IN VIVO ANALYSIS OF HUMAN *LHX3* GENE REGULATION

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ABSTRACT

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IN VIVO ANALYSIS OF HUMAN *LHX3* GENE REGULATION

LHX3 is a transcription factor important in pituitary and nervous system development. Patients with mutations in coding regions of the gene have combined pituitary hormone deficiency (CPHD) that causes growth, fertility, and metabolic problems. Promoter and intronic elements of *LHX3* important for basal gene expression *in vitro* have been identified, but the key regulatory elements necessary for *in vivo* expression were unknown. With these studies, I sought to elucidate how *LHX3* gene expression is regulated *in vivo*. Based on sequence conservation between species in non-coding regions, I identified a 7.9 kilobase (kb) region 3' of the human *LHX3* gene as a potential regulatory element. In a *beta galactosidase* transgenic mouse model, this region directed spatial and temporal expression to the developing pituitary gland and spinal cord in a pattern consistent with endogenous *LHX3* expression. Using a systematic series of deletions, I found that the conserved region contains multiple nervous system enhancers and a minimal 180 base pair (bp) enhancer that direct expression to both the pituitary and spinal cord in transgenic mice. Within this minimal enhancer, TAAT/ATTA sequences that are characteristic of homeodomain protein binding sites are required to direct expression. I performed DNA binding experiments and chromatin immunoprecipitation assays to reveal that the ISL1 and PITX1 proteins specifically recognize these elements *in vitro* and *in vivo*. Based on *in vivo* mutational analyses, two tandem ISL1 binding sites

are required for enhancer activity in the pituitary and spine and a PITX1 binding site is required for spatial patterning of gene expression in the pituitary. Additional experiments demonstrated that these three elements cannot alone direct gene expression, suggesting a combination of factors is required for enhancer activity. This study reveals that the key regulatory elements guiding developmental regulation of the human *LHX3* gene lie in this conserved downstream region. Further, this work implicates ISL1 as a new transcriptional regulator of *LHX3* and describes a possible mechanism for the regulation of *LHX3* by a known upstream factor, PITX1. Identification of important regulatory regions will also enable genetic screening in candidate CPHD patients and will thereby facilitate patient treatment and genetic counseling.

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Adrenocorticotropic hormone	ACTH
Alpha glycoprotein subunit	αGSU
Alpha melanocyte-stimulating hormone	αMSH
Arginine vasopressin	AVP
Base pair	bp
Basic helix loop helix	bHLH
Bone morphogenetic protein	BMP
CCAAT-enhancer-binding protein	C/EBP
CCCTC-binding factor	CTCF
Central nervous system	CNS
Chromatin conformation capture	3C
Combined pituitary hormone deficiency	CPHD
Conserved non-coding element	CNE
Corticotropin-releasing hormone	CRH
Days post coitum	dpc
Diaminobenzidine	DAB
Diencephalon	DIEN
Electrophoretic mobility shift assay	EMSA
Fibroblast growth factor	FGF
Follicle-stimulating hormone	FSH
Follicle-stimulating hormone beta	FSHβ

Forkhead box	FOX
Forebrain	FB
Gonadotropin-releasing hormone	GnRH
Gonadotropin-releasing hormone receptor	GnRH-R
Growth hormone	GH
Growth hormone-releasing hormone	GHRH
Growth hormone-releasing hormone receptor	GHRHR
High mobility group	HMG
Homeodomain	HD
Immunohistochemistry	IHC
Infundibulum	IF
Islet1	ISL1
Islet2	ISL2
Kilobases	kb
Luria-Bertani broth	LB
LHX3 binding consensus site	LBC
LIM homeobox 3	LHX3
LIM homeobox 4	LHX4
LIM homeodomain	LIM-HD
Luteinizing hormone	LH
Luteinizing hormone beta	LHβ
Magnocellular neurons	MCN
Medial ganglionic eminence	MGE

Multi-cloning site	MCS
Nuclear factor 1	NF1
Nuclear LIM interacting protein	NLI
Nuclear localization signal	NLS
Oral ectoderm	OE
Oxytocin	OT
Paired homeobox	PAX
Phosphate buffered saline	PBS
Polymerase chain reaction	PCR
Pro-opiomelanocortin	POMC
Prolactin	PRL
Prophet of Pit1	PROP1
Rathke's pouch	RP
Sex-determining region Y	SRY
Sine oculis homeobox	SIX
Single nucleotide variations	SNV
Sonic hedgehog	SHH
SRY-box	SOX
Specificity protein 1	SP1
Steroidogenic factor 1	SF1
Thyroid-stimulating hormone	TSH
Thyroid-stimulating hormone beta	TSHβ
Wingless/integrated protein	WNT

CHAPTER ONE

INTRODUCTION

1.1 Pituitary Structure and Function

The pituitary is located near the base of the brain in the sella turcica (a depression of the sphenoid bone), and secretes hormones which regulate many essential processes including development, the stress response, growth, reproduction, metabolism, and lactation. The pituitary has dual embryonic origins consisting of a posterior lobe originating from the neuroectoderm, or diencephalon, and the intermediate and anterior lobes developing from an invagination of the oral ectoderm known as Rathke's pouch. The release of pituitary hormones in response to physiological conditions is mediated by signals from the hypothalamus.

Two major hormones are secreted by the posterior lobe: arginine vasopressin (AVP) and oxytocin (OT). AVP controls osmotic balance by regulating water absorption in the kidneys and OT is required to stimulate muscle contractions during parturition and lactation. The posterior lobe connects directly to the hypothalamus via the infundibulum or pituitary stalk. Magnocellular neurons (MCN) originate in the supraoptic nuclei and paraventricular nuclei of the hypothalamus and extend through the pituitary stalk into the posterior lobe. AVP and OT are synthesized in MCN and transported along their axons to a capillary bed in the posterior lobe where they are secreted into the blood.

The intermediate lobe of the pituitary secretes α-melanocyte-stimulating hormone (αMSH) from melanotrope cells. αMSH is produced by proteolytic processing of its prohormone from the *pro-opiomelanocortin* (*POMC*) gene. Alpha MSH has functions in

skin pigmentation and dark adaptation in lower vertebrates. The human intermediate lobe is less pronounced than in other vertebrates consisting of only a thin layer of cells. Because of the diminutive size of the human intermediate lobe, humans produce little αMSH.

Five hormone-secreting cell types are found in the anterior pituitary: corticotropes, gonadotropes, thyrotropes, somatotropes, and lactotropes secreting adrenocorticotropic hormone (ACTH, a product of the *POMC* gene), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL), respectively. Glycoprotein hormones TSH, FSH, and LH are composed of a unique beta subunit (TSHβ, FSHβ and LHβ) and a common alpha-glycoprotein subunit (αGSU). Hormones secreted from the human anterior pituitary have key roles in development, the stress response (ACTH), reproduction (FSH, LH, and PRL), metabolism (TSH), growth (GH), and lactation (PRL). The hormone-secreting cell types are observed to differentiate in a distinct dorsal to ventral pattern in the developing pituitary. In the dorsal portion of the anterior pituitary, corticotropes, somatotropes, lactotropes are observed. Thyrotropes are found in the rostral tip and central portion of the lobe and gonadotropes arise ventrally (Dasen et al., 1999; Kioussi et al., 1999; Lin et al., 1994).

Hormone release by secreting cell types in the anterior and intermediate pituitary is positively and negatively regulated by hypophysiotropic hormones (e.g. release of GH: GH-releasing hormone, inhibition of GH: somatostatin). Hypophysiotropic hormones secreted from the median eminence of the hypothalamus are transported via the hypophyseal portal blood system and bind specific cell surface receptors (e.g. GH-

releasing hormone receptor, somatostatin receptor) in the anterior and intermediate pituitary resulting in hormone (e.g. GH) release or inhibition.

1.2 Early Signaling Events in Pituitary Development

Signaling gradients between multiple factors in the diencephalon and oral ectoderm result in the invagination of the oral ectoderm to form Rathke's pouch, the primordium of the anterior pituitary lobe (Figure 1.1). The first step in the formation of the anterior pituitary is a thickening of the oral ectoderm and invagination to form Rathke's pouch, the primordial structure of the anterior pituitary. Based on findings from multiple studies in mice, this initial step is dependent on bone morphogenetic protein (BMP) 4 signals originating in the adjacent ventral diencephalon (Davis and Camper, 2007; Sheng et al., 1997; Takuma et al., 1998). This invagination brings Rathke's pouch in close contact with the adjacent ventral diencephalon and promotes further the proliferation and differentiation signaling events required for the formation of the mature pituitary gland.

Subsequently, BMP2 and BMP7 expression is initiated in the ventral mesenchyme adjacent to Rathke's pouch and expands into the pouch in a ventral to dorsal pattern (Ericson et al., 1998; Gleiberman et al., 1999). Signaling gradients involving BMPs and fibroblast growth factors (FGF) 8, FGF10, and FGF18 have key roles in dorsal to ventral patterning of the pituitary gland. Dorsally, FGFs are thought to maintain Rathke's pouch cells in a proliferative state and prohibit cell cycle exit. As cells migrate ventrally, FGF levels are reduced and cells exit the cell cycle and differentiate into definitive hormone cell types. Ventrally, BMPs promote ISL1 (described in section 1.4)

and αGSU expression and ventral cell types in the anterior pituitary in part by opposing FGF signaling (Ericson et al., 1998; Kimura et al., 1996; Norlin et al., 2000).

Sonic hedgehog (SHH), expressed both in the ventral diencephalon and throughout the oral ectoderm, is excluded from Rathke's pouch (Treier et al., 1998; Treier et al., 2001). Studies have shown that SHH signaling has important roles in pituitary development. Blocking the pathway with the SHH antagonist hedgehog interacting protein in Rathke's pouch arrested pituitary development, and over expression of SHH in the developing pituitary of mice resulted in pituitary hyperplasia (Treier et al., 2001). Other signaling molecules and transcription factors in the ventral diencephalon important for proper pituitary development include the LIM homeodomain (HD) protein LHX2, SOX3, WNT5a, and NKX2.1 (Alatzoglou et al., 2009; Cha et al., 2004; Potok et al., 2008; Takuma et al., 1998; Zhao et al., 2010).

1.3 Transcriptional Regulation of Anterior Pituitary Development

Further differentiation and proliferation events controlled by a cascade of transcription factors results in development of the anterior pituitary and establishment of the hormone-secreting cell types (Figure 1.1) [reviewed in (Kelberman et al., 2009; Zhu et al., 2007)]. Signaling molecules and transcription factors found in the anterior pituitary required for these developmental events include GLI1, GLI2, EYA1, SIX1, SIX3, SIX6, PAX6, HESX1, SOX2, PITX1, PITX2, ISL1, LHX3, and LHX4 (described in section 1.4), and PROP1.

GLI1, GLI2, and GLI3 are downstream transcription factors expressed in Rathke's pouch in response to SHH signaling. *Gli1*$^{-/-}$ mice have variable loss of the pituitary while *Gli1*$^{-/-}$/ *Gli2*$^{-/-}$ double knockout mice have a more severe phenotype; in

addition to defects in the ventral diencephalon, all have aplastic pituitaries (Park et al., 2000). Heterozygous mutations within the human *GLI2* gene cause variable forms of holoprosencephaly with hypoplastic or absent pituitaries and variable defects in facial structures (Roessler et al., 2003).

The SIX gene family members are mammalian homologs of *Drosophila melanogaster* sine oculis homeobox containing genes and act as part of protein complexes containing the co-repressor recruiter DACH and the EYA phosphatase. SIX1, SIX3, and SIX6 are expressed in the developing pituitary [reviewed in (Kawakami et al., 2000)]. Studies in mice and zebrafish have shown that SIX1 and EYA1 have cooperative functions in pituitary development. Double knockdown of SIX1 and EYA1 in zebrafish results in a failure to develop corticotropes, melanotropes, and gonadotropes. Somatotropes and thyrotropes are present but fail to express GH and TSHβ (Nica et al., 2006). The *Six1*$^{-/-}$ / *Eya1*$^{-/-}$ double knockout mice have hypoplastic pituitary glands (Li et al., 2003). In *Six3*$^{-/-}$ mice, early inductive events are disrupted and Rathke's pouch fails to form and mice double heterozygous for *Six3* and *Hesx1* null alleles have hypopituitarism (Gaston-Massuet et al., 2008; Lagutin et al., 2003). *Six6* knockout mice have defects in retinal, optic nerve and pituitary development. SIX6 also represses transcription of cell cycle inhibitors thereby promoting cellular proliferation in the developing retina and pituitary (Li et al., 2002).

PAX6 is a paired HD transcription factor important in the development of several tissues including the eye, nervous system, pancreas, and pituitary (Bentley et al., 1999; Dohrmann et al., 2000; Terzic and Saraga-Babic, 1999). The Small eye *Pax6* mutants and *Pax6*$^{-/-}$ mice have defects in dorsal to ventral patterning of the pituitary that results in

reduced numbers of somatotropes and lactotropes dorsally and an increase in thyrotropes and gonadotropes ventrally (Kioussi et al., 1999). Recently the only surviving patient with a compound heterozygous mutation in the *PAX6* gene was described presenting with severe developmental defects consistent with single heterozygous mutations plus a hypoplastic pituitary (Solomon et al., 2009).

In mouse and humans, the paired-class HD transcription factor HESX1 is expressed first in the neural plate and later restricted to the forebrain, ventral diencephalon and Rathke's pouch by e9.5 (Hermesz et al., 1996; Sajedi et al., 2008; Thomas et al., 1995). LHX3 is required during early pituitary development to maintain HESX1 expression (Sheng et al., 1997). Then as differentiation proceeds of specific hormone-secreting cell types, *Hesx1* is down regulated by the PROP1 paired homeobox protein (described below) and becomes undetectable by e15.5 (Gage et al., 1996; Hermesz et al., 1996). HESX1 is capable of repressing *Prop1* gene expression by recruiting co-repressor complexes containing Groucho-like TLE proteins and histone deacetylases (Brickman et al., 2001; Carvalho et al., 2010; Dasen et al., 2001). *Hesx1*-null and human mutation knock-in mouse models have defects in eye, olfactory, and forebrain development and pituitary dysplasia (Dattani et al., 1998; Sajedi et al., 2008). Similarly, *HESX1* mutations in human patients are associated with septo-optic dysplasia and pituitary abnormalities (Dattani et al., 1998; Sobrier et al., 2005; Thomas et al., 2001).

The SRY-related high mobility group box (SOX) 2 transcription factor has important roles in anterior pituitary development. During pituitary development, SOX2 is first expressed in the ectoderm and by e11.5 throughout Rathke's pouch, but as cell

differentiation proceeds its expression is down regulated in a manner similar to HESX1. By e18.5 expression is found only in the lumen of Rathke's pouch and the mature gland, in the region thought to contain the adult stem cell population of the pituitary (Fauquier et al., 2008; Kelberman and Dattani, 2006). *Sox2*-null mice die shortly after implantation prior to pituitary development (Avilion et al., 2003). The roles of SOX2 in pituitary development have been partially elucidated in studies of heterozygous mice and humans. A portion of surviving $Sox2^{+/-}$ heterozygous mice have mild hypopituitarism and mild hypoplasia of the anterior pituitary with bifurcations in Rathke's pouch (Alatzoglou et al., 2009; Avilion et al., 2003; Ferri et al., 2004; Kelberman and Dattani, 2006). Humans with heterozygous mutations in *SOX2* display pleiotrophic symptoms including bilateral anophthalmia or severe microphthalmia, anterior pituitary hypoplasia and gonadotropin deficiency (Fantes et al., 2003; Kelberman et al., 2008; Kelberman et al., 2006; Williamson et al., 2006). Human mutations in either the *SOX2* or *LHX3* genes are also sometimes associated with sensorineural hearing loss in addition to pituitary defects. The two proteins have overlapping expression patterns in the developing ear and pituitary and SOX2 can bind and activate the *LHX3a* promoter *in vitro* suggesting a possible role in *LHX3* gene regulation (Rajab et al., 2008).

The bicoid-like HD transcription factors PITX1 and PITX2 are required for the proper development of multiple organs including the heart, limbs, and pituitary. PITX1 was first identified as a protein-protein partner of the pituitary transcription factor, PIT1 (Szeto et al., 1996). PITX1 also regulates expression of the *POMC* gene in early pituitary development (Lamonerie et al., 1996). $Pitx1^{-/-}$ mice have morphologically normal pituitaries; however there are reductions in the number of gonadotropes and thyrotropes

present and LHβ and TSHβ levels and an increase in ACTH levels (Szeto et al., 1999). Both PITX1 and PITX2 recognize and bind the hormone promoters *αGSU*, *TSHβ*, *LHβ*, *FSHβ*, *GnRHR*, *PRL*, and *GH* (Tremblay et al., 2000). Knock down of PITX1 *in vitro* causes a loss of both *Lhx3* and *αGSU* expression (Tremblay et al., 1998). Further *in vivo* experiments show PITX1 or PITX2 are required for activation of *Lhx*3 during early pituitary development (Charles et al., 2005).

PITX2 is found in both the developing and adult pituitary gland (Gage and Camper, 1997; Semina et al., 1996). *Pitx2* gene activation is induced by the WNT-activated beta-catenin pathways during early pituitary development (Baek et al., 2003; Kioussi et al., 2002) and PITX2 promotes cellular proliferation by activating transcription of critical cell cycle regulators (Baek et al., 2003; Kioussi et al., 2002). *Pitx2*$^{-/-}$ mice have developmental defects in the heart, tooth, eye and pituitary and disruption of normal left-right asymmetry (Lin et al., 1999; Logan et al., 1998; Lu et al., 1999; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). The pituitary defects of the *Pitx2*-null mice are more severe than the *Pitx1*-null mice and pituitary development is arrested at e12.5 (Gage et al., 1999). Further studies of *Pitx2* $^{neo/neo}$ hypomorphs demonstrated PITX2 is required for proper pituitary development and the differentiation of gonadotropes, thyrotropes, somatotropes, and lactotropes (Suh et al., 2002). Both PITX1 and PITX2 proteins are found primarily to co-localize with gonadotropes and thyrotropes in the adult pituitary. However, mice with tissue-specific knock out of *Pitx2* in adult gonadotropes are normal (Charles et al., 2008; Charles et al., 2005). This demonstrates that PITX2 is not required for gonadotrope function and maintenance. However, similar to the overlapping functions seen in early development, PITX1 may be compensating for the loss of PITX2 in this

mouse model. *PITX2* mutations in humans are a known molecular cause of Rieger syndrome, iridogoniodysgenesis syndrome, type 2 autosomal dominant iris hypoplasia, and Peter's anomaly (Alward et al., 1998; Doward et al., 1999; Kulak et al., 1998; Semina et al., 1996).

The paired-like HD transcription factor, Prophet of PIT1 (PROP1), is expressed exclusively in the developing pituitary and is required for its proper development and function (Sloop et al., 2000; Sornson et al., 1996). PROP1 can act as either a transcriptional activator or repressor (Nasonkin et al., 2004). For example, the PROP1/β-catenin complex has been shown to activate *Pit1* transcription and repress *Hesx1* transcription depending which cofactors are present (Olson et al., 2006). PROP1 expression in the developing pituitary is initiated at e10 to e10.5, peaks at e12.5 and then declines after e14.5 (Sornson et al., 1996). The *Ames* dwarf mouse is a naturally occurring mutant mouse found to have a point mutation resulting in a defective DNA binding HD. *Ames* and *Prop1*-null mice have identical phenotypes. Both have hypoplastic pituitaries with deficiencies in GH, TSH, LH, FSH, and PRL and fail to express PIT1 (Gage et al., 1996; Sornson et al., 1996; Tang et al., 1993). In these mouse models, proliferation of progenitors in the perilumenal region is not affected but the cells fail to migrate. This results in a pituitary which first appears enlarged at e14.5 with abnormal morphology, and then later as a result of increased apoptosis is hypoplastic (Ward et al., 2005). This wax and wane in pituitary size has also been observed in some human patients with *PROP1* mutations. *PROP1* gene mutations in humans are the most common known cause of combined pituitary hormone deficiency (CPHD) and patients have hormone deficiencies like those seen in the *Prop1* mutant mouse models (Cushman

et al., 2002; Wu et al., 1998). The results of several transgenic mouse over-expression studies have demonstrated that tight temporal control of *Prop1* gene expression is required for proper pituitary development. Expression of PROP1 early throughout Rathke's pouch ablates pituitary development and prolonged expression in gonadotropes and thyrotropes delays gonadotrope development and leads to pituitary tumors (Cushman et al., 2001; Dasen et al., 2001; Dasen and Rosenfeld, 2001). Double knockout of *Lhx4* and *Prop1* in mice more severely affects pituitary development than single knockout of either gene. Corticotrope differentiation is delayed and the other hormone-secreting cells fail to develop. This indicates LHX4 and PROP1 together regulate differentiation and expansion events in the developing pituitary gland (Raetzman et al., 2002).

Further actions by downstream transcription factors including PIT1, SF1 and TPIT are also required for differentiation and specification of specific hormone-secreting cell types. PIT1 (also POU1F1, and GHF1) is a POU-HD transcription factor required for specification of somatotropes, thyrotropes, and lactotropes. Steroidogenic factor (SF) 1 is essential for gonadotrope development. A T-box class transcription factor, known as TBX19 or TPIT, has key roles in specification of cortitropes and directly activates POMC expression with PITX1 (Figure 1.1) [reviewed in (Kelberman et al., 2009; Zhu et al., 2007)].

