ABSTRACT

Title of Dissertation: DIFFERENTIAL ABILITIES OF THE CHICKEN PIT1 ISOFORMS TO REGULATE THE CHICKEN GROWTH HORMONE PROMOTER

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Pit1, a pituitary-specific transcription factor, regulates differentiation of cells of the PIT1 lineage in the anterior pituitary. PIT1 also regulates the synthesis of peptide hormones from these cell types, including growth hormone (GH). A founding member of the POU-homeodomain family of transcription factors, PIT1 is characterized by a serine-threonine rich N-terminal transactivation domain and a C-terminal POU-domain. Alternative forms of PIT1, differing from each other in the N-terminal domain have been reported in several species, but the functional implication of having multiple isoforms is not known. Several *Pit1* isoform mRNAs exist in chickens which have not been characterized. The main aim of this study was to determine which, if any, of the chicken

PIT1 isoforms regulated the chicken *Gh* (*cGh*) promoter. PIT1β2, a novel isoform of chicken PIT1 was discovered, and known and novel isoforms (PIT1α, PIT1β1, PIT1β2 and PIT1γ) were characterized. A luciferase reporter construct containing 1775bp of the *cGh* promoter driving expression of firefly luciferase was used to determine the ability of the isoforms to regulate the target gene promoter activity in chicken LMH cells. We showed that three of the isoforms, PIT1α, PIT1β1 and PIT1β2, expressed from recombinant plasmids, regulated the *cGh* promoter, while PIT1γ did not. All the isoforms localized to the nucleus in both non-pituitary and pituitary cells. Results from gel-shift assays show that PIT1γ did not bind the proximal PIT1-binding site of the c*Gh* promoter as well as the other isoforms, suggesting a possible mechanism behind the inactivity. Our result did not suggest a negative regulatory role for this isoform. In contrast, we found a functional advantage for having multiple isoforms. PIT1β1, the isoform that activated the promoter most strongly, when co-transfected with other activating isoforms, such as PIT1α and PIT1β2, induced significantly higher level of activation than one isoform alone. Whether this increased activation required, or was facilitated by, heterodimerization of two isoforms is not known. Nevertheless, identification of isoforms with specific functions will facilitate identification of their respective interacting partners, which are essential for GH gene expression.

DIFFERENTIAL ABILITIES OF THE CHICKEN PIT1 ISOFORMS TO REGULATE THE CHICKEN GROWTH HORMONE PROMOTER

by

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Dedicated to the memory of my mother

Meena Mukherjee

Oct 22 1948-Sep 16 1997

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADG	average daily gain
ANOVA	analysis of variance
AP-2	activator protein-2
ATCC	American Type Culture Collection
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C	centigrade
cAMP	3’-5’-cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
cGH	chicken growth hormone
CORT	corticosterone
CPHD	combined pituitary hormone deficiency
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	corticotropin-releasing hormone
CS	chorionic somatomammotropins
d	post-hatch day
DAPI	4',6-diamidino-2-phenylindole
dCTP	Deoxycytidine triphosphate
Dex	dexamethasone
DMEM/F12	DMEM/Ham's nutrient mixture F12
DMP	Dimethyl pimelimidate dihydrochloride
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
e	embryonic day
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FBS	fetal bovine serum
FGF	fibroblast growth factor
GC	glucocorticoid
GFP	green-fluorescent protein
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone-releasing hormone
GHRH-R	growth hormone-releasing hormone receptor

GHRP	growth hormone releasing peptide
GLPI	glucagon-like peptide I
GR	glucocorticoid receptor
HA	hemagglutinin
HAT	histone acetyl transferase
HEK-293	human embryonic kidney-293
Hesx1	homeobox expressed in ES cells 1
Hr	hours
HRP	horse-radish peroxidase
IBMX	3-isobutyl-1-methylxanthin
IGF	insulin-like growth factor
Kbp	kilo base pairs
KDa	kilo Dalton
LMH	leghorn male hepatoma
MAPK	mitogen-activated protein kinase
MAPK	mitogen-activated protein kinase
MEK1/2	MAPK/ERK kinase1/2
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NGS	normal goat serum
NLS	nuclear localization signal
NMS	normal mouse serum
NPY	neuropeptide Y
NR	nuclear receptor
Opti-MEM I	optimized Eagle's minimal essentail medium
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pit-1	pituitary-specific transcription factor 1
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
POU-HD	POU homeodomain
POU-S	POU-specific domain
Prl	prolactin
Prop-1	prophet of Pit-1
PVDF	polyvinylidene fluoride
RACE	rapid amplification of cDNA ends
ras-dva	ras dorsal-ventral anterior
RNA	ribonucleic acid
RP	Rathke’s pouch
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean

Ser	serine
SF-1	steroidogenic factor-1
Shh	sonic hedgehog
shRNA	short hairpin RNA
SP1	specificity protein 1
Sport6	pCMV-Sport6.1
SST	somatostatin
TBS	tris-buffered saline
TBS/T	tris-buffered saline with tween-20
TE	Tris-EDTA
Thr	threonine
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TRH	thyrotropin-releasing hormone
TSH-β	thyroid-stimulating hormone β-subunit
TSS	transcription start site
UTR	untranslated region
VD	ventral diencephalon
VIP	vasoactive intestinal peptide
wt	wild type
Zn-15	zinc finger transcription factor 15

Chapter 1: Literature Review

Regulation of genes and metabolic pathways affecting production traits in agricultural animals have expectedly been an active area of research over several decades. The anterior pituitary gland is called a master regulator of homeostasis, because it regulates numerous physiological processes, several of which affect agricultural animal performance. A thorough understanding of the action of the genes involved in growth, lactation and reproductive performances will be a valuable tool to employ in agricultural biology to extract maximal performance with minimal input.

Growth hormone (GH), a peptide hormone released from the anterior pituitary gland, is essential for embryonic and posthatch growth in domestic chickens. In sex-linked dwarf chickens, a mutation of the GH receptor (GHR) results in a dwarf phenotype due to lack of GH signaling (Burnside et al., 1991; Huang et al., 1993). GH regulates metabolic processes such as lipolysis and muscle accretion. These two processes directly affect the quality of meat in broiler chickens. Posthatch administration of GH has little or no effect on growth rate in chickens. However, in ovo administration of GH at embryonic day 13 (e13) results in improved growth after hatch (Blumenthal et al., 1954), suggesting the establishment of a growth threshold during embryonic development. Hence, understanding the mechanisms that establish growth parameters in the embryo is essential for successful manipulation of this process to maximize agricultural yield.

Comparison of the regulation of Gh gene expression across vertebrate species has identified several common themes as well as differences, most of which will be addressed in this review. In the general theme, GH is synthesized and secreted from the somatotrophs (GH producing cells) of the anterior pituitary gland. Signaling pathways and transcription factors orchestrate the development of the pituitary gland and functional maturation of the hormone producing cells. Somatotroph differentiation and Gh gene expression are influenced by hypothalamic factors such as growth hormone-releasing hormone (GHRH) and somatostatin (SST) (Romero and Phelps, 1997), as well as hormones from peripheral glands (adrenal glucocorticoids and thyroid hormones). Several transcription factors, such as PIT1, Sp-1, AP-2 (activator protein-2), glucocorticoid receptor (GR), thyroid hormone receptor (TR) and cAMP (3'-5'-cyclic adenosine monophosphate) response element binding protein (CREB) coordinate to maintain the tissue-specific expression of the Gh gene. Absence of one or more key elements of this machinery leads to absence or significant reduction of GH synthesis and secretion. This review will provide background information on the key points mentioned above.

Development of the pituitary gland

Formation of Rathke's pouch (RP), a finger-like projection from the roof of the oral cavity toward the ventral diencephalon (VD) is the first step in pituitary development (Dasen & Rosenfeld, 2001). At about the same time, an outgrowth from the ventral diencephalon, called the infundibulum, starts developing toward RP. These two structures, after a series of developmental changes, give rise to the pituitary gland, with

RP forming the anterior lobe of the pituitary (adenohypophysis) and the infundibulum giving rise to the posterior lobe (neurohypophysis) (Wagner and Thomas, 2007). Physical contact between the RP and the infundibulum is essential for proper development of the pituitary gland, as the ventral diencephalon and infundibulum promotes growth of the anterior pituitary and final differentiation of the endocrine cell types (Ferrand 1972; Daikoku *et al.* 1982; Watanabe 1982(a,b); Fedtsova & Barabanov 1990).

The anterior pituitary differentiates into five distinct cell types: corticotrophs, gonadotrophs, somatotrophs, lactotrophs and thyrotrophs (Dasen & Rosenfeld, 2001). A number of signaling molecules play essential roles in pituitary development (Fig. 1). Development of RP at the very initial stage requires bone morphogenetic protein (BMP) 4 (Ericson *et al.* 1998, Treier *et al.* 1998; Takuma *et al.* 1998), but involvement of signals originating from the notochord has also been suggested. Sonic hedgehog (Shh), a secreted factor produced in the notochord is important for cell type specification and proliferation (Chiang *et al.* 1996; Fedtsova *et al.* 2003; Hammerschmidt *et al.*, 1997; Treier *et al.*, 1998). Members of the fibroblast growth factor (FGF) family, especially FGF8 and FGF10 are expressed in the VD. FGF10 is essential for cell survival, while FGF8 maintains proliferation and blocks BMP2 (discussed below).

Two members of the BMP family have documented roles in pituitary development. As mentioned previously, BMP4 is required at the beginning of RP formation. The other member, BMP2, is solely expressed in the VD (Ericson *et al.* 1998, Treier *et al.* 1998). Ventrally expressed BMP2 and dorsal FGF8 form an opposing gradient of transcription

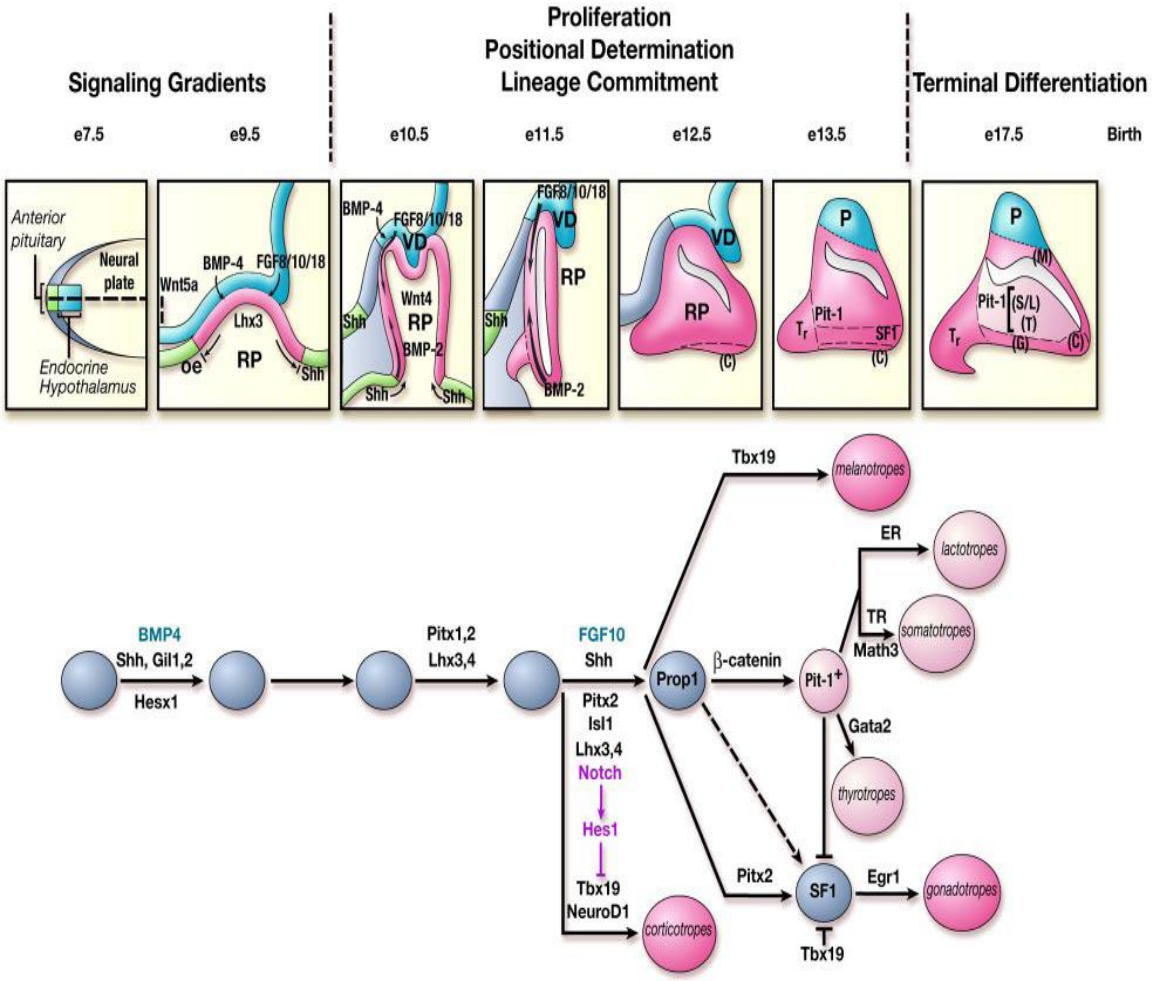


Fig. 1. Developmental stages, signaling molecules and selected transcription factors required for anterior pituitary development. Developmental stages denoted are for mouse. Top panel shows signaling molecules and morphological changes during pituitary development. Bottom panel shows transcription factors involved in initiation of pituitary development, lineage commitment and terminal differentiation. (Zhu et al., 2007)

factors leading to specification of cell lineages, after which BMP2 is down-regulated to achieve terminal differentiation of the pituitary gland (Dasen and Rosenfeld, 2001).

Notch signaling, required for early pituitary development, must be down-regulated during later phases to achieve terminal differentiation of cell lineages (Raetzman *et al.*, 2006; Zhu *et al.*, 2006). Constitutive expression of Notch leads to attenuated differentiation of all cell types of the anterior pituitary, as the factors Mash1 and Math3, both repressed by Notch, are critical for terminal differentiation (Raetzman *et al.*, 2006; Zhu *et al.*, 2006). The Wnt/β-catenin pathway is essential for terminal differentiation of cells of the PIT1 lineage and overall pituitary gland growth. It is activated somewhat late during embryogenesis, and aberrant early activation causes premature down-regulation of transcription factor Hesx1, leading to failure of pituitary gland formation (Olson *et al.*, 2006). Among the Wnts, Wnt4 and Wnt5a have been shown to be directly involved in anterior pituitary formation (Cha *et al.*, 2004; Treier *et al.*, 1998).

The list of transcription factors involved in pituitary development and differentiation is ever expanding but still far from complete. Some of them appear and act early, and may or may not need to be attenuated during further development. These early players usually show a more ubiquitous expression pattern throughout the developing embryo/fetus. Several members of the LIM Homeodomain proteins, for instance, *Isl1, Lhx3* and *Lhx4* are expressed early in very specialized regions of the developing pituitary gland. *Lhx3* is required for the formation of the definitive RP, but it also is needed later on for continued proliferation and prevention of apoptosis (Sheng *et al.*, 1996). *Isl1*, another member of

the LIM group, is induced by BMP2 and opposed by FGF8, the classic target of the dorsal-ventral gradient necessary for pituitary differentiation (Ericson *et al.*, 1998; Takuma *et al.*, 1998). *Hesx1*, a paired-homeodomain transcription factor expressed in the RP and VD, is also essential for formation of the definitive pouch but later must be down-regulated (Dattani *et al.*, 1998; Martinez-Barbera, 2000). *Lhx3*, *Hesx1*, and *Pitx1* (a bicoid-related transcription factor) are induced initially to form the definitive pouch, following which *Pitx2* is induced, *Lhx3* is maintained, and *Hesx1* is down-regulated to achieve terminal differentiation of the endocrine gland. *Prop-1*, another paired-like transcription factor, is the interaction partner of β-catenin in the complex that is responsible for the timely down-regulation of *Hesx1* (Olsen *et al.* 2006; Sheng *et al.* 1996; Sornson *et al.*, 1996).

After formation of the definitive pouch, a second set of transcription factors regulate further development, the most important being terminal differentiation of the hormone-producing cells. In mammals, the corticotrophs are the first cell type to reach final differentiation, followed by the thyrotrophs, somatotrophs, lactotrophs and gonadotrophs, in their order of appearance (Japon *et al.*, 1994; Simmons *et al.*, 1990). Tbx19, a T-box transcription factor, is essential for terminal differentiation of corticotrophs and also for repression of alternate cell fates (Pulichino *et al.*, 2003a, b). Three cell types, somatotrophs, lactotrophs, and thyrotrophs are characterized by the expression of one transcription factor, PIT1 (Cajiao *et al.*, 2004; Dasen *et al.*, 1999; Li *et al.*, 1990), while gonadotrophs are characterized by the absence of it. The transcription factor unique to gonadotrophs is steroidogenic factor-1 (SF1) (Ingraham *et al.*, 1994; Simmons *et al.*,

1990). Thyrotrophs and gonadotrophs both express GATA2 (Charles *et al.*, 2006), and it appears that the unique combination of PIT1 and GATA2 makes the cells thyrotrophs, and in the absence of PIT1, the cells adopt the fate of gonadotrophs. Conversely, gonadotrophs can be converted to thyrotrophs by the ectopic expression of PIT1 (Dasen *et al.*, 1999; Gordon *et al.*, 1997). These observations underline the importance and absolute requirements of these lineage-specifying transcription factors for proper pituitary development and function.

The development of the pituitary in chicken shares many of the general developmental pathways and factors mentioned above, but differs from mammalian systems in some details. For instance, development of the infundibulum and from it, the posterior pituitary, is much less pronounced compared to mammals (Zhu *et al.*, 2007). Expression of BMP4 differs between mouse and chicken: in chicken, it is expressed late, never in the RP, and never in levels comparable to that in mouse (Parkinson *et al.*, 2010). The order of appearance of the different hormone-producing cell types also differs, with the corticotrophs and gonadotrophs being the first two types to appear almost simultaneously, followed by thyrotrophs, somatotrophs, and finally, the lactotrophs (Sasaki *et al.*, 2003). Also, in mammals, somatotrophs and lactotrophs are thought to have originated from a common precursor, the somatomammotrophs (Asa *et al.*, 1983), while in chickens, the origin of these two cell types are clearly different (Fu *et al.*, 2004).

Functions of growth hormone in vertebrates

Growth hormone is involved in regulating a number of physiological processes in vertebrates, including long bone growth, lipid, carbohydrate and protein metabolism (Davidson *et al.*, 1987), immune function (Koojman *et al.*, 1996; Hooghe-Peters and Hooghe, 1998) and carcinogenesis. The "Somatomedin hypothesis," put forward in 1957 (Salmon and Daughaday, 1957), postulated that the effects of GH on its target organs are not direct, and that they involve one or more intermediate factors (termed somatomedins; later identified to be insulin-like growth factors or IGFs) (Van Wyk *et al.*, 1974). Decades of research in this area have proved this hypothesis to be partially true, and the hypothesis has been modified, with its present form stating that some actions of GH are direct while others are indirect (Le Roith *et al.*, 2001). For instance, the GH effect on long bone growth appears to be direct. It is generally seen that the anabolic effects of GH, such as those involving bone, cartilage and skeletal muscle growth are direct and in some of these IGFs act in concert with GH towards the same end, but the action of GH itself is IGF-independent. However, in certain catabolic processes, such as lipid and carbohydrate metabolism (gluconeogenesis and adipogenesis), IGFs and GH have opposite effects, and IGFs are thought to counteract the effects of GH, which would otherwise be harmful (Kaplan and Cohen, 2007).

In mammals, GH is the primary regulator of post-natal growth. Transgenic mice overexpressing GH grew almost twice as big as control littermates (Palmiter *et al.*, 1983). Exogenous administration of GH in swine results in increased average daily gain (ADG),

muscle accretion and lipolysis (Campbell *et al.*, 1989). GH also increases lactation in dairy cattle (Falaki *et al.*, 1997). Apart from its obvious effects on bone growth, GH is necessary for normal female reproductive function, steroid metabolism, tooth development and modulation of gut function.

In fish, apart from regulating growth throughout adult life, GH functions include regulation of metabolism by lipolysis and protein synthesis (Foster *et al.*, 1991; Leatherland and Farbridge, 1992; O'Connor *et al.*, 1996; Fauconneau *et al.*, 2000), reproduction, osmoregulation (Bolton *et al.*, 1987; Sakamoto *et al.*, 1991), and immune development (reviewed in Yada *et al.*, 2007). Osmoregulation by GH is brought about by morphological changes in gill chloride cells and by increasing the number of ion transporters in these cells, and this effect is believed to be IGF-dependent. Other vertebrate classes where GH exerts control over growth are reptiles (Denver and Licht, 1990) and amphibians (Huang and Brown, 2000).

Contrary to all other vertebrates, exogenous GH administration has no effect on growth and body weight in early post hatch broiler chickens (Burke *et al.* 1987; Cogburn *et al.*, 1989; Cravener *et al.*, 1989; Scanes *et al.* 1990). However, pulsatile infusion of GH after the decrease of endogenous levels did have positive effects on bone growth and other metabolic parameters such as weight gain and feed efficiency (Vasilatos-Younken, 1988; Leung *et al.*, 1986). Also, unlike in mammals, GH does not promote gluconeogenesis in avian species, and effects on lipid metabolism can be lipogenic or lipolytic depending on the age and mode of administration of GH.

While the pleiotropic nature of GH function is evident from the discussion above, regulation of body growth and development appears to be the major function of GH. GH synthesis and secretion is under the control of several hypothalamic and other endocrine factors, and the actions of GH, as mentioned previously, are mediated by IGFs produced either locally or by the liver (and carried by blood to the site of action). Hypothalamic regulators, GH, and IGFs constitute the somatotropic axis essential for post-natal growth. Evidence of the essentiality of the components and targets of the somatotropic axis comes mainly from mutant dwarf phenotypes, many of which will be discussed in this review. Classical pituitary ablation/replacement experiments have demonstrated that growth cessation resulting from hypophysectomy can be reversed by exogenous GH in chickens (Thommes *et al.*, 1992; Scanes *et al.*, 1986). In addition, both transgenic mice (Palmiter *et al.*, 1983) and fish (Houdebine and Chourrout, 1991) overexpressing the *Gh* gene show marked increases in growth when compared to control animals. Also, it is well known that in humans, GH hyposecretion leads to dwarfism and hypersecretion to gigantism (before puberty) or acromegaly (in the case of adult onset).

Gh gene structure

The *Gh* gene has been evolutionarily conserved from lower vertebrates to birds and mammals; however, the structural arrangement of the primate *Gh* gene is markedly different from other vertebrate species. In most mammals and all birds and fish, *Gh* is a single gene, but in primates GH is encoded by a cluster of 5 genes. Genes that comprise this cluster are thought to have arisen as a result of gene duplication. The cluster includes the normal and variant forms of GH, GH-N and GH-V, respectively, along with genes for

placental lactogens (also called chorionic somatomammotropins, or CS). Of the genes present in this cluster, only GH-N is expressed in the pituitary somatotrophs, while GH-V and the CSs are expressed in the syncytiotrophoblasts of the placenta (Chen *et al.*, 1989).

The mammalian *Gh* gene is made up of 5 exons and 4 introns within an approximately 3kb region (Tuggle and Trenkle, 1996). In fish, it can be either the same as mammals or contain 6 exons and 5 introns depending on the species. The *Gh* gene in chicken is located on chromosome 27, and, similar to mammals, comprises 5 exons and four introns, but the introns are larger than their mammalian counterparts, making the entire gene about 3.5kb. Alternative splicing of the h*Gh* gene yields proteins of 22kDa and 20kDa, with the 22kDa form being much more abundant. Among post-translational modifications, acylation, deamidation and glycosylation are known, and the first two display similar bioactivity, while the bioactivity of the glycosylated form is not known. Glycosylated forms have been reported for human, rat and chicken GH.

Neuroendocrine regulation of GH synthesis and secretion

GH is synthesized and secreted in a ultradian pulsatile manner (Winer *et al.*, 1990) from somatotrophs, one of the five cell types that make up the anterior pituitary. Somatotroph differentiation requires blood-borne signals from outside the pituitary in all species (Nogami *et al.*, 1995; Porter *et al.*, 1995). Somatotrophs are the third pituitary cell type to arise in most species, and they are one of the most numerous cell types in adults. Growth hormone-releasing hormone (GHRH) and somatostatin (SST) are two hypothalamic factors that regulate GH production, with GHRH stimulating the synthesis and secretion

of GH and SST inhibiting it. Both SST and GHRH are essential for maintaining the pulsatality of GH secretion (Wehrenberg *et al.*,1982, Cella *et al.*,1990, Katakami *et al.*,1988, Frohman *et al.*,1989) even though the exact mechanism behind the pulsatality remains unknown. SST, the inhibitor of GH release, probably negatively regulates GHRH to bring about the pulsatility.

GHRH belongs to the family of brain-gut peptides along with vasoactive intestinal peptide (VIP), glucagon, glucagon-like peptide I (GLPI), pituitary adenylate cyclase-activating peptide (PACAP) etc. (Campbell *et al.*, 1991). Functions of GHRH include stimulation of somatotroph differentiation, *Gh* gene transcription, biosynthesis, and secretion (Mayo *et al.*, 1995). GHRH is a peptide hormone produced in the arcuate nucleus of the hypothalamus (Bloch *et al.*,1983). The actions of GHRH are mediated through its receptor, GHRH-R, a seven transmembrane G-protein coupled receptor linked to a stimulatory G-protein (G_s) (Gaylinn *et al.*,1993, Lin *et al.*,1992, Narayanon *et al.* 1989, Spada *et al.*,1984), which activates adenylate cyclase (Spada *et al.*,1984), leading to an increase in intracellular cAMP and Ca^{2+} levels (Holl *et al.*,1988). The activated G-protein interacts with ion channels, leading to secretion of GH, whereas the elevated cAMP levels activate the protein kinase A (PKA) pathway that leads to increased transcription of the GH gene, possibly by phosphorylation of transcription factors and activators involved in the process (Holl *et al.*, 1988). Evidence of the essentiality of GHRH signaling in GH regulation comes from the GHRH-R mutant little mouse (*lit/lit*), with a mutation of a conserved aspartic acid residue (Asp60Gly) in the N-terminal ligand binding domain. This mutation changes the protein structure, and the mutant receptor is

incapable of increasing cellular cAMP levels (Lin *et al.*, 1993). Among vertebrates, the function of GHRH as a potent regulator of *Gh* has been established in mammals, birds, reptiles and one teleost, the goldfish.

