THE ROLE OF LIM HOMEODOMAIN TRANSCRIPTION FACTORS IN GLYCOPROTEIN HORMONE α-SUBUNIT GENE EXPRESSION

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THESIS ABSTRACT

Tissue-specific expression of the α-subunit gene of glycoprotein hormones involves an enhancer element designated the pituitary glycoprotein basal element (PGBE) which interacts with the LIM homeodomain transcription factor, Lhx2. In the present studies we have explored the function of the LIM domain of Lhx2 in stimulating α-subunit transcription. When fused to the GAL4 DNA binding domain, the LIM domain of Lhx2 was shown to contain a transcriptional activation domain. Furthermore, in the context of an α-subunit reporter gene in which a GAL4 binding site replaced the PGBE, the LIM domain enhanced both basal and ras-mediated transcription. In addition, a synergistic response to ras activation was observed when the Lhx2 LIM domain and the transactivation domain of Elk1 are directed to a minimal reporter gene. A yeast two-hybrid screen identified the recently described melanocyte specific gene related gene 1 (MRG1) as an Lhx2 LIM-interacting protein. MRG1 was shown to bind Lhx2 *in vitro* and a co-immunoprecipitation assay provided evidence that endogenous MRG1 forms a complex with Lhx2 in αT3-1 cells. Expression of MRG1 in αT3-1 cells enhanced α-subunit reporter gene activity. MRG1 was also shown to bind in vitro to the TATA binding protein (TBP) and the transcriptional coactivator, p300. These data suggest a model in which the Lhx2 LIM domain activates transcription through interaction with MRG1 leading to recruitment of p300/CBP and the TATA binding protein.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
ADH	antidiuretic hormone
bHLH	basic helix-loop-helix
cAMP	cyclic adenosine monophosphate
CBP	creb binding protein
CMV	cytomegalovirus
CRE	cAMP response element
Creb	cAMP response element binding protein
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EMSA	electrophoretic mobility shift assay
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotropin releasing hormone
GnRH-RE	gonadotropin releasing hormone response element
LH	luteinizing hormone
Lhx	LIM homeodomain
MAPK	mitogen activated protein kinase
MBP	Maltose binding protein
MLP	muscle LIM protein

MRG1	melanocyte specific gene related gene 1
MSG1	melanocyte specific gene 1
NLI	nuclear LIM interactor
PGBE	pituitary-specific glycoprotein basal element
PKC	protein kinase C
PMA	phorbol myristate acetate
PRL	prolactin
RNA	ribonucleic acid
SV40	Simian virus-40
TSH	thyroid stimulating hormone

CHAPTER I

INTRODUCTION

Overview

The mammalian endocrine system is regulated by the pituitary gland, which is responsive to both signals from the central nervous system and peripheral endocrine glands. The pituitary releases hormones, which govern the function of the adrenals, gonads, breasts, and thyroid. In addition, body growth, response to stress, water metabolism and birth are also regulated by the pituitary. The pituitary gland can be divided anatomically and functionally into posterior and anterior lobes. The posterior lobe, or neurohypophysis, is derived embryologically from neural tissue and secretes antidiuretic hormone (ADH) and oxytocin which function to regulate water metabolism and parturition, respectively (1). The anterior lobe, or adenohypophysis, is derived from Rathke's pouch and consists of five distinct cell types that are designated by the particular hormone(s) produced. The corticotrophs produce adrenocorticotropin hormone (ACTH) which promotes synthesis and secretion of adrenal cortical hormones. Somatotrophs release growth hormone (GH) which directs growth and metabolism. The lactotrophs secrete prolactin (PRL), which is important for milk production. Thyrotrophs produce thyroid stimulating hormone (TSH) which regulates thyroid hormone levels. The gonadotrophs secrete both follicle

stimulating hormone (FSH) and luteinizing hormone (LH) which regulate the function of the gonads (1).

The five cell types of the anterior pituitary are often divided into two groups based on histological characteristics when specialized stains are used. The somatotrophs and lactotrophs are termed acidophils, while the corticotrophs, thyrotrophs and gonadotrophs are called basophils. Individual cell types can be distinguished by the particular hormones that are produced using either immunohistochemistry or *in situ* hybridization techniques to detect protein or mRNA, respectively. These techniques have used to reveal the organization of the pituitary and have demonstrated the distinct regional distribution of each cell type within the pituitary in both the adult and in the developing animal.

The work presented in this thesis has focused on the mechanisms involved in expression of the gonadotropins, specifically, the common α-subunit of LH and FSH. Both FSH and LH are essential for reproductive function in both men and women, regulating the production and maturation of gametes and the synthesis of sex steroids (1). In addition, study of the α-subunit gene regulation has served as model system for gene expression. Previous studies have revealed the importance of members of the LIM homeodomain class of transcription factors, in α-subunit expression (2, 3). These studies have sought to extend this work and have focused on the structure-function relationships of the LIM domain.

Pituitary Gland Development

Many studies have been directed towards understanding the morphological and molecular events in pituitary gland development and differentiation. Much of this work has been done in mouse models where the ability of gene disruption studies has illustrated the importance of many factors. Several signaling molecules have been found to be essential, notably bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) (4). In addition, many transcription factors have also been shown to play a role in pituitary development (4, 5).

The first phase of pituitary development, as described by Kioussi et al. (4), involves commitment of the oral ectoderm, which lies below the developing diencephalon. The LIM homeodomain transcription factor, Isl1 is transiently expressed in the oral ectoderm that will become Rathke's pouch, and has been implicated in the early commitment of the anterior pituitary. In addition, Sonic Hedgehodge (Shh) is initially expressed uniformly across the oral ectoderm. As Rathke's pouch forms, representing the second and third phases of development, Shh expression becomes restricted, apparently as a result of the expression of BMP4 in the ventral diencephalon. Embryos that lack BMP4 function fail to develop the ectodermal placode that becomes Rathke's pouch (6). It is thought that the absence of Shh results in the induction of BMP2 in the ventral ectoderm, setting up a ventral-dorsal gradient. Later in development, expression of FGF8 in the diencephalon results in a dorsal-ventral gradient.

The dorsal-ventral gradients of BMP2 and FGF8, either directly or indirectly, result in the temporal and spatial expression of several transcription factors which are thought to promote the expression of genes required for continued pituitary development and differentiation. Interestingly, BMP4 can maintain the expression of Isl1, while FGF8 seems to extinguish expression. In addition, the LIM homeodomain transcription factor Lhx3, a factor of particular relevance to the work to be described in this thesis, is activated by FGF8 (7). Targeted gene disruption of Lhx3 results in the failure of the anterior pituitary to develop with the loss of thyrotrophs, gonadotrophs, somatotrophs and lactotrophs (8). Gene knockout studies of a related LIM homeodomain factor, Lhx4 (9) decreased the relative numbers of all five anterior pituitary cell types, a loss attributed to an early defect in cell proliferation (10). In mice lacking both Lhx3 and Lhx4 the severity of the proliferation defect was increased. These results indicate that Lhx3 and Lhx4 both play important roles in pituitary proliferation and cell fate. Another LIM homeodomain transcription factor, Lhx2 is also expressed in Rathke's pouch and in the adult pituitary, however, the role in pituitary development is not yet clear. Targeted disruption of the Lhx2 gene shows defects in eye, forebrain and erythrocyte development leading to early embryonic death. Analysis of the pituitary may be complicated by the defects in these other organ systems (11).

In the final phase of pituitary development, the distinct pituitary cell types arise within particular regions of the developing pituitary. The α-subunit of glycoprotein hormones, described below, can be detected prior to the formation

of Rathke's pouch, suggesting that cell differentiation occurs early in development (12). However, the first cell type to appear are the corticotrophs. Thyrotrophs can be detected next, followed by somatotrophs, gonadotrophs and finally lactotrophs (12). The pituitary cell types are also found in distinct spatial arrangements; the corticotrophs occupy the area adjacent to the intermediate lobe, the thyrotrophs are present at the rostral tip of the gland, the somatotrophs and lactotrophs are located in the caudal-medial region and the gonadotrophs are found at the anterior part of the pituitary (4). It is thought that the growth factor gradients and specific transcription factor expression help to guide the spatiotemporal differentiation of the pituitary. For example, the POU homeodomain transcription factor Pit1 has been shown to be essential for development of the somatotrophs, lactotrophs and thyrotrophs (13). Two naturally occurring mouse mutations in Pit1, *Snell* and *Jackson*, result in dwarf phenotype and the absence of these cell types (14). In addition, Pit1 regulates GH, PRL, and TSHβ gene expression. These results suggest that the somatotrophs, lactotrophs and thyrotrophs arise from a common precursor.

Glycoprotein hormones

FSH and LH share a similar structure and are classified as glycoprotein hormones. This family also includes TSH, which stimulates the synthesis and secretion of thyroid hormone and chorionic gonadotropin, which is synthesized in the trophoblasts of the placenta in primate and equine species. Each of these hormones are heterodimeric, formed by glycosylated, non-covalently associated

alpha (α) and beta (β) subunits (15). The α-subunit is common to all four hormones and is the product of a single gene, while each β subunit is unique and dictates the biological specificity of the heterodimer (16, 17).

Tissue specific and GnRH mediated expression of glycoprotein hormones

The common α subunit must be expressed in the pituitary in both thyrotrophs and gonadotrophs as well as in the placenta. In addition, both α- and β-subunit expression is subject to positive and negative regulation by hypothalamic hormones, sex steroids and thyroid hormone. The expression of glycoprotein hormones has been studied in primary pituitary cell cultures and in animal models including transgenic mice. However, studies of both tissue specific and hormone induced expression of the α-subunit have been greatly facilitated by the development of a gonadotropin derived immortal cell line (18). This cell line, termed αT3-1, was constructed by targeted oncogenesis of the anterior pituitary. Expression of the simian virus-40 (SV40) T-antigen using the promoter of the human α-subunit in transgenic mice resulted in pituitary adenomas from which stable cell lines were cultured. The αT3-1 cell line synthesizes and secretes the endogenous mouse α-subunit protein but fails to express the β-subunit of either FSH or LH. In addition, this cell line expresses receptors for the hypothalamic hormone, gonadotropin releasing hormone (GnRH) (19). αT3-1 cells were used

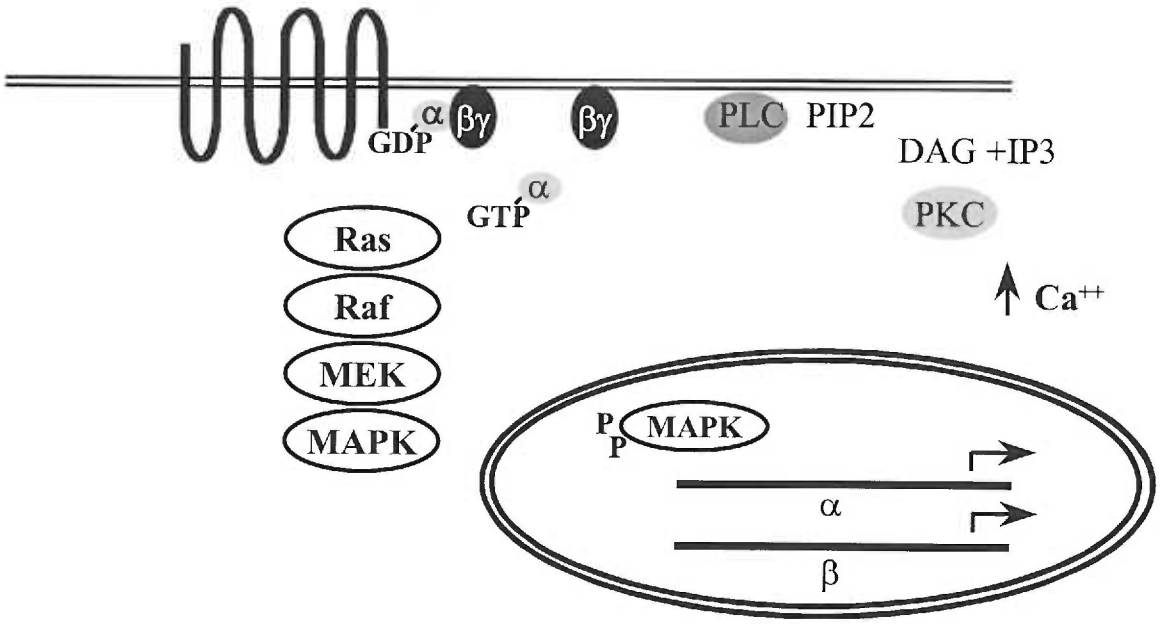
extensively in the studies performed in this thesis as a model system for the expression of the α-subunit gene.

Pituitary gonadotrophs synthesize and secrete FSH and LH in response to stimulation by the hypothalamic hormone, GnRH. GnRH is a decapeptide synthesized in the arcuate nuclei of the medial basal hypothalamus. It is released into the hypophyseal portal capillaries in a pulsatile fashion at intervals of one to three hours (1). This pulsatile release is essential for the maintenance of gonadotropin expression, and both α- and β-subunit mRNA levels have been shown to increase in response to pulsatile GnRH (20, 21). However, in αT3-1 cells, α-subunit gene expression can be induced by static treatment with GnRH and does not require pulsatile administration (19).

