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Transcriptional and Post-Transcriptional Regulation of the Growth Hormone-Releasing Hormone Receptor

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ABSTRACT

Transcriptional and Post-Transcriptional Regulation of the Growth Hormone-Releasing Hormone Receptor

Allison Therese McElvaine

In mammalian development, cell specification and organogenesis are achieved by the regulated expression and repression of specific subsets of genes. The anterior pituitary gland, comprised of five cell types that arise from a common precursor, is an excellent model system for understanding these developmental processes. Examination of factors regulating transcription of cell-specific genes leads to a better understanding of how cells differentiate from one another. One of the major goals of this thesis work is investigation of the transcriptional regulation of the growth hormone-releasing hormone (GHRH) receptor gene, which is expressed early in terminally differentiated somatotroph cells of the anterior pituitary gland. A proximal promoter including 1.6kb 5' to the transcriptional start site is sufficient to direct tissue- and cell-specific expression of the gene in transgenic mice. Further examination of the promoter in cell culture experiments led to the identification of ten binding sites for the pituitary-specific transcription factor Pit-1. Of these ten sites with varying affinities for Pit-1, a single proximal site is necessary and sufficient for Pit-1-activated expression of the gene.

The GHRH receptor is an integral molecule in regulating appropriate linear growth in vertebrates. Inappropriate expression or functioning of the GHRH receptor is associated with growth diseases, including dwarfism and gigantism or acromegaly. Another major goal of this thesis work is understanding signaling by the receptor. Post-transcriptional regulation, specifically alternative splicing, is a mechanism by which many protein products are produced

from a single gene. Examination of a truncated splice variant human GHRH receptor reveals a potential role for dimerization in GHRH receptor function. The truncated GHRH receptor cannot signal through the cAMP second messenger pathway and acts in a dominant negative fashion by forming a complex with the wild-type receptor that cannot bind GHRH. This work was the first to show a role for oligomerization in modulating signaling of this receptor.

Together, the work described in this thesis examines the requirements for transcriptional activation of expression of the GHRH receptor, and how the post-transcriptional process of alternative splicing affects signaling of this important molecule.

ACKNOWLEDGEMENTS

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Graduate school is a very long and challenging experience, and it would have been impossible for me to have successfully completed my degree without the love and support of my family and friends. My parents, Robert and Anne McElvaine, first instilled in me the enthusiasm that I have for learning and have always unconditionally supported me, as have all of my family members. I cannot thank them enough for their love and encouragement. Finally, I would like to acknowledge and thank the love of my life, Brian Turbyfill, who has endured this experience

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LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AMV	avian myeloblastosis virus
ATP	adenosine 5-prime-triphosphate
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine 3-prime, 5-prime-monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
ChIP	chromatin immunoprecipitation
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
dCTP	2-prime-deoxycytidine-5-prime-triphosphate
DNA	deoxyribonucleic acid
dNTP	2-prime-deoxynucleoside-5-prime triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-N,N,N’,N’-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor

FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRHR	growth hormone-releasing hormone receptor
GHS	growth hormone secretagogue
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinase
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor-1
IP	immunoprecipitate
kDa	kilodalton
MAPK	mitogen-activated protein kinase
mL	milliliter
mM	millimolar
mRNA	messenger RNA
PACAP	pituitary adenylyl cyclase activating polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PKA	protein kinase A
PMSF	phenylmethylsulfonylfluoride
PRL	prolactin
RLU	relative light units
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RT-PCR	reverse-transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
TSH	thyrotropin stimulating hormone
VIP	vasoactive intestinal peptide
□g	microgram
□L	microliter
□M	micromolar

CHAPTER ONE

GENERAL INTRODUCTION

Overview of Thesis

During the development of multicellular organisms, particular genes must be activated or repressed in each cell to lead to cell differentiation and organogenesis. The process of gene regulation is complex and tightly regulated.

Recent completion of the human genome project revealed relatively few genes in the human, given the complexity of the species (Lander et al. 2001; Venter et al. 2001). In large part, the explanation for how such complexity is achieved with so few genes lies in the processes of transcriptional and post-transcriptional regulation of genes. The introductory chapter begins with a description of these regulatory processes.

This thesis examines both transcriptional and post-transcriptional regulation of the Growth Hormone-Releasing Hormone (GHRH) receptor. The GHRH receptor is an integral molecule in transmitting information from the brain to the endocrine system to appropriately regulate growth in vertebrates. As such, understanding regulation of this gene is important to expanding our knowledge of its normal functioning and ways to treat pathologies involving misregulated expression of the gene. The remainder of the introductory chapter provides background on the GHRH receptor, its role in regulating growth hormone (GH) and select aspects of what is known about the regulation of G-protein-coupled receptors, the class of receptors that includes the GHRH receptor.

Eukaryotic Gene Regulation

Cell function and differentiation require specific proteins to be present in particular concentrations. The regulation of gene expression determines which genes are activated (transcribed to mRNA and translated to protein) and which are repressed in each cell. Gene

expression can be regulated at each step, the first of which is initiation of transcription. When transcription is initiated, a nascent RNA transcript of the gene is made. In eukaryotes, the RNA is processed by the addition of a poly(A) tail and splicing to remove non-coding regions called introns. The mature mRNA associates with ribonuclear proteins (RNPs) (Mehlin et al. 1992) that have a nuclear export signal (NES), which binds exportin1, a nuclear-export receptor (Ohno et al. 1998). This complex requires Ran-GTP (Ohno et al. 1998; Pemberton and Paschal 2005) to interact with proteins in the nuclear pore complex (NPC) and transport the mRNA from the nucleus to the cytoplasm (Doye and Hurt 1997). When an mRNA reaches the cytoplasm, its stability controls how quickly protein synthesis can be initiated and terminated. Most eukaryotic mRNAs have half-lives of many hours, but some that encode proteins that are required for only short periods, such as cytokines, have very short half-lives (Lodish et al. 2000). The sequence AUUUA disrupts mRNA stability through a process that involves sequence-specific binding of proteins that promote degradation (Shaw and Kamen 1986; Malter 1989; Chen et al. 1994; Sarkar et al. 2003). Degradation of some mRNAs is regulated by hormones or other factors. For example, prolactin increases stability of milk protein casein mRNA (Poyet et al. 1989). Each of these steps of mRNA processing is regulated and can alter the production of protein. Finally, the translated protein product can be modified to alter its functions, through processes such as glycosylation, phosphorylation and oligomerization, which will be described further in upcoming sections of the introductory chapter.

Transcriptional Regulation

The first evidence for protein-binding regulatory sequences associated with genes came from studies of the *lac* operon in bacteria. Within the *lac* operon, a specific DNA sequence

operator near the transcription start site was predicted to bind *lac* repressor. Mutations of this sequence performed by Jacob and Monod led to constitutive activity. The mutations in the region preceding the transcription start site, termed the promoter, block initiation of transcription by RNA polymerase (Jacob and Monod 1961).

In multicellular organisms, gene regulation serves the critical role of determining cell fate decisions during development, when specific genes must be activated in specific cells at specific timepoints. Regulation of transcription initiation is the predominant form of gene control in eukaryotes, determined by measurements of transcription rates of multiple genes (Weber et al. 1977; Derman et al. 1981; Powell et al. 1984). Eukaryotic DNA is associated with an approximately equal mass of protein called histones in a highly condensed structure called chromatin (Finch et al. 1977; Sperling and Klug 1977). The structural unit of chromatin is a nucleosome, which consists of a core octomer of histones H2A, H2B, H3 and H4 around which DNA is wound (Kornberg 1974; Olins and Olins 1974; Luger et al. 1997; Davey et al. 2002). Chromatin can exist in either an extended state or a condensed state. In their inactive state, eukaryotic genes are in condensed chromatin. This structure prevents RNA polymerase from binding to initiate transcription. The histone proteins contain 20-40 residues at their N-termini that consist of several positively charged lysine groups (Luger et al. 1997). These positively charged residues interact with the negatively charged phosphates in DNA to tightly wrap DNA around the histones. Positively charged histone residues can be acetylated, methylated or phosphorylated to neutralize the charge (Allfrey et al. 1964; Sanders et al. 1973), allowing the interaction with DNA to be weakened, and making DNA more accessible to the transcriptional machinery. Activator proteins bind both near the transcription start site and at sites kilobases away from it to decondense the chromatin and make the transcription initiation site accessible to

RNA polymerase. Transcriptional repressors bind to different regulatory regions of the promoter, and promote condensation of the chromatin.

Transcriptional coregulatory proteins do not bind DNA; rather, they interact with transcription factors to influence their ability to activate or repress transcription. Many coactivator and corepressor proteins function by modulating chromatin structure. Co-activators often have intrinsic histone acetyltransferase (HAT) activity (Panchenko et al. 2004; Valineva et al. 2005), while many co-repressors possess histone deacetylase (HDAC) activity (Alenghat et al. 2006; Gregoire et al. 2007). When HDAC proteins are recruited to the promoter, they deacetylate the amino-terminal tails of the core histones, allowing the chromatin to return to its condensed repressive state. Histone methylation is another modification that affects chromatin structure. Methylation is usually associated with repression (Hancock et al. 1974), though methylation of certain residues allows chromatin to decondense and transcriptional activation to occur (Wang et al. 2001). Another histone modification, phosphorylation, plays a role in regulation of processes such as transcription, DNA repair, apoptosis and chromosome condensation (Grant 2001). For example, phosphorylation of serine 10 in histone H3 correlates with gene activation (Nowak and Corces 2000). Like histone acetylation, phosphorylation of the histone tails neutralizes the basic charge and thereby reduces histone DNA affinity. Several acetyltransferases have increased HAT activity on serine 10-phosphorylated substrates (Cheung et al. 2000; Lo et al. 2000). Phosphorylation may influence transcriptional activation through the stimulation of HAT activity on the same histone tail. Acetylation, methylation and phosphorylation states are dynamic, and control access of DNA to the transcriptional machinery. The balance between activation and repression of genes at the appropriate time and in the appropriate cell is essential to proper development of multicellular organisms.

Many eukaryotic genes contain a TATA box about 25-35 base pairs upstream of the transcription start site (Bucher 1990). This sequence is involved in positioning RNA polymerase II at the start site to initiate transcription. Other genes may have an initiator-containing promoter, which is usually characterized by a cytosine at the –1 position and an adenine at the +1 position (Lodish et al. 2000). Alternatively, many genes lack these consensus sequences involved in transcription initiation. In these genes, transcription may begin at one of many possible start sites, often 20-200 base pairs in length (Lodish et al. 2000). These multiple transcription start sites give rise to mRNAs with alternative 5' ends. The GHRH receptor gene has of one of these so-called "TATA-less" promoters.

Transcription factors can interact with one another or with other gene regulatory factors to activate or repress transcription initiation. The ability to do so dramatically increases the capacity to control gene expression in a cell-type specific manner. Making the gene accessible to RNA polymerase is the first step of gene expression; therefore, the primary regulation of gene expression is transcriptional activation and repression.

Messenger RNA Processing

Post-transcriptional processing of genes provides an additional method of regulating gene expression. The nascent RNA transcript of a gene is non-functional and must undergo several levels of processing and translocation from the nucleus to the cytoplasm in order to be translated to functional protein. At each of these processing steps, regulation of expression can occur. These levels of regulation impart much greater diversity of gene products for a relatively small number of genes.

As RNA polymerase II initiates transcription of a gene, a 5' 7-methylguanosine cap is added to the primary transcript and this pre-mRNA is polyadenylated at the 3' end (Lodish et al. 2000). The final step in processing a mature mRNA is removal of noncoding introns and splicing together of exons. When different combinations of exons are spliced together or splicing occurs at alternative sites, different mRNAs are generated in a process called alternative splicing. The first indication of alternative splicing as a mechanism of gene regulation in cells was discovered in the immunoglobulin mu chain gene (Rogers et al. 1980; Maki et al. 1981). Regulation of alternative splicing allows different mRNAs to be expressed from a single gene in different cell types, at different times during development, or in response to changing physiological requirements. Alternative splicing is an emerging mechanism by which G-protein-coupled receptors (GPCRs) modulate their activity. Splice variant receptors often have altered signaling properties, and can influence ligand binding, signaling, expression or sub-cellular localization of wild-type receptors. For example, splice variants of the pituitary adenylate cyclase activating polypeptide (PACAP) receptor alter ligand affinity and subsequent signaling of the receptor (Spengler et al. 1993; Pisegna and Wank 1996). The studies reported in Chapter 4 of this thesis describe an important role for alternative splicing in regulation of the function of the GHRH receptor and a mechanism by which the signaling effect is achieved.

Post-translational Modifications

Protein synthesis, or translation of mRNA, occurs on ribosomes, either in the cytoplasm or associated with the rough endoplasmic reticulum (ER). Membrane and soluble secretory proteins cross the ER membrane as they are being translated. In the ER and the Golgi, these proteins are modified and properly folded before being transported to their destinations. First,

addition and processing of carbohydrates, called glycosylation, occurs. Most proteins that are synthesized on the rough ER, including the GHRH receptor, undergo this chemical modification. The addition of these oligosaccharides likely promotes proper folding and stability of the protein. Also in the ER, disulfide bonds are formed between cysteine residues. These bonds occur only in secretory proteins and in the extracellular domains of membrane proteins, including the GHRH receptor, and act to stabilize tertiary and quaternary structure of these proteins. Proteins are folded into their appropriate conformation in the ER, and a quality control system within the ER targets misfolded proteins to the cytosol for degradation.

After the production of the final protein product, additional modifications affect the function of the protein. Some examples relevant to the GHRH receptor include phosphorylation, palmitoylation and glycosylation. Phosphorylation occurs on serine, threonine, tyrosine and histidine amino acid residues, and the activity of many proteins is regulated by the protein's phosphorylation state. The dynamic palmitoylation reaction involves the addition of fatty acids to cysteine residues on membrane proteins (Magee and Courtneidge 1985; Schmidt et al. 1988). This reversible modification influences signaling, protein internalization and recycling, and is common among G-protein coupled receptors. Glycosylation of asparagine residues in the extracellular domains of transmembrane proteins has been shown to be important for stability, proper protein folding and cell-cell interactions (Lanctot et al. 2005).

An important functional modification that many proteins undergo is oligomerization. Oligomerization is the formation of a complex of multiple subunits of a protein or proteins. These complexes can encompass subunits of the same protein (homo-oligomer) or of different proteins (hetero-oligomer). Data presented in Chapter 4 show that the GHRH receptor can form

a complex with splice variant receptors, and suggest that this receptor normally functions as a homodimer or higher order complex.

The Growth Hormone Axis

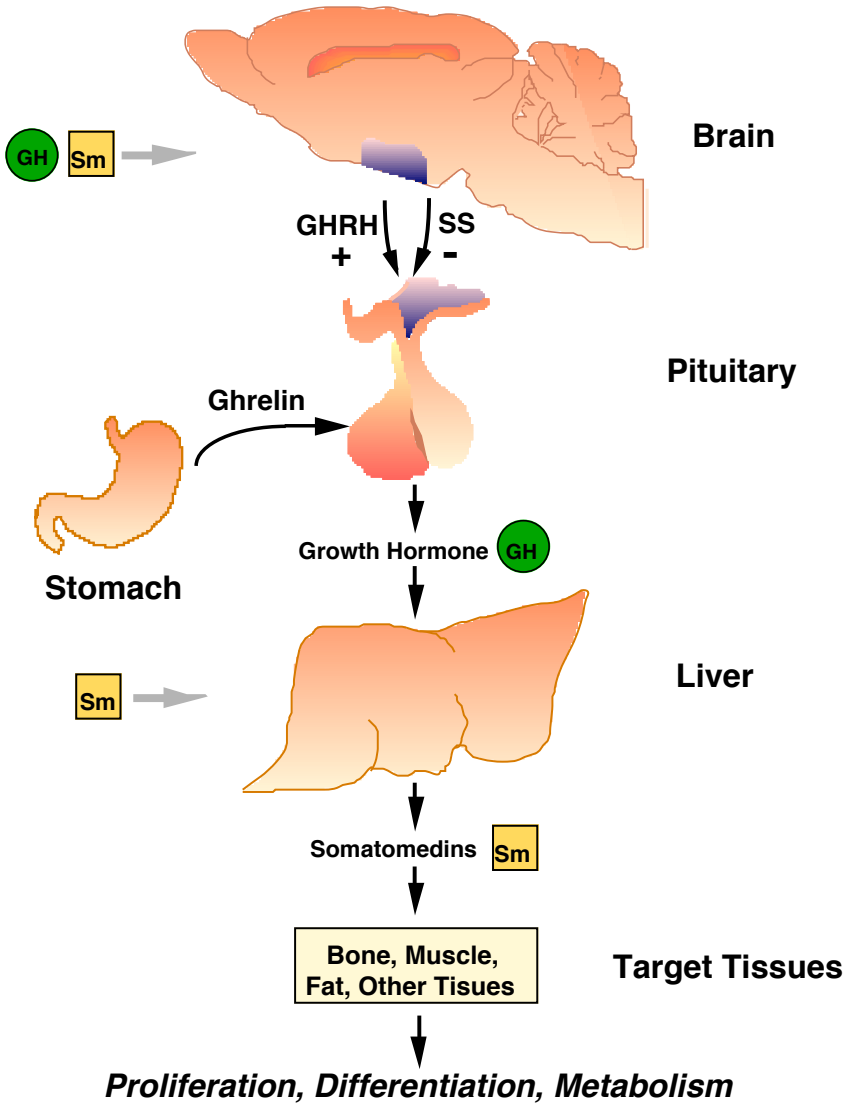
In vertebrates, GH stimulates proliferation (Smith 1930; Bullough 1954; Kember 1971; Thorngren and Hansson 1974; Isaksson et al. 1987), differentiation (Reddi and Sullivan 1980; Morikawa et al. 1982; Cobb et al. 1991; Campbell et al. 1992; Winston and Bertics 1992) and metabolism (Goodman 1965; Goodman 1968; Cheek and Graystone 1969; Lindahl et al. 1987) of target tissues, including bone, muscle, and fat. The production and secretion of GH is a tightly regulated process. The primary regulators of GH are the hypothalamic peptides GHRH, which stimulates the synthesis (Barinaga et al. 1983; Barinaga et al. 1985) and secretion (Guillemin et al. 1982; Rivier et al. 1982) of GH, and somatostatin, which inhibits its release (Brazeau et al. 1973). These peptide hormones act through G-protein-coupled receptors on the surface of the pituitary somatotroph cell. The receptor for GHRH interacts with G_s (Struthers et al. 1989), while the somatostatin receptor interacts with G_i (Lewis et al. 1986), altering production of the second messenger cAMP and subsequent signaling cascades. The GH axis is shown schematically in Fig. 1.1, and its components are further described in the following sections.

Growth Hormone and Growth Regulation

Following observations by Marie in 1886 that acromegaly was associated with an increased pituitary size (Welbourn 1986), the existence of a growth promoting factor in the pituitary gland was first demonstrated by Evans and Long in 1922, when they were able to

Figure 1.1: Schematic Representation of the Growth Hormone Axis

Growth hormone (GH) is made in and secreted from somatotroph cells in the anterior pituitary gland. Its secretion is regulated primarily by the hypothalamic peptide hormones, growth hormone-releasing hormone (GHRH), which stimulates (+) synthesis and secretion, and somatostatin (SS), which inhibits (-) its release. GH acts on target tissues throughout the body leading to proliferation, differentiation and metabolism. In the liver, GH induces the production of somatomedins (Sm), or insulin-like growth factors (IGFs), which mediate many of the effects of GH. Ghrelin, which is produced in the stomach, is an endogenous GH-secretagogue. Negative feedback occurs throughout the GH axis (grey arrows). (Adapted from Mayo et al. 1995).


GH
Sm
Brain
GHRH
+
SS
-
Pituitary
Ghrelin
Stomach
Growth Hormone
GH
Sm
Liver
Somatomedins
Sm
Bone, Muscle,
Fat, Other Tisues
Target Tissues
Proliferation, Differentiation, Metabolism

stimulate growth in rats with pituitary extracts (Evans and Long 1922). Subsequent work led to the purification of GH from the bovine pituitary (Li et al. 1945). Since the isolation of GH, the hormone has been extensively studied. The cDNA sequence of rat GH was determined in 1977 (Seeburg et al. 1977). Subsequently, sequencing of the GH gene in other species, including human, was performed. Cloning of human GH led to the production of recombinant GH for use in clinical cases of GH deficiency in humans, which has since become standard treatment for the disease.

Growth hormone is a polypeptide hormone of about 22 kilodaltons (Abdel-Meguid et al. 1987). Molecular cloning revealed that the GH gene has five exons (Seeburg et al. 1977), and that alternative splicing of exon three results in the production of a short variant form of the hormone that is about 20 kilodaltons (Abdel-Meguid et al. 1987). Growth hormone is made in somatotroph cells, which release the polypeptide from secretory vesicles via a pathway involving increases in intracellular calcium stores in response to GHRH binding its receptor (Holl et al. 1988). Somatostatin inhibits GH secretion (Brazeau et al. 1973; Holl et al. 1988). GH is also regulated by negative feedback pathways. Growth hormone, GHRH, somatostatin and insulin-like growth factor-I (IGF-1), which is stimulated in response to GH, act at the levels of the pituitary and the hypothalamus to inhibit GH and GHRH secretion and to stimulate somatostatin when GH levels are high (reviewed in Mayo 1999).

When GH levels are inappropriately regulated, which can result from misregulation or mutation of GH, GHRH, somatostatin or the receptors of these hormones, diseases of growth occur. Excessive levels of GH lead to gigantism when present in early post-natal development and acromegaly when present after the long bones have fused, usually resulting from pituitary adenomas that secrete GH. These diseases resulting from excess GH are rare. According to the

National Library of Medicine and the National Institutes of Health, only 100 cases of gigantism have been recorded in U.S. history. Acromegaly affects 6 in 100,000. At the opposite end of the spectrum, inadequate levels of GH result in dwarfism. GH deficiency is a much more common disease that is diagnosed in 4,000 new patients in the U.S. each year. Appropriate regulation of GH levels is necessary to achieve normal growth and functioning of target tissues.

Growth Hormone-Releasing Hormone

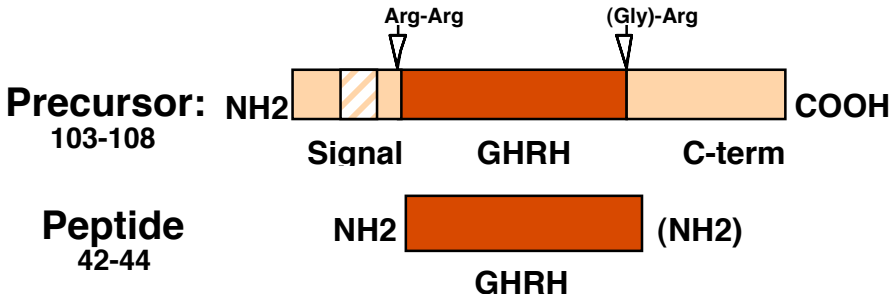
Growth Hormone-Releasing Hormone is the primary positive stimulus for GH synthesis and secretion. GHRH was first identified in pancreatic tumors in acromegalic patients (Guillemin et al. 1982; Rivier et al. 1982) and was later isolated from the hypothalamus (Spiess et al. 1983; Ling et al. 1984). GHRH expression has also been detected in the gonads, placenta, pancreas, gastrointestinal tract and lymphocytes (reviewed in Mayo et al. 1995). Though GHRH is known to stimulate GH synthesis and secretion, its broader expression pattern suggests other yet-unidentified roles for the peptide. Several cancers have been shown to express variant forms of the receptor for GHRH and exhibit mitogenic activity in response to GHRH (Halmos et al. 2000; Busto et al. 2002; Letsch et al. 2003; Chatzistamou et al. 2004; Engel et al. 2005). Recent clinical trials have involved using GHRH antagonists to curb proliferation of these cancers, with some success (Rekasi et al. 2000b; Letsch et al. 2003; Engel et al. 2005; Keller et al. 2005).

GHRH is a peptide hormone of 42-44 amino acids, depending on species, that is proteolytically processed from a larger precursor (Fig. 1.2; Mayo et al. 1983). The hormone belongs to a family of peptide hormones, including vasoactive intestinal protein (VIP), glucagon, PACAP, and secretin (Bell 1986; Campbell and Scanes 1992). These related peptides are

Figure 1.2: GHRH Biosynthesis and Expression

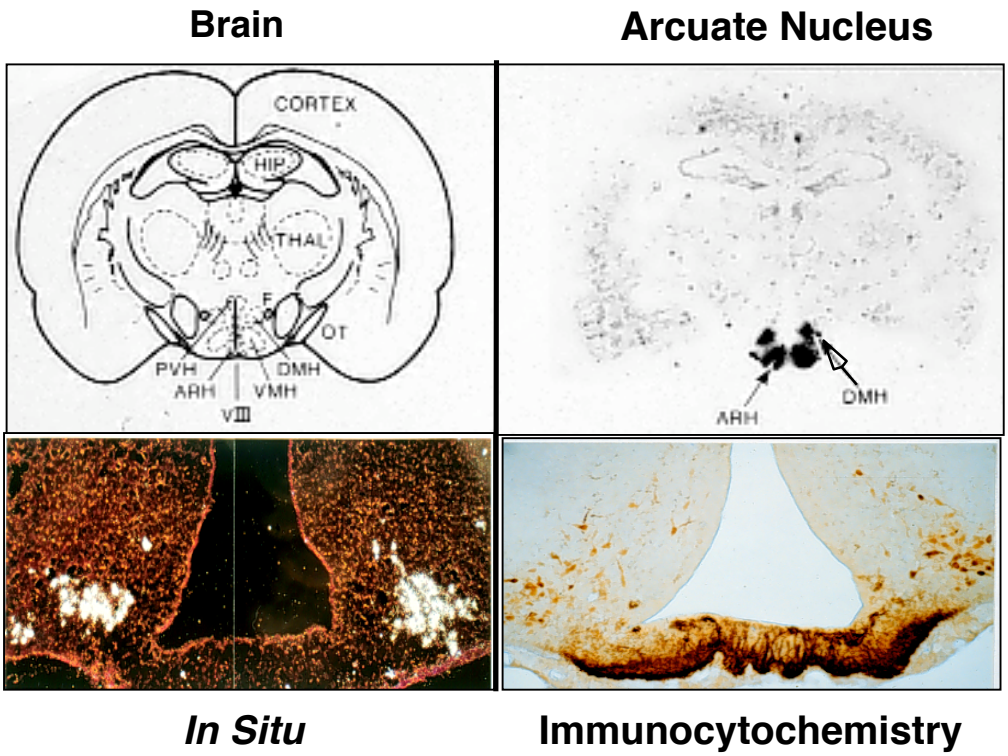
A: GHRH is proteolytically processed from a larger precursor protein. The mature form is 42-44 amino acids. **B:** Cell bodies of GHRH neurons are in the arcuate nucleus, shown by in situ hybridization and project to the median eminence, shown by immunohistochemistry. (From Mayo et al. 1995).

A.



rGHRH: HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQRSRFN

B.



thought to be derived from a common ancestor after gene duplication and divergence (Fig. 1.3). GHRH is released by neurosecretory cells of the arcuate nuclei of the hypothalamus (Fig. 1.2) in a pulsatile fashion (Merchenthaler et al. 1984; Sawchenko et al. 1985). GHRH travels through the pituitary portal system, where it acts by binding its receptor on the surface of pituitary somatotroph cells. In somatotroph cells, GHRH stimulates adenylate cyclase to produce intracellular cAMP (Bilezikjian and Vale 1983; Labrie et al. 1983; Horvath et al. 1995). The second messenger cAMP activates protein kinase A (PKA) (Kansaku et al. 1998), which leads to phosphorylation of CRE-binding protein (CREB) (Petersenn et al. 1995). GHRH also stimulates a cAMP-dependent increase in intracellular calcium (Schofl et al. 1987; Holl et al. 1988; Kato et al. 1992; Naumov et al. 1994). These signaling events lead to the synthesis and secretion of GH and to the transcriptional activation of the GHRH receptor. GHRH activates the mitogen-activated protein kinase (MAPK) pathway (Mayo et al. 2000; Zeitler and Siriwardana 2000), which leads to activation of genes involved in cell proliferation. Through the MAPK pathway, GHRH likely stimulates somatotroph cell proliferation. A schematic of GHRH signaling in the somatotroph cell is depicted in Fig. 1.4.

The Growth Hormone-Releasing Hormone Receptor

Just as GHRH is related to a family of peptide hormones, its receptor is related to the family of receptors for these hormones, the B-III subfamily of G-protein-coupled receptors (http://www.gpcr.org). The GHRH receptor is a seven-transmembrane GPCR expressed on the surface of pituitary somatotroph cells. Receptors for GHRH have been cloned in mouse, rat,

Figure 1.3: GHRH Homology to Related Peptides

A: The amino acid sequence for GHRH is aligned to those for the related peptide hormones VIP, secretin, glucagon, peptide histidine isoleucine (PHI), gastric inhibitory polypeptide (GIP) and PACAP. Residues boxed in red are conserved between GHRH and related peptides. **B:** GHRH and its related peptides are believed to be derived from a common ancestor exon through gene duplication and divergence (Campbell and Scanes 1992).