SST, the hypothalamic peptide hormone that inhibits GH synthesis (Brazeau *et al.*,1973), also acts through G-protein coupled receptors, of which there are six subtypes (Patel,1997). SST function has been conserved in vertebrates. In almost all species, SST does not decrease basal GH production at either RNA or protein levels; neither does it inhibit somatotroph proliferation, but rather it attenuates GHRH-induced GH expression (Barinaga *et al.*,1985, Billestrup *et al.*,1986, Fukata *et al.*,1985, Simard *et al.*,1986, Tanner *et al.*,1990). In chickens and teleosts, however, SST can affect both basal and GHRH-induced GH expression, while in reptiles and some amphibians, SST acts on thyrotropin releasing hormone (TRH)-stimulated GH expression (Hall and Chadwick, 1984; Jeandel *et al.*, 1998). SST possibly mediates its effect through decreasing intracellular cAMP levels and by affecting voltage-gated ion channels that lead to an increase in intracellular K^+ levels and simultaneous decrease in Ca^{2+} levels, but the precise mechanism is still unclear (Epelbaum, 1992, Patel *et al.*,1995).

Apart from these hypothalamic peptides, numerous growth hormone-releasing peptides, or GHRPs have been identified and characterized. GHRP-6 is the most potent peptide, being more potent than GHRH (Bowers, 1993; Bowers *et al.*, 1990). The potency of it has been evaluated in monkeys, sheep, pigs, chickens, steers, rats and humans (Bowers *et al.*,1984, Croom *et al.*,1984, Doscher *et al.*,1984, Kraft *et al.*,1984, Malozowski *et*

al.,1991, Bowers *et al.*,1990, Ilson *et al.*,1989). GHRP-6 can increase the level of *Gh* mRNA independent of GHRH, and the two together lead to a synergistic activation of *Gh* mRNA (Cheng *et al.*, 1989, Goth *et al.*,1992). Even though the precise mechanism of action of GHRPs is not known, it is postulated that GHRP binding to GHRP receptor leads to an increase in intracellular Ca^{2+} (Akman *et al.*,1993; Sartor *et al.*,1985), no change in intracellular cAMP (Cheng *et al.*,1989, Wu *et al.*,1994) and a possible activation of the protein kinase C (PKC) pathway (Cheng *et al.*, 1991; Cheng *et al.*,1993).

Several other hypothalamic peptides participate in the regulation of *Gh*, some of them having dual functions depending on age, pathological state, site of action, etc. For instance, thyrotropin releasing hormone (TRH) is stimulatory in fetal and neonatal stages before the somatotropic axis is established or in pathologies such as acromegaly (Cocchi *et al.*, 1983; Harvey, 1990), whereas it inhibits GH release by its action on the hypothalamus (Cocchi *et al.*, 1983; Müller, 1987). CRH, corticotrophin releasing hormone, and neuropeptide Y (NPY) both have an inhibitory effect (Katakami, 1985, Ono *et al.*, 1984; McDonald, 1985; Rettori *et al.*, 1990), and both are most likely mediated by SST (Katakami, 1985). PACAP, another brain-gut peptide has a pronounced role in GH release in fish (Montero *et al.*, 2000; Wong *et al.*, 2000) and amphibians (Martinez-Fuentes *et al.*, 1994), somewhat of a lesser effect in birds (Peeters *et al.*, 1998), and possibly no effect in mammals (Miyata *et al.*, 1989; Jarry *et al.*, 1992; Sawangjaroen and Curlewis, 1994; Chiodera *et al.*, 1996).

Among peripheral gland hormones, glucocorticoid (GC) regulation of *Gh* gene expression has been extensively studied in many vertebrate species. A part of the regulation is mediated through GC action on the hypothalamic regulators, GHRH (Seifert *et al.*, 1984; Michel *et al.*, 1984) and SST (Holl *et al.*,1988; Schonbrunn *et al.*,1982). However, GCs can also directly stimulate *Gh* gene transcription by acting through a ligand-dependent transcription factor (Thakore and Dinan,1994). These effects of GCs are true for physiologic doses. Physiological concentrations of thyroid hormones are also necessary for the maintenance of *Gh* gene expression (Giustina and Wehrenberg,1995). Thyroid hormones act in concert with GHRH by enhancing the effect of GHRH on somatotrophs (Martin *et al.*, 1985; Korythko *et al.*,1997).

Apart from the major regulators of *Gh* mentioned above, a host of other factors influence GH synthesis and secretion in vertebrates. Ghrelin, a stomach peptide hormone, has been shown to act as a stimulator of GH in all species. Neurotransmitters such as serotonin and norepinephrine, metabolic signals such as glucose and leptin, nonesterified fatty acids, nitric oxide, amino acids such as arginine, ornithine, lysine, and tryptophan, have all been shown to have specific roles in GH synthesis, secretion and feedback mechanisms, detailed explanations of which are beyond the scope of this review (Muller *et al.*, 1999). It suffices to say that *Gh* regulation is a complex process involving numerous players, which act in concert with tissue specificity to maintain normal GH supply and function in complex physiological systems, many aspects of such regulation being still unknown.

Transcription factor regulation of Gh gene expression

The regulatory regions of the *Gh* gene have been identified and characterized in several vertebrate species. In the species studied so far, PIT1 is undoubtedly the single most important transcription factor regulating *Gh* gene expression in the anterior pituitary somatotrophs. PIT1 is essential for terminal differentiation of cells of the PIT1 lineage (thyrotrophs, somatotrophs and lactotrophs) and for gene expression of the hormones secreted by these cells, β-subunit of thyroid-stimulating hormone (TSHβ), growth hormone (GH) and prolactin (Prl), respectively (Ingraham *et al.* 1988). Evidence of this essential role of PIT1 includes pituitary hypoplasia and lack of detectable levels of the hormones in *Pit1* mutant mice (Li *et al.*, 1990) and also clinical studies in humans (Radovick *et al.*, 2000). However, although necessary, PIT1 is not sufficient to regulate somatotroph-specific *Gh* gene expression. PIT1 interacts with other transcription factors, both general and specific, as well as with co-activators to bring about this highly cell-type specific gene expression. Several of the cellular factors that bind to and regulate the *Gh* promoter have been identified. Steroid hormone receptors, such as the glucocorticoid receptor (GR) and more extensively the thyroid hormone receptor (TR), binding to the *Gh* promoter has been studied, and the results have shown that the regulation of *Gh* varies considerably across species. Other transcription factors, such as Ets-1 (Yang *et al.*, 2010), Sp-1 (Melamed *et al.*, 1998), NF-1 (Norquay *et al.*, 2003), Ikaros (Ezzat *et al.*, 2005) and Zn finger 15 (Zn-15) (Lipkin *et al.*, 1993) are involved in tissue-specific transcription of *Gh*. The regulation of *Gh* expression in chickens will be discussed in subsequent sections,

and similarities and differences with mammalian species deemed noteworthy in the context of current work will be addressed.

Promoter analysis of the *Gh* gene across species brings out some common features. General transcription regulatory sequences, such as the TATA box, are present in representative species of all classes (teleosts, amphibians, birds, primate and non-primate mammals). In general, the fish species show maximum inter-species variation. Similarly, PIT1 and at least one AP site(s) are present in all species examined so far. The mammalian and chicken promoters have some similarities in terms of the binding sites present, marked by the presence of Sp1 and AP-2 binding sites, cAMP- and vitamin D response elements, and thyroid hormone response elements; however, the copy numbers and relative locations of these sites vary widely (Chuzhanova *et al.*, 2000).

Somatotroph differentiation and Gh gene expression in the chicken

Somatotroph differentiation takes place during the latter half of embryonic/fetal development in chickens and rats. In chickens, growth hormone-secreting cells are first detected on embryonic day 12 (e12) in the caudal portion of the pituitary gland, and they become a significant population around e16 (Porter *et al.*, 1995). Concomitant with this increase in abundance, *Gh* mRNA and protein levels increase and serum GH levels become detectable (Harvey *et al.*, 1979). Somatotroph differentiation is a highly regulated developmental process requiring the participation of signals emanating both from within and outside the pituitary gland (Fig. 2).

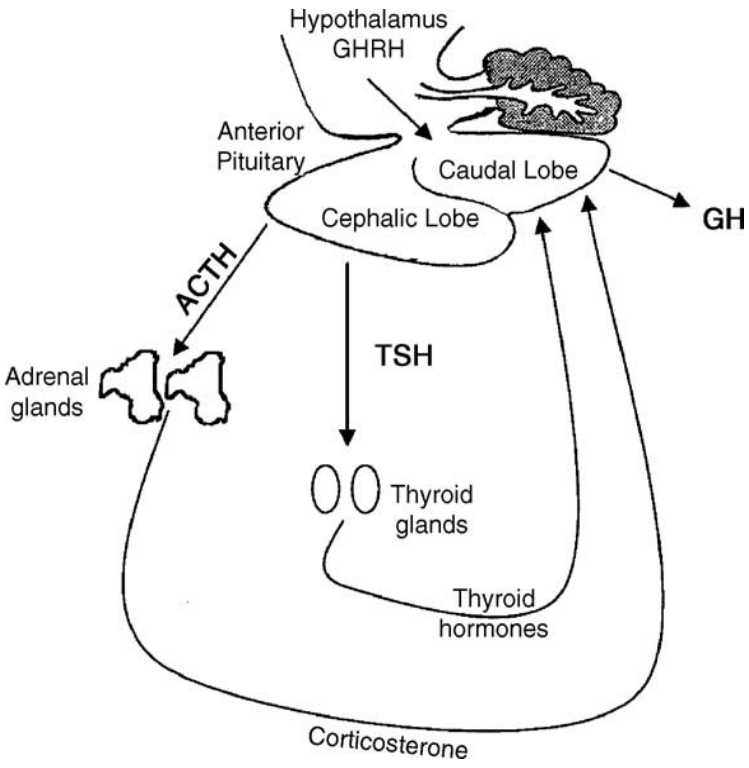


Fig. 2 Schematic representation of regulation of growth hormone in chickens by signals emanating from the hypothalamus as well as peripheral organs. (Porter, 2005)

A blood-borne signal is required for somatotroph differentiation. Anterior pituitary cells isolated from e12 to e16 chickens (prior to somatotroph differentiation) fail to differentiate into functional somatotrophs in culture in the absence of serum. Serum from e16 has the highest potency to induce somatotroph differentiation in cultured cells (Porter *et al.*, 1995). Heat-inactivated, but not ether-extracted serum maintains the ability to induce differentiation, suggesting the blood-borne factor is a steroid. This signal was later found to be the adrenal glucocorticoid, corticosterone (CORT), the level of which is known to increase dramatically with the onset of somatotroph differentiation (Morpurgo *et al.*, 1997; Jenkins and Porter, 2004).

In both rats and chicks, endogenous thyroid hormone, especially triiodothyronine (T_3), is necessary for somatotroph differentiation. However, unlike CORT, thyroid hormone is ineffective in increasing somatotroph abundance by itself and requires the presence of CORT (Liu *et al.*, 2003; Nogami *et al.* 1997). Also, the effect of T_3 is age-dependent, as at e20 T_3 actually inhibits GH secretion (Liu and Porter, 2004; Jenkins *et al.*, 2007).

Role of Glucocorticoids in somatotroph differentiation

Glucocorticoids (GCs) (cortisol in humans, CORT in rodents and birds) can induce premature differentiation of somatotrophs both *in vitro* and *in vivo*. Isolated chicken pituitary cells and whole rat pituitaries treated with GC show an increase in the number of somatotrophs, and these cells are characterized by increased *Gh* mRNA, protein and GH secretory capacity (Hemming *et al.*, 1988; Morpurgo *et al.*, 1997; Nogami *et al.*,

1997; Dean and Porter, 1999; Liu *et al.*, 2003). GCs show this effect only after the *Pit1* gene is expressed in the anterior pituitary and not before, suggesting the involvement of CORT in inducing the differentiation of cells already committed to become somatotrophs. GC induction of these committed cells cannot be blocked by a mitosis inhibitor, thus ruling out the possibility of CORT increasing the abundance of somatotrophs by acting as a mitogen and not a terminal signal (Porter *et al.*, 1995).

Treatment of pregnant rats with dexamethasone (dex), a synthetic glucocorticoid, results in premature appearance of GH cells in the corresponding fetuses on e17 and e18, before e19 when somatotrophs become a significant population (Nogami and Tachibana ,1993). Similarly, in chicken, treatment of e11 embryos with either e16 serum or CORT increased somatotroph abundance on e14 (Porter *et al.*, 1995; Dean *et al.*, 1999).

The mechanism of CORT induction of somatotroph differentiation and *Gh* gene expression is not known; however, the process appears to be indirect. In both rats and chickens, the ability of GC to induce somatotroph differentiation and increase *Gh* mRNA can be blocked by protein synthesis inhibitors (Nogami *et al.*, 1997; Bossis and Porter, 2003). This indicates the potential involvement of intermediary factors in the process. The transcription factor PIT1 was the first to be considered a likely candidate induced by GC. However, GCs do not increase the levels of *Pit1* mRNA, protein or the number of PIT1 expressing cells, ruling out the possibility that this factor might be PIT1 (Fu and Porter, 2004). Another possible candidate was the GHRH receptor (GHRH-R). In rats, GHRH-R levels increase in response to GC *in vitro* (Nogami *et al.*, 1999). However, the

following lines of evidence from chickens rule out the possibility of GHRH-R being involved in increasing *Gh* mRNA in response to CORT. GHRH treatment along with CORT stimulated *Gh* mRNA expression to a level above that obtained with CORT alone, but GHRH alone had no effect (Dean and Porter, 1999). GHRH-R stimulates GH synthesis and secretion by increasing cellular cAMP levels and by activating protein kinase A (PKA) (Anderson *et al.*, 2004). Cells treated with forskolin (which increases cAMP levels) and 3-isobutyl-1-methylxanthin (IBMX) and CORT mimicked the levels of *Gh* mRNA achieved with co-treatment of CORT and GHRH (Bossis and Porter, 2003). Increased cAMP levels could not induce *Gh* mRNA in the absence of CORT. Therefore, activation of GHRH-R is not an essential step in GC induction of *Gh* mRNA expression. Involvement of PKA and PKC in GC stimulation of *Gh* gene expression was ruled out by use of inhibitors; H-89 and calphostin C, respectively. Blocking the PKA pathway with H-89 resulted in obliteration of the synergistic effect of CORT and GHRH, indicating that the chicken GHRH-R acts by stimulating PKA. However, H-89 did not block CORT induction of *Gh* (Bossis and Porter, 2003, Porter, 2005). In contrast, treatment of e11 chicken pituitary cells with inhibitors of specific signaling pathways showed the involvement of MEK1/2, p38 MAPK, and Ras signaling pathways (Malkiewicz, 2003; Ellestad, 2010). Both *Gh* mRNA levels and the number of cells expressing GH protein were reduced in the presence of these inhibitors. The ability of CORT to activate *Gh* promoter activity in the presence of these inhibitors was also examined, and the results confirmed the involvement of MEK1/2, p38 MAPK and Ras signaling (Ellestad and Porter, unpublished results).

The glucocorticoid receptor

The cellular actions of GCs are mostly mediated through the glucocorticoid receptor (GR), which is a ligand-dependent transcription factor. GR belongs to the family of nuclear receptors (NRs), more specifically to a subset of NRs called steroid hormone receptors. Other members of this family are the androgen receptor, the mineralocorticoid receptor, the estrogen receptor and the progesterone receptor (Mangelsdorf *et al.*,1995). GR is ubiquitously expressed, but the sensitivity of the receptor to its ligand is not uniform across organs (Lim-Tio *et al.,* 1997).

GR is characterized by a number of specific functional domains, those responsible for dimerization, transactivation, ligand binding and DNA binding (Wurtz *et al.* 1996, Weatherman, 1999). In the absence of ligand, GR is present in the cytoplasm in a complex with chaperone proteins. Upon hormonal stimulation, GR dissociates from the complex, dimerizes and translocates to the nucleus, where it activates genes by binding to glucocorticoid response elements (GREs) in the promoter elements of target genes (Dostert and Heinzel, 2004) or represses genes by either binding to negative GREs or by binding to other transcription factors (Martens *et al.*, 2001).

More than 1700bp of the 5'-flanking region of the chicken *Gh* gene has been analyzed and functionally characterized (Ip *et al.* 2004) (Fig. 3). The organization of the proximal promoter region of the chicken *Gh* gene differs from that of the mammalian *Gh* genes, thereby opening up the possibility that the regulation of *Gh* in chickens might be unique

A)

Motif	Start	End	Sequence
AG-rich region	-1603	-1495	\
Pit-1	-541	-534	AGCTTCAT
TATA Box	-149	-143	TATAAAT
TRE	-137	-128	CAATGAGGTA
Pit-1	-111	-104	ATCTGCAT
TATA Box	-24	-30	TATATAA

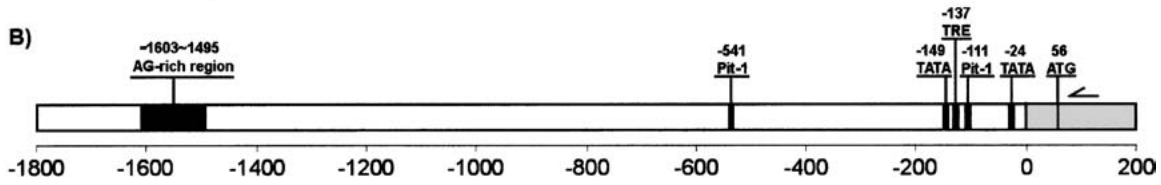


Fig. 3 Schematic representation of the chicken growth hormone promoter. Shown here are the TATA box and putative binding sites for thyroid hormone receptor (TRE), PIT1. Note the absence of classical glucocorticoid response element (GRE) or cAMP-response element (CRE) (Ip *et al.*, 2004)

in terms of activating and repressing factors employed. The promoter region of the chicken *Gh* gene has two PIT1 binding sites, a proximal site located at -113/-104 and a distal site at -541/-533, along with a putative thyroid hormone response element (TRE) at -137/-128. A TATA box is located at -24bp. Further functional characterization of the two PIT1 binding sites revealed the proximal site to be functional and able to bind PIT1, while the distal site was found to be either redundant or repressive, but only in the presence of the proximal site. Although no GRE was found, the region between -1727 and -1467 was found to be GC responsive in induction of *Gh* (Ip *et al.*, 2004).

PIT1: A POU-homeodomain transcription factor

Gh and *Prl* are two evolutionarily related genes, but they are expressed in phenotypically distinct cells within the same tissue. Experiments aimed to elucidate the mechanism behind such cell-type specific expression within the same organ identified a nuclear DNA-binding protein (initially named PIT1) as a transcription factor regulating both genes (Lefevre et al., 1987; Nelson et al., 1988). Promoter deletion analyses and DNase protection assays identified proximal and distal PIT1 binding sites as an imperfect palindrome with a core consensus A (A/T) (A/T) TATNCAT within the promoter of both genes. The pituitary-restricted expression and the ability to specifically activate *Gh* and *Prl* made PIT1 a possible positive transcription factor for *Gh* and *Prl* gene transcription (Nelson *et al.*, 1988; Cao *et al.*, 1987).

Purification of the factor(s) bound to proximal PIT1 binding sites in *Prl* and *Gh* promoters from rat pituitary G/C cells revealed a doublet of 31kDa and 33kDa (Nelson *et*

al., 1988). Soon after, the rat *Pit1* coding sequence was determined to be 873 nucleotides long, giving rise to a protein of approx. 33kDa (Ingraham *et al.,* 1988). The in vitro translated product also ran as a doublet of the same size as the endogenous protein, and they were found to be translated from multiple mature transcripts derived from the same gene. Structurally, the amino terminus of this transcription factor was found to be unique, but the carboxyl terminus had marked similarity to a homeodomain found in many Drosophila and vertebrate regulatory genes (Ingraham *et al.*, 1988).

At about the same time, three other transcriptional regulators, namely, a ubiquitously expressed octamer-binding protein Oct-1, a lymphoid tissue specific Oct-2, and a *C. elegans* developmental regulator *unc-86* were identified and found to contain a similar carboxyl terminus. Due to this sequence similarity they were thought to belong to a related family of transcription factors which was thereafter named the "POU family of transcription factors" (named after PIT1, Oct-1/2, unc-86) (Sturm *et al.*, 1988).

The POU proteins show remarkable sequence similarity over an approximately 160 amino acid length of sequence. The POU domain that characterizes this family can be subdivided into two subdomains: a C-terminally located POU homeodomain and an N-terminal POU-specific domain. The N-terminal domain (73-80 amino acids depending on species) is the transactivation domain required for transactivational properties of PIT1. The transactivation domain is characterized by a high percentage of serine and threonine residues but is otherwise not highly conserved (Theill *et al.*, 1989).

The homeodomain shares sequence similarity with other homeodomain proteins; however, the extent of similarity between the members of this family is higher than that between a POU factor and other homeodomain factors. This POU homeodomain (POU-HD) is about 60 amino acids in length, containing a tryptophan and a phenylalanine residue that are strictly conserved among all POU members and a cysteine that is not found in other homeodomain proteins except the POU proteins (Herr *et al.*, 1988).

Separated from the POU-HD by a linker of variable length and composition is the second POU sub-domain of about 80 amino acids. This domain is the most unique feature of this family of transcription factors and is referred to as the POU-specific domain (POU-S). Once again, there is high similarity between the members of this family in this domain. This domain can be further subdivided into two regions of sequence similarity that are higher than in any other region among the four members, with the exception of Oct-1: Oct-2 (Reviewed by Herr *et al.*, 1988). The function of the hinge region is not known, but it may be non-functional, as deletion of the hinge region did not affect PIT1 activation of the TSHβ promoter (Gordon *et al.*, 2002).

Both the POU-HD and POU-S domains are involved in DNA binding. Low affinity binding of PIT1 to its target region can be achieved by POUHD, but the POUS is required for site recognition and high affinity binding by virtue of a predicted alpha-helical structure within the region (Ingraham et al., 1990). Even though the N-terminal domain is primarily involved in transactivation of target genes, some residual transcriptional activation is also associated with the POUHD (Theill *et al.*, 1989,

Ingraham *et al.*, 1990). PIT1 is found in a monomeric state in solution, but usually binds DNA as a dimer. Protein-protein interactions with other transcription factors/coregulators are also mediated via the POUS domain (Theill *et al.*, 1989).

Regulation of target genes by PIT1

The well-studied targets of PIT1 include *Gh*, *Prl*, *TSHβ* and *GhRH-R*. These target genes contain one or more PIT1 binding sites in their promoter regions. The sequence of the PIT1 binding sites has been determined in mammals [(A/T)(A/T)(A/T)TATNCAT] (Nelson *et al.*, 1988) and in avian/teleost species [(A/T)NCTNCAT] (Ohkubo *et al.*, 1996). However, given the redundancy of the PIT1 binding site, it is very likely that several other pituitary genes are regulated by PIT1 at the transcriptional level. Indeed, results from our lab have shown that Ras-DVA is a novel PIT1 regulated gene (Ellestad, 2010). In addition, PIT1 autoregulates itself (Chen *et al.*, 1990; Rhodes *et al.*, 1993).

Multiple lines of evidence suggest a role of PIT1 in pituitary development and regulation of hormones secreted by cells of the PIT1 lineage. *Pit1* mRNA and protein are expressed in the anterior pituitary before the differentiation of these cell types (Dolle et al., 1990; Simmons et al., 1990). Mutations in the *Pit1* gene lead to severe pituitary hypoplasia, absence of the hormone producing cells, and complete lack of hormone gene transcription (Wilson and Wyatt, 1986; Radovick et al., 1992). Mice carrying mutations in the *Pit1* gene show dwarfism and severely reduced somatotrophs, lactotrophs and thyrotrophs. The Jackson dwarf mice are characterized by a gross alteration of the *Pit1* gene resulting from an insertion or inversion of a >4kb piece of DNA. The Snell mutant

has a point mutation in the POUHD resulting in a substitution of the tryptophan residue at position 261 with cysteine (Li et al., 1990). Mutations in Prop-1, a paired-like homeodomain protein required for *Pit1* gene activation and regulation also lead to dwarfism and absence of the cells of the PIT1 lineage (Sornson et al., 1996). In all the mutants, levels of PIT1 are nearly undetectable (Li *et al.*, 1990; Sornson et al., 1996). Knocking down Pit-1 expression by antisense oligonucleotides blocks *Gh* and Prl transcription along with inhibition of proliferation of somatotroph and lactotroph cell lines (Castrillo *et al.*, 1991). In teleosts, zebrafish *Pit1* mutant lacking 55 amino acid residues due to an internal truncation resulting from alternative splice site-usage shows severe dwarfism and lack of the three pituitary cell types (Nica *et al.*, 2004).

The regulation of cell-type specific expression of target genes by PIT1 has always been a conundrum. PIT1 is expressed in 3 cell types of the anterior pituitary; however, even within the cells of PIT1 lineage, there is specificity of hormone production. This cell type specificity is not just because the chromatin conformation is not conducive to transcription, because both in corticotrophs and in non-pituitary cell lines (Ingraham et al, 1988), where, for instance, the *Gh* promoter should be in closed conformation, transfected *Pit1* brings about expression of *Gh*. However, in lactotrophs, *Gh* gene transcription does not take place even in the presence of PIT1. In the lactotroph-derived cell line 235-1 (Nelson et al., 1988), transfected *Gh* promoter is not activated by PIT1 (Ingraham *et al.*, 1992), suggesting the presence of factors that either occupy the PIT1 binding site itself or the immediate vicinity, and/or modifies PIT1 in such a way that it is unable to bind *Gh* promoter PIT1 binding sites and/or recruit other essential transcription

factors, thus providing a negative regulation. An alternative mode of this regulation could be that factors otherwise ubiquitous are specifically absent from lactotrophs (Crenshaw et al., 1989).