Studies in both primary pituitary tissue culture and αT3-1 cells have defined many components of the GnRH signal transduction pathway (22). GnRH binds to a seven transmembrane, G-protein coupled receptor of the Gq class (23, 24). Binding of the receptor leads to activation of phospholipase C, resulting in the activation of protein kinase C (PKC), and an increase in intracellular Ca^{++} (19). Activation of PKC, as assessed by treatment with phorbol myristate acetate (PMA), has been shown to increase α-subunit gene expression (25). Increases in extracellular Ca^{++} influx have also been shown to increase human α-subunit transcription (26). In addition, GnRH treatment leads to mitogen activated protein kinase (MAPK) activation and MAPK activation is necessary for maximal GnRH induced transcription of the α-subunit gene (27, 28). Recently, Weck *et*

al. have shown that GnRH stimulation of rat LH β-subunit reporter gene activity is reduced in the presence of the Ca^{++} channel antagonist, nimodipine, in both the αT3-1 cell line and in transgenic mouse pituitary cells (29). However, LH β-subunit expression was less sensitive to PD98059, a selective inhibitor of MAPK kinase (MAPKK or MAP/ERK kinase, MEK) (30, 31). Conversely, while GnRH stimulated α-subunit expression was modestly reduced with nimodipine, the presence of the MAPK pathway inhibitor greatly decreased α-subunit promoter reporter gene activity. Thus, it is likely that GnRH stimulated α-subunit expression is mediated, at least in part, by activation of the MAPK pathway and that β-subunit expression is more dependent on increases in intracellular Ca^{++} (Fig. 1). In this way activation of distinct signal transduction pathways by GnRH, leads to the coordinated expression of the α-and β-subunits.

Figure 1. GnRH signal transduction pathways.



Transcriptional regulatory elements of the α-subunit gene

The DNA elements and transcription factors, which contribute to the regulation of the α-subunit, have been studied extensively. Results from both human and mouse promoter experiments indicate that tissue specific expression is mediated by distinct DNA elements. Many of the DNA elements required for expression in both the placenta and pituitary have been defined (Fig. 2).

Figure 2. Elements of the α-subunit gene.

A. Elements important in placenta expression.

B. Elements important in pituitary expression.

Elements necessary for expression of the human α-subunit gene in placenta are located within the proximal promoter between -180 and -100 base pairs upstream of the transcriptional start site. These elements include the upstream regulatory element (URE), tandem cAMP response elements (CRE), a junctional regulatory element (JRE), and a CCAAT box (32-37). While no single element is sufficient for trophoblast expression, the CREs contribute strongly to the promoter activity. Mutation of the core CRE sequence decreases promoter activity markedly in a trophoblast derived cell line (37-39). Indeed, comparison

of α-subunit gene promoter sequences from species which do not express chorionic gonadotropin reveals the presence of one variant non-palindromic CRE (40). It has been suggested that this variant accounts for the lack of placental expression in non-primate species.

The DNA sequences necessary for pituitary specific expression of the α-subunit gene are different than those required for placental expression. There are at least three elements in the mouse α-subunit gene promoter and others in the human promoter, which contribute to pituitary specific expression. These elements reside between -507 and -205 basepairs (bp) upstream of the transcriptional start site at a location more distal than those required for placental expression. A tissue specific enhancer element identified by Horn *et al.* and termed the gonadotropin specific element (GSE), is located at position -208 to –225 bp, and is required for expression in the αT3-1 gonadotroph derived cell line, (41). The orphan nuclear hormone receptor, steroidogenic factor-1 (SF-1) (42), has been shown to bind the GSE (43). Two elements, α basal element 1 and 2 (αBE1 and αBE2), were identified in the human α promoter by block mutation analysis (37). These elements contribute to basal expression of the human promoter in αT3-1 cells, however, it is not clear if analogous regions of the mouse α promoter are required. The OTX class homeodomain protein Ptx1 (P-OTX) (44, 45) has been shown to bind the -390 to -383 bp region of the α-subunit promoter (46, 47), however, deletion analysis indicates that multiple binding sites may be important for Ptx1 directed activation (44).

Deletion and clustered point mutation analysis of the mouse alpha promoter, by Schoderbek *et al.*, revealed two unrelated elements required for both basal and GnRH induced expression (25, 38). The first element located within the -406 to - 399 bp region was discovered to be sufficient to permit an increased transcriptional response to GnRH when tested as a multimer and so is named the GnRH-responsive element (GnRH-RE). The second element, located at position -343 to –330 bp, was found to be important for expression in both the αT3-1 gonadotroph and αTSH thyrotroph derived cell lines (48), but not placental cells. This element is designated the pituitary-specific glycoprotein hormone basal element (PGBE). Although mutation of this element in the context of the α promoter reduced both basal and GnRH induced transcriptional activity, multimers of the PGBE did not permit a response to GnRH. It has been proposed that the PGBE and the GnRH-RE function as a two-component GnRH responsive unit, and reporter genes containing mixed multimers of the PGBE and GnRH-RE show increased basal and GnRH responsiveness.

While the endogenous GnRH-RE binding factor has not yet been identified, analysis of the DNA element revealed a core binding site for an Ets transcription factor. *In vitro* DNA binding studies have shown that the DNA binding domain of Ets-2 is capable of binding the GnRH-RE in a sequence specific fashion (27). In addition, expression of the Ets-2 DNA binding domain, which lacks the transactivation domain, decreased the ability of a GnRH agonist to stimulate the α-subunit reporter gene in a dose specific manner. As a model for Ets factor mediated transcription, an expression vector for a fusion protein containing GAL4

DNA binding domain and the transactivation domain of the Ets factor Elk-1, was cotransfected with a GAL4 site minimal reporter gene in αT3-1 cells. Using this system, Roberson *et al.* demonstrated that treatment with GnRH agonist led to increased reporter gene activity (27). These results suggest that the increase in α-subunit gene transcription, in response to GnRH, is mediated at least in part, by a member of the Ets family of transcription factors, which is bound at the GnRH-RE site.

Further analysis of the PGBE element defined a 14 base pair imperfect palindrome (TACTTAGCTAATTA) that is required for the binding of a factor present in gonadotroph and thyrotroph cell lines but not in somato-lactotroph (GH3), corticotroph (AtT20) or placental cell lines (2). Point mutations of the palindromic site resulted in loss of factor binding as assayed by electrophoretic mobility shift assays (EMSA). These same mutations in the context of the α-subunit promoter result in decreased transcriptional activity.

Recently, an enhancer element has been identified upstream of the proximal promoter region of the α-subunit gene which directed expression in both thyrotrophs and gonadotrophs in transgenic mouse studies (49, 50). Interestingly, the ability of this enhancer region to increase α-subunit reporter gene activity in transgenic mice was decreased when a deletion corresponding to the PGBE region was made in the transgene. This result was confirmed in enhancer mediated reporter gene experiments in αT3-1 cells (51). Moreover, the PGBE deletion mutant transgene also displayed ectopic expression in pituitary somatotrophs, corticotrophs and lactotrophs as well as kidney and brain

tissue (50). Additional studies have indicated that the α-subunit enhancer may regulate promoter activity in a cell type specific manner. While the enhancer mediated activation of α-subunit promoter activity was seen in gonadotroph and thyrotroph cells, repression was demonstrated in the GH3 somatotroph-derived cell line (52). Thus, the enhancer studies taken together with the results of proximal promoter experiments demonstrate the importance of the PGBE element in facilitating both the expression of the α-subunit in pituitary gonadotrophs and thyrotrophs and repression in other cell types.

LIM homeodomain transcription factors in α-subunit expression

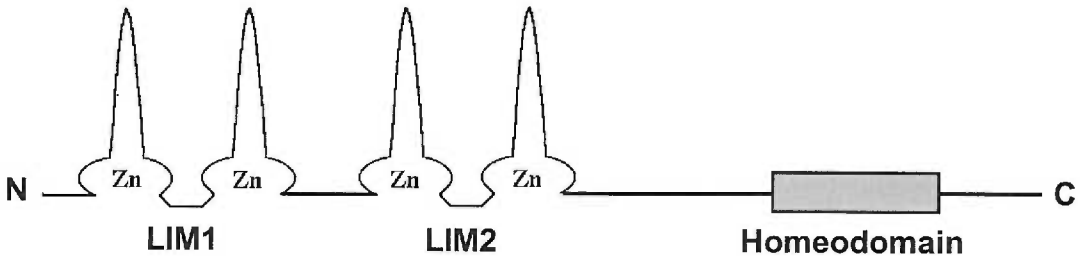
A PGBE binding factor was identified by Roberson *et al.* and was found to be a member of the LIM homeodomain transcription factor family (2). The factor, termed LIM homeodomain protein-2 (Lhx2 or LH-2), was also cloned independently by Xu *et al.* (53). Lhx2 is expressed in both αT3-1 and the αTSH, thyrotroph derived cell line, consistent with the observed requirement of the PGBE for α-subunit expression in these cell types. In addition, Lhx2 expression is detected in pituitary tissue but not in the placental cell line JEG-3 or in liver. Lhx2 is also expressed in the brain and in hematopoetic cells (53). Antibodies to Lhx2 supershift the endogenous PGBE binding proteins in electrophoretic mobility shift assays (EMSA). This indicates that Lhx2 or a protein immunologically related to Lhx2 binds the PGBE in αT3-1 cells. Importantly, overexpression of Lhx2 in heterologous cells increases the expression of an α-

subunit promoter reporter gene. There is also evidence that a related LIM homeodomain transcription factor, Lhx3 (also designated pLIM or LIM3), may be important in α-subunit gene expression. Bach *et al.* have shown that Lhx3 can mediate enhanced α-subunit reporter gene activity in heterologous cells (3, 47). It is also clear that LIM homeodomain transcription factors play an important role in pituitary development, as discussed above.

Structure and function of the LIM domain.

The structure of LIM homeodomain transcription factors reveals two LIM domains, designated LIM1 and LIM2, and a homeodomain. The homeodomain is a structural motif found in many DNA binding proteins (54, 55) and the homeodomains of Lhx2 and Lhx3 are sufficient to bind the PGBE sequence (2, 3). The LIM domain, named for the genes of the first three members of the family, *lin-11* (56) from *Caenorhabditis elegans*, *ISL-1* (57) from rat and *mec-3* (58) also from *C. elegans*, is characterized by its ability to bind zinc (59). The zinc atom is tetrahedrally coordinated by cysteine, histidine or aspartate residues spaced according to the amino acid consensus sequence ($CX_2C\ X_{17\pm1}\ HX_2C)\ X_2$ ($C\ X_{17\pm1}\ CX_2H/C/D$) (60). This consensus sequence forms two zinc finger motifs indicated by the parenthesis, each binding one atom of zinc (Fig. 3).

Figure 3. Schematic Representation of a LIM Homeodomain transcription factor.



The LIM domain has been identified in over 20 different proteins and two general classes have been defined (61-63). The first class, of which includes Lhx2, Lhx3 and Lhx4, has two LIM domains associated with a homeodomain. Members of this class function as transcription factors and many are thought to control differentiation of specific cell types (60). The second class of LIM proteins are known as LIM only proteins and are not associated with a homeodomain. Members of this group have one, two or three LIM domains and are found either in the nucleus where they are thought to participate in transcription or in the cytoplasm where they mediate other functions. In addition, a LIM kinase protein has been identified (64). Thus, the LIM domain is a protein module present in many classes of proteins.

Work from several laboratories suggests that the LIM domain mediates protein-protein interactions. Both the cytoplasmic and nuclear LIM only proteins have been shown to bind distinct protein partners. The LIM only protein, zyxin, which contains three LIM domains, has been shown to bind another LIM protein, called cysteine-rich protein (CRP) (65). Additionally, CRP homodimerizes in a

LIM dependent fashion (66). The LIM protein Enigma recognizes tyrosine containing tight turns of the glial cell line derived neurotrophic factor (GDNF) and insulin receptors (67). The muscle LIM protein (MLP) has been shown to bind the actin cytoskeleton (68, 69). The oncogenic nuclear protein LMO binds the basic helix loop helix (bHLH) transcription factor TAL1 in a multi-protein complex which mediates transcription (70). There is also evidence that members of the LIM homeodomain family can bind other classes of transcription factors. Lmx-1 has been shown to bind the bHLH factor, E47 (71, 72). Additionally, Lhx3 can bind the POU homeodomain factor Pit1 (3) as well as the homeodomain or carboxy-terminal region of the LIM factor, Isl1 (73).