A.

```
rGHRH       HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQRSRFN
VIP         HSDAVFTDNYTRLRKQMAVKKYLNSILN
Secretin    HSDGTFTSELSRLRDSARLQRLLQGLV
Glucagon    HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
PHI         HADGVFTSDFSRLLGQLSAKKYLESLI
GIP         YAEGTFISDYSIAMDKIRQQDFVNWLLAQQKGKKSDWKHNITQ
PACAP       HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
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B.

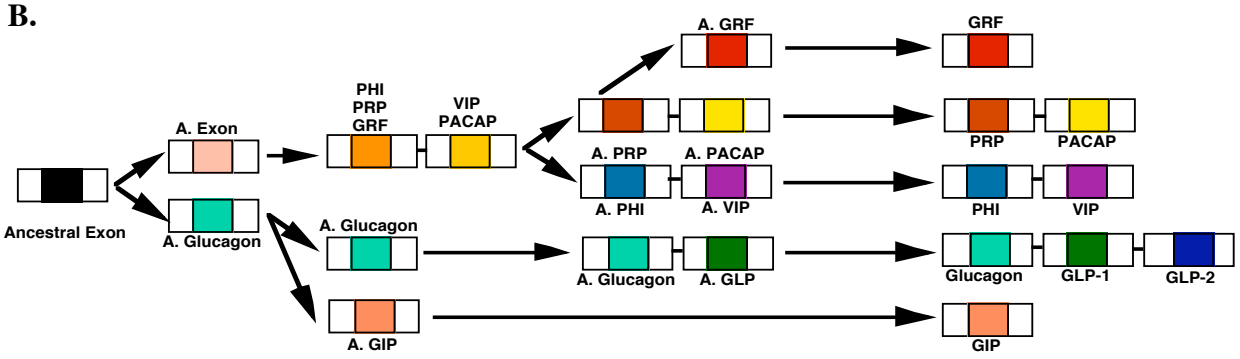
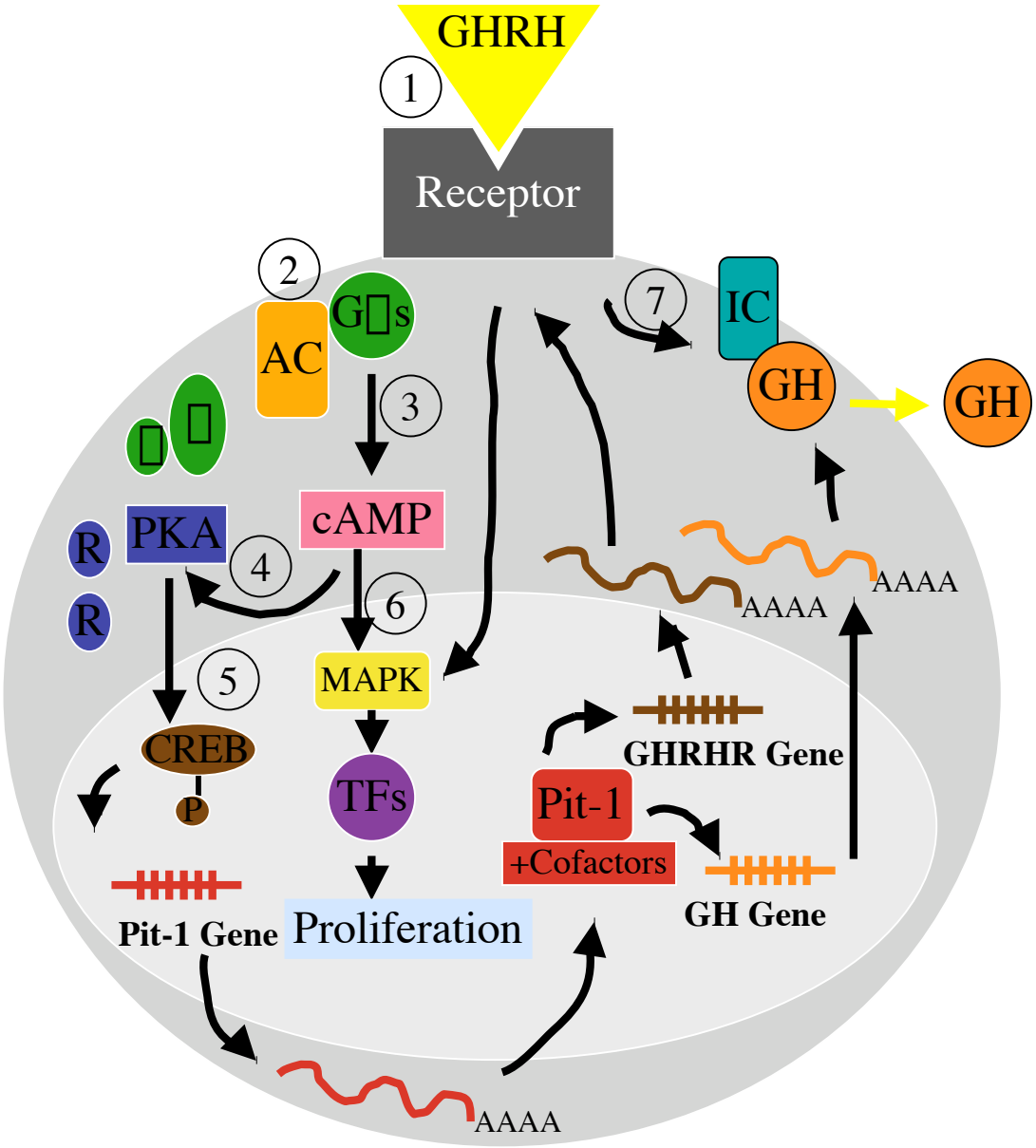


Figure 1.4: GHRH Signaling in the Somatotroph Cell

When GHRH binds its receptor (1), the receptor undergoes a conformational change that allows G-protein exchange on the alpha subunit (2). GTP loading of the alpha subunit leads to a dissociation of the heterotrimeric G-protein. The GTP-bound alpha subunit stimulates adenylyl cyclase (AC) to produce cAMP (3). cAMP activates the PKA pathway (4), leading to the phosphorylation of substrates that affect gene transcription (5). cAMP also activates the mitogen-activated protein kinase (MAPK) pathway (6). The GHRH-bound receptor stimulates intracellular calcium associated with release of GH from secretory vesicles through a pathway that is not well-understood (7) (Adapted from Mayo et al. 1995).


GHRH
1
Receptor
2
AC
Gs
3
cAMP
PKA
R
R
4
6
5
MAPK
CREB
P
TFs
Pit-1 Gene
Proliferation
7
IC
GH
GH
AAAA
AAAA
GHRHR Gene
Pit-1
+Cofactors
GH Gene
AAAA

human, pig, and most recently, chicken (Lin et al. 1992; Mayo 1992; Gaylinn et al. 1993; Hsiung et al. 1993; Toogood et al. 2006).

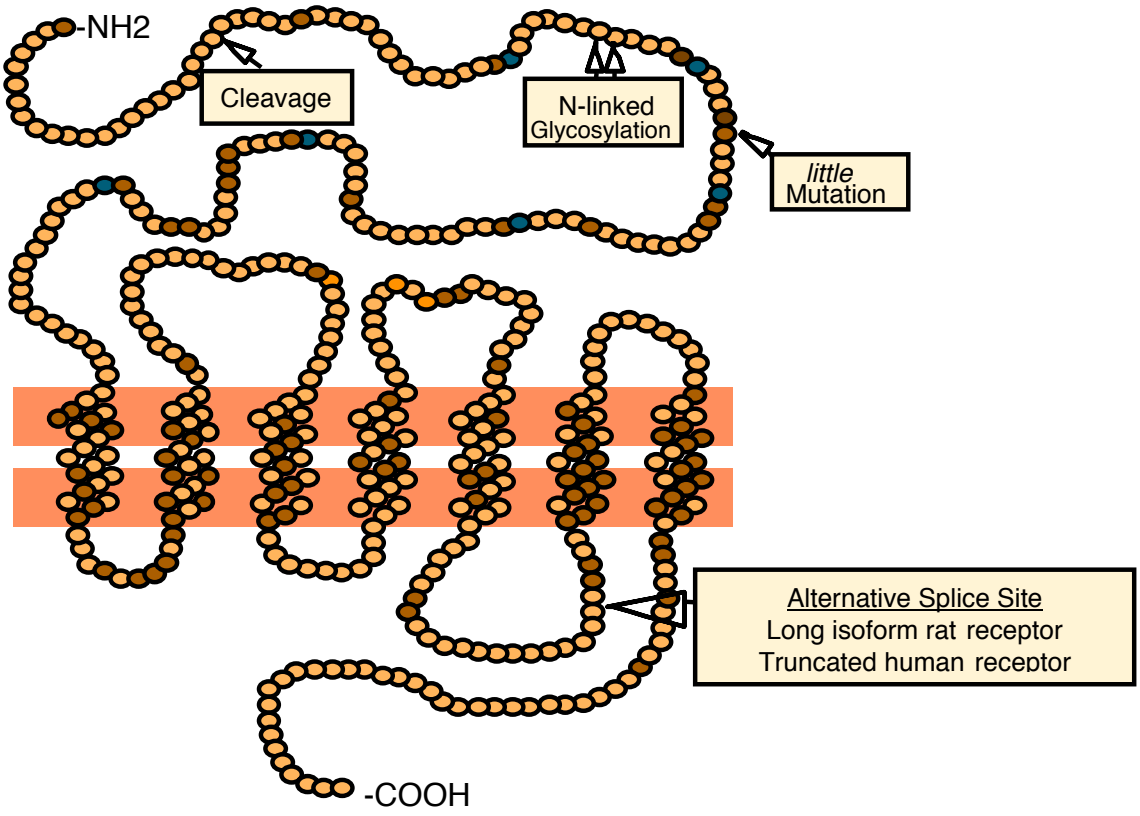
The GHRH receptor is 423 amino acids (Mayo 1992), coded by a gene containing 14 exons, spanning at least 15kb of genomic DNA (Miller et al. 1999). The exons are separated by introns ranging from 111 bp to more than 2 kb (Miller et al. 1999). Alternative splicing 3' of exon 11 leads to inclusion of intronic sequence that adds 41 amino acids in the third intracellular loop of the rat receptor, termed the long isoform (Miller et al. 1999). In the human, alternative splicing at the same intron/exon boundary leads to inclusion of an in-frame premature stop codon, encoding a protein truncated just before the sixth transmembrane domain (Hashimoto et al. 1995; Tang et al. 1995). Fig. 1.5 is a schematic of the GHRH receptor protein structure.

The rat GHRH receptor has four transcriptional start sites, identified by primer extension products from RNA hybridization experiments (Miller et al. 1999). The promoter does not contain a consensus "CCAT" or "TATA" sequence. In this thesis, the 5'-most transcriptional start site is referred to as "+1." In initial computational analysis of the rat GHRH receptor promoter, several putative binding sites for gene regulatory factors were identified (Miller et al. 1999). A review of the literature on gene regulation of the GHRH receptor will be presented in a later section of this introductory chapter.

The rat GHRH receptor mRNA is expressed at high levels in the pituitary and kidney (Lin et al. 1992; Mayo 1992; Gaylinn et al. 1993; Hsiung et al. 1993), and at lower levels in the placenta, gonads and spleen (DeAlmeida and Mayo 2001). Functional GHRH receptor protein has been detected only in the pituitary. The tissue specificity of expression is in part achieved by transcriptional activation of the gene by the pituitary-specific transcription factor, Pit-1. This

Figure 1.5: Key Features of the GHRH Receptor

The GHRH receptor is a seven-transmembrane G-protein-coupled receptor. It is glycosylated in the N-terminus. Alternative splicing in the region coding the third intracellular loop results in different protein products in rat and human, which are examined further in Chapter 4 and the appendix. The mutation leading to dwarfism of the *little* mouse has been mapped to amino acid 60 of the GHRH receptor. Dark brown circles represent residues conserved in the related B-III GPCR family members, VIP, PACAP and secretin receptors. Blue circles represent cysteines conserved in the B-III GPCR family (DeAlmeida and Mayo 2001).


-NH2
Cleavage
N-linked
Glycosylation
little
Mutation
Alternative Splice Site
Long isoform rat receptor
Truncated human receptor
-COOH

factor and other regulatory elements controlling GHRH receptor expression will be addressed in greater detail in the following sections.

Pituitary Development

The pituitary gland is an excellent model for understanding cell fate decisions during organogenesis in mammals. The anterior pituitary gland is comprised of five cell types, defined by the hormones they produce. These cells arise from a common pituitary precursor cell type and regulate many essential processes, including metabolism, growth, reproduction and behavior. The anterior pituitary primordium, Rathke's pouch, arises from oral ectoderm at embryonic day 8 (e8) in the mouse (Schwind 1928). The presumptive Rathke's pouch lies in direct contact with the floor of the diencephalon. Signals from the diencephalon are required for differentiation and proliferation of the anterior pituitary cell types (Etkin 1967; Ferrand 1972; Daikoku et al. 1982; Watanabe 1982b; Watanabe 1982a; Kawamura and Kikuyama 1995). These extrinsic factors are involved in regulating expression of intrinsic factors that are required for further steps of cell type differentiation. Rathke's pouch separates from the oral ectoderm by e12.5, and the five anterior pituitary cell types are defined by e16.5 (Japon et al. 1994). This section examines what is known about the factors involved in pituitary cell fate decisions, with an emphasis on the transcription factor (Pit-1) and cell type (somatotroph) that are major foci of this dissertation work. Unless otherwise noted, all developmental timepoints refer to the mouse.

Signaling Molecules and Transcription Factor Gradients in Anterior Pituitary Cell Specification

From very early in embryonic development, signaling events and expression of transcription factors are involved in the commitment and development of the pituitary gland.

These events leading to the differentiation of the anterior pituitary and its five cell types can be divided into four stages (Kioussi et al. 1999). The first stage takes place from e6-e8.5 and involves extrinsic signaling from the developing diencephalon. Stages 2 (e8.5-e10.5) and 3 (e10.5-e12.5) occur as Rathke's pouch is formed after invagination of the oral ectoderm. Stage 2 is characterized by the production of extrinsic signals that stimulate the intrinsic signals that characterize stage 3 development. The last stage takes place from e12.5 through birth, and is characterized by the sequential appearance of the five cell phenotypes of the anterior pituitary gland.

Bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 8 are expressed in the ventral diencephalon during initiation of Rathke's pouch development (Treier et al. 1998). BMP4 is required for organ commitment of the pituitary gland (Treier et al. 1998). Sonic Hedgehog (Shh) is expressed throughout the oral ectoderm, until e10.5, when it becomes restricted from the invagination of Rathke's pouch, thus establishing a molecular boundary at the transition between the oral ectoderm and the developing Rathke's pouch (Treier et al. 1998). This boundary establishes an opposing ventrodorsal gradient of BMP2/FGF8, which is involved in determination of ventral and dorsal cell phenotypes (Treier et al. 1998). These signaling molecules activate transcription factors in a ventral-dorsal and cell specific fashion that lead to cell type determination events. Recent findings have shown that Notch signaling is also required for proper cell commitment in pituitary development. The components of Notch signaling are expressed early in Rathke's pouch, and are down-regulated by e13.5, about the time Pit-1 expression is initiated (Zhu et al. 2006). Notch signaling is required to prevent premature differentiation to the corticotroph lineage, and to up-regulate expression of the transcription

factor Prop-1 (Zhu et al. 2006), which is required to establish Pit-1 expression and later differentiation of the Pit-1 lineages.

A large number of transcription factors is known to be expressed in temporal and spatial patterns in the developing hypothalamic-pituitary axis (Treier and Rosenfeld 1996; Watkins-Chow and Camper 1998; Burrows et al. 1999; Kioussi et al. 1999; Sheng and Westphal 1999; Dasen and Rosenfeld 2001). The homeobox gene Six3 is expressed at e6.5 at the anterior border of the neural plate, and is retained in Rathke's pouch and the hypothalamus, in addition to other structures (Oliver et al. 1995). Rpx (Rathke's pouch homeobox; also called Hesx1) is also expressed in the early anterior neural plate, but its expression is later restricted to Rathke's pouch (Hermesz et al. 1996). Expression of Rpx appears to be involved in the subdivision of Six3 positive structures, namely, determining anterior pituitary fate. Another homeobox transcription factor, Pax6, is expressed early in Rathke's pouch and in the developing anterior pituitary (Walther and Gruss 1991), with peak expression at e10 (Sornson et al. 1996). Expression of both Pax6 and Rpx is repressed after e12.5-13.5 as cell types are beginning to differentiate (Sornson et al. 1996). Pitx-1 (Lamonerie et al. 1996; Szeto et al. 1996), Pitx-2 (Gage and Camper 1997) and Otx-1 (Acampora et al. 1998) are additional homeobox containing genes that are expressed in the early anterior pituitary and maintain expression in some or all of the differentiated pituitary cells. These factors are presumed to have roles in early development and in later functioning of the specific cell types.

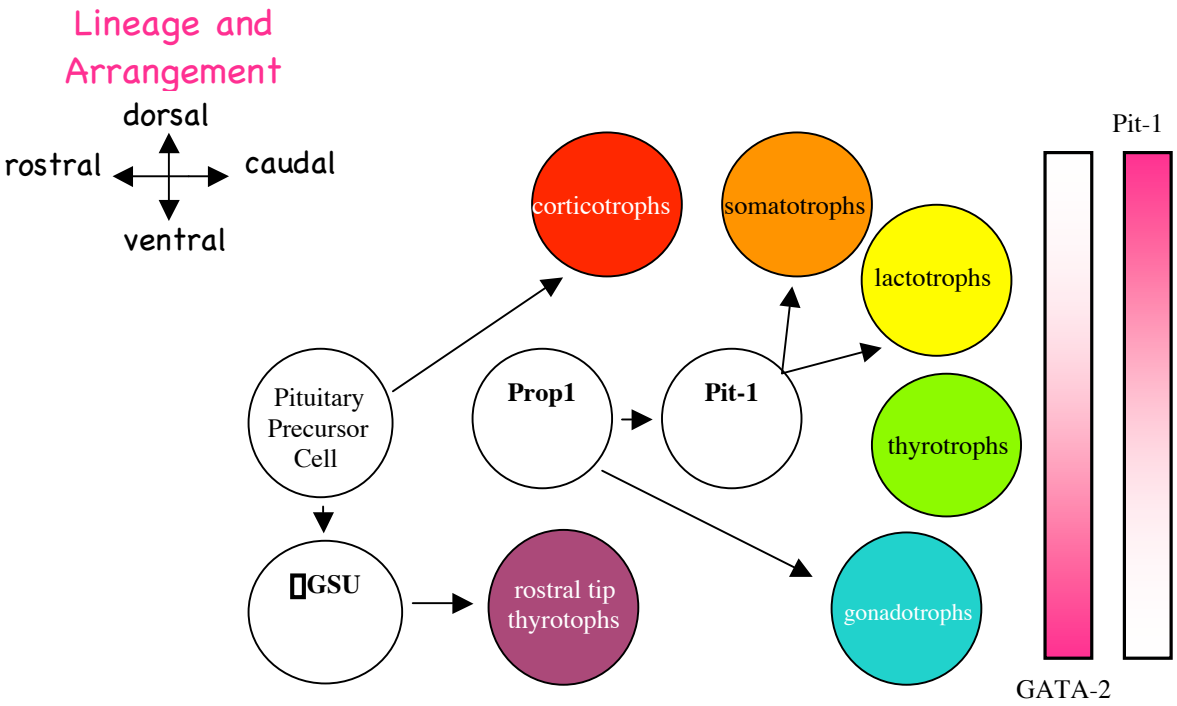
In addition to these early stage factors that are involved in the commitment of the anterior pituitary primordium, stage 3 factors are known to be present in the definitive Rathke's pouch and are involved in further differentiation of the pituitary. These include the LIM homeodomain factors Plim (also called Lhx3 and mLim3) (Seidah et al. 1994; Bach et al. 1995; Zhadanov et al.

1995) and Lhx4 (also called Gsh-4) (Li et al. 1994; Sheng et al. 1997). In addition, the paired like homeodomain factor Prophet-of-Pit-1 (Prop-1) is expressed in the cells that will later express Pit-1 (Gage et al. 1996; Sornson et al. 1996). Prop-1 is required for asymmetrical cell division, which is involved in generation of the Pit-1-positive lineage from a shared precursor. These factors are important to pituitary commitment and proliferation of the precursor cells.

Additional factors are required for cell differentiation. In vertebrate development, the pituitary cell types arise within different regions of the pituitary gland. The corticotrophs, which appear at e11 (Begeot et al. 1982) and a population of thyrotrophs in the rostral tip, which appear at e12 (Lin et al. 1994) are the first cell types to appear, This initial pool of thyrotrophs disappears by the time of birth (Lin et al. 1994). The four most caudal cell types, somatotrophs, lactotrophs, gonadotrophs, and the second population of thyrotrophs differentiate at later stages, based on transcription factor gradients. Reciprocal gradients of the transcription factors Pit-1 and GATA-2 are involved in the differentiation of these four cell types (Dasen et al. 1999). The zinc finger immediate-early response gene Droz-24 (also called NGFI-A, Egr-1 and zif268) is involved in somatotroph proliferation (Lee et al. 1996; Topilko et al. 1997). Examination of transcriptional regulation of cell-specific genes is revealing further information on what factors are required for differentiation of the pituitary cell types from their common precursor. A schematic of pituitary cell differentiation is shown in Fig. 1.6.

Figure 1.6: Anterior Pituitary Cell Differentiation

The five cell types of the anterior pituitary differentiate from a common precursor cell. Signaling molecules and reciprocal gradients of transcription factors are involved in establishing expression of the genes involved in cell differentiation. Corticotrophs and a transient population of thyrotrophs in the rostral tip develop first. The transcription factors Prop-1 and Pit-1 are required for differentiation of the somatotroph, lactotroph and thyrotroph lineages. The four most-caudal cell types differentiate based on a reciprocal gradient of Pit-1 and GATA-2 (Adapted from Dasen et al. 1999).


Lineage and Arrangement
dorsal
rostral
caudal
ventral
Pituitary Precursor Cell
corticotrophs
Prop1
Pit-1
somatotrophs
lactotrophs
thyrotrophs
GSU
rostral tip thyrotophs
gonadotrophs
Pit-1
GATA-2

The Pituitary-Specific Transcription Factor Pit-1

Pit-1, also known as GHF-1, is a pituitary-specific transcription factor and a founding member of the POU (Pit-1, Oct-1, Unc-86) homeodomain transcription factor family (Bodner et al. 1988; Ingraham et al. 1988). Although Pit-1 mRNA is expressed in all cell types of the anterior pituitary (Ingraham et al. 1988; Simmons et al. 1990) Pit-1 protein expression is restricted to three cell types, thyrotrophs, lactotrophs and somatotrophs (Simmons et al. 1990). Pit-1 has been shown to activate transcription of cell-specific hormones produced by each of these cell types (Bodner et al. 1988; Mangalam et al. 1989; Dolle et al. 1990; Fox et al. 1990; Steinfelder et al. 1992), in addition to other cell-specific genes.

This class of transcription factors is characterized by two highly conserved segments, a POU-Specific (POU_S) domain of approximately 75 amino acids and a POU-Homeodomain (POU_H) of 60 amino acids, separated by a variable linker region that can be 15 to 56 amino acids in length (Herr et al. 1988). Together, these segments are referred to as the POU domain, and the two structurally independent segments cooperate as a DNA-binding unit. For POU homeodomain transcription factors to be active, both of these domains are required make contact with the DNA. Because the bipartite POU domain is structurally flexible, POU domain factors can interact with DNA in various orientations, allowing versatility in transcriptional regulation.

Although Pit-1 exists as a monomer in solution, it usually binds DNA as a dimer (Jacobson et al. 1997). Pit-1 binding sites are not well conserved. A weak consensus binding sequence is indicative that Pit-1 is configured differently on the different sites it binds (Jacobson et al. 1997). The differences in spacing of nucleotides between the binding sites for the POU_H and POU_S domains in the promoters of different genes impart some degree of cell specificity. Structural analysis of Pit-1 binding both the prolactin and GH promoters revealed that a 2-base

pair difference in spacing of the Pit-1 binding sites in these promoters led to different orientations of Pit-1 on the promoters (Scully et al. 2000). Interestingly, these different orientations are associated with the ability of Pit-1 to recruit co-activators to the prolactin promoter and co-repressors to the GH promoter in lactotroph cells (Scully et al. 2000). Multiple Pit-1 binding sites have been identified within the promoters of many of the genes Pit-1 regulates (Mangalam et al. 1989; Nowakowski and Maurer 1994; Wood et al. 1996; Shewchuk et al. 1999), suggestive of the importance of this factor in appropriately regulating expression of many pituitary genes.

The Pituitary Somatotroph Cell

The pituitary somatotroph cell integrates signals from the brain to produce and secrete GH. Somatotroph cells are a late arising population of pituitary cells that are terminally differentiated at e17.5. Like thyrotrophs and lactotrophs, somatotrophs are a Pit-1-dependent linage, which arise from a precursor common to lactotroph cells. The bipotential mammosomatotroph cell expresses both prolactin and GH, and has been shown in the rat to be able to interconvert to the lactotroph or somatotroph fate (Porter et al. 1990; Porter et al. 1991), depending on changes in physiological requirements for the hormones these cells produce, for example, during gestation and lactation. Because three pituitary cell types require Pit-1 for differentiation, additional factors must be involved in somatotroph differentiation. Some candidates have been identified, including the steroid hormones glucocorticoids (Hemming et al. 1984; Kineman et al. 1992; Nogami and Tachibana 1993; Nogami et al. 1995), estrogen (Petersenn et al. 1998) and progesterone (Kineman et al. 1992), and the bHLH transcription factors Mash1 (unpublished data referenced in Zhu et al. 2006) and Math3 (Zhu et al. 2006).

The somatotroph cell population is initially small, and undergoes dramatic GHRH receptor-dependent expansion around the time of birth (Lin et al. 1993). Because GHRH receptor expression occurs early in terminally differentiated somatotroph cells and these cells respond to GHRH stimulation, understanding the transcriptional regulation of the GHRH receptor gene will help elucidate factors involved in differentiation of this cell type from the common pituitary precursor cells. Chapters 2 and 3 focus on examination of the transcriptional regulation of this gene, both *in vivo* and in cell culture models. The goal of these projects was to understand how expression of this integral component of the growth hormone axis is regulated.

GHRH Receptor Transcriptional Regulation

Pit-1 Regulation of GHRH Receptor Expression

GHRH receptor expression requires Pit-1. In Snell dwarf mice, which have a mutation in Pit-1, the GHRH receptor is not expressed, and the pituitary lacks somatotrophs, lactotrophs and thyrotrophs (Lin et al. 1992). Similarly, in Ames dwarf mice lacking Pit-1 expression due to a loss of the transcription factor Prop-1 which is required for Pit-1 expression, the somatotroph, lactotroph and thyrotroph lineages are absent (Gage et al. 1996; Sornson et al. 1996). In addition to animal models of decreased Pit-1 expression, a Pit-1 binding site mutation in the human GHRH receptor promoter has been identified in a patient with isolated GH deficiency type IB (Salvatori et al. 2002). The mutation prevents Pit-1 binding to one of the two identified Pit-1 binding sites in the human GHRH receptor promoter. When Pit-1 cannot bind this site, there is a decrease in GHRH receptor expression, further indicating the requirement of Pit-1 in activating transcription of the receptor gene.

Previous reports published by our laboratory and others have shown that Pit-1 can activate GHRH receptor expression in non-pituitary cells (Petersenn et al. 1998; Iguchi et al. 1999; Miller et al. 1999). Initial experiments examining this activation suggested a broad target for Pit-1 and several putative Pit-1 binding sites within the rat GHRH receptor promoter were identified by computational analysis (Miller et al. 1999). The mechanism for Pit-1 regulation of this gene has not been determined, and prior to the experiments described in Chapter 3 of this thesis, a comprehensive analysis of the rat GHRH receptor promoter had not been performed to examine Pit-1 regulation. Because Pit-1 is expressed in other pituitary cell types that do not express the GHRH receptor, cell-specific combinations of factors are likely to functionally interact with Pit-1 on the GHRH receptor promoter to achieve somatotroph-specific expression of the gene.

Other Regulatory Factors That Control GHRH Receptor Expression

In addition to Pit-1, several factors, including glucocorticoids (Lam et al. 1996; Korytko and Cuttler 1997; Miller and Mayo 1997; Petersenn et al. 1998; Gaylinn 1999; Nogami et al. 1999; Nogami et al. 2002), thyroid hormone (Miki et al. 1995; Korytko and Cuttler 1997; Nogami et al. 2002), sex steroids (Lam et al. 1996; Mayo et al. 2000), and GHRH (Girard et al. 1999; Mayo et al. 2000), are known to regulate GHRH receptor expression, though the mechanism of regulation has not been elucidated for most of these factors.

Glucocorticoids activate GHRH receptor gene transcription (Miller and Mayo 1997). In adrenalectomized rats, GHRH receptor expression is decreased, and can be restored to normal with corticosterone replacement (Miller and Mayo 1997). In addition, treatment of primary pituitary cells with glucocorticoids leads to a time- and dose-dependent increase in GHRH

receptor mRNA expression (Seifert et al. 1985; Korytko and Cuttler 1997; Miller and Mayo 1997). A recent report has shown synergism between Pit-1 and glucocorticoids on the rat GHRH receptor promoter (Nogami et al. 2005).

Thyroid hormone activates GHRH receptor expression in hypothyroid rats and in anterior pituitary cell cultures (Miki et al. 1995; Korytko and Cuttler 1997) and gonadal steroids have different effects on GHRH receptor expression. Oophorectomy leads to an increase in GHRH receptor mRNA levels, and estrogen treatment decreases GHRH receptor expression (Lam et al. 1996; Petersenn et al. 1998), while testosterone treatment of pituitary cells stimulates GHRH receptor expression (Ono et al. 1995).

GHRH itself has been reported to regulate receptor expression, though some studies suggest that it downregulates expression, while others suggest that GHRH is necessary for the activation of GHRH receptor expression. It is likely that other factors act in concert with GHRH to regulate expression of the GHRH receptor depending on physiological context, in some cases downregulating receptor expression, and in others activating its expression. This hypothesis is supported by a report that shows that GHRH-mediated regulation of the receptor varies during aging. In aged rats, GHRH stimulation can increase GHRH receptor mRNA expression, at a time when receptor levels are normally low (Girard et al. 1999). As previously mentioned, it is likely that a combination of these or yet-unidentified factors act in concert with Pit-1 to direct somatotroph-specific expression of the GHRH receptor gene.

Post-Transcriptional Processing & Post-Translational Modification of G-Protein-Coupled Receptors

Alternative Splicing in G-Protein-Coupled Receptors

While the human genome contains about 30,000 genes, over 100,000 proteins are synthesized in a cell at any given time (Liang et al. 2000; Wheeler et al. 2001). Alternative mRNA splicing accounts for this discrepancy by allowing the production of multiple transcripts from a single gene. In fact, genome-wide studies have revealed that at least 40-60 percent of human genes are alternatively spliced (Kan et al. 2001; Lander et al. 2001; Modrek et al. 2001). Alternative splicing can result in changes in mRNA stability and translatability and in protein structure and composition, thereby acting as an important mechanism for regulating protein function.

Alternative splicing of G-protein-coupled receptors (GPCRs) is an important mechanism by which many receptor subtypes modify their expression and function. Splice variant GPCRs affect many properties of these receptors. Variant GPCRs have been shown to affect ligand binding (Couvineau et al. 1994; Wellendorph et al. 2002; Elton and Martin 2003), signal transduction (Spengler et al. 1993; Moore et al. 1995; Grosse et al. 1997; Pierce and Regan 1998; Pindon et al. 2002), G-protein coupling (Pindon et al. 2002), internalization (Parent et al. 2001), desensitization (Pierce and Regan 1998), and localization of expression (Seck et al. 2003; Nakamura et al. 2004) of this class of receptors.

Several important GPCRs in the GH axis are alternatively spliced, and the variant receptors modulate signaling activity, suggesting that alternative splicing is an important mechanism by which receptors in this endocrine axis fine-tune their actions. For example, alternative splicing of the somatostatin receptor, which acts to inhibit GH release from the

pituitary, gives rise to isoforms that differ in expression pattern and response to cAMP (Patel et al. 1993). Alternative splicing of the growth hormone secretagogue (GHS) receptor results in a truncated isoform that affects signaling (Howard et al. 1996; McKee et al. 1997). The wild-type GHS receptor synergizes with the GHRH receptor, but the truncated GHS receptor has no effect on GHRH receptor signaling (Cunha 2002). The intersection of these pathways is examined further in experiments presented in Chapter 4. Splice variant GHRH receptors have been identified in pig (Hsiung et al. 1993; Hassan 2001), rat (Zeitler et al. 1998; Miller et al. 1999) and human (Hashimoto et al. 1995; Tang et al. 1995; Rekasi et al. 2000a). Conflicting data have been reported on the signaling effects of the truncated human GHRH receptor splice variant (Hashimoto et al. 1995; Motomura et al. 1998). Further examination of this receptor isoform led to the characterization of its dominant negative activity and a mechanism for the signaling effects, which are presented in Chapter 4.

Phosphorylation of G-Protein-Coupled Receptors

Several post-translational modifications are known to affect protein structure and function. Phosphorylation of GPCRs is an important mechanism for regulating receptor activity (Krupnick and Benovic 1998; Ferguson 2001). For many GPCRs, desensitization has been shown to be dependent on receptor phosphorylation. Conformational changes in the receptor upon agonist binding can induce phosphorylation by GPCR kinases (GRKs) (Krupnick and Benovic 1998; Ferguson 2001). For other GPCRs, second messenger-dependent kinases are involved in phosphorylation of the receptor (Krupnick and Benovic 1998; Ferguson 2001). Phosphorylation through both of these pathways results in reduced receptor-G protein coupling,

while GRK phosphorylation also enhances binding of arrestins, proteins that are involved in receptor desensitization and trafficking (Krupnick and Benovic 1998; Ferguson 2001).

Several members of the B family of GPCRs have been shown to exhibit agonist-induced C-terminal phosphorylation and down-regulation (Sibley et al. 1987; Holtmann et al. 1996; Nygaard et al. 1997; McDonald et al. 1998; Shetzline et al. 1998; Walker et al. 1999). The human GHRH receptor is phosphorylated in the C-terminus (Gaylinn et al. 1998), and putative phosphorylation sites for the GHRH receptor are conserved in multiple species. When the C-terminus of the ovine GHRH receptor is truncated, an enhanced cAMP activity at a lower dosage of GHRH is observed, indicating an inhibitory role of the C-terminus (Horikawa et al. 2001) . Six potential phosphorylation sites are removed in this hyperactive truncation (Horikawa et al. 2001), further supporting a role for phosphorylation in mediating GHRH receptor desensitization and internalization.

Palmitoylation of G-Protein-Coupled Receptors

Many GPCRs are post-translationally palmitoylated on cysteine residues in the carboxy tail (Loisel et al. 1999). Palmitate, a 16 carbon fatty acid, binds cysteine by a thioester link (Lodish et al. 2000). In GPCRs, this modification creates a fourth intracellular loop because the palmitate is inserted in the plasma membrane. This conformational change affects the accessibility of phosphorylation sites down-stream of the palmitoylated residue (Moffett et al. 1993), thereby modulating receptor down-regulation. The palmitoylation state of a GPCR has been shown to regulate recruitment of arrestin proteins to the receptor (Charest and Bouvier 2003), affecting receptor endocytosis.

The GHRH receptor contains C-terminal cysteine residues that could be targets for palmitoylation. Cys^{405} in the rat is replaced by an Arg residue in the human GHRH receptor (Mayo 1992). This substitution is suggested to play a role in directing the receptor to the endocytic pathway. The rat GHRH receptor is internalized in the caveolae pathway, while the human GHRH receptor is internalized via the clathrin pathway (Veyrat-Durebex et al. 2005). The difference in endocytic pathway appears to be driven by characteristics of the receptor. Because Cys^{405} in the rat is a potential palmitoylation site, it is proposed to be involved in the determination of different internalization pathways for the GHRH receptor of the two species (Veyrat-Durebex et al. 2005). The C-terminal truncation of the ovine GHRH receptor removes a cysteine that could be palmitoylated (Horikawa et al. 2001). The lack of this cysteine could be involved in the increased activity of the ovine receptor, by affecting structure and/or the phosphorylation state.

Glycosylation of G-Protein-Coupled Receptors