Regulation of GHRHR by PIT1 has been studied in mammals, but not in any other vertebrate class. The human and rat GHRHR gene promoters lack a TATA box, and have multiple PIT1 binding sites. Other transcription factor binding sites common between the two species are for estrogen receptor (ER) and CREB (Petersenn *et al.*, 1998; Miller *et al.*, 1998; Iguchi *et al.*, 1999; McElvaine *et al.*, 2007). Out of the multiple PIT1 binding sites present, usually a few are functional and even fewer contribute significantly to regulation of transcription. The purpose of having multiple sites is not known. In humans, GHRHR gene transcription is regulated by GCs, even though the 5'flanking region lacks a classical GRE. In rat, two GREs along with a PIT1 binding site and an internal silencer region make up the glucocorticoid response unit. It is hypothesized that PIT1 binding to its element leads to release of an unknown factor from the silencer, allowing GR to bind to the GRE (Nogami *et al.*, 2005).

TSHβ gene expression is regulated positively by TRH (Steinfelder *et al.*, 1992) phorbol esters (Haugen *et al.*, 1993) and forskolin (Kim *et al.*, 1993), and negatively by thyroid hormones and estrogen (Steinfelder *et al.*, 1992, Nagayama *et al.*, 2008). The 5'flanking region of this PIT1 regulated gene has been characterized in mammalian species (human, mouse, rat) (Steinfelder *et al.*, 1992; Haugen *et al.*, 1993; Mason *et al.*, 1993) and goldfish (Sohn *et al.*, 1999), but information from other groups of vertebrates is lacking.

In humans, 128bp of the 5'-flanking region is sufficient for the TRH response. This region has multiple functional PIT1 binding sites (Steinfelder *et al.*, 1992). TRH induction leads to increased cAMP, but the effects of cAMP are likely mediated through PIT1, as there is no cAMP response element (CRE) in this region. Also, the effect can be mimicked in non-pituitary cell lines only in the presence of transfected PIT1 (Steinfelder *et al.*, 1992). In rat, PIT1 binds to the minimal promoter required for TRH responsiveness (Mason *et al.*, 1993). In all species studied, PIT1 is necessary but not sufficient to facilitate thyrotroph-specific gene expression of TSHβ. While PIT1 binding to DNA is critical, there was evidence of the involvement of another factor, later identified to be GATA2 (Gordon *et al.*, 1997). Both PIT1 and GATA2 bind to the TSHβ promoter. In mouse, 5'-PIT1 and 3'GATA2 binding sites along with 16bp of spacer make up a composite unit. The spacer contains additional putative overlapping PIT1 and GATA2 binding sites, and the sequence of the spacer is functionally important. When only the spacer is mutated with the flanking PIT1 and GATA2 binding sites left intact, there is markedly reduced binding of both transcription factors and almost no formation of the ternary complex (Gordon *et al.*, 2002). It is hypothesized that binding of one factor causes the DNA to bend and attain a conformation that permits and facilitates the binding of the other factor. Also, PIT1 and GATA2 physically interact with each other; the POU homeodomain of PIT1 interacts with the zinc finger domain of GATA2 (Gordon *et al.*, 2002). PIT1 also interacts with CREB binding protein (CBP) on the TSHβ promoter, but the functional implication of such interaction is not clear (Hashimoto *et al.*, 2000).

Prl is a peptide hormone with diverse functions and expression in several tissues, including the lactotrophs of the anterior pituitary gland (reviewed by Bole-Feysot *et al.*, 1998). In humans, transcription of prolactin involves 2 independent promoters: proximal 5kb involved in pituitary-specific expression and a more distal promoter for regulation in other tissues (Berwaer *et al.*, 1991, 1994). Dopamine negatively regulates prolactin (McLeod, 1969; Elsholtz *et al.*, 1991). Factors that stimulate Prl include TRH, oxytocin, VIP, epidermal growth factor (EGF), estradiol and phorbol esters (Day and Maurer, 1989). Several of these pathways lead to increased cellular cAMP levels in both mammals and birds, and the cAMP effect is thought to be mediated by PIT1 if no CRE are found in the minimal promoter sufficient for activation of the Prl promoter in response to the stimulus (Peers *et al.*, 1991). Also, PIT1 may or may not need to be phosphorylated to bring about the effect, suggesting the involvement of other factor(s) that interact with PIT1 in mediating the effect. The interacting factor(s) of PIT1 in regulating the *Prl* gene are several, including, but not limited to, Ets-1, GR, and Oct-1. A composite PIT1/Ets-1 binding site in the rat Prl promoter is required for mediating the induction of Prl by several stimulatory factors (Howard and Maurer, 1995; Bradford *et al.*, 1997). PIT1 physically interacts with GR to inhibit human Prl transcription (Nalda *et al.*, 1997), while PIT1 heterodimerizes with Oct-1to produce synergistic activation of the rat Prl promoter (Voss *et al.*, 1991; Verrijzer *et al.*, 1992).

PIT1 is the major regulator of *Gh* gene expression by pituitary somatotrophs, but other nuclear factors are essential for the highly cell-type specific expression of GH. The Zn finger transcription factor Zn-15, binds to so-called “Z boxes” in the rat *Gh* promoter and

in other species and regulates *Gh* transcription, and a mutation in the Zn-15 binding site leads to a notable reduction of GH synthesis (Lipkin *et al.*, 1993). GC induction of *Gh* is a well-studied phenomenon, and in most species the effect is mediated through GR binding to GREs in the *Gh* promoter. In species where a canonical GRE is absent, GR is thought to mediate its effect by GR tethering to other protein(s) already present on the *Gh* gene promoter. GHRH induction of *Gh* is known to increase intracellular cAMP levels and activation of the PKA pathway. The exact target of PKA is not known, nor has the mechanism leading to increased *Gh* been elucidated. PIT1 is thought to be involved in the cAMP-induced response, whereby PKA phosphorylates PIT1, leading to an altered conformation and increased binding to the *Gh* promoter. However, evidence available showing the requirement for PIT1 phosphorylation has been conflicting. There are two hypotheses: in one scenario, increased cAMP leads to CREB phosphorylation and activation, CREB binding to CREs present in the promoter and activating transcription. In the absence of functional CREs, however, it is hypothesized that the mechanism is CREB-independent, and according to the alternative hypothesis, activated PKA instead leads to phosphorylation of CREB-binding protein (CBP), which can then act as a co-factor of PIT1 (Cohen *et al.*, 1999). PIT1 dependent negative regulation of *Gh* is mediated by activin by reducing PIT1 binding to *Gh* promoter (Struthers *et al.*, 1992).

Pit1 mutations as a cause of human pathologies

A tightly regulated spatiotemporal pattern of transcription factor expression is essential for initiating and maintaining the ontogeny of the pituitary gland and maintaining the pituitary cells in their state of terminal differentiation. In general, factors expressed late

during pituitary development are more pituitary-specific than ubiquitous. Mutations of these late-expressed pituitary specific transcription factors, especially *Prop1* and *Pit1* are the most common causes of combined pituitary hormone deficiency (CPHD) (Cohen et al., 1996). Phenotypes of patients of *Pit1* mutations are similar to those of dwarf mice. These mutations affect somatotroph differentiation, and as a result, dwarfism is the most common phenotype associated with *Pit1* mutations (Pfäffle and Klammt, 2011). Patients with a mutation in *Pit1* show complete lack of *Gh* and *Prl*, and a loss of *TSH* is common but not always found. This phenomenon can have 2 explanations; either the thyrotrophs arise independently of PIT1 expression, and/or since the PIT1 regulation of target genes is highly context dependent, it is possible that PIT1 might primarily affect *Gh* and *Prl* compared to *TSHβ* (Drolet *et al.*, 1991; Lin *et al.*, 1994).

In humans, mutations of *Pit1* have been found in 2 introns and all exons (except exon 2). Some of the dominant negative mutations are Ser179Arg, Lys216Glu and Arg271Trp (Cohen *et al.*, 1999; Miyata *et al.*, 2006; Radovick *et al.*, 1992). Of these, the last two are sporadic in nature. Arg271Trp, although located in the homeodomain, does not interfere with DNA-binding, because it is immediately 3' of the two alpha helices required for binding. However, even if it binds DNA just like the wild type, it does not transactivate the target gene promoters, but the mechanism behind the inactivity is not known (Radovick *et al.*, 1992). In the case of dominant negative mutations, patients who are heterozygous carriers may or may not manifest CPHD, and the extent of manifestation seems to vary (Reynaud *et al.*, 2004). Also, the lack of transactivation by the dominant

negative mutants is not always reproducible *in vitro*, thus making it an active area of clinical research (Pernasetti *et al.*, 1998).

Pit1 and production traits

Since the well-studied PIT1 regulated genes, *Gh*, *Prl* and *TSHβ*, are all involved in metabolic functions such as energy balance and homeostasis, it was tempting to predict a role of PIT1 in production traits. Indeed, several studies have been done in agricultural animals to identify polymorphisms associated with agricultural performances. Single nucleotide polymorphisms of *Pit1* have been identified as genetic markers for reproductive (fertilization) rate in cattle (Khatib *et al.*, 2009). Agricultural traits such as body weight, wither height (Zhang *et al.*, 2009), milk yield and duration of reproductive life (Huang *et al.*, 2009) are a few others that are influenced by PIT1. Similarly, in pigs, birth weight (Song *et al.*, 2007), fat thickness (Franco *et al.*, 2005), growth, meat quality and carcass composition (Brunsch *et al.*, 2002) are correlated with *Pit1* polymorphisms which can be either in the introns or exons. Cashmere wool production in goats is determined by a polymorphism located at the 3'-UTR of *Pit1* (Lan *et al.*, 2009). In chickens, growth traits such as growth rate, but not carcass composition or fat content are associated with *Pit1* polymorphisms (Jiang *et al.*, 2004; Nie *et al.*, 2005).

Alternative forms of Pit1

Isoforms, by definition, are structurally and functionally similar proteins which differ from each other in their amino acid sequence. Isoforms may arise from the same gene or from different genes. When isoforms are derived from the same gene, they may be the

result of alternative transcription initiation sites, alternative translation initiation sites, alternative splicing or any combination thereof. All three mechanisms are well established modes of genome expansion.

Alternative forms of *Pit1* have been reported in almost all species where PIT1 expression has been studied (Voss *et al.*, 1991; Morris *et al.*, 1992; Theill *et al.*, 1991; Haugen *et al.*, 1994; Kurima *et al.*, 1998; Tanaka *et al.*, 1999; Van as *et al.*, 2000; Bastos *et al.*, 1991). These isoforms arise as a result of alternative translation and/or alternative splicing. There is considerable variation in the number and types of isoforms reported across species, but it is entirely possible that not all isoforms from a particular species have been identified.

As mentioned previously, PIT1 protein was seen to run in gels as a doublet of 33KDa and 31KDa in extracts from both murine and human primary tissues. Pulse-chase experiments showed that there was no precursor-product relationship between the two, and the presence of downstream in-frame AUG start codons in the *Pit1* sequence gave rise to the possibility that these two bands represented alternative forms of *PIT1*. Site-directed mutagenesis proved this hypothesis to be true. Both the isoforms could bind DNA sequences equally well, and both isoforms could transactivate the rat Prl promoter in CV-1 (non-pituitary) cells, which was surprising because the shorter isoform was lacking 27 amino acids from the transactivation domain. The relative levels of the 33- and 31KDa isoforms were roughly 2:1; however, how these levels are regulated in pituitary cells is not known (Voss *et al.*, 1991; Ingraham *et al.*, 1991). Soon afterwards, an alternatively

spliced isoform was discovered. This isoform differed from the original PIT1 in having a 78-nucleotide exon present at position 142, immediately after exon 1. This alternative form was name PIT1a. This isoform differed considerably in both abundance and function; the transcript levels of this longer isoform were approximately $1/7^{th}$ of *Pit1*, and this isoform showed minimal to no transactivation of the Prl promoter in Chinese hamster ovary (CHO) cells, even though it bound equally well to PIT1 binding sites of this promoter as the shorter 33kDa form. However, the alternatively spliced protein product was not detected in CHO cells by overexpression, and only minimal detection was found in rat pituitary cells. Thus, this isoform was thought to be either translated less efficiently or degraded at a more rapid rate. The ability of this isoform to regulate the *Gh* promoter even more actively than PIT1 in both pituitary and non-pituitary cells has been reported by all groups except one (Morris *et al.*, 1991; Theill *et al.*, 1992; Konzak and Moore, 1992).

A thyrotroph-specific isoform, *Pit1T*, arising as an alternatively spliced form was detected in the mouse. This isoform has an additional 14 amino acids at the 5'-end of exon 2 and is required, along with PIT1, for activation of the TSHβ promoter in thyrotrophs (Haugen *et al.*, 1993). Identification of PIT1T raised the number of isoforms in mouse to four. However, PIT1T was not detected in humans and rhesus monkey.

Conclusion

Somatotroph differentiation and *Gh* gene expression are tightly regulated processes requiring interaction among a large number of transcriptional regulators and signaling

pathways. Adrenal glucocorticoids are essential for both processes, and act in concert with other peripheral hormones. PIT1, the transcription factor necessary for the processes, is involved in the differentiation of cells of the PIT1 lineage, and also regulates other pituitary hormones. However, PIT1, even though necessary, is not sufficient, and requires participation of other general and specific transcription factors. Alternative forms of *Pit1* have been reported in numerous species, but the functional implications of having several isoforms with similar functions are not clear. Studies aimed at functional characterization of PIT1 isoforms in avian species are particularly lacking. The present study was aimed at characterizing the chicken PIT1 isoforms for their ability to regulate the c*Gh* promoter, and elucidating the mechanism behind functional differences, if any.

Chapter 2: Regulation of chicken growth hormone by PIT1 isoforms

Introduction

Growth hormone (GH) is a peptide hormone produced in specialized cells called somatotrophs in the anterior pituitary gland. Even though GH has a diverse range of functions in essential physiological processes, regulation of bone and muscle growth and metabolism are the main functions associated with it. These particular functions make regulation of somatotroph differentiation and GH synthesis an active area of research in agriculture and human medicine.

In broiler chickens, GH levels are high post-hatch. Exogenous GH administration when endogenous levels are high has no effect on chicken growth. However, *in ovo* administration of GH during embryonic development leads to increased growth post-hatch (Blumenthal, 1954), suggesting establishment of a growth threshold in the egg. Current work in our laboratory focuses on elucidating how extrapituitary signals are involved in the regulation of somatotroph differentiation and GH synthesis in the embryonic chicken. Chickens have the dual advantage of being agriculturally important as well as model animals for biomedical research. In addition, chickens are an excellent model system for studies of embryonic development and endocrine regulation because i) it is relatively cheap to obtain a large number of samples for study; ii) the precise timing of development of a large number of embryos can be synchronised with relative ease; iii) eggs grow without maternal endocrine influence, and iv) embryos are easy to manipulate externally.

More than a decade of research by our laboratory and by others has contributed significantly to dissecting the mechanism of somatotroph differentiation and *Gh* gene expression in chickens. Somatotrophs first appear on embryonic day 12 (e12) during development, and become a significant population by e16 (Porter *et al.*, 1995). A blood-borne extra-pituitary signal is required for somatotroph differentiation and final maturation. The signal was identified to be the adrenal glucocorticoid corticosterone (CORT) (Morpurgo *et al.*, 1997), which can induce premature differentiation of somatotrophs at e11 (Dean and Porter 1999; Jenkins *et al.*, 2007). CORT induction of somatotroph differentiation involves both type I mineralocorticoid receptor (MR) and type II glucocorticoid receptor (GR) (Bossis and Porter, 2004).

Mechanisms underlying CORT induction of *Gh* gene expression are largely unknown. However, the process seems to require ongoing protein synthesis, as suggested by the delay (~ 4hr) between CORT treatment and *Gh* induction and evidenced by inhibition of the process by cycloheximide (Bossis and Porter, 2003). Also, lack of functional GREs (glucocorticoid response elements) in 10kbp upstream and 5kbp downstream of the chicken *Gh* (*cGh*) gene suggests the CORT effect is indirect. Additional features of this pathway include involvement of ras and possibly MEK1/2 pathways (Ellestad, 2010) and chromatin remodeling at the *cGh* promoter (Narayana, 2011).

The POU-homeodomain transcription factor PIT1 is essential for *Gh* gene expression. Even though the intermediary factor involved in CORT-induction of *cGh* synthesis is not PIT1, this transcription factor is essential for pituitary specific GH expression in all

vertebrate species. PIT1 also regulates expression of other genes in the pituitary, namely prolactin (Prl), β-subunit of thyroid-stimulating hormone (TSHβ) and growth hormone-releasing hormone receptor (GHRH-R).

Pit1 gene structure and function have been conserved across vertebrate species. Mammalian *Pit1* comprises 6 exons and 5 introns, and avian/teleost *Pit1* has 7 introns due to the presence of a unique 38 amino acid exon (designated 2a for avian species) located between exon 2 and exon 3 (Van as *et al.*, 2000). When compared with other avian species, chicken PIT1 shows 97% sequence similarity with turkey PIT1, 93% with mallard duck and 89% with zebra finch. Significant homology was also observed with non-avian species such as mouse (84%) and Carolina anole lizard (81%). The differences among species is mostly located in the N-terminal region, while the C-terminal POU domain shows maximum conservation.

Variant forms of PIT1 have been identified in several species that arise by alternative transcription, translation and/or splicing. The first report of a variant form was in rat, where PIT1 was seen to migrate in a gel as a doublet of 33KDa and 31KDa. The shorter isoform arose by translation initiation at a downstream in-frame AUG codon and lacked the first 27 amino acid residues present in the 33KDa variant, but did not differ in its ability to activate the Prl promoter in CV-1 cells (Voss *et al.*; 1991). The second alternative form in mammals, identified in the same year (Morris *et al.*; 1991, Theill *et al.*; 1992) arose by alternative splicing and possessed a short exon of 26 amino acids inserted after exon 1 in the transactivation domain. This isoform was termed PIT1a by the

authors, and was shown to be unable to activate rat Prl promoter in CHO cells. Soon afterwards, this isoform was also reported by Theill *et al.*, (1992) in rats and was unable to activate PIT1 and Prl, while retaining its ability to activate *Gh*. Later works (Konzak and Moore, 1992) showed PIT1a (subsequently named PIT1β) to be a dominant negative repressor of Prl in pituitary cells. However, PIT1β is a repressor of PIT1 function only in pituitary cells; in non-pituitary cells, it actually potentiates a greater activation of rPrl promoter by the PKA pathway (Diamond *et al.*, 1999). Another mammalian species where multiple isoforms have been identified and characterized is the sheep (Bastos *et al.*, 2006), and this is the only report of characterization of all known isoforms (PIT1α, PIT1β, PIT1γ, and PIT1δ) of PIT1 in an agricultural species for their ability to activate the rat Prl promoter in HeLa cells. A thyrotroph-specific isoform, PIT1T, reported only in a few mammalian species, is known to activate the TSH-β promoter both alone and synergistically with PIT1 in non-thyrotroph pituitary cells (Haugen *et al.*, 1994). In general, the variants differ from each other in their N-terminal transactivation domain, while the C-terminal POU domain is conserved among the isoforms in most species.

Among avian species, 3 turkey PIT1 variants were reported by Kurima *et al.* (1998). These authors designated the avian PIT1 as PIT1* to differentiate from mammalian PIT1. tPIT1beta* (turkey PIT1beta) was structurally similar to mammalian PIT1a in having arisen by alternative splicing and hence including a beta-specific exon encoding 28 amino acids (compared to 26 amino acids in mammals). The identification of tPIT1W* by these authors was the first report of a unique avian-specific isoform arising by use of a transcription start site in intron1 followed by inclusion of the beta-specific exon. The

presence of PIT1 variants in chicken was first reported by Tanaka *et al.* (1999). However, this report showed only the PIT1* and PIT1W isoforms in layer chickens. Later, Van as *et al.* (2000) reported the presence of all three isoforms, ggPIT1* (*Gallus gallus* PIT1), ggPIT1beta, and ggPIT1W* from Hybro G broiler strains (Fig. 4). Their findings also matched the turkey isoforms in their relative abundance, with PIT1* being most abundant, PIT1W* of intermediate abundance and PIT1beta* being least abundant. They also adopted the nomenclature PIT1α for the canonical form, PIT1β for the derivative of PIT1α with the β-specific exon, and PIT1γ for the equivalent of PIT1W* of Kurima *et al.* (1998), and this nomenclature will be followed in this report.

In fish, *Pit1* has been cloned so far in chum salmon (Ono and Takayama, 1992), rainbow trout (Yamada *et al.*, 1993), Chinook salmon (Majumdar *et al.*, 1996), Atlantic salmon (Lorens *et al.*, 1996), gilthead seabream (Martinez-Barbera *et al.*, 1997), ayu (Chiu *et al.*, 2002) and goldfish (referenced in Ip *et al.*, 2004). In all fish species for which *Pit1* has been cloned, only one variant has been identified. Chiu *et al.* (2002) tested the ability of ayu PIT1 to activate the zebrafish *Gh* promoter, and it was found that PIT1 activated zebrafish *Gh* promoter in NIH3T3 cells.

Even though multiple isoforms are found across many vertebrate species, the functional implications of having multiple isoforms are not yet fully known. Substantial research by the laboratory of Gutierrez-Hartmann in elucidating the function of the PIT1β in general and of the β-specific exon in particular in relation to the regulation of Prl has shown that the specific amino acid sequence of the β-specific domain is important for mediating the

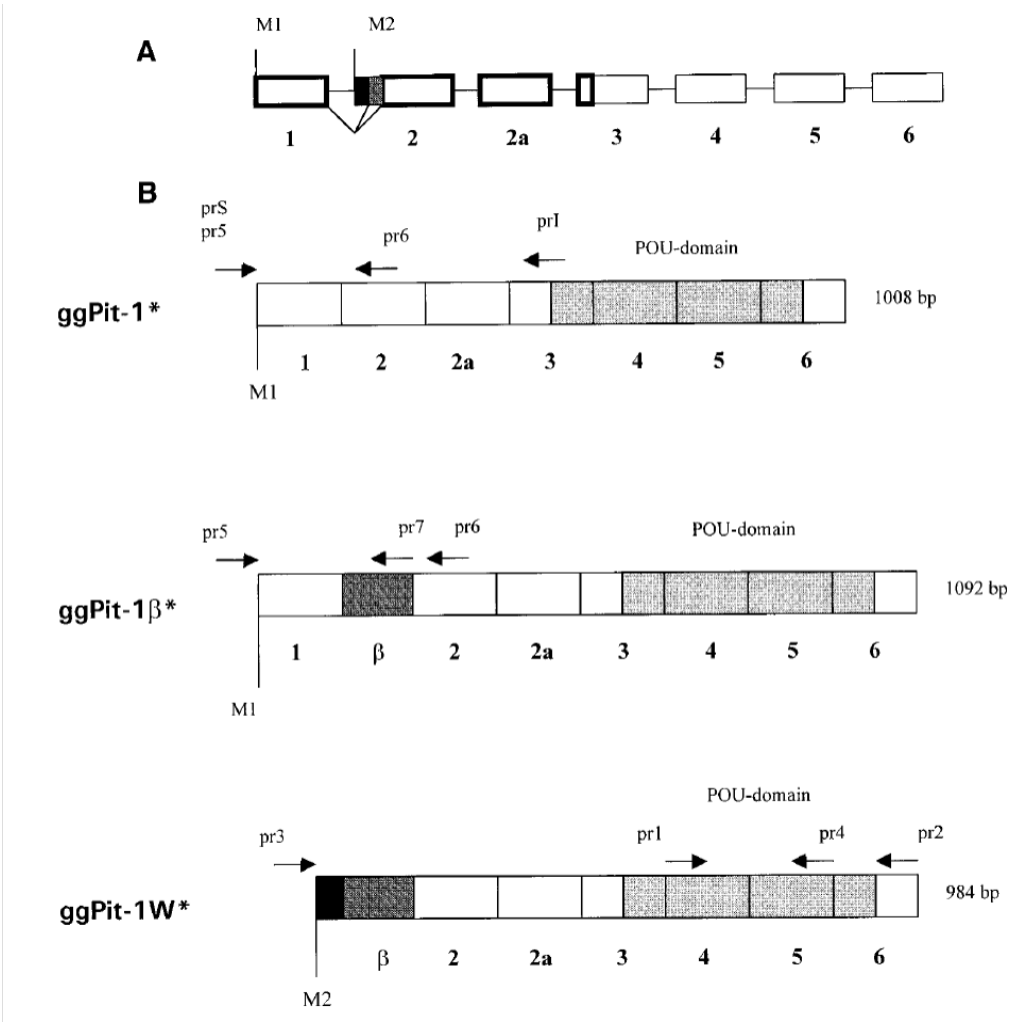


Fig. 4 Chicken *Pit1* gene and isoforms. (A) Chicken *Pit1* comprises of 7 exons and 6 introns, shown in (A). Region in bold encode the transactivation domain. The avian specific exon is numbered 2a. (B) Chicken *Pit1* isoform mRNAs. Alternative forms arise by using alternative transcription initiation and alternative splicing. β denotes 28amino acid insertion. Arrows denote the positions of primers used by authors in their study (Van As *et al.*, 2000)

dominant negative repression of Prl in pituitary cells (Diamond and Gutierrez-Hartmann, 1996). These same sequences make PIT1β a more potent PKA-mediated activator of Prl in non-pituitary cells (Diamond *et al.*, 1999). The same group later showed that the repression of Prl in pituitary cells is brought about by chromatin modification (specifically, reduction of acetylated state of histone H4) by 5 hydrophobic residues present in the β-domain (Diamond and Gutierrez-Hartmann, 2000) and also by inhibiting CREB-binding protein (CBP) recruitment to the Prl promoter (Ferry *et al.*, 2005). Genes regulated by PIT1 are characterized by the presence of one or more PIT1 binding site(s) in the proximal promoter. The *cGh* promoter has two avian-specific PIT1 binding sites at location -113/-104bp (proximal) and -541/-533bp (distal). Activation of this promoter was PIT1 dependent and of the two sites, only the proximal has been shown to be functional in PIT1 binding by mutational analysis (Ip *et al.*, 2004).

Even though a monomeric configuration is favored in solution, PIT1 is known to bind DNA as either a homodimer or a heterodimer. The bipartite structure of the PIT1 DNA-binding region consisting of a POU-homeodomain and POU-specific domain (POU-HD and POU-S, respectively) separated by a linker gives POU proteins the flexibility to orient themselves in various configurations on DNA. This flexibility makes it possible for PIT1 to regulate target genes in conjunction with other regulatory factors in various cell types. X-ray diffraction studies show that PIT1 contacts very few base pairs of the DNA, and that the two components of a dimer can bind to perpendicular faces of DNA by altering the spacing between them. In this respect, PIT1 differs from Oct-1, in which the POU-HD and POU-S bind on opposite faces of the DNA element (Jacobson *et al.*, 1996).