1.4 LIM-HD Transcription Factors ISL1, LHX3, and LHX4

ISL1

Islet (ISL) 1 is a member of the LIM-HD family of transcription factors. LIM-HD transcription factors contain two zinc finger LIM domains important for protein-protein interactions and a central DNA binding homeodomain domain [reviewed in (Hunter and

Rhodes, 2005)]. ISL1 was first found in the pancreas and was shown to regulate *insulin* gene expression via the *insulin* gene enhancer (Karlsson et al., 1990). ISL1 is expressed in a wide variety of tissues including the pituitary, thyroid, kidney, spinal cord, hypothalamus, diencephalon, telencephalon, inner ear and pancreas (Dong et al., 1991; Karlsson et al., 1990; Mitsiadis et al., 2003; Radde-Gallwitz et al., 2004; Thor et al., 1991). ISL1 is the first LIM-HD protein expressed during mouse pituitary development and is detectable at e8.5 throughout the oral ectoderm and Rathke's pouch (Ericson et al., 1998; Pfaff et al., 1996). Between e10.5 and e11.5 in mouse, *Isl1* is repressed dorsally in response to FGF8 signals from the neuroectoderm and becomes restricted to the ventral portion of the developing pituitary and is co-expressed with αGSU (Ericson et al., 1998). Rathke's pouch is formed but its development is blocked in *Isl1*-null mice. The pituitary defect in the *Isl1* knockout is similar to *Lhx3*-null mice and LHX3 expression is absent from the pituitary. However, ISL1 is thought to block differentiation at an earlier stage rather than acting directly upstream of *LHX3* (Takuma et al., 1998). In *Lhx3*-null mice, ISL1 expression is activated normally in the pituitary at e9.5, but is transiently lost at e12.5. Later ISL1 expression returns but is found ectopically in the dorsal region of the gland (Ellsworth et al., 2008). These experiments suggest LHX3 may regulate *Isl1* expression both positively at e12.5 and later negatively in the dorsal pituitary. ISL1 is found primarily in the gonadotropes of the adult pituitary and positively regulates *FSHβ* and *LHβ* transcription and mediates leptin regulation of their synthesis (Liu et al., 2005a; Liu et al., 2005b; Wu et al., 2010). ISL1 and LHX3 act together in gonadotropes to trans-activate the *gonadotropin releasing hormone receptor*, *GnRH-R* promoter (Granger et al., 2006b).

ISL1 also has important roles in neural development. Conditional *Isl1* motoneuron knockouts fail to develop motoneurons and a subpopulation of interneurons, and do not have any markers of motoneuron development (Pfaff et al., 1996). In the spinal cord, ISL1 functions as a part of a combinatorial code of regulatory transcription factors, including ISL2, LHX3, and NLI, that direct proper differentiation of neural progenitor cells into either motoneurons or interneurons (Jurata et al., 1998; Thaler et al., 2002; Tsuchida et al., 1994). Similarly, ISL1 is necessary for bipolar interneuron development in the retina. Mice with conditional knockouts of *Isl1* in the neural retina have vision loss and defects in biopolar interneuron differentiation. LHX3 and LHX4 are also expressed in bipolar interneurons at P9 and partially co-localize with ISL1. In the neural retina conditional knockout of *Isl1*, LHX4 expression is maintained however LHX3 expression is lost (Elshatory et al., 2007).

LHX3

The LHX3 LIM-HD protein consists of two N-terminal tandem repeat zinc finger LIM motifs followed by a DNA binding homeodomain and a proline rich C-terminus (Bach et al., 1995; Seidah et al., 1994; Zhadanov et al., 1995). The *LHX3* gene has seven coding exons and six introns, and produces two mRNAs, *LHX3a* and *LHX3b*, that result in three protein isoforms: LHX3a, LHX3b, and M2-LHX3 (Sloop et al., 2001). The two messages, *LHX3a* and *LHX3b,* are produced from alternative splicing of exon Ia and exon Ib. The LHX3a and LHX3b protein isoforms are translated from the first methionine of *LHX3a* and *LHX3b* mRNAs whereas the M2-LHX3 protein isoform results from translation from an internal start codon within *LHX3a* mRNA. The LHX3a and LHX3b isoforms have identical LIM domains, DNA binding homeodomain, and C-terminus, but

different amino termini. M2-LHX3 lacks the LIM domains (Sloop et al., 2001; Sloop et al., 1999). Transcription of the *LHX3* gene results from two TATA-less, GC-rich promoters upstream of exon Ia and exon Ib and involves the actions of specificity protein (SP) 1 and nuclear factor (NF) 1 (Yaden et al., 2005).

LHX3 is expressed throughout the developing pituitary at mouse e9.5 (Sheng et al., 1997). Maximal expression of mRNA in the pituitary is detected by *in situ* hybridization at e14. Expression in the anterior lobe decreases after e18, but is maintained in adult pituitary. The central nervous system shows expression in the ventral portion of the presumptive pons, the medulla, and the spinal cord in two thin strips along the longitudinal axis from e9.5-P1 with highest levels of expression at e13 (Bach et al., 1995; Seidah et al., 1994; Zhadanov et al., 1995). Similar expression patterns are seen in the developing human nervous system and pituitary (Sobrier et al., 2004).

LHX3 has important roles in the development of both motoneurons and the pituitary. Acting with ISL1 and LHX4, LHX3 directs axons ventrally from the neural tube in the developing nervous system (Sharma et al., 1998). LHX3 is required for the proper development of the anterior and intermediate lobes of the pituitary, and is necessary for the specification and differentiation of four of the five hormone-secreting cell types: somatotropes, thyrotropes, lactotropes, and gonadotropes, (Sheng et al., 1997; Sheng et al., 1996). In *Lhx3*-null mice, which die shortly after birth, a definitive Rathke's pouch forms but fails to develop further and lacks four of the five hormone-secreting cell types, containing only a small population of corticotropes. Rathke's pouch appears normal in the *Lhx3*$^{-/-}$ mouse at e11.5, but by e12.5, expansion of the pouch is arrested. The posterior lobe appears normal, however the anterior lobe is missing and the

intermediate lobe shows a reduction in size. The *Lhx3*$^{+/-}$ heterozygous mice have sufficient LHX3 for normal specification of the cell lineages and pituitary development (Sheng et al., 1996). Studies of *Lhx3* $^{Cre/Cre}$ mice revealed reduced expression of LHX3 in the pituitary, but near normal expression in the developing nervous system (Sharma et al., 1998; Zhao et al., 2006). In contrast to the *Lhx3*$^{+/-}$ mice, the *Lhx3* $^{Cre/Cre}$ mice displayed a pituitary phenotype similar to the null mouse. In these mice with reduced LHX3 action there is increased cell apoptosis in the ventral portion of Rathke's pouch, but similar levels of cell proliferation to wild type animals. Increased apoptosis is also noted in *Pitx1*/*Pitx2*-null mice which lack detectable LHX3 expression (Charles et al., 2005).

Several factors including FGF8, PITX1, PITX2, SOX2, LHX4 and FOXP1 have all been implicated in the regulation of *LHX3* gene transcription in pituitary and neural tissues. Expression of FGF8 in the adjacent diencephalon and Rathke's pouch is responsible for activation of *Lhx3* and *Lhx4*. Mice null for *T/ebp* fail to express FGF8 in this area and display a phenotype similar to *Lhx3/Lhx4* double knockout mice (Takuma et al., 1998). PITX1 or PITX2 is also required for activation of *Lhx*3 during early pituitary development. *Pitx1/Pitx2* double knockout mice fail to express *Lhx3* and have an analogous phenotype to *Lhx3*-null mice (Charles et al., 2005). LHX3 expression is maintained in both *Pitx1*-null and *Pitx2*-null mice suggesting an overlapping function of the two proteins with expression of either sufficient to activate *Lhx3* during pituitary development (Lanctot et al., 1999; Szeto et al., 1999). SOX2 has been shown to bind and activate the *LHX3a* promoter *in vitro* (Rajab et al., 2008). *In vivo* studies have shown LHX4 is required for timely activation of LHX3. In *Lhx4* knockout mice, LHX3 expression is delayed but returns to normal by e14.5 (Raetzman et al., 2002). The

winged-helix/ forkhead transcription factor, FOXP1, has been shown to repress LHX3 expression in neuroendocrine cell lines and occupy the *Lhx3a* promoter in cell lines and e13.5 spinal cords in chromatin immunoprecipitation (ChIP) assays suggesting a possible role for FOXP1 in the negative regulation of *Lhx3* gene transcription during spinal cord development (Morikawa et al., 2009).

LHX3 is required for activation and expression of FOXL2, a transcription factor expressed from e10.5 to e12.5 in mouse with suspected roles in promoting differentiation in the developing pituitary as well as possible maintenance roles in adult pituitary function (Ellsworth et al., 2006). Other known target genes of LHX3 include *αGSU*, *TSHβ*, *Pit1*, *FSHβ*, *GnRH-R*, and *PRL* (Granger et al., 2006a; McGillivray et al., 2005; Savage et al., 2003; West et al., 2004).

To date ten autosomal recessive mutations within the human *LHX3* gene have been described including missense mutations, intragenic deletions, nonsense mutations, and a complete gene deletion. All characterized patients have combined pituitary CPHD lacking GH, TSH, FSH, LH, and PRL. Two recently described mutations also have ACTH deficiency (Rajab et al., 2008). This is similar to the *Lhx3*-null mice that lose all hormone-secreting cell types, except a small population of ACTH-secreting corticotropes. Not unlike the $Lhx3^{+/-}$ mouse, heterozygous family members are unaffected. The majority of *LHX3* mutation patients have rigid cervical spine and limited neck rotation presumably related to LHX3's role in motoneuron development. Patient with *LHX3* mutations have variable pituitary morphology ranging from hypoplastic to enlarged pituitaries (Bhangoo et al., 2006; Kristrom et al., 2009; Netchine et al., 2000; Pfaeffle et al., 2007; Rajab et al., 2008). In addition to CPHD and limited neck rotation,

other neural defects have been observed including mental deficiency and deafness (Bhangoo et al., 2006; Kristrom et al., 2009; Rajab et al., 2008). Some patients exhibit CPHD plus spine and neck defects that are similar to patients with *LHX3* mutations despite normal coding regions for the gene. One possible explanation for this phenotype is mutation of regulatory or enhancer elements of *LHX3*. Regulatory and enhancer mutations have been identified previously in other human diseases including Hirschsprung disease, familial triphalangeal thumb and preaxial polydactyly, and IgA nephropathy for example (Aupetit et al., 2000; Emison et al., 2005; Gurnett et al., 2007).

LHX4

LHX4 is expressed in the developing hindbrain, cerebral cortex, pituitary gland and spinal cord (Li et al., 1994; Liu et al., 2002). The highly related proteins, LHX4 and LHX3, share 63% amino acid identity overall and 75%-95% homology within the LIM and HD domains (Hunter and Rhodes, 2005; Mullen et al., 2007). At e9.5, LHX4 is found throughout Rathke's pouch. In contrast to LHX3 which remains expressed in all areas of the developing pituitary, LHX4 is transiently expressed and is then restricted by e12.5 to the future anterior lobe and finally down regulated by e15.5 (Sheng et al., 1997). *Lhx4*$^{-/-}$ mice die shortly after birth due to defects in lung development, but similar to *Lhx3*$^{+/-}$ mice, *Lhx4*$^{+/-}$ mice are normal. In *Lhx4*$^{-/-}$ mice, Rathke's pouch forms, however it fails to develop properly resulting in a hypoplastic pituitary. All of the hormone-secreting cell types are present, but are greatly reduced in number (Li et al., 1994; Sheng et al., 1997). Although proliferation is also slightly reduced, a wave of apoptosis at e14.5 appears to be the major cause of the hypoplasia (Raetzman et al., 2002). LHX4 with PROP1 plays a role in cell survival and regulation of the *Lhx3* gene. Although delayed in

Lhx4$^{-/-}$ and *Lhx4*/*Prop1* double knockout mice, LHX3 expression is normal by e14.5 (Raetzman et al., 2002). Early in development LHX3 and LHX4 have overlapping functions. The presence of one functional allele of either results in the formation of a definitive Rathke's pouch. Pituitaries of mice with complete loss of both LHX3 and LHX4 proteins do not develop past an early rudimentary stage (Sheng et al., 1997). LHX4 also has important roles in the development of the ventral motoneurons in the spinal cord (Sharma et al., 1998). Similar to the *LHX3* gene, *in vitro* studies have shown that *LHX4* transcription is regulated by a TATA-less promoter(s) containing recognition sites for SP1 (Liu et al., 2008; Yaden et al., 2006). LHX4 binds and activates several pituitary target genes including *αGSU*, *GH*, *PRL*, *PIT-1,* and *FSHβ* (Castinetti et al., 2008; Kawamata et al., 2002; Machinis and Amselem, 2005; Sloop et al., 2001; West et al., 2004).

Five heterozygous mutations in the *LHX4* gene and a complete gene deletion have been identified that result in CPHD and other defects including hypoplasia of the anterior lobe, ectopic posterior pituitary, structural abnormalities of the sella turcica, chiari malformations in the brain, and respiratory distress syndrome. GH and TSH deficiencies are common to all patients, but deficiencies in LH, FSH, ACTH, and PRL are variable (Castinetti et al., 2008; Dateki et al., 2010; Machinis et al., 2001; Pfaeffle et al., 2008; Tajima et al., 2007; Tajima et al., 2009).

1.5 Central Hypothesis and Aims

The central hypothesis for this study was that enhancers found 3' of the *LHX3* gene are necessary for the proper expression of the protein in both the developing pituitary and spinal cord, and that mutations in these elements can result in CPHD.

This hypothesis was based on the following observations. First, a 7.9 kb region 3' of the LHX3 gene was found that directed expression to the pituitary and nervous system expression. In addition, this region was found to function independent of its position and the *LHX3* proximal promoters indicating that enhancer elements were contained in this region. Furthermore, these non-coding regions have a high degree of conservation in multiple vertebrate species which also often correlates with regulatory function. Additionally, regulatory and enhancer mutations have been identified previously in other human diseases (Aupetit et al., 2000; Emison et al., 2005; Gurnett et al., 2007). Moreover, some CPHD patients with the spine and neck defects that are similar to patients with *LHX3* mutations lack coding-region mutations suggesting an alternate defect in gene expression.

The key regulatory elements necessary for *in vivo* expression of *LHX3* were unknown. The overall goal of this study was to uncover the molecular mechanisms of *LHX3* regulation and the possible role of mutations in *LHX3* regulatory regions in CPHD. The specific aims of this study were to: characterize the temporal and spatial expression patterns of the identified 3' enhancer regions; identify trans-acting factors affecting LHX3 expression; and screen candidate CPHD patients for mutations in the identified regulatory regions.

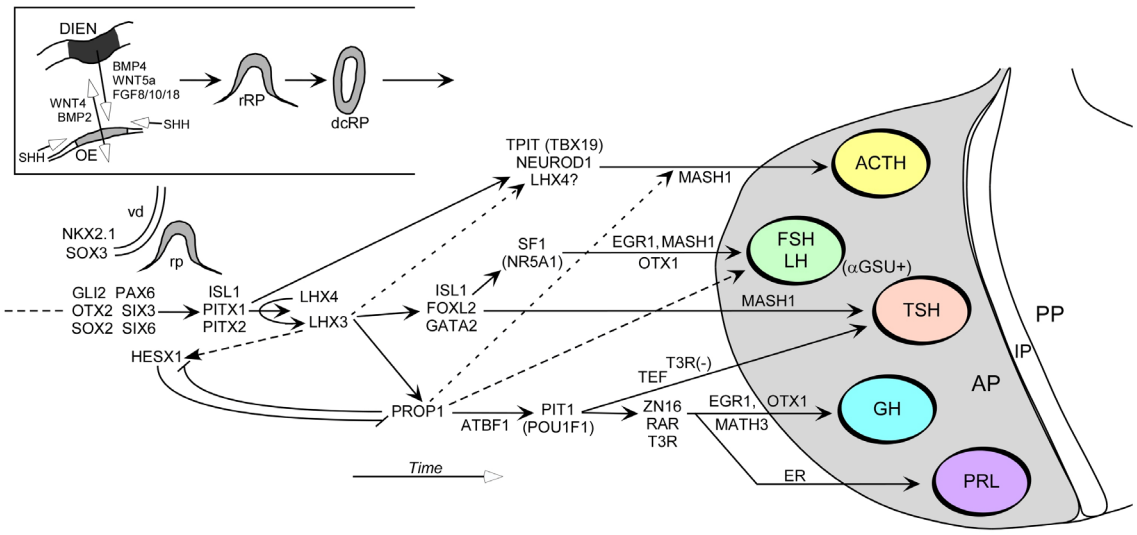


Figure 1.1. Regulation of anterior pituitary gland development by signaling proteins and transcription factors. Inductive signals between the ventral diencephalon (DIEN) and the oral ectoderm/anterior neural ridge (OE) precede formation of a rudimentary Rathke's pouch (rRP, the precursor of the adenohypophysis from which the anterior pituitary develops). Subsequently, a definitive, closed Rathke's pouch (dcRP) forms. Further differentiation and proliferation events controlled by a cascade of transcription factors results in development of the anterior pituitary and establishment of the hormone-secreting cell types. The mature pituitary gland has three main components: the anterior pituitary lobe (AP), the intermediate pituitary (IP), and the posterior pituitary (PP). Adapted from (Colvin et al., 2009)

CHAPTER TWO

MATERIALS AND METHODS

2.1 DNA Cloning and Vector Construction

Luciferase Reporter Constructs

The cloning and construction of the human -3.24 kb *LHX3a* promoter, -1.8 kb *LHX3b* promoter, and -2.5 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic constructs has been previously described (Yaden et al., 2006). To construct the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic vector, a region from the *NdeI* restriction site in the *LHX3a* promoter to the end of the *LHX3b* promoter, including *LHX3* Exon Ia, was cut from the -2.5 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic vector with *MluI* (blunted by incubating with 2 units of Klenow enzyme (Roche, Indianapolis, IN) for 20 m at room temperature) and *NdeI*, and inserted into the -3.24 kb *LHX3a* promoter pGL2-basic vector cut with *BamHI*, (blunted) and *NdeI*.

To construct the Full 3' enhancer pSC-B cloning vector, the 7.9 kb region 3' prime of the human *LHX3* gene was amplified in two fragments from 700 ng of BAC clone RP11-83N9/ALI38781 using *Pfu Ultra II HS* DNA polymerase (Stratagene, La Jolla, CA) and primers (5'-cgggatccgacccagttctgacctatcc-3' (S) and 5'-gaacagtcggcactttattaa ccacctgtcagc-3' (AS) for fragment I; 5'-ccaggtcgaaggcggaatttagggag-3' (S) and 5'-acgcg tcgaccactggcgacatcatctctg-3' (AS) for fragment II). PCR parameters were 2 m at 95°C, (20 s at 95°C, 20 s at 64°C, 1 m 15 s at 72°C) x 25, and 3 m at 72°C. PCR products were sub-cloned into pSC-B vector using Strataclone blunt cloning kit (Stratagene). Vector

fragment II- pSC-B and insert fragment I- pSC-B were cut at an overlapping *NotI* site and ligated together to form the Full 3' enhancer pSC-B vector. Vector was treated with Antarctic phosphatase (New England Biolabs, Ipswich, MA) prior to ligation.

The -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter-*luciferase*-Full 3' pGL2-basic vector was constructed by first excising the 7.9 kb Full 3' *LHX3* enhancer region from Full 3' pSC-B (*BamHI* and *SalI* sites) and ligating into the pGL2-basic vector (*BamHI* and *SalI* sites). Next the *LHX3a* plus *LHX3b* promoter region was excised from -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic (*SpeI* sites, blunted) and ligated into *luciferase*-Full 3' pGL2-basic (*BglII* site, blunted).

To construct the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter-*luciferase*-R3 pGL2-basic vector, the R3 enhancer was amplified using *Pfu Ultra HF* DNA polymerase (Stratagene) and primers (5'-cgggatccctgagactcctaggcctgacg-3' and 5'-acgcgtcgaccactggcgacatcatctctg-3'). PCR parameters were 4 m at 95°C, (30 s at 95°C, 30 s at 65°C, 30s at 72°C) x 30, and 7 m at 72°C. PCR products were sub-cloned into pSC-B vector using Strataclone blunt cloning kit (Stratagene). The R3 pituitary enhancer was excised from R3-pSC-B (*BamHI* and *SalI* sites) and ligated into -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic vector (*BamHI* and *SalI* sites).

The minimal -36 bp rat *prolactin* promoter was excised from the 3xPRDQ9 -36 *PRL luciferase* plasmid (described in Sloop et al., 2000) with *BglII* and *HindIII* and ligated into pGL4.1 (Promega, Madison, WI) upstream of the *luciferase* gene (*BamHI* and *HindIII* sites) to build the -36*PRL* pGL4.1 vector. The R3 pituitary enhancer was excised from R3 pSC-B (*BamHI* and *SalI* sites) and ligated into -36*PRL* pGL2-basic (*BamHI* and *SalI* sites) to construct the -36*PRL-luciferase*-R3 pGL4.1 vector.

LHX3 Promoter pWHERE Transgenes

The -3.24 kb *LHX3a* promoter pWHERE transgene was constructed by inserting the *LHX3a* promoter into the multi-cloning site (MCS) of the pWHERE vector (Invivogen, San Diego, CA). This vector contains a MCS upstream of a *beta galactosidase* transcription unit with a nuclear localization signal followed by untranslated region (UTR) and a polyadenylation signal from human *EF1 alpha* gene and flanked by murine *H19* insulator regions. The *LHX3a* promoter was cut from the -3.24 kb *LHX3a* promoter pGL2-basic construct (*SpeI* and *BglII*) and inserted into the pWHERE vector (*AvrII* and *BamHI* sites).

The -1.8 kb *LHX3b* promoter pWHERE transgene was constructed by inserting the *LHX3b* promoter into the MCS of the pWHERE vector (Invivogen). The *LHX3b* promoter was cut from the -1.8 kb *LHX3b* promoter pGL2-basic construct (*SpeI* and *HindIII*, blunted, sites) and ligated into the pWHERE vector (Invivogen) (*AvrII* and *SmaI* sites). The -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter modified pWHERE transgene was constructed in two steps. First, the pWHERE vector was modified to remove an additional *PstI* site in the MCS, leaving only the *PstI* site immediately after the poly-A tail. The pWHERE vector was digested with *SdaI* (cuts at the *PstI* site in the MCS) then blunted and re-ligated to remove the *SdaI* and *PstI* sites. Second, the *LHX3* promoter region was excised from the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic construct (*BamHI* sites) and ligated into the modified pWHERE vector (*AvrII* site).