Recently, a LIM binding protein was identified independently in several laboratories, which bound to both members of the LIM homeodomain protein family and the nuclear LIM only proteins (LMO). This LIM binding protein has been termed nuclear LIM interactor (NLI) (74), LIM binding factor (Ldb1) (75) and cofactor of LIM domain proteins (CLIM) (47). In addition, NLI has been shown to heterodimerize and may mediate homo- and heterodimerization of LIM domain transcription factors (73, 76). Specifically, crosslinking studies suggest that two molecules of the LIM factor, Lmx1, bind an insulin promoter element together with two molecules of the cofactor NLI (77).

The LIM domain and transcription.

The biological importance of LIM transcription factors in development and tissue specific expression is clear but the transcriptional mechanisms are not as

well understood (62, 63, 78). Moreover, while it is clear that the LIM domain mediates protein-protein interactions the specific function of the LIM domain in transcription is less clear. There is some evidence that suggests that the LIM domain functions to inhibit DNA binding of the associated homeodomain. Sánchez-García *et al.* found that LIM domains of Isl-1 inhibit DNA binding (79). Similar observations have been made for *Xenopus* XLim-1 (80, 81). There is also evidence that the LIM domain of MEC-3 inhibits DNA binding (82). In contrast full-length salmon Isl-2, containing both the LIM and homeodomain can bind DNA (83). Jurata and Gill also reported that the apparent affinity of Lmx1 for the FLAT DNA element on the insulin mini-enhancer was not increased by deletion of the LIM domains (77). These apparent discrepancies may be due to specific factor and DNA binding site requirements.

LIM domain transcription factors have been shown to mediate additive or synergistic activation with other transcription factor family members. Lhx3 has been shown to bind the POU homeodomain factor Pit1 and synergistically activated Pit1, thyroid stimulating hormone-β, and prolactin promoter reporter genes in a LIM dependent manner, as deletion of the LIM domain resulted in the loss of synergy (3). Similarly, in *C. elegans* the LIM factor mec-3 and unc-86 showed synergistic activation of transcription (84). Expression of Isl1 and the cyclic-AMP response element binding protein (CREB) also displayed greater than additive activation of the somatostatin gene (85-87). The LIM homeodomain factor, Lmx1 also displayed marked synergy when expressed with the bHLH factor E47 on the rat insulin mini-enhancer (71). In addition, synergy

was dependent on both the amino and carboxy terminal LIM domains of Lmx1, although the second LIM domain of Lmx1 was sufficient for E47 binding (72). From the above studies it seems likely that in general, LIM transcription factors function in combination with other factors that are bound to adjacent or distant promoter elements. However, the mechanisms by which synergy occurs is not well understood and may involve a change in DNA binding affinity, recruitment of co-activators to the promoter, or chromatin remolding and modification.

The LIM domain has also been proposed to function in a negative role in transcriptional regulation. Several LIM deletion and mutant studies in both *Xenopus laevis* expression systems and mammalian reporter gene assays suggest that the LIM domain negatively modulates a potential transactivation domain in the carboxy-terminal portion of the protein. Analysis of LIM homeodomain factor, Xlim1, in *Xenopus* development, has revealed a secondary axis formation defect when LIM deletion mutant constructs are injected into frog embryos (75). However, the wild type factor fails to induce any apparent defects. In addition, expression of the LIM deletion mutant, but not the full length protein, also upregulated the expression of several putative target genes (81). Similarly, it has been reported that deletion of the LIM domain of Lhx3 enhances transcriptional activation by this factor in heterologous cells (47). However, others have reported greater activity with full length Lhx3 than with the LIM deletion mutant in reporter gene assays (88).

Similarly to the LIM domain, the cofactor NLI has also been proposed to regulate transcription in both a positive and negative fashion. Bach *et al.* provide

evidence for the role of NLI in mediating the synergistic activation of the α-subunit glycoprotein gene by Lhx3, and the pituitary homeodomain transcription factor, Ptx (P-OTX) in heterologous cells (47). However, it is not clear that this synergy is dependent on the LIM domain. In contrast, Jurata and Gill have shown that NLI negatively regulates the synergy observed between Lmx-1 and E47 (77). Interestingly, NLI was also shown to bind Ptx1. It is not known if NLI binds E47, a potentially important mechanistic difference in these two responses. Recently, a role for NLI has also been proposed in the inhibitory model of LIM domain function. In this model, NLI is thought to bind the LIM domain and allow the complex to shift to a transcriptionally active state. In support of this model the secondary axis phenotype, observed when the Xlim1 LIM mutant is expressed, can be achieved with wild type Xlim1, if NLI is also co-injected (75). In addition, Breen *et al.* have shown that co-injection of Xlim1 and NLI is necessary to activate the expression of *goosecoid*, *chordin*, *nrp-1*, and *otx2* in Xenopus ectodermal explant experiments and that neither Xlim1 nor NLI alone is sufficient (76). Furthermore, the expression was dependent on both the LIM binding and the dimerization domains of NLI.

In the studies described in the following chapter the function of the LIM domain in transcription was further investigated. Specifically, the contribution of the LIM domains, of either Lhx2 or Lhx3, to the expression of the α-subunit gene in a gonadotroph cell cell line were assayed. In addition, the mechanisms by which α-subunit transcription is regulated by GnRH or MAPK pathway activation

were explored. Finally, the role of a potential LIM domain binding coactivator was investigated.

CHAPTER II

MRG1 BINDS TO THE LIM DOMAIN OF Lhx2 AND MAY FUNCTION AS A COACTIVATOR TO STIMULATE GLYCOPROTOEN HORMONE α-SUBUNIT GENE EXPRESSION

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INTRODUCTION

Members of the LIM homeodomain family of transcription factors have been shown to contribute to the regulated expression of the α-subunit of the glycoprotein family of hormones (2). The ability of the pituitary to secrete the gonadotropic hormones, follicle stimulating hormone and luteinizing hormone is crucial for normal reproductive function. The synthesis and secretion of the gonadotropins is regulated by the hypothalamic hormone, gonadotropin releasing hormone (GnRH), which acts to increase gonadotropin subunit mRNA levels (20, 21, 89-94) through effects at the transcriptional level (95, 96).

LIM homeodomain factors appear to play a role in both basal and GnRH-stimulated expression of the glycoprotein hormone α-subunit gene (2, 25). Initial studies demonstrated that LIM homeodomain factor-2 (Lhx2, also designated LH-2) can bind to a pituitary-specific, enhancer element designated the PGBE of the mouse α-subunit gene (2). As both the PBGE and a separate, structurally distinct DNA element designated the GnRH-RE are required for GnRH-responsiveness of the mouse glycoprotein hormone α-subunit promoter (25), the finding that Lhx2 binds to the PGBE implies that this LIM factor plays a role in transcriptional responses to GnRH. It has also been shown that a related LIM factor, Lhx3 (also designated pLIM or LIM3) can also enhance α-subunit gene expression (3). Targeted disruption of the Lhx3 gene in mouse results in loss of pituitary organogenesis (8) demonstrating that LIM factors also play an important developmental role in the formation of the pituitary.

The specific role that the LIM domains play in transcriptional activation is somewhat unclear. The LIM domain, named for the genes of the first three members of the family, lin-11 (56), Isl-1 (57) and mec-3 (58), is characterized by the presence of two zinc finger motifs which involve cysteine and histidine or aspartate residues which tetrahedrally coordinate a zinc atom (59, 97). There is evidence that some LIM domains can inhibit DNA binding of the associated homeodomain (79-81). This would suggest that the LIM domain may negatively regulate LIM factor activity. However, it is not clear that inhibition of DNA binding is a general phenomenon for LIM factors (83). Functional studies of Xlim-1 in *Xenopus laevis* have shown that deletion or mutation of the LIM domain of Xlim-1 results in the induction of secondary axis formation while the wild-type factor has no effect (75). This has been interpreted as evidence for a negative role for the LIM domain in regulating transcription. However, in the absence of more mechanistic information about Xlim-1 action, other interpretations are possible. In contrast to the view that LIM domains play a negative role in regulating DNA binding and transcription, some LIM factors have been shown to demonstrate synergistic transcriptional activation with other transcription factors (3, 72).

Recently, a putative co-activator was identified independently in several labs which binds to members of the LIM homeodomain protein family and nuclear LIM only proteins (47, 74, 75). This LIM binding protein has been termed nuclear LIM interactor (NLI), LIM domain binding factor and cofactor of LIM domain proteins. It has been suggested that NLI may have a positive effect on transcription by relieving the inhibitory effects of the LIM domain in the context of the full length

Xlim-1 *in vivo* (76). However, NLI has also been shown to inhibit the synergy between the LIM homeodomain factor Lmx-1 and the basic helix-loop-helix (bHLH) transcription factor, E47 (77). Thus, like the LIM domain itself, it is not yet clear if NLI plays a positive or negative role in mediating or regulating LIM factor function.

In the present studies we sought to further define the function of the LIM domain of Lhx2 in the transcription of the glycoprotein α-subunit gene. We have shown the LIM domain of Lhx2 is sufficient to activate transcription when directed to the PGBE of the α-promoter. Furthermore, we have identified a LIM interacting transcriptional activator, MRG1 which is capable of mediating enhanced transcription of the α-promoter.

MATERIALS AND METHODS

Reporter Genes and expression constructs.

Luciferase reporter genes containing the –507 to +46 region of the mouse glycoprotein hormone α-subunit gene or the -507 to -205 region linked to a minimal promoter have been described previously (25, 38). The PGBE and GnRH-RE mutant α-subunit luciferase reporter genes were constructed by subcloning double stranded oligonucleotides containing the GAL4 binding site, GGAAGACTCTCCTCCG, into the *NotI* restriction site of the previously described block PGBE and GnRH-RE mutant α-subunit promoter constructs (38). The 5X GAL4 binding site-luciferase reporter and the GAL4-Elk1 expression constructs

have been described previously (27). The mutant GAL4-Elk1 S383A expression vector was constructed by oligonucleotide directed mutagenesis using standard techniques to remove a major MAPK phosphorylation site (98). To prepare GAL4-LIM domain and GAL4-NLI expression constructs, the appropriate coding regions were isolated by polymerase chain amplification and subcloned into the pcDNA3 vector (Invitrogen) containing the GAL4(1-147) DNA binding domain downstream of the cytomegalovirus promoter (99). For construction of an MRG1 expression vector, the complete coding sequence for MRG1 was isolated by polymerase chain reaction amplification from αT3-1 cell cDNA using primers based on the known sequence (100) and the coding sequence was cloned into pcDNA3. The Lhx2 LIM and MRG1 maltose binding protein (MBP) fusion constructs were generated by subcloning appropriate fragments into the bacterial expression vector pMAL-c2 (New England Biolabs).

Cell culture and transfections.

αT3-1 and NS20Y cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL). For transient transfection assays cells were plated in 6-well plates, 24 hours prior to transfection and then treated with a suspension of 5-10 μL lipofectamine reagent (Gibco-BRL) and 1 to 3 μg total DNA in 1 mL serum free OPTI-MEMI (Gibco-BRL), according to the manufacturer's recommendations. After a 12-14 hour incubation, an equal volume of DMEM containing 20% fetal bovine serum was added to the transfected cells. Six hours later the cells were

lysed and assayed for luciferase activity (101). To assess transfection efficiency, cells were transfected with a CMV- β-galactosidase reporter gene (102) and β-galactosidase activity was determined and used to normalize luciferase light units.

Yeast two-hybrid screen.

To prepare a VP16- αT3-1 cDNA fusion library, duplex cDNA was prepared using RNA prepared from αT3-1 cells and reagents from Pharmacia following the manufacturer's recommendations. The cDNA termini were modified by ligation to an adapter containing a *NotI* restriction site and then the cDNA was amplified by the polymerase chain reaction. The amplified cDNA was digested with *NotI* and subcloned into the *NotI* site downstream of the VP16 activation domain in the yeast two-hybrid library vector, VP16 (103). A modified bait vector, BTMYeA, (Phyllis Goldman, unpublished data) which allows expression of both a LexA fusion and a second protein was used for the two-hybrid screen. The LIM domain of Lhx2 (residues 42-182) was isolated by the polymerase chain reaction and subcloned into *EcoRI* and *BamHI* sites which are downstream of the LexA DNA binding domain of BTMYeA. NLI was cloned downstream of the ADH promoter of the BTMYeA. The strategy of expression of both the LexA-Lhx2 LIM fusion protein and NLI was based on the possible identification of factors which interact with the LIM-NLI complex. However, subsequent analysis of the factors which were isolated in the screen demonstrated that none of the LIM-binding factors were dependent on NLI for binding. The bait and cDNA library plasmids

were transformed into the L40 yeast strain (MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2:: $(lexAop)_4$-His3 URA3::$(lexAop)_8$-lacZ GAL4 gal80) (103) and selected for positive, interacting clones using histidine minus media in the presence of 30 mM 3-aminotriazole (3-AT) to suppress the background activity of the bait. Further selection was performed on histidine minus media containing 50 mM 3-AT.

Immunoprecipitation and Immunoblot Analysis.

