tested using murine GHFT1-5 cells, a clonal somatotroph precursor cell line derived from pituitaries of transgenic mice with a Pit-1 promoter directing large T antigen expression *(29)*. These cells express mRNA for Pit-1 *(29)* and Zn-16 *(30)*, but not GH, PRL, POMC, or αSU *(29)*; Prop-1 was not tested in that previous report. In the present study, only Pit-1 and Zn-16 mRNAs were detected. Comparison of abundance of each mRNA in GHFT1-5 cells with those in normal pituitary showed a 190% increase in Zn-16 and a 133% increase for Pit-1, likely owing to the fact that the cells are a single type in contrast to the multiple cell types present in the pituitary. It is interesting that both Zn-16 and Pit-1 transcripts are present while GH is not, but this provides further evidence for the involvement of Zn-16 in somatotroph differentiation.

The wide range of transcript abundance in normal pituitaries largely reflected what is known about cell-type populations. The abundance of either GH or PRL mRNA was shown to be ≈4-fold higher than that of POMC mRNA and 25-fold more than αSU. By contrast, the abundance of Pit-1, Prop-1, or Zn-16 mRNAs was markedly lower than that of the hormones that they regulate. The amplification afforded by RT-PCR is critical for measuring transcription factors with very low abundance such as Zn-16 and Prop-1. *In situ* hybridization (ISH) has been useful for cellular localization of expression during pituitary development, but ISH may lack needed sensitivity for factors at very low abundance *(12)*. Other pituitary transcription factors, such as Rpx (also called Hesx1) *(39,40)* and Lim-3 (also called P-Lim or Lhx3) *(41–43)*, would likely also require the sensitivity of an RT-PCR assay.

GH mRNA was abundant in normal pituitary and varied in abundance in all the GH-altered mice, as predicted; i.e., undetectable in either **df/df** or **dw^J/dw^J** mice, reduced to 1.3% in **lit/lit** mice, and increased to 242% in GHRH-excess pituitaries. In previous reports, Northern blot analysis showed that GH mRNA was ≈8% of normal in **lit/lit** mice *(44)*, and GH mRNA assayed by RT-PCR in the GHRH-excess transgenic mice was increased to 153% of normal *(45)*.

PRL was as abundant as GH in normal pituitaries. In **dw^J/dw^J** and **df/df** pituitaries, PRL mRNA was not detected, as expected *(11)*. In **lit/lit** mice, the abundance of PRL mRNA was reduced to 52% of normal. There are no published measurements of PRL protein in **lit/lit** pituitaries, and several studies have shown disparate estimates of PRL in **lit/lit** mice, including increased circulating hormone by radioimmunoassay *(46)* and PRL absence using nonimmunogenic electrophoresis *(9)*. Lactotroph cell density by fluorescence immunocytochemistry has been reported as unaltered *(14)*, which may correlate with the present finding because the deficiency in somatotrophs in the **lit/lit** results in a gland of approximately half normal size. The lactotroph population in **lit/lit** mice might be reduced through the loss of precursor somatolactotrophs *(27,28)*. The GHRH-excess mice were found to have PRL mRNA abundance that was 158% that of normal littermates, similar to the reported 120% increase using an RT-PCR assay *(45)*. Thus, results in these two models suggest that GHRH has effect(s) on lactotrophs as well as somatotrophs *(47)*.

It was assumed that POMC would be expressed normally in the mutant pituitaries and would provide a normalization factor. POMC mRNA abundance was comparable with normal in both **lit/lit** and GHRH-excess pituitaries but was

reduced significantly in **df/df** and **dw^J/dw^J** mice. In a previous study, RT-PCR assay of POMC mRNA in GHRH-excess mice showed a significant reduction *(45)*. The reduction in POMC mRNA in dwarf mice may reflect the "highly variable" immunostaining reported for POMC in **dw/dw** pituitaries *(48)*. ACTH has been detected in dwarf mouse pituitaries by immunocytochemistry *(49)* but has not been quantified. Diminished ACTH has been noted in some Prop-1-deficient patients *(50)*. However, it is important to distinguish mRNA abundance from protein levels. Translational mechanisms may compensate for alterations in transcript copy numbers, especially for products such as ACTH that result from peptidase action on precursor proteins.