To build the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter-*nLacZ*-Full 3' modified pWHERE transgene (Figure 2.1), first, the Full 3' enhancer was excised

from Full 3' pSC-B (*BamHI* and *SalI* sites, blunted) and ligated into the modified pWHERE vector (*PstI* site, blunted). Next, the *LHX3* promoter region was excised from the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic construct (*SpeI* sites, blunted) and ligated into the *nLacZ*- Full 3' modified pWHERE vector (*BamHI* site, blunted).

To build the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter -*LacZ*- Full 3' + Far modified pWHERE, first the Far enhancer was amplified using *Pfu Ultra II HS* DNA polymerase (Stratagene) and primers (5'-gacagcagtgaagatttgtgac-3' and 5'-gag tgactgaaacagctccc-3'). PCR parameters were 2 m at 95°C, (20 s at 95°C, 20 s at 57°C, 15 s at 72°C) x 30, and 3 m at 72°C. PCR products were sub-cloned into pSC-B vector using Strataclone blunt cloning kit (Stratagene). Next, the Far enhancer was excised (*EcoRV* and *KpnI* sites) from pSC-B and ligated into the Full 3' enhancer pSC-B (*SmaI* and *KpnI* sites). The combined enhancer region (Full 3' + Far) was excised (*EcoRV* and *BAMHI*, blunted, sites) and ligated into modified pWHERE vector (*PstI* site, blunted). Lastly, the *LHX3* promoter region was excised from the -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter pGL2-basic construct (*SpeI* sites, blunted) and ligated into the MCS of *nLacZ*- Full 3' + Far modified pWHERE (*BamHI* site, blunted).

Human LHX3 Enhancer HSP68 Promoter pSC-B Transgenes

The *HSP68-Hand2-LacZ* pSK-Bluescript (a kind gift from Dr. Simon Conway, Indiana University School of Medicine, Indianapolis, IN) was first modified to remove the *Hand2* control enhancer by digestion with *XhoI* and *HindIII* followed by gel purification and re-ligation. Next, *HSP68-LacZ* was excised from *HSP68-LacZ* pSK-Bluescript (*KpnI* and *HindIII* sites, blunted) and ligated into the Full 3' enhancer pSC-B

(*EcoRV* site) to construct the Full 3'-*HSP68-LacZ* pSC-B vector. *HSP68-LacZ* (*KpnI* and *HindIII* sites, blunted) was ligated into Full 3' pSC-B (*BsaBI* and *EcoRV* sites) to construct the UTR *HSP68-LacZ* pSC-B vector. *HSP68-LacZ* (*KpnI* and *HindIII* sites, blunted) was ligated into Full 3' pSC-B (*MluI*, blunted, and *EcoRV* sites) to construct the UTR R1 *HSP68-LacZ* pSC-B vector. *HSP68-LacZ* (*KpnI* and *HindIII* sites, blunted) was ligated into Full 3' pSC-B (*NcoI* (blunted) and *EcoRV* sites) to construct the UTR R1 R2 *HSP68-LacZ* pSC-B vector. The R2 enhancer region was isolated from fragment II of the Full 3' enhancer by digestion with *NaeI* and *PmlI* and ligated into the cloning vector pSC-B (*SmaI* and *EcoRV* sites). *HSP68-LacZ* (*KpnI* and *HindIII* sites, blunted) was ligated into R2 pSC-B (*XhoI* site, blunted) to construct R2 *HSP68-LacZ* pSC-B plasmid. *HSP68-LacZ* (*KpnI* and *HindIII* sites, blunted) was ligated into Far 3' pSC-B (*BamHI* and *HindIII* sites, blunted) to construct the Far 3' *HSP68-LacZ* pSC-B vector.

Enhancer HSP68 pWHERE Transgenes

The Full 3' enhancer was excised from Full 3' enhancer pSC-B (*BamHI* and *SalI* sites) and ligated into the MCS of the modified pWHERE vector (*BamHI* and *SalI* sites). Next, the *HSP68* promoter was excised (*KpnI* and *NcoI* sites, blunted) and ligated into Full 3' pWHERE (*SalI* site, blunted) to construct the Full 3'-*HSP68* pWHERE (Figure 2.2). The UTR *HSP68* pWHERE, UTR R1 *HSP68* pWHERE, UTRR1R2 *HSP68* pWHERE, and R2 *HSP68* pWHERE transgenes were constructed by excising the respective enhancer-*HSP68* region from the pSC-B vector with *BamHI* and *NcoI* and ligating into the MCS of the modified pWHERE plasmid digested with the same enzymes. The Far 3'-*HSP68* pWHERE was constructed by excising the Far 3' enhancer-*HSP68* from the pSC-B vector with *NotI* (blunted) and *NcoI* and ligating into the MCS of

the modified pWHERE plasmid digested with *SmaI* and *NcoI*. To construct the R3-*HSP68* pWHERE transgene (Figure 2.3), the R3 enhancer was excised from the pSC-B vector with *BamHI* and *SalI* and ligated into R2-*HSP68* pWHERE digested with *BamHI* and *SalI*, thereby removing the R2 enhancer and replacing it with the R3 enhancer. The R3 enhancer was excised (*SalI* sites) and ligated 3' of the R2 region into the R2-*HSP68* pWHERE (*SalI* site) to construct the R2R3-*HSP68* pWHERE transgene. The Delta R2-*HSP68* pWHERE transgene was constructed by amplifying a region from directly downstream of the R2 enhancer to the 3' end of the Full enhancer using *Pfu Ultra HF* DNA polymerase (Stratagene) and primers (5'-cgggatccctgagactcctaggcctgacg-3' and 5'-acgcgtcgaccactggcgacatcatctctg-3'). The primers added *MluI* and *SalI* sites to the 5' and 3' end respectively. PCR parameters were 4 m at 95°C, (30 s at 95°C, 30 s at 65°C, 30 s at 72°C) x 30, and 7 m at 72°C. PCR products were sub-cloned into pSC-B vector using Strataclone blunt cloning kit (Stratagene). The insert was excised (*MluI* and *SalI* sites) and ligated into the Full 3' pWHERE (*MluI* and *SalI* sites) to construct Delta R2 pWHERE. The *MluI* site is 428 bp upstream of R2 and *SalI* is at the 3' end of the Full enhancer. *HSP68* (*KpnI* and *NcoI* sites, blunted) was then ligated into DeltaR2 pWHERE (*SalI*, blunted).

To construct the Core R3-*HSP68* pWHERE transgene, the 180 bp Core R3 enhancer region was amplified from the Full 3' enhancer using *Pfu Ultra HF* DNA polymerase (Stratagene) and primers (5'-cgggatcccaggcctctgctagggtggg-3' and 5'-acgcgtcgacatcccaatcccaccgccatc-3') and the PCR parameters 4 m at 95°C, (30 s at 95°C, 30 s at 65°C, 30 s at 72°C) x 30, and 7 m at 72°C. The primers added *BamHI* and *SalI* sites to the 5' and 3' end respectively. PCR products were sub-cloned into pSC-B vector

using Strataclone blunt cloning kit (Stratagene). The insert was excised (*BamHI* and *SalI* sites) and ligated into R3-*HSP68* pWHERE (*BamHI* and *SalI* sites) thereby removing the R3 enhancer and replacing it with the Core R3 enhancer region.

The Core R3 Fragment I-*HSP68* pWHERE was constructed as described for the Core R3-*HSP68* pWHERE transgene. Region was amplified with primers (5'-cgggatccca gtaatcctcggaatg-3' and 5'-tggtcgacgcgtcattccgaggattac-3'). The Core R3 Fragment II-*HSP68* pWHERE was constructed as described for the Core R3-*HSP68* pWHERE transgene. Region was amplified with primers (5'-cgggatcccagtaatcctcggaatg-3' and 5'-acgcgtcgacgaggagagtttgcg-3'). The Core R3 Fragment III-*HSP68* pWHERE was constructed as described for the Core R3-HSP68 pWHERE transgene. Region was amplified with primers (5'-cgggatccactctcctcattaaac-3' and 5'-acgcgtcgacatcccaatccc accgccatc-3').

R3 Binding Site Mutation Transgenes

Site-directed mutagenesis using the QuikChange II system (Stratagene) was used to mutate the R3 pSC-B construct. Oligonucleotides for mutagenesis were 5'-gctcct ctccctggcaaacg**ag**t**g**g**g**tcagagctcagtaatcctcg-3', 5'-cgaggattactgagctctga**cc**c**a**c**t**cgtttg ccagggagaggagc-3' ("SOX" mutation); 5'-gctttgttcagagctcagt**cgg**cctcggaatgacaagg-3', 5'-ccttgtcattccgagg**ccg**actgagctctgaacaaagc-3' (TAAT1 site mutation); 5'-cggaatgacaagg tttaaaatttt**cgg**tagcaggctcctcttacgc-3', 5'-gcgtaagaggagcctgcta**ccg**aaattttaaaccttgtcattccg-3' (TAAT/ATTA2 mutation); 5'-ggtttaaaatttaattagcaggctcctc**gg**acg**ggt**actctcctcattaaactaagtgt ccc-3', 5'-gggacacttagtttaatgaggagagt**acc**cgt**cc**gaggagcctgctaattaaatttttaaacc-3' ("C/EBP" mutation); 5'-ggctcctcttacgcaaactctcctc**cggc**aactaagtgtcccattagttaaagt-3', 5'-actttaactaat gggacacttagtt**gccg**gaggagagtttgcgtaagaggagcc-3' (ATTA3 mutation); 5'-ctctcctcattaaac

taagtgtccc**cggc**gttaaagtgaaacttgatggcggtg-3', 5'-caccgccatcaagtttcactttaac**gccg**gggacactta gtttaatgaggagag-3' (ATTA4 mutation) (Site mutations are bold underlined). The mutated R3 region was excised from the pSC-B vector and ligated into *HSP68* pWHERE (*BamHI* and *SalI* sites). R3 (ATTA3 Mutation and ATTA4 Mutation)-*HSP68* pWHERE was generated by site-directed mutagenesis of R3 (ATTA3)-pSC-B using the ATTA4 mutation oligonucleotides and ligation into *HSP68* pWHERE as described above.

Human Patient Sequencing

The R3 enhancer region was amplified from purified DNA of candidate patients using *Pfu Ultra II HS* DNA polymerase (Stratagene) and primers (5'-ctgagactcctaggcctga cg-3' and 5'-ctcactggcgacatcatctct-3') with the parameters; 2 m at 95°C, (30 s at 95°C, 30 s at 56°C, 1 m at 72°C) x 30, 10 m at 72°C. To sequence the PCR products in bulk, 20% of the total PCR product was digested with 0.5 U of exonuclease I (USB Corp., Cleveland, OH) for 60 m followed by heat inactivated for 15 m at 80°C. The PCR products were then purified by ethanol precipitation and resuspended in nuclease free water for DNA sequencing.

DNA Sequencing

DNA sequencing was performed with a Perkin Elmer DNA sequencer (Biochemistry Biotechnology Facility at the Indiana School of Medicine). The DNA templates were submitted using the recommended guidelines from the sequencing facility. The sequence alignment and analyses were done with the DNASIS (Hitachi Software Engineering, San Francisco, CA) software.

2.2 Protein Analyses

In Vitro Transcription/Translation

Human(h)LHX3, hLHX4, rat(r)ISL1, hPITX1 and hPITX2 proteins were synthesized *in vitro* from 0.5-1.0 µg expression vector substrates (LHX3 and LHX4 expression vectors, described in Pfaeffle et al. 2007 and 2008; rISL1 expression vector, a kind gift from Dr. Samuel Pfaff, Salk Institute, La Jolla, CA ; hPITX1 a kind gift from Dr. Marie-Hélène Quentien, Département de Neuroendocrinologie and Neuroimmunologie, Université de la Méditerranée; hPITX2 a kind gift from Dr. Micheal Walter, Department of Medical Genetics, University of Alberta) using T7 RNA polymerase, TNT rabbit reticulocyte lysates (Promega, Madison, WI), and cold or ^{35}S-cysteine (PerkinElmer, Waltham, MA). Parallel negative controls were programmed with empty vector. ^{35}S labeled proteins were separated in 12% SDS-PAGE gels, treated with Amplify fluorography reagent (GE Healthcare Biosciences, Piscataway, NJ) and visualized by autoradiography or using a Fujifilm FLA-5100 phosphorimager to confirm their correct size.

Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were performed using *in vitro* translated (see above) non-radiolabeled proteins. For LHX3, LHX4 and rISL1 EMSAs, equivalent amounts (7 µl) of TnT proteins and empty vector programmed cell lysates were incubated with 12 µl reaction mixture (5x reaction buffer [60% glycerol, 100 mM Hepes pH 7.9, 20 mM Tris-Cl pH 8.0, 300 mM KCl, and 3 mM EDTA], 1 µg/ml dIdC, 0.1 µg/µl salmon sperm DNA, 10 µg/µl BSA, and 100 mM DTT) to a final volume of 19 µl. Reactions were pre-blocked on ice for 10 m and then combined with 1 µl (40,000 cpm) of radiolabeled DNA probes and

incubated at 25°C for 30-45 m. Reaction complexes were resolved by gel electrophoresis in 5% polyacrylamide.

PITX1 and PITX2 EMSA experiments were performed using conditions modified from Amendt et al., 1999. Briefly, 10 µl of in vitro-translated protein lysate and ^{32}P labeled probes were incubated in 20 mM Hepes pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1.0 µg of poly(dI·dC) on ice for 30 m. The samples were electrophoresed for 2 ½ h at 250 V in 8% polyacrylamide gel with 0.25× TBE at 4 °C following pre-electrophoresis of the gels for 15 m at 200 V.

All EMSA were dried onto Whatman 3mm paper and visualized by autoradiography or using a Fujifilm FLA-5100 phosphorimager. DNA probe sequences were as follows; the LHX3 consensus binding site (LBC): 5'-gcgatcccagaaaattaattaattgtaa gcg-3'and 5'-cgcttacaattaattaattttctgggatcgc-3', the A3/4 ISL1 binding site in the rat *insulin* promoter: 5'-ccttgttaataatctaattacccta-3' and 5'-tagggtaattagattattaacaagg-3', the tandem bicoid element, Bcd2x5n, previously shown to bind PITX proteins (Saadi et al., 2003): 5'-atctaatcccgtcgtaatcgcat-3' and 5'-atgcgattacgacgggattagat-3', R3 enhancer site TAAT1: 5'-gttcagagctcagtaatcctcggaatg-3'and 5'-cattccgaggattactgagctctgaac-3', R3 enhancer site TAAT1 mutated: (Mutations are bold underlined.) 5'-gttcagagctcagt**cgg**cctc ggaatg-3'and 5'-cattccgagg**ccg**actgagctctgaac-3', R3 enhancer site TAAT/ATTA2: 5'-aaggtttaaaatttaattagcaggctcc-3'and 5'-ggagcctgctaattaaattttaaacctt-3', R3 enhancer site TAAT/ATTA2 mutated: 5'-aaggtttaaaattt**cgg**tagcaggctcc-3'and 5'-ggagcctgcta**ccg**aaattttaa acctt-3', R3 enhancer site ATTA3 and ATTA4: 5'-ctcattaaactaagtgtcccattagtta-3' and 5'-taactaatgggacacttagtttaatgag-3', R3 enhancer site ATTA3 mutated: 5'-ctc**gccg**aactaagtgtcc

cattagtta-3' and 5'-taactaatgggacacttagtt**cggc**gag-3', R3 enhancer site ATTA4 mutated: 5'-ctcattaaactaagtgtccc**gccg**gtta-3'and 5'-taac**cggc**gggacacttagtttaatgag-3', or R3 enhancer site ATTA3 and ATTA4 mutated: 5'-ctc**gccg**aactaagtgtccc**gccg**gtta-3' and 5'-taac**cggc**ggg acacttagtt**cggc**gag-3'.

Chromatin Immunoprecipitation (ChIP)

Chromatin cross-linking and immunoprecipitation (ChIP) analyses were performed on αT3 mouse pituitary cells with the EZ Chip™ Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA) following the manufacturers recommended protocol. Protein DNA chromatin complexes were fragmented by sonication with conditions optimized to obtain the majority DNA fragments within the range of 200 to 1000 bp. One million cells were used for each immunoprecipitation. Precleared protein DNA chromatin complexes were incubated overnight at 4°C with either 5 µg of PITX1 rabbit polyclonal antibody (Abnova Corporation, Walnut, CA) or a cocktail of ISL1 monoclonal antibodies (3 µg each) used previously by Du et al., 2009 for ChIP assay (Developmental Studies Hybridoma Bank 39.4D5, 39.3F7, 40.3A4, 40.2D6). Controls were incubated with normal mouse immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA) for ISL1 experiments or normal rabbit immunoglobulin (Sigma, St. Louis, MO) for PITX1 experiments. Quantitative real-time PCR was performed on 5 µl of the immunoprecipitated and input DNA using SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) and an ABI Prism 7900 instrument. The $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_{t,input} - \Delta C_{t,sample,}$ was calculated for each sample. Relative enrichment was calculated as the fold difference above the $2^{-\Delta\Delta Ct}$ for the control mouse or

rabbit normal immunoglobulin samples. Oligonucleotides used for quantitative PCR were; 5'-agccacccctcccaccatca-3' and 5'-ggagagtttgcataagagaaacctgct-3', or 5'-gcaggtttctc ttatgcaaactctcct-3' and 5'-tagctccaccccacccccac-3'.

2.3 Cell Culture and Transient Transfections

HEK 293T cells (1.5 x 10^5 cells/35 mm dish), mouse pituitary gonadotrope αT3 cells (5.0 x 10^5 cells/35 mm dish), and mouse pituitary gonadotrope LβT2 cells (2.5 x 10^5 cells/35 mm dish) were cultured in DMEM with 10% FBS (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). HEK 293T cells were transiently transfected using the CalPhos™ Mammalian Transfection Kit (Clontech). LβT2 and αT3 cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 0.5-1.0 μg of reporter gene plasmid and 0.1-1.0 μg of expression vector. Parallel control samples received equivalent amounts of empty expression vector DNA. All luciferase assays were performed in triplicate. Forty-eight hours following transfection, cells were lysed in 25 mM Tris pH 7.8, 2 mM DTT, 1% Triton X-100, 2 mM EDTA, and 10% glycerol assay buffer and luciferase activity was measured using a Beckman Coulter LD400 plate reader/luminometer (Beckman Coulter, Fullerton, CA) as described (Sloop et al., 2001). Following determination of total protein levels by the Bradford method (BioRad), luciferase activities were normalized to protein concentration. Experiments were repeated a minimum of three times.

2.4 Generation, Genotyping, and Breeding of Transgenic Mice

Transgenic Mouse Generation

One hundred micrograms of transgene plasmid DNA was linearized with *Pac I* digestion. Digest was submitted either to the Purdue Transgenic Mouse Core Facility (West Lafayette, IN) or the Indiana University Cancer Center Transgenic and Knock-out Mouse Core (Indianapolis, IN) for gel purification and microinjection. The linearized transgenes were microinjected into F2 zygotes from FVB/N or C3H parents at a concentration of approximately 2-3 ng/µl. After microinjection, two-cell stage embryos were transferred to 0.5 days post coitum (dpc) pseudopregnant females. Founder transgenic mice were harvested at embryonic day 12.5 (e12.5) or e14.5 for transient transgenic studies or used for breeding as adults for the generation of stable transgenic lines. Harvested embryos were designated e0.5 the day after microinjection of the transgene.

Breeding and Housing of Transgenic Mice

Transgenic founder animals and their progeny were bred to C3H mice (Harlan Laboratories, Indianapolis, IN) to generate heterozygotes. The morning after copulation, indicated by the presence of a vaginal plug, was considered embryonic day 0.5 (e0.5) and the day of birth was postnatal day 1 (P1).

Mice were housed in a specific pathogen-free environment under controlled conditions of temperature and light and were provided free access to tap water and commercial mouse chow. The Indiana University Committee on Use and Care of Animals approved all procedures done using the mice, and all experiments were

performed in agreement with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

Genotyping of Transgenic Mice

Genomic DNA was purified from mouse tail snips taken between 14 and 21 days of age using the genomic DNA solution set kit (Gerard Biotech, Oxford, OH) according to manufacturer's instructions. Genotyping for transgenic mouse lines was performed using a multiplex PCR amplifying the transgenic region and wild type control region with the following oligonucleotides: 5'-aggactgggtggcttccaactcccagacac-3', 5'-agcttctcatt gctgcgcgccaggttcagg-3' (wild type control, *Rapsn* gene) and 5'-tcatcagcagaaagacctacag-3', 5'-tcagaagggaacacataaggg-3' (pWHERE modified *beta galactosidase* gene). Expected amplicon sizes were 591 bp and 252 bp respectively. PCR parameters used for genotyping experiments were 2 m at 94°C, (30 s at 94°C, 30 s at 56 °C, 30 s at 72°C) x 30, and 7 m at 72°C.

2.5 Histology and Immunohistochemistry

Fixation and Sectioning

Embryos for immunohistochemistry were fixed on ice in 4% paraformaldehyde in 1X PBS for 1 to 24 h. Adult pituitaries and embryos used for beta galactosidase staining were fixed on ice in 2% paraformaldehyde and 0.2% glutaraldehyde in 1X PBS (pH 7.2) for 1 h. All harvested tissues were washed three times in 1X PBS (pH 7.2; quick rinse, 1 h, and 30 m) and placed in 20% sucrose overnight. Next tissues and embryos were embedded in O.C.T. compound (Sakura Finetek, Torrence, CA) on dry ice and stored at -80°C until cryosectioned at a thickness of 7 µm.