One of the most common forms of post-translational modification of proteins is glycosylation. About half of all proteins and 95 percent of all GPCRs are known to be glycosylated (Lanctot et al. 2005). Two types of glycosylation occur in eukaryotes, N-linked glycosylation and O-linked glycosylation (Lodish et al. 2000). N-glycosylation of asparagine side chains occurs on extracellular proteins or in the extracellular domain of transmembrane proteins (Lodish et al. 2000). O-glycosylation involves the addition of N-acetylglucosamine to the beta-hydroxyl of serine or threonine residues on intracellular proteins (Lodish et al. 2000).

N-glycosylation is considered a co-translational modification, as it occurs on nascent peptides as they emerge from the ribosome (Lodish et al. 2000). This modification appears to

be important for regulation of several functions of proteins, including proper protein folding, targeting to the lyzosome, ER-associated degradation, protection from protease-mediated degradation and cell-cell interactions (Lanctot et al. 2005). The N-terminus of the rat GHRH receptor contains two consensus sites for N-linked glycosylation, and both the long and short isoforms of the rat receptor are glycosylated (Miller et al. 1999). The importance of N-glycosylation to proper activity of the GHRH receptor is indicated by its conservation in species as distant from mammals as the teleost fish *Fugu rubripes* (Cardoso et al. 2003). Mutational analyses of the secretin (Pang et al. 1999) and VIP receptors (Couvineau et al. 1996) have shown different roles for glycosylation in ligand binding and cell surface trafficking, respectively. Given the close relationship of the B-III family of receptors, it is likely that glycosylation of the GHRH receptor plays a role in one or both of these functions.

Dimerization of G-Protein-Coupled Receptors

Though GPCRs were classically thought to act as monomers, studies in recent years have shown that many GPCRs form oligomers, and that the state of oligomerization of GPCRs dramatically affects their function. The number and diversity of GPCRs that have been shown to homo-oligomerize is so vast, that dimerization is now considered a general phenomenon among this class of receptors (Filizola and Weinstein 2002; Kroeger et al. 2003; Fotiadis et al. 2006; Herrick-Davis et al. 2006). For example, the serotonin receptor has recently been shown to form homodimers within the endoplasmic reticulum that translocate to the cell surface (Fotiadis et al. 2006; Herrick-Davis et al. 2006). Dimer formation is necessary for function of this and other GPCRs. Heterodimerization between members of the GPCR superfamily, including between different classes of GPCRs, has also been reported for many receptors (Devi 2001; Kroeger et al.

2003; Ciruela et al. 2006; Levoye et al. 2006; Modzelewska et al. 2006). Recently discovered examples of heterodimerization in GPCRs include rhodopsin and arrestin receptors (Modzelewska et al. 2006) and the MT1 melatonin receptor in complex with the orphan GPR50 (Levoye et al. 2006). The interaction between GPCRs greatly expands the capability of these receptors to respond to different physiological and pathophysiological conditions. Hetero-oligomerization of wild-type receptors with splice variant or mutant receptors is a key way by which signaling of many GPCRs is modulated.

Though the GHRH receptor has not definitively been shown to homodimerize, studies from our laboratory have revealed that it can interact with other GPCRs in the GH axis (Cunha 2002) and with splice variant GHRH receptors (described in Chapter 4). These interactions affect the signaling properties of the wild-type receptors in the complex. The ability of the GHRH receptor to hetero-oligomerize suggests that it normally functions by the formation of homodimers or higher order complexes.

Specific Aims of Thesis Project

Appropriate regulation of gene expression and modification of gene products are essential to determining cell fate decisions in development and responding to changing physiological conditions in multicellular organisms. In order to achieve the proper expression of gene products, tightly regulated and highly complex processes are employed by the cell. Beginning with regulation of when a gene is transcribed into mRNA, and ending with quality control checks on protein structure and folding and chemical modifications of mature protein products, these processes ensure that the necessary gene products are present at the appropriate

times in each cell. The goal of this thesis is to contribute to our understanding of these regulatory processes through the examination of a particular gene, the GHRH receptor gene.

The GHRH receptor is expressed in somatotroph cells of the anterior pituitary gland. Its cell-type specific expression makes it an ideal model for understanding factors involved in differentiation of this cell type. In order to better understand the regulatory factors required to activate transcription of this gene, two projects were pursued. First, in data reported in Chapter 2, the proximal promoter of the GHRH receptor was examined *in vivo* in transgenic mice. Using the mouse model, the studies were able to examine expression of the GHRH receptor under the control of the promoter. The expression profile of the transgene closely matches what is known for the endogenous receptor, and confirms that the 1.6kb promoter is sufficient for directing appropriately regulated expression of the GHRH receptor gene.

In experiments presented in Chapter 3, the promoter was further analyzed in cell culture. The pituitary-specific transcription factor Pit-1 is known to be required for activation of GHRH receptor expression *in vivo*. Because initial experiments comparing binding of nuclear proteins from pituitary and non-pituitary cells showed that Pit-1 binds multiple sites in the GHRH receptor promoter, subsequent studies focused on identifying functional binding sites for Pit-1 in the GHRH receptor proximal promoter. Though Pit-1 can specifically bind 10 sites within this promoter, mutational analyses of the binding sites show that one site in the proximal promoter is necessary and sufficient for Pit-1 activation of this gene.

Chapter 4 focuses on a post-transcriptional regulation of the GHRH receptor gene, specifically alternative splicing. A splice variant of the GHRH receptor occurs at the intron/exon boundary 3' of exon 11. The human GHRH receptor protein is truncated as a result of an in-frame premature stop codon in the alternatively spliced mRNA, and its signaling properties are

affected by this alteration. The truncated GHRH receptor is characterized in Chapter 4 with respect to its own signaling and its effects on signaling of the wild-type receptor. These studies were carried further by examining the role of the truncated receptor on signaling of the growth hormone secretagogue (GHS) receptor and of the splice variant rat GHRH receptor on wild-type receptor signaling. These studies suggest that oligomerization of the GHRH receptor is involved in signaling and that alternative splicing is an important regulator of GHRH receptor signaling.

Taken together, the studies presented in this thesis describe how the gene product of the GHRH receptor is generated and how signaling of the receptor is regulated by post-transcriptional alternative splicing. The data expand on the knowledge of how cells differentiate in mammalian organogenesis and how activity of this molecule that integrates signaling between the brain and the endocrine system is modulated. Chapter 5 describes the implications of the results and the future directions to be pursued in light of the results.

CHAPTER TWO

IN VIVO CHARACTERIZATION OF THE 1.6KB RAT GHRH RECEPTOR PROXIMAL PROMOTER IN LUCIFERASE REPORTER TRANSGENIC MICE

Introduction

The anterior pituitary gland is comprised of five cell types, defined by the hormones they secrete, which arise from a single pituitary precursor cell. Development of the anterior pituitary is characterized by a series of inductive signals from the neural ectoderm of the ventral diencephalon (Ferrand 1972; Daikoku et al. 1982; Watanabe 1982a; Kawamura and Kikuyama 1995; Takuma et al. 1998) that stimulates the growth and differentiation of the anterior pituitary primordium, Rathke's pouch, around embryonic day 8.5 in the mouse (Takor and Pearse 1975; elAmraoui and Dubois 1993; Osumi-Yamashita et al. 1994). Once established, Rathke's pouch creates an ectodermal boundary that allows subsequent signaling gradients (Treier et al. 1998), which stimulate the expression of transcription factors in overlapping patterns (Simmons et al. 1990; Sornson et al. 1996; Treier et al. 1998; Dasen et al. 1999), ultimately determining which genes are expressed in each cell type, leading to the differentiation of the various pituitary cell types, which are specified by E17.5 (Japon et al. 1994). An opposing ventrodorsal gradient of BMP2 and FGF8 (Ericson et al. 1998; Treier et al. 1998) 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 (Dasen et al. 1999).

Pituitary somatotroph cells, which secrete growth hormone (GH), 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 from the hypothalamus (Mayo et al. 2000). Because GHRH receptor expression is an early event in the somatotroph cell lineage (Lin et al. 1992), understanding regulation of this gene may be

important to determining yet unidentified factors involved in the process of differentiating this cell type from the somato-lactotroph precursor cells.

Mutations in the GHRH receptor in human (Baumann and Maheshwari 1997; Salvatori et al. 1999; Hayashida et al. 2000; Salvatori et al. 2001) and in animal models (Godfrey et al. 1993; Lin et al. 1993; Gaylinn et al. 1999) 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 important role of the GHRH receptor in mediating the neuroendocrine pathway regulating GH levels, understanding regulation of the GHRH receptor gene itself is important. Several studies have examined the GHRH receptor promoter from different species *in vitro*, and clinical cases of growth hormone deficiency in patients with mutations in the promoter have determined important regulatory regions of the promoter; however, a determination of which promoter elements are required to direct appropriately regulated expression *in vivo* has not previously been made.

In order to identify a region of the GHRH receptor promoter sufficient to direct tissue- and cell-specific expression *in vivo*, a transgenic mouse model was used. The transgene consists of 1.6kb of the proximal rat GHRH receptor promoter directing expression of a luciferase reporter gene. This chapter characterizes the *in vivo* expression profile directed by the 1.6kb rat GHRH receptor promoter. The data show that this 1.6kb proximal promoter is sufficient to direct pituitary-specific expression, somatotroph cell expression, sexually dimorphic expression, and age-dependent expression, closely matching the profile of reported data for the endogenous GHRH receptor gene.

Materials and Methods

Generation and Maintenance of Transgenic Mice

An NcoI fragment of the rat GHRH receptor promoter (-1673 to +286, relative to the farthest upstream transcriptional start site) was previously cloned into the SmaI site of pGEM7Z after cutting with BstXI and filling in the ends with T4 DNA polymerase to remove the ATG initiation codon (Miller et al. 1999). The NcoI promoter fragment was inserted into the KpnI site of pA3-Luc. The transgene was produced by digesting this construct with NcoI to linearize the DNA. Transgenic mice were generated at Case Western Reserve University by Andrew Korytko and Leona Cuttler, according to standard procedures, as described (Bokar et al. 1989) . Transgene DNA was injected into the pronucleus of zygotes. Microinjected eggs were transferred to the oviducts of pseudopregnant foster mothers. Animals used to generate founders were F_1 hybrids of C57/Bl6 x SJL. Two founder male mice were identified and used to establish two separate transgenic lines. The transgenic lines were maintained as heterozygotes by crossing transgenic offspring to C57/Bl6 mice. Genotyping was performed on genomic DNA extracted from tail biopsies by PCR using primers to amplify a 900 bp product of the luciferase reporter ((5'-GATTCTAAAACGGATTACCAG-3') and (5'-GTGTTGTAACAATATCGATTC-3')). All animal protocols were approved by the animal care and use committee of Northwestern University (Evanston, IL), and animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 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).

Luciferase Assays

Pituitary glands were removed following euthanasia by CO_2 asphyxiation and decapitation. Pituitaries were placed in 100µl 1X Reporter Lysis Buffer (Promega Corp., Madison, WI) and homogenized using a handheld motorized grinder. Samples were stored on ice for 15 minutes, cleared by centrifugation, and frozen at –80°C for at least 1 hour to facilitate efficient cell lysis. Thawed samples were re-centrifuged and 25µl of the supernatant was used in a luciferase assay by injecting 100µl of Luciferase Assay Reagent (Promega Corp., Madison, WI) with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Protein assays were performed using the Bio-Rad protein assay dye reagent (BioRad 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 following euthanasia by CO_2 asphyxiation and decapitation. Tissues were immediately transferred to Wash/Collection Media (3g/L BSA; 25mM 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) (1X Hanks Balanced Salts Solution (Invitrogen Life Technologies, Inc., Gaithersburg, MD) and 25mM Hepes, pH 7.4) in a 15mL conical tube. HDB was removed, and pituitaries were dissociated in 5mL Collagenase II solution (0.4%

Collagenase, type II (Invitrogen Life Technologies, Inc., Gaithersburg, MD) 0.4% BSA Fraction V (Sigma, St. Louis, MO), 0.2% Glucose, 80U/mL DNase II (Sigma, St. Louis, MO) in HDB; filter-sterilized aliquots were stored at –20°C before use) in a 15mL conical tube by shaking at 100 RPM in a 37°C waterbath for two hours. 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 1X Pancreatin (Sigma, St. Louis, MO) (2.5mg/ml in HDB) was added to the cells, and the cells were incubated at 37°C, 100 RPM for an additional 7 minutes. 2mL charcoal-stripped Fetal Bovine Serum (Invitrogen Life Technologies, Inc., Gaithersburg, MD) was added to stop the action of pancreatin and cells were centrifuged at 1000 RPM for 10 minutes. Cells were washed twice with 420 l Culture Media (low-glucose DMEM with 10% horse serum and 2.5% FBS 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 8-well chamber slides (BD Biosciences. San Jose, CA) at approximately 1.5 x 10^5 cells per well.

Immunocytochemistry

Immunocytochemistry was performed on dispersed pituitary cells after culturing for 16-24 hours at 37°C in culture media. Cells were fixed in 1% paraformaldehyde for 30 minutes at 4°C, permeabilized in 0.02% Triton X-100 for 5 minutes at room temperature, and blocking was performed by incubation in 6% BSA for 30 minutes at room temperature. Primary antibodies were diluted in 1%BSA in 1X PBS. Mouse anti-Luciferase (Zymed Laboratories, Invitrogen, San Francisco, CA) was used at 30 g/ml, and Guinea Pig anti-rat Growth Hormone (National

Hormone & 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 hours. 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 minutes at room temperature in a 1:200 dilution of Rabbit anti-Mouse conjugated to Alexa Fluor 488 and a 1:200 dilution of Goat anti-Guinea Pig conjugated to Alexa Fluor 546 (Molecular Probes) in 1%BSA in 1X PBS. To amplify the signal of the luciferase primary antibody, a second step was performed. Cells were incubated for 30 minutes at room temperature in a 1:200 dilution of Goat anti-Rabbit conjugated to Alexa Fluor 488 and a 1:200 dilution of Goat anti-Guinea Pig conjugated to Alexa Fluor 546. Vectashield mounting media with DAPI (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 400X.

Results

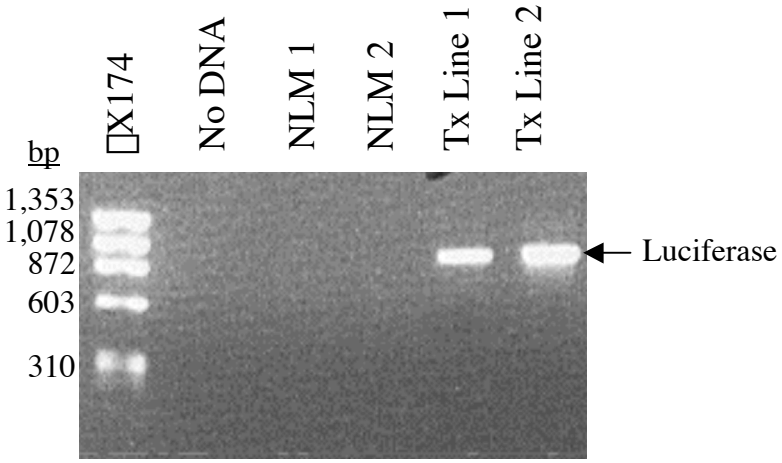
Pituitary-Specific Expression of the Luciferase Transgene in Two Independent Mouse Lines

Two independent lines of transgenic mice were generated in which the 1.6kb GHRH receptor promoter directs expression of a luciferase reporter. PCR of genomic DNA from tail biopsies was used to identify transgenic and normal littermate animals (Fig. 2.1A) using primers to amplify luciferase (Fig. 2.1B). Luciferase assays were performed on pituitaries taken from normal littermate and transgenic mice for each line. Luciferase expression is significant in

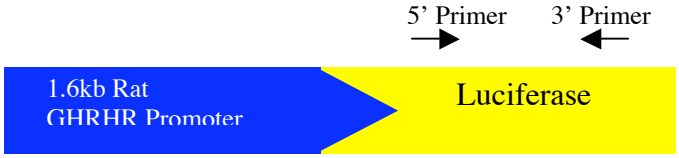
Figure 2.1: Generation of Transgenic Mice Expressing the 1.6kb Rat GHRH Receptor Promoter Directing a Luciferase Reporter

A: Genotyping by PCR of genomic DNA taken from tail biopsies detects luciferase expression in transgenic (Tx) mice from two lines, but not in normal littermate (NLM) mice. Genotyping was performed by Signe Kilen. **B:** A schematic of the transgene shows primer locations in the luciferase gene.

A.


X174
No DNA
NLM 1
NLM 2
Tx Line 1
Tx Line 2
bp
1,353
1,078
872
603
310
Luciferase

B.


5' Primer
3' Primer
1.6kb Rat
GHRHR Promoter
Luciferase

transgenic pituitaries compared to normal littermates in each line (Fig. 2.2). Transgenic mice in Line 2 show significantly higher transgene expression than those in Line 1 (Fig. 2.2). Due to the robust transgene expression level in the pituitary of transgenic mice in Line 2, this line was used for further analysis and data from Line 2 are reported here.

To determine whether the 1.6kb GHRH receptor promoter is sufficient to direct tissue-specific expression, multiple endocrine and non-endocrine tissues were examined for luciferase expression in both normal littermate and transgenic mice. In addition to pituitary the tissues tested include medial-basal hypothalamus, cerebellum, pancreas, cerebral cortex, testes, 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 to the normal littermate sample (Fig. 2.3).

Transgene Expression is Targeted to Somatotroph Cells

In order 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. 2.4A-H). An antibody to growth hormone was used to localize somatotroph cells. A luciferase antibody was used to examine transgene expression localization. The growth hormone antibody, visualized on the Texas Red channel, shows that somatotroph cells in both normal littermate (Fig. 2.4J) and transgenic (Fig. 2.4N) pituitaries occur at equivalent numbers. As expected, the luciferase antibody, visualized on the FITC channel, shows luciferase expression in pituitary cells of only the transgenic mice (Fig. 2.4K & O).

Figure 2.2: Luciferase Transgene Expression in the Pituitary of Two Independent Mouse Lines

Luciferase assays were performed on pituitaries from normal littermate (NLM) and transgenic (Tx) mice from two independent lines. Statistical analysis was performed using a two-way ANOVA comparing genotype and tissue expression (*, $p<0.05$; ***, $p<0.001$; n=5 for each group).

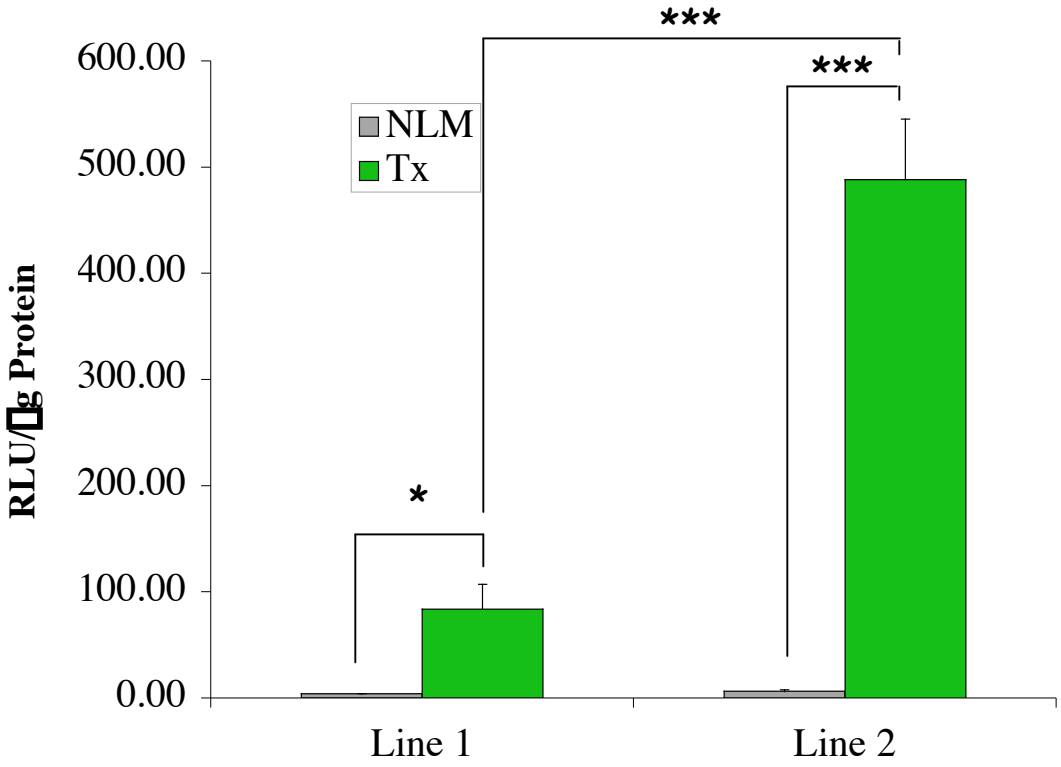

***
***
*
600.00
500.00
400.00
300.00
200.00
100.00
0.00
NLM
Tx
RLU/µg Protein
Line 1
Line 2

Figure 2.3: Tissue-Specific Expression of the GHRHR-Luciferase Transgene

Luciferase assay of 15 homogenized tissues from normal littermate (NLM) and transgenic (Tx) mice, normalized to protein. Statistical analysis was performed using a two-way ANOVA comparing genotype and tissue expression (***, $p<0.001$; $n\geq3$ for each group; mbh: medial basal hypothalamus).

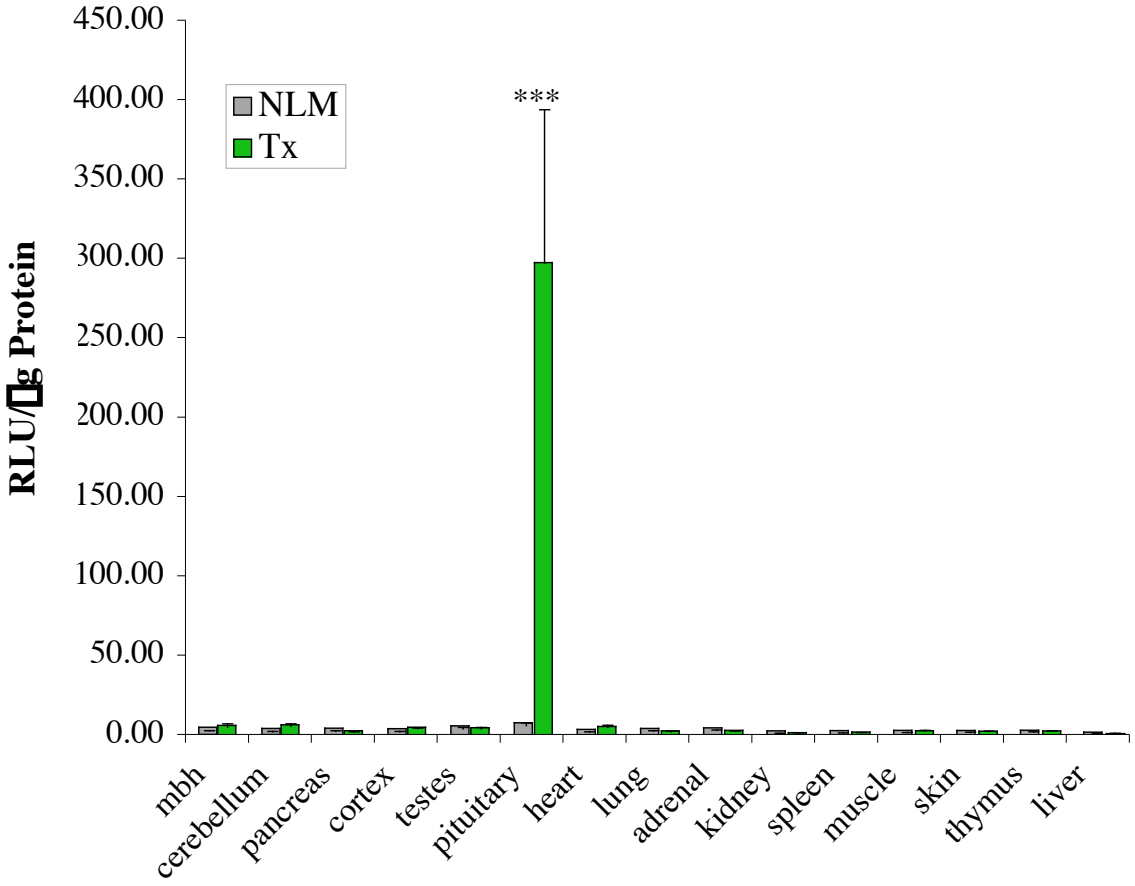
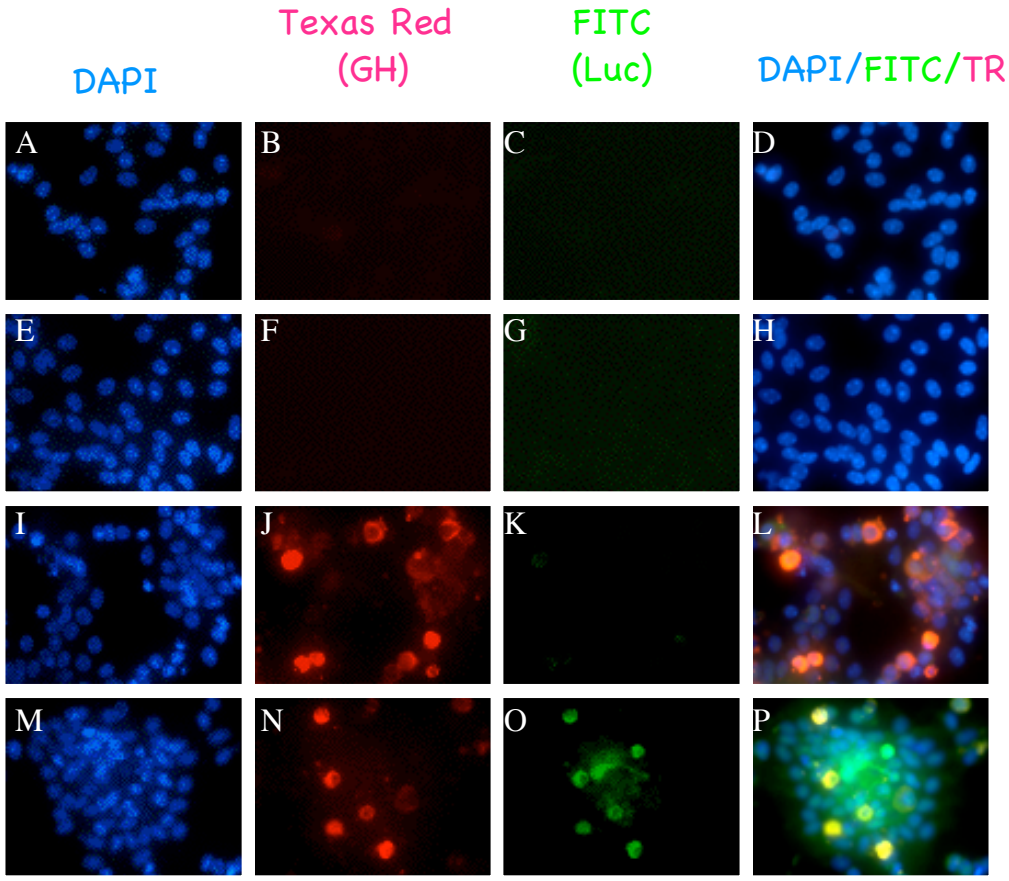

450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
RLU/µg Protein
NLM
Tx
***
mbh
cerebellum
pancreas
cortex
testes
pituitary
heart
lung
adrenal
kidney
spleen
muscle
skin
thymus
liver

Figure 2.4: Immunocytochemistry of Pituitary Cells to Localize Transgene Expression

Pituitaries from male mice, age 21-45 days, were removed and cells were dispersed. Immunocytochemistry was performed using primary antibodies to growth hormone and luciferase. Additional samples were processed without primary antibody incubation (normal littermate: A-D; transgenic: E-H). A DAPI stain identifies cell nuclei (A, E, I & M). Growth hormone-positive cells are visualized on the Texas Red channel (B, F, J & N) and luciferase-positive cells are visualized on the FITC channel (C, G, K & O). Overlays show where co-localization of growth hormone and luciferase occurs (D, H, L, & P). Data are representative of at least three independent experiments.


DAPI
Texas Red
(GH)
FITC
(Luc)
DAPI/FITC/TR
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Overlays of the channels detecting growth hormone and luciferase antibodies reveal that the two antibodies co-localize in the transgenic pituitary (Fig. 2.4P), indicating that the luciferase transgene is expressed in somatotroph cells of the anterior pituitary gland. The experiment was performed twice with six animals of each genotype used in each experiment. Quantification of 850 cells from the immunocytochemistry experiments shows that 100 percent of the somatotroph (GH-positive) cells co-express the luciferase transgene and that in fewer than 5 percent of cells expressing the transgene, growth hormone is not detected. This small number of cells is likely not significant enough to represent an entire population of an additional pituitary cell type expressing the transgene.

Transgene Expression is Sexually Dimorphic and Regulated during Post-Natal Development

Transgene expression was examined in males compared to 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 more than five-fold difference (Fig. 2.5). Age-dependent analysis of transgene expression shows that low, but detectable levels of expression are present in the first postnatal day, with rising expression levels as the animal approaches puberty (Fig. 2.6). Expression of the transgene was not consistently altered during post-pubertal aging.

Discussion

The goal of this project was to assess *in vivo* regulation of the proximal promoter of the rat GHRH receptor. In previous cell culture experiments, 1.6kb of the rat promoter was able to drive expression of reporter constructs in a pituitary cell line (Miller et al. 1999). In order to

Figure 2.5: Sexually Dimorphic Expression of the 1.6kb Rat GHRH Receptor Promoter Luciferase Transgene

Pituitaries were removed from transgenic and normal littermate male and female mice and luciferase assays were performed to measure transgene expression. Statistical analysis was performed using a two-way ANOVA comparing genotype and sex-specific expression ($n \geq 5$; *, $p<0.05$; **, $p<0.01$).

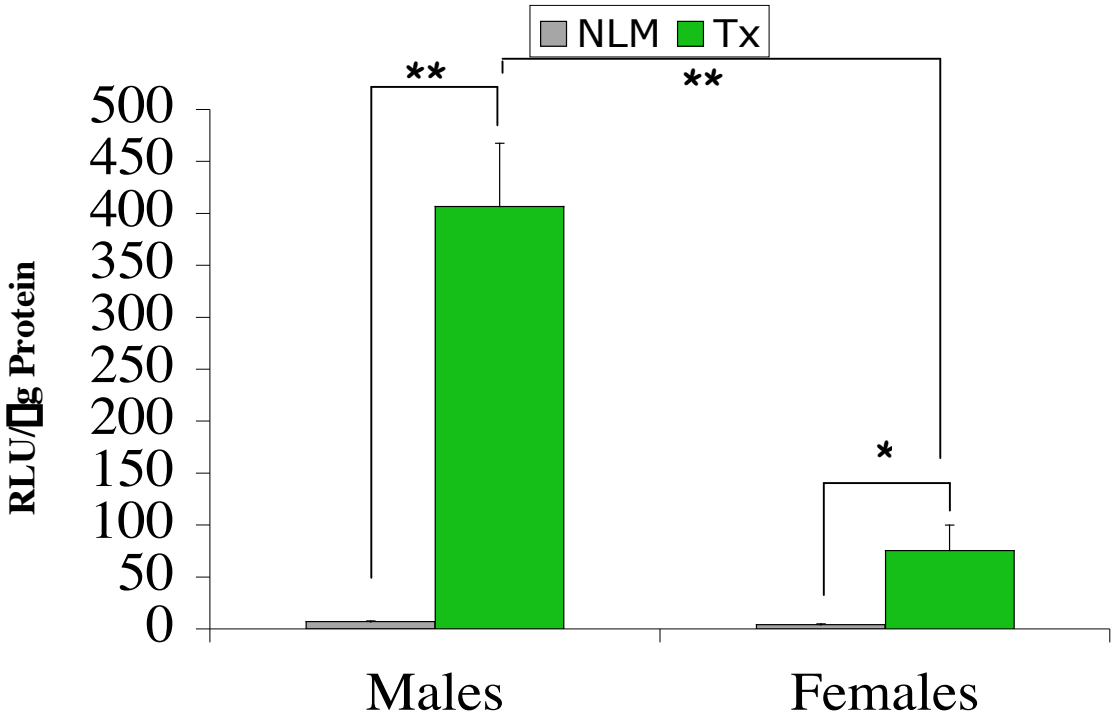
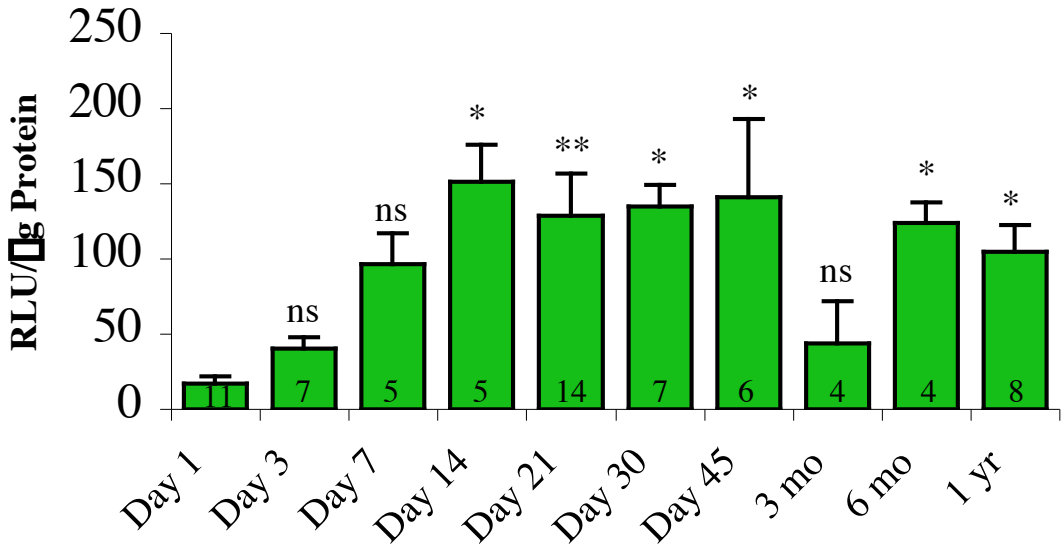

NLM
Tx
**
**
*
500
450
400
350
300
250
200
150
100
50
0
RLU/µg Protein
Males
Females

Figure 2.6: Transgene Expression During Post-Natal Development and Aging

Luciferase assays were performed on transgenic pituitaries from animals of age 1 day through 1 year to determine changes in expression during development and aging. N (number of animals) for each age group is indicated on the bottom of the bar in the graph. Statistical analysis was performed by one-way ANOVA (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$).


RLU/µg Protein
250
200
150
100
50
0
ns
ns
*
**
*
*
ns
*
*
11
7
5
5
14
7
6
4
4
8
Day 1
Day 3
Day 7
Day 14
Day 21
Day 30
Day 45
3 mo
6 mo
1 yr

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 the endogenous GHRH receptor is expressed specifically in the pituitary (Mayo 1992), transgene expression is restricted to the pituitary in transgenic mice, suggesting that tissue-specificity of expression is driven by elements within the 1.6kb promoter. Interestingly, no transgene expression is observed in the kidney, where an alternative GHRH receptor mRNA is expressed from a distinct promoter in the rat (J. Zheng and K. Mayo, unpublished). A report has suggested that GHRH receptor expression is restricted to somatotroph cells of the anterior pituitary gland (Morel et al. 1999) in experiments in which cell type was morphologically determined. Though a negligible percentage of non-somatotroph cells express the GHRH receptor promoter transgene, all somatotroph cells, defined by growth hormone antibody 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 1.6kb proximal promoter.

The literature reports conflicting data on whether GHRH receptor expression is dependent on sex. A couple of reports suggest that males express the receptor at significantly higher levels than females (Ono et al. 1995; Mayo et al. 2000), while other reports have failed to see a difference in receptor expression between males and females (Carmignac et al. 1996; Kamegai et al. 1999). The explanation for the conflict in the literature is not clear. One hypothesis is that female cycle status should be taken into account, considering that the GHRH

receptor is known to be regulated by estrogen (Petersenn et al. 1998; Yan et al. 2004). Expression of the GHRH receptor promoter transgene is consistently higher in males than females, with an approximate five-fold difference. The data from the transgenic mouse support sexual dimorphism of GHRH receptor expression and indicate that it is controlled by elements in the 1.6kb receptor promoter. The differences in GHRH receptor expression are likely to result in part from differences in pituitary somatotroph cell numbers between males and females, which differ by about 20 percent in adult mice (Kineman et al. 1996). The five-fold difference in transgene expression suggests that there is also a difference in GHRH receptor expression levels per somatotroph cell between males and females. Research has shown that lactotrophs and somatotrophs can bidirectionally interconvert based on physiological state, such as in pregnancy and lactation when prolactin requirements are higher (Porter et al. 1990; Porter et al. 1991; Kineman et al. 1992). The relatively higher number of somatotroph cells and higher expression of GHRH receptor is likely responsible for the higher levels of circulating GH in males compared to 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, with high expression in newborns that declines during early development. GHRH receptor transcript expression increases dramatically at puberty, before again declining throughout aging (Korytko et al. 1996). Subsequent reports have further substantiated that GHRH receptor mRNA expression dramatically decreases in the aged animal (Carmignac et al. 1996; Kamegai et al. 1999). In the transgenic mouse, expression of the promoter-transgene is detectable in the postnatal day 1 animal, and increases leading up to puberty. In stark contrast to reported data on the endogenous gene, no consistent decline in

expression of the transgene occurs throughout aging, likely indicating that, while the 1.6kb promoter contains the elements necessary to regulate the increased expression leading up to puberty, it does not contain the elements involved in repressing expression as the animal ages. Alternatively, the profile of GHRH receptor protein expression may differ from RNA expression, which was measured for the endogenous gene. Finally, regulation of expression may differ between rat and mouse.

In summary, the data in this chapter show that 1.6kb of the rat GHRH receptor promoter is sufficient to target tissue- and cell-specific expression of the receptor. In addition, sex-dependent expression and the pubertal rise in expression level of the receptor is regulated by elements within this 1.6kb proximal promoter. Results from experiments with the transgenic animal suggest that elements not included in the 1.6kb promoter may be involved in repression of gene transcription during the aging process. Given the importance of this region of the proximal promoter in regulating GHRH receptor expression *in vivo*, the 1.6kb promoter was evaluated further in order to understand what elements bind to and activate the rat GHRH receptor promoter. Chapter 3 describes experiments that examine the identical promoter sequence *in vitro* in an attempt to identify important regulatory elements.

CHAPTER THREE

ACTIVATION OF RAT GHRH RECEPTOR TRANSCRIPTION BY THE PITUITARY-SPECIFIC POU-HOMEODOMAIN TRANSCRIPTION FACTOR PIT-1

Introduction

The experiments described in Chapter 2 showed that the 1.6kb proximal promoter of the rat GHRH receptor gene directs appropriate expression *in vivo*. The 1.6kb promoter was therefore further analyzed in cell culture experiments to examine important regulatory factors. Factors known to be involved in the regulation of the GHRH receptor gene include the pituitary-specific transcription factor Pit-1 (Petersenn et al. 1998; Iguchi et al. 1999; Miller et al. 1999; Nogami et al. 1999; Salvatori et al. 2002), glucocorticoids (Miller and Mayo 1997; Petersenn et al. 1998; Nogami et al. 1999; Nogami et al. 2002), thyroid hormone (Stahl et al. 1999; Nogami et al. 2002) and estrogen (Petersenn et al. 1998; Yan et al. 2004). The mechanism of regulation has not been elucidated for any of these factors. Initial experiments were set up to broadly examine pituitary-specific factors involved in regulating expression of the GHRH receptor gene.