PIT1 was shown to bind as a monomer and dimer to Prl and GH binding sites, respectively (Holloway *et al.,* 1992). However, this result is not in agreement with later work by Sporici *et al.,* (2005). Apart from structural studies, PIT1 homo- and hetero-dimerization has been demonstrated by GST- pull down assays and gel-shift assays with both wild type and dimerization-deficient mutants (Ingraham *et al.,* 1990, Sporici *et al.,* 2005).

The promoter of the *Gh* gene has been characterized in several species, including human, mouse, dog, cow, rat, chicken and several fish species, and the characteristic features that are similar or distinct among these vertebrates have been discussed in the literature review. One aspect of *Gh* regulation that will be reiterated here is that GH synthesis and secretion are regulated by signaling pathways leading to elevated intracellular cAMP levels. If the *Gh* promoter in question contains functional cAMP response element (CRE) motifs, the effect is most likely mediated via activated protein kinase A (PKA), which phosphorylates CREB (CRE-binding protein), leading to CREB binding to CRE and recruiting CREB-binding protein (CBP) (Chrivia *et al.,* 1993; Arany *et al.,* 1994; Kwok *et al.,* 1994) . CBP regulates transcription by its histone acetyl transferase (HAT)-activity by interaction with the basal transcription machinery (Ozryzko *et al.,* 1996; Kee *et al.,* 1996). However, in the absence of functional CRE motifs, as is the case of chicken *Gh*, rat *Gh* and several other cAMP-regulated promoters, the mechanism is thought to be CREB-independent, involving direct interaction of CBP to specific transcription factors (in the case of *Gh*, PIT1) (Gonzalez and Montminy, 1989; Chawla *et al.* 1998, Zanger *et*

al., 1999). Surprisingly, in spite of the presence of functional CREs, cAMP regulation of the human *Gh* promoter is CREB-independent (Cohen *et al.,* 1999).

Since almost all target genes of PIT1 are activated by signals that involve activation of kinases, a pertinent question to ask was if PIT1 is a target of phosphorylation. Initial investigations identified two threonine residues in the PIT1 homeodomain (Thr-219, Thr-220) and a serine residue (Ser-115) of rat PIT1 as targets of phosphorylation (Kapiloff *et al.*, 1991). Among these residues, Thr-220 was initially determined, and later confirmed, to be the predominant site (Howard and Maurer, 1994). Phosphorylation of PIT1 at Thr-220 altered PIT1 binding to proximal and distal binding sites on the *Gh* and *Prl* promoters, as well as decreased dimerization of PIT1 on some of them. Altered binding and dimerization was attributed to altered conformation of PIT1, brought about by phosphorylation. Steinfelder *et al.* (1992) reported similar results for phosphorylation of PIT1 by TRH and cAMP for the activation of the human TSHβ promoter. In all cases, the signaling pathways involved were PKA and PKC, and sequences immediately upstream of the PIT1 binding sites were thought to dictate binding of phosphorylated PIT1. However, PIT1 phosphorylation mutants, in which all three phosphorylation sites were mutated to alanine, retained most of the transactivational properties of wild type PIT1 to activate the rat *Prl* and *Gh* promoters in HeLa cells (Fischberg *et al.*, 1994). Similarly, it was shown that even though PIT1 was essential for activation of the Prl promoter by cAMP, PIT1 phosphorylation was not required (Okimura *et al.,* 1994), suggesting that other phosphorylation-dependent components may be involved in the process. Caelles *et al.* (1995) suggested a mitosis-specific phosphorylation of PIT1 by a cell-cycle specific

kinase. Such specific phosphorylation is believed to be required for PIT1-dependent proliferation of cells of the PIT1 lineage. Subsequent identification of *c-fos* as a phospho-PIT1 regulated gene (Gaiddon *et al.*, 1999) lends traction to a role of PIT1 phosphorylation in cell cycle regulation.

While some body of knowledge exists regarding PIT1 isoform-specific regulation of the Prl promoter, not much is known about the *Gh* promoter. For instance, it is not even known what other general or specific factors interact with PIT1 to regulate cell-type specific expression of the *Gh* gene. Recent results from our laboratory have suggested the involvement of Ets-1 as a regulator of CORT-induced *Gh* gene expression (Knubel, 2010). At the time this project was started, PIT1 isoforms had not been functionally characterized in any avian species. Therefore, we decided to characterize the chicken PIT1 isoforms for their ability to regulate the *cGh* promoter. Our specific objectives included:1) Define the transcription start site of all chicken *Pit1* isoforms; 2) Demonstrate that all known isoform mRNAs can be translated into functional proteins; 3) Determine the sub-cellular localization of the isoforms; 4) Determine which, if any, of the known isoforms regulate the chicken *Gh* promoter; 5) Demonstrate PIT1 isoform protein binding to the *cGh* promoter; 6) Investigate the effects of simultaneous expression of multiple isoforms on activation of the *cGh* promoter; 7) Determine if the isoforms bind DNA as homo- or heterodimers; 8) Identify which, if any of the isoforms interact with CBP; and 9) Determine if PIT1 is phosphorylated in response to CORT in chicken embryonic pituitary cells. Successful completion of this project will be the first report of PIT1 isoform-specific regulation of a gene regulating agriculturally important traits in an

agriculturally important avian species and enrich our knowledge of regulation of *Gh* gene expression in broiler chickens.

Materials and methods

Animal use

Broiler (Ross x Ross) chicken eggs, when used, were obtained from Allen's Hatchery (Seaford, DE). Embryonated eggs were incubated at 37.5°C in a humidified incubator (GQF Manufacturing, Savannah, GA) with rotation every hour. The day that the eggs were placed in the incubator, was designated e0, and eggs were removed on various stages of development during the 21 day incubation period according to experimental requirements. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland. For some experiments, cells from several embryos were pooled depending on the number of cells required.

Cell Culture and Transfections

The anterior pituitary gland of the embryos was removed using a dissecting microscope and dispersed to single cells using trypsin digestion and mechanical agitation as described (Porter *et al.*, 1995). For endogenous total protein extraction, cells were allowed to attach to poly-L-lysine coated cell culture plates overnight. Treatments, if any, were applied 24 hr after the cells were plated. For transient transfection experiments, cells were transfected following dispersion, plated on poly-L-lysine coated plates and allowed to express the transfected gene. Dispersed chicken embryonic pituitary cells were maintained at 37.5°C/5% CO_2 atmosphere in Dulbecco's modified Eagle's medium:Ham's nutrient mixture F12 (DMEM/F12) without serum, supplemented with 0.1% BSA, 100U/ml penicillin, and 100µg/ml streptomycin, and 5µg/ml human insulin.

Leghorn Male hepatoma (LMH) cells [American Type Culture Collection (ATCC), Manassas, VA], a chicken carcinoma cell line, were maintained in Waymouth's medium supplemented with 10% fetal bovine serum (FBS) with 100U/ml penicillin and 100µg/ml streptomycin in a humidified incubator at 37.5°C and 5% CO_2 atmosphere. Cells were split by recovering with 0.25% trypsin/ 0.03% ethylenediamine tetraacetic acid (EDTA) when confluent and plated in cell culture flasks (Corning Life Sciences, Lowell, MA) coated with 0.1% gelatin (Sigma, St. Louis, MO). All cell culture media and media supplements were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. For transient transfection in cell lines, cells (LMH) were plated in either 6-well or 24-well culture plates (Corning Life Sciences) coated with 0.1% gelatin (Sigma) and allowed to grow overnight to a confluence of 90-95%. Typically, $3.5x10^5$ cells were used for 24-well plates and $1.0x10^6$ cells were used for 6-well plates. The following day, cells were transfected using Lipofectamine-2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol in OPTI-MEM for 6 hr, following which the medium was replaced with Waymouth's medium with 0.01% BSA supplemented with penicillin and streptomycin.

Cloning of untagged and tagged isoform constructs

The primers used for cloning full-length *Pit1* isoforms are listed in Table 1. All primers were obtained from Sigma-Aldrich (St. Louis, MO) unless mentioned otherwise. Chicken pituitary cDNA (e14) was amplified using Phusion Flash proof-reading polymerase (Finnzymes Inc., Woburn, MA), and the gel-extracted amplicon was digested with

Table 1. Primer names and sequences used for cloning

Name	Sequence
Pit1_ab_HindIII_F	CCCAAGCTTGGGGCCACCATGACTTGCCAAGCGTTTGCTTCATC
Pit1_g_HindIII_F	CCCAAGCTTGGGGCCACCATGTATCTTGAATCCTCATGCATTTTCTT ACC
Pit1_abg_HindIII_R	CCCAAGCTTGGGTTACCGGCACTCGTGGTGCTC
Pit1_b_28end_R	ACACGTTTCCCGAAGTCATC
HindIII-ATG-HA-PIT1a/b	CCCAAGCTTGGGGCCACCATGTACCCATACGACGTCCCAGACTACG CTATGACTTGCCAAGCGTTTGCTTCATC
HindIII-ATG-myc-PIT1a/b	CCCAAGCTTGGGGCCACCATGGAACAAAAACTTATTTCTGAAGAAG ATCTGATGACTTGCCAAGCGTTTGCTTCATC
HindIII-ATG-HA-PIT1Beta2	CCCAAGCTTGGGGCCACCATGTACCCATACGACGTCCCAGACTACG CTATGCGACCAATGTTGTCTCCACAGAC
HindIII-ATG-myc-PIT1Beta2	CCCAAGCTTGGGGCCACCATGGAACAAAAACTTATTTCTGAAGAAG ATCTGATGCGACCAATGTTGTCTCCACAGAC
HindIII-ATG-HA-PIT1g	CCCAAGCTTGGGGCCACCATGTACCCATACGACGTCCCAGACTACG CTATGTATCTTGAATCCTCATGCATTTTCTTACC
HindIII-ATG-myc-PIT1g	CCCAAGCTTGGGGCCACCATGGAACAAAAACTTATTTCTGAAGAAG ATCTGATGTATCTTGAATCCTCATGCATTTTCTTACC
cCBP-1674-2447-HindIII-F	CCCAAGCTTGGGGCCACCATGTGGGAGTTCTCCTCGCTGCGC
cCBP-1674-2447-myc-HindIII-R	CCCAAGCTTGGGCTACAGATCTTCTTCAGAAATAAGTTTTTGTTCCA AACCCTCCACAAATTTTTCTAACG
cCBP-1674-2447-Test-F	AGCTGCGATCTGATGGATG
cCBP-1674-2447-Test-R	ATAAAAGTGATGCTCCCATAATGC

HindIII (Invitrogen, Carlsbad, CA) at 37°C for 4 hr in appropriate buffer. DNA from the restriction digestion reactions was purified and quantified by comparing to the intensity of the band(s) of the DNA ladder I (Genesee Scientific, San Diego, CA). Different insert:vector ratios (typically 1:1 and 3:1) were ligated non-directionally into pCMV-Sport6.1 mammalian expression vector. Successful ligation was determined by diagnostic PCR using vector specific M13-forward and M13-reverse primers, and 2μl of the ligation reaction was transformed into DH5α Max Efficiency chemically competent cells (Invitrogen). Plasmid DNA was extracted using Nucleobond PC-500 Plasmid DNA purification kit (Macherey-Nagel, Bethlehem, PA) from ampicillin-resistant bacterial colonies grown overnight in the presence of 100μg/ml ampicillin, screened for directionality by PCR, and sequenced at the University of Maryland Center for Biosystems Research. Each clone was sequenced in two directions in its entirety to ensure absence of mutations.

For generating N-terminally hemagglutinin (HA)- and c-myc-tagged clones, forward primers were designed to contain a Hind-III site, a Kozak sequence, and the HA or c-myc sequence in that order. A single primer amplified *Pit1*α and *Pit1*β1, while two other primers were designed for *Pit1*β2 and *Pit1*γ. The forward primers were used in conjunction with a common anti-sense primer. For C-terminal tagging, a similar approach was used, but only one antisense primer was designed which contained the tag, a stop codon and a HindIII site. These tagging primers were used with isoform specific forward primers. For all tagging, the plasmid containing untagged isoform cDNA was used as template.

For cloning cDNA encoding C-terminal 784 amino acids of chicken CREB-binding protein (cCBP), primers were designed to amplify cDNA corresponding to the 3'-2402bp of the cCBP (Genbank Accession no. XM_414964). Chicken pituitary cDNA was amplified using these primers. The product of PCR was purified and further amplified with a forward primer encoding a HindIII site and Kozak sequence and a reverse primer encoding region a c-myc tag, stop codon and a HindIII site. Product obtained from the second round of PCR was purified and ligated into pSport6.1 expression vector and transformed into XL10B maximum efficiency competent cells. Plasmid DNA was isolated from positive clones, and the insert sequenced in its entirety in two directions.

5'RACE of Pit1isoforms

Primers used for 5'-RACE are listed in Table 2. Total RNA was extracted from e11 chicken pituitaries using the RNeasy mini kit (Qiagen, Valencia, CA) and quantified using Quant-iT Ribogreen RNA Quantitation Reagent (Invitrogen, Carlsbad, CA). Total RNA (1μg) was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen) in a 20μl reaction using the common reverse primer (GSP1) used for cloning *Pit1* isoforms. A negative control containing no reverse transcriptase was performed alongside to ensure there was no genomic DNA contamination. Synthesized cDNA was purified with Wizard SV® Gel and PCR Clean-Up System (Promega, Madison, WI). Half of the cDNA obtained was tailed with dCTP by Terminal Transferase (TdT) (New England Biolabs, Ipswich, MA), while the other half served as a control reaction without TdT. Five microliters of the tailed reaction was amplified with forward Abridged Anchor Primer (AAP) (Invitrogen) and GSP1 (reverse primer). The first round of PCR was

Table 2. Primers used for 5'RACE

Name	Sequence
5'RACE_GSP1	TTACCGGCACTCGTGGTGCTC
5'RACE_GSP2	GCTCAGCTGCAAGTTCTCAA
5'RACE_GSP3-alpha	GAGTAGTGTAGTCCTGTGGAGACAACA
5'RACE_GSP4/5-Beta/gamma	GGCACAGAGTAGTGTAGTCCTGCTG
5’RACE_Diag_Beta2	GGAACATTTAGGAGTCTGTGGAGACAAC

followed by a second round of PCR in which AAP was replaced with Abridged Universal Anchor Primer (AUAP) (Invitrogen), while two different reverse primers were used as antisense: GSP3, annealing to a sequence present in *Pit1*α only, and GSP4/5, annealing to *Pit1*β1, *Pit1*β2 and *Pit1*γ. Specific *Taq*-amplified bands obtained in these amplification reactions were gel-purified and cloned into pGEM-T Easy Vector (Promega) by T/A cloning. Ligation reactions were transformed into JM109 cells and screened with blue/white screening. Colonies picked were grown overnight in TB broth with ampicillin in 96-well plates. After about 16 hr of growth, 100µl of the culture were transferred to a 96-well plate and centrifuged at 1000xg for 5 min at 4°C. The supernatant was discarded, and the cells were resuspended in 50µl deionized water. The plates were sealed and cells were lysed in a thermocycler at 95°C for 5 min. These crude bacterial lysates were used as template in PCR with SP6/T7 primers to determine which clones contained an insert. Plasmid DNA was extracted from clones containing insert by the alkaline lysis method (Sambrook *et al.*) and sequenced as above.

Immunocytochemistry

Cells (LMH) were plated at $1x10^5$ per chamber in Lab-Tek II 2-chamber slides (Nalge Nunc International, Naperville, IL) and allowed to reach ~90% confluency. Cells were transfected with 2µg *Pit1* isoform expression vector with Lipofectamine-2000. Empty expression vector was used as negative control. The following day, cells were washed twice with ice-cold PBS. For each wash, media in slides were decanted; PBS was added, and slides were rocked for 10 min at room temperature. Following washing, cells were fixed with freshly prepared 4% formaldehyde solution pH 7.5 (Thermo Scientific,

Rockford, IL) for 20 min. Slides were washed two more times, and permeablized in PBS with 0.1% Tween-20 and 0.1% Triton-X 100 for 8 min at room temperature. Chambers were removed, and the slides were washed 2 more times with PBS. Blocking was done for 1 hr at room temperature in PBS with 5% normal goat serum (NGS) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Cells were washed twice with PBS and incubated with PIT1 antiserum (1:1000 dilution) at 4°C overnight in PBS/ 2% NGS. Primary antibody was excluded from one additional chamber transfected with *Pit1α* as a control. The next day, cells were washed first in PBS then in PBS/0.1% Tween-20, and incubated with Rhodamine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:50 in PBS/0.025% Tween-20 with 2.5% NGS for 1 hr at room temperature in the dark. Following secondary antibody incubation, cells were washed with PBS and incubated with 5ng/ml 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific) for 10 min in the dark. Cells were then washed thoroughly, mounted in fluorescent-dye compatible mounting medium Fluoromount-G (Southern Biotech, Birmingham, AL) and stored in the dark. Cells were examined with a Zeiss AxioObserver.Z1 inverted microscope, and images were acquired using the Zeiss Axiovision Re1 4.6 software with the Zeiss Axiocam HRC camera.

Western blot

To determine expression of untagged and tagged constructs of *Pit1* and C-terminal c-myc tagged cCBP fragment (1677-2442), LMH cells were plated at $1x10^5$ per well in 6-well plates overnight until the cells were ~90% confluent. Cells were transfected with 4µg expression plasmid using Lipofectamine 2000 (Invitrogen) according to

manufacturer's instructions. Cells were allowed to express the transfected gene for 24 hours, and then cells were lysed in100µl per well of lysis buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100)] containing 1mM phenylmethylsulfonyl fluoride (PMSF) and HALT™ Protease and Phosphatase inhibitor cocktail (Thermo Scientific) by rocking on ice for 15 min. Plates were scraped, and all contents were transferred to microfuge tubes and incubated on ice for 15 min with vortexing every 3-4 min. Lysed cells were then centrifuged at 14000xg for 15 min at 4°C. Supernatants were transferred to a fresh tube and stored at -80°C. For total protein extraction from whole pituitaries, pooled (3-4) pituitaries were homogenized with a Dounce homogenizer in 200µl lysis buffer and centrifuged as above. Protein samples were quantified with Coomassie Plus (Bradford) Protein Assay kit (Thermo Fisher Scientific, Inc., Rockford, IL). Desired amounts of protein samples were diluted with 50mM Tris-HCl, pH 7.2, and then further diluted in an equal volume of Laemmli Sample Buffer (LSB) (BioRad, Hercules, CA) with 5% β-mercaptoethanol and resolved in 10% (for PIT1) or 15% (for cCBP) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) in Tris-glycine buffer (25mM Tris, 192mM glycine, 0.1% SDS). Electrophoresed proteins were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) using a Trans-Blot SD Semi Dry Electrophoretic Transfer Cell (Bio Rad), blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS/T) for 2 hr at room temperature or overnight at 4°C. Primary antibody incubation duration and dilutions were according to manufacturer's instructions. The PIT1 antiserum used in all experiments was raised in rabbit against bacterially expressed full length rat PIT1 and was a kind gift from

Dr. Simon J. Rhodes of Indiana University School of Medicine, Indianapolis, Indiana. For over-expressed proteins, the PIT1 antibody was used at a dilution of 1:1000 overnight in TBS/T with 1% non-fat dry milk and 1% normal goat serum (NGS) (Jackson Immunoresearch) at 4°C. For endogenous PIT1 from chicken pituitary cells, a 1:500 dilution was used. Rabbit anti-HA antibody (#H6908, Sigma) and mouse monoclonal anti-c-myc antibody (#A00704, Genscript, Piscataway, NJ) were both used at 1:1000 dilution in TBS/T with 1% non-fat dry milk and 1% NGS. The next day, membranes were washed thoroughly in TBS/T with 1% milk and incubated with horse-radish peroxidase (HRP) conjugated secondary antibody (1:5000) in TBS/T with 1% non-fat dry milk for 2 hr at room temperature at the manufacturer's recommended dilution. After extensive washing, blots were developed using Lumi-Glo enhanced chemiluminescent detection reagent (Cell Signaling Technologies, Danvers, MA). If required, blots were stripped in buffer containing 62mM Tris-HCl (pH 6.7), 100mM β-mercaptoethanol and 2% SDS at 50°C for 30 min with occasional rocking. Membranes were washed in large volumes of TBS/T. Blocking and subsequent steps were performed as mentioned previously.

Proteasome inhibitor assay

For proteasome inhibitor assay, LMH cells were plated at $1x10^6$ per well in 6-well plates and allowed to attach overnight. Cells were transfected with 4μg *Pit1* isoform expression vector or empty vector and allowed to express the transfected gene for 24 hours, following which cells were treated with 20μM MG-132 (Cayman Chemical, Ann Arbor, MI) for 0, 1.5 hr or 6 hr. At the end of incubation, cells were washed with ice-cold PBS

and lysed in cell lysis buffer, and total protein was extracted and quantified as mentioned previously. Equal amounts of total protein from each treatment were separated by 12% SDS-PAGE, transferred to PVDF membrane and blotted with PIT1 antiserum (1:1000). HRP-conjugated goat anti-rabbit IgG (1:5000; KPL Inc., Gaithersburg, MD) incubation was for 2 hr at room temperature in TBS/T with 1% non-fat dry milk. Detection of immunoreactive bands was performed as mentioned previously.

Analysis of promoter activity

For analysis of promoter activity, LMH cells were used. Cells were plated at $3x10^5$ per well in 24-well plates in Waymouth's medium with 10% FBS and penicillin/streptomycin and allowed to attach for 6 hr in plates coated with 0.1% gelatin. Cells were then visually inspected to ensure proper attachment. Medium was replaced with Opti-MEM, and cells were transfected with Lipofectamine-2000 according to the manufacturer's instructions. Typically, each well received 3 plasmids: *Pit1* isoform expression vector (or empty vector), *cGh* promoter reporter vector pGL3-1727 (or empty reporter vector, pGL3-basic) and normalization vector. Empty reporter plasmid and normalization plasmids were purchased from Promega. *cGh* promoter reporter vector was a kind gift from Dr. F.C. Leung (University of Hong Kong). Cells were transfected in Opti-MEM for 6 hr, after which transfection medium was replaced with serum-free Waymouth's medium supplemented with BSA and Penicillin/Streptomycin. Cells were allowed to express transfected genes for 24 hr, after which cells were washed once with PBS and lysed in 100µl passive lysis buffer (Promega) with rocking for 15 min. Cell lysates were collected in a fresh microfuge tube and stored at -20°C until assay. Samples were assayed for

firefly luciferase (reporter) and renilla luciferase (normalization) activity using the Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. For each sample, the ratio of firefly over renilla was determined and reported as such. Data are presented as mean + SEM of at least three replicate experiments., and $p < 0.05$ was considered significant.

Electrophoretic mobility shift assay (EMSA)

5'-infra-red labeled EMSA probes for the proximal chicken PIT1 binding site were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 3. Equal amounts of 20μM sense and anti-sense probe dilutions were annealed in a thermocycler (95°C 5 min, followed by slow cooling in thermocycler) to obtain 10μM double-stranded DNA stock solutions. Probe stocks were diluted 1:200 in TE buffer to make working dilutions (50nM). Total protein (5μg) from LMH cells transfected with titrated amounts of individual *Pit1* isoform expression vectors to yield equal protein expression were used for EMSA. Protein samples were incubated in the dark with either labeled PIT1 probe, labeled PIT1 scrambled probe, or a labeled PIT1 probe with 100-fold excess of unlabeled probe in binding buffer for 30 min at room temperature (RT). Binding reactions contained buffer (10mM Tris, 50mM NaCl, 1 mM DTT, pH 7.5), 50ng sheared salmon sperm DNA, 5mM $MgCl_2$, 2.5mM DTT, 0.25% Tween 20 and 0.2% NP-40. At the end of incubation, 2μl of 10x Orange G loading dye (LI-COR Biosciences, Lincoln, NE) was added to each reaction, and the entire binding reaction was loaded onto 6% non-denaturing polyacrylamide gels pre-run for 30 min in the cold room. Electrophoresis was performed at 70V at 4°C in the dark, after which the gels were

Table 3. EMSA Probes

Name	Sequence
PIT1_Proximal_S	ATGGCGAACACATCTGCATTTATGCAAGGA
PIT1_Proximal_AS	TCCTTGCATAAATGCAGATGTGTTCGCCAT
PIT1_Proximal_Scrambled_S	ATGGCGAACAACCGAAGCCAGATGCAAGGA
PIT1_Proximal_Scrambled_AS	TCCTTGCATCTGGCTTCGGTTGTTCGCCAT

scanned by Odyssey infra-red scanner (LI-COR) at intensity 4. For statistical analysis, background-subtracted average intensity of 3 replicate experiments was subjected to ANOVA after blocking for gel.

Co-immunoprecipitation

For co-immunoprecipitation to detect interaction between isoforms, LMH cells were plated at $1x10^6$ cells per well in 6-well plates. Cells were transfected with 4µg of N-terminally HA- or c-myc tagged *Pit1* isoform expression vectors using Lipofectamine 2000 according to manufacturer's instructions. Twenty four hours after transfection, cells were washed twice with ice cold PBS with 1mM $MgCl_2$, 0.1mM $CaCl_2$ and lysed in lysis buffer (10mM HEPES, 150mM NaCl, 1mM EGTA, 0.1mM $MgCl_2$) supplemented with PMSF and HALT™ Protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Cells were rocked in lysis buffer for 30 min on ice, scraped and centrifuged at 14000xg for 15 min. The supernatant was transferred to a fresh 1.5mL tube, and an aliquot was quantified using Coomassie Plus™ protein assay reagent. For each transfection, equal amounts of fresh whole cell lysate (typically 300µg) were immunoprecipitated using either 1.0µg mouse monoclonal anti-c-myc antibody (Genscript) or normal mouse IgG (Jackson Immunoresearch). Lysates were incubated in 500µl total volume for 1 hr at 4°C with end-over-end turning. Protein A/G agarose beads (Cell Signaling Technologies Inc.) (20µl per tube) were added, and the entire reaction was incubated at 4°C overnight with turning. The beads were then washed four times in lysis buffer (composition as before, but with no protease and phosphatase inhibitor). Briefly, tubes were centrifuged at 1000xg for 5 min at 4°C, and the supernatant was

carefully aspirated out. Fresh lysis buffer (1ml) was added to each tube, and tubes were rotated at 4°C for 10 min. At the end of the last wash, beads were resuspended in 20µl LSB with β-mercaptoethanol, boiled, and resolved by SDS-PAGE (10% gels). PVDF membranes containing the electrophoresed proteins were blocked with 5% non-fat dry milk in TBS/T overnight at 4°C. The following day, membranes were washed in TBS/T and incubated with primary antibody [1:1000 rabbit anti-HA (Sigma)] overnight at 4°C. After washing extensively with 1% non-fat dry milk in TBS/T, membranes were incubated with 1:5000 dilution of HRP-conjugated goat anti-rabbit IgG (KPL). Immunoreactive bands were detected as mentioned previously. Membranes were then stripped, and re-probed with anti-PIT1 anti-serum (1:1000) as described previously.