Whole Mount Beta Galactosidase Activity Staining

After fixation and washing as described above, embryos were incubated at room temperature overnight with gentle agitation in X-gal solution (in 1X PBS (pH 7.2), 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.2% each of Triton X-100, Nonidet P-40 and Tween 20, and 1 mg/ml X-gal diluted in dimethylformamide). Following staining, specimens were washed at room temperature overnight with gentle agitation in 1X PBS (pH 7.2). After washing, embryos were imaged for surface staining. To clear, embryos were dehydrated in 70% (30 m), 95% (30 m), and 100% ethanol (30 m twice or overnight) and then incubated with gentle agitation in 100% methyl salicylate at room temperature for 1 h. Imaging was done immediately following clearing. Wild type embryos in litters served as negative controls. After whole mount staining, e12.5 embryos were paraffin embedded and sectioned at a thickness of 6 μm. Sections were deparaffinized, dehydrated in ethanol washes (95% then 100%; 1 m two times) and then eosin counterstained (1:1 mixture of 100% ethanol and eosin stain; 30 s) and then washed in 100% ethanol and allowed to dry. After drying, slides were cover-slipped using permamount (Fisher Scientific, Pittsburg, PA).

Cryosection Beta Galactosidase Activity Staining

Cryosections were air dried and fixed for 10 m with 0.5% glutaraldehyde in 1X PBS (pH 7.2) then washed three times in 1X PBS (pH 7.2; quick rinse, 10 m, and 5 m) followed by staining in X-gal solution (described above) at room temperature in the dark overnight with gentle agitation. Following staining, the slides were washed overnight in 1X PBS (pH 7.2). After washing, slides were dehydrated in ethanol washes (95% then 100%; 1 m two times) and then eosin counterstained (1:1 mixture of ethanol and eosin

stain; 1 time 30 s) and then washed in 100% ethanol and allowed to dry. After drying, slides were cover-slipped using permamount (Fisher Scientific, Pittsburg, PA).

Immunohistochemistry

Cryosections were rehydrated in 1X PBS (pH 7.2) and antigen unmasked by citrate boil [(10 m boil in 10 mM citric acid (pH 6.0)] before immunostaining. Double immunohistochemistry was performed using polyclonal antibodies against *E. coli* β-galactosidase (1:500 Fluor.) (MP Biomedicals, Solon, OH) and mouse LIM-3 (1:100 Fluor.) (Chemicon, Temecula, CA) or mouse LIM-3 (1:100 Fluor.) (Chemicon, Temecula, CA) and human ACTH (1:500 Fluor.) (AFP-39032082), or rat αGSU (1:100 Fluor.) (AFP-66P9986), or rat GH (1:500 Fluor.) (AFP-5672099), or rat LHβ (1:400 Fluor.) (AFP-571292393), or rat TSHβ (1:500 Fluor.) (AFP-1274789) (National Hormone and Pituitary Program, Torrance, CA). To determine the co-localization pattern of enhancer directed transgenes and the hormone secreting cell types, cryosections were first stained for β-galactosidase activity and then immunostained with the antibodies against human ACTH (1:1000 DAB) (AFP-39032082), or rat αGSU (1:500 DAB) (AFP-66P9986), or rat GH (1:1000 DAB) (AFP-5672099), or rat LHβ (1:800 DAB) (AFP-571292393), or rat TSHβ (1:1000 DAB) (AFP-1274789) (National Hormone and Pituitary Program). Bound biotinylated secondary antibodies were detected using avidin and biotinylated peroxidase or fluorescent avidin only (Vectastain rabbit and M.O.M. kits; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) (Sigma, St. Louis, MO) was used as the chromogen for some immunostaining reactions. Normal serum was substituted for primary antibody in parallel negative control experiments.

2.6 Microscopy

Light and fluorescent images of embryo and pituitary sections were obtained with a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY) with DAPI, FITC, and TRITC filter cubes. Canvas (ACD Systems of America, Inc., Miami, FL) and NIS Elements (Nikon Instruments, Inc.) were used to process the images. Light images of whole embryos and adult pituitaries were obtained using a Leica MZ 6 microscope and a CCD camera (PL A662, PixeLINK, Ottawa, Ontario, Canada) with PixeLINK Capture software.

2.7 Bioinformatics Analyses

NCBI (www.ncbi.nlm.nih.gov/sites/genome) and the Ensembl genome browser (www.ensembl.org) were used to retrieve the human *LHX3* gene and enhancer sequences. The ECR, UCSC**,** and VISTA Genome browsers were used to identify conserved non-coding elements (CNEs) between 10 kb upstream of the transcription start site and 10 kb downstream of the stop codon of the human *LHX3* gene (Couronne et al., 2003; Kent et al., 2002; Ovcharenko et al., 2004). Putative transcription factor binding sites were predicted with TRANSFAC and rVISTA tools (Loots et al., 2002; Matys et al., 2003).

2.8 General Molecular Techniques

Ligations and Transformations

DNA fragments to be ligated were visualized for size and concentration by agarose gel electrophoresis. DNA fragments were joined using 0.5 µl T4 DNA ligase (Roche) in a total volume of 10 µl buffered solution. Typically, ligation reactions are allowed to proceed overnight at 16°C. Five µl of the ligation reaction or 2-5 ng of plasmid preparation were transformed into bacterial cells for preparation of clones.

Plasmid DNA was added to 50 µl of chemically competent *E. coli* DH5α cells (Invitrogen) on ice. This mixture was heat shocked at 42°C for 45 s and placed on ice again for a minimum of 2 m. The addition of 800 µl of LB broth supplemented with 0.2% glucose and 10 mM $MgCl_2$ preceded incubation at 37°C for 45-60 m with shaking at 180 rpm. Transformed cells were plated on LB agar plates containing the appropriate selective antibiotic.

Small Scale Alkaline Lysis Plasmid Preparation

E. coli DH5α transformants were incubated at 37°C with shaking overnight in LB broth with appropriate antibiotics. To pellet cells, 1.5 ml of liquid culture was centrifuged at 16,000 x g for 1 m. The supernatant was aspirated and the pellet was resuspended in 100 µl hypertonic solution 1 (50 mM Tris-HCl, pH 8.0, 0.9% glucose, 10 mM EDTA) followed by incubation on ice for 5 m. Cells were lysed by the addition of 200 µl solution 2 (0.2 M NaOH, 0.5% SDS), followed by gentle mixing and incubation on ice for 5 m. One hundred seventy five µl solution 3 (3 M KOAc, 11.5% (v/v) glacial acetic acid) was added followed by an additional incubation on ice for 5 m. The solution was centrifuged at 16,000 x g for 10 m followed by the addition of 300 µl of phenol/chloroform (1:1) solution and centrifugation at 16,000 x g for 10 m. The upper aqueous phase was removed and 1 volume of room temperature isopropanol was added to precipitate nucleic acids. The sample was centrifuged at 16,000 x g for 10 m and the pellet washed with 75% ethanol. The pellet was dried at 37°C for 5 m and resuspended in 40 µl TER (100 µg/ml RNaseA in 1X TE buffer [10 mM Tris-Cl, pH 7.4, 1 mM EDTA]).

One to two microliters of the plasmid preparations were digested with restriction endonucleases using the recommended buffer conditions (New England Biolabs). Restriction digests were separated on agarose/1X Tris-borate gels to select properly constructed plasmids.

Gel Purification of DNA Fragments

DNA fragments to be gel purified were separated on 0.7%, 1.0%, or 1.5% agarose/Tris-borate gels. Gel slices containing the fragments of interest were excised as quickly as possible using razor blades and long wavelength ultraviolet light. Gel purification was performed with regular Qiaquick or MinElute gel extraction columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. Elution from the column was performed with either 10 µl (MinElute) or 30 µl (Qiaquick) of the supplied buffer.

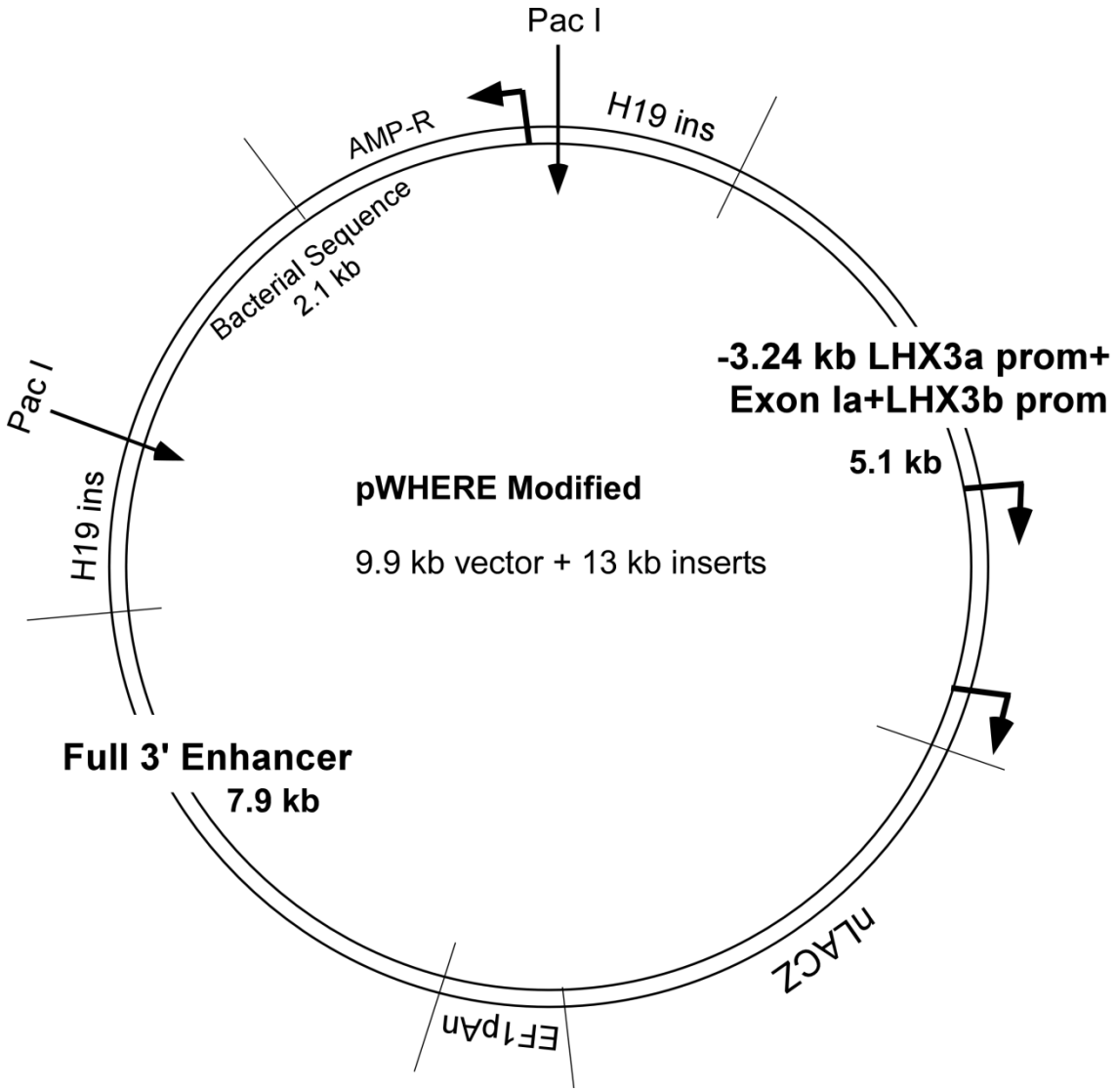


Figure 2.1. *LHX3a* promoter-*LHX3* exon Ia-*LHX3b* promoter-*nLACZ*- Full 3' modified pWHERE transgene. The -3.24 kb *LHX3a* promoter-*LHX3* Exon Ia-*LHX3b* promoter region and the full 3' enhancer region were inserted into the modified pWHERE vector as shown. *Beta galactosidase* transcription unit with a nuclear localization signal (nLACZ); polyadenylation signal from human *EF1 alpha* gene (EF1pAn); murine *H19* insulator regions (H19 ins).

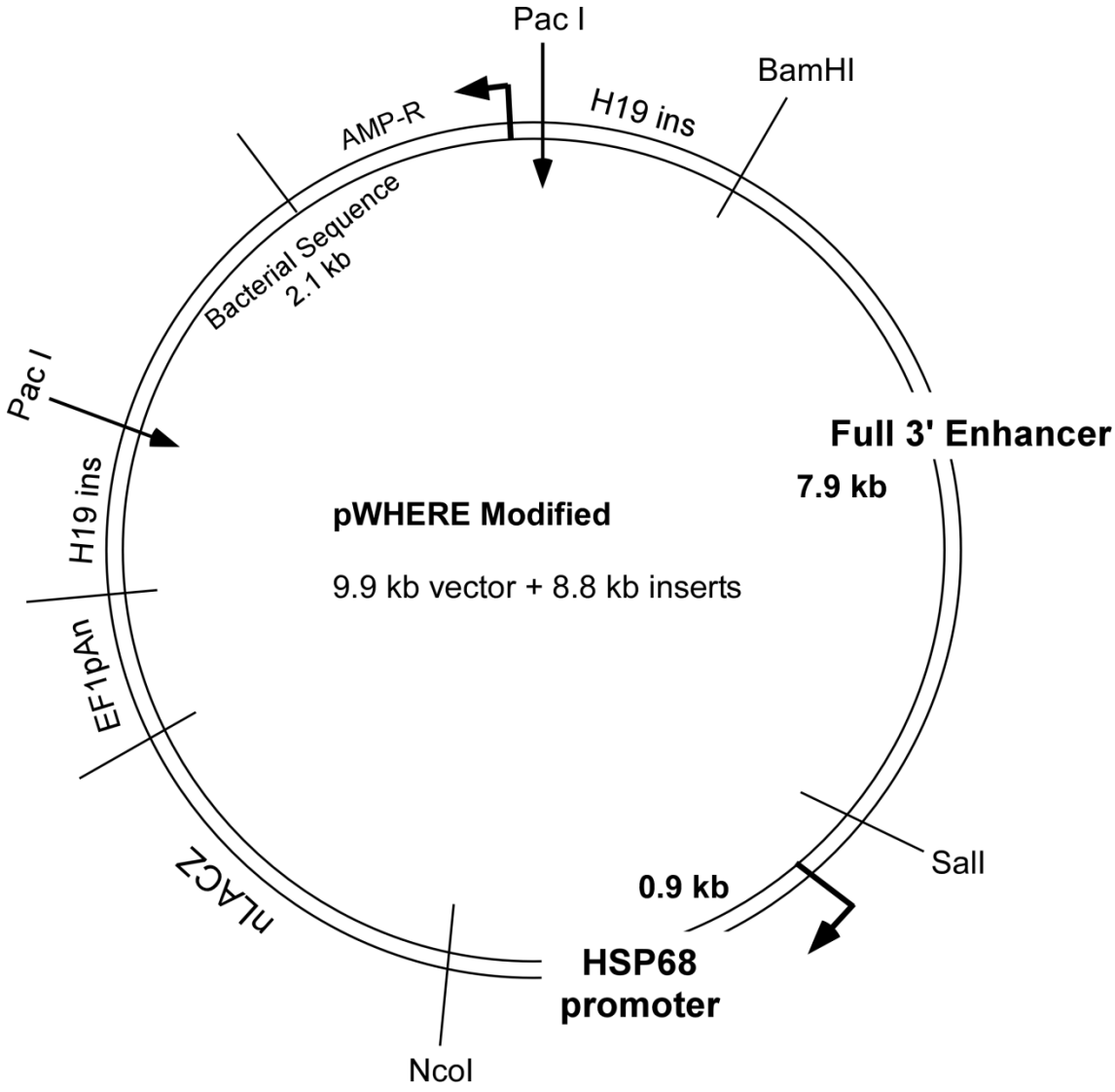


Figure 2.2. Full 3'-*HSP68 nLACZ* pWHERE transgene. The full 3' enhancer region and the *heat shock protein 68* (*HSP68*) promoter were inserted into the modified pWHERE vector as shown. *Beta galactosidase* transcription unit with a nuclear localization signal (nLACZ); polyadenylation signal from human *EF1 alpha* gene (EF1pAn); murine *H19* insulator regions (H19 ins).

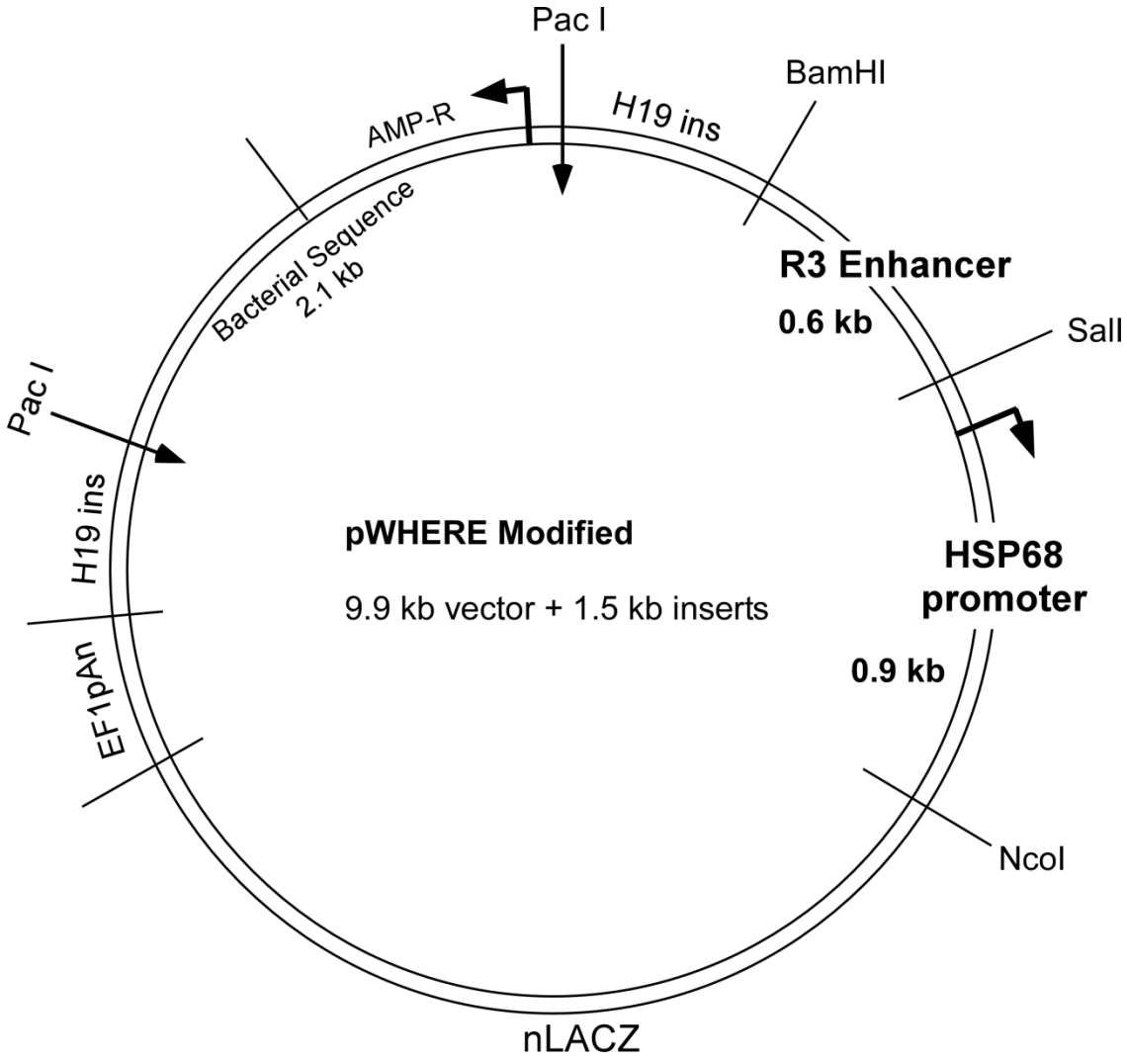


Figure 2.3. R3-*HSP68 nLACZ* pWHERE transgene. The R3 enhancer region and the *heat shock protein 68* (*HSP68*) promoter were inserted into the modified pWHERE vector as shown. *Beta galactosidase* transcription unit with a nuclear localization signal (nLACZ); polyadenylation signal from human *EF1 alpha* gene (EF1pAn); murine *H19* insulator regions (H19 ins).

CHAPTER THREE

IN VIVO ANALYSIS OF HUMAN *LHX3* GENE REGULATION

3.1 Results

Conserved Distal Elements Downstream of the Human LHX3 Gene Direct Pituitary and Nervous System Expression

Our lab previously characterized two human *LHX3a* and *LHX3b* promoters with basal activity *in vitro*. To test the function of the promoters *in vivo*, I used a *beta galactosidase* reporter gene mouse model. Transgenes were constructed by placing the promoters in a vector containing a *beta galactosidase* reporter gene with a nuclear localization signal followed by an untranslated region (UTR) and a polyadenylation signal from the human *EF1 alpha* gene flanked by murine *H19* insulator regions. *H19* insulators have been shown to help reduce inappropriate repression or activation of gene expression caused by the site of integration of the transgene (Touw et al., 2007). Transient transgenics were analyzed at e14.5, a time point when mouse *Lhx3* gene expression is high in both the developing pituitary and spinal cord (Bach et al., 1995; Seidah et al., 1994; Zhadanov et al., 1995). Neither promoter alone was able to drive expression in any tissue including the pituitary and spinal cord (Figure 3.1 B, C; I and II). To test the hypothesis that interactions between the two upstream promoter regions were required to guide gene expression *in vivo*, I also examined the activity of the combined promoter regions in the mouse model (Figure 3.1 B; III). This transgene was also unable to direct reporter gene expression (Figure 3.1 C; III). A minimum of six independent founders were analyzed for each promoter transgene (Figure 3.1 C; I, II, and III).

Together, these results indicate that regions outside of the basal promoters are required to direct *LHX3* gene expression *in vivo*.