Abundance of αSU mRNA was reduced significantly in **df/df** and **dw^J/dw^J** mice; this may reflect deficient thyrotrophs, and reduction in gonadotrophs has also been reported in dwarf mice *(22)*. Expression of αSU in **lit/lit** mice also was reduced significantly, but in the GHRH-excess mice, expression was normal. The reduced abundance of αSU mRNA in **lit/lit** mice was unexpected because the GHRH-R signaling pathway has not been thought to influence glycoprotein hormone–producing populations *(51)*.

Pit-1 mRNA abundance was in agreement with the known mutational status of **df/df** and **dw^J/dw^J** mice, in which expression is abrogated either directly *(11)* or indirectly *(12)*, as well as in the GHRH-excess "giants," in which previous studies have shown that Pit-1 protein was increased and highly localized to nuclei *(52)*. On the other hand, the reduction in Pit-1 mRNA in **lit/lit** mice was greater than that previously measured using Northern blot analysis *(15)*. It is likely that methodologic considerations account for this difference. It is important to note that the reduced Pit-1 mRNA abundance in the present study correlated significantly with the reduction in both GH and PRL expression in the **lit/lit** pituitary, in agreement with the role of Pit-1 in transcriptional activation for both hormones *(11)*.

That Prop-1 is needed to activate Pit-1 expression has been shown in murine and human dwarfism *(4,12,18)*, but it is not known whether other roles exist for this protein. It was previously reported that there was no detectable Prop-1 mRNA in adult mouse pituitaries assessed by ISH *(53)*. However, Prop-1 mRNA has been detected in human adenomas and normal pituitaries *(54)*. Prop-1 mRNA was low but detectable in all four types of normal mouse pituitaries assayed in the present report. Changes in correlation with GH and PRL in pituitaries of the four types of GH-altered mice studied suggest that Prop-1 has a functional role in addition to the developmental initiation of Pit-1 expression *(12)*.

Little is known about the transcription factor Zn-16. Zn-16 showed the same low mRNA abundance as Prop-1 in the present study. In all the models examined, Zn-16 mRNA abundance was correlated significantly with GH and PRL mRNA. This is the first evidence from in vivo models to suggest that Zn-16 functions in the physiology of pituitary hormone expression. The low abundance of Zn-16 and Prop-1 mRNAs in normal adult pituitaries implies that the localization of these factors to specific cell types using ISH methods may not be possible. Thus, it may be difficult to characterize the functions of Zn-16 by the methods employed so successfully for Pit-1, for which ≈30-fold more transcripts (2000 vs 70) are present in the normal mouse pituitary. However, the findings of expression in somatotroph precursor cells and significant correlation with GH mRNA in models of over- and underexpression describe a pattern of Zn-16 expression that would be predicted for a transcription factor synergizing with Pit-1 in the somatotroph *(55)*.

Materials and Methods

Mice and Cell Culture

Male mice of 3 to 4 mo of age were either phenotypically normal (heterozygous or homozygous dominant) littermates or homozygous mutants. Ames dwarf (**df/df**) mice were from the colony of Dr. Carol Phelps; Snell Jackson (**dw^J/dw^J**) and little (**lit/lit**) mice were purchased from Jackson (Bar Harbor, ME). Transgenic GHRH-excess mice *(56)* were the generous gift of Dr. James Hyde, formerly at the Department of Anatomy and Neurobiology, University of Kentucky Medical Center. Mice were euthanized with CO_2 according to procedures approved by the Tulane University Institutional Animal Care and Use Committee. Individual pituitaries were removed using instruments washed between dissections and stored in separate tubes at –70ºC. The presomatotroph cell line GHFT1-5 was provided by Dr. Pamela Mellon, University of California, San Diego, and cultured as described previously *(29)*.