Co-immunoprecipitation for detecting interaction of isoforms with cCBP(C-terminal myc-tagged C-784 amino acids) was done essentially the same way, except that 15% gels were used for electrophoresis. Lysates from cells transfected with empty vector, *Pit1* isoforms, C-terminal cCBP or both *Pit1* isoforms and C-terminal cCBP were immunoprecipitated using c-myc antibody, and membranes were blotted with anti PIT1 antiserum.

Detection of phosphorylated PIT1

Chicken pituitaries (e11, 120 per replicate) were dissected out and dispersed as described previously. The cells obtained were plated as 2 wells per treatment in a 6-well plate and cultured for 24 hr in DMEM/F12 supplemented with BSA, Penicillin/Streptomycin and insulin. The following day, cells were treated for 0, 1.5 or 6 hr with 10^{-7}M CORT. At the

end of CORT treatment, cells were washed twice with ice-cold PBS/Ca/Mg and lysed in 250µl lysis buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100)] containing 1mM PMSF and HALT™ Protease and Phosphatase inhibitor cocktail (Thermo Scientific) per well for 15 min on ice with rocking. Lysates were collected and centrifuged at 14000xg for 20 min at 4°C. The supernatant was transferred to a fresh tube and protein yield was quantified as mentioned previously.

For antibody-bead crosslinking, 70µl of Protein A/G agarose (Cell Signaling Technologies, Inc.) beads were washed twice in PBS and incubated in the presence of 20µl PIT1 antiserum diluted in 910µl PBS for 4 hr at 4°C with end-over-end turning. At the end of incubation, beads were washed once with ice-cold PBS to wash off unbound antibody. The antibody-bead mixture was crosslinked with 20mM Dimethyl pimelimidate dihydrochloride (DMP; Sigma) in 0.2M triethanolamine (Sigma) pH 8.7 for 30 min at room temperature with rotation. Beads were centrifuged at 1000xg for 5 min, and the cross-linking was repeated with fresh triethanolamine/DMP solution. The reaction was quenched with 0.2M ethanolamine pH8.0 (Santa Cruz Biotechnology) for 1 hr at room temperature with rotation. After quenching, beads were aliquoted into 3 tubes.

Typically, 200µg of total protein was immunoprecipitated in IP buffer (composition same as lysis buffer except with 0.1% Triton X-100) with either PIT1 antiserum (cross-linked to beads) or normal rabbit serum in 500µl total volume at 4°C overnight with rotation. The next day, immunoprecipitates were washed 4 times with IP buffer. For each wash, reactions were centrifuged at 1000xg for 5 min at 4°C, the supernatant was aspirated off,

and beads were rotated for 10 min at 4°C in fresh buffer. At the end of the final wash, 20µl of LSB was added to the beads and incubated at 37°C in a water bath for 30 min. For input, 10µg (5%) protein was diluted in LSB and incubated the same as IP reactions. Immunoprecipitated samples were separated in 10% polyacrylamide gels with SDS. Electrophoresed proteins were transferred to PVDF membranes and blocked with TBS/T with 2% normal goat serum. Blots were probed with 1:500 dilution of rabbit anti-phospho-threonine antibody (Cell Signaling Technology) in TBS/T with 1% normal goat serum overnight at 4°C. HRP-conjugated goat anti-rabbit secondary antibody (1:5000) treatment was in TBS/T+1% normal goat serum for 2 hr at room temperature. Immunoblot detection was performed as before. Blots were stripped and blotted for PIT1 as mentioned previously, except a 1:500 dilution of primary antibody was used.

Statistical analysis

For luciferase reporter assays, the ratio of firefly to renilla luciferase was subjected to 2-way analysis of variance (ANOVA) using the MIXED procedure in the Statistical Analysis System (SAS; SAS Institute, Cary, NC). For determining significant differences between band intensities in EMSA, mean average intensities from 3 replicate experiments were subjected to 1-way ANOVA after blocking for gel. For both analyses, differences in Least Squares Means (LSM) between treatments were determined by option PDIFF in SAS (version 9), and $p<0.05$ was considered statistically significant.

Results

Cloning of Pit1 isoform cDNAs into expression vectors: Identification of a novel isoform mRNA

At the start of this project, three *Pit1* isoform mRNAs had been identified in chickens (Van As *et al.*, 2000). However, translation of these isoform mRNAs to stable and functional proteins had not been demonstrated. To demonstrate translation of chicken *Pit1* mRNAs into full length proteins, the isoforms were cloned into expression vectors. Chicken *Pit1* isoforms *Pit1*α and *Pit1*β were amplified from chicken pituitary cDNA (e14) using forward and reverse primers (Table 1), and the amplicons were cloned into the p-CMV-Sport6.1 mammalian expression vector. Reverse primer binding to the 84-bp β-specific exon was used to differentiate between *Pit1*α and *Pit1*β. A different forward primer was used, along with the common reverse primer, to amplify *Pit1*γ. Since the same primer pair amplified the *Pit1*α and *Pit1*β isoforms (Fig. 5), it is possible to predict *Pit1*α mRNA to be present in e14 chicken pituitaries at a level substantially higher than *Pit1*β1. In fact, out of a total of 215 colonies screened, only 2 were found to contain the *Pit1*β1 clones. Recombinant plasmid constructs for each isoform were sequenced in both directions to confirm sequence integrity. Sequence comparison of *Pit1* isoform cDNAs amplified from Ross broiler strain cDNA revealed few points of difference when compared with the sequence available at Ensembl, which is from Red Jungle Fowl.

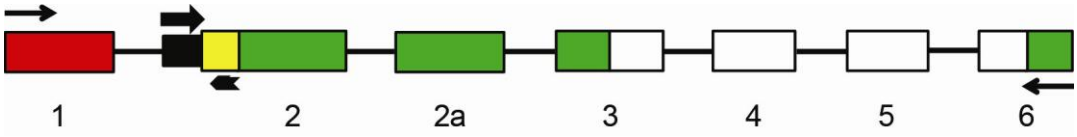


Fig. 5. *Pit1* gene structure. Exons are represented as boxes, and introns are represented as lines. Exons and introns not to scale. Line arrows denote forward and reverse primer for isoforms *Pit1*α and *Pit1*β. Filled arrow denotes forward primer for *Pit1*γ. Chevron denotes β-specific reverse primer used for identification of β-clones. β-specific exon is in yellow. Unique exon 1 of *Pit1*γ is in black. White represents region of gene encoding POU domain. (Adapted from Van As *et al.*, 2000)

Sequencing results also revealed the presence of an isoform that lacked the first 26bp of the β-specific exon (Fig. 6). We predict this is a yet unidentified and novel isoform. To confirm that this was not a cloning artifact, we sequenced several other chicken pituitary mRNA samples of different ages (e11, e17, d32) which also showed the presence of this isoform. Initiation of translation of this novel isoform from the same site as *Pit1*α and *Pit1*β1would result in a completely different protein product that would not have amino acid sequence related to the reported PIT1 isoforms, except at the very N-terminal region up to the middle of the β-specific exon. However, if this isoform used a different ATG codon, it is possible to yield a protein product with a POU-domain similar in sequence as the other isoforms. Scrutiny of the cDNA sequence revealed only one such downstream ATG codon in the +2 frame which, if used, and if the transcript had been alternatively spliced, would result in a novel isoform of 315 amino acids, making this isoform the shortest of all known chicken PIT1 isoforms. This isoform is thus predicted to result from a combination of using an alternative translation start site as well as alternative splicing. A comparison of the mRNA structure of the isoforms is shown in Fig. 7.

Determination of transcription start site of the isoforms by 5'RACE.

It has been reported by Van As *et al.,* 2004 that the *Pit1* gene is transcribed as a single mRNA which then undergoes alternative splicing to derive isoforms *Pit1*α and *Pit1*β1, while a different mRNA, derived by alternative transcription start site (TSS) usage, leads to the *Pit1*γ isoform. To determine the TSS of the novel *Pit1*β2 isoform, and also to confirm if the TSS for the other isoforms is the same in Ross broiler strains as the Hybro G strain used by Van As *et al.,* (2000), 5' RACE was performed. Chicken pituitary e11

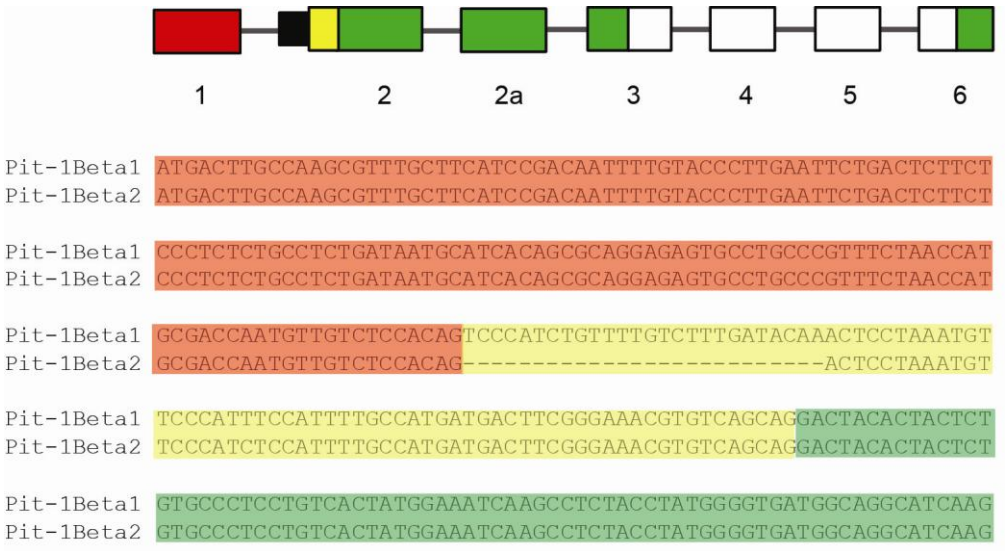


Fig. 6. Sequence alignment of *Pit1*β1 and *Pit1*β2. *Pit1* gene structure is shown on top. Sequences were aligned using CLUSTALW. Dashed line in *Pit1*β2 denotes portion of β-specific exon not present in *Pit1*β2. Only relevant portion showing difference is represented. Exon 1 is in red. Green shading represents beginning of portion of gene common to all isoforms.

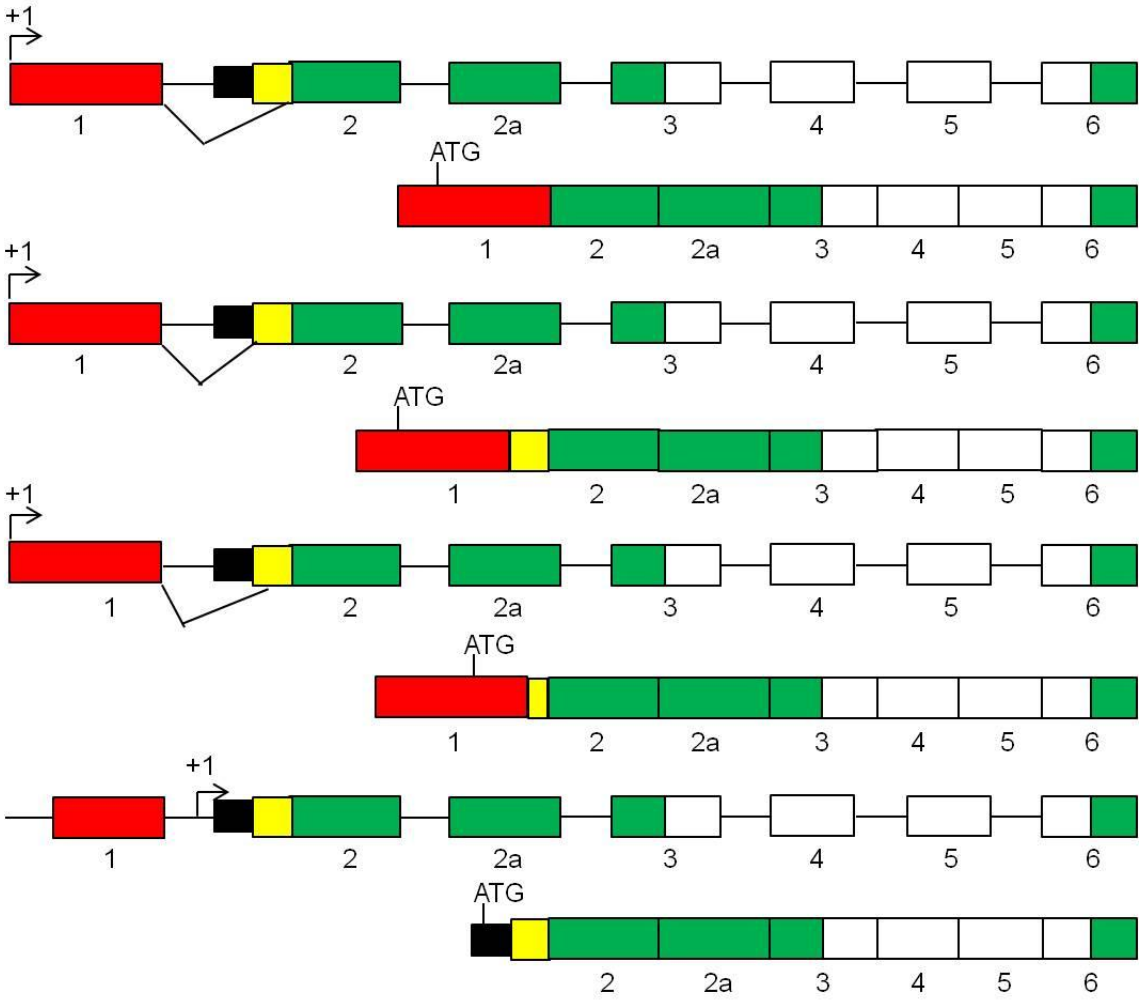


Fig. 7. Comparative structure of *Pit1* isoforms. For each isoform, the gene is represented in the form of introns (lines) and exons (boxes) along with transcription start site (TSS), 5'-untranslated region and mode of splicing, if present. Coding sequence is shown for all isoforms as exons (boxes). White boxes represents portion of gene encoding the POU-domain. ATG shows the position of translation start site.

total RNA was reverse transcribed with reverse primer annealing to a common sequence located at the 3'-end. After tailing, cDNAs were amplified with two sets of primers to separate out *Pit1*α from *Pit1*β1, *Pit1*β2 and *Pit1*γ. These two sets of reactions were cloned into pGEM vector and multiple clones obtained and sequenced. Any insert not aligning to chromosome 1 in BLAST was not pursued further. Correct inserts were aligned with the *Pit1* gene in Ensembl and the TSS determined. We found the TSS of the mRNA giving rise to *Pit1*α and *Pit1*β1 to be 78base pairs upstream of the site previously reported, while our findings for *Pit1*γ showed the TSS to be 8base upstream from the site previously reported (Fig. 8). No clones containing *Pit1*β2, the novel isoform we discovered, were found in this screen. We derived cDNA from total pituitary RNA by reverse transcription with a primer that annealed to an exon-exon junction found only in *Pit1*β2 (Primer 5'RACE_Diag_Beta2, table 3). This cDNA was tailed, amplified with AAP/PIT1beta2-rev and sequenced. We found *Pit1*β2 also to be processed from the same TSS as *Pit1*α and *Pit1*β1, and we predict this isoform arises as a result of an alternative translation start site usage, from an mRNA that has been alternatively spliced downstream of the new start site (Fig. 8).

PIT1 isoform protein expression in LMH cells

To determine whether or not all isoform mRNAs identified can be translated into proteins, recombinant plasmids expressing cPIT1 isoforms were transfected into LMH cells. While this report was under preparation, expression of PIT1α and PIT1γ in non-pituitary cells was reported by Murase *et al.*, 2011. We demonstrated expression of all

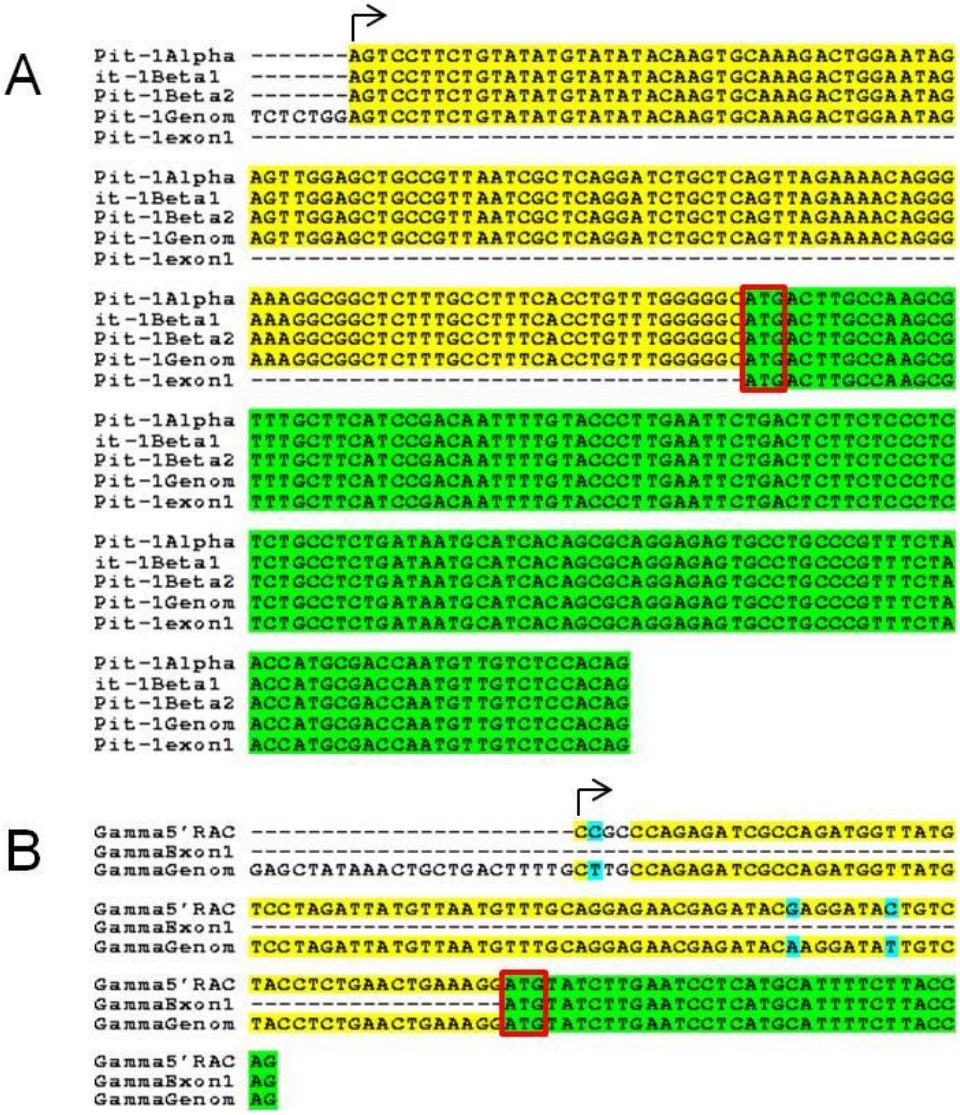


Fig. 8. 5'Rapid Amplification of cDNA Ends (RACE) of *Pit1* isoforms. (A) Alignment of 5'RACE products of *Pit1α*, *Pit1*β1 and *Pit1*β2 with genomic sequence.. (B) Alignment of 5'RACE product of *Pit1*γ and genomic sequence. Transcription start site predicted from our results is denoted with arrow.Yellow denotes 5-untranslated region, green denotes translated sequence. Red box denotes translation start site (ATG).

four isoforms by Western blot with rabbit PIT1 antiserum, as shown in Fig. 9. However, we found differences in the levels of expression between the isoforms in both HEK-293 (Fig. 9A) and LMH (Fig. 9B) cells. There were robust expression levels for isoforms alpha and beta2, while isoforms beta1 and gamma showed reduced levels with equal amounts of plasmid DNA transfected. In fact, we failed to detect PIT1γ expression in HEK293 cells (Fig. 9A). To address the possibility that one or more isoforms were subjected to proteosomal degradation, we conducted a proteasome inhibitor assay. LMH cells transfected with PIT1 isoform expression plasmids were allowed to express the transfected gene for 24 hours, following which cells were treated with MG-132 (20μM) for 0, 1.5 and 6 hr. However, we found no difference in expression after 6 hr of 20μM MG-132 treatment (Fig. 10). It should be mentioned that we tried using longer incubation periods (12 hr, and 24 hr) in the presence of MG-132, but these led to massive cell death at the concentration used.

Also, we detected multiple bands, especially for PIT1α and PIT1β1 using the PIT1 antiserum. These can be shorter isoforms resulting from premature translation termination, alternative downstream translation initiation site usage, shorter cleavage products derived from a full-length precursor, or products of partial degradation. To determine whether these were shorter peptides arising from the use of downstream translation start sites or whether they were degradation products, we used N- and C-terminally HA tagged isoform constructs. As seen in Fig. 11B & C, multiple bands were not detected when an N-terminally tagged construct was used, but present when the tags were placed at the C-terminus (Fig. 11D & E), thus suggesting these were protein

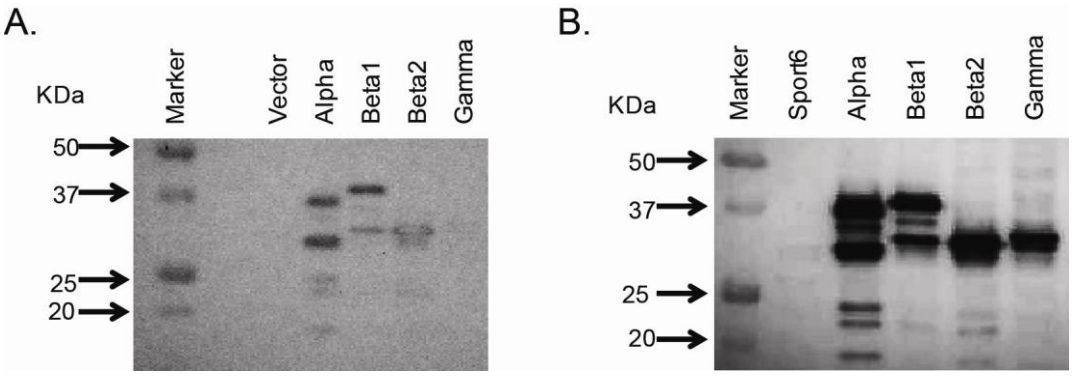


Fig. 9. PIT1 isoform protein expression in HEK-293 (A) and LMH (B) cells. For each cell type, PIT1 isoform expression vector or empty vector (4μg) were transfected using Lipofectamine-2000. Total protein was extracted, resolved by SDS-PAGE and blotted with anti PIT1 antiserum. Images are representative of 3 separate experiments.

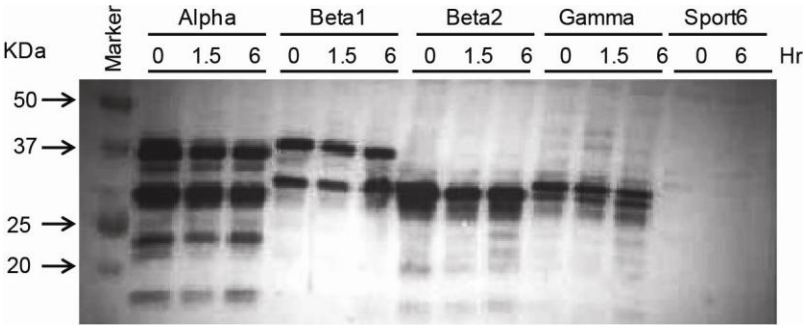


Fig. 10. Proteasome inhibitor assay. LMH cells were plated at $1x10^6$ per well in 6-well plates and transfected with 4µg *Pit1* isoform expression vector or empty vector and allowed to express the transfected gene for 24hours, following which cells were treated with 20µM MG-132 for 0, 1.5 hr or 6 hr. Equal amount of total protein from each treatment was resolved by SDS-PAGE, and blotted with PIT1 antiserum. Results shown are representative of two separate experiments.