For immunoprecipitation studies, αT3-1 cells were cultured in 150 mm^2 plates and transfected using lipofectamine with either pcDNA3 or pcDNA3 directing the expression of FLAG epitope-tagged Lhx2 as described above. 48 hours after transfection the cells were collected and nuclei isolated and extracted as previously described (2). Nuclear extracts were incubated with M2 FLAG antibody immobilized on agarose beads (Kodak) overnight at 4° C and subsequently washed 5 times in 1 mL each of 50 mM Tris pH 7.8, 150 mM NaCl, and 0.5% NP-40 (Sigma). The immunoprecipitates were resolved on a denaturing 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using a semi-dry transfer apparatus following the manufacturer's instructions (Biorad). Lhx2 and Lhx3 were detected using polyclonal antibodies. The Lhx2 antibody was prepared by immunizing rabbits with a glutathione S transferase fusion to a fragment of the coding sequence of mouse Lhx2. The antibody to mouse Lhx3 was a generous gift of Dr. S. Pfaff (104). MRG1 was detected using a polyclonal antibody to MRG1 at a dilution of 1:1000, a generous

gift of T. Shioda. GAL4 fusion proteins were detected using monoclonal antibodies (Santa Cruz). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies (Sigma), followed by detection with an enhanced chemiluminescence reagent (Dupont-New England Nuclear).

In vitro binding assays.

MBP fusion proteins were expressed in *E. coli* grown at 30 °C and immobilized on amylose resin as described (105). *In vitro* binding reactions were performed in 10 mM HEPES pH 7.4, 150 mM NaCl, and 0.1 % Tween-20. Radiolabeled proteins for analysis of binding were generated by *in vitro* transcription using bacteriophage SP6 or T7 RNA polymerase (106) and translation in a reticulocyte lysate in the presence of [^{35}S]methionine using reagents from Promega. FLAG-epitope tagged p300 was expressed and purified from baculovirus. After incubation of the binding reactions for 2 hours at 4° C, the resin was washed 5 x 1 ml in the binding buffer. Bound proteins were resolved on 10% polyacrylamide denaturing gels and visualized by autoradiography or by Western blotting using the anti FLAG monoclonal antibody as described above.

RESULTS

Lhx2 and Lhx3 are both present in the αT3-1 gonadotrope-derived cell line and both LIM factors can activate the α-subunit promoter.

Previous studies from this laboratory have provided evidence that Lhx2 can bind to the PGBE of the α-subunit gene and stimulate α-subunit promoter activity (2). The Lhx2 cDNA was isolated from a library prepared from the gonadotrope derived, αT3-1 cell line (18), and this cell line was confirmed to contain Lhx2 mRNA (2). More recently it has been shown that αT3-1 cells also contain Lhx3 mRNA and that Lhx3 can also activate the α-subunit promoter (3). These separate studies raise the possibility that both Lhx2 and Lhx3 may contribute to activation of the α-subunit gene. To further explore this possibility we examined the expression of Lhx2 and Lhx3 in αT3-1 cells at the protein level. Immunoblots demonstrated that both Lhx2 and Lhx3 are indeed present in extracts of αT3-1 cells (Fig. 4A). The ability of these LIM factors to activate the α-subunit promoter in heterologous cells was then compared (Fig. 4B). Expression vectors for Lhx2 and Lhx3 were both able to stimulate expression of an α-subunit reporter gene. Interestingly, deletion of the LIM domain of Lhx2 eliminated activation of the α-subunit reporter gene. Although the role that the LIM domain plays in transcriptional activation has been somewhat controversial, these findings provide evidence that within this specific context, the LIM domain of Lhx2 is

necessary for transcriptional activation. The finding that both Lhx2 and Lhx3 are present in αT3-1 cells and that both factors can activate the α-subunit promoter provides evidence that both Lhx2 and Lhx3 may play a role in α-subunit regulation in this cell line. In the present studies we have focused our attention on further characterization of Lhx2. However, it should noted that both Lhx2 and Lhx3 appear to contribute to transcription of the α-subunit gene.

The LIM domain of Lhx2 contains a transcriptional activation domain.

The preceding experiments offered evidence that the LIM domain of Lhx2 may contain a transcriptional activation domain. To directly evaluate the transcriptional activation potential of the LIM domain of Lhx2, we constructed expression vectors in which the yeast GAL4 DNA binding domain was fused to the LIM domain of Lhx2. The activity of the GAL4-LIM fusion constructs was assessed by transfection of αT3-1 cells. Transcription of the α-subunit gene in these cells is stimulated by GnRH (25) through a pathway which involves activation of the mitogen activated protein kinase (27). In the present studies we have used an expression vector for constitutively active ras (107) to activate MAPK and stimulate α-subunit transcription. In the initial studies, a reporter gene containing 5 copies of a GAL4 binding site upstream of a minimal promoter linked to luciferase was used to assess the ability of the GAL4-Lhx2 LIM construct to activate transcription (Fig. 5A). We found that the GAL4-Lhx2 LIM construct stimulated reporter gene expression and that reporter gene activity was not further stimulated by ras (Fig. 5B). Similar results were obtained using a

GAL4-Lhx3 LIM construct . Thus, when tested with this simple reporter gene, the Lhx2 LIM domain appears to contain a transcriptional activation domain that is not responsive to the MAPK pathway. To examine the specificity of activation, we prepared a GAL4 fusion with muscle LIM protein (MLP). MLP has been shown to bind to the actin cytoskeleton (68, 69) and enhance myogenesis (68, 108). The GAL4-MLP construct had very little effect on reporter gene activity. Thus, transcriptional activation is a property of the LIM domain of the nuclear transcription factor, Lhx2, which is not shared with the LIM domain of MLP. It is likely that structural differences in these LIM domains leads to unique protein-protein interactions mediating different responses. To determine whether the transcription stimulating activity of the Lhx2 LIM domain was dependent on the intact structure of the LIM domain, cysteine residues 52 and 55 which are involved in zinc binding (59, 97) were mutated to alanine. This mutation which presumably disrupts the structure of the LIM domain was found to substantially decrease reporter gene activity. All of the GAL4-fusion proteins were expressed at comparable levels (Fig. 5C).

We also assessed the ability of the LIM domains of Lhx2 to activate transcription in the context of the α-subunit promoter. For these studies the wild type PGBE element of α-subunit promoter was replaced with one copy of a GAL4 binding site (Fig. 5D). This allowed GAL4-LIM fusion proteins to be directed to the mutated PGBE in transient transfection studies. As described previously (27), expression of the wild-type α-subunit reporter gene was induced several fold by activated ras (Fig. 5E). Consistent with previous studies (2),

replacement of the PGBE with a GAL4 binding site reduced basal expression of the α-subunit reporter gene and also reduced the ability of activated ras to stimulate reporter gene expression in the absence of the GAL4-Lhx2 LIM domain construct . Neither the GAL4 DNA binding domain alone, nor the GAL4-MLP fusion were able to activate basal expression or support a ras response (Fig. 5F). In contrast, the GAL4-Lhx2 LIM fusion construct increased basal reporter gene activity and also supported a ras response which is similar in magnitude to that obtained with the wild type α-subunit construct (compare Fig. 5E and 5F). The ability of the GAL4-Lhx2 LIM construct to permit a ras response with the α-subunit reporter is in contrast to failure of this same construct to support a ras response with the simple 5xGAL4 reporter (Fig. 5B). This observation suggests that within the context of the α-subunit gene, the Lhx2 LIM domain cooperates with other factors to enhance the response to ras and activation of MAPK. The activity of the GAL4-Lhx2 LIM construct was dependent on an intact LIM structure as mutation of crucial cysteine residues within the one zinc finger greatly reduced both basal and ras-stimulated reporter gene activity. We also compared the ability of the LIM domain of Lhx2 and Lhx3 to activate the modified α-subunit reporter gene (Fig 5G). Expression of GAL4-LIM domain fusions of both Lhx2 and Lhx3 allowed ras-induced activation of the α-subunit reporter gene (Fig 5G).

As both cytoplasmic and nuclear LIM domains have been shown to function as protein-protein interaction domains (63, 78) it seems likely that the LIM domain of Lhx2 functions as a transcriptional activation domain through

recruiting other factors to the α-subunit gene. As an initial characterization of this possibility, we sought to determine if the Lhx2 LIM domain could inhibit α-subunit expression (Fig. 6). For these studies, the wild type α-subunit reporter gene was co-transfected with the GAL4-LIM constructs. As the wild type α-subunit reporter gene does not contain GAL4-binding sites, we anticipated that any effects of the GAL4 fusion proteins would be indirect through sequestration of specific proteins. Transfection of GAL4-Lhx2 LIM domain vector substantially inhibited ras-induced expression of the α-subunit reporter in a concentration-dependent manner (Fig. 6A). Comparable effects were seen for the GAL4-Lhx3 LIM construct but not the Lhx2 LIM mutant . This effect was specific for the α-subunit reporter as the GAL4-Lhx2 LIM construct had little or no effect on the thymidine kinase promoter (Fig. 6B). Similarly, an expression vector for GAL4-MLP had no effect on α-subunit gene expression , again indicating the specificity of this effect.

To further define the structure-function relationship for the Lhx2 LIM domain, the effects of GAL4 fusion constructs containing the individual LIM domains, designated LIM1 and LIM2 were also tested (Fig. 7B.). The second LIM domain (LIM2) was sufficient to permit a response to activated ras, although the total activity is considerably reduced as compared to the activity of the intact LIM domain. Both LIM domains were expressed at comparable levels (Fig. 7C). This result is consistent with reports suggesting that one LIM domain may be

sufficient for interaction with a binding partner while the other LIM domain may function to potentiate binding (69, 72).

Direction of GAL4-NLI to the α-subunit gene does not mimic the effects of the GAL4-Lhx2 LIM domain.

Recent studies have identified NLI as a putative co-activator which binds to the LIM domain of both LIM homeodomain transcription factors and LIM-only nuclear factors (70, 73-77). While it is clear that NLI binds to a group of nuclear LIM factors, the functional role of NLI has not yet been fully explored. It is possible that LIM-dependent recruitment of NLI to a promoter directly leads to transcriptional activation. To test this possibility a GAL4-NLI fusion vector was co-transfected with the mutant α-subunit reporter in which the PGBE was replaced with a GAL4 binding site (Fig. 8A). GAL4-NLI had little effect on basal reporter gene activity and did not support a ras response (Fig. 8B). GAL4-NLI also did not increase the activity of the simple 5xGAL4 reporter gene consistent with the observation that NLI does not function as a transcriptional activator in yeast (76). Thus, direction of NLI to the PGBE of the α-subunit gene is not sufficient to activate transcription. This finding implies that the function of the LIM domain is not limited to the recruitment of NLI to the promoter. Of course, it remains possible that binding of NLI contributes to the transcriptional activity of the LIM domain.

The Lhx2 LIM domain functionally cooperates with an Ets transactivation domain.

The preceding experiments provide evidence that binding of Lhx2 to the PBGE site contributes to basal and ras-stimulated transcription of the α-subunit gene. Previous studies have demonstrated that a different DNA element, the GnRH-RE, also contributes to GnRH-stimulated and presumably ras-stimulated activation of the α-subunit gene (25). The endogenous factor which binds to the GnRH-RE has not been determined but the element does contain a core binding site for the Ets family of transcription factors. A role for an Ets factor in mediating responses to GnRH and ras/MAPK activation is consistent with the known ability of several Ets factors to be phosphorylated and activated by the mitogen activated protein kinase (98, 109, 110). Therefore, we sought to determine whether binding of an Ets transcription factor at the GnRH-RE site is capable of supporting ras-stimulated transcription. For these studies an α-subunit reporter gene was constructed in which the GnRH-RE sequence was replaced with a GAL4 binding site in the context of a wild type PGBE sequence (Fig. 9A). GAL4 fusion genes were constructed with the transactivation domains of Elk1 or a mutant Elk1 in which a crucial MAPK phosphorylation site at Serine-383 was mutated to alanine (98). As expected (25), replacement of the GnRH-RE with a GAL4 binding site eliminated the ability of the α-subunit gene reporter to respond to activated ras (Fig. 9C). Transfection of a GAL4-Elk1 fusion vector was able to restore ras-activated reporter gene activity while the MAPK phosphorylation mutant, Elk1-S383A, was unable to restore the ras response. Similar results

were obtained for GAL4 fusions containing the transactivation domains of two other MAPK-responsive Ets factors, Ets1 (109, 111) and Net (112) . Of course this experiment does not identify the endogenous GnRH-RE binding factor. However, the findings demonstrate that when directed to a site corresponding to the GnRH-RE, Ets factors are capable of contributing to ras responsiveness of the α-subunit gene presumably through a mechanism involving functional cooperation with a LIM factor binding to the PGBE.