Conserved non-coding elements (CNE) are associated with gene enhancer activity in multiple tissues including neural tissues and the developing heart (Blow et al., 2010; Visel et al., 2007). To search for regulatory regions, I performed bioinformatic searches using the ECR browser of sequences ± 10 kb surrounding the human *LHX3* gene and discovered multiple CNEs within a 7.9 kb region directly 3' of the gene (Bray et al., 2003; Couronne et al., 2003). The CNEs in the 3' region show ≥ 70% conservation between humans and multiple other vertebrates (Figure 3.1 A). In order to examine the function of these CNEs *in vivo*, I next tested the combined human promoter region plus the 3' 7.9 kb region in the transgenic mouse model. Unlike the promoter constructs, this construct was able to drive tissue-specific expression in the developing pituitary and spinal cord at e14.5 (Figure 3.1 C; IV). This result indicates that the 3' region contains one or more enhancers required for spatial and temporal expression of the *LHX3* gene.

An additional CNE located far downstream (Far 3') was also identified from the VISTA enhancer browser computational dataset and tested *in vivo* (Visel et al., 2007). This element was able to direct some expression to the developing spinal cord but not the pituitary gland (Figure 3.1 C; VI). The Far 3' CNE lies ~63 kb 3' of *LHX3* and 31 kb 5' of the *nucleus accumbens-associated protein 2 (NACC2)* gene. *NACC2* is expressed in the developing nervous system but not the pituitary (Visel et al., 2004). It is therefore possible the Far 3' is an enhancer of *NACC2* expression. In mouse, *Lhx3* expression begins at approximately e9.5. By e11.5, robust *Lhx3* expression is detected in both the developing spinal cord and pituitary. Additional Far 3' transient transgenic founders were

evaluated at e11.5 and no beta galactosidase activity was detected (data not shown). This result and the fact that the nearest gene, *NACC2*, is expressed in neural tissues makes the role of the Far 3' CNE in *LHX3* gene regulation difficult to interpret.

One recognized characteristic of an enhancer is the ability to act independent of position to guide expression from a heterologous basal promoter (Blackwood and Kadonaga, 1998). Construct IV containing the *LHX3* promoters and the 3' region in its native position exhibits tissue-specific transgene expression. To determine if the 3' region acts as an independent enhancer, we tested a transgene containing the 3' region upstream of the *HSP68* promoter, a basal promoter which lacks activity in the absence of an enhancer in transgenic mice (Kothary et al., 1989; Pennacchio et al., 2007) (Figure 3.1 B; V). The construct V expression pattern recapitulates that seen with the *LHX3* construct IV (Figure 3.1 C; V). Therefore the 3' enhancer region has transferrable activity independent of its native promoters and genomic position.

Endogenous LHX3 Expression Correlates with the Expression Pattern Guided by the 3' Enhancer Region During Development

To more completely dissect the spatial and temporal expression patterns of the enhancer region, stable transgenic lines were generated using the *HSP68* promoter downstream of the full 3' enhancer region (Figure 3.1 B; V). Whole mount beta galactosidase staining of the embryos showed that the enhancer element was activated at about e9.5 to e10.0 in Rathke's pouch, the primordium of the pituitary. Spinal cord expression preceded pituitary expression slightly and was easily detectable at e9.0 to e9.5 (Figure 3.2 B; and data not shown). In previous studies it has been shown that mouse *Lhx3* is activated by e9.5 in both the pituitary and spinal cord (Bach et al., 1995; Seidah

et al., 1994; Zhadanov et al., 1995). To examine whether the 3' enhancer region activation coincided with endogenous *Lhx3* activation, we used serial sections from the same embryo to control for differences in activation of *Lhx3* expression between embryos. Serial sections were stained for either beta galactosidase activity or immunohistochemistry was performed using antibodies against mouse LHX3 protein. LHX3 expression and the spatial pattern of enhancer-directed transgene activity were similar, consistent with a role of the 3' enhancer region in *LHX3* gene activation (Figure 3.2 A). LHX3 protein expression appeared stronger in the developing spinal cord than in the pituitary matching the pattern of expression seen for the enhancer directed transgene (Figure 3.2 A).

Whole mount staining at e12.5 revealed high levels of transgene expression in the developing spinal cord and pituitary in a pattern consistent with the expression pattern of endogenous mouse LHX3 protein (Figure 3.2 B). Some ectopic expression was found in the epidermis and the nasal cavity (Figure 3.2 B). LHX3 has not been shown to be expressed in these areas. This activity may be attributed to effects from the site of transgene integration or might also be because other regulatory elements not contained within the transgene are needed to more tightly regulate *LHX3* expression.

Expression of the transgene in the pituitary was consistent with a subset of the endogenous LHX3 expression pattern. The transgene was expressed throughout the unclosed Rathke's pouch, recapitulating LHX3 expression patterns, but became restricted to the ventral portion of the pituitary by e14.5 (Figure 3.2 C). In contrast, LHX3 is more strongly expressed in the dorsal pituitary with weaker expression in the ventral pituitary

by e12.5 (Raetzman et al., 2002). At postnatal day 1 (P1), expression was absent from the intermediate lobe (Figure 3.2 C).

Next to examine the expression pattern on a cellular level; I wanted to determine which hormone-secreting cell types also showed enhancer directed expression. Because the co-expression pattern of endogenous LHX3 had not previously been determined, I also did co-labeling experiments for the hormone subunits and LHX3. Both native LHX3 and enhancer-directed β-galactosidase co-localize with αGSU expressing cells but not GH-positive cells in P1 pituitaries (Figure 3.3).

The 3' Region Contains Several Nervous System Enhancers and a Pituitary Enhancer

The identified 3' region contains several conserved sub-regions that were designated regions R1 (355 bp), R2 (680 bp) and R3 (557 bp) (Figure 3.4 A). To evaluate the importance of these sequences, a series of systematic deletions was generated. Regulatory regions R1, R2, R3 were each independently sufficient to direct expression to the spinal cord, but not transgenes containing the UTR region alone (Figure 3.4 A, B).

In the absence of the UTR R1 region both the R2 and R3 enhancer separately directed robust expression to the developing cerebral cortex (Figure 3.5 E, F). Cerebral cortex expression is not detected in transgenics containing the UTR R1 (~ 4500 bp) element indicating a neural silencing element is contained in this region. Interestingly, at e14.5 in any of the deletion constructs tested containing either R1, R2, or R3, transgene expression is detected in the medial ganglionic eminence (MGE) that was not observed in transgenic mice containing the full 3' enhancer region (Figure 3.5 A, B, C, D; data not shown). However at e17.5, Full-*HSP68* (transgene V) mice have reporter gene expression in the MGE (data not shown). One possible explanation for these results is an element

needed for temporal control of MGE expression is contained or partially contained in the only non-overlapping region immediately 3' of R2 (Figure 3.4).

Only transgenic founders generated using reporter genes containing the R3 element directed expression to both the developing spinal cord and pituitary (Figure 3.4 A, B; X, XI, and XIII). Transgenes containing the R3 element alone were expressed in the spinal cord in a similar pattern to the full 7.9 kb region (Figure 3.1; V). However, pituitary expression was expanded to include the dorsal portion of the developing anterior pituitary (Figure 3.4 B; XIII). Intriguingly, the pattern of pituitary expression in a construct with a deleted R2 sub-region (transgene X) shows repression in the dorsal anterior pituitary thereby recapitulating the expression of the full enhancer element (Figure 3.4 B). This result suggests that a pituitary silencing or repressive element is contained within either the UTR R1 region or in the region between R2 and R3.

A 180 Bp Minimal Region Is Sufficient To Direct Expression to the Developing Pituitary

Sequence alignment of the R3 pituitary enhancer region from several species using the ECR browser identified a highly conserved region of 180 bp (designated Core R3, Figure 3.6 A). We generated transgenic reporter gene containing the Core R3 region (Figure 3.6 A; XIV). Of six transgenic embryos analyzed at e14.5, four had strong levels of transgene expression and directed expression to the pituitary (Figure 3.6 A, B; XIV). Of these, two directed weak (compared to the R3 or full 3' enhancer transgenic mice) expression in the developing spinal cord (Figure 3.6 A, B). Additional transgenic mice were generated expressing reporter genes containing overlapping fragments of the Core R3 enhancer. These fragments were unable to direct pituitary expression (Figure 3.6 B;

XV, XVI, and XVII). Fragments 2 and 3 directed some weak spinal cord expression in a pattern similar to the Core R3 enhancer transgenic mice (Figure 3.6 B; XIV, XVI, and XVII). Overall, these data indicate that key elements required to direct pituitary expression are contained in the 180 bp Core R3 enhancer.

Core R3 enhancer sequences for multiple species were aligned and conserved putative trans-acting factor binding sites were identified (Figure 3.7). The Core R3 enhancer contained four conserved TAAT/ATTA sequences that are characteristic of homeodomain protein recognition sites (Figure 3.7; sites AT1 to AT4). These sites were compared to the consensus binding sites of candidate factors with suggested roles in pituitary development and *LHX3* gene regulation. AT1 matches the pituitary homeobox (PITX) consensus binding site. PITX1 and PITX2 are required for activation of *Lhx3 in vitro* and *in vivo* (Charles et al., 2005; Tremblay et al., 1998). AT2 was identified as a possible binding site for LHX3 and LHX4 proteins (Bridwell et al., 2001). This sequence might be important for autoregulation by LHX3 or regulation by LHX4. *In vivo* studies have shown that *Lhx4* is required for proper activation of *Lhx3*: in *Lhx4* knockout mice, LHX3 expression is delayed but returns to normal by e14.5 (Raetzman et al., 2002). AT3 and AT4 sequences are consistent with a tandem binding site for ISL1 (Karlsson et al., 1990). Importantly, *Isl1* expression precedes and then overlaps *Lhx3* expression in the developing spine and pituitary in a pattern consistent with a possible role in *Lhx3* regulation (Ericson et al., 1998; Pfaff et al., 1996). The pituitary defect in the *Isl1* knockout is similar to *Lhx3*-null mice and LHX3 expression is missing; however, previous studies have suggested ISL1 blocks differentiation at an earlier stage of development independent of *LHX3* gene regulation (Takuma et al., 1998). Conditional

motoneuron knockouts of *Isl1* do not have any markers of motoneuron development including *Lhx3* (Pfaff et al., 1996). LHX3 and LHX4 are expressed in the developing bipolar interneurons of the eye at P9 and partially co-localize with ISL1. Loss of ISL1 in the neural retina causes loss of *Lhx3* expression, but *Lhx4* expression is maintained (Elshatory et al., 2007).

Additional transcription factor binding sites within the Core R3 included putative elements for CCAAT enhancer binding protein alpha (CEBPA) and SRY-box 2 (SOX2) sites (Figure 3.7). CEBPA has not been implicated in *LHX3* regulation, but is important in differentiation and proliferation events. Mouse *Cebpa* is expressed in the developing pituitary and has been shown to regulate the *prolactin* promoter with PIT1 (Enwright et al., 2003). SOX2 is known to bind to the human *LHX3a* promoter (Rajab et al., 2008) and human patients and mouse models heterozygous for *SOX2* gene mutations have anterior pituitary hormone deficiencies (Kelberman et al., 2006).

To determine whether these elements are required to direct expression *in vivo*, transient transgenic founders were generated with individual mutations in the putative SOX, AT1, AT2 and CEBPA binding sites or a combined mutation of the tandem sites AT3 and AT4 in the context of the 557 bp R3 enhancer (modeled in Figure 3.8 A). Founders with mutations in AT1 lost expression in the dorsal portion of the pituitary in comparison to the wild type R3 expression pattern (Figure 3.8 B). This is similar to the expression pattern of the full 3' enhancer. This suggests a role for the AT1 element in spatial control of pituitary *LHX3* expression. Mutation of the tandem AT3 and AT4 sites abolished nearly all expression in both the pituitary and the developing spinal cord (Figure 3.8 B) demonstrating that AT3 and AT4 are required for enhancer directed

expression. However, since fragment 3 of the core R3 containing both AT3 and AT4 did not alone direct pituitary expression, the AT3 and AT4 elements are not sufficient for enhancer function (Figure 3.6 B; XVII). Individual mutation of the SOX, AT2, or CEBPA sites failed to alter the expression pattern of the R3 enhancer at e14.5 (Figure 3.8 B). These sites are not required to direct expression at e14.5 however it is possible that they may have function at other developmental time points.

To test whether candidate factors could bind to the AT elements in the core R3 enhancer *in vitro*, electrophoretic mobility shift assays (EMSAs) were performed using *in vitro* translated proteins and ^{32}P-labeled enhancer DNA sequences. ISL1 protein was able to bind probes containing sites AT2, AT3, and AT4 and mutation of these sites abolished binding (Figure 3.8 C). PITX1 and PITX2 protein bound to probes containing AT1 but not to the mutated AT1 probe or other AT sites (Figure 3.8 D, and Figure 3.9 A). LHX3 and LHX4 proteins were able to bind probes containing AT2 (Figure 3.9 B, C, D). Lysates programmed with empty vector did not bind to the probes specifically (Figure 3.8 C, D and Figure 3.9). Similarly, chromatin immunoprecipitation (ChIP) experiments using alpha T3 pre-gonadotrope cells confirmed *in vivo* occupancy of PITX1 and ISL1 proteins in a cellular context (Figure 3.8 E). In *luciferase* reporter gene assays in either α-T3 or LβT2 pituitary cell lines, addition of the R3 enhancer upstream of the *LHX3* promoters or the minimal *prolactin* promoter failed to increase gene activation above basal levels. Consistent with this lack of R3 mediated reporter gene activation, co-transfection of PITX1 and ISL1 proteins did not synergistically activate the construct (n=1; data not shown). EMSA and ChIP experiments were done in collaboration with graduate student Soyoung Park.

Genetic Analysis of Regulatory Regions in CPHD Patients

Sequencing of the R3 region of thirty three CPHD patient DNA samples previously screened in our lab for coding region mutations revealed no variations. Additionally our collaborator from the Hospital for Children and Adolescents at the University of Leipzig, Dr. Roland Pfaeffle screened an additional 100 candidate CPHD patients for mutations in the *LHX3a* and *LHX3b* promoters and the R2 and R3 enhancers. Patients with previously identified mutations in the coding regions of *PROP1*, *PIT1* or *LHX3* gene were excluded. Samples from patients with CPHD were screened by denaturing gradient high pressure liquid chromatography (dHPLC) (WAVE DNA Fragment Analysis System, Transgenomic, Omaha, NE). Fragments showing abnormal retention patterns on the HPLC column were subjected to Dideoxy-Sequencing to specify the sequence abnormality. No homozygous sequence aberrations were detected. Three heterozygous single nucleotide variations (SNV) were identified (Table 3.1). These SNVs were not found in 50 unaffected individuals sequenced. The SNV within the R3 enhancer region lies outside of the minimal Core R3 pituitary enhancer.

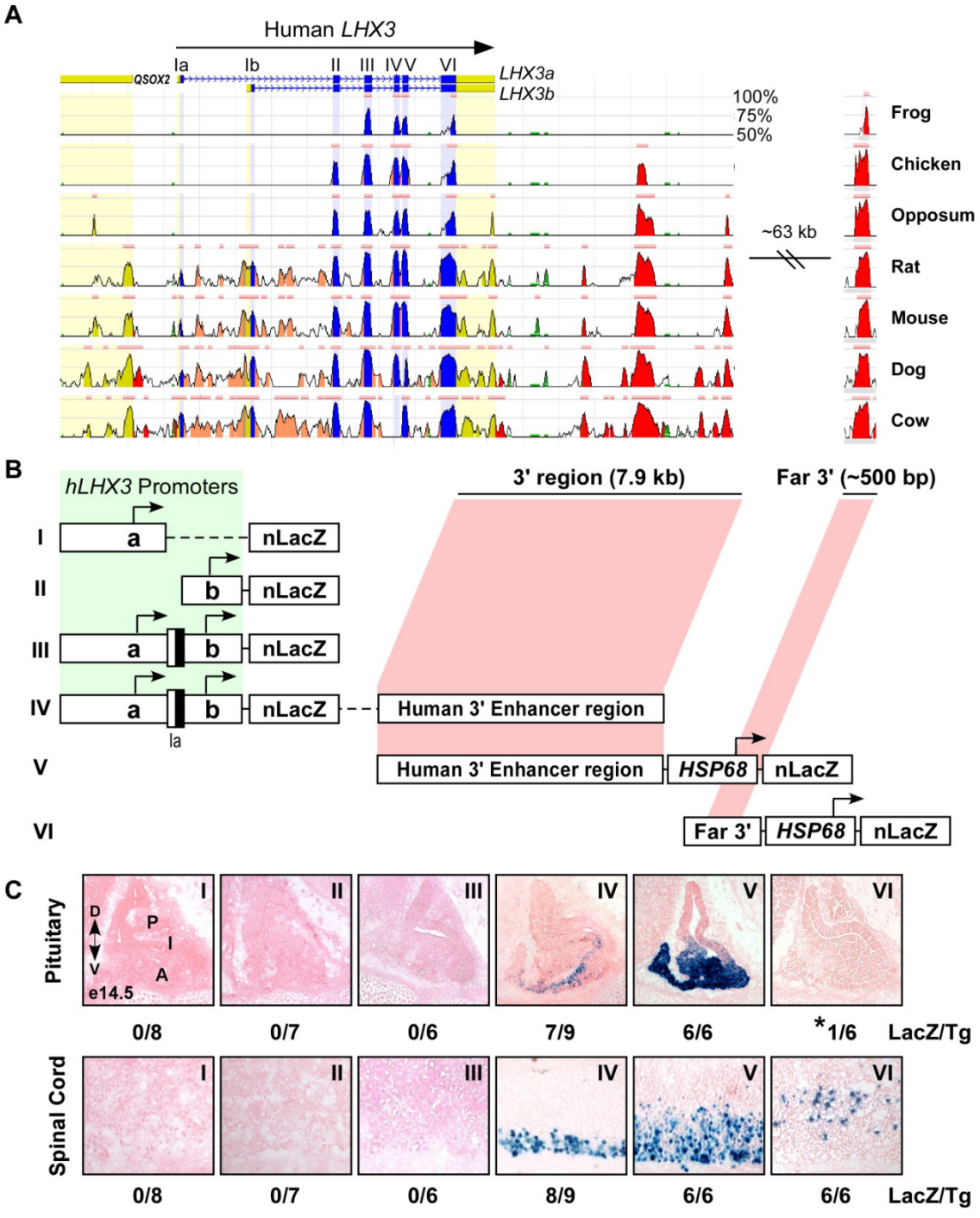

A
Human LHX3
Ia
Ib
II
III
IV
V
VI
QSOX2
LHX3a
LHX3b
100%
75%
50%
~63 kb
Frog
Chicken
Opposum
Rat
Mouse
Dog
Cow
B
hLHX3 Promoters
3' region (7.9 kb)
Far 3' (~500 bp)
I
II
III
IV
V
VI
a
b
nLacZ
Ia
Human 3' Enhancer region
HSP68
Far 3'
C
Pituitary
Spinal Cord
D
V
P
I
A
e14.5
0/8
0/7
0/6
7/9
6/6
*1/6
LacZ/Tg
0/8
0/7
0/6
8/9
6/6
6/6
LacZ/Tg

Figure 3.1. Distal downstream regions of the human *LHX3* gene direct expression to the developing pituitary and spinal cord. (A) Comparative genomic analysis. The ECR browser was used to compare sequences surrounding human *LHX3* and a Far 3' region identified using the VISTA enhancer browser computational dataset to frog, chicken, opossum, rat, mouse, dog, and cow. Conserved non-coding elements (CNE) were defined as regions ≥70% identity and ≥100 base pairs: *red* color indicates CNE; (*blue*) coding exons; (*salmon*) conserved intronic regions; (*yellow*) UTR; (*green*) transposable elements and simple repeats. (B) Reporter gene constructs I - VI used to generate transgenic mice. (C) Sagittal cryosections of e14.5 founder embryos stained for beta galactosidase activity. The fraction of transgenic embryos expressing beta galactosidase in the pituitary or spinal cord is shown below each respective image. The asterisk indicates ectopic expression. D, dorsal; V, ventral; P, posterior lobe; I, intermediate lobe; A, anterior lobe.