RNA Isolation and Reverse Transcription

Total RNA was extracted from individual mouse pituitaries or from independent aliquots of ≈1×10^7 GHFT1-5 cells using a modified phenol/chloroform/ guanidinium protocol according to the manufacturer's directions (Ultraspec; Biotecx, Houston, TX). The RNA pellet was briefly dried before resuspension in 0.1 μ*M* oligo-dT and aliquots used in subsequent reverse transcription of poly-A RNA (SuperScript II Preamplification System; Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The reaction was carried out in a 20-μL vol containing 200 U of Moloney murine leukemia virus reverse transcriptase at 45ºC for 60 min. Then the enzyme was denatured by heating at 65ºC for 15 min. Hybridized RNA was removed by digestion with 2 U of *Escherichia coli* RNase H. The volume was then brought to 50 μL with diethylpyrocarbonate-treated water and stored at –20ºC. Transcriptions in vitro using RNA polymerase were performed as previously described *(57)*. In assays used to evaluate the kinetic parameters of each primer set, cDNA was pooled from

several reactions to provide adequate template for repeated tests. Ultraviolet absorbance at 260 nm was measured on a GeneQuant spectrophotometer (Pharmacia, Piscataway, NJ) in a 50-μL cuvet.

Primers

Primers were selected for specificity for mouse mRNAs, high annealing temperature, and absence of secondary structure using the computer programs Right Primer (BioDisk, San Francisco, CA) and Oligo 5.0 (National Biosciences, Plymouth, MN). Primer sequences and the accession numbers of derivation from the GenBank database were as follows:

1. POMC, NM008895.1; upper, GATGTCTGGAGCTGGTG CCTG; lower, TTGACGTACTTCCGGGGGTT.
2. αSU, NM009889.1; upper, TGTGGCCAAAGCATTTAC TAAGG; lower, TTTCTGACGGAACCGTGGTAA.
3. GH, NM008117.1; upper, AAGAGTTCGAGCGTGCCT AC; lower, ACAGGAGAGTGCAGCAGAGACACTGG.
4. PRL, NM011164.1; upper, AGGGAGTTGAGAAGATAAT TAGCCAG; lower, AAGAGGAGACCCAATTGCACCCA.
5. Pit-1, NM008849.1; upper, TGGAGCAGTTTGCCAACGA; lower, TTCTCTCTGCCTTCGGTTGC.
6. Prop-1, NM008895.1; upper, TGGTACAGAGCTGGGGA GACCTAAG; lower, GGCTATCGGCTGGAGAAGTGAC.
7. Zn-16, NM013889.1; upper, TGAAAGGCCATAAGGAT ATCGAA; lower, GATGCTCCTTTCGGTGGGCTAA.

PCR Amplification

Standard PCR conditions for cDNA amplification included each dNTP at a concentration of 0.2 m*M* and 1.25 U of *Taq* polymerase in a final volume of 50 μL. The fluorescent dye-labeled dUTPs ([F]dUTPs) used for labeling were [R110], [R6G], or [TAMRA] (Applied Biosystems, Foster City, CA). [F]dUTPs were diluted for a constant addition volume of 0.1 μL per reaction for a final concentration of 0.2%. Amplifications were performed in a Model TC-1 thermal cycler (Applied Biosystems). Amplification reactions were initiated with a hot start using AmpliWax PCR Gem 50 beads (Applied Biosystems) to separate the primers, $MgCl_2$, and dNTPs (lower master mix) from the DNA template and *Taq* polymerase (upper master mix). Thermal cycling conditions consisted of initial denaturation for 60 s at 94ºC, followed by a three-step profile (94ºC for 60 s, 54ºC for 45 s, and 72ºC for 90 s) for the desired number of cycles, and a terminal extension step of 72ºC for 5 min. For some reactions, a final, nontemplate-dependent extension was carried out at 60ºC for 30 min. After amplification, samples were diluted with water, and the wax was remelted to facilitate accurate withdrawal of sample. Unincorporated primers and dNTPs were removed by centrifugation through Centricon-50 or Microcon-30 filters (Amicon, Beverly, MA).

Visualization and Detection of Product

PCR products were analyzed on 12-cm well-to-read gels composed of 10% Long Ranger (FMC BioProducts, Rockland, ME) with a 373A instrument using GeneScan 1.2 software. Electrophoresis was performed with power limiting at 12 W in 0.5X TBE buffer. Samples were mixed with an ROX-dye-labeled size marker (ROX-500; Applied Biosystems) and a sucrose– or Ficoll–bromophenol blue dye solution before loading on the gel.