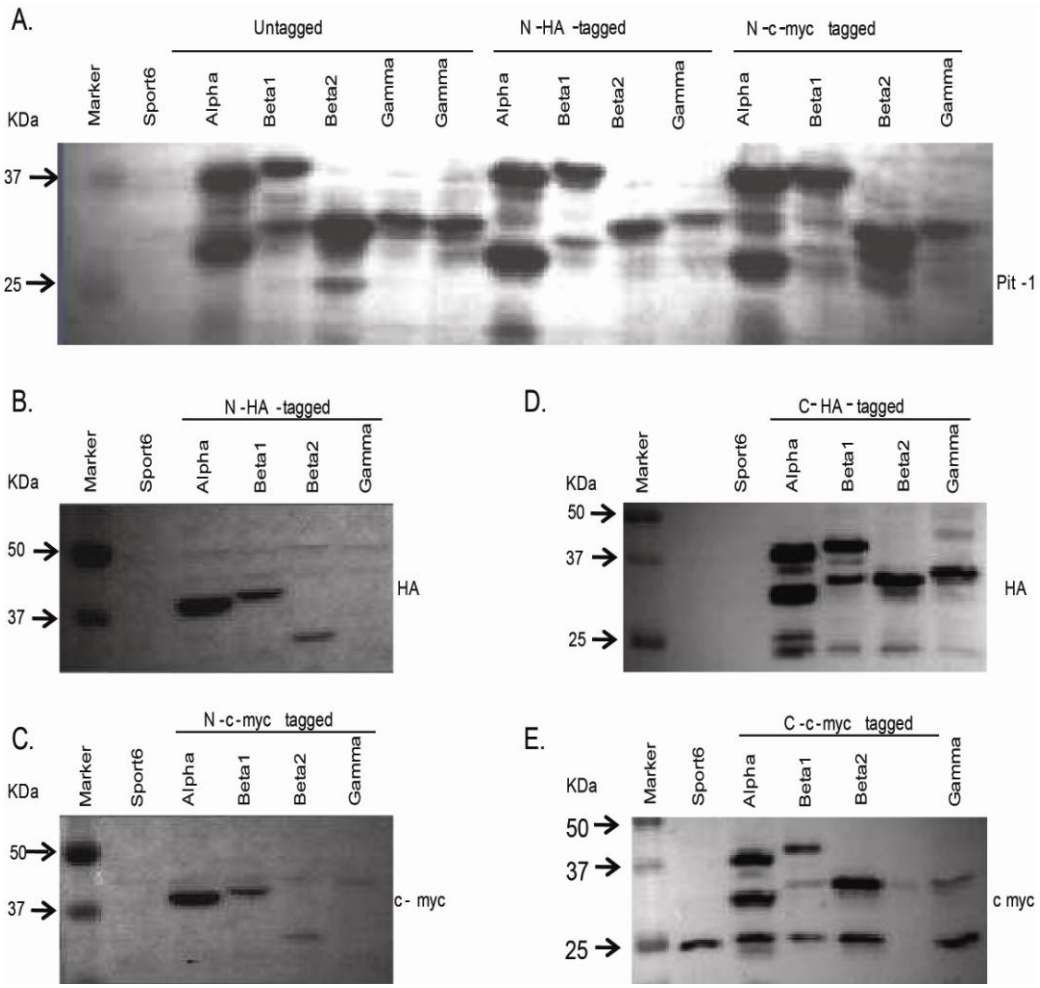


Fig. 11. Expression of N-terminal and C-terminal HA and c-myc tagged PIT1 isoforms in LMH cells. LMH cells were transfected with N-terminal HA- and c-myc tagged PIT1 isoform expression vectors. Total protein was isolated, separated by SDS-PAGE, and blotted with anti-PIT1 antiserum (A), rabbit anti-HA antibody (1:1000) (B & D) and mouse anti-c-myc antibody (1:1000) (C & E).

products resulting from the use of a downstream in-frame translational start site. We examined the sequences of the isoforms to look for such ATG codons with Open Reading Frame Finder (ORF Finder, www.ncbi.nlm.nih.gov/projects/gorf/) and found several present. However, the possibility that these bands represent degradation products cannot be ruled out.

An interesting observation was that an N-terminal tag on PIT1γ was not detectable by antibody directed against the tag. As a comparision of Fig. 11A vs. Fig. 11B & C reveals, even though detectable amounts of the PIT1γ isoform are present when blotted with the PIT1 antiserum, blotting with either anti-HA or anti-c-myc antibody failed to detect N-terminally tagged PIT1γ. This suggests that the N-terminally located tag is either cleaved off or the N-terminus of the PIT1γ isoform is folded such that the tag is masked.

Detection of endogenous PIT1 in chicken embryonic pituitary cells

Even though all reported *Pit1* isoform mRNAs are detectable in chicken embryonic pituitary cells by quantitative real-time PCR (Ellestad *et al.*, 2011), whether or not all yield functional proteins is not known. Unfortunately, no antibodies are available that detect only one isoform but not others, making it impossible to answer this question. Hence, we took an alternate approach and resolved LMH whole cell extracts transfected with single PIT1 isoform expression vectors alongside e17 whole pituitary extracts by SDS-PAGE. The results can be seen in Fig. 12. From the results, it is difficult to conclude which isoforms are expressed. Endogenous PIT1α, the isoform most readily detected is resolved at the same position of the gel as overexpressed PIT1α, and the isoform slightly heavier than PIT1α in molecular mass is most likely PIT1β1; however, we cannot

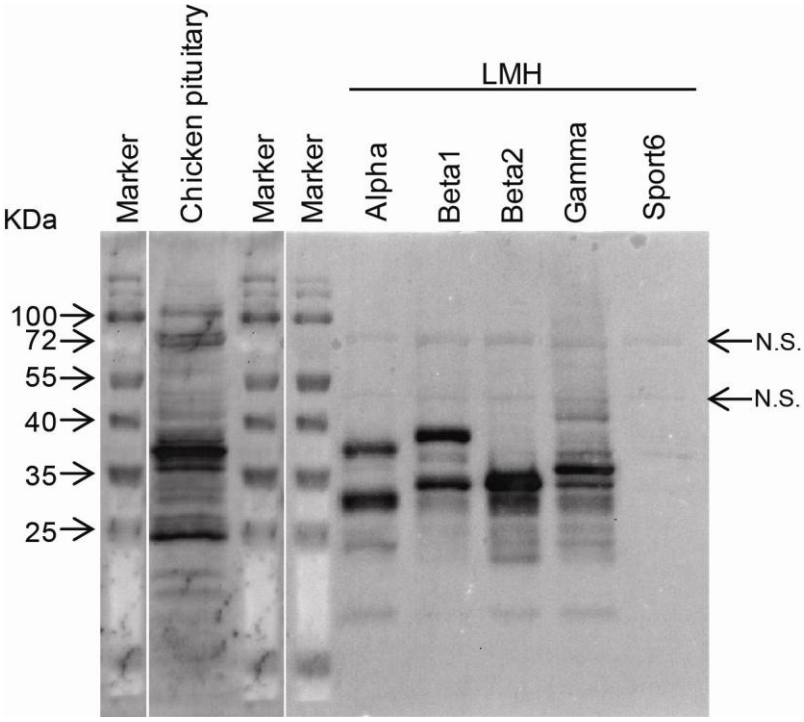


Fig. 12. Endogenous PIT1 from embryonic day 11 chicken pituitary cells. Whole cell lysates from e11 primary cells were electrophoresed on the same gel with lysates from LMH cells transfected with either empty vector or a single PIT1 isoform. Anti-PIT1 antiserum (1:500) was used for immunoblotting. (N.S. denotes non-specific bands).

conclude whether the shorter bands represent isoforms β2 and γ or they are shorter products arising from downstream ATG codons. Some of the higher molecular weight bands possibly represent homo- and heterodimers between endogenous PIT1 isoforms. It is also possible that the endogenous expression level of one or more isoform is below the level of detection without overexpression.

PIT1 isoform protein localization in LMH cells

So far, only one study has looked at nuclear localization abilities of wild type and mutant human PIT1 (Kishimoto *et al.*, 2002). A cluster of 6 basic residues in the POU domain of Oct-1 is known to determine nuclear localization. This cluster (RKRKRR) is conserved in all isoforms of PIT1 and is considered to be the putative nuclear localization signal (NLS). However, sub-cellular localization of none of the PIT1 isoforms had been demonstrated empirically in chicken at the time this experiment was planned and performed. Recently, Murase *et al.*, 2011 reported the localization of PIT1α and PIT1γ to the nucleus in Cos-7 cells, and their data are in agreement with our results, which show localization of all the isoforms to the nucleus in LMH cells (Fig. 13).

PIT1 isoform protein (C-terminal HA tagged) localization in chicken embryonic pituitary cells

To determine the sub-cellular localization of PIT1 isoforms in chicken pituitary cells, plasmid constructs expressing epitope-tagged isoforms were used. Cells were transfected with C-terminal HA tagged PIT1 isoforms along with expression plasmid for golgi-directed green fluorescent protein (GFP). Due to the low transfection efficiency of

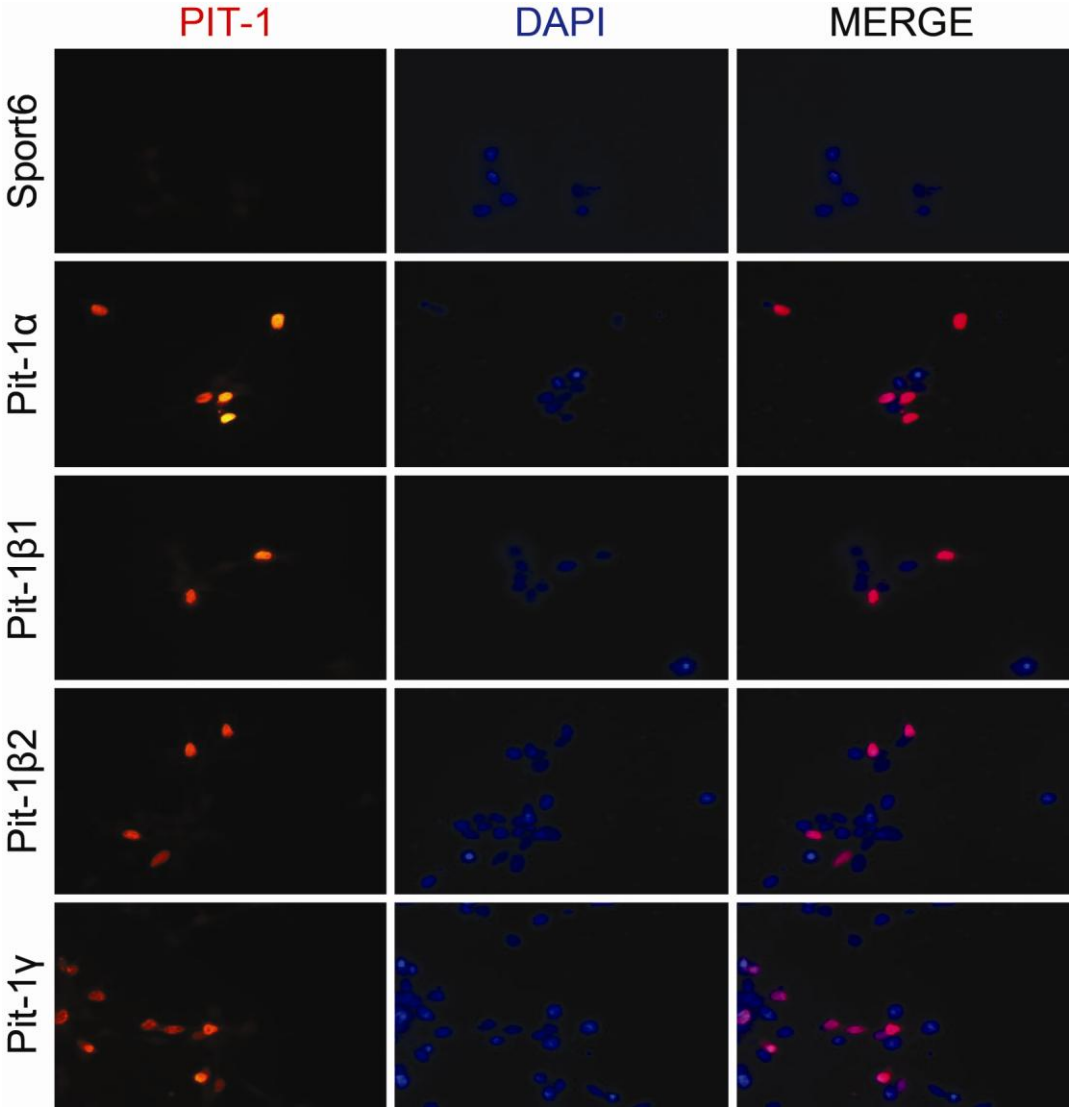


Fig. 13. Sub-cellular localization of PIT1 isoforms in LMH cells. Cells were plated in 2-chamber slides and transfected with 2µg *Pit1* isoform expression vector. Cells were fixed, permeablized, incubated with PIT1 antiserum (1:1000 dilution) at 4°C overnight in PBS/ 2% NGS. Following secondary antibody (Rhodamine-conjugated goat anti-rabbit IgG) incubation, cells were incubated with DAPI, mounted in Fluoromount-G and visualized with a Zeiss fluorescent microscope using Axiovision LE software version 4.7. Results shown are representative of two separate experiments.

primary chicken pituitary cells, transfection was performed in suspension. High cell numbers, close to $2x10^6$ cells per chamber in 8-chambered slides were used to get high cell density. GFP expression was observed as punctuate green staining outside the nucleus. Cell nuclei were stained with DAPI. Localization of tagged PIT1 isoforms in e19 chicken pituitary cells was also predominantly in the nucleus (Fig. 14). We also examined if CORT had an effect on the localization. Transfected cells were treated with CORT (10^{-7}M) 24 hr after transfection, but CORT had no effect on localization of the isoforms in chicken embryonic pituitary cells (data not shown). It should be noted that even though almost all PIT1 expression in both chicken pituitary cells and when over-expressed in LMH cells was nuclear, isoform proteins were readily detectable in whole cell lysate.

Activation of the chicken Gh promoter by PIT1 isoforms in LMH cells

To determine which, if any, of the PIT1 isoforms can activate the chicken *Gh* promoter, we performed a dual-luciferase reporter assay with a pGL3-1727 reporter vector in which the firefly luciferase gene is driven by -1727 to +48bp of the *cGh* promoter. This region of 1774bp of the *cGh* 5'flanking region has been shown to be sufficient for mediating glucocorticoid activation of *cGh* gene expression both in rat pituitary GH4C1 cells by dexamethasone (Ip *et al.*, 2004) and chicken pituitary primary cells by CORT (Knubel, 2010). However, for our purposes it was essential to perform the experiments in a situation where only one isoform of PIT1 would be present. We could not use a rat or mouse pituitary cell line as the *cGh* reporter has been shown to be activated in mammalian GH4C1 cells without added chicken PIT1. Non-pituitary chicken LMH cells

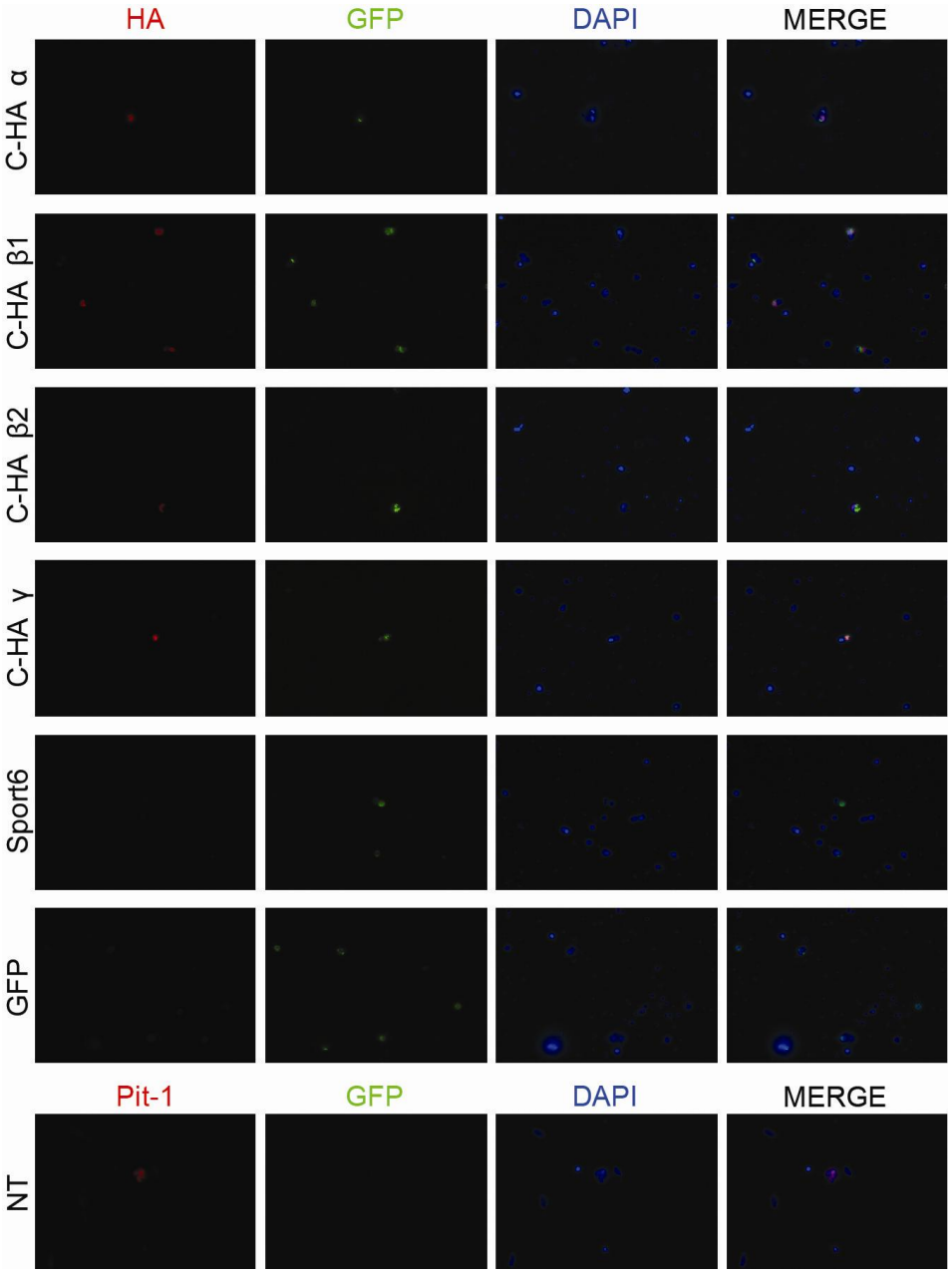


Fig. 14. Sub-cellular localization of PIT1 isoforms in chicken pituitary cells. Cells were plated in 8-chamber slides and transfected with 2µg C-terminal HA-tagged *Pit1* isoform expression vector. Cells were fixed, permeablized incubated with anti-HA antibody (1:1000 dilution) at 4°C overnight in PBS/ 2% NGS. Following secondary antibody (Rhodamine-conjugated goat anti-rabbit IgG) incubation, cells were incubated with DAPI, mounted in Fluoromount-G and visualized with Zeiss fluorescent microscope using Axiovision LE software version 4.7. Images shown are representative of two separate experiments.

were plated ($3x10^5$ per well in 24-well plates) on gelatin-coated plates. Cells were transfected with 1μg of either empty reporter vector backbone (pGL3-Basic) or the *cGh* reporter vector (pGL3-1727) along with specified amounts of *Pit1* isoform expression vector and 20ng pRL-SV40 renilla luciferase normalization vector. The amount of expression vector transfected was kept constant (2μg) with empty expression vector (pCMV-Sport6.1). Initially, a dose-response study was conducted with 1ng, 10ng, 100ng and 1000ng of expression vector. Ratios obtained by dividing the firefly luciferase activity by renilla luciferase activity are shown in Fig. 15. Different letters denote significant differences of Least Squared Means between treatment groups ($n=4$, $p<0.05$). The isoforms PIT1α, PIT1β1 and Pit-β2 showed a dose-dependent activation of the *cGh* promoter, while PIT1γ did not activate the promoter even at the highest dose. The three activating isoforms showed lower activation at a dose of 100ng. However, no significant activation was seen at 10ng or below. In a pilot study, similar luciferase reporter assays (using 1000ng expression vector) were conducted in presence and absence of 10^{-7}M CORT for 24 hr. We saw no difference in the activation of *cGh* promoter in presence of CORT, regardless of which PIT1 isoform was tested (data not shown).

Since we had previously detected differences in the level of isoform protein expression from recombinant plasmid in LMH cells, we transfected additional wells with identical amounts of reporter, expression and normalization vectors and extracted total protein at the same time the other wells were lysed to obtain samples for reporter assays. Samples were resolved by SDS-PAGE and blotted with PIT1 antibody. As seen previously, amount of PIT1 protein expressed with same amount of plasmid transfected varied

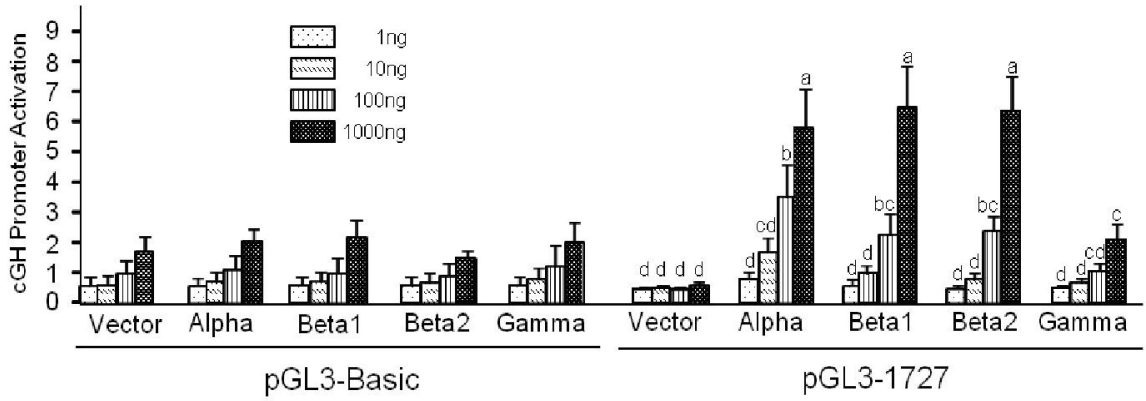


Fig. 15. Activation of the chicken *Gh* promoter by PIT1 isoforms in LMH cells. Figure shows dose-dependent activation of the *cGh* promoter by PIT1 isoforms. For each dose, specified amount of expression plasmid was transfected along with either 1µg pGL3-Basic or pGL3-1727 along with 20ng pSV-40 renilla. Different letters denote significant differences of Least Squared Means between treatment groups Data shown are mean±SEM of 4 replicate experiments.

widely among the isoforms (Fig. 16). Blots were stripped and reprobed with mouse anti-α-tubulin antibody (1:1000; sc-32293; Santa Cruz Biotechnology, Santa Cruz, CA) which confirmed that equivalent amounts of total protein were loaded.

Since a difference in level of expression of PIT1 proteins was apparent, we titrated the amount of DNA transfected to obtain equal levels of PIT1 protein expression (Fig. 17). For each isoform, a lower and a higher dose was selected and protein expression determined by Western blot (Fig. 17B). The amounts of plasmids obtained from titrations that yielded equivalent amounts of PIT1 protein, were transfected into LMH cells and the promoter activity measured the same way as before. The total amount of plasmid DNA transfected was kept constant with empty pCMV-Sport6.1 vector. Once again, PIT1γ failed to activate the *cGh* promoter, even when the amount of PIT1 protein present was comparable to the other isoforms (Fig. 17A). A significant effect was seen when PIT1γ(3000ng)/pGL3-1727 is compared to pSport6.1/pGL3-1727, but we believe this result is due to an effect that the large amount of transfected PIT1γ has on renilla luciferase, as we saw a significant decrease of renilla activity at this dose. PIT1β1 gave the maximum activation of approximately 15-fold over empty expression vector. The amounts of PIT1α transfected were 10ng and 50ng, and neither of these doses activated the *cGh* promoter similar to the result seen in the dose-response experiment. Only the higher dose of PIT1β2 activated the *cGh* promoter. Taken together, these results indicate that all known isoforms of PIT1 except PIT1γ can activate the *cGh* promoter in non-pituitary chicken hepatoma cells.

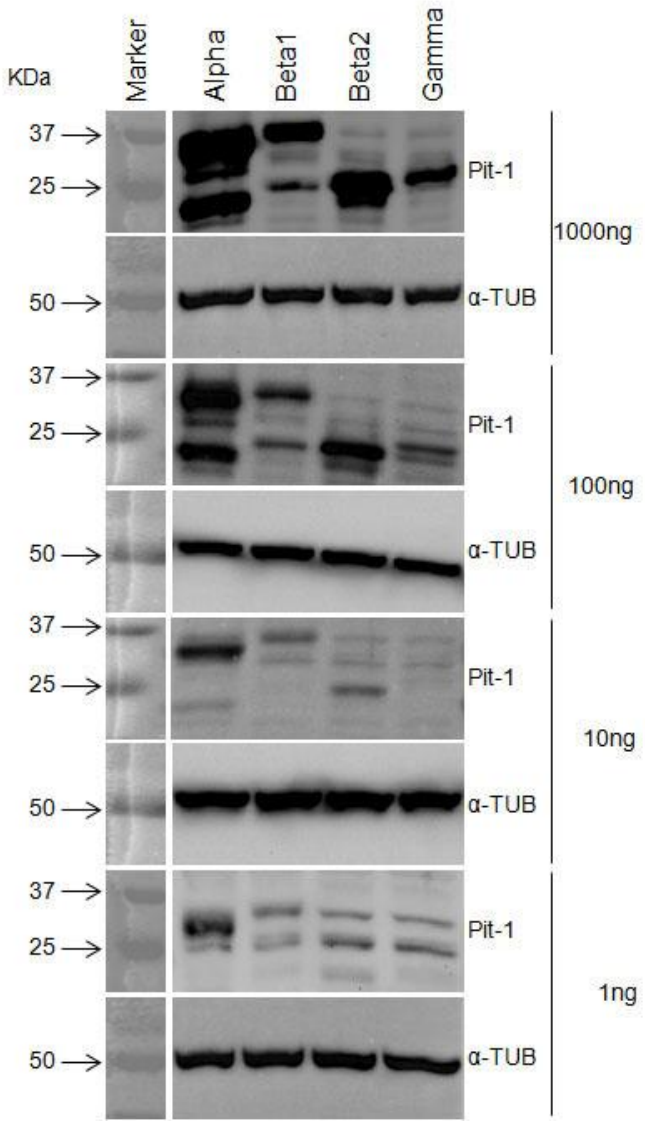


Fig. 16. Protein expression of PIT1 isoforms transfected with 1ng, 10ng, 100ng and 1000ng *Pit1* expression vector. PIT1 antiserum (1:1000) was used for immunoblotting. Blots were then stripped and reprobed with antibody against alpha tubulin (1:1000) which served as a loading control.

