ABSTRACT

Title of Document: IDENTIFICATION OF A NON-CLASSICAL GLUCOCORTICOID-RESPONSIVE ELEMENT IN THE 5'-FLANKING REGION OF THE CHICKEN GROWTH HORMONE GENE

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Growth hormone (GH) effects growth and contributes to a lean phenotype in broiler chickens. GH secretion by the anterior pituitary begins on embryonic day (e) 14, concomitantly with a rise in adrenal glucocorticoids (GC) or corticosterone (CORT) secretion. CORT treatment of chicken embryonic pituitary (CEP) cells induces GH secretion prematurely. GC induction of the GH gene requires on-going protein synthesis or an intermediary protein, but the gene lacks a classical GC-response element. We hypothesized that a GC-responsive intermediary protein is necessary for the CORT induced increase in GH. Characterization of the upstream region of the gene may help identify such a protein. To this end, a fragment of the GH gene (-1727/+48) was cloned into a luciferase reporter and characterized in e11 CEP cells. CORT treatment increased luciferase activity and mRNA. Inclusion of CHX blocked CORT induction of luciferase mRNA. Through deletion analysis, we found

that a GC-responsive region (GCRR) is located at -1045 to -954. By defining the GC-responsive region and *cis*-acting elements located within, *trans*-acting proteins involved in GC induction of the GH gene may be identified. The GCRR is CORT-responsive in either orientation, but it is context-dependent. Potential transcription factor motifs in the GCRR include ETS-1 and a degenerate GRE (GREF). Nuclear proteins bound to a GCRR probe in a CORT-regulated manner and unlabeled competitor DNA competed off binding. Mutation of the central portion of the DNA probe resulted in a significant decrease in protein binding. Mutation of the ETS-1 site or GREF site in the -1045/+48 GH construct resulted in ablation of luciferase activity. ETS-1 and GR are associated with the endogenous gene under basal and 1.5 h CORT-treated conditions, while GR recruitment increased after CORT treatment. GC regulation of the GH gene during chicken embryonic development requires *cis*-acting elements located 1 kb upstream from the transcription start site and includes recruitment of ETS-1 and GR. This is the first study to demonstrate involvement of ETS-1 in GC regulation of the GH gene during embryonic development. Characterization of GC regulation of the GH gene during embryonic development enhances our understanding of growth regulation in vertebrates.

IDENTIFICATION OF A NON-CLASSICAL GLUCOCORTICOID-RESPONSIVE ELEMENT IN THE 5'-FLANKING REGION OF THE CHICKEN GROWTH HORMONE GENE

By

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List of Abbreviations

Ab	antibody
ACTH	adrenocorticotropin
ANOVA	analysis of variance
bp	base-pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CORT	corticosterone
Ct	cycle threshold
CV	coefficient of variation
d	day
kDa	kilo-Dalton
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
e	embryonic day
EMSA	electrophoretic mobility shift assay
ETS-1	e-twenty six
FACS	fluorescence activated cell sorting
GC	glucocorticoid
GCRR	glucocorticoid responsive region
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GREF	glucocorticoid responsive and related elements
h	hour
IgG	immunoglobulin G
LCR	locus control region
M	molar
min	minute
mAb	monoclonal antibody
mL	milliliter
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
µg	micrograms per milliliter
ng	nanograms per milliliter
NRS	normal rabbit serum
nm	nanometer
nM	nanomolar
PBS	phosphate buffered saline

PCR	polymerase chain reaction
Pit-1	pituitary specific factor 1
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
RPA	ribonuclease protection assay
RHPA	reverse hemolytic plaque assay
SFM	serum free culture media
siRNA	small interfering ribonucleic acid
SP1	specificity protein 1
TBE	Tris boric acid EDTA buffer
TSHβ	thyroid stimulating hormone β
TR	thyroid hormone receptor
T_4	thyroxine
T_3	triiodothyronine
UTR	untranslated region
ZK98	ZK98299