Development of Fluorescence-Based RT-PCR Assay

A kinetic RT-PCR assay was developed using fluorescence detection and molecular weight determination on an automated sequencing instrument. Amplifications were performed in the presence of fluorescence-labeled dUTP so that products were detectable during electrophoresis using an Applied Biosystems 373A DNA sequencer in the "GeneScan" mode. Peaks of fluorescence intensity from amplification products were sized by comparison to labeled standards present in each lane as an internal control, and the amount of product determined from peak height. To measure samples in the linear range of the instrument response (data not shown), amplification products were sometimes diluted before analysis. This also ensured that all products were measured over the same range, avoiding variation arising from the differences in initial abundance. For quantification of amplification products, peak height was found to be a more sensitive measure of product amount than peak area (data not shown).

Kinetic quantitative RT-PCR analysis required the determination of two values for each primer set *(32,33)*. First, peak heights were correlated with the amount of DNA in the sample by pooling multiple amplification products and determining the concentration of DNA in the sample. Then, peak heights of samples that were diluted from this known concentration were determined and used to generate calibration curves of final copy number (*FCN*) vs measured peak height. The slopes and intercepts of these curves are given in Table 3. Second, amplification with each primer set was performed at different numbers of initial input molecules from cloned templates. Average efficiencies (*EF*) were then calculated from 20 to 35 repetitions. Efficiency was constant for a primer set at levels of mRNA abundance that were varied over a 1000-fold range. Table 3 shows *EF* values determined empirically for each of the seven primer pairs, as well as optimized primer and $MgCl_2$ concentrations, and product sizes. Multiple bands or other indications of nonspecific amplification were not detected. Each product was cloned and sequenced and found to be identical to the designated target sequence (data not shown). The determined sizes of the products obtained were close to those predicted, although some variation was caused by the size difference of fluorescent dye–labeled nucleotides. The deviation found for PRL apparently was owing to the A+T richness of the region of amplification, since trials using lower ratios of dye-labeled to unlabeled nucleotides resulted in a size in agreement with the predicted value (data not shown). GH gave a size 10% less than predicted, apparently owing to standards being less

Table 3
Summary of Primer Optimization Conditions and Kinetic Parameters
Used for Each mRNA in Calculation of Initial Product Expression in Pituitary Samples

mRNA	Optimum primer (m*M*)	Optimum $MgCl_2$ (m*M*)	Predicted product size (bp)	Measured product size (bp)	Size difference (%)	Efficiency (%)	Slope ($\times 10^{-7}$)	Intercept
POMC	0.80	2.0	170	162	4.7	88.9	1.17	16.50
αSU	0.60	4.0	299	303	–1.3	81.7	2.07	–16.60
PRL	0.60	3.5	330	349	–5.8	81.7	1.01	12.20
GH	0.40	2.0	516	465	9.9	88.2	2.78	8.13
Pit-1	0.60	2.0	400	372	7.0	88.9	1.95	–10.90
Prop-1	0.40	2.0	237	253	–6.8	68.0	8.42	65.7
Zn-16	0.60	3.0	218	230	–5.5	76.3	1.65	–16.10

effective at >500 bp, the limit of the sizing mixture. Sizing with other standards of higher molecular weight gave a size closer to that predicted (data not shown).

From *EF* and *FCN*, the initial number of template (*IT*) molecules present in a sample before amplification can be calculated using the mathematical relationship of factors *IT*, *FCN*, *EF*, and number of amplification cycles (*CN*) *(32,33)*:

$$FCN = IT \times (EF + 1)^{CN} \quad (1)$$

Equation 1 may be rearranged to solve for *IT*:

$$IT = e^{\left(\frac{\ln FCN}{CN \ln(EF + 1)}\right)} \quad (2)$$

In Eq. 2, *IT* can be calculated from *FCN* as determined by calibration curves and from the measured values of *EF* shown in Table 3. Using these values, the reproducibility of the assay was tested using known amounts of RNA transcribed in vitro as input template. In 10 independent repetitions of all enzymatic steps of the assay, the SEM ranged from 7.8% for Pit-1 to 10.5% for Zn-16.