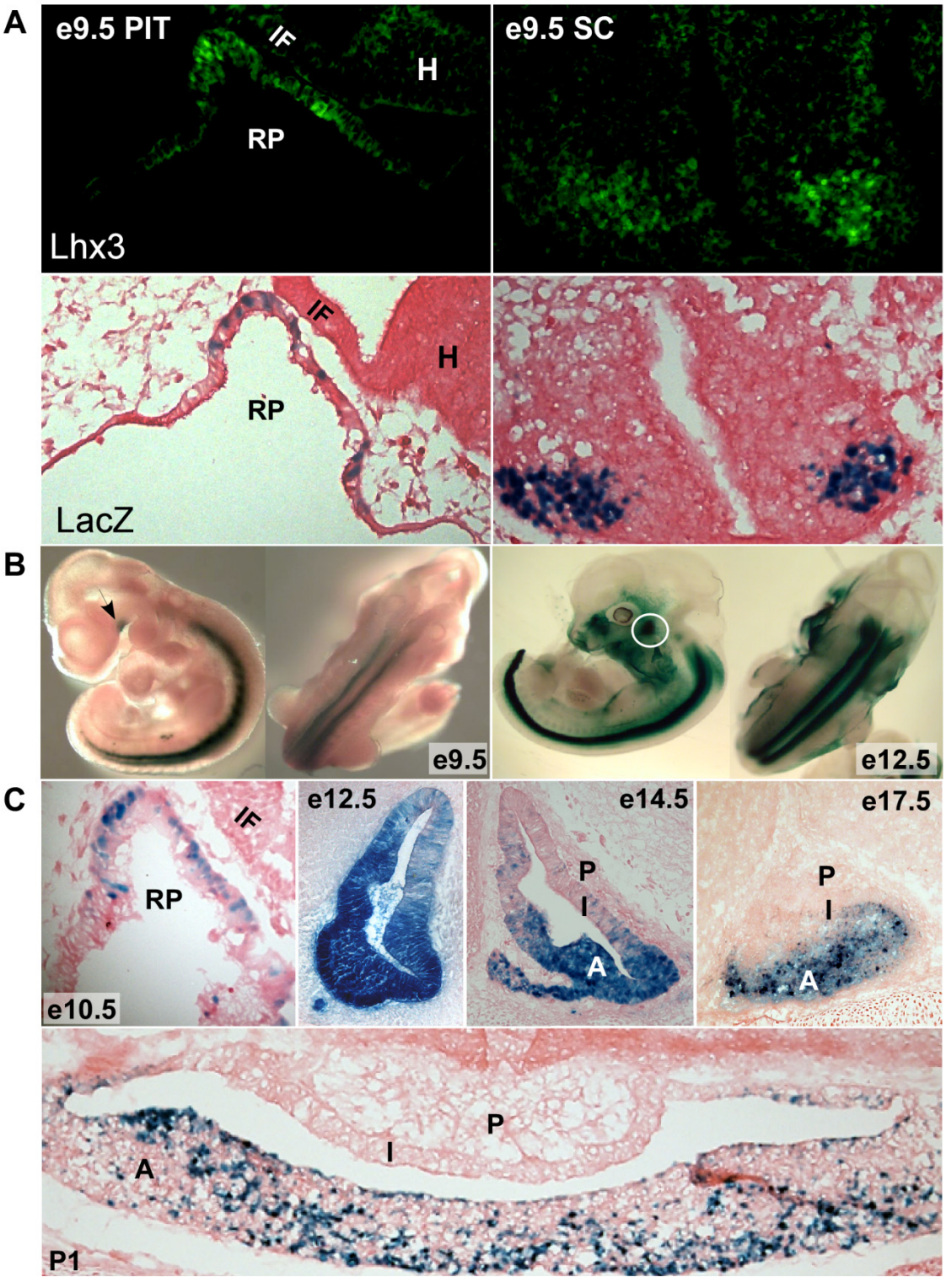

A
e9.5 PIT
IF
H
RP
Lhx3
e9.5 SC
IF
H
RP
LacZ
B
e9.5
e12.5
C
IF
RP
e10.5
e12.5
e14.5
P
I
A
e17.5
P
I
A
P
I
A
P1

Figure 3.2. Expression patterns guided by the 7.9 kb 3' enhancer region during development correlate with endogenous LHX3 expression. (A) Antibody staining of mLHX3 and *beta galactosidase* activity staining of serial sections at e9.5 in a stable transgenic line containing construct line V. The onset of *beta galactosidase* activity is consistent with the onset of mLHX3 protein expression detected by antibody staining in Rathke's pouch (RP) and in the developing spinal cord. (B) X-gal stained embryos at e9.5 and e12.5 show strong staining in the developing pituitary (arrowhead and circle) and spinal cord consistent with known *Lhx3* expression patterns. (C) X-gal staining of sagittal cryosections of the developing pituitary at e10.5, e12.5, e14.5, e17.5 and coronal cryosections at postnatal day 1(P1). The 3' enhancer directs expression through out the developing anterior pituitary at e10.5 and e12.5. At e14.5, e17.5 and P1 expression is restricted to the anterior lobe and is absent from the intermediate and posterior lobes. RP, Rathke's pouch, IF, infundibulum, H, hypothalamus; P, posterior lobe; I, intermediate lobe; A, anterior lobe.

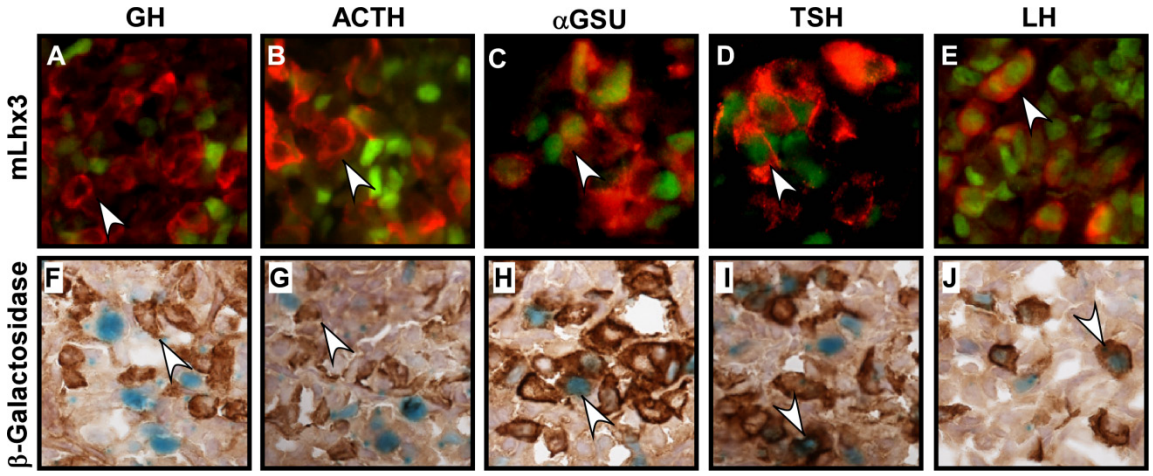
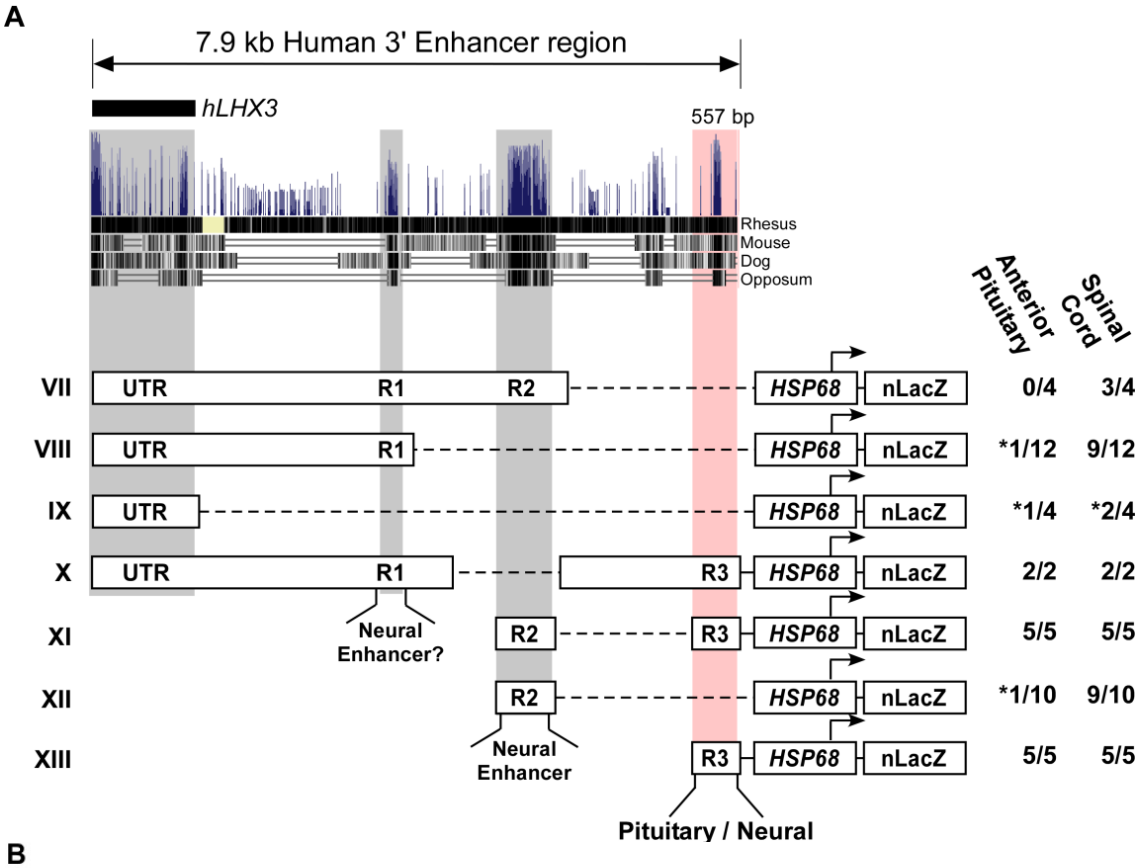


Figure 3.3. Native LHX3 and enhancer directed beta galactosidase expression co-localization pattern is similar in the hormone-expressing cell types of P1 mouse pituitaries. Coronal pituitary sections from newborn (P1) Full-*HSP68*-nLacZ transgenic mice (transgene V) were stained by immunohistochemistry (*green*) for LHX3 (A–E) or stained for beta galactosidase activity (*blue*) (F–J) and double-labeled [*red (upper panel) or brown (lower panel)*] for pituitary hormones: GH (A and F), ACTH (B and G), αGSU (C and H), TSH (D and I), LH (E and J). Co-localization was highest (*arrowheads*) for gonadotropes and thyrotropes (C, D, E, H, I and J) and nearly absent (*arrowheads*) for corticotropes and somatotropes (A, B, F, and G).


A
7.9 kb Human 3' Enhancer region
hLHX3
557 bp
Rhesus
Mouse
Dog
Opposum
Anterior Pituitary
Spinal Cord
VII
UTR
R1
R2
HSP68
nLacZ
0/4
3/4
VIII
UTR
R1
HSP68
nLacZ
*1/12
9/12
IX
UTR
HSP68
nLacZ
*1/4
*2/4
X
UTR
R1
R3
HSP68
nLacZ
2/2
2/2
XI
Neural Enhancer?
R2
R3
HSP68
nLacZ
5/5
5/5
XII
R2
HSP68
nLacZ
*1/10
9/10
XIII
Neural Enhancer
R3
HSP68
nLacZ
5/5
5/5
Pituitary / Neural

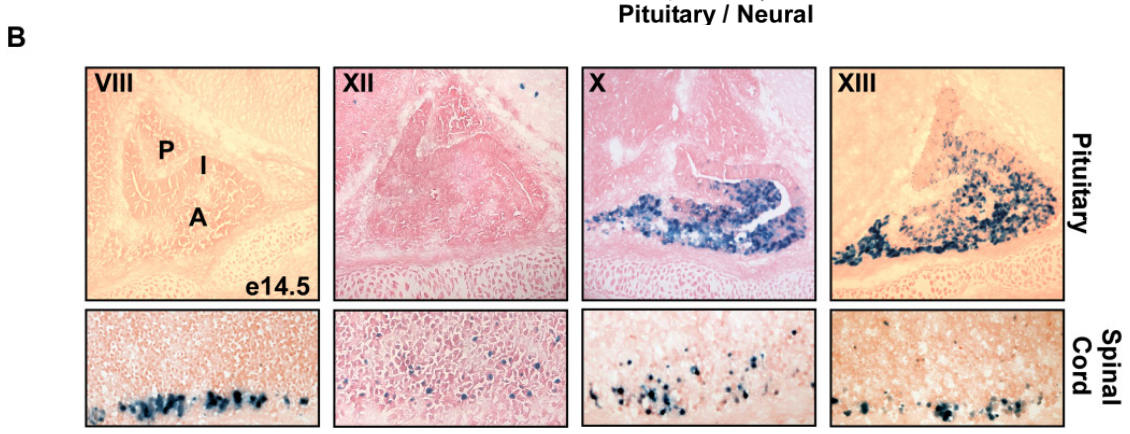

B
VIII
P
I
A
e14.5
XII
X
XIII
Pituitary
Spinal Cord

Figure 3.4. Deletion analysis of the 3' region reveals several nervous system enhancers and a pituitary enhancer. (A) Alignment of the 3' prime region across the indicated species from the UCSC Genome Browser (http://genome.ucsc.edu). The fraction of transgenic embryos expressing *beta galactosidase* in the pituitary or spinal cord is shown beside each construct. One founder each from lines UTR, UTR R1 and R2 showed expression in the majority of tissues including the spinal cord and pituitary. The pattern was inconsistent with other founder lines and was considered ectopic expression and likely due to affects from the site integration of the transgene. Asterisks indicate non-specific ectopic expression. (B) Sagittal sections of e14.5 embryos harboring constructs VIII (UTR R1), XII (R2), X (delta R2), and XIII (R3) stained for *beta galactosidase* activity. P, posterior lobe; I, intermediate lobe; A, anterior lobe.

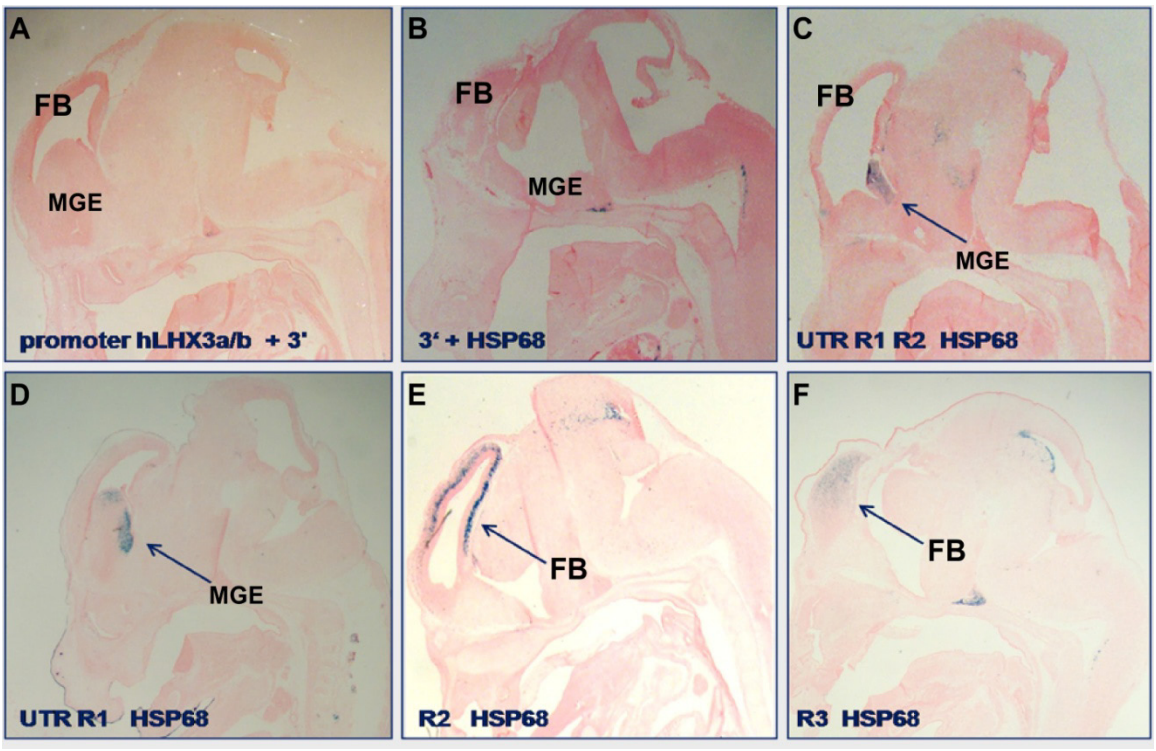


Figure 3.5. UTR R1 (~4500 bp) contains a silencing element for the developing forebrain. (A) *hLHX3a/b* promoters -7.9 kb 3′ enhancer (B) Full-*HSP68* (C) UTR R1 R2-*HSP68* (D) UTR R1-*HSP68* (E) R2-*HS68* (F) R3-*HSP68.* Arrows show additional forebrain expression in the developing cerebral cortex (E, F) and in the medial ganglionic eminence (C, D). Sagittal sections at e14.5. n≥3 for all lines. FB, forebrain; MGE, medial ganglionic eminence.

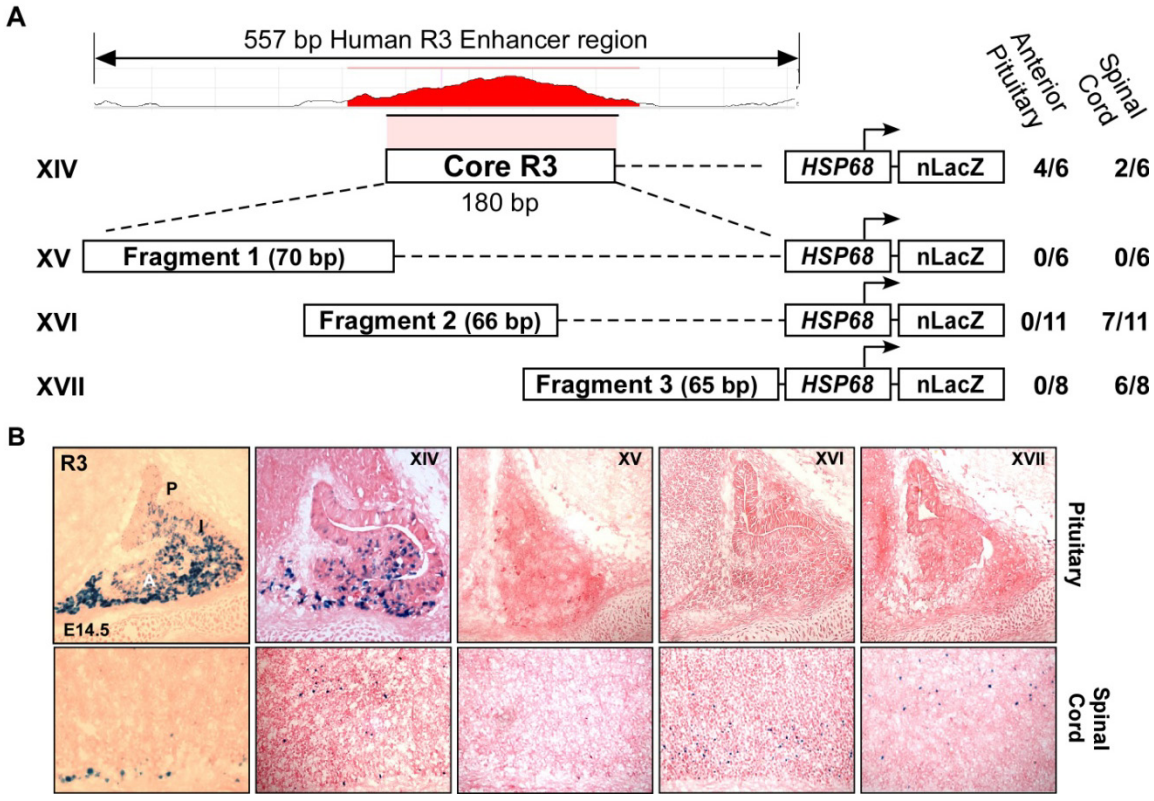


Figure 3.6. A highly conserved 180 bp minimal region (Core R3) is sufficient to direct expression to the developing pituitary. (A) The ECR browser indicated a conserved non-coding element (CNE, ≥70% identity and ≥100 base pairs); shown in *red* color. Constructs XIV through XVII were used to generate transgenic mice and the fraction of transgenic embryos expressing *beta galactosidase* in the pituitary or spinal cord is shown beside each construct. (B) Sagittal cryosections of e14.5 embryos stained for *beta galactosidase* activity. P, posterior lobe; I, intermediate lobe; A, anterior lobe.

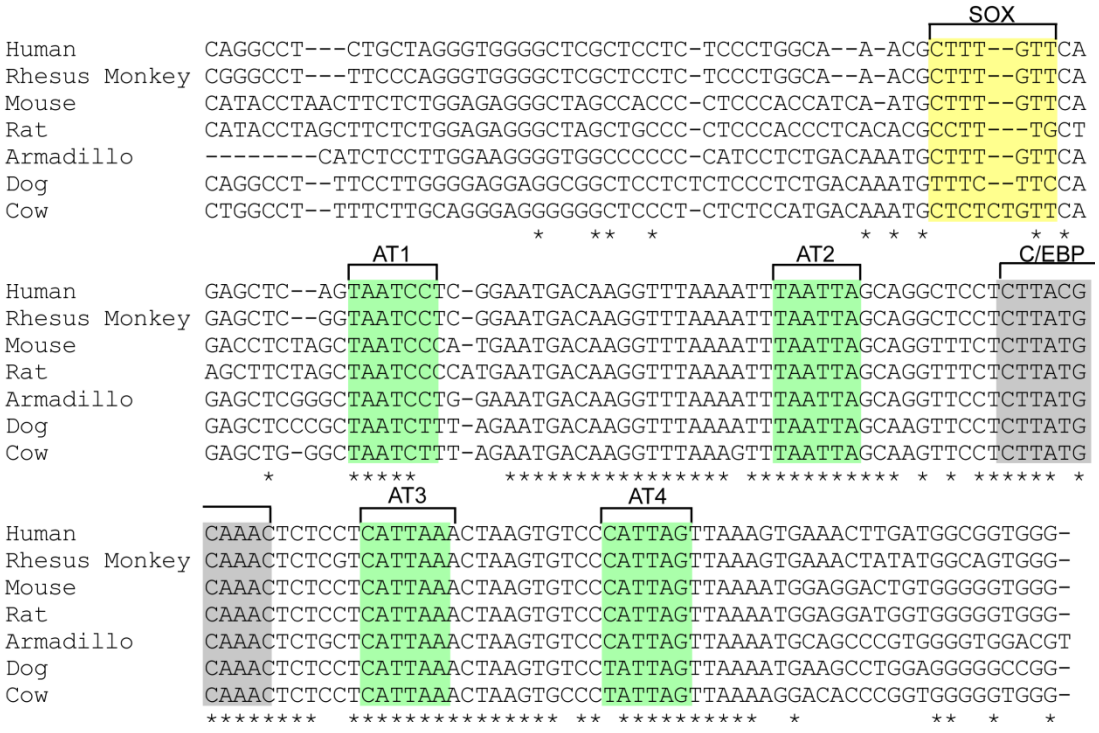


Figure 3.7. Alignment of human Core R3 enhancer sequences with multiple species reveals conserved putative transcription factor binding consensus sequences. Putative SOX sites (yellow); four potential homeodomain factor sites (AT1-AT4, green); and a possible C/EBP site (gray) are indicated. Analyses were performed with ClustalW2.0 and rVISTA.