The transcription factors Prop-1 (Sornson et al. 1996) and Pit-1 are required for the differentiation of the thyrotroph (Lin et al. 1994), lactotroph (Crenshaw et al. 1989) and somatotroph (Lira et al. 1993) cell lineages from a common pituitary precursor. Pit-1 is a pituitary-specific POU homeodomain transcription factor (Bodner et al. 1988; Ingraham et al. 1988) that is involved in the transcriptional activation of the growth hormone gene in somatotroph cells and of the prolactin gene in lactotroph cells (Mangalam et al. 1989; Day et al. 1990; Fox et al. 1990). Previous work from our laboratory and others has shown that Pit-1 also leads to activation of the GHRH receptor promoter (Petersenn et al. 1998; Iguchi et al. 1999; Miller et al. 1999), and has suggested a broad target for Pit-1 activity. Further indicative of the important role Pit-1 plays in stimulating GHRH receptor expression, a recessive inactivating mutation in the GHRH receptor Pit-1 binding site results in decreased expression of the receptor in a compound heterozygote patient exhibiting GH deficiency (Salvatori et al. 2002). Pit-1 has

recently been shown to be required for glucocorticoid regulation of the rat GHRH receptor (Nogami et al. 2005), suggesting that cooperation between Pit-1 and other factors may lead to differential cell-specific gene regulation.

In initial electrophoretic mobility shift assay (EMSA) experiments examining pituitary-specific factors binding to the GHRH receptor promoter, it was determined that Pit-1 binds to regions across the proximal promoter. Pit-1 is known to be required for activation of GHRH receptor expression (Lin et al. 1992; Miller et al. 1999). On the promoters of many of the genes Pit-1 regulates, Pit-1 binding to multiple sites acts cooperatively with other factors to confer cell-specific expression (Nowakowski and Maurer 1994; Wood et al. 1996; Shewchuk et al. 1999). A first step to understanding cell-specific expression of the GHRH receptor is identification of the functional Pit-1 binding sites in the promoter.

To understand Pit-1 regulation of the GHRH receptor, a comprehensive examination of the 1.6kb rat GHRH receptor promoter was pursued. Transcriptional activation of the GHRH receptor requires Pit-1 binding to the promoter (Miller et al. 1999). Though Pit-1 is known to bind to A/T rich sites (Ingraham et al. 1988; Jin et al. 1999), it has no real consensus binding sequence, rendering computational binding-site predictions imprecise. To determine where Pit-1 binds the rat GHRH receptor promoter and which of the binding sites are important for activation of the promoter, EMSAs, promoter-luciferase activity assays and mutational analysis of Pit-1 binding sites were performed. Pit-1 binds specifically to ten sites throughout the 1.6kb promoter. Functional analyses show that the high-affinity Pit-1 binding site downstream of two of the four identified transcriptional start sites (+129 to +164, relative to the 5'-most transcriptional start site) is required for Pit-1 activation of this gene. This site, which was previously identified as a functional Pit-1 activation site in the rat GHRH receptor (Nogami et al. 2002), is highly

conserved across multiple species, and corresponds to the site that contributes most to Pit-1 activation of the human receptor gene (Iguchi et al. 1999). In addition, four other novel sites contribute to full Pit-1-stimulated activation of the GHRH receptor gene. Of these, a site at –888 to –857 contributes greatest.

Materials and Methods

Vaccinia Transfection System

HeLa T4 cells, maintained in DMEM with 4.5g/L glucose and L-glutamine (Mediatech, Inc., Herndon, VA) and 5 percent fetal bovine serum (Mediatech, Inc., Herndon, VA), were transfected with constructs using the vaccinia virus-T7 polymerase expression system (obtained under license from Dr. Bernard Moss, NIH, Bethesda, MD), as described (Fuerst et al. 1987). For transfection, the cells were incubated with vaccinia at a multiplicity of infection of 10 in PBS/0.1% BSA for 30 minutes. 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 (Felgner et al. 1987; Campbell 1995) at 5 g lipid/ g DNA in OptiMEM media (Gibco BRL, Grand Island, NY) for 15-20 minutes at room temperature. After infection, the virus was aspirated, and the DNA/transfectAce was added. Cells were transfected for 6 hours at 37°C in 5 percent CO_2 and allowed to recover overnight in DMEM.

Extraction of Nuclear Proteins

After transfection, nuclear extracts were collected, as described (Dignam et al. 1983). HeLa cells were washed twice with cold 1X PBS and scrape collected in 1mL 1X PBS with protease inhibitors (1 g/ml aprotinin, leupeptin, antipain, and pepstatin) in microcentrifuge tubes. Cells were pelleted by spinning for 5 minutes at 500x*g* at 4°C. The cell pellet was resuspended in 5X volume of Buffer A (10mM Hepes, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol (DTT), 0.5mM phenylmethylsulphonylfluoride (PMSF) with 1 g/ml aprotinin, leupeptin, antipain and pepstatin) and incubated on ice for 15 minutes, followed by homogenization by 10 strokes with a glass-glass Dounce homogenizer. Samples were spun at 1,500x*g* for 2 minutes at 4°C. The nuclear pellet was resuspended in two-thirds volume Buffer C (20mM Hepes, pH 7.9, 1.5mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, 25% glycerol, 0.5mM DTT, 0.5mM PMSF with 1 g/ml aprotinin, leupeptin, antipain and pepstatin) while constantly stirring with a micro stirbar on ice. After stirring for 30 minutes samples were spun at 13,000x*g* for 10 minutes. The supernatant was aliquoted and stored at -80°C. Protein concentration was determined by Bradford Protein Assay using BioRad dye reagent (BioRad Laboratories, Hercules, CA).

Western Blot Analysis

Ten g nuclear extracts were boiled for 5 minutes 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 minutes at room temperature with shaking,

followed by incubation with a 1:100 dilution of Pit-1 antibody (PRB-230C, Covance, 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-anti-rabbit conjugated to horseradish peroxidase (Promega, Madison, WI) in blocking buffer for 90 minutes. The blot was washed twice with water, then with PBS/0.05% Tween for 5 minutes, 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).

Electrophoretic Mobility Shift Assays

DNA probes for EMSAs were generated either by polymerase chain reaction incorporating ^{32}P-dCTP (GE Healthcare) for probes longer than 90bp (see Table 3.1 for probe locations and primer sequences) or by end labeling annealed complimentary oligonucleotides in a T4 kinase reaction using ^{32}P-γATP (MP Biomedicals, Solon, OH), for shorter probes (Pit-1 binding probe sequences and mutations listed in Table 3.2). Labeled probes were gel-purified on 5-12% acrylamide/TBE gels and eluted from the gel in 0.5M NH_4OAc and 1mM EDTA. Probe eluates were spun through nylon wool, ethanol precipitated twice, and resuspended in 50μl ddH_2O. 1μl of each probe was counted on a scintillation counter and probes were diluted for use at $1.0x10^4$ cpm per reaction. In each reaction, 5μg nuclear extract was used, unless otherwise indicated. Nuclear extracts were incubated with 1X EMSA buffer (5X EMSA Buffer: 5mM DTT, 5mM MgCl2, 50mM Tris, pH 7.5), 0.2μg/μl poly dI/dC double strand (Amersham Biosciences, Piscataway, NJ) and radiolabeled probe in a final volume of 10-15μl for 20 minutes at room temperature. For supershift experiments, 2μl Pit-1 antibody (200μg/ml) (sc-16289, Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extract for 30 minutes on ice before the addition of the probes. TBE loading dye (30% glycerol/70% TBE with

16mg/ml bromophenol blue and 16mg/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, Sunnyvale, CA) for quantification using a STORM 860 PhosphorImager (GE Healthcare) and ImageQuant 5.0 software (Molecular Dynamics).

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

Rat GHRH receptor promoter 5' deletion constructs were generated by PCR using 5' primers with a KpnI site and a 3' primer with either an NheI site (-1672, -1355, -1115) or a HindIII site (-840, -601, -396, -131) using the –1672 rat GHRH receptor promoter construct as a template. Primers were generated by Integrated DNA Technologies (Coralville, IA). Primer sequences follow: -1672 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 NheI 3', 5'-GGTGCTAGCCAAA GAGCCGTGGCCCTTCCCTCCCA-3' and +264 HindIII 3', 5'-GGAAGCTTCCAAAGAGCC 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, unpublished). The –1672 luciferase clone was used as a template for generation of Pit-1 binding site mutation clones. Mutations are shown in Table 3.2 and were generated using the

Quickchange (Stratagene, La Jolla, CA) protocol by PCR with complimentary oligonucleotides incorporating the desired mutations. The parental DNA was digested by DpnI for 1 hour 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 co-transfected 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 g each plasmid (2 g total DNA per well) in Opti-MEM containing lipofectamine 2000 (2 l/ g DNA) (Invitrogen, Carlsbad, CA) for 4-6 hour. Cells transfected with an RSV-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 1X PBS and lysed in 150 l 1X Reporter Lysis buffer (Promega, Madison, WI) for 20 minutes on ice. Cells were scrape-collected and lysis was completed by freezing at –80°C for at least one hour. For the luciferase assay, 100 l 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 BioRad dye reagent (BioRad Laboratories, Hercules, CA).

Results

Identification of Pituitary Specific Factor Binding Regions within the 1.6kb Rat GHRH Receptor Promoter

In order to examine which regions of the 1.6kb rat GHRH receptor promoter bind to pituitary specific factors, electrophoretic mobility shift assays (EMSAs) were performed using radiolabeled probes spanning the promoter (Table 3.1). The promoter probes were incubated with nuclear extracts from either a pituitary cell line, MtT/S cells, which expresses the GHRH receptor (Miller et al. 1999; Nogami et al. 2000; Voss et al. 2001), or from a non-pituitary cell line, HeLa T4 cells. In addition, binding was competed with a cold oligonucleotide probe to a consensus Pit-1 binding sequence, in order to differentiate Pit-1 binding from other pituitary-specific factors. Fig. 3.1 shows that pituitary specific factors bind to all but the most 3' region of the promoter, and that each of these probes appears to bind Pit-1 (bands marked with red arrows), in addition to other pituitary-specific factors (bands marked with green arrows). Because Pit-1 is known to be required for transcriptional activation of the GHRH receptor, subsequent experiments focused on identifying functional Pit-1 binding sites within the 1.6kb promoter.

Pit-1 Binding to the 1.6kb Rat GHRH Receptor Promoter

To determine where Pit-1 binds to the rat GHRH receptor promoter, electrophoretic mobility shift assays (EMSAs) were performed using overlapping probes spanning the proximal promoter (Table 3.1). DNA probes are shown schematically in Fig. 3.2. In the rat, four transcriptional start sites for the GHRH receptor gene have been identified (Miller et al. 1999); these are depicted as arrows in the schematic (Fig. 3.2A). We refer to the 5'-most of these sites

Table 3.1: EMSA Probe Primers for PCR-Generated Probes

Probe endpoints, primer sequences and product lengths for each probe are listed.

Probe Location	Product Size	5' Primer	3' Primer
-1673 to –1563	110 bp	rGHRHR –1673 5' (5'- ccatggcctctgcatcaactt-3')	rGHRHR –1563 3' (5'- gagtttgcttggcatacactt-3')
-1588 to –1478	110 bp	rGHRHR –1588 5' (5'- cttgaagtgtatgccaagcaa-3')	rGHRHR –1478 3' (5'- tgagcttctattccggcacct-3')
-1503 to -1393	110 bp	rGHRHR –1503 5' (5'- ttacaggtgccggaatagaag-3')	rGHRHR –1393 3' (5'- aaactgaactaacctacctgt-3')
-1418 to -1323	94 bp	rGHRHR –1418 5' (5'- aggaacaggttggttagttca-3')	rGHRHR –1323 3' (5'- ctgctggtatgcagtagggag-3')
-1355 to –1255	100 bp	CJY1 (5'-ctcagcacggttgtgggagct-3')	rGHRHR –1255 3' (5'- aactatgtctggcaaggtgtt-3')
-1280 to –1180	100 bp	rGHRHR –1280 5' (5'-gtagtaaaacaccttgccagacat-3')	rGHRHR –1180 3' (5'- aatcaataccaagaattcata -3')
-1205 to –1092	113 bp	rGHRHR –1205 5' (5'- ataataggaactgctatgaat-3')	GHRHR –1100 (5'- aggggacagaggctgccacac-3')
-1115 to –1000	115 bp	CJY2 (5'- gggtgtggcagcctctgtccc-3')	rGHRHR –1000 3' (5'- tgggtggcacacagcacacag-3')
-1025 to –900	125 bp	rGHRHR –1025 5' (5'- agccctgtgtgctgtgtgcca-3')	rGHRHR –900 3' (5'- tgcatctgggaatcatttgac-3')
-925 to –808	117 bp	rGHRHR –925 5' (5'- tgatgtcaaatgattcccaga-3')	GHRHR –800 (5'- cacttacagagctgttgtgct-3')
-840 to –740	100 bp	CJY3 (5'- tggcacccccccagcacaacag-3')	rGHRHR –740 3' (5'- gaatgtcacttttgacaaccc-3')
-765 to –665	100 bp	rGHRHR –765 5' (5'- ttcagggttgtcaaaagtgac-3')	rGHRHR –665 3' (5'- tacttgagaatacaagctctc -3')
-690 to –574	116 bp	rGHRHR –690 5' (5'- ccaatggagagcttgtattct-3')	GHRHR –600 (5'- gaggactctcagagggatctc -3')
-601 to –511	90 bp	CJY4 (5'- agccccagagatccctctgag-3')	rGHRHR –511 3' (5'- tcacatgcgttaggcaaagtt-3')
-536 to –446	90 bp	rGHRHR –536 5' (5'-cagaaactttgcctaacgcat -3')	rGHRHR –446 3' (5'- acagtgtttgcctgacgggga-3')
-471 to –367	104 bp	rGHRHR –471 5' (5'- agcgtccccgtcaggcaaaca-3')	GHRHR –400 (5'- ggctccaggttcaaatccctg-3')
-396 to –281	115 bp	CJY5 (5'- tgtggtgccagggatttgaac-3')	rGHRHR –281 3' (5'- ataagatttgaattagcctta-3')
-306 to –191	115 bp	rGHRHR –306 5' (5'- ttcataaggctaattcaaatc-3')	rGHRHR –191 3' (5'- ccggtttgttgatgctctaag-3')
-216 to -104	112 bp	rGHRHR –216 5' (5'- gagacttagagcatcaacaaa-3')	GHRHR –200 (5'- ctcagagaggcctcctatacc-3')
-131 to –1	130 bp	CJY6 (5'- cccgtgcaggtataggaggcctc-3')	rGHRHR –1 3' (5'- cctgagaacacagatagggca-3')
-26 to +104	130 bp	rGHRHR –26 5' (5'- tctgtgccctatctgtgttct-3')	rGHRHR +104 3' (5'- cagccccaaatgggactctgt-3')
+79 to +208	129 bp	rGHRHR +79 5' (5'- ggacacagagtcccatttggg-3')	GHRHR +200R (5'- cctcaccttcccagtaactgc-3')
+148 to +219	71 bp	CJY7 (5'-ttcagctgggtgtccctcct -3')	rGHRHR +219 3' (5'- catctctgctacctcacctcc-3')
+194 to +264	70 bp	rGHRHR +194 5' (5'- ctgggaaggtgaggtagcaga-3')	KM2a (5'- gtggcccttccctcccagggct-3')

Figure 3.1: Pituitary Specific Factors Binding the 1.6kb Rat GHRH Receptor Proximal Promoter

EMSAs were performed using overlapping probes spanning the proximal promoter. Radiolabeled probes were incubated with nuclear extracts from non-pituitary HeLa T4 cells (lanes 2 and 3) and from pituitary MtT/S cells (lanes 4 and 5). Lanes 1 are probe without nuclear extracts. Lanes 3 and 5 inclued a cold oligonucleotide probe containing a consensus Pit-1 binding site. Red arrows highlight Pit-1 binding (pituitary-specific bands that are competed by the Pit-1 cold competitor), while green arrows show pituitary-specific factors other than Pit-1 that bind this promoter (pituitary-specific bands that are not competed by the Pit-1 cold competitor).

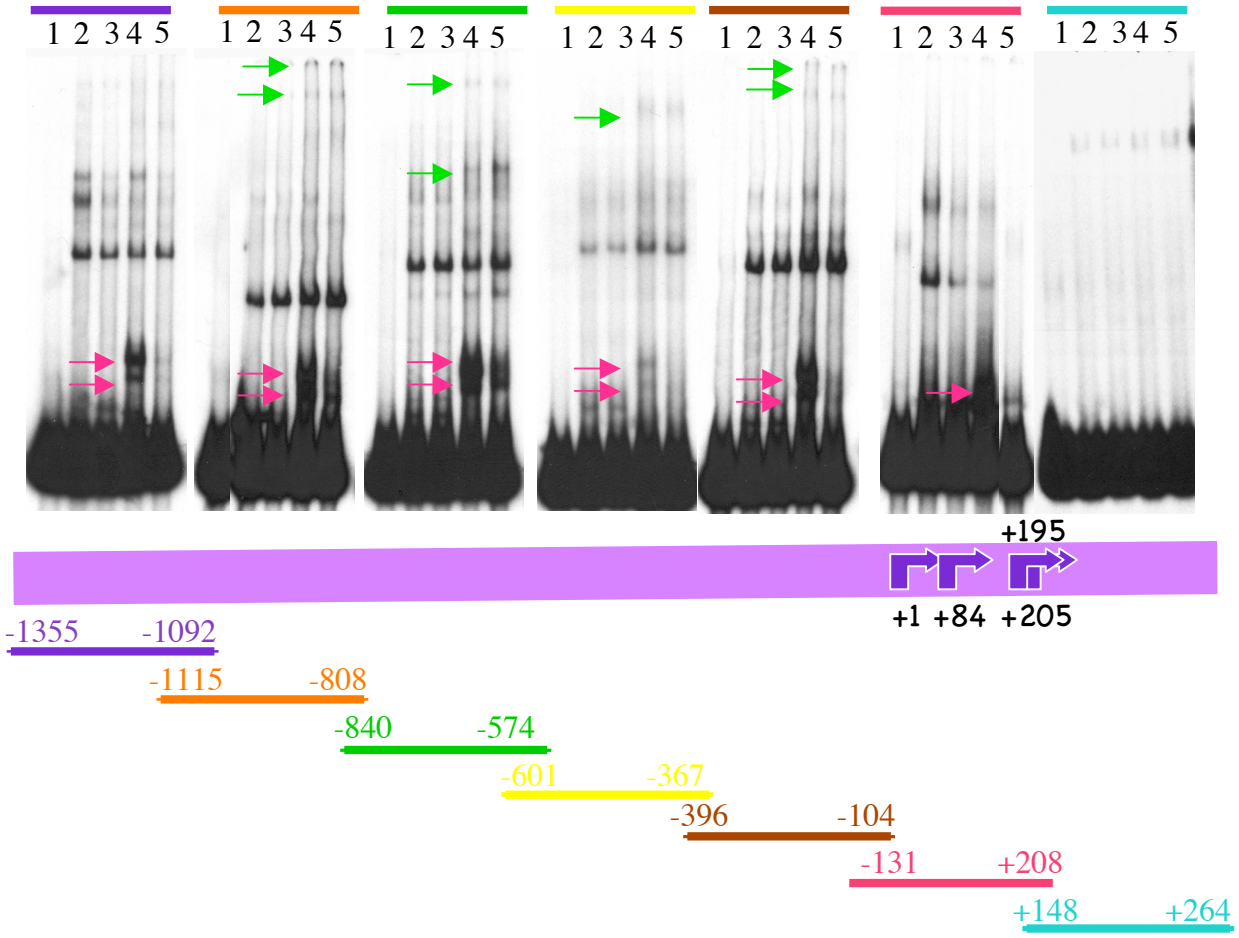
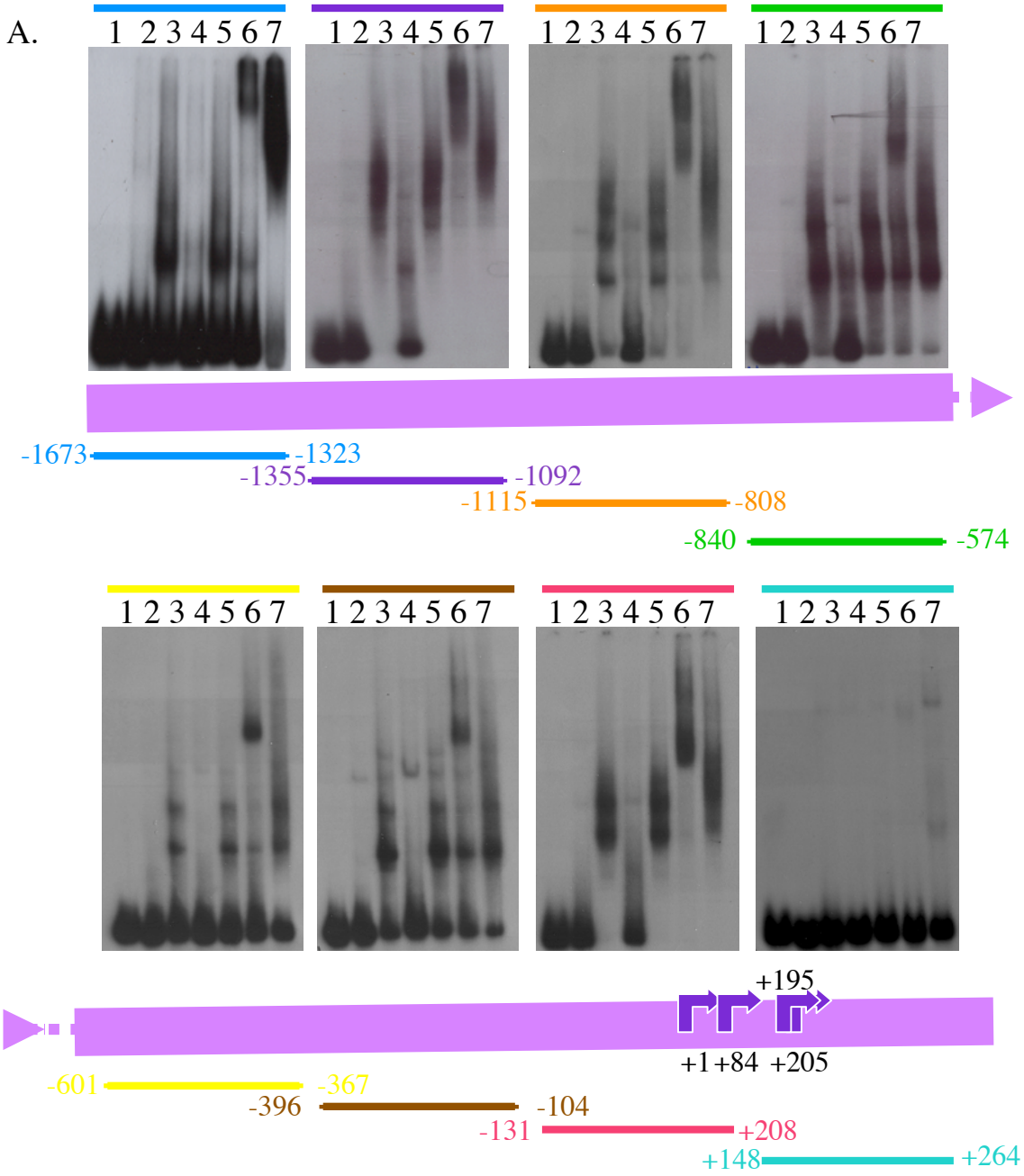

1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
+195
+1 +84 +205
-1355 -1092
-1115 -808
-840 -574
-601 -367
-396 -104
-131 +208
+148 +264

Figure 3.2: Pit-1 Binding Across the 1.6kb Rat GHRH Receptor Promoter

A: EMSAs were run by incubating nuclear extracts with radiolabeled DNA probes spanning the promoter. Lane 1 in each panel is probe without nuclear extracts. 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 a cold competitor Pit-1 consensus binding sequence oligonucleotide. Lane 5 includes a cold competitor mutated Pit-1 binding sequence oligonucleotide. Lane 6 includes an antibody to Pit-1 and lane 7 includes normal serum. Data are representative of at least three independent experiments. A schematic represents the overlapping DNA promoter probes and the four transcriptional start sites in the rat GHRH receptor promoter. **B:** Nuclear extracts from HeLa T4 cells either overexpressing Pit-1 (lane 1) or mock-transfected (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. Data are representative of at least three independent experiments.


A.
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
-1673
-1323
-1355
-1092
-1115
-808
-840
-574
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
+195
+1
+84
+205
-601
-367
-396
-104
-131
+208
+148
+264
B.
Pit-1
Mock
35.9 kDa
24.5 kDa

as "+1." Only the 3'-most probe was eliminated for Pit-1 binding (Fig. 3.2A). Pit-1 binds specifically to each of the other seven probes, as nuclear extracts from cells overexpressing Pit-1 shift the mobility of the probes (Fig. 3.2A, lanes 3). An unlabeled oligonucleotide encompassing a consensus Pit-1 binding site competes away Pit-1 binding (Fig. 3.2A, lanes 4), but an unlabeled mutated Pit-1 oligonucleotide has no effect on binding (Fig. 3.2A, lanes 5). Incubation of the nuclear extracts with an antibody to Pit-1 supershifts the Pit-1-binding band (Fig. 3.2A, lanes 6), while normal serum does not affect binding (Fig. 3.2A, lanes 7). A western blot confirms 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. 3.2B).

Pit-1 Specifically Binds Ten Short Regions in the 1.6kb Rat GHRH Receptor Promoter

In order to determine short DNA regions that bind Pit-1 specifically, overlapping PCR (Table 3.1) and oligonucleotide (Table 3.2) probes were generated and used in EMSAs. Fig. 3.3 provides an example of the approach using successively shorter probes in EMSA assays to identify short, specific binding sites for Pit-1 within the rat GHRH receptor promoter. In a previous report, four putative Pit-1 binding sites in the 1.6kb promoter were identified computationally (Miller et al. 1999). The binding experiments show that, in fact, ten short regions of the 1.6kb rat GHRH receptor promoter bind Pit-1 specifically (Fig. 3.4, lanes 3). The binding of Pit-1 to these probes is competed by an unlabeled Pit-1 site oligonucleotide (Fig. 3.4, lanes 4), not competed by a mutated Pit-1 site oligonucleotide (Fig. 3.4, lanes 5), supershifted by incubation with an antibody to Pit-1 (Fig. 3.4, lanes 6) and unaffected by incubation with normal rabbit serum (Fig. 3.4, lanes 7). When key nucleotides are mutated from A/T to G (Table 3.2),

Figure 3.3: Example of the Approach Used to Identify Short, Specific Binding Sites for Pit-1

A series of EMSAs was performed using successively shorter PCR and oligonucleotide probes to identify short regions of the promoter that bind Pit-1 specifically. The experiments that led to the identification of the –1178 to –1153 and –1148 to –1123 sites are shown. In each panel, lane 1 is probe alone, lane 2 is probe incubated with mock-transfected HeLa cell nuclear extracts and lanes 3-7 are probe incubated with nuclear extracts from HeLa cells over-expressing Pit-1. Lane 4 includes cold competitor with a Pit-1 consensus binding sequence. Lane 5 includes a mutated cold competitor. Lane 6 includes antibody to Pit-1 and lane 7 includes normal serum (* identified Pit-1 binding regions).

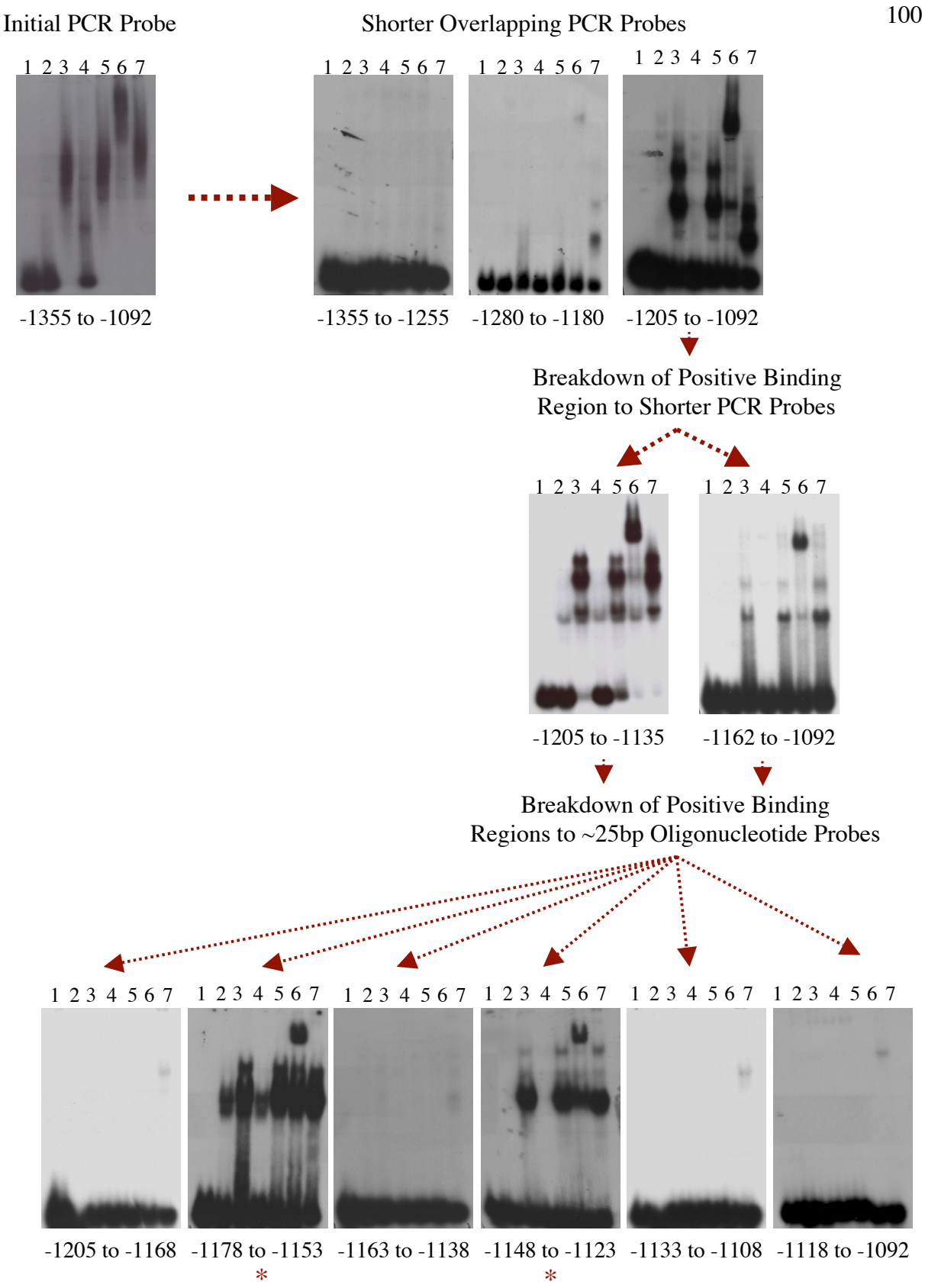

Initial PCR Probe
Shorter Overlapping PCR Probes
1 2 3 4 5 6 7
-1355 to -1092
-1355 to -1255
-1280 to -1180
-1205 to -1092
Breakdown of Positive Binding Region to Shorter PCR Probes
-1205 to -1135
-1162 to -1092
Breakdown of Positive Binding Regions to ~25bp Oligonucleotide Probes
-1205 to -1168
-1178 to -1153
-1163 to -1138
-1148 to -1123
-1133 to -1108
-1118 to -1092
*
*

Figure 3.4: Identification of Ten Sites in the 1.6kb Rat GHRH Receptor Promoter that Specifically Bind Pit-1

EMSAs were performed using progressively shorter radiolabeled oligonucleotide probes and nuclear extracts from HeLa T4 cells (either mock-transfected or overexpressing Pit-1). Shown are the ten probes that specifically bind Pit-1. Lane 1 in each gel is probe without nuclear extracts. 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 a cold competitor Pit-1 consensus binding sequence oligonucleotide. Lane 5 includes a cold competitor mutated Pit-1 binding sequence oligonucleotide. Lane 6 includes an antibody to Pit-1 and lane 7 includes normal serum. The second gel in each panel is an identical experiment with a mutated probe. Data are representative of at least three independent experiments.

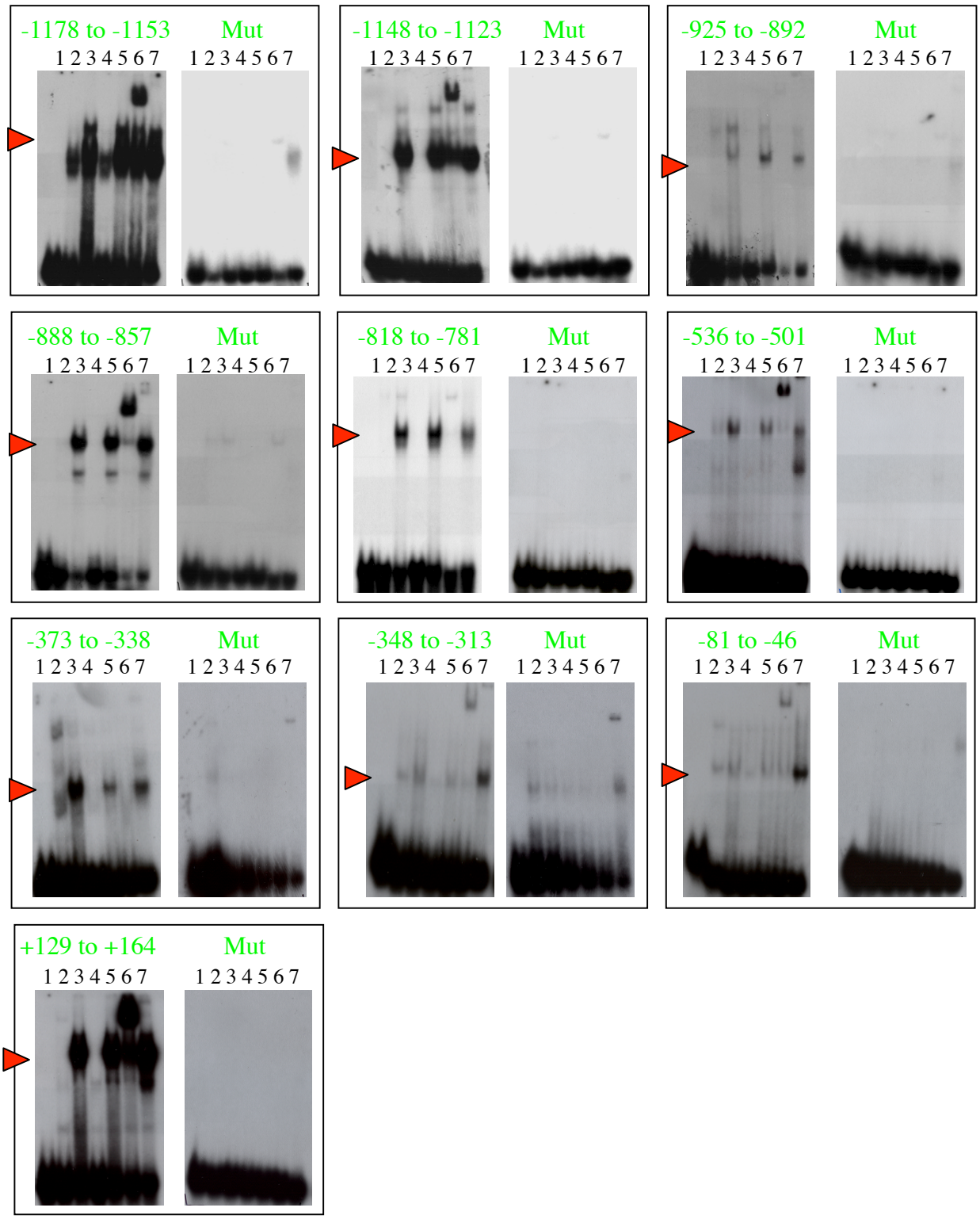

-1178 to -1153
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-1148 to -1123
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-925 to -892
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-888 to -857
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-818 to -781
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-536 to -501
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-373 to -338
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-348 to -313
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
-81 to -46
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7
+129 to +164
1 2 3 4 5 6 7
Mut
1 2 3 4 5 6 7

Table 3.2: Rat GHRH Receptor Promoter Pit-1 Binding Sequences and Mutations

Each of the identified Pit-1 binding sequences and promoter locations are shown, along with the mutations that affect binding, which were also examined in subsequent functional experiments. The nucleotides marked with asterisks were mutated to guanines for binding and functional assays.

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 ** **

Pit-1 binding to these sequences is completely obliterated (Fig. 3.4, Mut panels). The –1673 to –1323 region was not successfully narrowed down, as binding sites within this region were not detectable in short probes, possibly indicative of the existence of multiple weak-affinity sites within this region. The sequences of the ten identified sites and the locations of the mutations are listed in Table 3.2.

There is No Strong Consensus Pit-1 Binding Sequence in the Rat GHRH Receptor Promoter

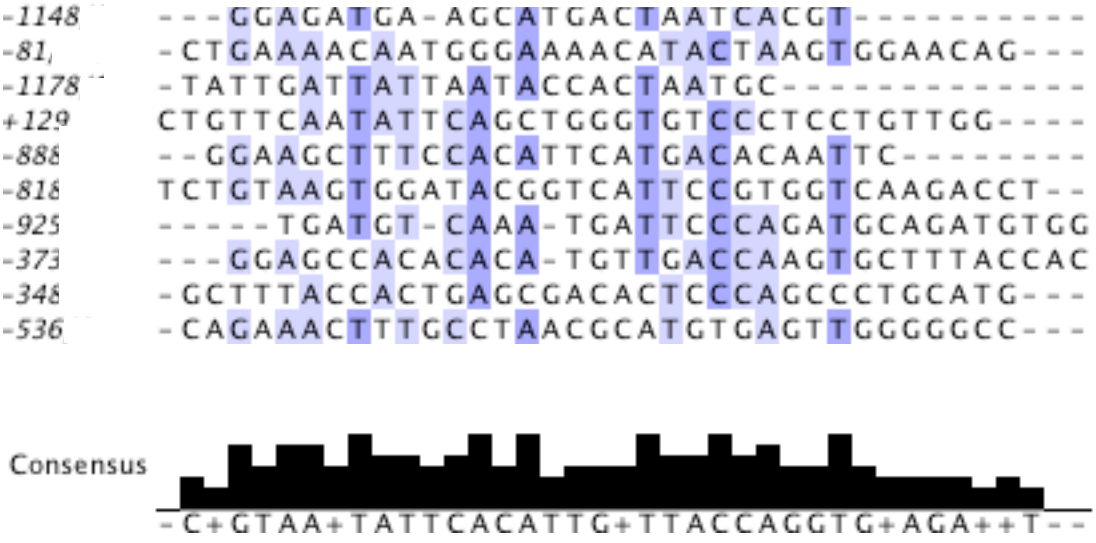
As previously mentioned, no strong consensus binding sequence for Pit-1 has been defined. Pit-1 is known only to bind A/T rich sequences, and reported binding sites differ for nearly all promoters. To determine whether a promoter with many binding sites for this transcription factor itself has a consensus binding site for Pit-1, alignment of each of the identified binding probe sequences was performed using ClustalW (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/; (Thompson et al. 1994)). Alignments were assembled using Jalview to determine consensus residues (Clamp 2004) (Fig. 3.5). Increasing shades of blue represent greater degrees of conservation. Clearly, there is no consensus binding sequence, even for Pit-1 binding sites within this single promoter. In the absence of a consensus binding site, mutational analysis was performed by selecting A/T nucleotides in A/T rich regions of the probes (Table 3.2) and replacing them with guanines, which successfully abolished Pit-1 binding (Fig. 3.4).

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

The ten 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

Figure 3.5: Alignment of Pit-1 Binding Probe Sequences

To determine whether a consensus for Pit-1 binding sites exists within the rat GHRH receptor promoter, the ten sequences identified by binding assays were aligned using ClustalW. Increasing shades of blue represent greater degrees of conservation.


-1148 ---GGAGATGA-AGCATGACTAATCACGT----------
-81, -CTGAAAACAATGGGAAAACATACTAAGTGGAACAG---
-1178 -TATTGATTATTAATACCACTAATGC--------------
+129 CTGTTCAATATTCAGCTGGGTGTCCCTCCTGTTGG----
-888 --GGAAGCTTTCCACATTCATGACACAATTC---------
-818 TCTGTAAGTGGATACGGTCATTCCGTGGTCAAGACCT--
-925 ------TGATGT-CAAA-TGATTCCCAGATGCAGATGTGG
-373 ---GGAGCCACACACA-TGTTGACCAAGTGCTTTACCAC
-348 -GCTTTACCACTGAGCGACACTCCCAGCCCTGCATG---
-536 -CAGAAACTTTGCCTAACGCATGTGAGTTGGGGGCC---
Consensus
-C+GTAA+TATTCACATTG+TTACCAGGTG+AGA++T--

made in initial experiments that different sites seem to bind Pit-1 with different affinities, relative binding affinity was examined more closely. EMSAs were performed by incubating increasing amounts of Pit-1 nuclear extracts, ranging from 0 M to 2000 M, with each of the labeled probes (Fig. 3.6A). EMSA gels were exposed to PhosphorImager screens and arbitrary densitometry units were calculated for each (Fig. 3.6B). Average relative affinities were compared to the affinity of a known Pit-1 binding sequence (Shewchuk et al. 1999), which is referred to as the Pit-1 consensus sequence. Relative affinities for the ten sites are highly variable, with EC50 values ranging from 280.9 M for the +129 to +164 site to greater than 2000 M for six of the sites (Fig. 3.6C). Affinity ranks are listed for comparison (Fig. 3.6C).

An attempt was made to define actual affinities of these sites using recombinant Pit-1; however, the commercially available Pit-1 does not bind even the consensus sequence in EMSA experiments.

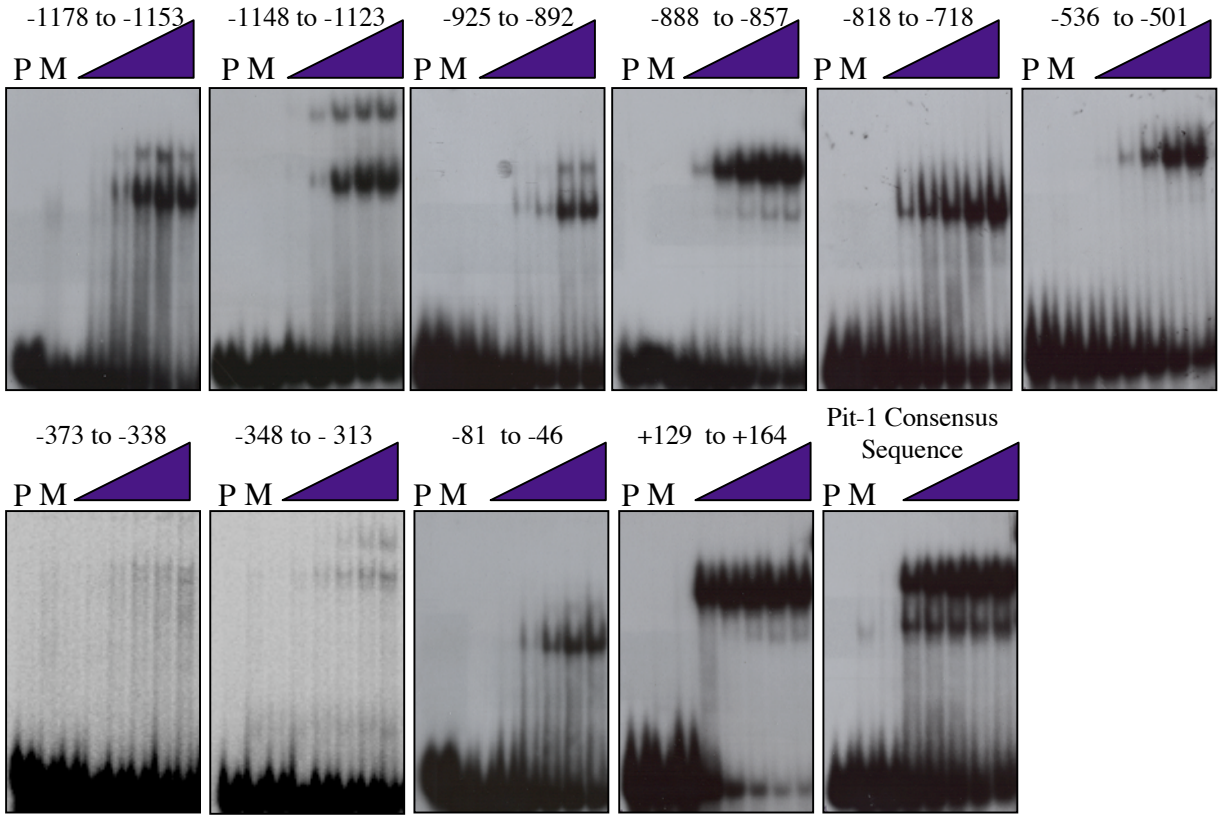
Conservation of Pit-1 Binding Sequences Across Multiple Species

The 1.6kb rat GHRH receptor promoter sequence was entered into Ensembl BLAST (http://www.ensembl.org/Multi/blastview) to look for high-scoring hits among all species for which genome sequence is known. High-scoring hits were defined for mouse, human, chimpanzee, dog and rhesus monkey. These sequences were aligned to the rat promoter sequence using ClustalW (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/; (Thompson et al. 1994)). Alignments were assembled using Jalview to determine consensus residues (Clamp 2004), and the alignments for the Pit-1 binding sites are shown (Fig. 3.7, black boxes). Increasing shades of gray indicate higher degrees of conservation across the species. The relative affinity rank is given for each sequence for comparison. The two lowest-affinity

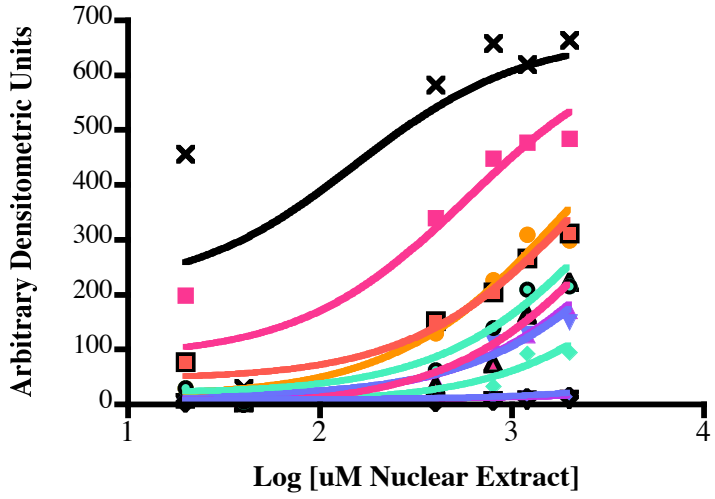
Figure 3.6: Varying Relative Affinities for Pit-1 Among the Ten Binding Sites in the Rat GHRH Receptor Promoter

Relative Pit-1 binding affinities were calculated from EMSAs using increasing amounts of Pit-1 nuclear extract. **A:** In each panel, the first lane, marked "P" is probe without nuclear extract. 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 (1 □g, 5 □g, 10 □g, 20 □g, 30 □g and 50 □g). 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 setting the Pit-1 consensus binding sequence to 100 percent. Percentages are calculated for each probe and represent the average of three independent experiments ± SEM. A relative affinity rank is also provided, with the rank of 1st having the highest affinity and the rank of 10th having the lowest.

A.



B.

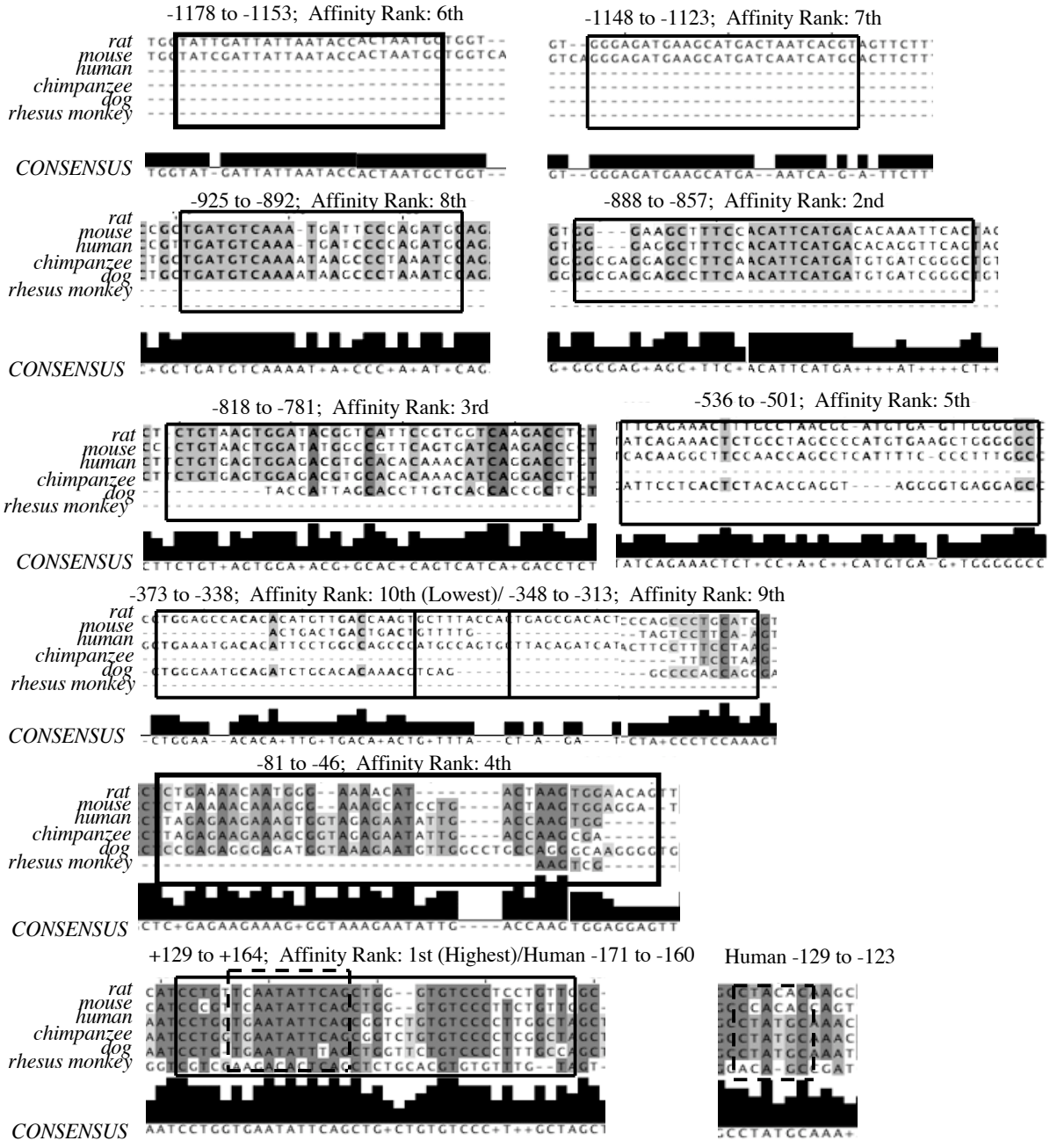


C.

	EC_{50} (□M)	Affinity Rank
Pit-1 Consensus	390.8	-
-1178 to -1153	>2000	6th
-1148 to -1123	>2000	7th
-925 to -892	>2000	8th
-888 to -857	792.1	2nd
-818 to -781	1193	3rd
-536 to -501	>2000	5th
-373 to -338	>2000	10th
-348 to -313	>2000	9th
-81 to -46	1631	4th
+129 to +164	280.9	1st

Figure 3.7: Conservation of Rat GHRH Receptor Promoter Pit-1 Binding Site Sequences Across Multiple Species

The 1.6kb proximal promoter of the rat GHRH receptor was entered into the ENSEMBL BLAST program (http://www.ensebml.org/Multi/blastview) to identify high-scoring hits to other species. High-scoring hits were identified for mouse, human, chimpanzee, dog and rhesus monkey. The corresponding sequences were aligned using the ClustalW program (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/). Alignments were assembled using Jalview to determine consensus residues. Shown are the sequences corresponding to the identified Pit-1 binding sites of the rat GHRH receptor promoter. Percent sequence identity among species is represented by increasing shades of gray for highly conserved residues. The Pit-1 binding sites from the rat GHRH receptor promoter are boxed with a solid line, and those identified in the human GHRH receptor promoter are boxed with a dotted line.


-1178 to -1153; Affinity Rank: 6th
-1148 to -1123; Affinity Rank: 7th
-925 to -892; Affinity Rank: 8th
-888 to -857; Affinity Rank: 2nd
-818 to -781; Affinity Rank: 3rd
-536 to -501; Affinity Rank: 5th
-373 to -338; Affinity Rank: 10th (Lowest)/ -348 to -313; Affinity Rank: 9th
-81 to -46; Affinity Rank: 4th
+129 to +164; Affinity Rank: 1st (Highest)/Human -171 to -160
Human -129 to -123
rat
mouse
human
chimpanzee
dog
rhesus monkey
CONSENSUS