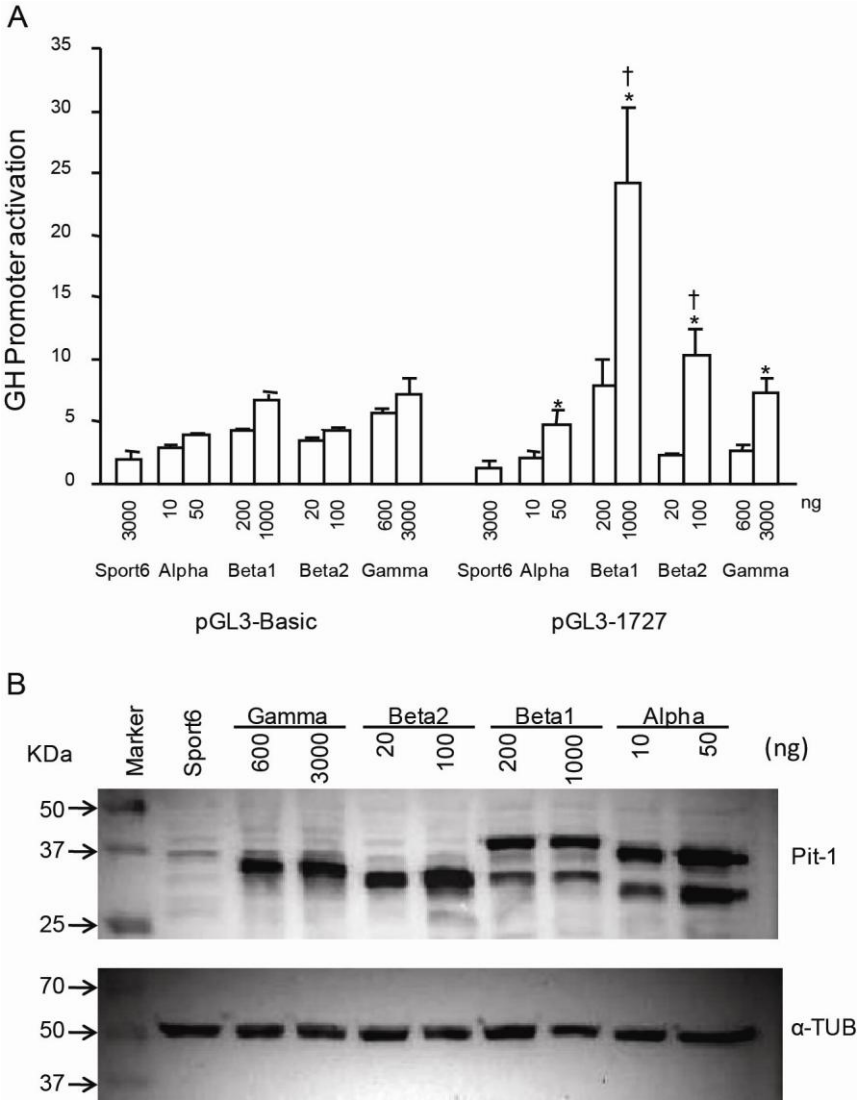
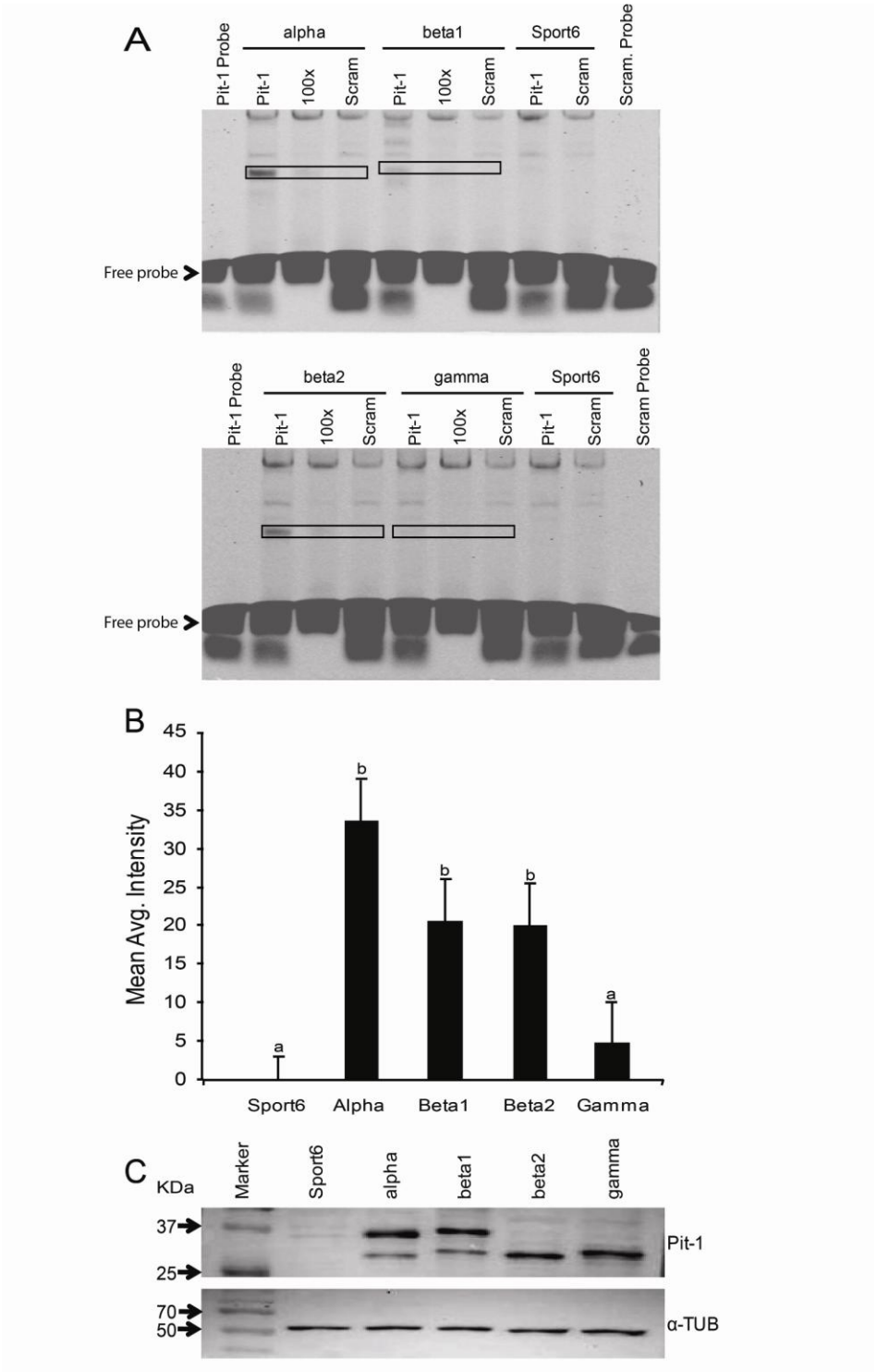


Fig. 17. Activation of the chicken *Gh* promoter by PIT1 isoforms in LMH cells. (A) shows activation of the *cGh* promoter by PIT1 isoforms when comparable amount of protein is expressed. The amount of PIT1 isoform expressed was determined by Western blot (B). Alpha tubulin was used as a loading control. For each dose, specified amount of expression plasmid was transfected along with either 1µg pGL3-Basic or pGL3-1727 along with 20ng pSV-40 renilla. Asterisk denotes significant difference from cells transfected with empty expression vector. Dagger (†) denotes significant difference from cells transfected with expression vector along with empty reporter vector. Data shown is mean±SEM of 3 replicate experiments.

Isoform protein binding to the proximal PIT1 binding site on the cGh promoter

Since we detected no activation of the *cGh* promoter by PIT1γ, we hypothesized that this inactivity may be due to the fact that PIT1γ cannot bind to the proximal PIT1 binding site. There are two putative PIT1 binding sites within 1kb of the *cGh* promoter. The proximal site is located at -104/-113 bp, and the distal site is located further upstream at -533/-541bp. Of the two PIT1 binding sites present, we decided to test for binding of the isoforms to the proximal site, because that is the site which is known to be functionally important (Ip *et al.*, 2004). Mutations in either both or only the proximal site abolish promoter activation in rat pituitary GH4C1 cells. Also, GH4C1 cell extracts bound only to the proximal site *in vitro*. DNA probes were designed spanning the proximal PIT1 binding site and incubated with total protein lysates of LMH cells transfected with single *Pit1* isoform expression vectors. The amount of expression vector transfected varied according to isoform to obtain comparable PIT1 protein expression levels. In our experiments, we found specific significant binding of all isoforms except PIT1γ to the proximal PIT1 binding site (Fig. 18A). The binding showed by PIT1γ was less than others that was not statistically significant. This binding was successfully competed off using a 100-fold molar excess of unlabeled probe. A labeled mutant probe, with the proximal PIT1 binding sequence and neighboring bases mutated to a GC-rich sequence as opposed to the AT-rich sequence recognized by PIT1, showed no specific binding. The same mutation has been shown to result in no binding by rat PIT1 to the proximal site of the *cGh* promoter (Ip *et al.*, 2004). Whole cell extract from cells transfected with empty expression vector showed some binding which was not competed off with excess unlabeled probe, confirming non-specific binding. The experiment was replicated 3

Fig. 18. Isoform protein binding to the proximal PIT1 binding site on the *cGh* promoter. (A)EMSA showing PIT1 isoform protein binding to the proximal PIT1 binding site on the *cGh* promoter (Top: Pit1-α and PIT1β1; Bottom: PIT1β2 and PIT1γ). Infra-red labelled DNA probes were designed spanning the proximal PIT1 binding site and incubated with total protein lysates of LMH cells transfected with single PIT1 isoforms expression vectors. DNA-Protein complexes were resolved in 6% non-denaturing polyacrylamide gels. Boxes denote specific bands for each PIT1 isoforms. (B) Quantitation of PIT1 isoform binding to *cGh* promoter. Means of background-subtracted average intensity of 3 replicate experiments were subjected to ANOVA after blocking for gel ($p<0.05$). The amount of expression vector transfected varied according to isoform to obtain comparable protein expression levels. (C) Western blot showing level of protein expression obtained with specifed amount of plasmid DNA transfected in samples used for EMSA. Alpha-tubulin was used as loading control.


A
Pit-1 Probe
alpha
beta1
Sport6
Scram. Probe
Pit-1
100x
Scram
Free probe
beta2
gamma
Scram Probe
B
Mean Avg. Intensity
45
40
35
30
25
20
15
10
5
0
a
b
b
b
a
Sport6
Alpha
Beta1
Beta2
Gamma
C
KDa
Marker
Sport6
alpha
beta1
beta2
gamma
37
25
70
50
Pit-1
α-TUB

times, and average binding for each isoform was quantified and compared with binding by pSport6.1-transfected LMH cell extract (Fig. 18B). Since comparable amounts of all the isoform proteins were present in the cell lysates (Fig. 18C), we conclude that PIT1γ, in spite of possessing the POU-homeodomain and POU-specific domain identical to the other isoforms, lacks the ability to bind to the proximal PIT1 binding site of the *cGh* promoter.

Effect of co-transfection of an isoform on the activity of another

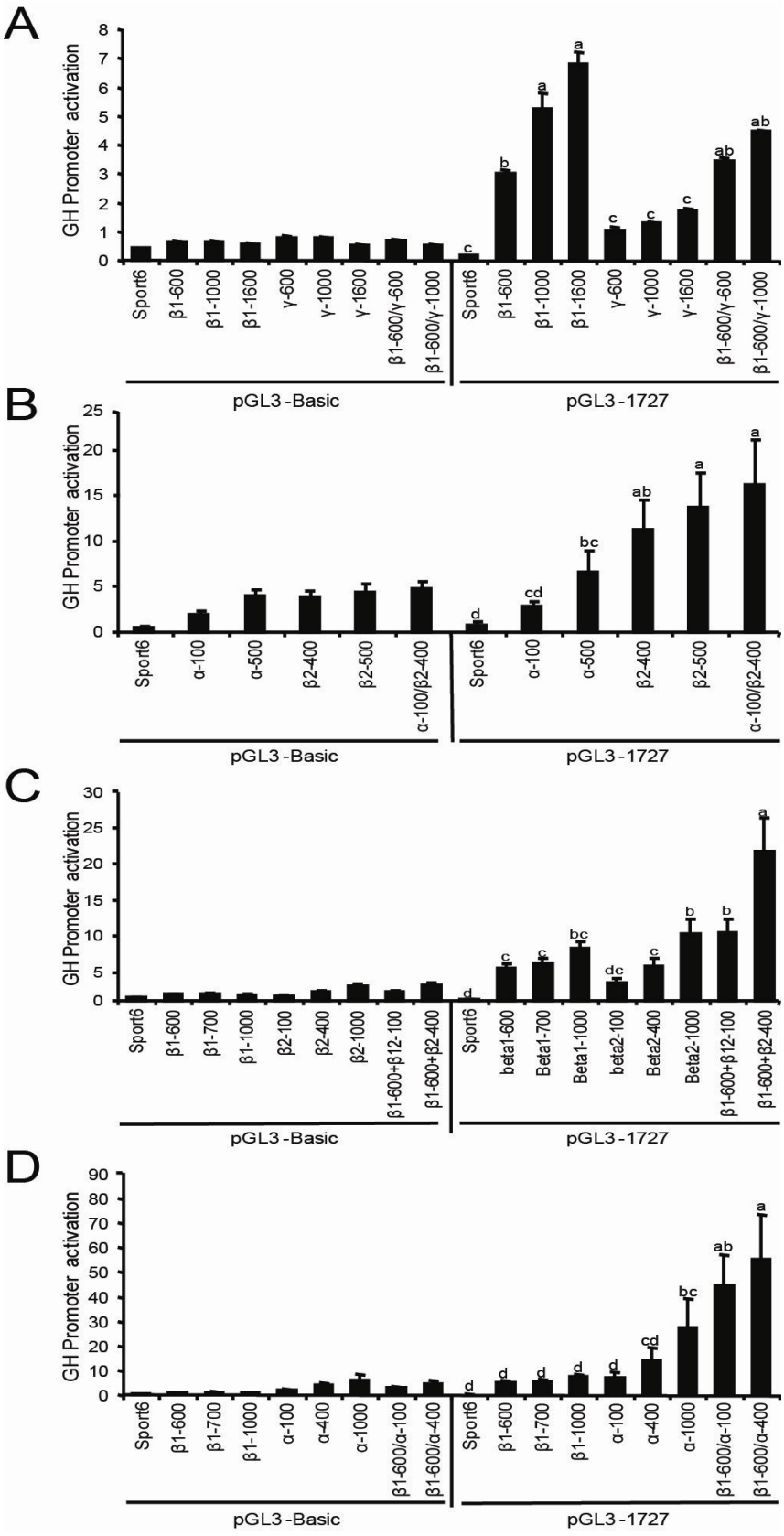
Expression of multiple protein products from a single gene by alternative transcription, translation and/or splicing is a mode of expansion of the genome. However, one would expect a functional aspect to this. Based on what our results showed so far, we found that isoforms PIT1α, PIT1β1 and PIT1β2 can activate the *cGh* promoter, while PIT1γ cannot. Among those isoforms that had an activating role, PIT1β1 seems to activate the promoter to a higher level when the amount of isoform protein present is equal for all isoforms. Arbitrarily classifying the isoforms as most activating (PIT1β1), activating (PIT1α and PIT1β2) and not activating (PIT1γ), we intended to see what effect the isoforms might have on the activity of each other.

There are very few studies done looking at the effect of co-transfection of PIT1 isoforms on promoter activation. In sheep, two isoforms PIT1γ and PIT1δ, which lack exon 3 and exons 3, 4 and 5, respectively, act as dominant negative inhibitors of PIT1wt in regulation of the rat Prl promoter in HeLa cells (Bastos *et al.*, 2006). Based on our results showing inability of PIT1γ to activate the *cGh* promoter, PIT1γ was hypothesized instead to have a negative and/or regulatory role. There are two possible mechanisms by which

that could be employed: i) PIT1γ occupies the PIT1 binding site, thus preventing activating isoforms from binding and ii) PIT1γ heterodimerizes with activating isoforms, thus preventing them from binding to the promoter. Our EMSA results showed that PIT1γ does not bind the *cGh* promoter, thus eliminating the first possibility. To test if PIT1γ sequesters an activating isoform, we co-transfected PIT1β1 and PIT1γ in LMH cells and evaluated *cGh* promoter activation. The amount of expression vector transfected for each isoform was such that they yielded comparable protein expression, and also the co-transfection had as much DNA transfected as the individual transfections. As seen in Fig. 19A, the presence of PIT1γ did not affect the ability of PIT1β1 to activate the *cGh* promoter. The level of activation achieved by PIT1β1 in the presence of PIT1γ was comparable to that by PIT1β1 alone ($n=3$, $p<0.05$). Thus, PIT1γ does not negatively regulate activation of the *cGh* promoter by PIT1β1 in LMH cells.

As mentioned previously, both PIT1α and PIT1β2 activate the *cGh* promoter. However, the level of activation achieved by these two isoforms is less than that of PIT1β1 if comparisons are made when comparable amounts of isoform protein are expressed. We asked the question if expressing them together leads to an additive or synergistic activation of the promoter. The amount of plasmid DNA transfected was determined from the results of titration experiments (Fig. 17A & B) to yield equal protein expression. As seen in the results presented in Fig. 19B, co-expressing PIT1α and PIT1β2 did not have an additive effect on *cGh* promoter activation. Another combination of activating isoforms, PIT1β1 and PIT1β2, was tested. In this experiment, like the others, amounts of plasmid DNA transfected were such that the doses activated the *cGh* promoter as well as gave comparable amounts of PIT1 protein expression. Results (presented in Fig. 19C)

Fig.19. Effect of co-transfection of an isoform on the activity of another. Two isoforms at a time were co-transfected in LMH cells and cGh promoter activation was evaluated. The combinations tested were (A) PIT1β1 and PIT1γ; (B) PIT1α and PIT1β2; (C) PIT1β1 and PIT1β2; and (D) PIT1α and PIT1β2. The amount of expression vector transfected for each isoform was such that they yielded comparable protein expression, and also the co-transfection had as much DNA transfected as the individual transfections. For each dose, specified amount of expression plasmid was transfected along with either 1μg pGL3-Basic or pGL3-1727 along with 20ng pSV-40 renilla. Data shown is mean±SEM of 3 replicate experiments.


A
GH Promoter activation
Sport6
β1-600
β1-1000
β1-1600
γ-600
γ-1000
γ-1600
β1-600/γ-600
β1-600/γ-1000
pGL3-Basic
pGL3-1727
B
GH Promoter activation
Sport6
α-100
α-500
β2-400
β2-500
α-100/β2-400
pGL3-Basic
pGL3-1727
C
GH Promoter activation
Sport6
β1-600
β1-700
β1-1000
β2-100
β2-400
β2-1000
β1-600+β12-100
β1-600+β2-400
beta1-600
Beta1-700
Beta1-1000
beta2-100
Beta2-400
Beta2-1000
pGL3-Basic
pGL3-1727
D
GH Promoter activation
Sport6
β1-600
β1-700
β1-1000
α-100
α-400
α-1000
β1-600/α-100
β1-600/α-400
pGL3-Basic
pGL3-1727

show an additive and possibly synergistic activation of the *cGh* promoter in the presence of both isoforms. The level of activation achieved by either 1000ng of *Pit1*β1 or 1000ng of *Pit1*β2 is significantly less than that obtained when 400ng of *Pit1*β2 and 600ng of *Pit1*β1 are simultaneously transfected ($p<0.05$, n=3). A fourth possible combination was tested in which *Pit1*β1 was transfected along with *Pit1*α (Fig. 19D). With this combination, significantly higher activation of the promoter was obtained with 600ng *Pit1*β1 transfected with 400ng *Pit1*α than with 1000ng of either isoform transfected alone ($p<0.05$, n=3). Taken together, these results suggest that PIT1β1, if expressed along with either PIT1α or PIT1β2, leads to an increased activation of the *cGh* promoter than either isoform expressed alone. In contrast, co-expression of PIT1α and PIT1β2, showed no additive or synergistic activation of the *cGh* promoter.

Physical interaction between PIT1 isoforms co-expressed in LMH cells

Even though PIT1 exists predominantly as a monomer in solution, it has been suggested that PIT1 binds DNA as a dimer, either as a homodimer or a heterodimer. Encouraged by our results showing increased activation of the *cGh* promoter by isoform combinations over isoforms expressed alone, we sought to determine if this was brought about by physical protein-protein interactions between the isoforms. LMH cells were transfected with expression vectors encoding N-terminal c-myc tagged PIT1β1 alone and in combination with N-terminal HA-tagged PIT1α or PIT1β2. Cells transfected with empty expression vector, HA-tagged *Pit1*α and HA-tagged *Pit1*β2 were also included in the experiment. Fresh lysates from each transfection were divided into two parts and immunoprecipitated with either mouse anti-c-myc antibody or normal mouse serum

(NMS). Immunoprecipitated samples were resolved by SDS-PAGE, blotted onto PVDF membrane and probed with rabbit anti-HA antibody. We performed this experiment in the presence and absence of DNA containing the PIT1 binding site(s). Employing this strategy, we were not able to demonstrate any protein-protein interaction between the isoforms by co-immunoprecipitation (Fig. 20). While individual isoforms were immunoprecipitated by antibodies directed against the tag, the potential dimerization partners were not co-immunoprecipitated. We have also performed this experiment in rat pituitary GH4C1 cells, with the same result. However, in the absence of a positive control, we cannot conclude if the isoforms do not interact or if the interaction is transient and does not withstand the manipulation of protein extraction and immunoprecipitation.

Physical interaction between PIT1 isoforms and chicken CREB-binding protein (cCBP)

cAMP-mediated gene regulation can be either CREB-dependent or CREB-independent, and regulation of *Gh* in humans, despite the presence of functional CREs on the h*Gh* promoter, appears to belong to the latter category (Cohen *et al.*, 1999). The mechanism of CREB-independent activation postulated involves interaction of CREB-binding protein (CBP) with a gene-specific transcription factor bound to the promoter. CBP, by its intrinsic histone acetyltransferase (HAT) activity, remodels the local chromatin and also interacts with the general transcriptional machinery. Both rat and chicken *Gh* promoters lack a classical CRE core motif, but cAMP is involved in regulation of *Gh* in both species, and in rats the cAMP response is no longer present if the PIT1 binding sites are mutated (Treacy *et al.*, 1991). This information pointed strongly towards a mechanism

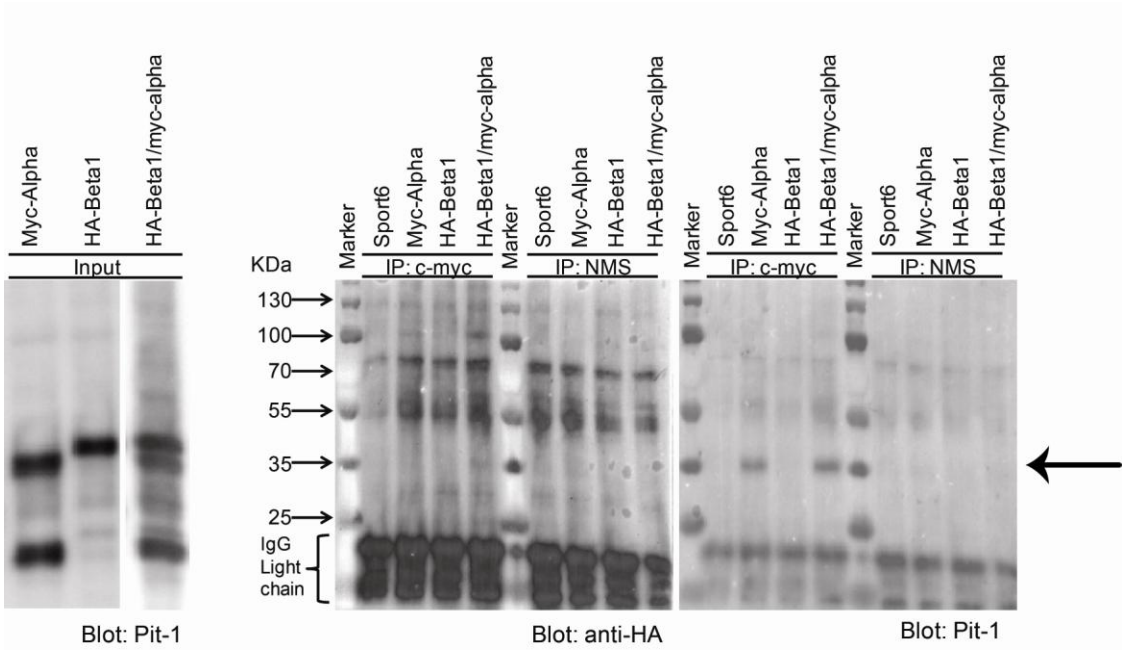


Fig. 20. Assays for physical interaction between co-transfected isoforms. LMH cells were transfected with N-terminal c-myc tagged Pit-α alone and in combination with N-terminal HA-tagged PIT1β2. Cells transfected with empty expression vector, and HA-tagged PIT1β1 were also included in the experiment. Fresh lysates from each transfection were divided into two parts and immunoprecipitated with either mouse anti-c-myc antibody or normal mouse serum (NMS). Immunoprecipitated samples were resolved by SDS-PAGE, blotted into PVDF membrane and probed with rabbit anti-HA antibody. Large arrow indicates the location of where PIT1α and PIT1β1 should be located. Blot on extreme left shows 5% input blotted with anti-PIT1 antiserum. Results shown are representative of 3 trials.

involving an interaction of PIT1 with CBP, a known interaction partner of several other transcriptional regulators and a target of phosphorylation by protein kinase A (PKA) and protein kinase C (PKC). We hypothesized that PIT1 regulation of c*Gh* will involve interaction of PIT1 with CBP, and that the most activating isoform (PIT1β1) will show a higher level of interaction than either PIT1α or PIT1β2. The region of CBP essential for regulating *Gh* has been shown to be located at the C-terminus and contain a functionally active Histidine rich domain (C/H3). We cloned this domain of the chicken CBP into pCMV-Sport6.1 mammalian expression vector with a C-terminal c-myc tag. The translation product obtained from this vector should lack the CREB-binding domain of CBP, and any interaction seen in the presence of *cGh* promoter DNA, then, must be mediated by PIT1. We transfected LMH cells with c-myc-cCBP (1677-2442), and a band of the correct size (90KDa) was detected using anti-c-myc antibody (data not shown). For co-immunoprecipitation, LMH cells were transfected with empty vector, PIT1, cCBP (1677-2442), and PIT1/cCBP(1677-2442) with pGL3-1727 reporter vector. Lysates were immunoprecipitated with anti-c-myc antibody, run onto SDS-PAGE gel and blotted with anti-PIT1 antibody. Despite several attempts, we were not able to demonstrate interaction between PIT1 isoform(s) and cCBP (data not shown).

Detection of phosphorylation of PIT1

Phosphorylation of PIT1 appears to modulate its ability to bind cognate sequences in promoter regions of target genes. There is evidence of PIT1 phosphorylation in regulation of Prl and TSHβ expression (Steinfelder *et al.*, 1992; Howard & Maurer, 1994).

However, there are conflicting reports about the requirement of PIT1 phosphorylation in Prl gene regulation.