To further investigate the functional cooperation of Lhx2 and Elk1, a simple reporter gene was constructed which contained a multimer of the binding site for the bacterial repressor LexA adjacent to a GAL4 binding site. This construct permits analysis of the combined effect of the Elk-1 and LIM domain fusion proteins on a single promoter (Fig. 9D). The GAL4 and LexA DNA binding domains alone have little or no transcriptional activity when co-transfected with this reporter. When the GAL4-Elk1 or LexA-Lhx2 LIM fusion constructs were transfected separately, there was little if any increase in either basal or ras-stimulated activity. It should be noted that when GAL4-Elk1 is tested on a reporter gene containing 5 copies of a GAL4 binding site, the reporter gene is ras/MAPK responsive (98). It is not clear if the lack of ras responsiveness with the GAL4-Elk1 construct and this reporter is due to the decreased number of binding sites or the different arrangement of the binding sites. Nonetheless, when both GAL4-Elk1 and LexA-Lhx2 LIM were expressed, a substantial synergistic response was observed. This activation was not observed when the GAL4-Elk1-S383A MAPK phosphorylation mutant was co-expressed with LexA-

Lhx2 LIM. Thus when the Lhx2 LIM domain and the activation domain of Elk1 are directed to this reporter gene the response mimics that of the wild type α-subunit reporter. Again, these findings are consistent with a model in which transcriptional responses of the α-subunit gene to ras/MAPK activation involves the functional cooperation of Lhx2 and Elk1 or another Ets transcription factor.

Identification of a factor which interacts with the Lhx2 LIM domain.

In order to identify proteins which may be involved in mediating transcriptional responses to the Lhx2 LIM domain, the yeast two-hybrid assay (103, 113-115) was used to screen for Lhx2 LIM domain-interacting proteins. Approximately 12 million clones representing the αT3-1 fusion cDNA library were screened for factors which can interact with the LIM-domain of Lhx2. Among the positive, LIM-interacting factors, NLI was detected several times, consistent with previous reports (47, 74, 75). The detection of NLI served as a positive control for the quality of the αT3-1 cDNA fusion library and suggested that appropriate conditions were used for the screen. In addition to NLI, a VP16-fusion cDNA corresponding to melanocyte specific gene related gene 1 (MRG1) amino acids 1-145 (100) was isolated from the screen. MRG1 is widely expressed in both adult tissues and in the later stages of the developing embryo (116). MRG1 and the closely related melanocyte specific gene (MSG1) are 24 and 27 kD nuclear proteins, respectively, which have a highly conserved transcriptional activation domain but lack any known DNA binding domain (100). In addition, MSG1, but

not MRG1, has been shown to interact with the transcription factor Smad4, and may function as a co-activator (117).

The ability of MRG1 to bind to the LIM domain of Lhx2 was tested by several different approaches. An *in vitro* binding assay was used to determine whether the interaction of the Lhx2 LIM domain and MRG1 was direct. This approach provided evidence that *in vitro*, an MBP-Lhx2 LIM domain fusion protein was able to bind to full-length MRG1 and NLI, but not CREB (Fig. 10). The ability of Lhx2 and MRG1 to associate *in vivo* was examined by a co-immunoprecipitation experiment. An expression vector for FLAG-epitope tagged Lhx2 was transfected into αT3-1 cells and the tagged Lhx2 was isolated from nuclear extracts by immunoprecipitation. Subsequent immunoblot analysis with anti-MRG1 antibodies (100) demonstrated that endogenous MRG1 was associated with the tagged Lhx2 (Fig. 11A). MRG1 was not immunoprecipitated with the FLAG antibody in cells which were not transfected with the FLAG-Lhx2 construct. Thus, the immunoprecipitation experiments provide evidence that Lhx2 and MRG1 can associate *in vivo*. As it is possible that the association of Lhx2 and MRG1 could have occurred after disruption of the cells, we also tested for *in vivo* interaction using a mammalian two-hybrid assay. GAL4 fusions with the Lhx2-LIM domain or the Lhx3-LIM domain were activated by the VP16 fusion protein containing MRG amino acids 1-145 (Fig. 11B). A GAL4 fusion with MLP or the POU transcription factor, Pit1, were not activated by the VP16-MRG1. Thus, both co-immunoprecipitation studies and the mammalian two-hybrid assay

provide evidence for a selective *in vivo* interaction of MRG1 and the LIM domain of Lhx2 and Lhx3.

MRG1 may function as a co-activator to stimulate α-subunit glycoprotein gene expression.

Transfection of αT3-1 cells with an expression vector for MRG1 was found to substantially activate the wild type α-subunit reporter construct (Fig. 12A). Importantly, this finding suggests that MRG1 can interact with endogenous factors to enhance α-subunit expression. Mutation of the PGBE to a GAL4 binding site disrupted the ability of MRG to stimulate the reporter gene and MRG-responsiveness was restored by transfection of either a GAL4-Lhx2 or -Lhx3 LIM domain expression vector (Fig. 12B). Thus the ability of MRG to activate the α-subunit reporter gene was dependent on the presence of the LIM domain at the PGBE site. MRG1 also failed to activate a TK-luciferase construct (Fig. 12C) providing additional evidence that the response is specific.

To begin to understand the mechanisms by which MRG1 functions as a potent transcriptional activator we tested the ability of MRG1 to bind to the Ets-1 transcription factor, the TATA binding protein (TBP) and the widely utilized co-activator, CBP/p300 (Fig. 13). We found that immobilized MRG1 bound both TBP and p300 but not Ets-1. The ability of MRG1 to bind to CBP/p300 is consistent with a report which appeared while this manuscript was in preparation demonstrating that an alternatively spliced isoform of MRG1, designated p35srj, binds CBP/p300 both *in vitro* and *in vivo*. (118).

DISCUSSION

These studies provide evidence that the LIM domain of Lhx2 can function as a transcriptional activation domain and enhance both basal and MAPK-pathway stimulated transcription of the α-subunit gene. Interestingly, the LIM domain is a zinc finger structure which forms a highly ordered structure (97, 119). While there are only a few examples where the structures of transcriptional activation domains have been determined, in at least some cases the activation domain may not be highly structured. For instance the transcriptional activation domain of CREB consists largely of random coil and β turns (120) until a specific structure is induced after binding to the co-activator, CBP (121, 122). Presumably the ability of the LIM domain to function as a transcriptional activation domain involves the ability of the zinc finger structure to recruit specific proteins to a promoter.

Several laboratories have demonstrated transcriptional synergy between members of the LIM homeodomain transcription factor family and other transcription factors. Lmx-1 and the basic helix-loop-helix (bHLH) factor, E47, synergistically enhance the activity of the rat insulin mini-enhancer (71, 72). Lhx3 has been shown to bind to the POU factor, Pit-1, and synergistically activate prolactin and thyroid stimulating hormone gene transcription (3). Previous studies from this laboratory have provided evidence that the PBGE of the α-subunit gene, which contains an Lhx2 binding site, synergizes with a

separate DNA element, the GnRH-RE, to support both basal and GnRH-stimulated transcription (2, 25). The present studies extend the understanding of how Lhx2 supports synergistic activation of transcription. Our studies provide evidence that the LIM domain in the context of the α-subunit promoter is able to enhance ras-activated transcription in the absence of other functional domains of Lhx2. These findings are consistent with a model in which the LIM domain contacts other transcription factors or co-activators leading to synergistic activation. We have also demonstrated the ability of the LIM domain to synergize with members of the Ets family of transcription factors to mediate ras-responsiveness. In the context of the α-subunit gene, the LIM domain of Lhx2 is able to enhance MAPK-pathway activated transcription. However, when directed to a simple promoter containing multiple GAL4 binding sites, a GAL4-Lhx2 LIM domain construct was unable to support a ras response. These results imply that the ras response depends on the ability of the Lhx2 LIM domain to functionally cooperate with other factors which are recruited to the α-subunit gene promoter.

Several previous studies have concluded that the LIM domain plays a negative role in modulating transcriptional activation. However, the function of the LIM domain has largely been inferred from analysis of the activity of LIM domain deletion mutants. For instance, expression vectors for full length Xlim-1 have little or no effect on *Xenopus laevis* development, while deletion of the LIM domain results in the formation of a secondary axis (75). Similarly, deletion of the LIM domain of Lhx3 enhances transcriptional activation by this factor in

heterologous cells (47). In contrast, the present study as well as recent findings from another laboratory (88) have demonstrated that deletion of the LIM domain can decrease transcription stimulating activity of LIM homeodomain factors. While the Xenopus studies described above are consistent with a possible negative modulatory role for the LIM domain, other interpretations are possible. If the LIM domain is the major transcriptional activation domain, then deletion of the LIM domain could create an inactive transcription factor. Displacement of a wild type, active LIM homeodomain factor by the inactive LIM-deleted factor would then inhibit transcription. Deletion of the LIM domain of Xlim-1 may then cause formation of a secondary axis through inhibition of a LIM factor-dependent target gene. However, it has been demonstrated that Xlim-1 contains a carboxy-terminal transcriptional activation domain (76) distinct from the LIM domain and therefore it is not clear that deletion of the LIM domain would create a dominant negative form of Xlim-1. In any case, the present studies provide evidence that the LIM domain of Lhx2 can function as a transcriptional activation domain and this function should be considered in evaluating the activity of LIM homeodomain deletion mutants.

The ability of the LIM domain to function as a transcriptional activation domain likely involves the ability of this structure to recruit transcription factors or co-activators. Several laboratories have identified NLI as a LIM-binding, putative co-activator (47, 74, 75). As with the functional properties of the LIM domain itself, the role of NLI has not been clearly established. The results of several studies suggest that NLI acts to stimulate transcription (47, 76). In contrast, NLI

has been shown to inhibit the synergy between Lmx-1 and E47 (77). Moreover, recent genetic studies of apterous in *Drosophila* suggest that the relative stoichiometry of LIM transcription factors and Chip, the *Drosphila* ortholog of NLI (123), is critical for proper function (78, 124, 125). While the present studies do not resolve this issue, the results provide evidence that recruitment of NLI is not sufficient for transcriptional activation. Forced recruitment of GAL4-NLI to a mutant α-subunit reporter in which the PGBE was replaced with a GAL4 site was not sufficient for transcriptional activation. If NLI functions as a co-activator, its mechanism of action is probably substantially different than the well studied co-activator, CBP, which contains several transcriptional activation domains (126-128).

We have used a yeast two-hybrid screen to search for LIM-interacting factors that might serve as LIM domain co-activators. The screen led to the identification of MRG1 as a LIM-interacting factor. MRG1 was previously isolated based on sequence similarity to the melanocyte specific gene, MSG1 (100). Initially, the function of MRG1 and MSG1 was unknown although both are nuclear proteins containing a conserved transcriptional activation domain and neither contain a known DNA binding domain. Recently, evidence has been obtained that MRG1 and MSG1 may function as co-activators which bind to other transcription factors (117, 118)

MRG1 has a number of properties that are consistent with a possible function as a co-activator for Lhx2. MRG1 binds directly to the LIM domain of Lhx2 as determined by an *in vitro* binding assay. A co-immunoprecipitation assay

provided evidence that endogenous MRG1 interacts with Lhx2. Importantly, MRG1 expression enhanced α-subunit reporter gene activity in a LIM domain-dependent manner. We found that MRG1 is able to bind to the TATA binding protein and p300/CBP. Our findings are consistent with a model in which Lhx2 recruits MRG1 to the α-subunit promoter which enhances recruitment of p300/CBP and the TATA binding protein leading to transcriptional activation. Our findings have similarities and important differences with a report which appeared while this manuscript was in preparation (118). Similar to our findings, it was reported that an alternatively spliced isoform of MRG1, which is termed p35srj, binds p300. However, rather than leading to transcriptional activation, p35srj/MRG1 was able to compete with the hypoxia-inducible factor, HIF-1, for binding to p300 leading to a reduction in HIF-1 activity. Thus, MRG1 and its isoforms may function as a co-activator modulator, capable of mediating p300/CBP recruitment and transcriptional activation in some circumstances or blocking the recruitment of p300/CBP depending on the nature of specific interactions with individual transcription factors.

ACKNOWLEDGMENTS

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Figure 4. Lhx2 and Lhx3 are present in αT3-1 cells and both LIM factors can enhance α-subunit reporter gene activity in heterologous cells. Nuclear extracts from αT3-1 cells were resolved by denaturing gel electrophoresis and transferred to a membrane before immunostaining with antibody to either Lhx2 or Lhx3 as indicated (A). To examine the ability of Lhx2 and Lhx3 to enhance α-subunit promoter activity, NS20Y cells were transfected with 0.2 μg of a reporter construct containing the –507 to +46 region of the mouse α-subunit promoter (B). The cells were also transfected with 0.2μg of either an empty expression vector or expression vector for Lhx2, Lhx3, or the LIM deletion mutant of Lhx2 as indicated. A CMV-β-galactosidase reporter construct , 0.5 μg, was transfected to assess differences in transfection efficiency. Data are reported as the relative luciferase activity from three transfections ± standard error normalized to β-galactosidase activity.