sequences, -373 to –338 and –348 to –313, exhibit very low conservation across multiple species (Fig. 3.7). Interestingly, the sequence with the highest relative affinity for Pit-1, +129 to +164, is also the most highly conserved sequence (Fig. 3.7). The dotted-line boxes represent the previously identified Pit-1 binding sites from the human GHRH receptor promoter (Fig. 3.7 (Iguchi et al. 1999)). The more-5' of these human sites, which contributes most to Pit-1 activation of the human GHRH receptor, is highly conserved and is included within the high-affinity +129 to +164 rat sequence (Fig. 3.7), indicating a possible functional importance for this site in regulating the rat GHRH receptor gene.

Interestingly, this approach yielded no high-scoring hits to non-mammalian species. To directly examine a non-mammalian species, the chicken GHRH receptor promoter was aligned to the rat promoter sequence. Very little sequence conservation between these promoters exists (not shown). When the rat Pit-1 binding site sequences are aligned to the 2kb chicken GHRH receptor promoter sequence individually, some degree of sequence conservation is detected (Fig. 3.8). However, the rat sequences align out of order to the chicken promoter, and six of the rat sequences align to only three different locations in the chicken promoter (Fig. 3.8). Given these results, it is not clear how significantly the chicken promoter sequence is conserved in mammals.

Pit-1 Activation of the Rat GHRH Receptor Promoter

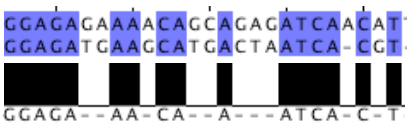
A rat GHRH receptor promoter-luciferase reporter construct containing all ten Pit-1 binding sites (rGHRHR –1673) is not expressed in a non-pituitary cell line, HeLa T4 cells, but is expressed in a Pit-1 expressing pituitary cell line, GH3 cells (Fig. 3.9). In non-pituitary HeLa cells, expression of Pit-1 by transfection leads to activation of the rGHRHR –1673 construct, but Pit-1 activation of this promoter in HeLa cells is about 8-fold lower than in GH3 cells, indicating

Figure 3.8: Pit-1 Binding Site Conservation in Chicken GHRH Receptor Promoter

The ten identified Pit-1 binding site sequences in the rat promoter were aligned individually to the 2kb chicken GHRH receptor promoter using the ClustalW program (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/). Alignments were assembled using Jalview to determine consensus residues. Conserved residues are highlighted in blue.

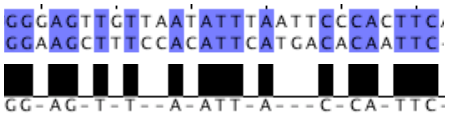
Rat -1178 to -1153
Chicken -120 to -90

Rat -1148 to -1123
Chicken -1200 to -1180

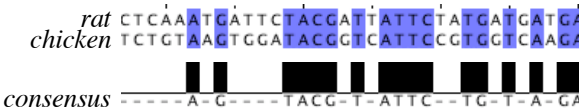


Rat -925 to -892
Chicken -700 to -680

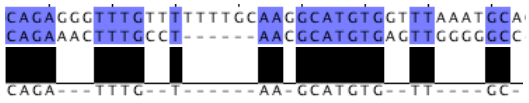
Rat -888 to -857
Chicken -120 to -95



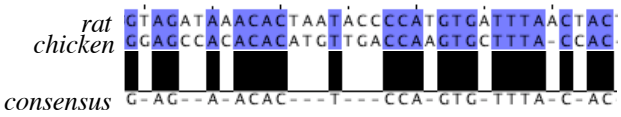
Rat -818 to -781
Chicken -700 to -670



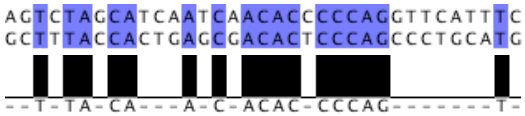
Rat -536 to -501
Chicken -340 to -310



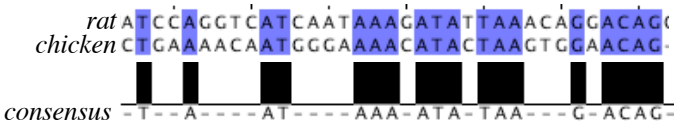
Rat -373 to -338
Chicken -1350 to -1320



Rat -348 to -313
Chicken -1765 to -1735



Rat -81 to -46
Chicken -1690 to -1665



Rat +129 to +164
Chicken -1220 to -1190

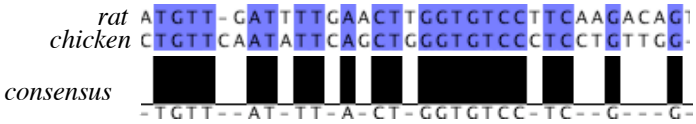
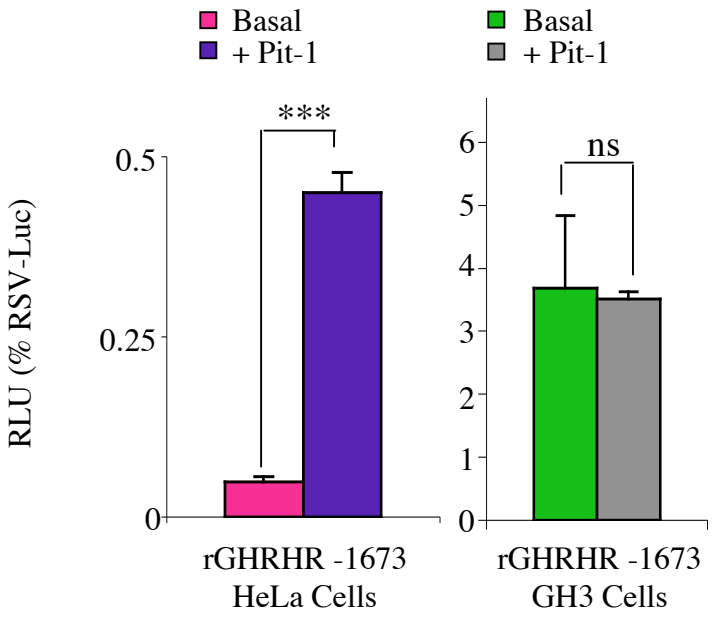


Figure 3.9: Pit-1 Activation of the Rat GHRH Receptor Promoter

Non-pituitary HeLa T4 cells and pituitary GH3 cells were transfected with the 1.6kb Rat GHRH Receptor Promoter-Luciferase construct and luciferase assays were performed to measure promoter activation. In HeLa cells, the promoter is inactive. With the co-expression of Pit-1, the 1.6kb promoter is significantly activated in HeLa cells. Co-expression of Pit-1 does not increase basal promoter activity in pituitary GH3 cells. Data are plotted as a percentage of RSV-Luciferase activity, in an attempt to normalize for different transfection efficiencies between the cell types. Data are representative of at least three independent experiments. Statistical analysis was performed using a t-test (***, $p<0.001$; ns, $p>0.05$).


Basal
+ Pit-1
Basal
+ Pit-1
***
ns
RLU (% RSV-Luc)
0.5
0.25
0
6
5
4
3
2
1
0
rGHRHR -1673
HeLa Cells
rGHRHR -1673
GH3 Cells

that factors in addition to Pit-1 are required for full activation of this promoter (Fig. 3.9). Transfection of a Pit-1 expression construct leads to little or no further activation of the GHRH receptor promoter in GH3 cells. For this reason, the contribution of Pit-1 to regulation of the promoter was examined in HeLa cells, and compared to basal activity in GH3 cells.

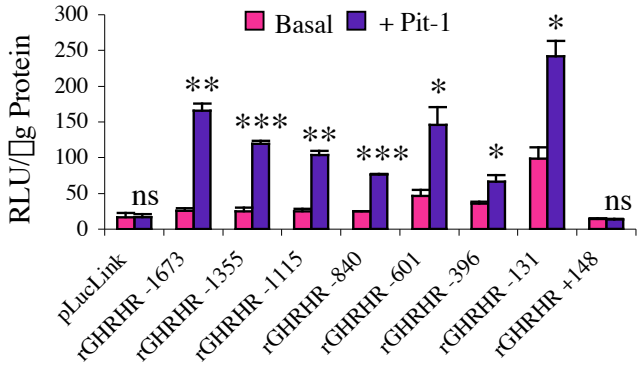
In order to determine where functional Pit-1 binding sites reside, a series of 5'-deletion constructs was analyzed in transient transfection assays. Significant Pit-1 activation of the promoter constructs is observed up to the rGHRHR –131 construct, which still contains the high-affinity, highly conserved +129 to +164 site (Fig. 3.10A). When this site is removed, in the rGHRHR +148 construct, Pit-1 activation is lost (Fig. 3.10A). Examination of fold increase in reporter expression with Pit-1 co-transfection for each of the promoter deletion constructs shows that Pit-1 significantly increases promoter activity for each except the shortest construct which lacks Pit-1 binding sites (Fig. 3.10B). Pit-1 co-expression has the greatest effect on the full –1673 construct, suggesting that multiple binding sites are necessary for full Pit-1 responsiveness of this gene (Fig. 3.10B).

In order to more closely examine the contributions of the identified Pit-1 binding sites, mutated reporter constructs were generated. The mutations were shown to prevent Pit-1 binding to these sites (Fig. 3.4). Each of the mutations was examined relative to the full-length –1673 promoter construct. Mutation of five of the Pit-1 binding sites leads to a significant reduction in promoter activation, relative to the –1673 rGHRHR construct (Fig. 3.10C). 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 co-expression was examined for these mutation constructs. The fold increase is significant for each of the mutations except the +129 to +164 site (Fig. 3.10D),

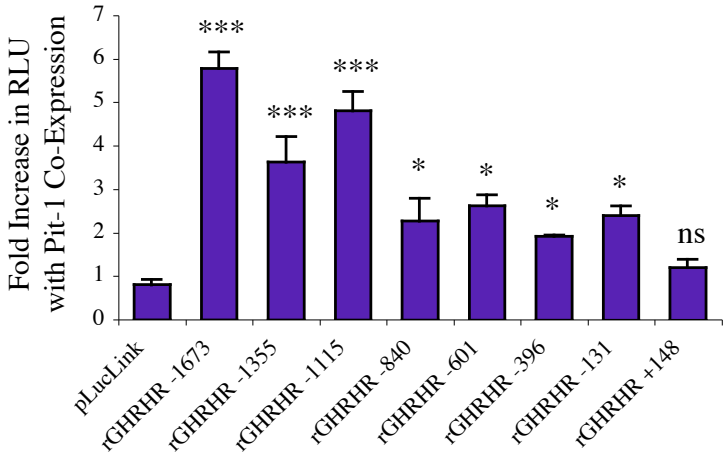
Figure 3.10: Pit-1 Activated Expression of Rat GHRH Receptor Promoter 5'-Deletion and Mutation Luciferase Reporter Constructs in Non-Pituitary HeLa Cells

A: HeLa T4 cells were transfected with 5'-deletion rat GHRH receptor promoter constructs alone or with a co-transfected 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. **B:** The fold increase in reporter activity with Pit-1 co-expression 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 Student's t-test. **C:** Mutation of the Pit-1 binding sites was examined by transfection and luciferase assay in HeLa cells. Data are representative of three independent experiments performed in triplicate. Error bars represent the SEM. Statistical analysis was performed using a Student's t-test. **D:** Fold increase with Pit-1 co-expression is plotted for the wild-type and each of the Pit-1 binding mutation constructs relative to the empty vector. Data represent the average of three independent experiments. Error bars represent the SEM. Statistical analysis was performed using a Student's t-test. (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

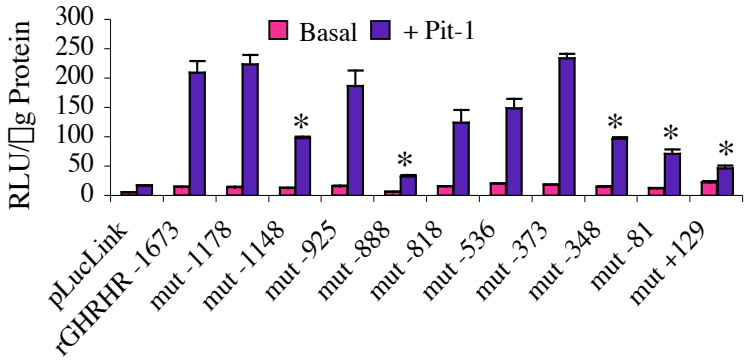
A.


Basal
+ Pit-1
RLU/μg Protein
300
250
200
150
100
50
0
ns
**
***
**
***
*
*
*
ns
pLucLink
rGHRHR -1673
rGHRHR -1355
rGHRHR -1115
rGHRHR -840
rGHRHR -601
rGHRHR -396
rGHRHR -131
rGHRHR +148

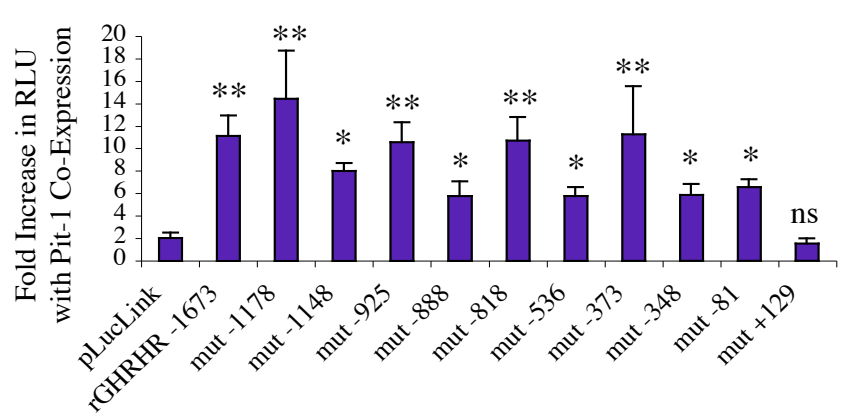
B.


Fold Increase in RLU with Pit-1 Co-Expression
7
6
5
4
3
2
1
0
***
***
***
*
*
*
*
ns
pLucLink
rGHRHR -1673
rGHRHR -1355
rGHRHR -1115
rGHRHR -840
rGHRHR -601
rGHRHR -396
rGHRHR -131
rGHRHR +148

C.


Basal
+ Pit-1
RLU/μg Protein
300
250
200
150
100
50
0
*
*
*
*
*
*
pLucLink
rGHRHR -1673
mut -1178
mut -1148
mut -925
mut -888
mut -818
mut -536
mut -373
mut -348
mut -81
mut +129

D.


Fold Increase in RLU with Pit-1 Co-Expression
20
18
16
14
12
10
8
6
4
2
0
**
**
**
*
**
*
**
*
**
*
*
ns
pLucLink
rGHRHR -1673
mut -1178
mut -1148
mut -925
mut -888
mut -818
mut -536
mut -373
mut -348
mut -81
mut +129

supporting that Pit-1 binding to this site is essential for activating the rat GHRH receptor promoter.

Basal expression of the 5'-deletion and mutation constructs was further examined in pituitary GH3 cells. Interestingly, the basal expression pattern in GH3 cells closely matches the Pit-1-activated expression pattern in HeLa cells (Fig. 3.11A). In GH3 cells, mutation of seven of the Pit-1 binding sites significantly reduces expression relative to the full-length –1673 rGHRHR (Fig. 3.11B). These seven sites include the five mutations that reduced Pit-1-activated expression in HeLa cells, and two additional sites that might represent sites where factors other than, or in addition to, Pit-1 bind and activate this promoter in pituitary somatotroph cells. As in HeLa cells, the +129 to +164 mutation completely abolishes expression of the promoter in GH3 cells (Fig.3.11B). Fig. 3.12 shows the effect of mutation preventing Pit-1 binding to the +129 to +164 site on activation of the promoter in both non-pituitary and pituitary cells. This site is required for Pit-1 activated expression of the receptor in non-pituitary cells and for activation of receptor expression in pituitary cells.

Discussion

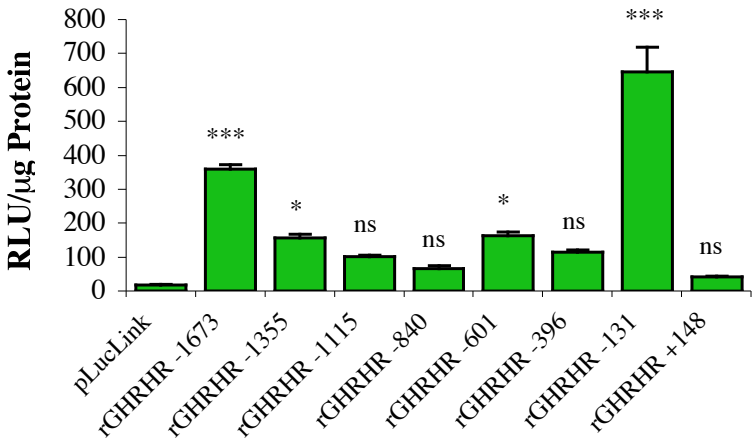
The 1.6kb proximal promoter of the rat GHRH receptor was established as sufficient *in vivo* for appropriate expression in the studies presented in Chapter 2. In this chapter, the promoter was examined in cell culture experiments to determine factors important to achieving cell- and tissue-specific expression.

The cell-specificity of expression driven by the 1.6kb promoter is mimicked in cell culture. Co-expression of Pit-1 leads to activation of this promoter reporter construct in non-pituitary HeLa

Figure 3.11: Expression of Rat GHRH Receptor 5'-Deletion and Mutation Luciferase Reporter Constructs in Pituitary GH3 Cells

A: GH3 cells were transfected with 5'-deletion rat GHRH 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 Student's t-test (ns, $p>0.05$; *, $p<0.05$; ***, $p<0.001$). **B:** Mutation of the Pit-1 binding sites was examined by transfection and luciferase assay in GH3 cells. Data are representative of three independent experiments performed in triplicate. Error bars represent the SEM. Statistical analysis was performed using a Student's t-test (*, $p<0.05$).

A.


RLU/µg Protein
800
700
600
500
400
300
200
100
0
***
*
ns
ns
*
ns
***
ns
pLucLink
rGHRHR -1673
rGHRHR -1355
rGHRHR -1115
rGHRHR -840
rGHRHR -601
rGHRHR -396
rGHRHR -131
rGHRHR +148

B.

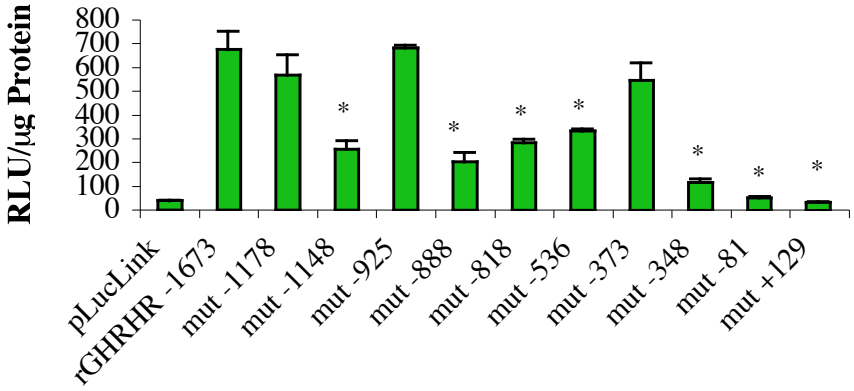
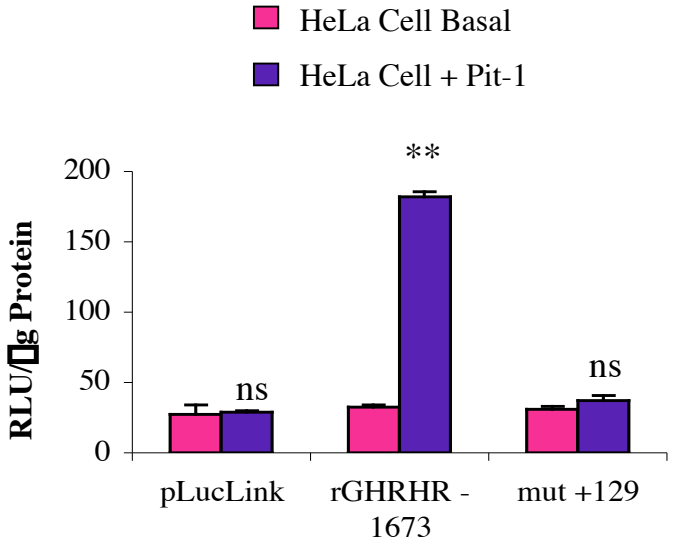

RLU/µg Protein
800
700
600
500
400
300
200
100
0
*
*
*
*
*
*
*
pLucLink
rGHRHR -1673
mut -1178
mut -1148
mut -925
mut -888
mut -818
mut -536
mut -373
mut -348
mut -81
mut +129

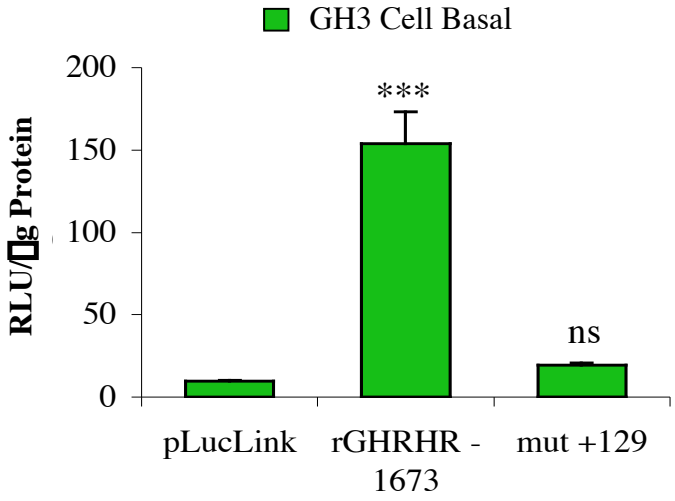
Figure 3.12: Summary of Functional Effects of the +129 to +164 Pit-1 Binding Mutation

Non-pituitary HeLa cells ***(A)*** and pituitary GH3 cells ***(B)*** were transfected with the full-length –1673 rGHRHR construct or the construct containing a Pit-1 binding mutation in the +129 to +164 region (mut +129) and examined by luciferase assay.

A.


HeLa Cell Basal
HeLa Cell + Pit-1
RLU/μg Protein
200
150
100
50
0
**
ns
ns
pLucLink
rGHRHR -1673
mut +129

B.


GH3 Cell Basal
RLU/μg Protein
200
150
100
50
0
***
ns
pLucLink
rGHRHR -1673
mut +129

cells, suggesting an important role for Pit-1 as a tissue-specific factor regulating pituitary-specific expression of this gene. Pit-1 regulation of the 1.6kb rat GHRH receptor promoter was examined with a goal of identifying the functional Pit-1 binding sites.

Ten 25-35 base pair regions were effectively narrowed down using sequentially shorter oligonucleotide probes in EMSAs. Only two of the ten sites correspond to computationally predicted Pit-1 binding sites (Miller et al. 1999), illuminating the lack of consensus for Pit-1 binding sequences. In order to determine whether a single or few sites contributes greatest to Pit-1 activation of the rat GHRH receptor promoter, relative binding affinities and sequence conservation were examined 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 rat GHRH 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 rat GHRH receptor promoter (Nogami et al. 2005). In addition, this site corresponds to one of the human sites identified by Iguchi et al (Iguchi et al. 1999), which contributes greatest 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 (Salvatori et al. 2002). These data confirm that the +129 to +164 site is essential to Pit-1 activation of the GHRH receptor gene. Furthermore, the studies have led to the identification of four additional functional Pit-1 binding sites that contribute to activation of the rat promoter. Of these, the –888 to –857 site contributes greatest

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.

In combination with the data presented in Chapter 2, these data confirm that the 1.6kb promoter is sufficient for tissue- and cell-specific expression of the rat GHRH receptor gene. Pit-1 is an important activator of the 1.6kb promoter. 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, including the TSH gene (Steinfelder et al. 1992), the GH gene (Lira et al. 1993; Shewchuk et al. 1999), and the prolactin gene (Nowakowski and Maurer 1994), which seems to be indicative of the importance of this factor in regulating expression of cell-specific genes in the thyrotroph, somatotroph and lactotroph lineages.

A report on estrogen regulation of the prolactin promoter shows a requirement for multiple Pit-1 binding sites to facilitate estrogen responsiveness (Nowakowski and Maurer 1994), which confers cell-specific expression of this gene. It is likely that the Pit-1 binding sites identified in the rat GHRH 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.

Transcriptional regulation is an important mechanism by which expression of all genes is regulated. Many genes are also regulated post-transcriptionally, which dramatically increases the diversity and levels of gene products from a relatively small number of genes. While Chapters 2 and 3 focus on transcriptional regulation of the GHRH receptor promoter, Chapter 4 transitions to examine a post-transcriptional regulation, alternative splicing, and how splice

variant receptors affect signaling of the GHRH receptor and related receptors in the GH axis.

CHAPTER FOUR

A MECHANISM OF ACTION FOR A DOMINANT NEGATIVE HUMAN GROWTH HORMONE-RELEASING HORMONE RECEPTOR SPLICE VARIANT

Introduction

As discussed in Chapter 1, alternative splicing is an important mechanism by which genes are regulated. After transcription, gene products can be modified to change the structure and function of the resulting protein. Alternative splice variants can be different from the wild-type protein in expression, composition and signaling, and can modify these characteristics of the wild-type receptor. This chapter examines alternative splice variants of the rat and human GHRH receptors.

The GHRH receptor is a seven transmembrane G-protein coupled receptor (Mayo 1992) that, in rat and human, is 423 amino acids in length (Mayo 1992; Gaylinn et al. 1993). The GHRH receptor is a member of the B-III subfamily of G-protein coupled receptors, which includes the secretin/glucagon peptide receptors (Mayo et al. 2000). Alternative splicing in G-protein coupled receptors is one of many emerging mechanisms by which this class of receptors diversifies its activities. Splice variants that result in changes in signaling or protein expression have been identified in many G-protein coupled receptors, such as the GnRH receptor (Zhou and Sealfon 1994; Grosse et al. 1997), the $GABA_B$ receptor (Schwarz et al. 2000), the angiotensin II type 1 receptor (Elton and Martin 2003), and the luteinizing hormone receptor (Nakamura et al. 2004). In a particularly relevant example, several splice variants of the PACAP receptor, which is closely related to the GHRH receptor, have been identified that differ in their signal transduction properties (Spengler et al. 1993; Nicot and DiCicco-Bloom 2001). These PACAP receptor splice variants differ in the third intracellular loop of the protein (Pisegna and Wank 1993; Spengler et al. 1993; Nicot and DiCicco-Bloom 2001), which is important to G protein interactions (O'Dowd et al. 1988) and consequently represents an excellent target for altering the signaling properties of the protein.

Alternative splicing of the GHRH receptor in the rat and human occurs at the intron/exon boundary 3' of exon 11, and results in distinct predicted protein products with differential signaling capacities. In the rat, the alternative splicing results in inclusion of 41 amino acids in the third intracellular loop (Miller et al. 1999). This long isoform of the rat receptor is capable of binding ligand, but incapable of signaling through cAMP production (Miller et al. 1999). The human splice variant that occurs at the same intron/exon junction leads to inclusion of intronic sequence that has an in-frame premature stop codon, and this mRNA encodes a protein truncated just before the sixth transmembrane domain (Hashimoto et al. 1995; Tang et al. 1995). This human splice variant was originally identified in GH-producing pituitary adenomas, though it is also present at lower levels in normal pituitaries (Hashimoto et al. 1995; Tang et al. 1995). The identification of a splice variant of the GHRH receptor present in acromegalic cancer patients unveils a potential role for alternative splicing in response to changing physiological or pathophysiological conditions. One of the initial reports identifying this splice variant suggests that the truncated splice variant cannot signal through the cAMP pathway, but that its expression has no effect on wild-type receptor signaling (Hashimoto et al. 1995). A later report suggests that the truncated receptor acts as a dominant negative repressor of the wild-type receptor, as measured by cAMP accumulation (Motomura et al. 1998).

In order to further investigate the role of these GHRH receptor splice variants, experiments were performed to assess the functional consequences of co-expressed wild-type and splice variant GHRH receptors. Existing HA epitope-tagged long rat GHRH receptor and HA epitope-tagged wild-type human GHRH receptor constructs were used and a FLAG epitope-tagged truncated human GHRH receptor construct was cloned. Expression and signaling of the wild-type and alternative splice variant receptors were examined. Results show that the variant

receptors of the two species, which encode very different protein products, have very different signaling effects. In a study performed with a summer undergraduate student, Bradford Paul, we show that the long rat GHRH receptor has no effect on signaling of the wild-type receptor. Examination of the truncated human receptor reveals a dominant negative effect on wild-type receptor signaling. These data led to further examination of cellular localization and ligand binding of the truncated GHRH receptor alone, and of the HA-tagged wild-type GHRH receptor in the presence and absence of the truncated splice variant receptor. The data show a novel mechanism for GHRH receptor splice variant function, in which the truncated receptor can form a complex with the wild-type receptor and inhibit normal GHRH binding, thereby altering the signaling activity of the wild-type receptor.

In addition, a study performed with undergraduate Bradford Paul looking at the effects of truncated GHRH receptor expression on wild-type growth hormone secretagogue (GHS) receptor signaling is described. Previous work from our laboratory has shown that co-expression of the GHS receptor leads to potentiation of wild-type GHRH receptor signaling (Cunha and Mayo 2002), strongly suggesting an interaction between these two types of receptors in the GH axis. Further indicative of an interaction between these receptors is that a short form of the GHS receptor leads to a dominant negative signaling effect on the wild-type GHRH receptor (Cunha 2002). The functional interaction of these receptors was examined further by looking at the effects of co-expression of the truncated human GHRH receptor on signaling of the wild-type GHS receptor.

Materials and Methods

Generation of the Flag Epitope-Tagged Truncated Human GHRH Receptor

The full-length human GHRH receptor plasmid was used as a template to PCR a FLAG epitope-tagged receptor with an engineered premature stop codon at amino acid 325, which corresponds to the predicted stop codon in the human splice variant that has been identified in pituitary adenomas, using oligonucleotide primers ((5'-GCT CTA GAC CTT GTC ATC GTC GTC CTT GTA GTC CCA ATA CTG AGA CTG-3'and 5'-GCG GTA CCC ATG GAC CGC CGG ATG-3') (Integrated DNA Technologies, Coralville, IA)). PCR products were cloned into pcDNA3 (Invitrogen, Carlsbad, CA) downstream of the T7 promoter using the Kpn I and Xba I sites. The full-length human GHRH receptor and long rat GHRH receptor with influenza hemagglutinin (HA) tags had been previously cloned in the laboratory (DeAlmeida and Mayo 1998; Miller et al. 1999).

Vaccinia Infection/Transfection System

The vaccinia virus infection/transfection system was used to overexpress receptor expression constructs in HeLa T4 cells, as described in Chapter 3.

Immunofluorescence Localization of Epitope-Tagged Receptors

HeLa T4 cells were cultured on 12mm round glass coverslips in 24-well plates and transfected as described with 500ng/well DNA. Cells were washed with 1X PBS and fixed in 1% paraformaldehyde for 30 minutes at 4°C. The cells were washed twice in PBS and incubated for 4-6 hours at 4°C with 1 g/ml of the HA-specific 12CA5 ascites fluid (a gift from Dr. Robert

Lamb, Northwestern University) or the anti-M2 monoclonal antibody against the flag epitope (Sigma Co., St. Louis, MO) in PBS/0.1% BSA containing 0.1% saponin to permeabilize cells. After extensive washing in PBS, the cells were incubated for 1 hour at room temperature with 2 g/ml fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) in PBS/0.1% BSA containing 0.1% saponin. Coverslips were mounted in VectaShield mounting media with DAPI (Vector Laboratories, Burlingame, CA), and images were taken at 40X with a Leica DM5000 fluorescence microscope, using OpenLab software (Improvision, Lexington, MA).

Measurement of Intracellular Cyclic AMP Levels

HeLa T4 cells were transfected using the vaccinia infection/transfection system with 2 g DNA per well in 12-well plates, or stably transfected cells were plated to confluency in 12-well plates. Cells were washed twice with 1X PBS and incubated in serum-free media with 0.1mM isobutylmethylxanthine (IBMX) for 20 minutes at 37°C to inhibit phosphodiesterase. Cells were then treated with 10^{-7} M hormone (Peptides International, Louisville, KY), or incubated with media alone for unstimulated control conditions, at 37°C for 20 minutes. Cells were lysed in 150 l cold 0.1N HCl. The lysates were collected and neutralized in an equal volume of 50mM Tris-HCl, pH 8.0 with 4mM EDTA. 25 l of neutralized lysates were used in a competitive protein-binding assay to measure intracellular cAMP levels (Tovey et al. 1974). [8-^{3}H] cAMP (Amersham, Piscataway, NJ) was used as a tracer in this assay. The assays were performed with triplicate samples, and a linear standard curve was performed in each experiment. Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

Metabolic Labeling of Transfected Cells and Immunoprecipitation of Epitope-Tagged Receptors

HeLa T4 cells were grown in 6-well plates, transfected with 4 g total DNA per well for 12-15 hours using the vaccinia transfection system, starved in cysteine/methionine-deficient DMEM (Gibco BRL, Grand Island, NY) for 30 minutes, and labeled with 50 Ci/well Trans [^{35}S] Label (ICN Biomedical Inc., Irvine, CA) for 3 hours at 37°C in 5% CO_2. The cells were harvested in 1X PBS, pelleted, and resuspended in 400 l RIPA buffer (150mM NaCl, 50mM Tris-HCl, pH 7.5, 1% Igepal CA-630 (Nonidet P 40), 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate) containing 0.1mM phenylmethylsulfonylfluoride, 1 g/ml leupeptin, and 1 g/ml aprotinin. Cells were lysed by a series of five freeze-thaw cycles in a dry ice-ethanol bath. The lysates were centrifuged for 10 minutes to pellet cellular debris, and the supernatant was divided into two fractions. 1 g/ml of the HA-specific 12CA5 ascites fluid or the anti-M2 monoclonal antibody against the FLAG epitope was added. Immunoprecipitation went overnight at 4°C on a hematology mixer. 30 l of a 50% suspension of protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ) in PBS was added to the tubes, and the incubation was continued for 1 hour. The beads were washed eight times with 500 l cold RIPA buffer and resuspended in 30 l 2X SDS-PAGE sample buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100mM dithiothreitol, 0.1% bromophenol blue). The samples were boiled for 5 minutes, then separated by SDS-PAGE using a Tris-glycine buffer with the Benchmark Pre-stained Protein Ladder (Invitrogen, Carlsbad, CA) as a size marker. Gels were fixed in 20% methanol/7% acetic acid for 30 minutes, saturated with glacial acetic acid (two five-minute washes), impregnated with 22% (w/v) 2, 5 diphenyl-ox-axole in acetic acid for 45 minutes, dried, and exposed to Kodak X-

OMAT AR film (Rochester, NY). Quantification of expression was performed using the Image J program provided by the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Generation of Stable Cell Lines

Human embryonic kidney 293 cells were transfected with the FLAG tagged truncated hGHRH receptor construct alone or with equivalent amounts of the HA tagged full-length hGHRH receptor construct, both of which are cloned in pcDNA3, which is neomycin-resistant. Transfections were performed using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and 10□g DNA per 10cm plate. Transfected cells were selected in 400mg/L G418 (Gibco BRL, Grand Island, NY), and individual clones were isolated and proliferated for analysis. Previously generated HPR9B cells were used for full-length receptor-expressing cells (Mayo 1992).

Detection of RNA Expression in Stable Cell Lines

RNA was isolated from stable cell lines using the RNeasy kit (Qiagen, Valencia, CA). RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase in the presence of 1mM deoxynucleosidyltriphosphates and random hexameric oligonucleotides. Complementary DNA was amplified by PCR incorporating [^{32}P] radiolabeled-dCTP. Human ribosomal protein L19 primers were used as an internal control (5'-CTG AAG GTG AAG GGG AAT GTG-3' and 5'-GGA TAA AGT CTT GAT GAT CTC-3'). Full-length and truncated hGHRH receptor expression were detected by RT PCR with a shared 5' primer (5'-CGT GGG TGA GCT GCA AAC TGG-3') and one of two 3' primers specific to each sequence (5'-CTC ACC TCT TGG TTG AGGG AAG-3' or 5'-GTC CTT GTA GTC CCA ATA CTG-3'). PCR products were separated on 5 percent polyacrylamide gels by electrophoresis. Dried gels were

exposed to Kodak X-OMAT AR film (Rochester, NY) and PCR products were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Fluorescence Activated Cell Sorting

Stable cells were grown to confluency in 6cm plates. Cells were fixed in 1% paraformaldehyde in PBS for 30 minutes at 4°C. Cells were blocked in 1%BSA/0.02% sodium azide in PBS for 30 minutes at 4°C for 30 minutes. Cells were washed three to five times in PBS containing 0.02% sodium azide. Primary antibody incubation was performed at 4°C for 4 hours using a polyclonal antibody to the N-terminus of the GHRH receptor (a gift from Dr. Bruce Gaylinn, The University of Virginia, Charlottesville, VA (Gaylinn et al. 1999)) (1:750 dilution). Cells were washed three to five times in PBS containing 0.02% sodium azide followed by secondary antibody incubation (donkey anti-rabbit conjugated to fluorescein isothiocyanate at a 1:500 dilution) for 30 minutes at 4°C. Following three washes in PBS containing 0.02% sodium azide, cells were scrape-collected in 50mM EDTA/1X PBS and run through a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) in Dr. Robert Lamb's laboratory at Northwestern University. 10,000 cells were counted and analysis was performed using the Cell Quest program (BD Biosciences). Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

Measurement of Ligand Binding

Assays to measure binding to membrane fractions were performed on stably transfected cells grown in 10cm plates. Cells were washed with PBS and homogenized by 20 strokes with a Teflon-glass homogenizer on ice in 50mM Tris-HCl, pH 7.4, 5mM $MgCl_2$, 2mM EGTA, and