Unpublished results from our lab (Malkiewicz, 2003; Ellestad, 2010) have shown the involvement of p38 MAPK and MEK1/2 pathways in glucocorticoid (GC) induction of somatotroph differentiation and *Gh* gene expression. PIT1 is a known target of Ras, PKA and PKC pathways in mammals. Based on these results, we predicted an involvement of these signaling pathways in CORT-induced *Gh* expression. We hypothesize that the GC activates PIT1 protein by phosphorylation at a threonine residue, conserved in all chicken PIT1 isoforms (Thr263 in PIT1α; Thr291 in PIT1β; Thr242 in PIT1β2 and Thr255 in PIT1γ), as well as in PIT1 found in other mammalian species and in the related transcription factor Oct-1. To test this hypothesis, we sought to determine if CORT induces phosphorylation of PIT1. Chicken pituitary cells (e11, $1x10^7$ per treatment) were cultured in the presence of CORT (10^{-7}M) for 0 (basal), 1.5 and 6 hr. These time points were chosen as previous results from our laboratory have shown that activation of signaling pathways and *Gh* expression begins at 1.5 hr and continues up to 6 hr. Total protein extracted from these cells was immunoprecipitated using anti-PIT1 anti-serum or normal rabbit serum. Immunoprecipitated proteins were washed and resolved by SDS-PAGE. Proteins were transferred onto PVDF membrane and probed with rabbit polyclonal anti-P-Thr antibody. Membranes were stripped and probed with anti PIT1 antiserum to show the presence of immunoprecipitated PIT1. This experiment was replicated 4 times, and even though we could detect phosphorylated PIT1 in one experiment after 6 hr CORT treatment, we could not detect this effect consistently (Fig. 21).

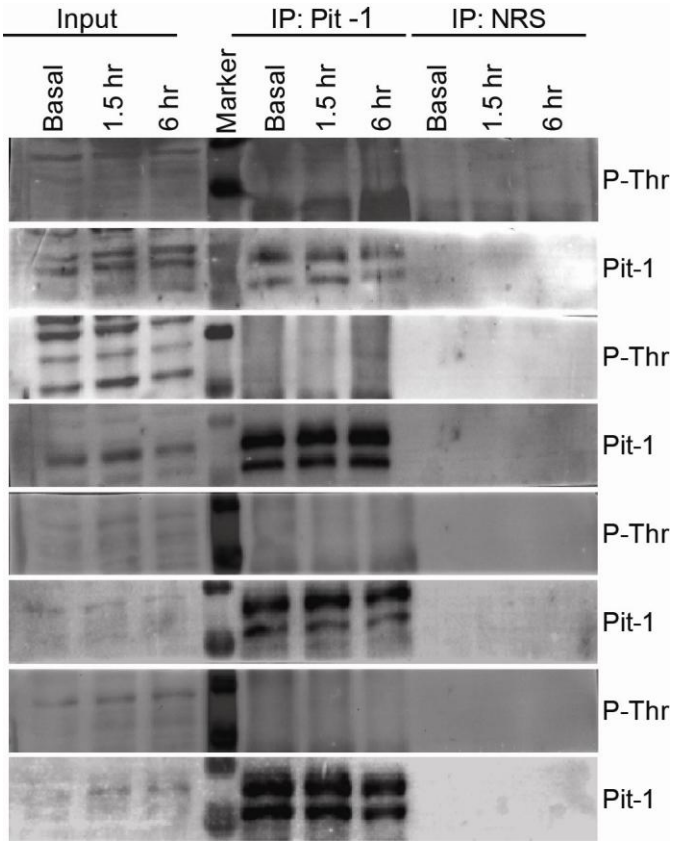


Fig. 21. Assay for phosphorylation of PIT1 in response to corticosterone (CORT). Pituitary cells (e 11) cells were plated ($1x10^7$ per well) in 6-well plates and treated with CORT (10^{-7} M) for 0, 1.5 or 6 hr. Total protein extracted from these cells was immunoprecipitated using anti-PIT1 anti-serum or normal rabbit serum. Immunoprecipitated proteins were washed and resolved by SDS-PAGE. Proteins were transferred onto PVDF membrane and probed with rabbit polyclonal anti-P-Thr antibody. Membranes were stripped and probed with anti PIT1 antiserum to show the presence of immunoprecipitated PIT1. This experiment was replicated 4 times.

Discussion

The overall objective of this study was to characterize chicken Pituitary Specific Transcription Factor I (cPIT1) isoforms for their ability to regulate *cGh* gene expression. PIT1 has been shown to be essential for differentiation of somatotrophs and other anterior pituitary cells of the PIT1 lineage, as well as for spatial and temporal regulation of *Gh* gene expression. Multiple PIT1 isoform proteins are known to co-exist in anterior pituitary somatotrophs, lactotrophs and thyrotrophs, but the significance of having multiple isoforms is not known.

The presence of three *Pit1* isoform mRNAs in chicken was reported by Van As, *et al.* (2000). At the time this project was started, verification of chicken PIT1 isoform protein expression and functional characterization had not been done. Recently, Murase, *et al.* (2011) reported cloning and partial characterization of two PIT1 isoforms, PIT1α and PIT1γ for their ability to transactivate the *cGh* gene. However, our work is the first to report expression, functional characterization and investigation of the mechanism leading to differences in activation potential of the *cGh* promoter for all the known isoforms. We hypothesized that PIT1γ, due to a grossly different N-terminal transactivation domain completely lacking exon 1, will not transactivate the *cGh* promoter.

The results reported here, with two exceptions, are from experiments performed in a non-pituitary chicken cell line. Since multiple isoforms of PIT1 are present in cells of the PIT1 lineage, it was imperative to perform the experiments in cells that do not express

PIT1 endogenously. Gonadotrophs and corticotrophs are known to express *Pit1* mRNA, which is suppressed at the level of translation (Simmons *et al.*, 1990; Asa *et al.*, 1996), thus making them unsuitable for our studies. Use of a chicken cell line was necessary, as initial trial experiments in a human cell line (HEK-293) showed suppression of protein expression of one of the isoforms (PIT1γ). A luciferase reporter gene driven by 1774bp of the *cGh* promoter was used to evaluate transactivation properties of the cPIT1 isoforms. This reporter construct gave specific dose-dependent activation of the *cGh* promoter in the presence of over-expressed PIT1. Thus, we conclude this to be an appropriate system for our studies.

In our attempt to clone full-length isoform cDNAs into an expression vector, we identified a novel isoform (PIT1β2) that lacks 26bp at the 5'-end of the β-specific 84bp exon present in PIT1β1 and PIT1γ. We predict that this isoform is translated from an initiation codon located in exon 1 downstream of the start site used by *Pit1*α and *Pit1*β1 from an mRNA that is alternatively spliced (Fig. 7). This isoform, which we named *Pit1*β2, differs from the other known isoforms in the N-terminal transactivation domain, while the C-terminal POU-domain is conserved, and PIT1β2 is the shortest of all PIT1 isoforms, comprising 315 amino acids.

5'RACE was performed to identify the TSS of the isoforms. PIT1 isoforms α, β1 and β2 were found to have a common TSS. Thus, these three isoforms were transcribed as a single mRNA, which was then alternatively spliced and translated from alternate Met codons to give rise to the isoforms mentioned. However, our results indicate this TSS to

be located 128bp upstream of the translation start site. The TSS of the avian-specific isoform *Pit1*γ is different, and our findings for the TSS of *Pit1*γ show the initiation of transcription within introns 1, 90bp upstream of translation start site.

Protein expression of chicken PIT1 isoforms from recombinant plasmid revealed certain interesting points. PIT1γ protein was either not expressed or very unstable in non-chicken cell lines. Even when detectable in a chicken cell line, the level of expression was lower than other isoforms when the same amount of plasmid was transfected. Based on our results, it does not appear likely that this isoform is subjected to proteosomal degradation, and the impaired translation is not due to an imperfect Kozak sequence, because expression was also lower than the other isoforms when the endogenous sequence was replaced by a perfect Kozak sequence. A possible mechanism behind such regulated expression might be phosphorylation of PIT1γ, a modification that unmasks a hidden degradation signal. However, in absence of isoforom specific antibody this could not be tested empirically. Also, post-translational modifications of this isoform appear to be different than the others, as demonstrated by either masking or cleavage of an epitope tag placed at the N-terminal end of the protein. It should be mentioned that the level of expression of PIT1β1 was also lower than that observed for PIT1α and PIT1β2 in our transient transfection experiments.

A consistent feature of over-expression of PIT1 isoforms in LMH cells was the presence of multiple bands detected when blotted with the PIT1 antiserum. The identities of these peptides are not known. The presence of multiple in-frame start codons in the PIT1

protein sequence gives rise to the possibility that these are stable, shorter peptides which differ from each other in their N-terminus. While the possibility that these are products of proteolytic degradation exists, results from Western blots for N- and C-terminally epitope-tagged proteins strongly indicate otherwise. As shown in Fig. 11, we detected single bands when N-terminally tagged isoforms were probed with antibody against the tag, but multiple bands were detected when the same lysates were probed with the PIT1 antiserum. Multiple bands were also detected when the tag was placed at the C-terminus. If an endopeptidase was involved, we would expect to see multiple bands with N-terminally tagged constructs with antibody directed against the tag. We detected these shorter products in both fresh and freeze-thawed lysates, thus suggesting these are not degradation products arising after denaturation of protease inhibitors used in protein extraction. Protein sequencing (N-terminal sequencing) might be used as a tool to determine the identity of these bands.

The functional significance of these shorter proteins is not known. In mammals, a functional isoform arises from using a downstream in-frame Met codon that activates the Prl promoter just like PIT1, while it has no effect on *Gh* promoter activation (Voss *et al.*, 1991). Future studies are warranted to find out whether these shorter proteins in chicken are functional and can be designated as novel isoforms. However, we are not certain if these shorter isoforms are stable in pituitary somatotrophs.

Luciferase reporter assays with a construct containing -1727/+48 of the *cGh* promoter driving expression of a reporter gene showed that cells expressing any PIT1 isoform

activated the *cGh* promoter significantly over cells expressing no PIT1. The response was found to be specific and dose-dependent. For the highest dose transfected, the isoforms PIT1α, PIT1β1 and PIT1β2 gave a 10-fold activation of the *cGh* promoter over cells expressing empty vector, while for PIT1γ the activation was about 3-fold. These results are in agreement with comparable work done with PIT1α and PIT1γ by Murase *et al.* (2011) in CHO-K1 cells. However, even though the activation caused by PIT1γ is statistically significant compared to that caused by empty expression vector, it is not significant when compared with that achieved with an empty reporter vector. This may indicate that the activation is non-specific and thus, we should exercise caution in interpreting it. At a lower dose of 100ng, PIT1γ lost its ability to transactivate the *cGh* promoter, while the other isoforms still exhibited more than 5-fold activation of the promoter.

Due to the lower abundance of PIT1γ protein in transfected cells, we wanted to ensure expression of comparable levels of proteins when promoter activities were tested. In such titration experiments, we had to transfect very low amounts of PIT1α and PIT1β2 to obtain protein expression comparable to that obtained with much more *Pit1*β1 and *Pit1*γ expression vectors. At such doses, PIT1γ (3000ng) resulted in a 3-fold activation of the promoter, but this was not significantly different than when an empty reporter vector was used. We compared these two results, because expressing 3000ng of PIT1γ had resulted in a suppression of the renilla luciferase. Similar suppression was not seen with the doses of other isoforms transfected. When comparable protein expression was obtained, PIT1β2 resulted in a 20-fold activation of the promoter, significantly higher than those by PIT1α

and PIT1β2, while the last two were not significantly different from each other. Overall, our results indicate that PIT1γ is not as capable of transactivating the *cGh* promoter as the other isoforms. This is actually what we hypothesized based on the fact that the structure of the N-terminal transactivation domain of PIT1γ is markedly different from the other isoforms. A limited activation is possible because even though the N-terminal domain is primarily involved in transactivation of target genes, some residual transcriptional activation is also associated with the POUHD (Theill *et al.*, 1989; Ingraham *et al.*, 1990). However, in our experiments, activation of the *cGh* promoter by PIT1γ was comparable to the effect of PIT1γ on an empty reporter construct. Thus, the activity of PIT1γ, if any, on the *Gh* promoter is questionable.

All the isoform proteins were found to localize to the nucleus in both LMH and embryonic chicken pituitary cells. Localization of PIT1 to the nucleus has been shown in turkey (Weatherly *et al.*, 2001), and recently, Murase *et al.* (2011) demonstrated nuclear localization of PIT1α and PIT1γ in Cos-7 cells. We demonstrated nuclear localization of all known chicken PIT1 isoforms to the nucleus of LMH cells and chicken embryonic pituitary cells. Thus, inability to localize to the nucleus is not responsible for the lack of activation of *cGh* promoter by PIT1γ.

The current model of PIT1 regulated gene transcription involves PIT1 binding, via the POU-domain, to AT-rich sequences in the target gene promoter, followed by recruitment of other factors to facilitate transcription. Therefore, we decided to test if all PIT1 isoforms bind to the functional, proximal PIT1 binding site to determine if reduced

activation by PIT1γ is due to lack of binding to the *cGh* promoter. The proximal PIT1 binding site (ATCTGCAT) (Ohkubo *et al.*, 1996) located at -104/-113 of the *cGh* promoter is the functional site (Ip *et al.*, 2004, Murase *et al.*, 2011). Previous results from our laboratory have shown that PIT1 binds the proximal site with increased affinity after 1.5 hr of CORT treatment (i.e., before the initiation of *Gh* gene expression) and this decreases at 6 hr (Narayana and Porter, unpublished). However, these experiments were done with endogenous PIT1 in chicken pituitary cells, and since the PIT1 antibody recognizes all the isoforms, the results are not isoform-specific. Results of EMSA with whole cell extracts of LMH cells transfected with individual PIT1 isoforms showed robust binding of PIT1α, PIT1β1 and PIT1β2 to the proximal PIT1 binding site contained within an infra-red labeled probe. This binding was specific, as no binding was detected with a scrambled probe, and the specific binding was competed off with 100-fold molar excess of unlabeled probe. Surprisingly, PIT1γ exhibited significantly reduced binding even when the level of protein expression was comparable among the isoforms. This result was contrary to our hypothesis, which was based on the fact that DNA-binding is mediated by the POU-homeodomain which is perfectly conserved among all the isoforms. Thus, it appears that the reduced activation of the *cGh* promoter by PIT1γ is due, at least in part, to reduced binding of the same to the proximal PIT1 binding site.

Since multiple PIT1 isoforms (PIT1α, PIT1β1 and PIT1β2) activated the *cGh* promoter but to different levels (PIT1β1>PIT1α/Pit-β2>PIT1γ), we wanted to see if any isoform had a regulatory effect on another. Very few studies have looked at the effects of the presence of one isoform on the other(s) in regulating target genes (Sporici *et al.*, 2005), a

condition that is encountered in the PIT1 expressing cells of the anterior pituitary. Specifically, we were interested in a possible negative regulatory role of PIT1γ on one of the activating isoforms. In a previous report, Bastos *et al.* (2006) have shown that ovine PIT1γ and PIT1δ, which showed no activation of the rat Prl promoter in HeLa cells, functioned as dominant negative repressors of PIT1 (wild type) when expressed together. To this end, we co-expressed PIT1β1 and PIT1γ in LMH cells to investigate a possible effect on *cGh* promoter activity. According to our results, co-transfection of PIT1γ did not have an effect on the transactivation potential of PIT1β1. The two other activating isoforms, PIT1α and Pit-β2, were also co-expressed individually with Pit-β1 to see if the combination leads to a synergistic activation of the *cGh* promoter. As seen from our results, co-expressing either PIT1α or PIT1β2 with PIT1β1 led to stimulation of the *cGh* promoter to a level significantly higher than that achieved with either isoform transfected alone. In these experiments, the doses were chosen such that they activated the *cGh* promoter and at the same time led to comparable levels of protein expression. For the combination PIT1α with PIT1β1, there is a possibly synergistic activation of the *Gh* promoter, while for PIT1β1 and PIT1β2, the combined effect is likely additive. Our results are the first to demonstrate such effects of co-transfection of PIT1 isoforms in chicken for any PIT1 regulated gene.

Since PIT1 is known to bind DNA as a dimer (Holloway *et al.*, 1995; Jacobson *et al.*, 1997), we wanted to test if the additive or synergistic effects shown by isoform co-transfection are results of physical protein-protein interactions between the isoforms. We employed co-immunoprecipitation to detect PIT1β1/PIT1β2 and PIT1β1/PIT1α

heterodimerization. Co-immunoprecipitations in our conditions failed to capture any such physical interaction between the isoforms. However, in the absence of a positive control for known protein-protein interactions, we are reluctant to conclude that the isoforms do not form a heterodimer. It is entirely possible that the interaction may not be strong enough to withstand the physical stress of cell lysis and subsequent immunoprecipitation. Other reports of heterodimerization (Sporici *et al.*, 2005) have employed bacterially expressed PIT1 protein with GST tags. In these experiments, in vitro translated PIT1 proteins were purified and incubated with their dimerization partners that were obtained in a similar manner. These conditions provide highly enriched proteins and are not physiologically similar to conditions in vivo.

Involvement of cAMP in GH synthesis has been shown in several species (Copp and Samuels, 1989; Tansey *et al.* 1993; Shepard *et al.* 1994, Argenton *et al.* 1996, Wong *et al.* 1996), and for promoters lacking a core CRE motif, the cAMP effect is thought to be mediated by PIT1 through phosphorylation and recruitment of CBP to the transcription complex (Xu *et al.*, 1998, Zanger *et al.*, 1999; Cohen *et al.*, 1999). As seen from our results, PIT1β1 resulted in significantly higher activation as compared to PIT1α and PIT1β2. We hypothesized that enhanced interaction of PIT1 with CBP, resulting in faster recruitment of the same to the transcription initiation complex, is the mechanism responsible for the effect. To test this, we transfected c-myc-tagged C-terminal 773 aa of cCBP (cCBP773) along with PIT1β1 into LMH cells, but once again we failed to detect protein-protein interaction between PIT1 and cCBP by co-immunoprecipitation. Thus, our co-immunoprecipitation results were inconclusive.

The phosphorylation status of PIT1 response to CORT in e11 chicken pituitary cells was investigated using an anti-P-threonine antibody. PIT1 is a target of phosphorylation by PKA and PKC pathways in mammals. Previous (Bossis and Porter, 2003) and unpublished results from our lab have shown that GC induction of chicken somatotroph differentiation and *Gh* gene expression requires the involvement of p38 MAPK and MEK1/2 pathways (Malkiewicz, 2003; Ellestad, 2010). PIT1 is known to be phosphorylated at a conserved Thr residue located in the POU-domain that is conserved across isoforms. Also, CORT induces increased binding of PIT1 to the proximal Pit-binding site of the *cGh* promoter (Narayana and Porter, unpublished), which can be a result of phosphorylation-induced conformational change. However, the requirement of phosphorylation seems to vary according to the gene being regulated and the species being tested. No report of PIT1 phosphorylation exists for any avian species. Unfortunately, we were not able to consistently detect phosphorylation of endogenous PIT1 from e11 chicken pituitary cells, either under basal or CORT-stimulated conditions.

This project began with the hypothesis that the PIT1 isoforms will differ in their ability to activate the *cGh* promoter, and that this difference will be due to structural variations in the N-terminal transactivation domain. The first part of the hypothesis was supported, as three isoforms exhibited significant activation of the promoter, while PIT1γ showed minimal activation, if any. Even though we initially thought the isoforms will not differ in their ability to bind to the *cGh* promoter proximal PIT1 binding site, we were proven

wrong. PIT1γ showed no binding to this site, thus providing a possible explanation as to why it may not function as the other isoforms. Co-expression of PIT1γ did not lead to suppression of activation by PIT1β1, while co-expression of either PIT1α or PIT1β2 with PIT1β1 led to increased activation of the *cGh* promoter. The mechanism leading to this increased activation is not known. In conclusion, in this study we have identified a novel isoform of chicken PIT1 (PIT1β2) and have provided a comparative account of the transactivational properties of chicken PIT1 isoforms to regulate the *cGh* promoter. We have also postulated a possible mechanism behind the difference noted, and speculated on the purpose of having multiple isoforms of the same protein in the same cell type involved in the regulation of a single gene.

Chapter 3: Conclusion and future directions

Conclusion

PIT1 is a POU-homeodomain transcription factor essential for pituitary-specific growth hormone (*Gh*) gene expression. Several PIT1 isoform mRNAs exist in chickens which have not been characterized. The main aim of this study was to determine which, if any, of the chicken PIT1 isoforms regulated the *Gh* promoter. Our study is the first to report a comparative functional characterization of all known PIT1 isoforms in an avian species. We showed that three of the isoforms, PIT1α, PIT1β1 and PIT1β2 regulated the *cGh* promoter, while PIT1γ did not. Results from gel-shift assays show that PIT1γ did not bind the proximal PIT1-binding site of the *cGh* promoter as well as the other isoforms, suggesting a possible mechanism behind the inactivity. Our results, did not suggest a negative regulatory role for this isoform. In contrast, there seemed to be a functional advantage for having multiple isoforms expressed simultaneously. PIT1β1, the isoform that activated the promoter to the greatest extent, when co-transfected with another activating isoform (PIT1α or PIT1β2) brought about a significantly higher level of activation than any isoform alone. Whether this increased activation required or was facilitated by heterodimerization of two isoforms is not known. However, identification of isoforms with specific functions will facilitate identification of their interacting partners essential for *Gh* gene expression.

Future directions

Our study aimed to determine the ability of the chicken PIT1 isoforms to regulate the *cGh* promoter. Since chicken pituitary cells express all known isoforms, and possibly unknown ones, it was essential to perform the experiments in a non-pituitary cell line. After performing trial experiments in mammalian and chicken cell lines, LMH cells (a chicken liver carcinoma cell line) were deemed to be suitable for our purposes. However, this is not the native environment of PIT1. Hence, even though we obtained specific activation of the *Gh* promoter using our reporter construct, our findings need to be confirmed in pituitary cells. We have weighed in on the option of using a rat pituitary cell line. However, previous work from our laboratory has shown that the *cGh* promoter can be activated in the rat pituitary GH4C1 cell line in absence of transfected chicken PIT1 expression construct. Moreover, our trial experiments have shown significant downregulation of PIT1β1 protein expression from transfected plasmid in GH4C1 cells. Pituitary cells, in both mammals and birds, normally have about 10-fold less abundance of PIT1β mRNA and protein (Morris *et al.*, 1991; Kurima *et al.*, 1999; Van As *et al.*, 2000), and it seems that such comparative levels are regulated in a pituitary-specific manner.

Testing the effect of individual isoforms in cells of pituitary lineage will involve selective knockout of the others. Given the fact that the isoforms are encoded from a single gene, knockout of the entire gene is not a possible solution. Short hairpin RNA (shRNA)-mediated gene silencing appears to be the most attractive way to achieve this. However,

there are two main impediments that could make achieving this goal difficult. Normally, multiple shRNAs are tested, and often used simultaneously, to obtain substantial silencing of a gene. Since the differences between the isoforms encompass relatively short regions and all are located toward the 5'end, designing sufficiently unique shRNAs will prove to be a challenge. Furthermore, shRNAs against multiple isoforms will need to be delivered into chicken pituitary cells, which have very low (5-10%) transfection efficiency. No chicken pituitary cell line exists. A way around this problem might be the use of adenoviral constructs encoding the short RNAs or some other mechanism of direct delivery of the shRNA into pituitary cells in culture.

Binding of endogenous PIT1 to the endogenous *Gh* promoter in chickens under both basal and CORT-stimulated conditions has been demonstrated by our laboratory (Narayana, 2010). However, isoform-specific binding has been demonstrated by us only with PIT1 isoforms over-expressed in a heterologous system. Isoform-specific monoclonal antibodies will be required to demonstrate endogenous PIT1 binding, and producing such antibodies, while not impossible, will be difficult, as they would need to be designed against minimal N-terminal differences among the isoforms, reducing the number of unique sequences that can serve as epitopes.

Since the main area of research by our group is regulation of *Gh*, we have not tested the ability of the isoforms to regulate other PIT1 regulated genes, such as prolactin, thyroid-stimulating hormone β-subunit, or growth hormone-releasing hormone receptor. Testing

activational abilities of PIT1 isoforms for these promoters will provide an estimate of the specificity of isoform function.

It will also be of interest to know which isoform mRNA and proteins are expressed in which cells of the PIT1 lineage. Co-localization experiments using antibodies against hormones unique to cell types would have been a simple approach but for the fact that isoform-specific antibodies are not available. We are currently working on determining cell-type specific isoform mRNA expression. The approach involves FACS sorting of pituitary cells based on fluorescence emitted by antibodies directed against pituitary hormones to obtain homogeneous populations of pituitary cell types. RNA extracted from these populations will be reverse transcribed, and the presence of PIT1 isoform cDNAs will be determined using quantitative real-time PCR. However, experiments demonstrating the presence of mRNA should always be supplemented by those showing protein expression.

Our results have shown an additive or possibly synergistic effect of co-transfection of two isoforms. Since PIT1 is known to bind DNA as a dimer, we tested if two activating isoforms bound as a dimer. Also, while investigating a possible mechanism underlying high activation by PIT1β1 compared to others, we tried to determine if PIT1β1 showed increased and tighter interaction with CREB-binding protein (CBP). However, we could not detect any interaction between the isoforms or between PIT1 and CBP by co-immunoprecipitation. Previous reports of dimerization of PIT1 have all employed purified *in vitro* translated proteins, conditions that do not represent the actual cellular

environment (Ingraham *et al.*, 1990; Holloway *et al.*, 1995; Sporici *et al.*, 2005). Reports of interactions of PIT1 with other transcription factors or transcriptional activators, with one exception of PIT1 interaction with GATA2 (Gordon *et al.*, 1997), have all employed *in vitro* translated purified protein as well (Voss *et al.*, 1991; Verrijzer *et al.*, 1992; Nalda *et al.*, 1997) . We do not know if the isoforms do not dimerize, or if the interaction is dynamic, and therefore, transient. It will be a sensible approach to first test dimerization *in vitro* by other methods such as FRET or surface plasmon resonance, and then use a functional assay such as a two-hybrid system to detect interactions *in vivo.*

In conclusion, while our work made a significant contribution in elucidating the fine details of regulation of *Gh* by chicken PIT1 isoforms, much remains to be known about the process. Future advancements in technology will hopefully aid in circumventing the problems that are faced currently and help elucidate the mechanism of regulation of *Gh* gene expression in detail.

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