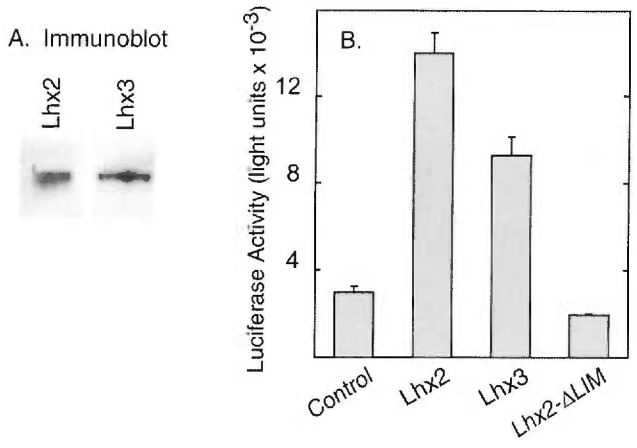

A. Immunoblot
Lhx2
Lhx3
B.
Luciferase Activity (light units x 10-3)
12
8
4
Control
Lhx2
Lhx3
Lhx2-ΔLIM

Figure 5. The LIM domain of Lhx2 contains a transcriptional activation domain. αT3-1 cells were transfected with either 0.2 μg of a reporter construct containing five copies of a GAL4 binding site upstream of the E1b TATA box luciferase reporter (A and B) or an α-subunit reporter gene containing the -507 to -205 region of the mouse glycoprotein hormone α-subunit gene placed upstream of a minimal TATA box linked to luciferase (D and E) or a mutant α-subunit reporter gene in which the PGBE was replaced with a GAL4 binding site (D, F and G). The cells were also transfected with 0.2 μg of either an empty expression vector control or an expression vector for constitutively active ras and 0.2 μg of an expression vector for the GAL4 DNA binding domain alone (GAL4) or a GAL4 DNA binding domain fusion with muscle LIM protein (GAL4-MLP), with Lhx2 or Lhx3 LIM domains (GAL4-Lhx2 LIM or GAL4-Lhx2 LIM) or an Lhx2 LIM domain mutant in which cysteine residues 52 and 55 which interact with a zinc atom were replaced with alanine (GAL4-LIM mut). The cells were also transfected with 0.5 μg of a CMV-β-galactosidase vector to assess differences in transfection efficiency. Data are reported as the relative luciferase activity from three transfections ± standard error normalized to β-galactosidase activity. The relative expression of the GAL4 fusion proteins was assessed by immunoblot analysis of nuclear extracts from αT3-1 cells which were transfected with the indicated constructs (C).

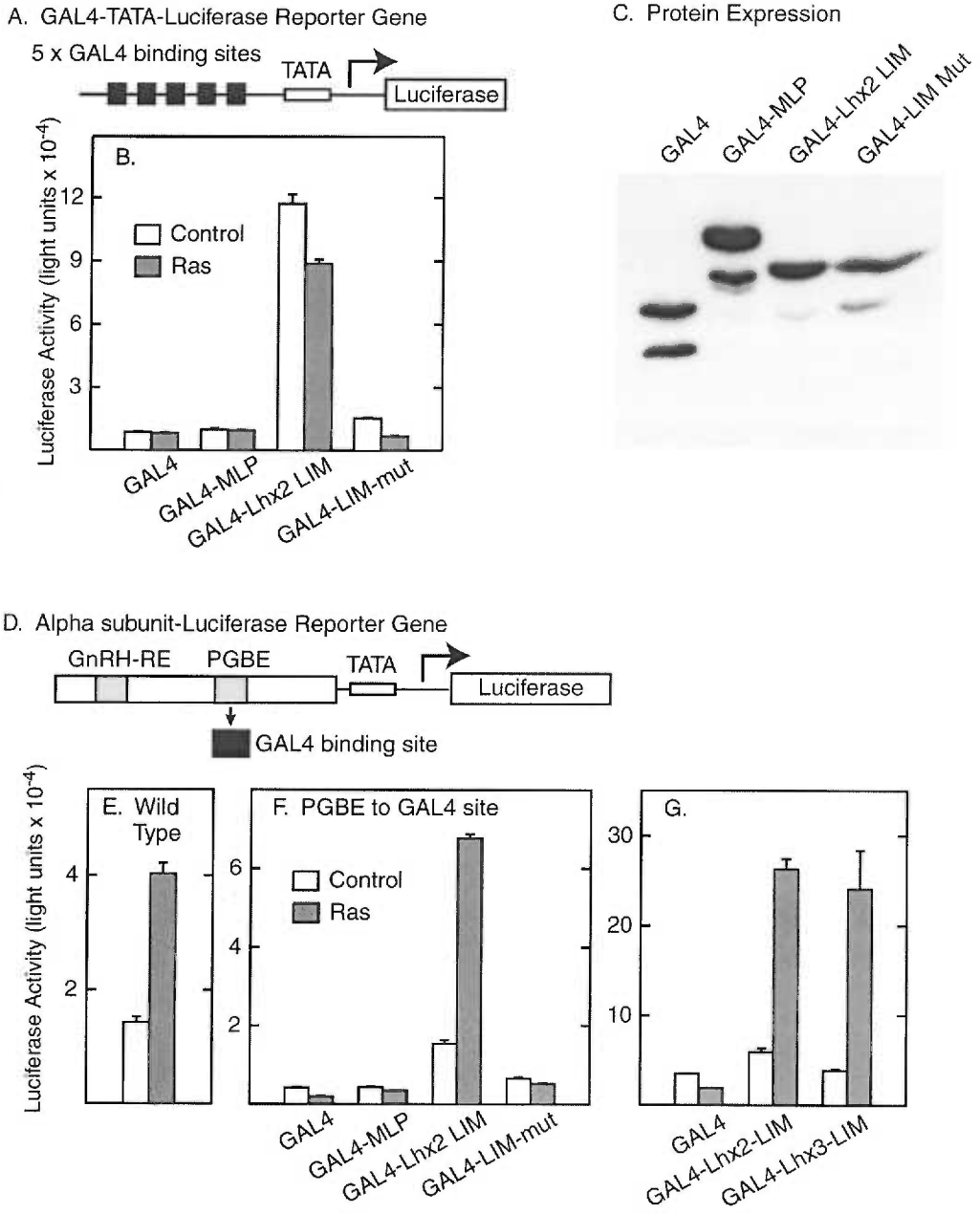

A. GAL4-TATA-Luciferase Reporter Gene
5 x GAL4 binding sites
TATA
Luciferase
B.
Luciferase Activity (light units x 10⁻⁴)
12
9
6
3
Control
Ras
GAL4
GAL4-MLP
GAL4-Lhx2 LIM
GAL4-LIM-mut
C. Protein Expression
GAL4
GAL4-MLP
GAL4-Lhx2 LIM
GAL4-LIM Mut
D. Alpha subunit-Luciferase Reporter Gene
GnRH-RE
PGBE
TATA
Luciferase
GAL4 binding site
E. Wild Type
F. PGBE to GAL4 site
G.
Control
Ras
GAL4
GAL4-MLP
GAL4-Lhx2 LIM
GAL4-LIM-mut
GAL4-Lhx2-LIM
GAL4-Lhx3-LIM

Figure 6. Expression of the LIM domain of Lhx2 inhibits the ability of ras to stimulate α-subunit reporter gene activity in a concentration dependent manner. αT3-1 cells were transfected with 0.2 μg of either the -507 to -205 wild type α-subunit reporter gene (A) or a thymidine kinase luciferase reporter (B) and either 0.2 μg of an empty expression vector control or vector for constitutively active ras and the indicated amount of a GAL4-Lhx2-LIM domain expression vector. Data are reported as relative luciferase activity from three transfections ± standard error corrected for transfection efficiency.

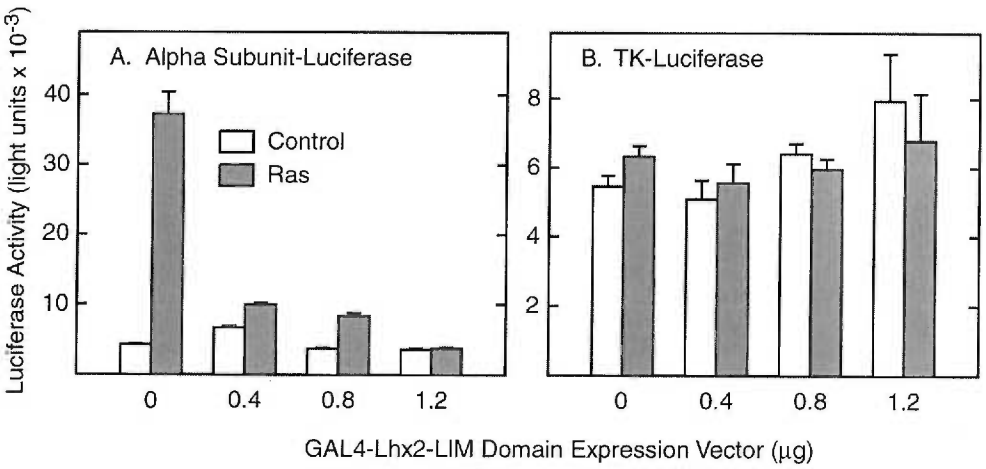

A. Alpha Subunit-Luciferase
B. TK-Luciferase
Control
Ras
Luciferase Activity (light units x 10-3)
40
30
20
10
8
6
4
2
0
0.4
0.8
1.2
GAL4-Lhx2-LIM Domain Expression Vector (μg)

Figure 7. The second LIM subdomain of Lhx2 is sufficient to support ras-mediated enhancement of α-subunit transcription. The wild type α-subunit reporter gene (A) or the PGBE to GAL4 site mutant α-subunit (B) reporter genes (0.2 μg) were transfected into αT3-1 cells with either an empty expression vector control or a vector for constitutively active ras (0.2 μg). The cells also received 0.2 μg of an expression vector for the GAL4 DNA binding domain (GAL4), or fusion of the GAL4 DNA binding domain with the Lhx2 LIM domain (GAL4-Lhx2 LIM) or the first LIM subdomain of Lhx2 (GAL4-LIMa) or the second LIM subdomain of Lhx2 (GAL4-LIMb) as indicated. Data are reported as the relative luciferase activity from three transfections ± standard error corrected for transfection efficiency. The relative expression of the GAL4 fusion proteins was assessed by immunoblot analysis of nuclear extracts from αT3-1 cells which were transfected with the indicated constructs (C).

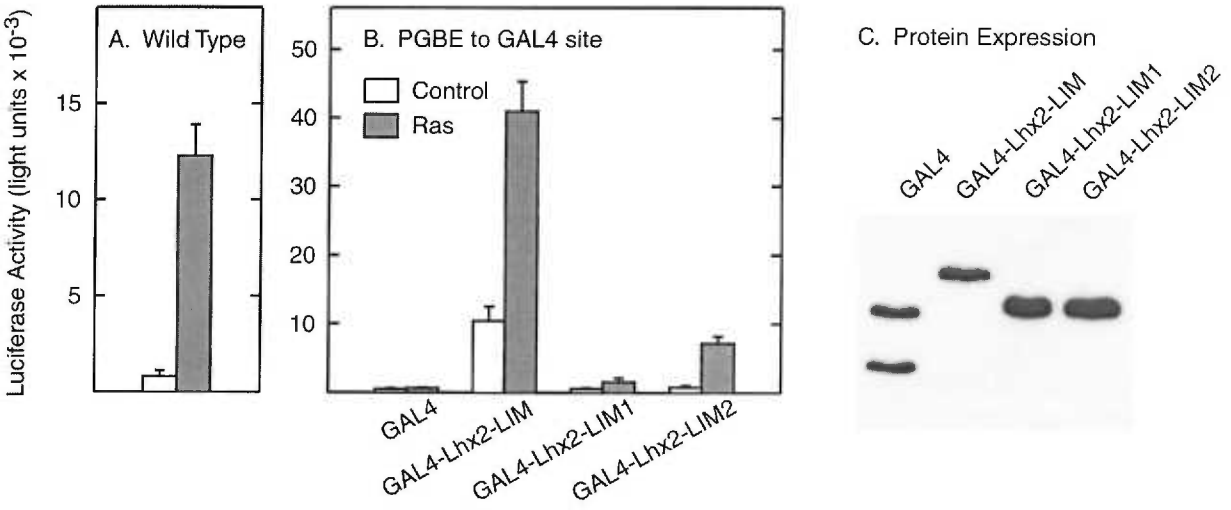

Luciferase Activity (light units x 10-3)
A. Wild Type
15
10
5
B. PGBE to GAL4 site
Control
Ras
50
40
30
20
10
GAL4
GAL4-Lhx2-LIM
GAL4-Lhx2-LIM1
GAL4-Lhx2-LIM2
C. Protein Expression
GAL4
GAL4-Lhx2-LIM
GAL4-Lhx2-LIM1
GAL4-Lhx2-LIM2

Figure 8. Directed binding of the nuclear LIM-interacting protein to the PGBE of the α-subunit gene, does not support a transcriptional response to activated ras. αT3-1 cells were transfected with 0.2 μg of the wild type α-subunit (A) or the PGBE to GAL4 mutant α-subunit (B) luciferase reporter genes and 0.2 μg of either an empty expression vector control or a vector for constitutively active ras and 0.2 μg of an expression vector for the GAL4 DNA binding domain (GAL4) or fusion of the GAL4 DNA binding domain with the Lhx2 LIM domain (GAL4-Lhx2-LIM) or the nuclear LIM-interacting protein coding sequence (GAL4-NLI) as indicated. Data are reported as the relative luciferase activity from three transfections ± standard error corrected for transfection efficiency. The relative expression of the GAL4 fusion proteins was assessed by immunoblot analysis of nuclear extracts from αT3-1 cells which were transfected with the indicated constructs (C).