0.1mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged for 5 min at 100 x *g*, and the supernatant was recentrifuged at 4000 x *g* for 10 min. Membrane pellets were resuspended in binding buffer (25mM HEPES, pH 7.4, 50mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 1mg/ml bacitracin, and 0.1% BSA). Approximately 50 g membrane protein was used for each reaction in a volume of 300 l with 75pM [^{125}I] hGHRH (1-44)-amide (Amersham Biosciences, Piscataway, NJ) in the presence or absence of unlabeled hormone at a concentration of 10^{-6}M. Binding reactions were performed at 25°C for 60 min and were terminated by centrifugation for 10 min at 4°C. Membrane pellets were washed with binding buffer and the bound radioligand was measured using a Micromedic 4/600 Plus Automatic Gamma Counter (Micromedic, Horsham, PA). Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

Measurement of Intracellular Calcium Release

Intracellular calcium mobilization in response to hormone stimulation was measured by fluorimetry. Transfected HeLa T4 cells were trypsinized and washed with DMEM containing 5% FBS. The cells were pelleted at 400 x g for 1 minute, then washed with loading buffer (145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 10mM HEPES, 10mM glucose, 1mM $CaCl_2$, 1% BSA containing 147 g/ml probenecid). Cells were resuspended in 1.5ml loading buffer with 3.5 g Fluo-3AM (Molecular Probes, Eugene, OR) and incubated on a hematology mixer for 30 minutes. Excess calcium indicator dye was removed by washing the cells with loading buffer. Cells were resuspended in flux buffer (loading buffer without $CaCl_2$ and BSA) and an ISS PC1 photon counting spectrofluorometer was used to measure calcium release. Cells were excited at

515nm and the intensity of emission at 530nm was determined. Before the hormone was added, basal emission of the cells was recorded for 1 minute. The results indicate intensity of emission at 530nm per second.

Results

Splice Variant GHRH Receptor Isoforms in Rat and Human

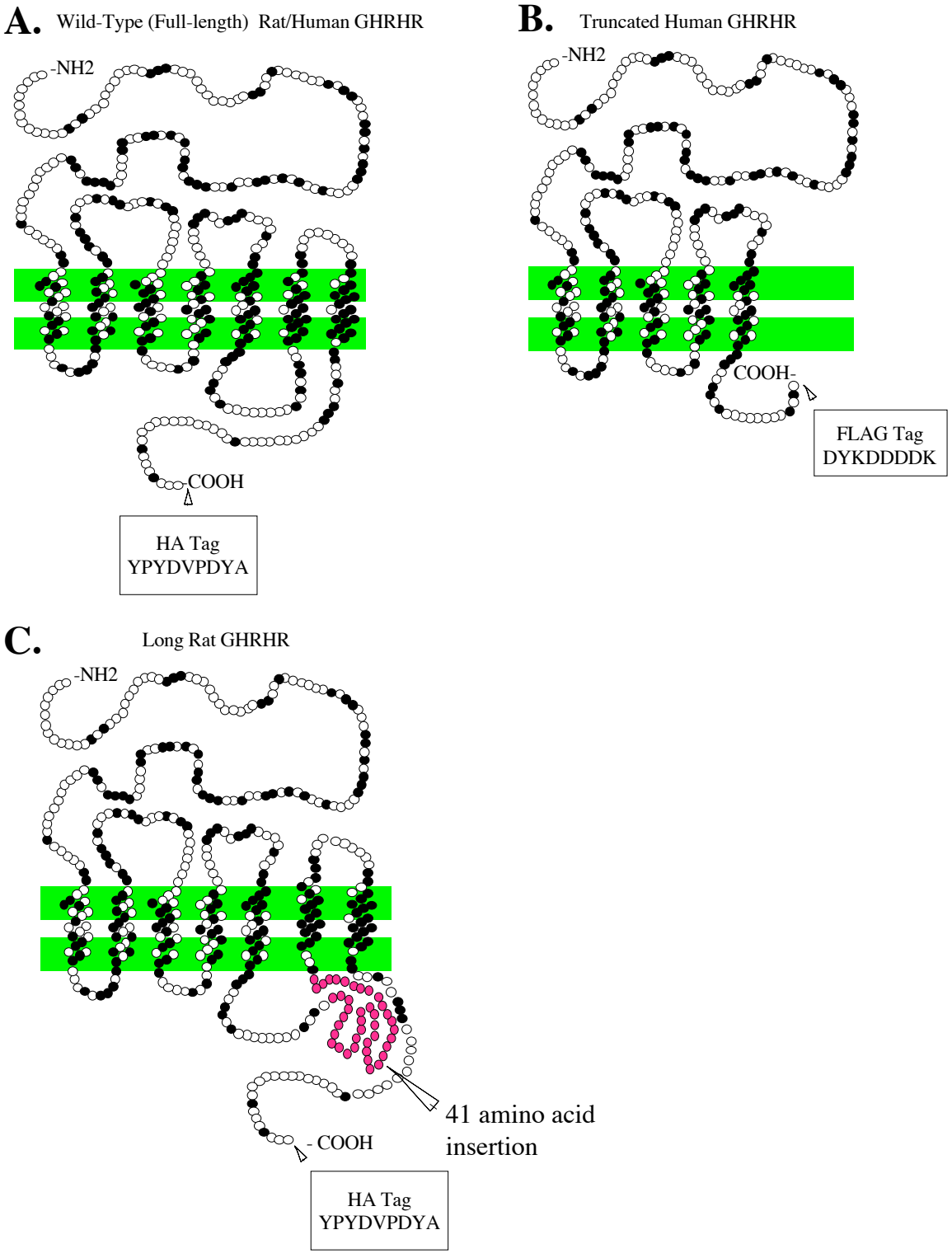
The wild-type human and rat GHRH receptors are 423 amino acids in length. Alternative splicing 3' of exon 11 leads to insertion of normally intronic sequence in both species. In the rat, this alternative splicing leads to a 41 amino acid insertion in the third intracellular loop of the receptor to generate a protein of 464 amino acids. In the human, there is an in-frame premature stop codon resulting in a truncation in the third intracellular loop, generating a protein of 325 amino acids. The variant isoforms are shown schematically in Fig. 4.1. The current chapter focuses on examination of signaling effects of these splice variant receptors on wild-type receptors in the GH axis.

Expression and Signaling of Wild-Type and Long Rat GHRH Receptors

As mentioned, previous work from our laboratory had shown that the long rat GHRH receptor cannot signal through the cAMP pathway. With a summer undergraduate student, Bradford Paul, the long rat GHRH receptor was examined in co-transfection experiments to determine whether it has a dominant negative effect on wild-type receptor signaling. HeLa cells expressing either the wild-type rat GHRH receptor, the long rat GHRH receptor, or co-expressing equal amounts of both receptor isoforms were examined for signaling through the

Figure 4.1: Schematic Representation of Wild-Type and Splice Variant Rat and Human GHRH Receptors

A: The full-length GHRH receptor is epitope tagged with influenza HA on the C terminus. **B:** The truncated human GHRH receptor is tagged with a C-terminal FLAG-epitope. C: The long rat GHRH receptor has a 41-amino acid insertion in the third intracellular loop (pink residues) and is tagged with a C-terminal HA tag. Conserved residues of the closely related VIP receptor are shaded in *black*.


A.
Wild-Type (Full-length) Rat/Human GHRHR
-NH2
COOH
HA Tag
YPYDVPDYA
B.
Truncated Human GHRHR
-NH2
COOH-
FLAG Tag
DYKDDDDK
C.
Long Rat GHRHR
-NH2
- COOH
41 amino acid
insertion
HA Tag
YPYDVPDYA

cAMP pathway. First, expression of the receptor constructs was verified by indirect immunofluorescence (Fig. 4.2A). Expression of the wild-type and long forms of the receptor is localized similarly in the transfected cells. Measurement of intracellular cAMP levels was examined for cells expressing either the wild-type receptor, the long splice variant receptor, or co-expressing both wild-type and long rat GHRH receptors. Stimulation of transfected cells with GHRH led to a dramatic increase in cAMP production for cells expressing the wild-type receptor, as expected (Fig. 4.2B). Cells expressing the long rat GHRH receptor did not produce cAMP in response to GHRH stimulation, consistent with published data from our laboratory (Miller et al. 1999). Interestingly, when cells co-expressing the wild-type and long GHRH receptors were stimulated with GHRH, a significant increase in cAMP is observed. The levels of cAMP produced in cells co-expressing both receptor isoforms is not different from the levels produced in cells expressing only the wild-type form, indicating that this splice variant does not exert a dominant negative effect on signaling of the wild-type receptor.

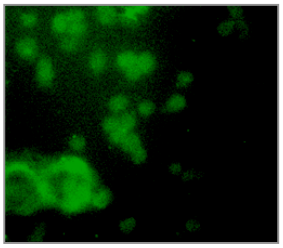
Expression of The Full-Length and Truncated Human GHRH Receptor Splice Variants in Transiently Transfected Cells

Alternative splicing in the human GHRH receptor produces a receptor protein truncated at amino acid 325 (Fig. 4.1). The truncated receptor is identical to the full-length receptor up to amino acid 325, where a premature in-frame stop codon results in a protein terminated just before the sixth transmembrane domain. Given the structural differences in splice variants in rat and human, the truncated human GHRH receptor was also examined to determine whether it can signal through the cAMP pathway and whether its expression affects signaling of the wild-type receptor. In order to develop a system in which expression of the wild-type and truncated

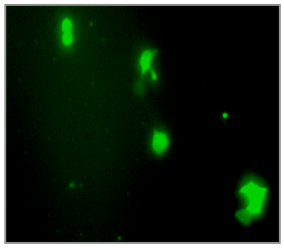
Figure 4.2: Expression and Signaling of Wild-Type and Long Isoforms of the Rat GHRH Receptor

A: Both wild-type and long rat GHRH receptors were transfected into HeLa T4 cells and expression was examined via immunofluorescence detecting the intracellular HA epitope tag. **B:** Cells expressing the wild-type GHRH receptor, the long GHRH receptor, or co-expressing both receptors were stimulated with GHRH and examined for cAMP production. Experiments were performed by summer undergraduate student Bradford Paul.

A.

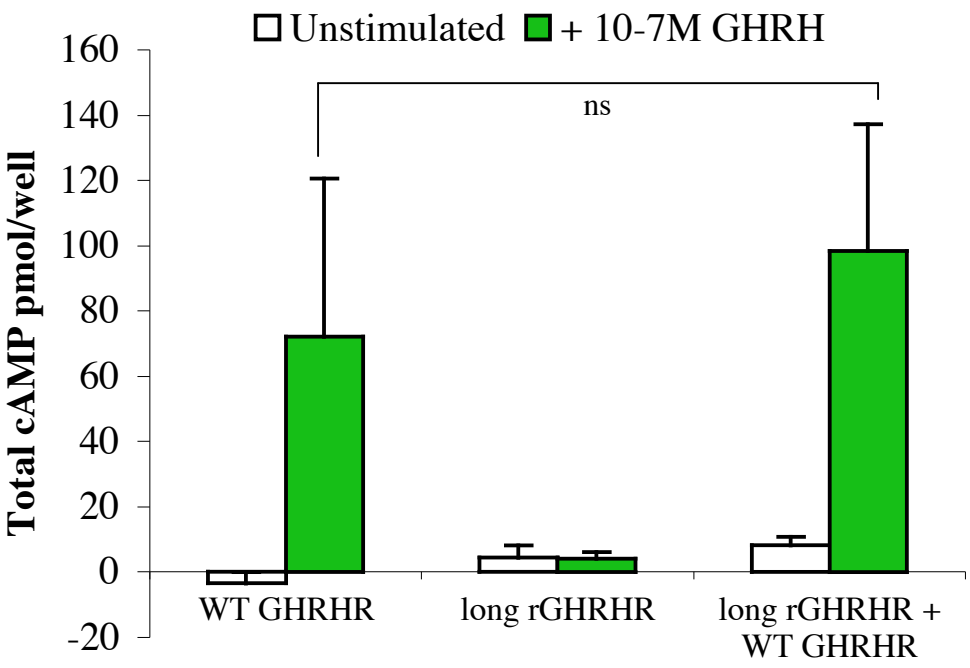


□ HA
Long rGHRH-R



□ HA
Wild-Type hGHRH-R

B.



GHRH receptors could be independently examined, the truncated GHRH receptor was cloned with a carboxyl-terminal FLAG epitope tag (DYKDDDDK). The full-length GHRH receptor was previously cloned with a carboxyl-terminal influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA).

In immunoprecipitation of metabolically labeled transfected cells, a specific band corresponding to the size of the full-length GHRH receptor is pulled down with the antibody recognizing the HA epitope tag (Fig. 4.3A, lane 3), and a specific band corresponding to the predicted size of the truncated GHRH receptor is pulled down with the anti-FLAG antibody (Fig. 4.3A, lane 6). Furthermore, both constructs are localized similarly in transfected HeLa T4 cells, as shown by immunofluorescence (Fig. 4.3B). In permeabilized cells, diffuse expression of the receptors on the cell surface and in intracellular compartments, including the endoplasmic reticulum and Golgi apparatus, is detected. Expression in intact cells cannot be examined because the epitope tags are intracellular. Extracellular epitope tags have been shown to disrupt receptor function (DeAlmeida and Mayo 1998), and were therefore not used in this study.

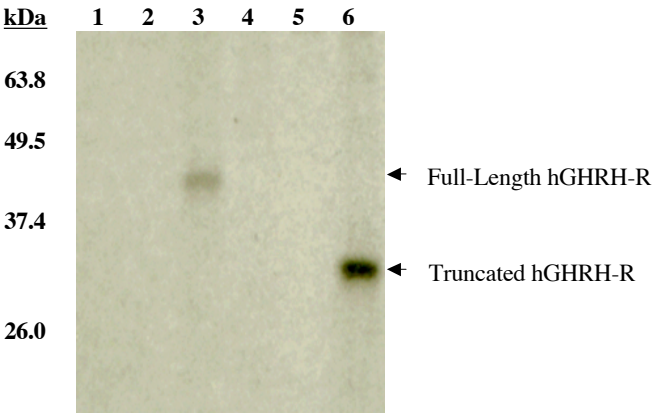
Effect of the Truncated Receptor on GHRH Receptor Signaling

To determine the signaling properties of the truncated GHRH receptor splice variant, basal and GHRH-stimulated cAMP production were measured in cells transfected with the full-length GHRH receptor, the truncated GHRH receptor, or both receptors. Cells transfected with the full-length GHRH receptor have measurable cAMP that increases approximately six-fold upon stimulation with 10^{-7}M GHRH (Fig. 4.4A). In contrast, cells transfected with the truncated GHRH receptor show no increase in cAMP levels when stimulated with GHRH, indicating that the truncated GHRH receptor is not capable of signal transduction through the cAMP pathway

Figure 4.3: Expression of Full-length and Truncated Human GHRH Receptors

A: Immunoprecipitation of metabolically labeled vaccinia-infected/transfected HeLa T4 lysates. Cells were either mock transfected (lanes 1 and 2), transfected with the HA-tagged full-length GHRH receptor (lanes 3 and 4), or transfected with the FLAG-tagged truncated GHRH receptor (lanes 5 and 6) and immunoprecipitated using either the 12CA5 monoclonal antibody (□HA) to the HA epitope tag or the anti-M2 antibody (□FLAG) to the FLAG epitope tag and separated by SDS-PAGE on a 10% gel. **B:** Immunofluorescence localization of epitope tagged full-length and truncated human GHRH receptors. Because the receptor constructs are C-terminally tagged, the cells were permeabilized with 0.1% saponin to detect the receptors using the □HA or □FLAG antibodies. Data are representative of three independent experiments.

A.



Full hGHRH-R			+	+		
Trunc hGHRH-R					+	+
HA Ab	+		+		+	
FLAG Ab		+		+		+

B.

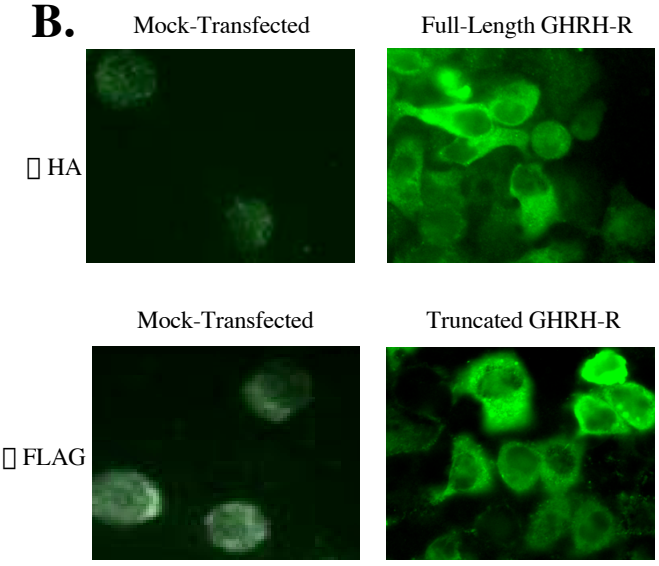
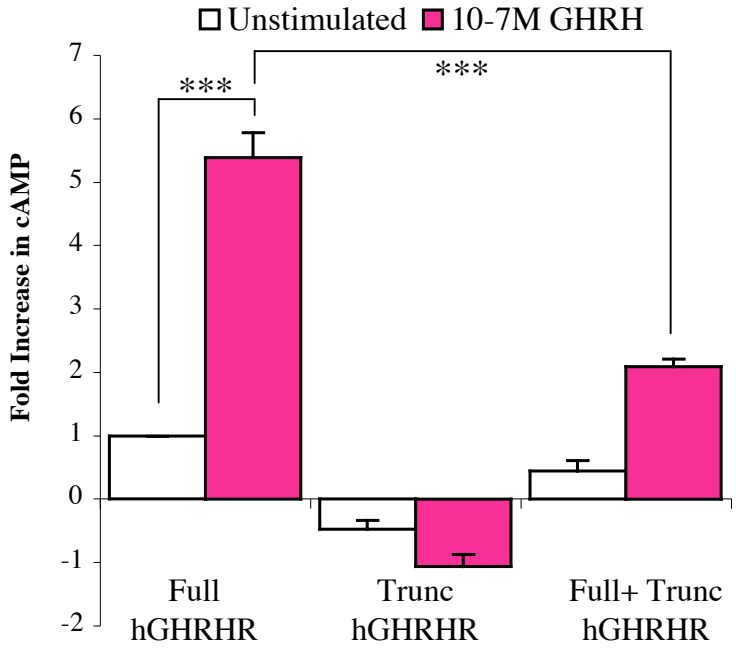


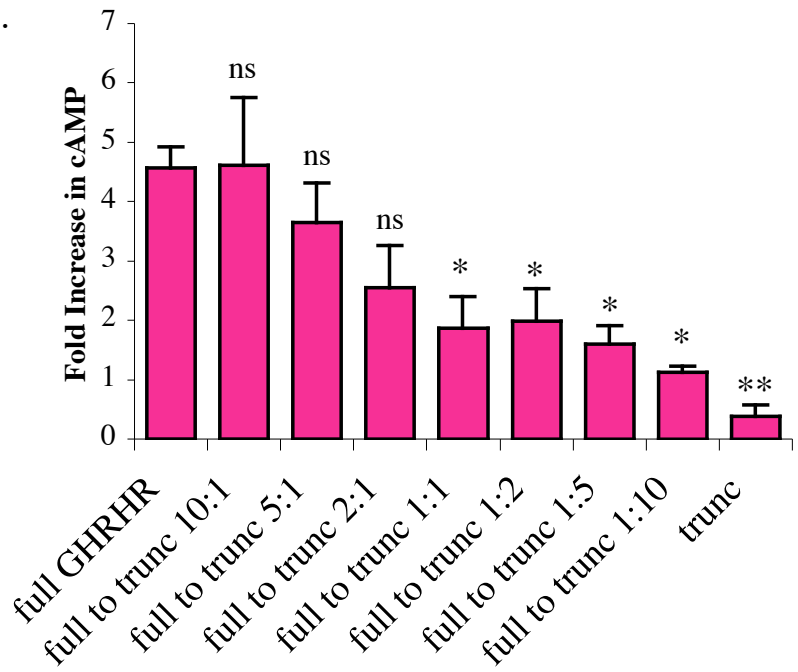
Figure 4.4: Stimulation of cAMP Production by GHRH in Cells Expressing Full-length and Truncated Human GHRH Receptors

A: Lysates from vaccinia-infected/transfected HeLa T4 cells were used to measure basal and GHRH-stimulated cAMP levels in cells expressing either the full-length receptor, the truncated receptor, or coexpressing equivalent amounts of the full-length and truncated receptors. Shown is the average fold increase in cAMP production from nine experiments with triplicate samples in each experiment. *Error bars* represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and GHRH stimulation (***, $p<0.001$). **B:** Cells were transfected with varying doses of full-length or truncated receptors, and cAMP assays were performed. Shown is the average fold increase in cAMP production from four experiments with triplicate samples in each experiment. Statistical analysis was performed using a t-test comparing all values to the value for GHRH-stimulated full length GHRHR (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$). For both panels, the values from unstimulated cells transfected with the full-length GHRH receptor were used as basal, which was set equal to one.

A.


Unstimulated
10-7M GHRH
Fold Increase in cAMP
7
6
5
4
3
2
1
0
-1
-2
***
***
Full hGHRHR
Trunc hGHRHR
Full+ Trunc hGHRHR

B.


Fold Increase in cAMP
7
6
5
4
3
2
1
0
ns
ns
ns
*
*
*
*
**
full GHRHR
full to trunc 10:1
full to trunc 5:1
full to trunc 2:1
full to trunc 1:1
full to trunc 1:2
full to trunc 1:5
full to trunc 1:10
trunc

(Fig. 4.4A). When the truncated GHRH receptor is co-transfected with the full-length GHRH receptor, GHRH-stimulated cAMP signaling by the full-length receptor is repressed by about 60 percent, indicating that the truncated GHRH receptor acts as a dominant negative inhibitor (Fig. 4.4A). The effect on signaling of the full-length receptor is dependent on the dose of co-transfected truncated receptor (Fig. 4.4B). The dominant negative signaling effect is lost when full-length receptor is expressed at a two-to-one or higher ratio with truncated receptor. Further, the reduction in cAMP signaling is greater with increasing ratios of truncated receptor expression (Fig. 4.4B).

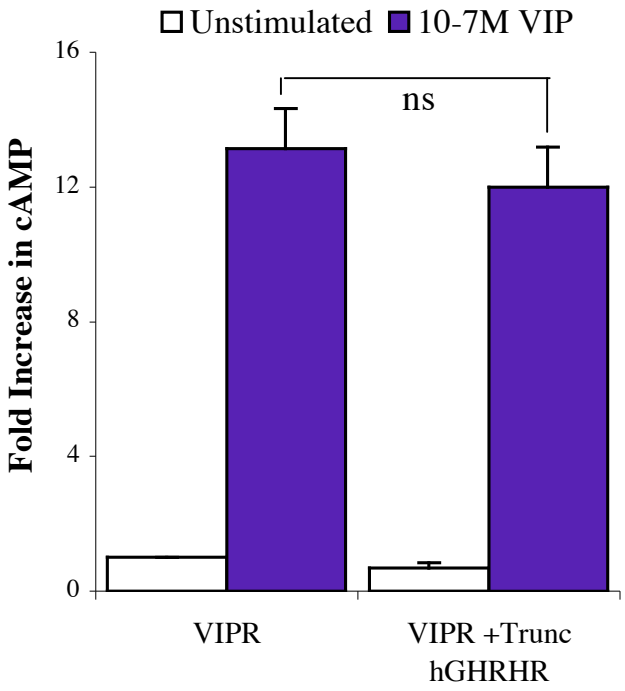
In order to determine the specificity of the dominant negative effect of the truncated GHRH receptor, signaling of the VIP receptor, a member of the same family of G-protein coupled receptors with 42 percent identity to the GHRH receptor, was examined when co-expressed with the truncated GHRH receptor. The wild-type VIP receptor produces about a thirteen-fold increase in cAMP levels when stimulated with 10^{-7}M VIP (Fig. 4.5). When the truncated GHRH receptor is co-transfected with the wild-type VIP receptor, cAMP levels are increased about twelve-fold upon 10^{-7}M VIP stimulation (Fig. 4.5), which is not significantly different from the wild-type VIP receptor alone. These results suggest that the signaling effect of the truncated GHRH receptor is specific to the full-length GHRH receptor.

Co-Expression of Full-length and Truncated GHRH Receptors

To investigate the mechanism of the dominant negative signaling effect, lysates from metabolically labeled cells transfected with the full-length and truncated GHRH receptor constructs were immunoprecipitated, using both anti-HA and anti-FLAG antibodies. To examine the hypothesis that decreased expression of the full-length receptor is responsible for

Figure 4.5: Stimulation of cAMP Production by VIP in Cells Expressing VIP and Truncated Human GHRH Receptors

Lysates from vaccinia-infected/transfected HeLa T4 cells were used to measure basal and VIP-stimulated cAMP levels in cells expressing either the VIP receptor or equivalent amounts of the VIP receptor and truncated GHRH receptor. Data represent the average fold increase in cAMP production from nine experiments with triplicate samples in each experiment. The values from unstimulated cells transfected with the VIP receptor were used as basal, which was set equal to one. *Error bars* represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and VIP stimulation (ns, $p>0.05$).

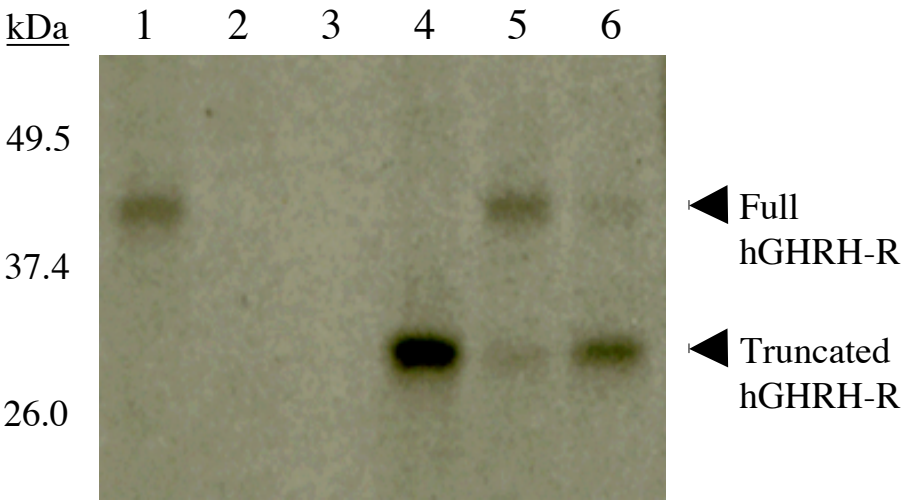

Unstimulated
10-7M VIP
Fold Increase in cAMP
16
12
8
4
0
ns
VIPR
VIPR +Trunc hGHRHR

the observed reduction in cAMP signaling, the expression level of the full-length GHRH receptor was examined when the truncated GHRH receptor is co-expressed. The bands corresponding to the full-length receptor are approximately of equal intensity, whether the full-length receptor is transfected alone, or co-transfected with the truncated GHRH receptor (Fig. 4.6, lanes 1 & 5). When the truncated GHRH receptor is co-transfected, the band corresponding to the full-length receptor is 94 percent the intensity of the band that is pulled down from cells transfected with the full-length receptor alone, indicating that the dominant negative signaling effect is not caused by a change in the amount of full-length GHRH receptor protein expression.

In order to test whether the truncated GHRH receptor interacts with the full-length GHRH receptor, co-immunoprecipitation experiments were performed. Immunoprecipitation of one receptor also brings down the second when both are co-expressed, indicating that the two receptors form a complex. When co-transfected lysates are immunoprecipitated with the HA antibody, 29 percent of the truncated receptor is pulled down with the full-length receptor. Similarly, when co-transfected lysates are immunoprecipitated with the FLAG antibody, 31.6 percent of the full-length receptor is pulled down with the truncated receptor (Fig. 4.6, lanes 5 & 6). Formation of the complex is ligand-independent, as the cells in the experiment were unstimulated. To test whether ligand stimulation alters the extent of complex formation, immunoprecipitation of lysates from stimulated versus unstimulated cells was performed. When cells are stimulated with GHRH, there is no significant difference in the amount of receptor involved in complex formation. In unstimulated cells 31.8 percent of the full-length GHRH receptor is pulled down with the antibody to the truncated receptor. In GHRH stimulated cells 28.8 percent of the full-length GHRH receptor is pulled down with the antibody to the truncated receptor. Similarly, in unstimulated cells 29 percent of the truncated GHRH receptor is pulled

Figure 4.6: Expression of Full-length and Truncated Human GHRH Receptors in Co-transfection Experiments

Lysates from metabolically labeled vaccinia-infected/transfected HeLa T4 cells were immunoprecipitated with either the 12CA5 monoclonal antibody to the HA epitope tag or the anit-M2 antibody to the FLAG epitope tag, and separated by SDS-PAGE on a 10% gel. Lanes 1 and 2 are lysates from cells transfected with the full-length GHRH receptor. Lanes 3 and 4 are lysates from cells transfected with the truncated GHRH receptor. Lanes 5 and 6 are lysates from cells cotransfected with equal amounts of full-length and truncated GHRH receptors. Data are representative of three independent experiments.



Full hGHRH-R	+	+			+	+
Trunc hGHRH-R			+	+	+	+
□ HA Ab	+		+		+	
□ FLAG Ab		+		+		+

down with the antibody to the full-length receptor, while in stimulated cells 29.3 percent of the truncated receptor is pulled down with the antibody to the full-length receptor (data not shown).

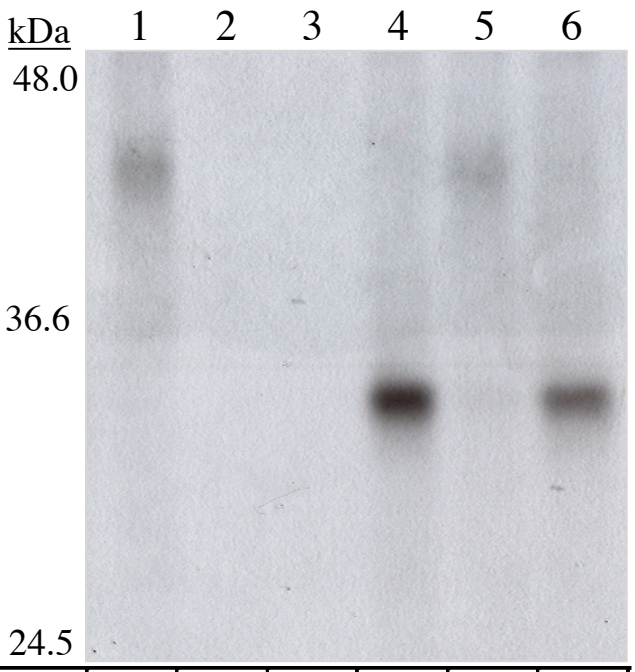
As a control, immunoprecipitations on lysates from metabolically labeled cells transfected with the truncated GHRH receptor and the VIP receptor, which is epitope tagged with HA, were performed. Bands corresponding to multiple glycosylation states of the VIP receptor are immunoprecipitated with the anti-HA antibody (Fig. 4.7, lane 1), and a specific band corresponding to the size of the truncated GHRH receptor is detected by immunoprecipitation with the anti-FLAG antibody (Fig. 4.7, lane 4). In co-expressing cells, no complex formation is detected between the wild-type VIP receptor and the truncated GHRH receptor (Fig. 4.7, lanes 5 & 6). These data are consistent with the signaling studies and again suggest a specific interaction between the truncated GHRH receptor and the full-length GHRH receptor.

Expression and Signaling of Full-length and Truncated GHRH Receptors in Stably Transfected Cell Lines

To investigate the specific mechanism by which this complex exerts its dominant negative effect on signaling of the full-length GHRH receptor, it was desirable to examine cell surface expression by fluorescence-activated cell sorting (FACS). Because preliminary experiments revealed that FACS was not sensitive enough to detect expression in transiently transfected cells, stable cell lines were generated to examine cell surface localization and ligand binding. Cells expressing full-length (HPR9B), truncated (TGR4), or both (FTGR10) receptor constructs were analyzed for RNA expression (Fig. 4.8). The clones used for analysis express the receptors at high levels, and the co-expressing FTGR10 cells express equivalent levels of both receptor mRNAs. To confirm the dominant negative signaling effect of the truncated

Figure 4.7: Expression of Wild-type VIP and Truncated Human GHRH Receptors in Co-transfection Experiments

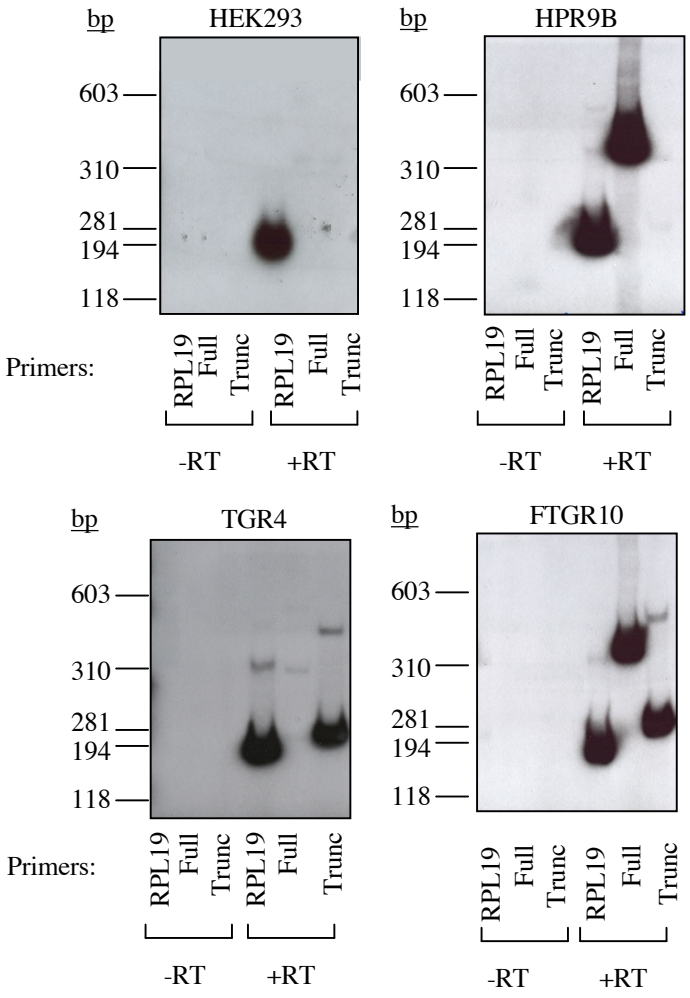
Using metabolic labeling of vaccinia-infected/transfected HeLa T4 cells and SDS-PAGE as described, lanes 1 and 2 are lysates from cells transfected with the wild-type VIP receptor. Lanes 3 and 4 are lysates from cells transfected with the truncated receptor. Lanes 5 and 6 are lysates from cells cotransfected with the wild-type VIP receptor and the truncated GHRH receptor at a two-to-one ratio to normalize protein expression. Data are representative of three independent experiments.



rVIP-R	+	+			+	+
Trunc hGHRH-R			+	+	+	+
□ HA Ab	+		+		+	
□ FLAG Ab		+		+		+

Figure 4.8: Expression of Full-length and Truncated Human GHRH Receptors in Stably Transfected Cells

RNA was isolated from stable cell lines and used in RT-PCR to examine expression of GHRH receptor constructs. HPR9B cells express the full-length GHRH receptor, TGR4 cells express the truncated GHRH receptor, and FTGR10 cells express both the full-length and truncated GHRH receptors. Primers amplifying either the full-length receptor (Full) or the truncated receptor (Trunc) or control primers to ribosomal protein L19 (RPL19) were used, as described in Materials and Methods. Data are representative of three independent experiments.


bp
HEK293
603
310
281
194
118
Primers:
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
HPR9B
603
310
281
194
118
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
TGR4
603
310
281
194
118
Primers:
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
FTGR10
603
310
281
194
118
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT

receptor, stable cell lines were used in a cAMP measurement assay. Untransfected HEK293 cells do not produce cAMP in response to GHRH stimulation (Fig. 4.9). HPR9B cells, which express only the full-length receptor, show a four-fold increase in cAMP upon GHRH stimulation (Fig. 4.9). The truncated receptor does not signal through the cAMP pathway in response to GHRH in stably transfected TGR4 cells, and acts as a dominant inhibitor of full-length receptor signaling in co-expressing FTGR10 cells (Fig. 4.9). The stable cell lines were subsequently used to examine the mechanism of the dominant negative signaling effect of the truncated GHRH receptor.

Cell Surface Localization of the Full-length and Truncated GHRH Receptors

Cell surface localization of the variant receptor expressed alone and co-expressed with the wild-type receptor was examined in stably transfected cell lines using FACS. The full-length GHRH receptor is expressed on the cell-surface, as shown by a shift in fluorescence in HPR9B cells compared to untransfected HEK293 cells (light gray line) using an antibody that recognizes the N-terminus of the GHRH receptor (Fig. 4.10A). The same antibody recognizes the truncated GHRH receptor, which is also detected on the cell-surface of TGR4 cells (Fig. 4.10B). When the full-length and truncated GHRH receptors were co-transfected in FTGR10 cells, cell-surface expression was also detectable (Fig. 4.10C), as expected, though it is not possible to differentiate full-length from truncated receptors on the cell surface because it is not possible to place epitope tags in the N-terminus, as N-terminal tags disrupt receptor function (DeAlmeida and Mayo 1998). Quantification of FACS experiments shows that the percent of cells expressing surface receptors is not significantly different for TGR4 cells expressing the splice variant or FTGR10 cells co-expressing both receptors (Fig. 4.10D). Similarly, there is no significant

Figure 4.9: Stimulation of cAMP Production by GHRH in Stably Transfected Cells Expressing Full-length and Truncated Human GHRH Receptors

Lysates from stably transfected cells were used to measure basal and GHRH-stimulated cAMP levels in cells expressing either the full-length receptor alone, the truncated receptor alone, or equivalent amounts of the full-length and truncated GHRH receptors. Data are representative of three independent experiments with triplicate samples in each experiment. *Error bars* represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and GHRH stimulation (**, $p<0.01$; ***, $p<0.001$).

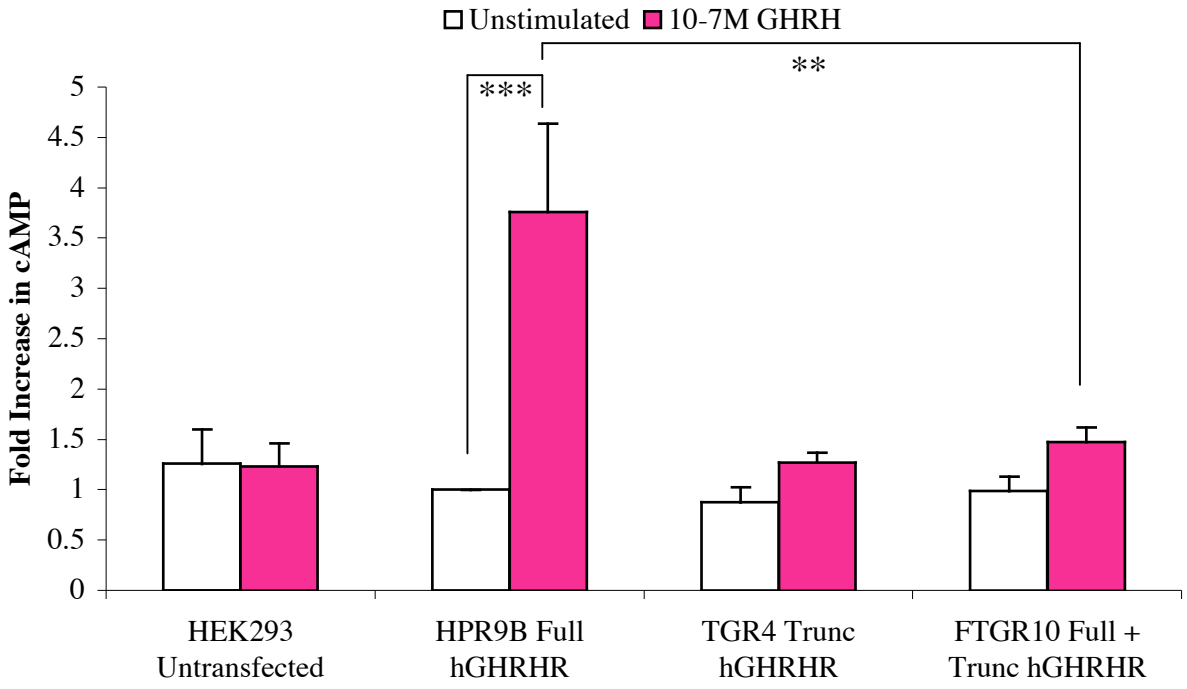
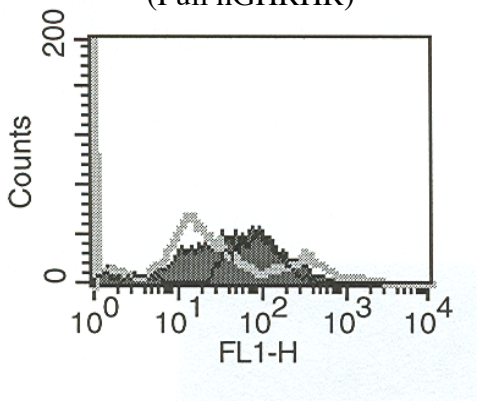

Unstimulated
10-7M GHRH
***
**
Fold Increase in cAMP
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
HEK293 Untransfected
HPR9B Full hGHRHR
TGR4 Trunc hGHRHR
FTGR10 Full + Trunc hGHRHR

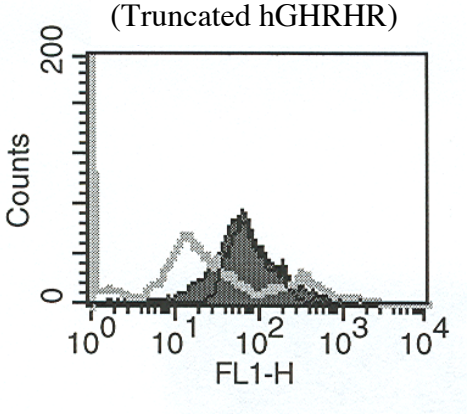
Figure 4.10: Cell Surface Localization of Truncated and Full-length Human Receptors in Stably Transfected Cells

A-C: Stably transfected cells were fixed in 0.1% paraformaldehyde and incubated with a primary polyclonal antibody to the GHRH receptor followed by secondary antibody conjugated to FITC. Cells were then run through a flow cytometer for FACS analysis. Data are representative of three independent experiments. The x-axis represents intensity of the fluorescein fluorophor for each cell. **D:** Quantification of cells expressing surface receptors and average fluorescence is shown. Data represent the average of three independent experiments. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and either the percentage of cells expressing surface receptor or average mean fluorescence. For percent cells expressing surface receptor as well as fluorescence intensity, there is no significant difference for all conditions ($p>0.05$).

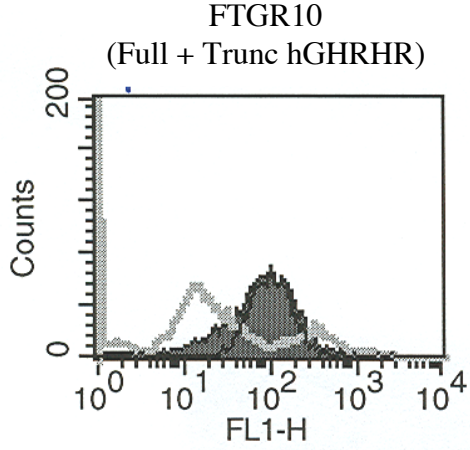
A.



B.



C.



D.

	HPR9B	**TGR4**	**FTGR10**
Average % Cells Expressing Surface Receptor (±SEM):	43.64 ± 2.93	58.2 ±2.3	45.02 ±9.48
Average Mean Fluorescence (±SEM):	109.47 ±11.68	108.42 ±16.06	74.6 ±17.57

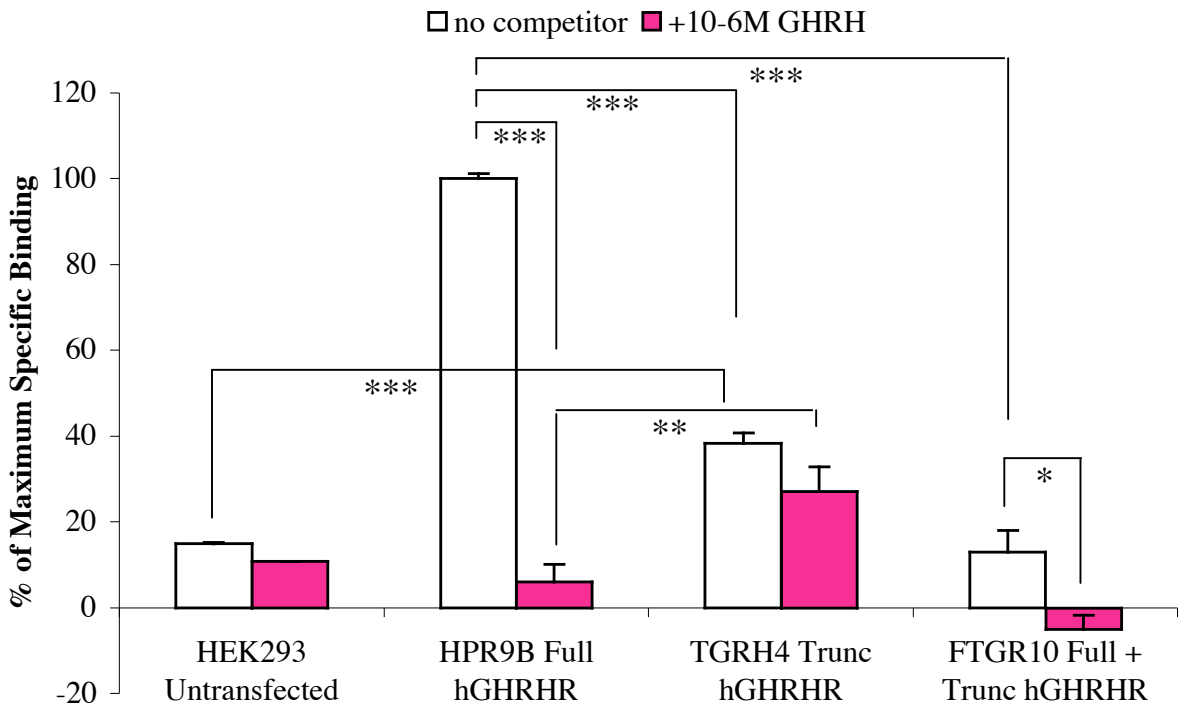
difference in mean fluorescence per cell (Fig. 4.10D), which is an index of receptor density. These data indicate that equivalent numbers of receptors reach the cell surface of cells expressing the full-length receptor and cells expressing the truncated receptor or both receptors. Because the antibody detects all receptors in the FTGR10 co-expressing cells and the truncated and full-length receptors are expressed at equivalent levels in these cells, it is not possible to determine the ratio of the two species on the cell surface. It is possible that approximately half of each receptor species reaches the cell surface, or that only the truncated receptor reaches the cell surface. Since both receptor isoforms localize to the cell surface, ligand binding in these stable cell lines was examined.

Ligand Binding to Full-length and Truncated GHRH Receptors

Membrane fractions were isolated from each stable cell line and incubated with iodinated GHRH in the presence or absence of 10^{-6}M unlabeled GHRH. Untransfected HEK293 cells bind a small amount GHRH non-specifically, probably through the VIP receptor (DeAlmeida and Mayo 1998) (Fig. 4.11). HPR9B cells expressing the full-length receptor bind GHRH, which is fully competed in the presence cold GHRH (Fig. 4.11). The TGR4 cell line, expressing the truncated receptor, shows significantly higher binding levels in the absence of competitor than those seen in the HEK293 cells, but binding is not competed with cold GHRH (Fig. 4.10). These results are perhaps indicative of variable non-specific binding or binding with a slow off-rate. The FTGR10 cell line that co-expresses the wild-type and truncated GHRH receptors clearly shows reduced binding compared to HPR9B cells expressing the full-length receptor, though cold GHRH still significantly competes iodinated ligand binding in FTGR10 cells (Fig. 4.11). The effective abolishment of GHRH binding in co-expressing cells indicates that the

Figure 4.11: GHRH Binding to Full-length and Truncated Human GHRH Receptors in Stably Transfected Cells

Cell membrane fractions were incubated with 75pM ^{125}I-labeled GH-releasing factor in the presence or absence of 10^{-6} M cold GHRH competitor and collected for counting on a □-counter. In cells expressing only the full-length receptor, cold GHRH significantly reduces iodinated GHRH binding ($p<0.001$). In cells coexpressing full-length and truncated receptors, cold GHRH significantly reduces iodinated GHRH binding ($p<0.05$), but overall binding levels are significantly reduced with respect to full-length receptor alone ($p<0.001$). Data represent the average of three independent experiments. Error bars represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and cold GHRH competition (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).


no competitor
+10-6M GHRH
% of Maximum Specific Binding
120
100
80
60
40
20
0
-20
***
***
***
***
**
*
HEK293 Untransfected
HPR9B Full hGHRHR
TGRH4 Trunc hGHRHR
FTGR10 Full + Trunc hGHRHR

predominant mechanism for the dominant negative signaling effect of the truncated GHRH receptor involves an inability to bind ligand.

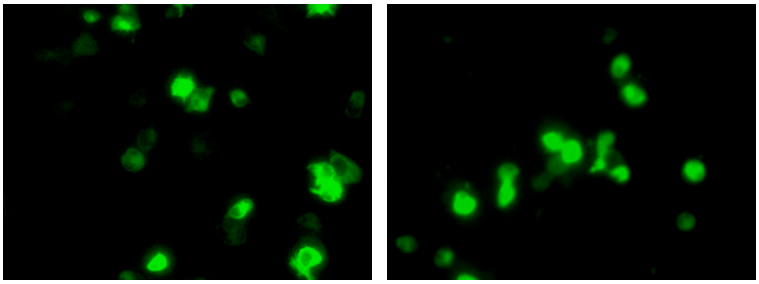
Expression and Signaling of GHS and Truncated GHRH Receptors