Efficiency Measurements

Efficiency was determined using amplification of a series of diluted templates under standard conditions for increasing numbers of cycles. The results were used to plot cycle number and the logarithm of product intensity. The measured logarithmic range of amplification for each reaction covered 6–12 cycles depending on relative *IT*. On average, 3.3 more cycles were needed to amplify the 10-fold more-dilute samples to a given intensity. Given exponential amplification, this 9.98 ($2^{3.3}$)-fold difference is equal to the known 10-fold difference in template concentration. Using the values from 20 to 30 repetitions, the measured slope of the exponential portion of each amplification profile, the efficiency of amplification was calculated from the following relationship *(32,33)*:

$$\text{Efficiency} = 10^{(\text{slope}-1)} \quad (3)$$

Statistical Analysis

GHFT1-5 and normal samples were compared using analysis of variance followed by Dunnett one-tailed test. Student's *t*-test was used to compare values in GH-altered and normal pituitaries. Linear regression was used to determine correlation of hormone and transcription factor abundance. Testing was performed using either SuperANOVA (Abacus Concepts, Berkeley, CA) or StatWorks (Abacus Concepts) software. A significant difference was considered present at $p < 0.05$.

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References

1. Dasen, J. S. and Rosenfeld, M. G. (1999). *Curr. Opin. Genet. Dev.* **9,** 566–574.
2. Giustina, A. and Veldhuis, J. D. (1998). *Endocr. Rev.* **19,** 717–797.
3. Muller, E. E., Locatelli, V., and Cocchi, D. (1999). *Physiol. Rev.* **79,** 511–607.
4. Parks, J. S., Brown, M. R., Hurley, D. L., Phelps, C. J., and Wajnrajch, M. P. (1999). *J. Clin. Endocrinol. Metab.* **84,** 4362–4370.
5. Phelps, C. J. and Hurley, D. L. (1999). *Proc. Soc. Exp. Biol. Med.* **222,** 39–58.
6. Snell, G. D. (1929). *Proc. Natl. Acad. Sci. USA* **15,** 733,734.
7. Eicher, E. M. and Beamer, W. G. (1980). *J. Hered.* **71,** 187–190.
8. Schaible, R. and Gowen, J. W. (1961). *Genetics* **46,** 896.
9. Eicher, E. M. and Beamer, W. G. (1976). *J. Hered.* **67,** 87–91.
10. Mayo, K. E., et al. (1988). *Mol. Endocrinol.* **2,** 606–612.
11. Li, S., et al. (1990). *Nature* **347,** 528–533.
12. Sornson, M. W., et al. (1996). *Nature* **384,** 327–333.
13. Gaylinn, B. D., et al. (1999). *Endocrinology* **140,** 5066–5074.
14. Lin, S. C., et al. (1993). *Nature* **364,** 208–213.
15. Godfrey, P., et al. (1993). *Nat. Genet.* **4,** 227–232.
16. Pfäffle, R. W., et al. (1992). *Science* **257,** 1118–1121.