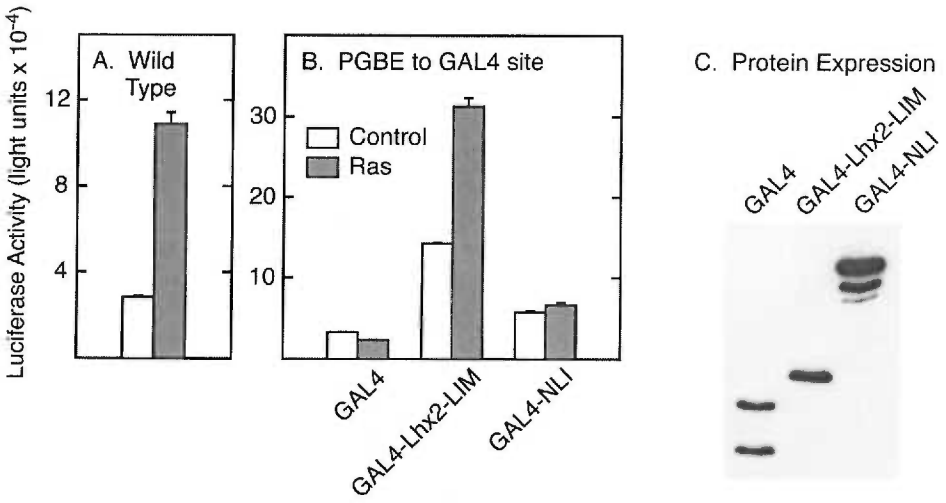

Luciferase Activity (light units x 10^{-4})
A. Wild Type
12
8
4
B. PGBE to GAL4 site
30
20
10
Control
Ras
GAL4
GAL4-Lhx2-LIM
GAL4-NLI
C. Protein Expression
GAL4
GAL4-Lhx2-LIM
GAL4-NLI

Figure 9. The Elk1 carboxy-terminal transcriptional activation domain can contribute to the ras-responsiveness of an α-subunit reporter gene and synergize with the LIM domain of Lhx2 to mediate ras-responsiveness. αT3-1 cells were transfected with 0.2 μg of either the wild type α-subunit reporter gene (A and B) or the GnRH-RE to GAL4 site mutant α-subunit reporter (A and C) or a simple reporter gene containing 3 copies of a composite LexA+GAL4 binding site upstream of minimal promoter linked to luciferase (D and E). The cells were also transfected with 0.2 μg of either an empty expression vector control or a vector for constitutively active ras and with 0.2 μg of an expression vector for the GAL4 DNA binding domain (GAL4) or the LexA DNA binding domain (LEX) or fusion of the GAL4 DNA binding domain with the Elk1 carboxy-terminal transcriptional activation domain (GAL4-Elk) or the Elk1 activation domain in which a crucial phosphorylation site at serine 383 has been mutated to alanine (GAL4-Elk-mut) or a fusion of the LexA DNA binding domain with the Lhx2 LIM domain (LEX-LIM) as indicated. Data are reported as the relative luciferase activity from three transfections ± standard error corrected for transfection efficiency.

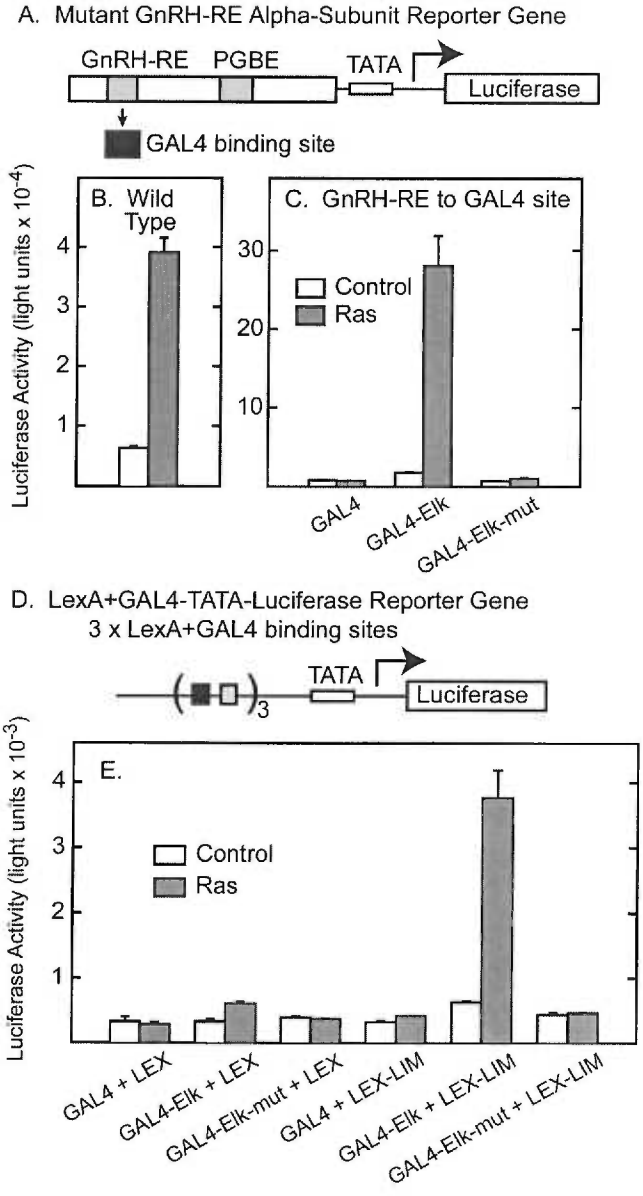

A. Mutant GnRH-RE Alpha-Subunit Reporter Gene
GnRH-RE
PGBE
TATA
Luciferase
GAL4 binding site
B. Wild Type
C. GnRH-RE to GAL4 site
Control
Ras
Luciferase Activity (light units x 10-4)
GAL4
GAL4-Elk
GAL4-Elk-mut
D. LexA+GAL4-TATA-Luciferase Reporter Gene
3 x LexA+GAL4 binding sites
TATA
Luciferase
E.
Control
Ras
Luciferase Activity (light units x 10-3)
GAL4 + LEX
GAL4-Elk + LEX
GAL4-Elk-mut + LEX
GAL4 + LEX-LIM
GAL4-Elk + LEX-LIM
GAL4-Elk-mut + LEX-LIM

Figure 10. Lhx2 can bind to MRG1 *in vitro.* Radiolabeled MRG1 (A), NLI (B), or CREB (C) were prepared by *in vitro* transcription and translation and then assayed for the ability to bind to either the maltose binding protein (MBP) or an MBP-Lhx2 LIM fusion protein immobilized on amylose resin. After binding and subsequent washes the reactions were resolved by denaturing gel electrophoresis and the proteins visualized by autoradiogrphy. For comparison, 15% of the input labeled protein is also shown (input).

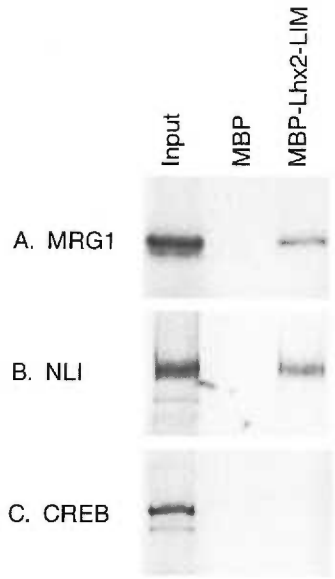
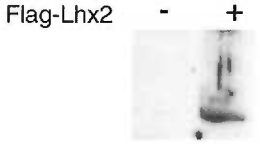

Input
MBP
MBP-Lhx2-LIM
A. MRG1
B. NLI
C. CREB

Figure 11. *In vivo* interaction of Lhx2 and MRG1. A co-immunoprecipitation assay was used as a test for the *in vivo* interaction of the Lhx2 and MRG (A). αT3-1 cells were transfected with an expression vector for FLAG-epitope tagged Lhx2 or an empty expression vector. Nuclear extracts were prepared and incubated with monoclonal M2 FLAG antibody immobilized on agarose beads. After binding and subsequent washes the immunoprecipitates were resolved by denaturing gel electrophoresis and transferred to a membrane before immunostaining with antibody to MRG1. The interaction of MRG1 with LIM domains was also tested using a mammalian two-hybrid assay (B and C). αT3-1 cells were transfected with 0.2 μg of a reporter construct containing five copies of GAL4 binding sites upstream of the E1b TATA luciferase reporter and 0.2 μg of either an expression vector for the transactivation domain of VP16 only or a fusion of VP16 with residues 1-145 of MRG1 (VP16-MRG1) and with 0.2 μg of an expression vector for the indicated GAL4 fusion proteins. Data are reported as relative luciferase activity from three transfections ± standard error corrected for transfection efficiency.

A. Co-Immppt. of MRG with Flag-Lhx2



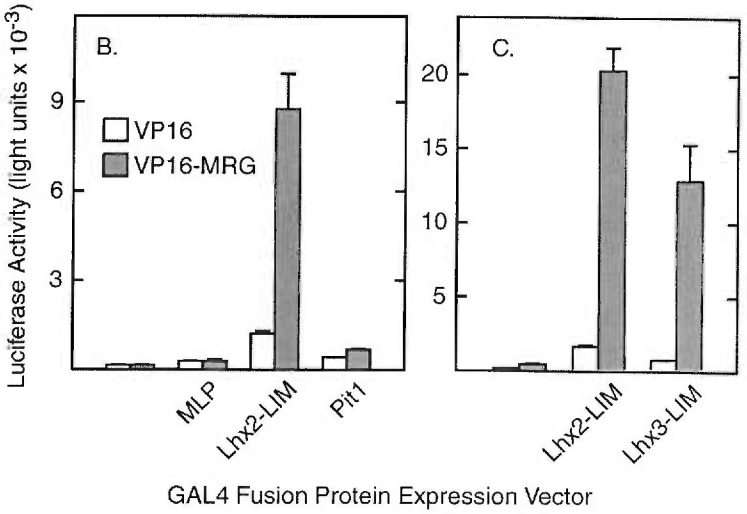


Figure 12. An MRG1 expression vector stimulates expression of an α-subunit reporter gene. The wild type α-subunit luciferase reporter gene (A) or the PGBE to GAL4 site mutant α-subunit reporter gene (B) or a reporter gene containing the herpes simplex virus thymidine kinase reporter (C) were transfected into αT3-1 cells with either 2.0 μg of an empty expression vector control or a vector directing expression of full length MRG1. The cells also received 0.2 μg of an expression vector for GAL4 DNA binding domain (GAL4), or a fusion of the GAL4 DNA binding domain with the Lhx2 LIM domain (GAL4-Lhx2 LIM) or the Lhx3 LIM domain (GAL4-Lhx3-LIM) as indicated. Data are reported as the relative luciferase activity from three transfections ± standard error corrected for transfection efficiency.

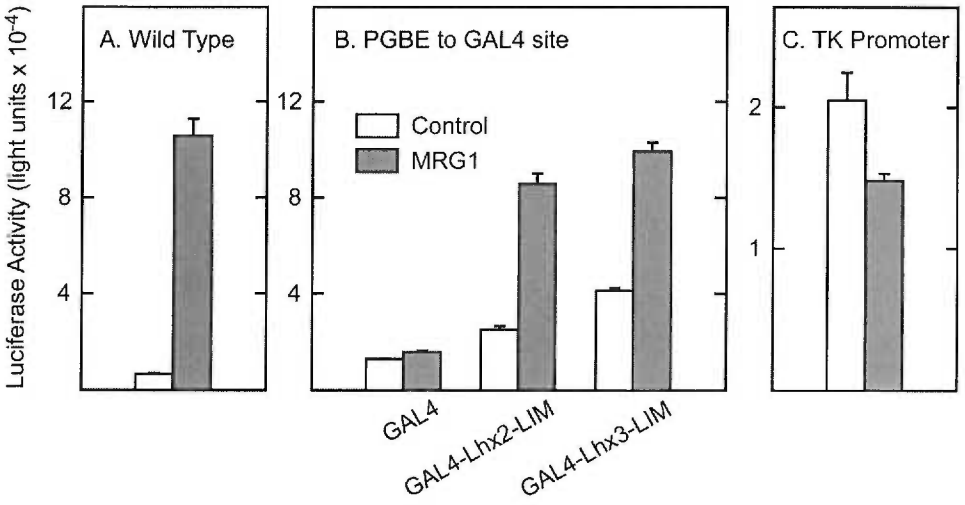
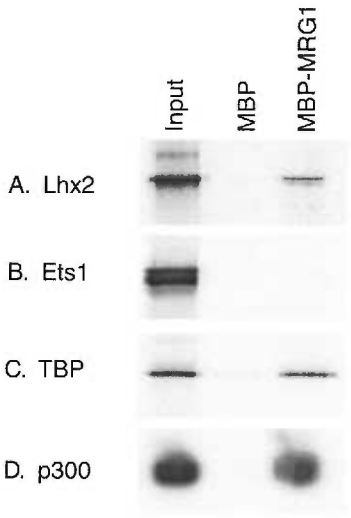

Luciferase Activity (light units x 10⁻⁴)
A. Wild Type
B. PGBE to GAL4 site
C. TK Promoter
Control
MRG1
12
8
4
2
1
GAL4
GAL4-Lhx2-LIM
GAL4-Lhx3-LIM

Figure 13. MRG1 can bind to the TATA binding protein and p300 *in vitro*. MBP or an MBP-MRG1 fusion protein was immobilized on amylose resin and incubated with (A) radiolabeled Lhx2 (A), radiolabeled Ets-1 (B), radiolabeled TBP (C) or flag-epitope-tagged p300 (D). After washing the resin, the bound proteins were analyzed by denaturing gel electrophoresis and detected by autoradiography (panel A, B and C) or immunostaining (panel D). For comparison, 15% of the input was also analyzed (input).