To further evaluate the effects of variant receptor isoforms on wild-type receptor signaling, the truncated human GHRH receptor was examined to determine whether it affects signaling of the GHS receptor. Because activation of the GHS receptor synergizes with GHRH receptor activation, and because this synergy is lost in the presence of a splice variant GHS receptor, we considered the possibility of a similar effect for the GHRH receptor on signaling of the GHS receptor. HeLa cells expressing the wild-type GHS receptor, the truncated GHRH receptor, or co-expressing both receptors were first examined by undergraduate student Bradford Paul for expression of the constructs. Immunofluorescence shows that both receptors are expressed with similar cellular localizations in these cells (Fig. 4.12A). Bradford Paul examined transfected cells for intracellular calcium mobilization, an indicator of GHS receptor activation. Cells expressing the GHS receptor have low basal emission at 530nm, that is stimulated by the addition of Ghrelin, an endogenous GHS (Fig. 4.12B, first panel). A spike in the readings occurs upon the addition of hormone because of light entering the machine. This initial spike does not reflect a change in calcium signaling. In cells expressing the truncated GHRH receptor, stimulation with Ghrelin does not increase intracellular calcium mobilization, as expected (Fig. 4.12B, second panel). Cells co-expressing the wild-type GHS receptor and truncated GHRH receptor also do not exhibit a Ghrelin-induced increase in intracellular calcium mobilization (Fig. 4.12B, bottom panel), revealing that the truncated GHRH receptor exerts a dominant negative effect on signaling of the GHS receptor, as it does on the wild-type GHRH receptor.

Figure 4.12: Expression and Signaling of the GHS and Truncated Human GHRH Receptors

A: Expression of the GHS receptor and truncated GHRH receptor was evaluated by immunofluorescence using an antibody to the HA or FLAG tag, respectively. **B:** Measurement of intracellular calcium mobilization in response to Ghrelin stimulation for cells expressing the wild-type GHS receptor, the truncated GHRH receptor, or co-expressing both receptors. The initial spike in the readings upon Ghrelin stimulation is the product of light entering the machine when Ghrelin was added and does not reflect a change in calcium signaling. Experiments were performed by summer undergraduate student Bradford Paul.

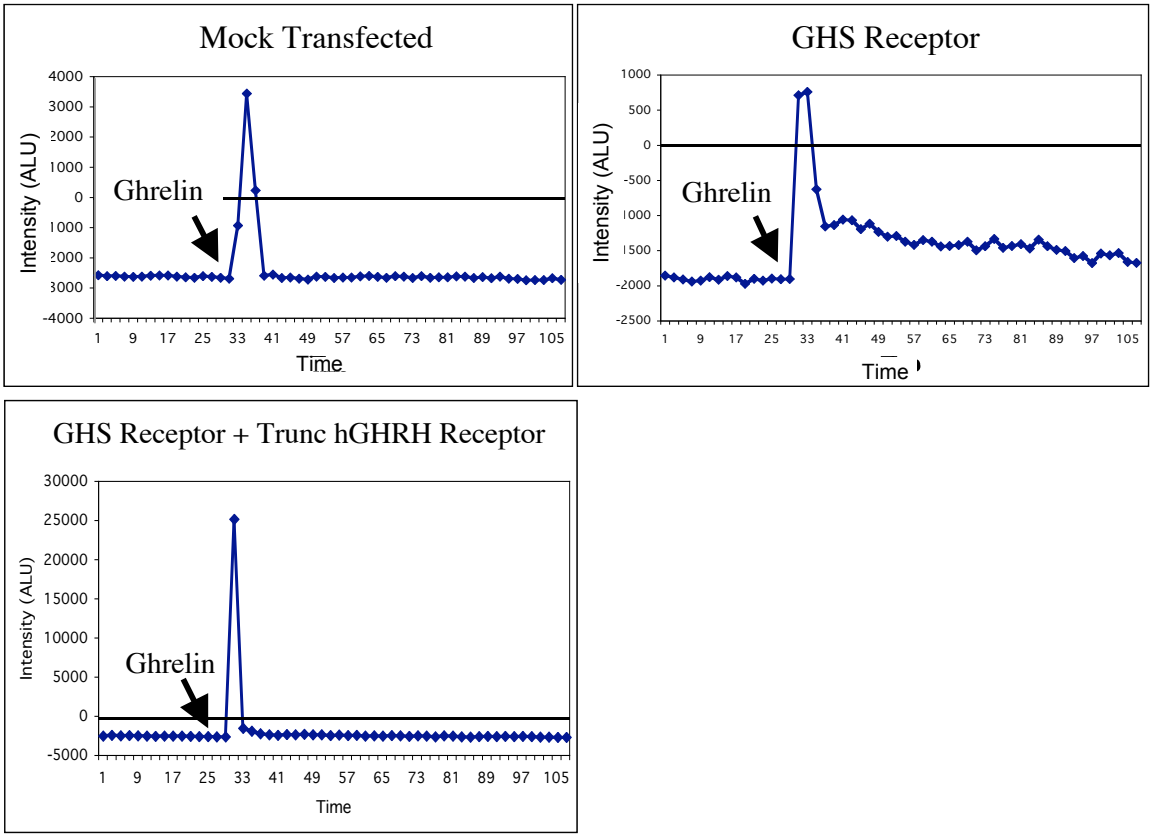
A.



□ HA
Wild-Type GHSr

□ FLAG
Truncated GHRHr

B.



Discussion

The discovery of splice variant receptors that have diverse signaling activities indicates further complexity in the way that GHRH regulates the production and release of growth hormone. While the long rat receptor is incapable of signaling through the cAMP pathway, it does not have a dominant negative effect on signaling of the wild-type receptor. The truncated human receptor does exert a dominant negative signaling effect. Given this effect, the truncated receptor was further examined to determine a mechanism of action. The characterization of this major splice variant of the human GHRH receptor lends insight into the role that this isoform of the receptor may play in normal development and in pathological conditions. In addition, elucidating the mechanism of how this splice variant receptor affects signaling of the wild-type receptor has revealed a possible new role for oligomerization in normal functioning of the wild-type GHRH receptor.

Examination of the signaling properties of the truncated GHRH receptor indicates that the splice variant receptor is unable to signal through the cAMP pathway. That the truncated GHRH receptor is incapable of cAMP signaling is not surprising, given that the splice variant receptor has an incomplete third intracellular loop and lacks the cytoplasmic tail, which have been shown to be essential for proper G-protein coupling in other G-protein coupled receptors (O'Dowd et al. 1988), and lacks the third extracellular loop, which is involved in high-affinity GHRH binding and receptor activation (DeAlmeida 2000).

The dominant negative activity that the truncated GHRH receptor exerts on the wild-type receptor is intriguing. To establish whether the effect was specific to the GHRH receptor, signaling of a closely related G-protein coupled receptor, the VIP receptor, was examined, and it was determined that the truncated GHRH receptor has no effect on signaling of this close family

member, which is 42 percent identical to the GHRH receptor. These data indicate that the truncated GHRH receptor has some specific effect on the full-length GHRH receptor, and is not acting by competing for G-proteins, for example. The specificity of the effect alluded to a mechanism for dominant negative activity that involved an interaction between the truncated and full-length GHRH receptors, which was confirmed by co-immunoprecipitation studies.

The proposed model for the dominant negative activity of the truncated receptor is that the truncated receptor forms a complex with the wild-type receptor. These truncated receptor containing complexes reach the cell surface, but do not bind GHRH at wild-type levels, perhaps due to a conformational change in the structure of the complex. The truncated receptor may bind GHRH at lower levels, but cannot signal through the cAMP pathway, and likely competes for dimerization with the wild-type receptor, downregulating GHRH signaling of the wild-type receptor, changing the predominant species on the cell surface.

Though the GHRH receptor has not yet been shown to homodimerize, the data in this chapter show that it can form a complex with a splice variant receptor that is identical to the wild-type receptor for the first 325 amino acids. While we do not know if this complex includes directly interacting GHRH receptors, this would be the simplest explanation, given the wealth of evidence supporting such interactions in other G-protein coupled receptors (Cvejic and Devi 1997; Angers et al. 2000; Franco et al. 2000; Rocheville et al. 2000; Robbins et al. 2001; Terrillon et al. 2003; Mandrika et al. 2005). The fact that the truncated GHRH receptor, missing the last 98 amino acids of the full-length GHRH receptor, can form a complex with the wild-type GHRH receptor indicates that the interaction motif is located somewhere within the first 325 amino acids of the protein. Alternatively, a domain inhibiting interaction may be located within the last 98 amino acids of the wild-type GHRH receptor, and, when removed, the receptor may

be allowed to interact. Interestingly, the VIP receptor, which is 42 percent identical to the GHRH receptor in amino acid sequence, does not interact with the truncated GHRH receptor in co-transfected cells.

Oligomerization of G-protein coupled receptors is increasingly recognized as a mechanism by which this class of receptors, classically thought to act as monomers, alters its functions under different physiological conditions. The interaction between the truncated and full-length GHRH receptors is ligand-independent. This result conflicts with previous computer models that suggested that the GHRH receptor might form a dimer when bound to its ligand, when an α-helical region of the N-terminus of a family B G-protein coupled receptor interacts with an α-helical region in the C-terminus of the hormone to form a coiled-coil (Momany and Bowers 1996). Oligomerization of GPCR's in general has been suggested to provide a mechanism for masking hydrophobic patches or retention signals that would keep receptors in the ER (Reddy and Corley 1998). Given that the truncated GHRH receptor is identical to the wild-type receptor for the first 325 amino acids, it is reasonable to suggest that a truncated receptor could mask ER retention signals of the wild-type receptor, allowing the nonfunctional complex to be transported to the cell surface. Many mutants and splice variants of G-protein-coupled receptors that exert dominant negative effects on wild-type receptors act by decreasing or preventing cell-surface localization (Colley et al. 1995; Zhu and Wess 1998; Karpa et al. 2000; Brothers et al. 2004; Tao et al. 2004), often retaining the receptor complex in the ER, probably through an inability to mask ER retention signals; however, a model exists for allowing heterodimers to reach the cell surface and differentially regulate signaling (Terrillon and Bouvier 2004). Dimers can form in the ER and be trafficked to the cell surface, where ligands might alter oligomerization state or bind differentially to heterodimers. A truncated splice variant of the

$GABA_{B(1)}$ receptor acts similarly to the truncated GHRH receptor described here, heterodimerizing with the wild-type receptor, forming a complex with reduced ability to bind hormone and signal (Schwarz et al. 2000).

The differences between the human and rat GHRH receptor splice variants both in protein structure and in signaling properties may be informative to dissecting how the normal receptor functions. The long rat receptor can bind GHRH, not surprisingly, considering the intact extracellular domains, but cannot signal through the cAMP pathway (Miller et al. 1999), though it does not exert a dominant negative effect on wild-type receptor signaling. The insertion in the third cytoplasmic loop of the long rat receptor may disrupt G-protein coupling, as the third intracellular loop is important to G-protein activation (O'Dowd et al. 1988). Given the structural difference between splice variant receptors in these two species and the fact that the long isoform does not exert a dominant negative signaling effect, I would hypothesize that the insertion in the long rat receptor prevents oligomerization with the wild-type receptor. This possibility and others are elaborated upon in the general discussion, Chapter 5.

The effect of the truncated GHRH receptor on signaling of the full-length GHRH receptor and the fact that it is expressed in both normal pituitary and GH-secreting pituitary adenoma raises the question of its potential roles in physiological and pathological conditions. One hypothesis involves a preferential expression of the truncated splice variant receptor during physiological and pathological states that require a dampening of GH signal, such as in the excess GH secretion observed in pituitary adenomas. In this view, the truncated splice variant receptor would be preferentially expressed in response to excess GH secretion, representing a mechanism to downregulate GHRH signaling, and, thereby, GH production. Understanding the regulation of splice variant expression and determining the interaction domains of interacting G-

protein coupled receptors will prove essential for learning the structures of the complexes, and for potentially manipulating the oligomerization states of G-protein coupled receptors, such as the GHRH receptor.

This chapter examines the signaling properties of splice variant GHRH receptors in rat and human and determines a specific mechanism by which the dominant negative activity of the truncated human GHRH receptor splice variant is achieved. In addition, the discovery of complex formation between the truncated splice variant receptor and the wild-type receptor supports a role for dimerization or higher order oligomerization in GHRH signaling, and elucidates a new mechanism by which this receptor can modify its activity, forming complexes with other isoforms of the GHRH receptor, and possibly with different G-protein coupled receptors, such as the GHS receptor.

The fact that the truncated GHRH receptor exerts a dominant negative effect on the GHS receptor is very interesting, and further indicates a role for dimerization between these types of receptors in normal functioning. As mentioned, while the wild-type GHS receptor synergizes with the GHRH receptor to increase signaling through the cAMP pathway, the short isoform of the GHS receptor exerts a dominant negative effect on GHRH receptor signaling in cells that express both receptors. The functional interaction between these receptors suggests that the two pathways are regulated in part through a physical interaction between variant forms of GHS and GHRH receptors. To date, a physical interaction has not been detected experimentally. These results highlight a role for alternative splicing and receptor interactions in regulating the GH axis, and establish a need to further examine these receptors for physical interactions. These data support the idea that interactions between receptors of this class represents an important means by which a single type of receptor can diversify its signaling properties.

CHAPTER FIVE

GENERAL DISCUSSION

Mammalian gene regulation is a complex and tightly regulated process by which appropriate genes are activated or repressed in each cell. The regulated expression of genes is essential to the development of multicellular organisms and the proper functioning of specific cell-types. Transcriptional regulation is the first step by which the process of gene expression is controlled. All genes are transcriptionally regulated. Repressed genes exist in an inactive state in highly condensed heterochromatin. Repression is a regulated state, enhanced by the binding of repressor transcription factors and co-repressor proteins. Activating transcription factors bind to DNA, helping to unwind the DNA from the condensed chromatin and allowing for the transcriptional machinery to access the DNA and begin transcribing the mRNA. The balance between these states is the primary mechanism by which gene expression is regulated.

Organogenesis in mammals is achieved by expression of signaling molecules that establish gradients of transcription factors during embryonic development. The presence or absence of specific transcription factors in different zones determines which genes will be activated and which will be repressed as the cells divide and differentiate. Development of the anterior pituitary gland represents an excellent model for understanding mammalian organogenesis, in general. The anterior pituitary is comprised of five distinct cell types that arise from a common precursor cell and differentiate based upon signaling molecules and transcription factor gradients. While corticotrophs and a transient population of thyrotrophs in the rostral tip differentiate first (Lin et al. 1994; Rosenfeld et al. 2000), the four most caudal cell types differentiate based on a reciprocal gradient of the transcription factors Pit-1 and GATA-2 (Dasen et al. 1999), which is involved in the transcriptional activation of cell-specific genes for these cell types. The thyrotroph, lactotroph and somatotroph lineages are dependent upon the expression of Pit-1 (Lin et al. 1994). Other cell-specific factors must be involved in further

differentiating these cell types from their common precursors. A precedent exists for examining transcriptional regulation of early-expressed cell-specific genes to determine what factors are involved in cell fate decisions. Because terminally differentiated somatotroph cells express the GHRH receptor and respond to GHRH stimulation (Mayo et al. 1996), examination of the transcriptional regulation of the GHRH receptor gene was undertaken with a goal of identifying factors required for differentiation of this cell lineage.

The GHRH receptor is an integral molecule in mediating signaling in the neuroendocrine system regulating linear growth, metabolism and proliferation of bone, fat and muscle. Analyses of animal models of dwarfism and clinical cases of growth-hormone deficiency have implicated mutations in the GHRH receptor itself, its promoter, or in factors involved in expression of the GHRH receptor as causative. The Ames dwarf mouse has a mutation in the gene encoding Prop-1 (Prophet of Pit-1), (Gage et al. 1996; Sornson et al. 1996) which is necessary for expression of Pit-1, as its name suggests, and for the extinction of Rpx, allowing further differentiation of the pituitary cells. In Snell and Jackson dwarf mice, inactivating mutations in the Pit-1 gene are associated with GH-deficiency and pituitary hypoplasia due to an absence of the Pit-1 derivatives, thyrotrophs, lactotrophs and somatotrophs (Lin et al. 1994; Barlier et al. 1997). Similarly in humans, a congenital combined GH, prolactin, TSH deficiency syndrome has been mapped to a mutation in the DNA binding domain of Pit-1 (Barlier et al. 1997). Correspondingly, Pit-1 is expressed in pituitary adenomas that secrete GH, prolactin and TSH (Barlier et al. 1997). The *little* mouse has a missense mutation in the GHRH receptor, preventing the mutant receptor from binding GHRH, and leading to GH-deficiency and pituitary hypoplasia with an absence of somatotroph cells (Gaylinn et al. 1999). This mutation reveals a role for the receptor in somatotroph cell proliferation. GHRH receptor-activation of the MAPK pathway has

been implicated in somatotroph proliferation (Mayo et al. 2000; Zeitler and Siriwardana 2000; Lee et al. 2001; Cunha 2002). Multiple inactivating mutations in the GHRH receptor have been identified in clinical cases of GH-deficiency (Netchine et al. 1998; Salvatori et al. 1999; Hayashida et al. 2000; Salvatori et al. 2001). In addition, a mutation in one of the binding sites for Pit-1 in the human GHRH receptor promoter is associated with decreased GHRH receptor expression in a case of isolated GH deficiency type IB (Salvatori et al. 2002). These examples of GH deficiency associated with the development of somatotroph cells and/or the proper expression and signaling of the GHRH receptor highlight the importance of the receptor in mediating proper control of the GH axis.

Further indicative of the role the GHRH pathway plays in regulating GH are an animal model expressing excess GHRH and human cases of pituitary adenomas that secrete GH. The transgenic mouse model overexpressing GHRH exhibits pituitary hyperplasia and gigantism (Mayo et al. 1988). GH-producing pituitary adenomas that express wild-type and splice variant forms of the GHRH receptor have been isolated, indicating a role for alternative splicing in regulating the GH pathway. In addition, many other types of tumors have been shown to express variant forms of the GHRH receptor (Rekasi et al. 2000a; Othman et al. 2001; Halmos et al. 2002; Kiaris et al. 2002; Plonowski et al. 2002; Garcia-Fernandez et al. 2003; Chatzistamou et al. 2004), which appears to be involved in promoting proliferation of these cancer cells. GHRH antagonists are the focus of new anti-proliferative treatment for these types of cancer (Kahan et al. 2000; Rekasi et al. 2000b; Letsch et al. 2003; Engel et al. 2005; Keller et al. 2005).

Given the importance of this receptor in regulating appropriate GH synthesis and secretion and its role as a target for anti-proliferative cancer drugs, understanding the regulation and signaling of the GHRH receptor is extremely valuable. From the studies presented in this

thesis, we have a better understanding of the regulatory elements in the gene's promoter that control appropriate expression of the GHRH receptor *in vivo*. Further analysis of this promoter has led to the identification of ten binding sites for Pit-1, a factor known to be required for GHRH receptor expression. Functional analyses of these sites show that, while the most proximal site is required for Pit-1 activation, the highest level of Pit-1 activation of the gene is achieved when all ten sites are intact.

A second level of regulation, alternative splicing, is a key mechanism by which this receptor modulates its signaling properties. Analysis of a splice variant receptor first identified in human GH-producing adenomas (Hashimoto et al. 1995; Tang et al. 1995) revealed that this post-transcriptional modification affects signaling of the wild-type receptor in a way that had not previously been shown for this receptor. These findings are important to understanding the role of this splice variant, which is also expressed at low levels in the normal pituitary, in regulating the GH axis. The remainder of this chapter discusses the implications of the data reported in the thesis and future directions for this line of research.

Analysis of a 1.6kb GHRH receptor proximal promoter

Characterization of transgenic mice expressing a transgene comprised of the 1.6kb proximal promoter of the rat GHRH receptor directing a luciferase reporter led to the discovery that this promoter sequence is sufficient to direct pituitary- and somatotroph-specific expression of the gene *in vivo*. Furthermore, the data show that transgene expression is sexually dimorphic. This result was interesting, given the inconsistency of reports in the literature on sex differences in expression of the endogenous GHRH receptor. Clearly and consistently, the transgene is more highly expressed in males than in females, with a difference of about five-fold. The sex

difference in expression may in part be the result of greater numbers of somatotroph cells in the pituitary of males versus females, though the difference in cell numbers (20 percent) is much lower than the difference in transgene expression (500 percent). In these experiments, it was also noted that transgene expression levels in females was much more highly variable than in males. This result possibly reflects differences in cycle status of the females, given that GHRH receptor expression is regulated by estrogen. Analysis of transgene expression during post-natal development and aging revealed an interesting result. Though there is an increase in expression of the transgene leading up to puberty, consistent with the expression pattern for the endogenous gene, there is no consistent decrease in transgene expression as the animal ages. This result suggests that regulatory factors not contained within the 1.6kb promoter are involved in the downregulation of GHRH receptor expression with aging. For example, certain regulatory factors might bind upstream of the 1.6kb promoter. These factors themselves could be expressed in an age-dependent manner, thereby regulating expression of the GHRH receptor differently throughout aging.

For nearly every parameter examined, the regulated expression pattern of the 1.6kb promoter transgene closely matches what is known for the endogenous GHRH receptor gene. These experiments were the first to examine regulation of the GHRH receptor promoter in its physiological context, which is important to interpreting existing and forthcoming data from *in vitro* systems.

With the data establishing appropriately regulated expression of the 1.6kb promoter in transgenic mice, this promoter sequence was further evaluated in cell culture experiments aimed at identifying the factors involved in directing pituitary-specific expression of the GHRH receptor. The TESS transcription factor site (http://www.cbil.upenn.edu/tess) identified 227

predicted transcription factor and gene regulatory elements and 1,061 binding sites in the 1.6kb rat GHRH receptor promoter. Of these factors, forty are known to be expressed in the pituitary gland, though only Pit-1 is restricted to the pituitary. The pituitary-expressed factors with predicted binding sites within this promoter are listed in Table 5.1. This list of factors provides a starting point for examining other factors involved in transcriptional regulation of the GHRH receptor gene. My work focused on identifying functional binding sites within the GHRH receptor promoter for a pituitary-specific transcription factor, Pit-1, known to be required for GHRH receptor expression. While I did not identify novel factors involved in somatotroph cell development, I did determine a mechanism by which the known pituitary-specific factor Pit-1 regulates expression of the GHRH receptor gene and established the 1.6kb promoter as sufficient to direct tissue- and cell-specific expression *in vivo*. These experiments make the future work of identifying additional factors that work with Pit-1 more easily achievable.

Pit-1 regulation of GHRH receptor expression

In situ hybridization experiments on pituitaries from Snell and Jackson dwarf mice, which have an inactivating mutation in the Pit-1 gene, show that the GHRH receptor is not expressed in these animals (Lin et al. 1992). This experiment was the first to suggest that GHRH receptor activation requires expression of Pit-1. Subsequent experiments from our laboratory and others have shown that Pit-1 stimulates expression of the GHRH receptor (Petersenn et al. 1998; Gaylinn 1999; Miller et al. 1999; Nogami et al. 2002; Salvatori et al. 2002), though a comprehensive examination of the promoter for functional Pit-1 binding sites had never been

Table 5.1: Pituitary-Expressed Factors Predicted to Bind the 1.6kb Rat GHRH Receptor Promoter

The –1673 to +285 rat GHRH receptor promoter sequence was entered into the TESS transcription factor database. Of the 1,061 predicted binding sites, 227 factors were predicted to bind this sequence. The Gene Expression Database was used to determine which of these factors are known to be expressed in the pituitary, and these are shown here.

Pituitary-Expressed Factors with Predicted Binding Sites within the 1.6kb Rat GHRH Receptor Promoter

Factor	Description	Known Roles
AP1	Activating Protein 1	Dimerizes with other bZIP proteins to regulate transcription
AP2	Activating enhancer binding protein 2	Carcinogenesis, differntiation, proliveration, survival
AR	Androgen Receptor	Binds testosterone/DHT; regulates transcription; signal transduction
c-Fos	Proto-oncogene	Dimerizes with Jun to form AP1 complex
c-Jun	Proto-oncogene	Dimerizes with Fos to form AP1 complex
c-Myb	Proto-oncogene	Proliferation of hematopoietic cells
CBP	CREB binding protein	Acetyl transferase enzyme; activates transcription
C/EBP	CAAT Enhancer Binding Protein Alpha	Functioning and differentiatin of hepatocytes and adipocytes
COUP	Chicken Ovalbumin Upstream Promoter	Steroid receptor/activator
CREB	CAMP Response Element Binding Protein	Interacts with CBP to regulate transcription
DBP	D site of albumin promoter binding protein	Regulation of nucleic acid metabolism
Elk-1	E-26 like protein 1	Tumorigenesis; apoptosis; differentiation
ER	Estrogen Receptor	Binds estrogen; regulates transcription; signal transduction
Ets-1	Phosphoprotein	Lymphoid activation
Ets-2	Phosphoprotein	Cell proliferation; macrophage activation
GATA-2/3	Binds WGATAR sequence	Hematopoesis; proliferation
GR	Glucocorticoid Receptor	Binds glucocorticoids; induces transcription; glucose, protein, fat metabolism
Hes-1	Helix-loop-Helix Factor	Gene regulation; cell communication; signal transduction
Ik-1-4	Ikaros 1-4	Represses GH gene in mammosomatotrophs; activates GHRH expression; somatotroph development; hematopoesis
IL-6	BZIP transcription factor	Regulation of cytokine and adhesion molecules
IRF1	Interferon Regulatory Factor 1	Cell proliferation; regulation of growth inhibitory interferon genes
Isl-1	LIM Homeodomain transcription factor	Pituitary development; programmed cell death
MAF	BZIP transcription factor	Tissue-specific gene expression
MAT1	Cdk-activating kinase assembly factor	Regulation of proliferation genes
NGFI-B	Nerve Growth Factor Induced B	Orphan steroid hormone receptor; transcriptional regulation
NF1	Nuclear Factor 1	Regulation of milk genes in response to PRL signaling
Pit-1	Pituitary Specific Transcription Factor 1	Regulation of genes in somatotroph, lactotroph, thyrotroph lineages
PR	Progesterone Receptor	Binds progesterone; regulates gene transcription; signal transduction
RAR/ /	Retinoic Acid Receptors	Gene regulation in development
SP-1		Proliferation; signal transduction
T3R	Thyroid Hormone Receptor	Binds Thyroid hormone; regulates gene transcription
TCF	T Cell Factor	Regulation of developmental genes
TEF2	Translational Elongation Factor 2	Binding aminoacyl tRNA

performed previously. The 1.6kb rat GHRH receptor promoter was examined in a cell culture system to determine where Pit-1 binding sites are located, and which of the binding sites are required to achieve Pit-1 activation of GHRH receptor expression. Ten sites that specifically bind Pit-1 were identified. Further functional analyses of these binding sites revealed that, while full Pit-1 activation of GHRH receptor expression is achieved when all sites are intact, a single proximal site within +129 to +164 relative to the 5'-most transcriptional start site is required for Pit-1 activation of GHRH receptor expression.

Several other Pit-1 target genes are known to have multiple binding sites for Pit-1. For example, the thyroid receptor □2 (TR□2), which is expressed only in the pituitary, has six identified Pit-1 binding sites in the 500bp proximal promoter (Wood et al. 1996). Promoter deletions lead to progressively decreased activity for the first four sites, but the most proximal site is the most critical for Pit-1 activation (Wood et al. 1996), closely matching the data described in Chapter 3 for the GHRH receptor. In the prolactin promoter Pit-1 binding to multiple sites is required for estrogen responsiveness (Nowakowski and Maurer 1994) and lactotroph-specific expression of the gene. The GH proximal promoter binds Pit-1 at two sites, and binding at both sites is necessary for Pit-1 activation of this promoter *in vivo* (Lira et al. 1993; Shewchuk et al. 1999). However, the presence of these binding sites is not sufficient for full GH promoter activation in transgenic mice (Shewchuk et al. 1999). The addition of upstream promoter sequences confers higher GH promoter activity *in vivo* (Shewchuk et al. 1999), suggesting that Pit-1 synergizes with other factors to activate transcription of the GH gene. Given the expression pattern of Pit-1 in three cell types in the anterior pituitary, other factors are required to lead to cell-specific expression of target genes.