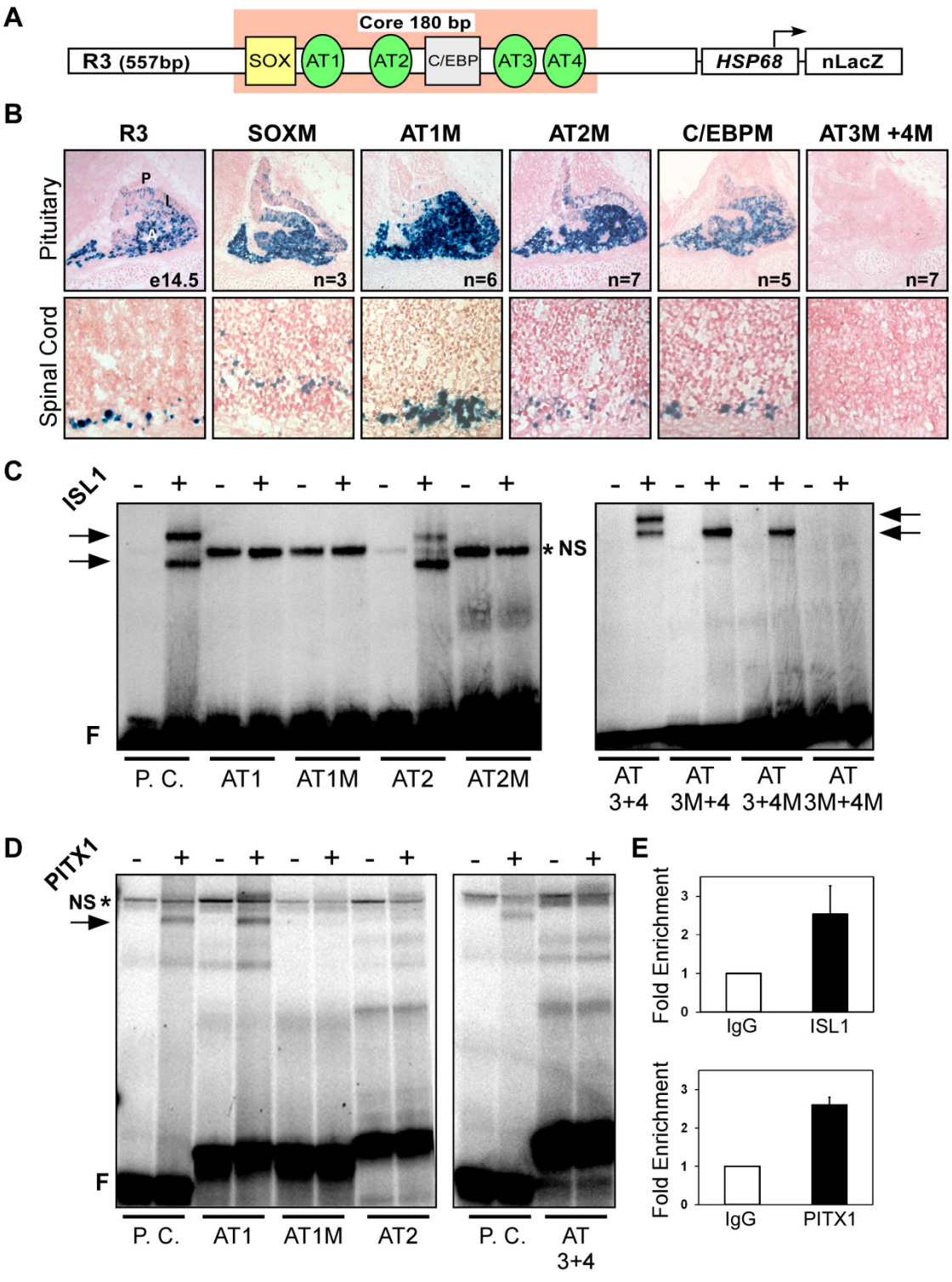

A
Core 180 bp
R3 (557bp)
SOX
AT1
AT2
C/EBP
AT3
AT4
HSP68
nLacZ
B
R3
SOXM
AT1M
AT2M
C/EBPM
AT3M +4M
Pituitary
Spinal Cord
P
L
A
e14.5
n=3
n=6
n=7
n=5
n=7
C
ISL1
- + - + - + - + - +
* NS
F
P. C.
AT1
AT1M
AT2
AT2M
AT 3+4
AT 3M+4
AT 3+4M
AT 3M+4M
D
PITX1
NS *
F
P. C.
AT1
AT1M
AT2
P. C.
AT 3+4
E
Fold Enrichment
IgG
ISL1
Fold Enrichment
IgG
PITX1

Figure 3.8. ISL and PITX binding sites in the Core R3 enhancer are critical for expression in the developing pituitary and spinal cord. (A) Potential transcription factor binding sites in the R3 region are shown in the context of the R3-*HSP68*-nLACZ transgenic reporter gene. (B) Mutation analyses in transgenic mice demonstrate that AT3 and AT4 are required for pituitary and spinal cord expression *in vivo* and AT1 is required for dorsal pituitary expression. (C) ISL1 binds specifically to AT2, AT3 and AT4 in electrophoretic mobility shift assays (EMSA). The A3/A4 element from the enhancer of the rat *insulin* promoter was used as the positive control (Karlsson et al., 1990). P.C., positive control; –, empty vector programmed lysate; +, PITX1 or ISL1 expression vector programmed lysate; Arrows, specific bound complex; *NS, nonspecific band; F, free probe. (D) PITX1 binds specifically to AT1 sites in EMSA. A Bcd2x5n bicoid element was used as the positive control (Saadi et al., 2003). (E) ChIP experiments show occupancy of the Core R3 enhancer by ISL1 and PITX1 proteins in αT3 pituitary cells. Pull-down and input DNAs were characterized for the presence of the Core R3 enhancer element by quantitative PCR. Immunoprecipitation with non-immune species-appropriate IgG was carried out as a control and relative enrichment was calculated as the fold difference above the $2^{-\Delta\Delta Ct}$ for the normal immunoglobulin samples. Values are mean ± s.e.m. for three independent experiments.

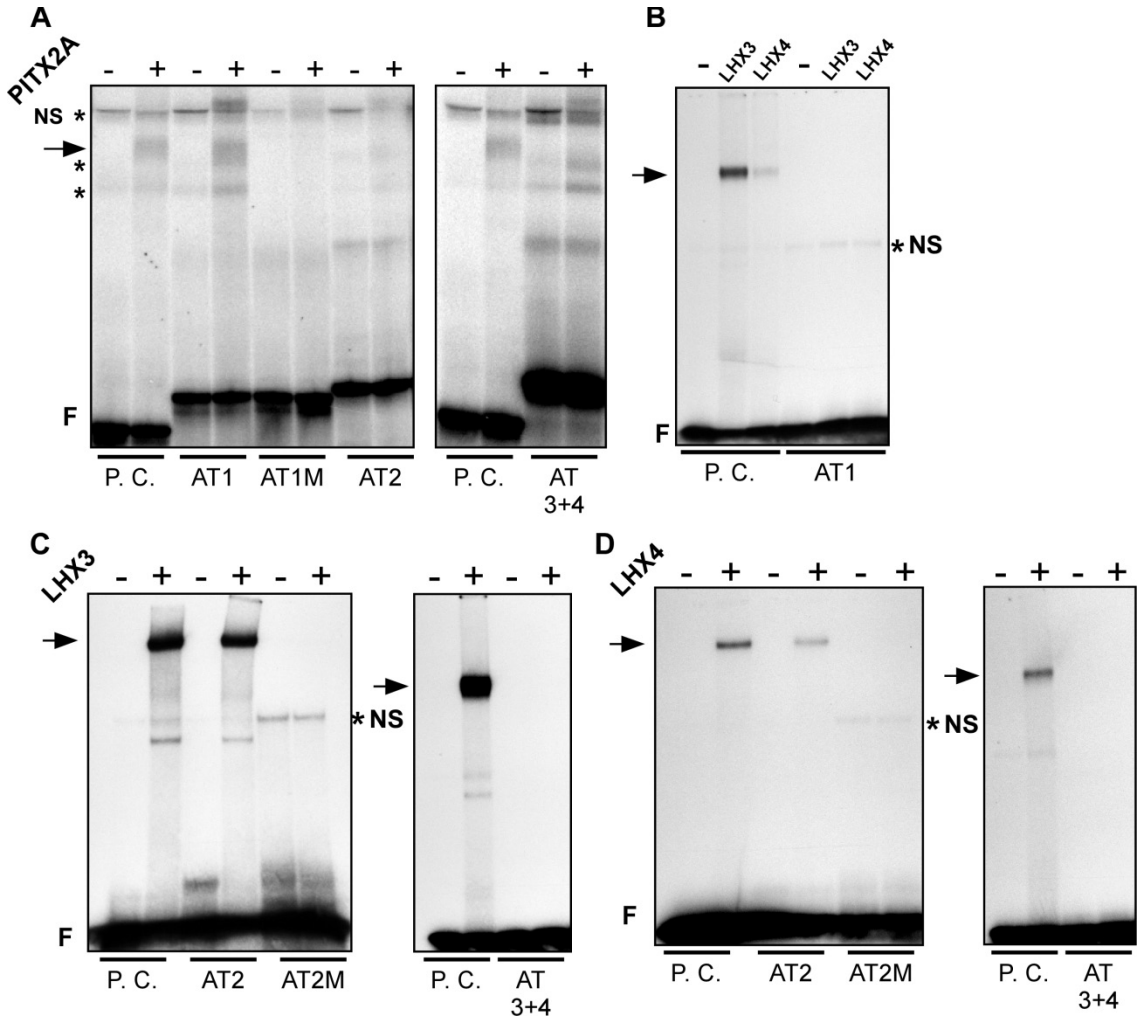


Figure 3.9. EMSA analysis of PITX2A, LHX3, and LHX4 binding of TAAT elements in Core R3. (A) PITX2A binds specifically to AT1 sites in EMSA. A Bcd2x5n bicoid element was used as the positive control (Saadi et al., 2003). (B) LHX3 and LHX4 fail to bind to AT1 sites in EMSA. (C and D) LHX3 and LHX4 bind to AT2 in EMSA, but not AT3 or AT4. The LHX3 consensus binding (LCB) element was used as the positive control (Bridwell et al., 2001). P.C., positive control; –, empty vector programmed lysate; +, LHX3 or LHX4 expression vector programmed lysate; Arrows, specific bound complex; *NS, nonspecific band; F, free probe.

Table 3.1. Single nucleotide variations identified in human *LHX3* regulatory regions

Patient	**Region**	**Genetic Variation**[1]		**additional SNP**
A	R3 enhancer	g.+7757C>G	heterozygous	
B	*LHX3a* Promoter	g.-31G>A	heterozygous	
C	*LHX3a* Promoter	g.-119C>T	heterozygous	db SNP rs10858245 heterozygous
D	*LHX3a* Promoter	g.-119C>T*	heterozygous	db SNP rs11103377 heterozygous

[1] Numbering in reference to the *LHX3a* isoform (genomic DNA (g); bp upstream (-) of the first start methionine; bp downstream (+) of *LHX3* STOP codon); * This patient was later identified with a compound heterozygous *PROP1* mutation.

CHAPTER FOUR

DISCUSSION

The pituitary gland is essential for the function and control of many processes in the body including development, growth, metabolism, reproduction and the stress response. Numerous studies have demonstrated that LHX3 is required for proper pituitary development and function. While a great deal is known about the actions of the LHX3 protein, information about the transcriptional regulation of the *LHX3* gene was limited to *in vitro* studies identifying promoter and intronic elements important for basal gene expression (Rajab et al., 2008; Yaden et al., 2005). The crucial regulatory elements required for *in vivo* expression were unknown. This is the first *in vivo* description of *LHX3* gene regulation. This study reveals that multiple enhancers 3' of the *LHX3* gene are involved in regulation of *LHX3* pituitary and spinal cord expression. Further, this work presents evidence that ISL1 and PITX1 are direct transcriptional regulators of *LHX3*, functioning in part through interactions with the Core R3 enhancer element.

These results show that PITX proteins are capable of binding to the AT1 element *in vitro*, occupy the core R3 enhancer in cells, and mutation of the PITX recognized AT1 element affects the spatial pattern of the R3 enhancer *in vivo*. This suggests a mechanism for PITX1 in regulation of the spatial pattern of *LHX3* expression in the pituitary. This study showed that ISL1 binds specifically to the AT2, AT3 and AT4 elements *in vitro* and occupies the core R3 enhancer *in vivo*. Mutations of the AT3 and AT4 elements resulted in almost complete loss of expression in both the pituitary and spinal cord. However, alone these elements were insufficient to direct any pituitary expression

suggesting a combination of homeodomain and perhaps other factors is required for enhancer activity. This implicates ISL1 for the first time as a regulator of *LHX3* gene expression in the ventral portion of the anterior pituitary and the spinal cord. Results of this study are summarized in Figure 4.1.

Screening of 100 CPHD candidate patients in the *LHX3a* and *LHX3b* promoters and R2 and R3 enhancers and of 33 additional candidate patients in the R3 enhancer has revealed no homozygous variations in these regulatory regions to date. There are several possible explanations for this. First, the cohort of patients screened was relatively small in number and mutations may yet be found in other patients. It is also possible that additional enhancers play roles in pituitary development in particular in somatotrope development. Support for this includes the observation that the R3 enhancer region was found to be primarily expressed ventrally where gonadotropes and thyrotropes develop and not dorsally where somatotropes are found. Alternately the low degree of variation observed suggests a high level of evolutionary constraint and may indicate mutations in these regions are detrimental to survival. Considering that patients with *LHX3* coding region mutations survive this seems unlikely (Bhangoo et al., 2006; Kristrom et al., 2009; Netchine et al., 2000; Pfaeffle et al., 2007; Rajab et al., 2008). Although several known regulatory mutations are associated with human disease, it is a relatively rare cause of human disease and most have been found in proximal promoters. Less than 1.8% of the mutations documented in the Human Gene Mutation Database are point mutations in non-coding regions (Noonan and McCallion, 2010). This percentage likely reflects the difficulty in correlating mutations often found far from the coding regions of genes to specific human diseases.

Though PITX1 and PITX2 are known upstream factors in the regulation of *LHX3* no mechanism had been previously identified (Charles et al., 2005; Tremblay et al., 1998). PITX1 and PITX2 are expressed strongly in the dorsal pituitary with weak expression ventrally in a pattern similar to LHX3 during development. However in P1 and adult pituitaries these PITX proteins are mostly found to co-localize predominantly with the αGSU expressing cells found more ventrally (Charles et al., 2005; Lanctot et al., 1999). This is nearly identical to the LHX3 expression patterns shown in this study at P1 and previous studies at earlier time points (Bach et al., 1995; Seidah et al., 1994; Zhadanov et al., 1995).

The full 3' enhancer directs expression throughout Rathke's pouch at e9.5 but by e12.5 it is expressed more ventrally and by e14.5 the expression is strongly ventral and nearly absent dorsally (Figure 3.2). There are a few possible explanations for this difference between previously observed LHX3 expression patterns and enhancer-directed reporter gene expression patterns. Because this study uses the human enhancer region, it is possible that the differences in observed pituitary expression represent differences between mouse and human expression patterns or differences in control mechanisms between the two species. Equally plausible is that this enhancer is not responsible for all regions of LHX3 pituitary expression. Perhaps this points to one or more additional enhancers that direct expression in the dorsal pituitary. It is possible that pituitary expression of LHX3 is regulated by multiple enhancers and the identified 3' enhancer region acts as an activating enhancer with later roles in the ventral anterior pituitary.

Another key difference in the expression pattern directed by the enhancer and the endogenous gene is intermediate lobe expression. The enhancer fails to direct expression

to the intermediate lobe, but both the human and mouse intermediate lobes express LHX3 (Bach et al., 1995; Seidah et al., 1994; Sobrier et al., 2004; Zhadanov et al., 1995). However, the human intermediate lobe is very small or even absent in adults. The expression pattern directed by this enhancer may be an indication of the level of human LHX3 expression in the intermediate lobe. The difference could possibly represent morphological differences between the human intermediate lobe and that of mice. If there is a marked reduction in LHX3 levels in the human intermediate lobe it is possible this could affect its development. It is conceivable that apoptosis as a result of low LHX3 expression in the intermediate lobe is a key factor in loss of all but just a few cells of the adult human intermediate lobe. Reductions in LHX3 action are known to increase apoptosis in the ventral portion of Rathke's pouch during development and the intermediate lobes of the *Lhx3*$^{-/-}$ mice and *Lhx3* $^{Cre/Cre}$ hypomorph mice are reduced in size at e15.5 (Charles et al., 2005; Sheng et al., 1996; Zhao et al., 2006). To explore this idea, first the mouse full 3' enhancer would need to be tested in the reporter gene mouse model. Then, if marked differences in expression are observed between the human and mouse enhancer region, a knock-in of the human enhancer region could be used to determine what affect this change would have on pituitary morphology. Changes in conserved non-coding elements have been established as one way evolution of morphological change occurs (Carroll, 2008). This would be a high risk set of experiments because redundancy is very common with regulatory elements. A number of enhancers with confirmed ability to direct expression *in vivo* have been knocked out with no observable phenotype (Ahituv et al., 2007; Nobrega et al., 2004).

In this study, ISL1 was identified as a likely regulator of *LHX3* gene transcription based on ChIP and EMSA analysis. From the *in vivo* mutational analysis, R3 enhancer-directed expression in both the developing pituitary and spinal cord was shown to be dependent on a single tandem ATTA site. ISL is expressed in both these tissues in a temporal and spatial pattern consistent with a role in activation of the R3 enhancer (Ellsworth et al., 2008; Ericson et al., 1998).

Several pieces of evidence in the literature point to a role for ISL1 in the regulation of *LHX3* gene expression. ISL1 protein expression precedes LHX3 expression in Rathke's pouch and at the time of *Lhx3* activation ISL1 is co-localized with *Lhx3* in Rathke's pouch (Ellsworth et al., 2008; Ericson et al., 1998). ISL1 expression later primarily co-localizes with αGSU expressing cells similar to what was found in this study for LHX3 and the enhancer-directed transgene (Liu et al., 2005a; Liu et al., 2005b; Wu et al., 2010). *Isl1*$^{-/-}$ mice display a similar pituitary phenotype to *Lhx3*$^{-/-}$ and lack LHX3 expression (Takuma et al., 1998). Tissue specific knock-outs of *Isl1* in motoneuron and neural retina also lack LHX3 expression (Elshatory et al., 2007; Pfaff et al., 1996). So why has ISL1 not previously been identified as a potential upstream factor of LHX3? Probably the primary reason for this is that after the early overlap of expression, the two proteins segregate into a largely inverse expression pattern with ISL1 restricted to the most ventral region and LHX3 expressed dorsally. This led to the interpretation that the phenotypes of the *Isl1* knock-out in both the pituitary and motoneurons blocked development at an earlier stage and was not the result of LHX3 loss (Elshatory et al., 2007; Pfaff et al., 1996; Takuma et al., 1998). Although ISL1 likely has other early roles in the development of neural retina, pituitary and spinal cord it is also possible it acts as

an activator of *LHX3*. Earlier roles of ISL1 in differentiation do not necessarily exclude a direct role in the activation of specific factors. The regulatory relationship between LHX3 and ISL1 is likely complex. Loss of LHX3 in mice does not affect PITX1 or PITX2 expression however the spatial and temporal expression pattern of ISL1 is affected. In *Lhx3*$^{-/-}$ mice, ISL1 is expressed normally at e9.5, but is transiently lost at e12.5 (Ellsworth et al., 2008; Zhao et al., 2006). At e16.5 and e18.5, ISL1 expression returns but its expression is shifted dorsally (Ellsworth et al., 2008). This and the largely inverse expression pattern of the two proteins after e9.5 have suggested LHX3 is a both a positive and negative regulator of *Isl1*. Work presented in this study suggests that ISL1 may also regulate *LHX3* activation and later its activity in αGSU expressing cells.

LHX3 has important roles in somatotrope development based on both the mouse models and the human CPHD patients with *LHX3* coding regions mutations (Colvin et al., 2009). Although LHX3 is not co-expressed at P1 with somatotropes it seems likely it is found in somatotrope progenitors based on its expression throughout Rathke's pouch and importance in somatotrope development. An alternative hypothesis is that LHX3's action on somatotropes is not direct. To more completely answer where enhancer-directed expression is found during pituitary development and by extension LHX3 expression, a cre recombinase lineage tracing approach is being used. Two mouse lines have been generated containing the human full 3' region upstream of the *HSP68* promoter directing cre recombinase, and these have been crossed to floxed stop reporter strains. Unfortunately the expression patterns of the two founders are too variable to draw any conclusions without examining more transgenic lines. This variability is most easily explained by site of transgene integration effects. Additional transgenics are being

generated. Some early data however indicates the enhancer does not direct expression in somatotrope progenitors. Unlike a positive result in which enhancer-directed transgene co-localization would suggest LHX3 co-localization in progenitors, this result gives us no information as to whether LHX3 is expressed in somatotrope progenitors. Additional experiments will be needed to determine the lineage specific expression of LHX3. Possible methods include generating a *LHX3* bacterial artificial chromosome-cre mouse model or co-labeling with somatotrope lineage specific transcription factors. Two transcription factors PIT1 and MATH3 could be used to identify somatotrope progenitors prior to GH expression at e15.5. PIT1 is known to be required for somatotrope, lactotrope, and thyrotrope development. MATH3, downstream factor to PIT1, is expressed at e13.5 and required for maturation and expansion of somatotropes (Zhu et al., 2006).

The R1, R2, and R3 enhancers were found to direct nervous system expression in the developing mouse embryo (Figure 4.1). Whether these enhancers are functionally redundant or direct unique expression patterns in the developing spinal cord will require further study. Additional co-localization experiments with lineage specific transcription factors HB9 (motoneurons) and CHX10 (interneurons) are also needed to determine on a cellular level where the enhancer regions direct expression in the developing spinal cord (Thaler et al., 2004).