17. Radovick, S., et al. (1992). *Science* **257,** 1115–1118.
18. Wu, W., et al. (1998). *Nat. Genet.* 18, 147–149.
19. Maheshwari, H. G., Silverman, B. L., Dupuis, J., and Baumann, G. (1998). *J. Clin. Endocrinol. Metab.* **83,** 4065–4074.
20. Phillips, J. A. III, Beamer, W. G., and Bartke, A. (1982). *J. Endocrinol.* **92,** 405–407.
21. Phelps, C. J. (1994). *Proc. Soc. Exp. Biol. Med.* **206,** 6–23.
22. Bartke, A., Goldman, B. D., Bex, F., and Dalterio, S. (1977). *Endocrinology* **101,** 1760–1766.
23. Bartke, A. (1964). *Anat. Rec.* **149,** 225–236.
24. Roti, E., Christianson, D., Harris, A. R. C., Braverman, L. E., and Vagenakis, A. G. (1978). *Endocrinology* **103,** 1662–1667.
25. Bartke, A. and Lloyd, C. W. (1970). *J. Endocrinol.* **46,** 321–326.
26. Bartke, A. (1971). *J. Reprod. Fertil.* **27,** 121–124.
27. Behringer, R. R., Mathews, L. S., Palmiter, R. D., and Brinster, R. L. (1988). *Genes Dev.* **2,** 453–461.
28. Borrelli, E., Heyman, R. A., Arias, C., Sawchenko, P. E., and Evans, R. M. (1989). *Nature* **339,** 538–541.
29. Lew, D., et al. (1993). *Genes Dev.* **7,** 683–693.
30. Vander Heyden, T. C., et al. (2000). *Mol. Cell. Endocrinol.* **159,** 89–98.
31. Lipkin, S. M., Naar, A. M., Kalla, K. A., Sack, R. A., and Rosenfeld, M. G. (1993). *Genes Dev.* **7,** 1674–1687.
32. Wang, A. M., Doyle, M. V., and Mark, D. F. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 9717–9721.
33. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 2725–2729.
34. Merzouki, A., et al. (1994). *J. Virol. Methods* **50,** 115–128.
35. Zimmermann, K. and Mannhalter, J. W. (1996). *Biotechniques* **21,** 268–279.
36. Souazé, F., Ntodou-Thomé, A., Tran, C. Y., Rosténe, W., and Forgez, P. (1996). *Biotechniques* **21,** 280–285.
37. Tsai, S.-J. and Wiltbank, M. C. (1996). *Biotechniques* **21,** 862–866.
38. Hurley, D. L., Wojtkiewicz, P. W., and Phelps, C. J. (1995). *Recent Prog. Horm. Res.* **50,** 443–448.
39. Hermesz, E., Mackem, S., and Mahon, K. A. (1996). *Development* **122,** 41–52.
40. Dattani, M. T., et al. (1998). *Nat. Genet.* **19,** 125–133.
41. Seidah, N. G., et al. (1994). *DNA* **13,** 1163–1180.
42. Bach, I., et al. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 2720–2724.
43. Sheng, H. Z., et al. (1996). *Science* **272,** 1004–1007.
44. Cheng, T. C., et al. (1983). *Endocrinology* **113,** 1669–1678.
45. Moore, J. P. Jr., et al. (2000). *Endocrinology* **141,** 81–90.
46. Amador, A. G., Parkening, T. A., Beamer, W. G., Bartke, A., and Collins, T. J. (1986). *Endocrinologia Experimentalis* **20,** 349–358.
47. Miller, T. L., Godfrey, P. A., Dealmeida, V. I., and Mayo, K. E. (1999). *Endocrinology* **140,** 4152–4165.
48. Wilson, D. B. and Wyatt, D. P. (1993). *Cell Tissue Res.* **274,** 579–585.
49. Roux, M., Bartke, A., Dumont, F., and Dubois, M. P. (1982). *Cell Tissue Res.* **223,** 415–420.
50. Pernasetti, F., et al. (2000). *J. Clin. Endocrinol. Metab.* **85,** 390–397.
51. Mayo, K. E., Godfrey, P. A., Suhr, S. T., Kulik, D. J., and Rahal, J. O. (1995). *Recent Prog. Horm. Res.* **50,** 35–73.
52. Osamura, R. Y., et al. (1993). *Endocr. J.* **40,** 133–139.
53. Cushman, L. J., et al. (2001). *Hum. Mol. Genet.* **10,** 1141–1153.
54. Nakamura, Y. (1999). *J. Clin. Endocrinol. Metab.* **84,** 1414–1419.
55. Gutierrez-Hartmann, A. (1994). *Mol. Endocrinol.* **8,** 1447–1449.
56. Hammer, R. E., Brinster, R. L., Rosenfeld, M. G., Evans, R. M., and Mayo, K. E. (1985). *Nature* **315,** 413–417.
57. Voss, T. C., Flynn, M. P., and Hurley, D. L. (2001). *Mol. Endocrinol.* **15,** 1549–1558.