Pit-1 is known to synergize with several factors on different target gene promoters. On the GH promoter, Pit-1 synergy with CEBP/ (Schaufele 1996) and thyroid hormone receptor (Lira et al. 1993; Palomino et al. 1998) have been reported. A synergy domain in Pit-1 that is required for activation of the prolactin gene by Pit-1 and ER, but not required for synergistic activation of the GH promoter has been identified (Holloway et al. 1995). This domain requires the presence of two tyrosine residues that determine whether Pit-1 binds DNA as a monomer or a dimer (Holloway et al. 1995). In the prolactin enhancer, Pit-1 binds as a monomer, while it binds the GH promoter as a dimer (Holloway et al. 1995) . The differences in alternative synergy domains change the binding properties to sequence-specific DNA sites and provide an additional regulatory strategy by which different target genes are activated or repressed in different cell types. The different states of Pit-1 binding to the promoter likely correlate to the recruitment of different co-factors and binding partners. The sequence variation among the Pit-1 binding sites in the GHRH receptor promoter reported in Chapter 3 might serve to alter binding to the promoter as monomers or dimers and thereby recruit different factors to the promoter at different times, depending on physiological requirements for gene expression. In fact, analysis of Pit-1 mutations isolated from patients with the disease Combined Pituitary Hormone Deficiency (CPHD), determined that CBP/p300 recruitment and Pit-1 dimerization are necessary for appropriate activation of the GH promoter (Cohen et al. 2006).

An additional level of regulation of target genes by Pit-1 is achieved by an alternative splice variant of the transcription factor, Pit-1. Pit-1 contains a repression domain within the transactivation domain of Pit-1. Promoter-specific repression by Pit-1 is achieved by a hindrance of the recruitment of CBP and a reduction of histone acetylation of the promoter (Ferry et al. 2005). The transcription factor Ets-1 physically interacts with Pit-1 to synergize

activation of the prolactin promoter in lactotroph cells (Howard and Maurer 1995). While Pit-1 interacts with Ets-1, it fails to synergize with it to activate transcription of the prolactin gene (Bradford et al. 2000), leading to repression of the gene.

It is likely that the multiple Pit-1 binding sites in the GHRH receptor coordinate with other factors to regulate somatotroph-specific expression. One factor that is known to synergize with the required proximal Pit-1 site in the rat GHRH receptor promoter is glucocorticoid receptor (Nogami et al. 2005). Bioinformatic analysis of the Pit-1 binding site probe sequences was used to determine whether known factors bind close regions, which would suggest possible interactions between other factors and Pit-1 (Fig. 5.1). These candidate factors provide a starting point for evaluating additional factors that work with Pit-1 to achieve cell-specific expression of the GHRH receptor. Particularly interesting candidates include estrogen receptor, for which binding sites are present in the –818 to –781 and –373 to –338 sites, Ik 1-4, for which binding sites are present in the –925 to –892, -888 to –857, and –81 to –46 sites, and Isl-1, which has a binding site in the –1178 to –1153 sequence. Glucocorticoid receptor has been shown by another group to synergize with Pit-1 at the +129 to +164 site, and also has a potential binding site in the –888 to –847 sequence. These factors are intriguing because they are known to influence GHRH receptor expression (estrogen and glucocorticoids) or are known to be involved in pituitary development (Ik 1-4 and Isl-1).

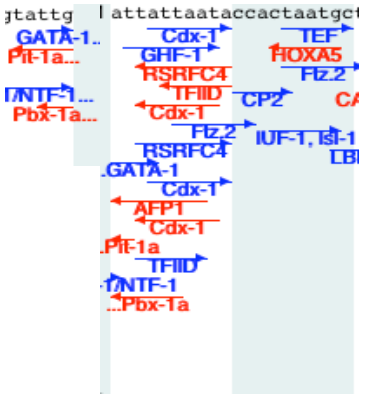
Interactions of alternative splice variant receptors

Alternative mRNA splicing is a common mechanism by which many gene products are regulated. The isoforms produced by alternative splicing often have variable functions resulting

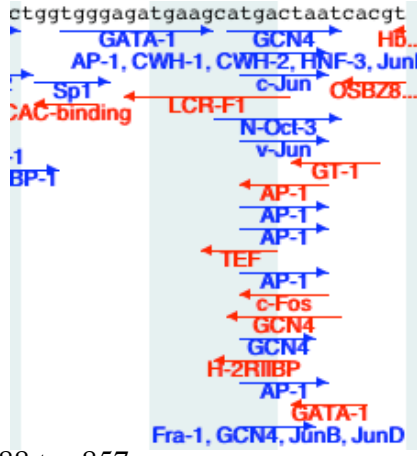
Figure 5.1: Bioinformatic Analysis of Potential Gene Regulatory Factors Binding the Rat GHRH Receptor

The 1.6kb Rat GHRH receptor promoter sequence was entered into the TESS transcription factor binding site prediction tool (http://www.cbil.upenn.edu/tess). The sequences corresponding to the identified Pit-1 binding probes are shown with predicted binding factors.

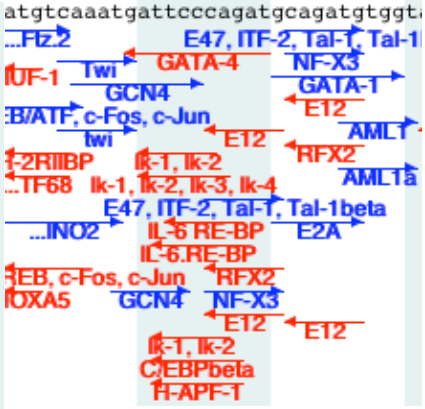
-1178 to -1153



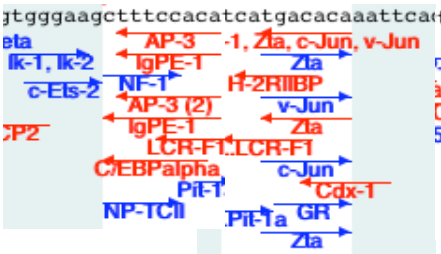
-1148 to -1123



-925 to -892

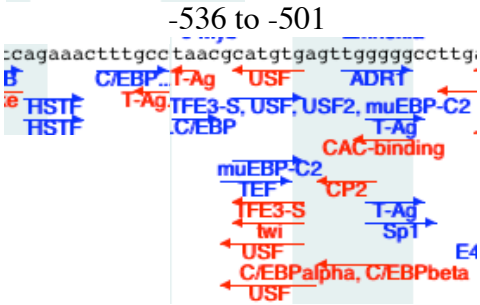


-888 to -857

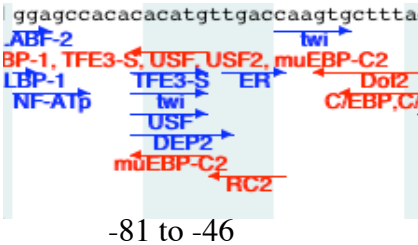


-818 to -781

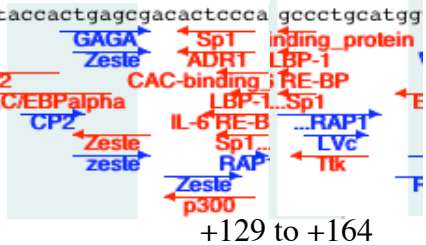
-536 to -501



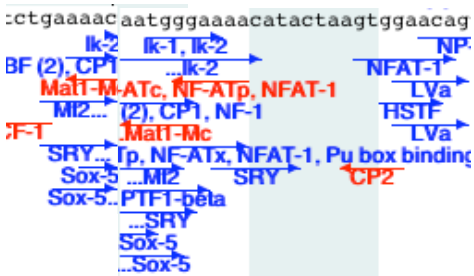
-373 to -338



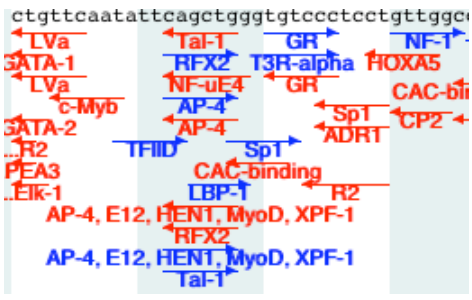
-348 to -313



-81 to -46



+129 to +164



from differences in protein structure. Alternative splicing of GPCRs is an emerging mechanism by which this class of receptors modulates its functions by changing such properties as ligand binding, G-protein coupling, cellular localization, signal transduction, internalization and desensitization compared to the wild-type isoforms. Several examples of alternative splice variants that affect these functions have been identified within the different classes of GPCRs. Examples of splice variant receptors that cannot bind ligand at levels similar to wild-type isoforms include the 5-HT receptor 4 variant (Pindon et al. 2002), the histamine H3 receptor (Wellendorph et al. 2002) and a variant of the VIP receptor (Couvineau et al. 1994). The 5-HT receptor 4 variant is also deficient in G-protein coupling (Pindon et al. 2002). Several splice variant GPCRs do not properly localize to the cell surface, including variants of the dopamine D3 receptor (Karpa et al. 2000), the calcitonin receptor (Seck et al. 2003) and the LH receptor (Nakamura et al. 2004). Deficits in signaling have also been reported in variant receptor isoforms that do reach the cell surface, but do not effectively transduce signaling. Some of these non-functional variant receptors differ from wild-type receptors in internalization, such as the thromboxane A2 receptor truncated splice variant (Parent et al. 2001), and in receptor desensitization, such as an isoform of the prostanoid receptor (Pierce and Regan 1998). These properties lead to a difference in signal transduction of the variant receptors. Other GPCRs with identified signal transduction deficits include variants of the PACAP (Pisegna and Wank 1993; Spengler et al. 1993; Journot et al. 1995; Pantaloni et al. 1996; Pisegna and Wank 1996; Scaldaferri et al. 2000; Nicot and DiCicco-Bloom 2001), calcitonin (Moore et al. 1995) and GnRH receptors (Grosse et al. 1997). Known splice variants for the B-III subfamily of GPCRs are described in Table 5.2.

Table 5.2: GPCR Subfamily B-III Splice Variants

Known splice variants of family B-III GPCRs and the effects of their expression are listed.

Receptor	Structural Variation	Function/Disease Association
GHRH Receptor	Truncation in 3rd intracellular loop; insertion in 3rd intracellular loop; C-terminal insertion	Alterations in ligand binding, signaling; truncated isoform acts as dominant negative; expression in pituitary adenomas (Zeitler et al. 1998; Miller et al. 1999; McElvaine and Mayo 2006)
GIP Receptor	27 amino acid insertion in C-terminal tail	Binds GIP and activates AC; inappropriately expressed in Cushing's syndrome, adrenal adenoma, bilateral macronodular hyperplasia (N'Diaye et al. 1998)
PACAP Receptor	N-terminal alterations; 3rd intracellular loop variants	Differential regulation in suprachiasmatic nucleus; alternately associated with neuroblast proliferation and inhibition of cortical precursors; changes in ligand affinity (Spengler et al. 1993; Pantaloni et al. 1996; Nicot and DiCicco-Bloom 2001)
Secretin Receptor	N-terminal deletion	Dominant Negative; found in ductal pancreatic adenocarcinomas (Korner et al. 2005)
VIP Receptor	Truncation of 6th and 7th transmembrane domains	Expression in prostate cancer (Bokaei et al. 2006)

Expression of inactive splice variant receptor isoforms represents a mechanism by which this class of proteins can fine-tune its actions during development or as physiological requirements for their signaling change. Splice variants of the PACAP receptor, for example, are expressed in tissue- or lineage-specific patterns, and influence differentiation or function of the tissues in which they are expressed (Pisegna and Wank 1996; Scaldaferri et al. 2000; Nicot and DiCicco-Bloom 2001) based on differences in their signaling properties. Many inactive GPCR isoforms have been shown to act as dominant negatives by physically interacting with wild-type receptors and reducing signal transduction of the wild-type receptors. A variant dopamine D3 receptor heterodimerizes with the wild-type receptor and the complexes fail to reach the cell surface (Karpa et al. 2000). Because the wild-type receptor becomes sequestered to the intracellular compartments, it cannot signal. Similarly, a variant LH receptor forms a complex with the wild-type form of the receptor and reduces the density of wild-type receptors on the cell surface (Nakamura et al. 2004). In an example particularly relevant to the data reported in this thesis for the truncated GHRH receptor, an isoform of the GnRH receptor reaches the cell surface, though at lower levels than the wild-type GnRH receptor, and cannot bind its ligand or transduce signal (Grosse et al. 1997).

The data reported in Chapter 4 show that truncated GHRH receptor reaches the cell surface but is unable to bind GHRH and transduce signaling through the cAMP pathway. This truncated receptor isoform acts as a dominant negative by forming a complex with the wild-type receptor. Based on data reported for GHRH receptors of different species, some hypotheses exist as to the normal functioning of this receptor. Potential roles for the C-terminus, which is lacking in the truncated splice variant, include palmitoylation and phosphorylation. The C-terminal truncation of the ovine GHRH receptor removes a cysteine that may be palmitoylated (Horikawa

et al. 2001). Removal of this cysteine could be correlated to the increased activity of the ovine receptor, as palmitoylation affects structure and/or phosphorylation state. The human GHRH receptor is phosphorylated in the C-terminus (Gaylinn et al. 1998). The C-terminal truncation in the ovine GHRH receptor leads to enhanced cAMP activity at a lower dosage of GHRH, supporting a role for phosphorylation in mediating GHRH receptor desensitization and internalization (Horikawa et al. 2001) . Though the truncated GHRH receptor lacks the C-terminus, it is inactive due to an inability to effectively bind GHRH. The absence of C-terminal palmitoylation and phosphorylation sites in the truncated GHRH receptor may lead to decreased internalization of the non-functional truncated receptor homodimers and heterodimers, thereby further dampening wild-type GHRH receptor signaling.

Implications for functional domains of the GHRH receptor based on different properties of splice variant receptors

Though many examples of G-protein-coupled receptors that modulate signaling by forming homo- or hetero-oligomers have been reported, the GHRH receptor had not previously been shown to physically interact with other receptors. The complex formed by truncated and full-length GHRH receptors reported in Chapter 4 represents the first definitive evidence that the GHRH receptor can physically interact with other subunits in a complex. Taken together, data on signaling properties of different splice variant isoforms of the GHRH receptor suggest potential functions of different domains of the receptor.

Though they are generated from alternative splicing at the same intron/exon boundaries, the protein products of the truncated human GHRH receptor and the long rat GHRH receptor are very different, as their names indicate. Inclusion of normally intronic sequence in these splice

variants encodes a premature in-frame stop codon just before the sixth transmembrane domain in the human and a 41-amino acid insertion in the third cytoplasmic loop in the rat. Neither of these protein products signals through the cAMP pathway in response to GHRH stimulation. Chapter 4 describes the dominant negative signaling effect of the truncated human GHRH receptor on the wild-type receptor in cells that express both and defines a mechanism by which this effect is achieved. The long rat GHRH receptor does not signal through the cAMP pathway (Miller et al. 1999), but it was unknown whether this splice variant produced a dominant negative signaling effect. Additional experiments presented in Chapter 4 show that the long rat GHRH receptor does not affect signaling of the wild-type receptor in cells co-expressing both receptor isoforms, though the co-expression data need to be strengthened. This important functional difference suggests roles for different domains of the receptor that can be deduced based on the structural differences of the isoforms.

The complex formed between the truncated human receptor isoform and the wild-type GHRH receptor was the first indication of oligomerization of the GHRH receptor. The fact that the splice variant receptors form hetero-oligomers suggests that the wild-type receptor normally functions by formation of homodimers or higher order complexes. The truncated human GHRH receptor is identical to the wild-type receptor for the first 325 amino acids. Assuming direct interactions between subunits of the GHRH receptor, the dimerization domain exists within the first 325 amino acids of the protein. Further comparison between residues of the closely related VIP receptor and the GHRH receptor, which are 42 percent identical, but do not form complexes with one another, could help narrow down potential domains important for dimerization. The fact that the long rat GHRH receptor does not impair signaling of the wild-type receptor indicates that it may not form heterodimers with the wild-type receptor. According to this

hypothesis, wild-type receptor subunits are not competed for dimerization by the splice variant receptor, and signaling of the wild-type receptor is therefore unaffected by expression of the long rat GHRH receptor isoform. The 41 amino acid insertion in the third intracellular loop may interfere with dimerization, or it may lead to a change in structural conformation that is not conducive to oligomerization. Alternatively, the long rat GHRH receptor may form heterodimers with the wild-type receptor that are still active.

Another difference between the splice variant GHRH receptors from the two species is that the long GHRH receptor binds GHRH (Miller et al. 1999), while the truncated human receptor does not. This difference indicates an important role for the third extracellular loop, which is intact in the long rat receptor but absent from the truncated human receptor, in binding GHRH, supporting the conclusions from previous chimeric receptor studies in our laboratory (DeAlmeida 2000). That neither of the splice variant isoforms can signal in response to GHRH stimulation is not surprising, given the lack of structural integrity within the third intracellular loop of both isoforms. The third intracellular loop of GPCRs has been shown to be essential to proper G-protein coupling (O'Dowd et al. 1988), and disruptions to this region in the rat and human GHRH receptor splice variants lead to inactive protein products. The signaling properties of both receptor isoforms support the fact that this region of GPCRs is essential to proper signaling of this class of receptors.

Combined with the known structural differences between receptor splice variant isoforms, the signaling properties of these receptors strongly suggest roles for domains of the receptor that are differently altered in the different species. Further experimentation is necessary to definitively determine roles for these domains, but the signaling data illuminate regions of the receptor to target in future studies.

Significance of the thesis work and future directions

Appropriate temporal and spatial regulation of gene expression is essential to the development of multicellular organisms. The particular gene that is the focus of this thesis is expressed in a specific cell type and is required for appropriate control of the signaling pathway leading to regulation of GH synthesis and secretion. Understanding regulation of the GHRH receptor is important for several reasons. Regulation of expression of this gene serves as a model for how cells differentiate from a common precursor during organogenesis. Because expression of the GHRH receptor is an early event in terminally differentiated somatotroph cells, its expression is activated by the presence of a unique combination of activators or absence of repressors in this developing cell type. By learning what factors are involved in transcriptional activation of the gene, we will have a greater understanding of the requirements for cell differentiation in the anterior pituitary, which may be applicable to mammalian organogenesis in general.

When this gene is misregulated, diseases of growth occur. Hypersecretion of GH during early post-natal development is very rare, and results in gigantism. Only 100 cases of gigantism have been recorded in the U.S. More common is acromegaly, which results from hypersecretion of GH after the long bones have fused, usually associated with GH-secreting tumors. Acromegaly affects 6 in 100,000 people. Hyposecretion of GH, resulting in dwarfism, is a much more prevalent disorder. Currently, 70,000 cases of GH deficiency exist in the U.S. and 4,000 new childhood diagnoses are made each year. Statistics are from the National Library of Medicine. Because the GHRH receptor modulates signaling between the nervous system and the endocrine system in controlling proper differentiation, proliferation and metabolism of target tissues, understanding regulation of this gene and its signaling properties is essential.

To further our understanding of appropriate regulation of the GHRH receptor, both transcriptional and post-transcriptional regulation, specifically, alternative splicing, were examined. The data in this thesis report the first examination of regulation of the GHRH receptor *in vivo* in transgenic mice. The results show that the 1.6kb proximal promoter appropriately directs tissue- and cell-specific expression that is sexually dimorphic and age-dependent. Further analysis revealed that the pituitary-specific transcription factor Pit-1 binds to several sites within this promoter, and that, while the greatest activation is achieved when all sites are intact, a single site is required for Pit-1 activation of gene expression.

These data lay the groundwork for further analysis of cell-specific combinations of factors involved in appropriate expression of this gene. Future work will focus on identification of factors in addition to Pit-1 that are involved in appropriately regulating GHRH receptor expression. One method of identification of pituitary-specific factors involved in regulation of this gene is to perform EMSAs using probes spanning the receptor promoter and nuclear extracts from a cell line expressing the receptor, such as MtT/S cells, and from a non-pituitary cell line, such as HeLa cells. Narrowing down regions that specifically bind the MtT/S nuclear proteins, short promoter sequences can be entered into transcription factor prediction databases to determine whether known factors bind these sites. Expression of predicted factors in MtT/S cells would be assessed using RT-PCR. If expressed, the contribution of these factors to regulation of the GHRH receptor gene would be examined by co-expressing the factor(s) with promoter-reporter constructs in the presence or absence of co-expressed Pit-1. In addition to discovering new binding sites for known factors, future work should aspire to uncover novel factors involved in the cell-specification of somatotroph cells. If a critical site is identified through these studies and a pituitary-specific protein binds to the site, identification and cloning the novel protein will

be important. Oligonucleotide screening of cDNA expression libraries or DNA affinity chromatography are approaches to consider for identifying novel factors. A new proteomics technique for identifying transcription factor-interacting proteins has been reported (Meng et al. 2006) and would be useful in identifying factors that interact with Pit-1 on the GHRH receptor promoter. This technique involves isolation of transcriptionally active Pit-1 by binding Pit-1 nuclear extracts to streptavidin-coupled double-stranded biotinylated oligonucleotide DNA. The DNA-bound Pit-1 and its associated proteins are then precipitated and the proteins are analyzed and identified via mass spectrometry. While the data presented in the thesis show an important role for the pituitary specific transcription factor Pit-1 and determine elements of the promoter required for appropriately regulated expression of the GHRH receptor *in vivo*, the data from the proposed studies will be insightful in uncovering what factors may be involved in differentiation of somatotrophs and lactotrophs from a common Pit-1 positive precursor cell type, and are expected to expand current understanding of pituitary development.

The GHRH receptor splice variant data reported in this thesis show for the first time that this receptor forms complexes, and are an important contribution to the field of GH regulation. The data from these experiments suggest a role for homo-oligomerization of the GHRH receptor in its normal functioning. The focus of future work should include determination of whether the GHRH receptor in fact forms homodimers or higher order complexes. Several techniques may be employed in this analysis, including fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), co-immunoprecipitation, IP-Westerns, and GST-pulldown assays. One limitation of each of these techniques is that a negative result does not necessarily indicate that the proteins do not interact. In fact, FRET has inconclusively been attempted in our laboratory to assess whether dimerization occurs for this receptor. Functional

data, including those reported in this thesis, indicate that oligomerization is important to normal functioning of the GHRH receptor, and determining the formation of homodimers will be important to understanding appropriate regulation of receptor signaling.

In addition, the data reported in this thesis can be useful in expanding our understanding of proliferation of certain types of cancer, as splice variant GHRH receptors are expressed in many cancers. In fact, the truncated human GHRH receptor was first identified in acromegalic patients with pituitary adenomas that express excess GH. Though it seems counterintuitive that this variant receptor that cannot signal in response to GHRH is most highly expressed in a pathological condition in which GH is hypersecreted, the most plausible explanation is that the splice variant is preferentially expressed in response to conditions in which GH secretion needs to be down-regulated. To further evaluate this hypothesis, examination of the regulation of splice variant receptor expression is required. Future work should focus on determination of whether variant receptor isoforms are preferentially expressed during different times of development or with changing physiological conditions and what factors may be involved in regulating expression of the different isoforms. Results from the proposed experiments will be important to expanding our knowledge of appropriately regulated GHRH receptor expression and how misregulation results in disease. Already, cancer treatments using GHRH antagonists are being developed and employed. The greater our knowledge of the expression, signaling and regulation of these splice variant receptor isoforms, the better our opportunity to effectively treat and cure related diseases.

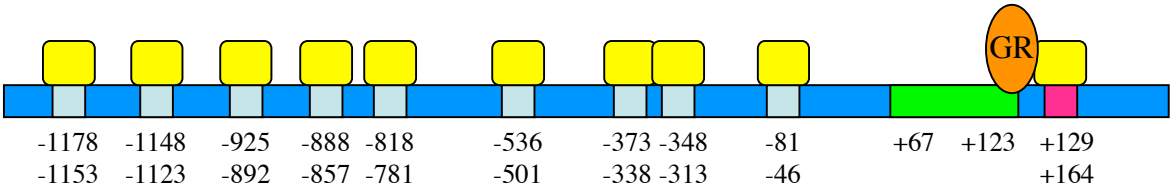
Taken together, the data presented in this thesis provide novel information on regulation of an important modulator of signaling in the GH axis, the GHRH receptor. For the first time, elements controlling regulation of GHRH receptor expression were examined in their

physiological context in transgenic mice. Determination that the 1.6kb proximal promoter directs appropriately regulated expression of the GHRH receptor *in vivo* led to further analysis of the promoter and the discovery of a mechanism for Pit-1 regulation of the gene, through binding to multiple sites throughout the promoter, including a required site within the proximal region (Fig. 5.2). Furthermore, examination of the functional effects of a post-transcriptional regulation revealed a role for alternative splicing in modulating signaling of this receptor and showed that oligomerization likely functions in normal signaling of this receptor. A model summarizing the data for signaling by dimerization with variant receptor isoforms is shown in Fig. 5.3. The data reveal important roles for both transcriptional and post-transcriptional regulation of the GHRH receptor gene, and provide details of particular mechanisms involved in these regulatory processes. This information will be invaluable in future work examining cell-specific regulation of expression and the involvement of homo-oligomerization in normal functioning of the GHRH receptor.

Figure 5.2: Schematic of Pit-1 Regulation of GHRH Receptor Transcription

Pit-1 (yellow blocks) binds ten sites (blue and pink rectangles) in the rat GHRH receptor proximal promoter. While Pit-1 binding to the combination of these sites yields the greatest activation of GHRH receptor expression, a single site (pink rectangle) is necessary and sufficient for Pit-1 activation of the promoter. A composite hormone response element (green box) contains response elements for T_3 and glucocorticoid receptor and contributes to synergistic activation with Pit-1 of the rat GHRH receptor promoter (Nogami et al. 2002). Glucocorticoid receptor (orange oval) activation of gene transcription requires the Pit-1 binding site at +129 to +164 (Nogami et al. 2005). In the human GHRH receptor promoter, two sites have been shown to bind Pit-1 (Iguchi et al. 1999). The upstream site, which corresponds to the conserved +129 to +164 required site in the rat, contributes most to Pit-1 activated transcription (Iguchi et al. 1999), but a mutation in the second site is associated with decreased GHRH receptor expression in a clinical case of isolated GH deficiency type IB (Salvatori et al. 2002).

Rat GHRH Receptor Promoter



Human GHRH Receptor Promoter

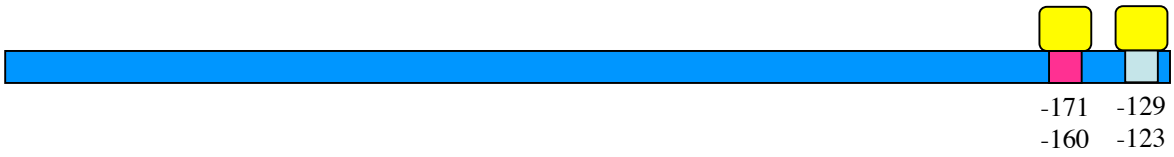
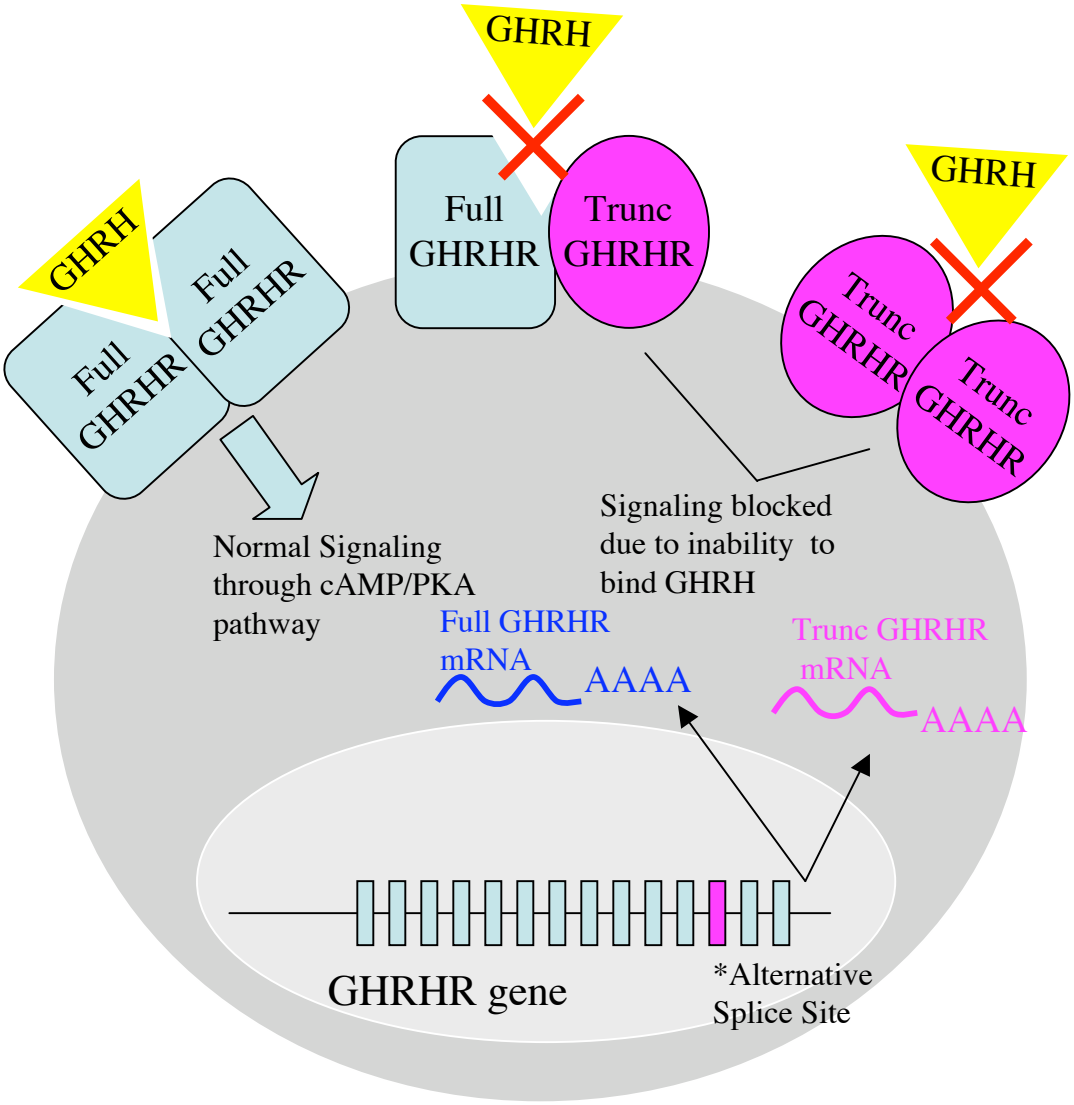


Figure 5.3: Interactions and Signaling Effects of GHRH Receptor Isoforms

Signaling of two isoforms of the receptor are examined in this thesis. The site of alternative splicing of Exon 11 is shown in pink. Variant mRNAs are produced from alternative splicing. In the human, a truncated isoform of the receptor is produced (pink ovals) that can heterodimerize with the wild-type receptor (blue rectangles). The truncated receptor cannot bind GHRH (yellow triangles) with high affinity (red x's blocking GHRH), and reduce ligand binding to the wild-type receptor. The data suggest that the wild-type GHRH receptor normally functions through the formation of homodimers and that heterodimers with the truncated receptor or truncated receptor homodimers act to dampen GHRH signaling by populating the cell membrane with non-functional complexes that potentially lack internalization domains.


GHRH
Full GHRHR
Full GHRHR
Full GHRHR
Trunc GHRHR
GHRH
Trunc GHRHR
Trunc GHRHR
GHRH
Normal Signaling through cAMP/PKA pathway
Signaling blocked due to inability to bind GHRH
Full GHRHR mRNA
AAAA
Trunc GHRHR mRNA
AAAA
GHRHR gene
*Alternative Splice Site

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