Another interesting finding from this study is the presence of potential silencing elements. Future work will be needed to isolate these elements and determine how they are functioning mechanistically. Insulators or repressors are known to block enhancer activity or serve as a barrier between areas of open and closed chromatin and in some

cases function in both manners (Gaszner and Felsenfeld, 2006). Numerous studies have shown the CCCTC-binding factor (CTCF) is found at many insulators but exactly how CTCF functions in enhancer insulating and blocking is unknown (Noonan and McCallion, 2010). Insulators repressing expression in the dorsal pituitary and in developing cerebral cortex were contained within either the UTR R1 region or in the region between R2 and R3 (Figure 4.1). R1 may contain these insulator elements. Potential CTCF binding sites were identified using TRANSFAC in R1, but these did not strongly match the known consensus sequence of CTCF. Further experiments are needed to determine if R1 is an insulator element, what trans-acting factors are needed for its functions, and if CTCF binding is required for R1 insulator function.

The AT1, PITX binding site, was identified as important in spatial control of the enhancer-directed pituitary expression. This pattern could indicate a function of this site in dorsal pituitary repression of the larger full 3' enhancer. A potential mechanism for this observed activity is that an unknown dorsal factor bound to the insulator element facilitates chromatin looping between the R3 enhancer and the insulator that is mediated by PITX protein bound to the AT1 element and this looping isolates the R3 enhancer away from the proximal promoter (Figure 4.2). In the context of the R3-*HSP68* transgene, without the insulator regions, the enhancer is not sequestered away from the proximal promoter and is able to direct dorsal expression in the pituitary. Chromatin conformation capture (3C) experiments will be needed to test this hypothesis after a minimal insulator element is identified. A similar mechanism of function has been identified for the H19ICR insulator. In 3C experiments, H19ICR enhancers and the

promoter region were shown to form loops with the insulator that blocked gene expression (Yoon et al., 2007).

Several chromatin markers have been associated with active enhancers including monomethylation on histone H3 lysine 4 and acetylation of histone H3 lysine 27 (Heintzman et al., 2009; Heintzman et al., 2007). Active enhancers have also been associated with binding of the transcriptional co-activator p300. Recent global ChIP sequencing experiments using e11.5 mouse forebrain, midbrain, and limb tissues were able to highly correlate p300 binding with active enhancers which when tested directed tissue-specific enhancer expression in the same tissue with *in vivo* occupancy of p300 (Gotea et al., 2010).

Further, this same study, showed homotypic clusters of transcription factor binding sites were found to be abundant in both promoter regions and developmental enhancers and were more highly correlated with functional activity than CNEs not containing homotypic sites (Gotea et al., 2010). Intriguingly, I have found that both the R2 and R3 enhancers contained multiple conserved TAAT sites (numbering 9 and 4 respectively). A combination of the TAAT elements in R3 was also found to be needed for proper enhancer activity. This discovery of homotypic TAAT sites within the *LHX3* enhancers may have broader implications for the regulation of other LIM-HD transcription factors. Similar to the *LHX3* gene, a 15 kb region 3' of the *LHX4* gene contains conserved regions with multiple TAAT elements (Sequences analyzed using the ECR browser). Considering the close relationship of these genes it is highly probable this 3' region of the *LHX4* gene may also function as an enhancer of neural and pituitary expression. Additionally, the less related LIM-HD transcription factor *LHX2* is expressed

primarily in the neural ectoderm and developing cerebral cortex and shows areas of high conservation 5' of the gene with multiple TAAT elements (Sequences analyzed using ECR browser) (Hunter and Rhodes, 2005). These findings suggest TAAT homotypic clusters may play broad and important role in the regulation of LIM-HD genes in multiple tissues.

Interestingly, a NOBOX binding site [TAATT(G/A)] was highly associated with forebrain activity in the p300 ChIP sequencing study. NOBOX is not found in the forebrain. However, LIM-HD transcription factors are important in transcriptional regulation of multiple systems and have consensus binding sequences similar to the NOBOX binding site. LHX2 is highly expressed in the developing forebrain and could potentially be the factor binding to these identified sites (Gotea et al., 2010; Hunter and Rhodes, 2005). In the absence of the insulator regions, R2 and R3 both have very robust forebrain expression. This suggests the default for enhancers with homotypic TAAT sites is to be active in the forebrain, and insulator regions likely play key roles in the repression of ectopic forebrain expression. This result also highlights an important caveat to these types of studies. Many studies look at enhancer elements based on conservation and analyze these in isolation (Visel et al., 2007). It is important to keep in mind the true activity of the enhancer is dependent on interactions with other regulatory regions in its native position and many of these interacting regions are found many kb away (Noonan and McCallion, 2010).

The studies presented in this report describe and characterize the first known pituitary and spinal cord enhancers of *LHX3* and are the first step in uncovering the mechanisms required for proper spatial and temporal expression of the *LHX3* gene. In

addition to identifying other important regulatory regions, future research will need to explore the interactions between the *LHX3* proximal promoters, insulator regions, and enhancers. Uncovering how these elements interact to direct tissue-specific gene expression will be essential to understanding the mechanisms behind *LHX3* gene regulation and understanding pituitary development on a molecular level. Information gathered about *LHX3* may have implications in the regulation of other LIM-HD genes and give us insight into how gene regulation works in general. The molecular cause of most cases of CPHD is unknown (Dattani, 2005). Further understanding and identification of important *LHX3* gene regulatory regions will allow for the identification of novel genetic defects responsible for CPHD and will facilitate patient treatment and enable genetic counseling.

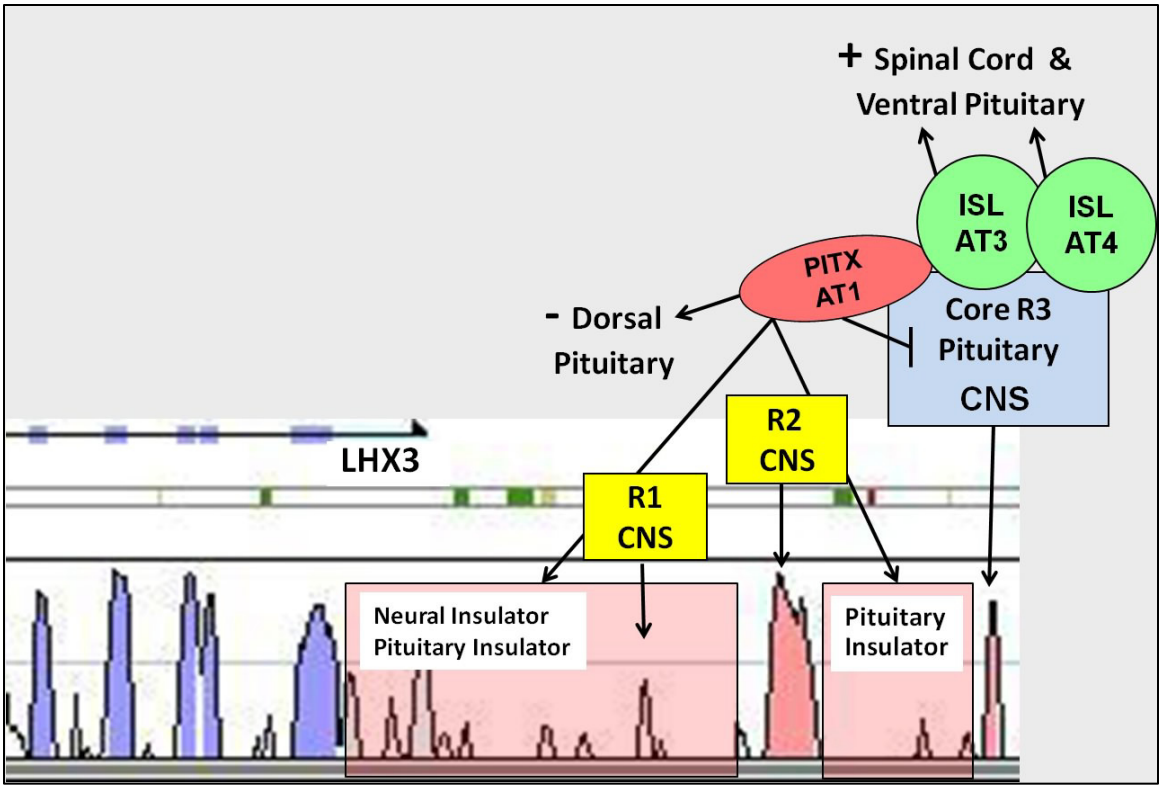


Figure 4.1. A schematic summary of findings. Regulatory regions R1, R2, and R3 are sufficient to independently direct expression to the central nervous system (CNS). Only R3 directs pituitary and CNS expression. PITX proteins bind to the AT1 element *in vitro* and occupy the Core R3 enhancer. Mutation of the PITX recognized AT1 element affects the dorsal pituitary expression pattern of the R3 enhancer *in vivo*. ISL1 binds specifically to the AT2, AT3 and AT4 elements *in vitro* and occupies the Core R3 enhancer *in vivo*. Mutations of the AT3 and AT4 elements resulted in almost complete loss of expression in both the pituitary and spinal cord. Pituitary and neural insulators are contained within either the UTR R1 region or in the region between R2 and R3.

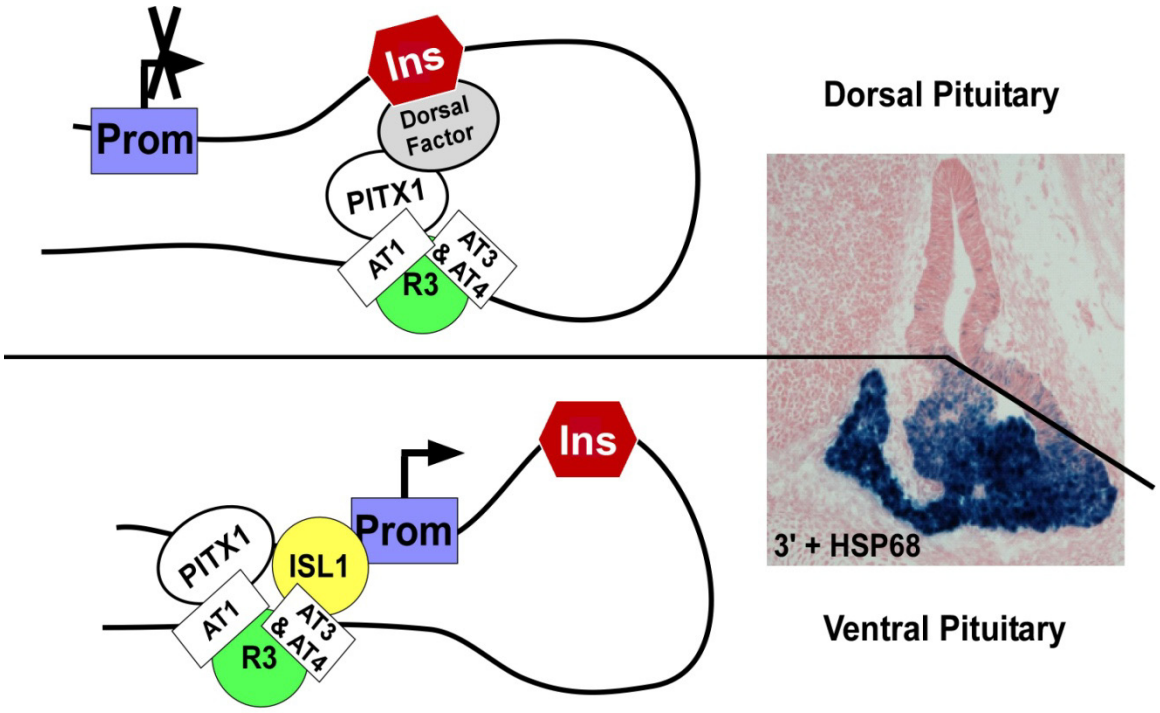


Figure 4.2. A hypothetical mechanism for regulation of the spatial expression pattern in the developing pituitary. In the dorsal pituitary, an unknown factor bound to the insulator element facilitates chromatin looping between the R3 enhancer and the insulator that is mediated by PITX protein bound to the AT1 element. This looping isolates the R3 enhancer away from the proximal promoter and silences dorsal pituitary expression. In the ventral pituitary, because the dorsal factor is absent the interaction does not occur and the R3 enhancer is able to loop to the promoter and activate gene expression. Prom, promoter; Ins, insulator element.

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Yaden, B. C., Savage, J. J., Hunter, C. S. and Rhodes, S. J. (2005). DNA recognition properties of the LHX3b LIM homeodomain transcription factor. *Mol Biol Rep* **32**, 1-6.

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Zhao, Y., Mailloux, C. M., Hermesz, E., Palkovits, M. and Westphal, H. (2010). A role of the LIM-homeobox gene Lhx2 in the regulation of pituitary development. *Dev Biol* **337**, 313-23.

Zhao, Y., Morales, D. C., Hermesz, E., Lee, W. K., Pfaff, S. L. and Westphal, H. (2006). Reduced expression of the LIM-homeobox gene Lhx3 impairs growth and differentiation of Rathke's pouch and increases cell apoptosis during mouse pituitary development. *Mech Dev* **123**, 605-13.

Zhu, X., Gleiberman, A. S. and Rosenfeld, M. G. (2007). Molecular physiology of pituitary development: signaling and transcriptional networks. *Physiol Rev* **87**, 933-63.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E. H., Guillemot, F., Kageyama, R. and Rosenfeld, M. G. (2006). Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. *Genes Dev* **20**, 2739-53.

CURRICULUM VITAE

Rachel Diane Mullen

Education

Ph.D., Biochemistry and Molecular Biology
Indiana University, Indianapolis, IN
Dissertation: *In Vivo* Analysis of Human *LHX3* Gene Regulation
Advisor: Simon J. Rhodes Ph.D.
Committee: B. Paul Herring Ph.D., David Skalnik Ph.D., Debbie Thurmond Ph.D., Emily Walvoord M.D.
Graduate Certificate in Biotechnology, July 2005
Indiana University, Indianapolis, IN
Bachelors of Science, Biology; Minor, Chemistry, May 2002
Indiana University, Richmond, IN

Fellowship, Awards & Honors

Graduate Fellowship in Translational Research
Indiana University School of Medicine, 2007-2008
Sigma Xi Graduate Research Competition
Third Place
Indiana University Medical Center Chapter, 2010
Educational Enhancement Travel Grant
Graduate Student Organization
Indiana University Purdue University Indianapolis, 2010
Biochemistry & Molecular Biology Department Research Day
First Place Poster Award
Indiana University School of Medicine, 2010
Sigma Xi Graduate Research Competition
First Place
Indiana University Medical Center Chapter, 2008
Biochemistry & Molecular Biology Department Research Day
Honorable Mention Poster Award
Indiana University School of Medicine, 2008
Biochemistry & Molecular Biology Department Research Day
First Place Poster Award
Indiana University School of Medicine, 2007
Judy White Award
Academic Excellence in the Biotechnology Certificate program
Indiana University School of Medicine, 2006
Distinguished Biology Student
Indiana University East, 2002

Professional Experience

Research Technician (2004-2006)
Department of Cellular and Integrative Physiology
Indiana University School of Medicine, Indianapolis, IN

- Primary research project was analyzing the phenotype of transgenic mice over-expressing LHX3 transcription factor isoforms in the pituitary (Savage, Mullen et al., 2007).
- Lab manager duties included safety compliance, supply ordering, and supervision of undergraduates. Responsible for coordinating move of the lab across campus and setting up the new lab space in 2005. Lab was fully operational within one week.
- Performed mouse colony genotyping and husbandry.

Quality Control Chemist (2002-2004)
Gilchrist & Soames, Indianapolis, IN

- Responsible for all quality control of products and raw materials.
- Checked samples for viscosity, total solids, pH, microbiological testing and maintained records.
- Wrote and updated laboratory standard operating procedures.
- Implemented quality control program for incoming raw materials and goods.
- Assisted formulating chemist in development of new product lines.

Lab Working Foreman/ QA Associate (1996-2002)
Smith Dairy/Wayne Div., Richmond, IN

- Directed flow of work.
- Maintained the Indiana State Board of Animal Health certification.
- Tested raw milk and milk products for bacteria, antibiotics, butterfat and total solids.
- Communicated batch corrections, and trained new lab associates

Publications

Mullen R.D., Park S., and Rhodes S.J. *In Vivo* Expression of the Human *LHX3* Gene in the Developing Pituitary and Central Nervous System is Mediated by Multiple Distal Enhancers. *In preparation*

Prince K.L., **Mullen R.D.**, Colvin S.C., and Rhodes S.J. (Updated 12/18/2009). LHX3, LIM homeobox protein 3. The Transcription Factor Encyclopedia, http://www.cisreg.ca/tfe. Review

Prince K.L., **Mullen R.D.**, Colvin S.C., and Rhodes S.J. (Updated 12/18/2009). LHX4, LIM homeobox protein 4. The Transcription Factor Encyclopedia, http://www.cisreg.ca/tfe. Review

Colvin S.C., **Mullen R.D.**, Pfaeffle R.W., Rhodes S.J. LHX3 and LHX4 transcription factors in pituitary development and disease. Pediatr Endocrinol Rev. 2009 Jan;6 Suppl 2:283-90. Review

Pfaeffle R.W., Hunter C.S., Savage J.J., Duran-Prado M., **Mullen R.D.**, Neeb Z.P., Eiholzer U., Hesse V., Haddad N.G., Stobbe H.M., Blum W.F., Weigel J.F., Rhodes S.J. Three Novel Missense Mutations within the *LHX4* Gene Are Associated with Variable Pituitary Hormone Deficiencies. J Clin Endocrinol Metab. 2008 Mar;93(3):1062-71.

Savage J.J., **Mullen R.D.**, Sloop K.W., Colvin S.C., Camper S.A., Franklin C.L., Rhodes S.J. Transgenic mice expressing LHX3 transcription factor isoforms in the pituitary: effects on the gonadotrope axis and sex-specific reproductive disease. J Cell Physiol. 2007 Jul;212(1):105-17.

Mullen R.D., Colvin S.C., Hunter C.S., Savage J.J., Walvoord E.C., Bhangoo A.P., Ten S., Weigel J., Pfäffle R.W., Rhodes S.J. Roles of the LHX3 and LHX4 LIM-homeodomain factors in pituitary development. Mol Cell Endocrinol. 2007 Feb;265-266:190-5. Review

Invited Talks

Genes Controlling Pituitary Development: Applications in Pediatric Hormone Deficiency Diseases. Seminar in Biomedical Research, Biology 491. Nov. 2008. California State University Dominguez Hills, Los Angeles, CA.

Regulation of the Human *LHX3* Transcription Factor Gene: Translation to Hormone Deficiency Diseases. Keynote Speaker, Research Day. March 2008. Indiana University East, Richmond, IN.

Abstracts

Mullen, R.D., Park, S., and Rhodes, S.J. (2010) Multiple *Cis*-Acting Enhancers Regulate Temporal and Spatial Expression of the Human *LHX3* Gene in the Developing Endocrine and Nervous Systems. Endocrine Society Meeting. San Diego, CA.

Mullen, R.D., Park, S., and Rhodes, S.J. (2009) Distal Enhancers Regulate Temporal and Spatial Expression of the Human *LHX3* Gene in the Developing Endocrine and Nervous System. IU Medical School Department of Cellular and Integrative Physiology Department Retreat. Indianapolis, IN.

Mullen, R.D., and Rhodes, S.J. (2008) Identification of Regulatory Elements of the Human *LHX3* Gene and Investigation of Their Possible Role in Pediatric Combined Pituitary Hormone Deficiency Diseases. IU Medical School Department of Biochemistry and Molecular Biology Research Day. Indianapolis, IN.

Mullen, R.D., and Rhodes, S.J. (2008) Regulation of the Human *LHX3* Transcription Factor Gene: Translation to Hormone Deficiency Diseases. Sigma Xi Graduate Research Competition. Indiana University Medical Center Chapter. Indianapolis, IN.

Mullen, R.D., Savage, J.J., Colvin, S.C., Sloop, K.W., Camper, S.A., Franklin, C.L., and Rhodes, S.J. (2006). Sex-specific reproductive disease and loss of viability in transgenic mice over-expressing LHX3 protein isoforms. Endocrine Society Meeting, Boston, MA.

Hunter, C.S., Savage, J.J., **Mullen, R.D.**, Colvin, S.C., Walvoord, E.C., Bhangoo, A.P.S., Ten, S., Pfäffle, R., Weigel, J., and Rhodes, S.J. (2006). Gene regulation by LIM homeodomain transcription factors: association with hormone deficiency diseases. Adrenal 2006/Molecular Steroidogenesis 5 (AMS) Meeting, Boston, MA.

Savage, J., Hunter, C., Garcia, M., **Mullen, R.**, Colvin, S., Clark, S., Jacob, T., Sloop, K., Smith, T., Franklin, C., Pfaffle, R., Rhodes, S. (2005) LIM-Homeodomain Transcription Factors in HPG Axis Function. Endocrine Society Meeting. San Diego, CA.

Cain, C., Savage, J., **Mullen, R.**, Rhodes, S., and Franklin, C. (2005) Acute Mortality of Male Transgenic Mice Over-expressing hLHX3. AALAS National Meeting. St. Louis, MO.

Research Techniques

Murine Transgenesis: transgene construction, animal husbandry, genotyping, sera collection, Cre/lox technology, reporter gene analysis (*β-galactosidase*)

Histology: embryo harvesting (e9.5→), tissue harvesting, tissue processing (paraffin and cryosectioning), antigen retrieval, immunohistochemistry, tissue homogenization and extraction, light and immunoflourescence microscopy

Molecular Biology: DNA cloning, plasmid construction, agarose and polyacrylamide gels, PCR techniques, site-directed mutagenesis, DNA sequence analyses, southern blotting, plasmid preparation, genomic DNA purification, gel purification, DNA and protein bioinformatics (sequence alignments, functional predictions, database searching)

RNA Analyses: RNA extraction, cDNA synthesis, quantitative and semi-quantitative PCR analyses of gene expression

Protein Analyses: *in vitro* transcription/translation, western blotting, electrophoretic mobility shift assays, SDS-PAGE, glutathione s-transferase fusion protein purification, chromatin immunoprecipitation

Cell Culture: cell line maintenance, transient transfection (calcium phosphate and lipofection), reporter gene analyses (*luciferase* and *β-galactosidase*)