Input
MBP
MBP-MRG1
A. Lhx2
B. Ets1
C. TBP
D. p300

CHAPTER III

SUMMARY AND CONCLUSIONS

It is clear that LIM homeodomain transcription factors are important in both pituitary development (6, 8, 10, 44, 129, 130) and expression of pituitary hormone genes, including the α-subunit of glycoprotein hormones (2, 3, 131). Recently, attention has been focused on the role of the LIM domain in transcription, and several studies have led to the proposal of at least two functional models. In the first, the LIM domain is believed to inhibit the transactivation capability of the full-length protein, through either inter- or intramolecular interactions (62, 76). In the second model the LIM domain is thought to mediate interactions with other transcription factors leading to synergistic activation of transcription (62, 63). It is unclear, at present, which of these two models best represents the action of LIM homeodomain factors. Of course, these models are not necessarily mutually exclusive and may depend on specific transcription factors and promoter context. With this in mind, the overall goal of the work described in this thesis was to further characterize the function of the LIM domain in α-subunit gene expression.

Previous studies of LIM homeodomain transcription factors have depended largely on comparison of the full length factor to that of the LIM deletion mutant. An alternative approach was taken in many of the studies performed in this

thesis, such that constructs containing the LIM domain fused to a heterologous DNA binding protein were used. This allowed the LIM domain to be directed either to a simple reporter gene promoter or to a specific site on the α-subunit promoter. Of course, the results obtained from fusion proteins may not fully reflect the activity of full-length proteins.

Using fusion constructs, the LIM domain of Lhx2 and Lhx3 were shown to be sufficient to allow both basal and MAPK-pathway stimulated transcription when directed to the α-subunit promoter. However, outside the context of the α-subunit promoter, the LIM domain did not a support a MAPK-pathway mediated increase in reporter gene activity, unless the transactivation domain of Elk1 was also directed to the promoter. It is important to note that although the LIM and Elk1 transactivation domains appear to mediate enhanced reporter gene activity in response to ras-activation, the mechanism remains unclear. Specifically, it is not known whether the LIM domain and Elk1 bind directly and or if the synergy is mediated through an indirect mechanism. Future binding experiments may help to clarify this issue. However, further characterization may have to await the identification of the endogenous factor that binds the GnHR-RE *in vivo*. Attempts to use a GnRH-RE DNA probe to identify or clone the endogenous binding factor have been unsuccessful thus far.

Interestingly, the cofactor NLI has been shown to affect the synergy observed between LIM factors and other transcription factors in both a positive and a negative fashion (47, 74, 75). At present the role of NLI in the apparent synergy between LIM homeodomain and Ets factor is unknown. The results do imply that

simple recruitment of NLI to the promoter is not sufficient to mediate MAPK-pathway stimulated α-subunit gene expression. However, we have not directly examined whether NLI binding is necessary for the activation function of the LIM domain. Presumably, endogenous NLI is bound to the LIM domain fusion protein. Additional study will be necessary to resolve this question as well as the larger role of NLI in α-subunit transcription. It may be that the function of NLI will require a different approach than transient transfection and reporter gene assays. For instance, studies in *Drosophila* suggest that NLI may mediate long distance enhancer-promoter interactions (123). It is interesting that the recently identified α-subunit distal enhancer (49-52) is dependent on the proximal promoter PGBE element, and presumably LIM homeodomain factors, for full transcriptional activity. Future experiments may reveal a role for NLI in α-subunit enhancer-promoter communication.

To better understand the transactivation function of the LIM domain, we searched for additional LIM binding partners and identified MRG1 as a potential binding partner for Lhx2. Recently, MSG1, but not MRG1, was shown to enhance reporter gene activity mediated by the transcription factor, smad4 (117). While it is not known if MSG1 binds the LIM domain, it is likely that MRG1 and MSG1 bind distinct transcription factors, but facilitate transcription through similar mechanisms. Comparison of MRG1 and MSG1 revealed two conserved regions, designated conserved region one and two (CR1 and CR2) by Shioda *et al.* (100). While the function of CR1 is unknown, the CR2 region has been shown to be a potent transactivation domain in reporter gene assays. The CR2 amino acid

sequence is acidic and has been predicted to form an amphipathic helix, which is thought to be critical to its transcriptional activation function. However, MRG1 may also inhibit transcription of some p300 dependent genes. Bhattacharya *et al.* reported that a differentially spliced isoform of MRG1, which they termed p35srj, bound the CH1 region of p300 and competitively inhibited the binding of the transcription factor HIF1. Structure-function analysis of the transactivation domain may confirm the importance of the CR2 domain in transcription.

There are several unresolved issues concerning the role of MRG1 in α-subunit gene expression. Perhaps most importantly, it is not known if MRG1 is necessary for α-subunit gene expression. In initial attempts to address this question, MRG1 deletion mutants were tested for function as putative dominant negative constructs. However, these mutants failed to block α-subunt reporter gene activity. It may be possible to use either anti-sense RNA or a specific ribozyme techniques (132, 133) to prevent translation of MRG1 and assay the effects on α-subunit gene expression. In addition, antibody microinjection strategies have been used by others to block the action of specific transcriptional cofactors (134) and could be employed using anti-MRG1 antibodies. Alternatively, targeted disruption (135) of the MRG1 gene in mice could also clarify the role of MRG1 in α-subunit gene expression.

Future studies will also be necessary to determine what role NLI may play in MRG1 mediated transcription. At present is it is not known whether binding of MRG1 and NLI at the LIM domain are mutually exclusive or whether they are both capable of binding the LIM domain as part of a large complex. Attempts to

purify a complex containing Lhx2, NLI and MRG1 using a PGBE DNA probe from αT3-1 nuclear extracts were unsuccessful. However, gel mobility shift assays using purified proteins could be performed in the future in order to answer this question. Interestingly, the LIM only protein, LMO2 has been shown to participate in a large complex including TAL1, E47, GATA-1 and NLI (70).

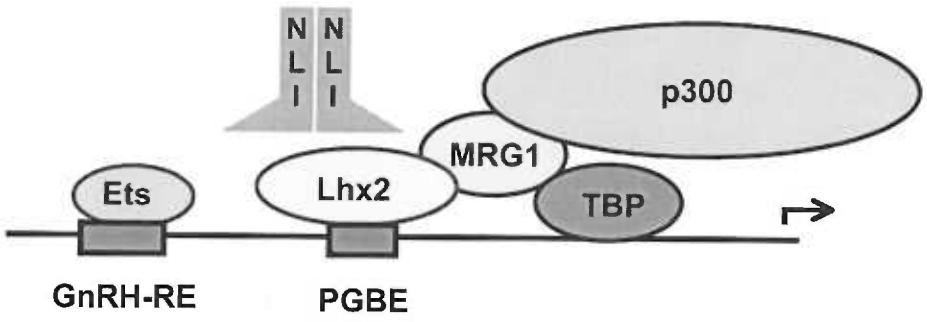
MRG1 expression has been shown to be induced by a number of cytokines and growth factors, suggesting a possible mechanism for transcriptional regulation by extracellular signaling molecules (136). Future studies may reveal a role for GnRH in the regulation of MRG1 expression or function. In particular, it would of interest to know if GnRH treatment affected the expression or stability of either MRG1 mRNA or protein. In addition, MRG1 function might be affected by phosphorylation by MAPK or other kinases. To begin to address this question putative phosphorylation sites in MRG1 could be mutated and tested for altered transcriptional or LIM binding activity.

MRG1 has been shown to bind p300 (118) and TATA binding protein (TBP) *in vitro*. The interaction between specific activators of transcription and components of the basal transcriptional machinery, specifically TBP and TBP-associated factors (TAFs), has been proposed as a general model for gene expression (137-142). The transcriptional cofactors p300 (143) and CBP (126) have been shown to bind many different classes of transcription factors (144). In addition, p300 and CBP posses an intrinsic histone acetyl transferase activity and may promote gene expression through modification of histones (145, 146). Although, additional studies are necessary to establish the functional

significance of MRG1 and both TBP and p300 interactions, the *in vitro* binding results do suggest a mechanism by which MRG1 may enhance transcription. Recently, Bach *et al.* identified a novel LIM domain binding protein, termed Rlim, which was also shown to bind NLI (147). They have shown that Rlim inhibits the activation of several Lhx3 dependent reporter genes in transient transfection assays. Furthermore, they propose that the inhibitory function of Rlim is mediated through recruitment of the Sin3a/histone deacetylase complex to the promoter. These results, taken together with the proposed role of MRG1 and p300, suggest that LIM homeodomain transcription factors could positively or negatively regulate the activity of genes, through recruitment of either histone acetylase or deacetylase complexes.

In summary, the studies presented in this thesis support and extend a model in which the LIM domain, together with other transcription factors and cofactors, plays a positive role in the transcriptional activation of the α-subunit gene. The LIM domain was shown to be a transcriptional activation domain, which can functionally cooperate with a member of the Ets family of transcription factors. In this way, either Lhx2 or Lhx3, bound to the PGBE and an Ets factor bound to the GnRH-RE, form a two component system allowing GnRH or MAPK pathway mediated transcription of the α-subunit gene. In addition, a putative LIM domain binding cofactor, MRG1, was identified and shown to enhance α-subunit reporter gene activity. Finally, MRG1 was found to bind TBP and p300 in *in vitro* binding assays, suggesting a possible model for LIM domain mediated transcriptional transactivation (fig. 14).

Figure 14. A proposed model of α-subunit gene activation.



Future studies

It is clear that additional experiments are needed to further examine the role of LIM homeodomain proteins, Ets factors, NLI and MRG1 in α-subunit gene expression, and several directions are outlined above. One of the first steps towards better understanding the transcriptional function of Lhx2 or Lhx3 would be to further define the binding complex present on the promoter, particularly the role of NLI and MRG1. It is possible that the LIM domain binds either NLI or MRG1 exclusively. Alternatively, they may both bind the LIM domain as part of a larger complex. Preliminary competition experiments, using the α-subunit reporter gene in transient transfection assays, seem to indicate that MRG1 and NLI bind the LIM domain in a mutually exclusive fashion. However, other interpretations of this data are possible, including mislocalization of the LIM factor with increased expression of either MRG1 or NLI. In order to overcome this limitation, gel shift studies using purified Lhx3, NLI and MRG1 and the PGBE

containing α-subunit DNA probe, could be conducted. Purified Lhx3, NLI and MRG1 have been prepared using the baculovirus system of expression. Ideally, the affinity of MRG1 and NLI for the LIM domain could also be determined using these purified proteins. It may also be possible to determine the identity of the complex that forms on the PGBE DNA using αT3-1 nuclear extract and specific antibodies to Lhx2, Lhx3, NLI and MRG1. If these factors are present, the addition of antibody should supershift or disrupt the complex in a specific manner. In addition, mutational analysis of the LIM domain may also reveal the distinct requirements for NLI and MRG1 binding. Several point mutations were made in the LIM domain of Lhx2, however none of the mutations seemed to disrupt NLI or MRG1 binding as assayed in a mammalian two-hybrid assay. It may be that several amino acid residues are necessary for binding and multiple combinations could be tried in the future. In addition, the use of a genetic screen to detect mutations that disrupt the binding of two factors could also be employed. Such a system has been described by Shih et al. and is termed the yeast split-hybrid (148). This approach could be used to detect the specific residues required for MRG1 and NLI binding of the LIM domain. Ultimately, the function of such mutants in reporter gene assays could be tested. These proposed experiments should begin to clarify the role of MRG1 and NLI in LIM mediated transcription.

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