Chapter 1: Literature Review

Introduction

Growth hormone (GH) is a protein hormone produced in the anterior pituitary (Giustina & Veldhuis, 1998). The pituitary is composed of two lobes: anterior and posterior, also known as the adenohypophysis and the neurohypophysis, respectively. The anterior pituitary can be considered the master regulator of homeostasis because it contains five different cell types that secrete hormones which are essential for growth, reproduction, and metabolism in vertebrates. GH is produced by the somatotroph cell type, and has several important functions in the body, most notably, bone and muscle growth, muscle accretion, lipolysis, and promotion of immune function (Fig. 1). It is under strict regulation at the gene, mRNA, and protein levels, through feedback loops from the hypothalamus, liver, adrenal glands, and thyroid gland (Giustina & Veldhuis, 1998). Understanding of the regulation of GH will aid in the overall understanding of growth and metabolism in vertebrates. Major regulators of the GH gene include the pituitary specific factor, Pit-1, glucocorticoids, thyroid hormone, specificity protein 1 (Sp1), and cAMP response element binding protein (CREB). Pit-1 is necessary for the differentiation of somatotrophs during development, but it is not sufficient. Other transcription factors are necessary for the final induction of the GH gene in all species; however, the necessary transcription factor(s) and their associated upstream signaling pathways have not been fully elucidated. Most species studied, such as mouse, rat, dog, fish, and chicken, have one GH gene; however, the human GH gene lies within a cluster

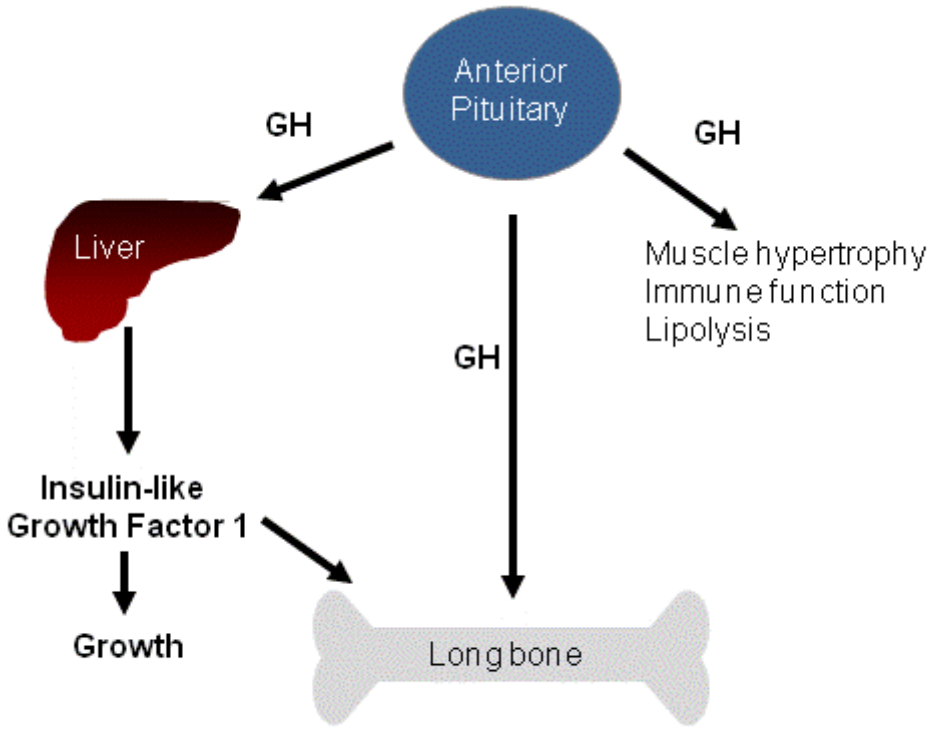


Figure 1: Functions of GH. GH is produced in the anterior pituitary gland and directly effects long bone growth. It travels to the liver to induce secretion of insulin-like growth factor 1 to effect overall body growth. GH's secondary functions include muscle hypertrophy, immune function and lipolysis.

of 5 related genes, and these genes are under the control of a locus control region (LCR) that directs tissue-specific expression. It is important to highlight the species-specific similarities and differences in the regulation of the GH gene, as this may lead to the discovery of the unknown necessary differentiating transcription factors. The review here will focus on pituitary development, GH gene regulation, glucocorticoid mechanism of action, and glucocorticoid regulation of the GH gene.

Anterior Pituitary Development

Introduction

In the brain, the pituitary resides ventral to the hypothalamus in the *sella turcica* and is connected to the hypothalamus through the highly vascularized hypophyseal stalk. Hormones produced in the hypothalamus are released into the stalk and are quickly transported to the pituitary via a portal blood system. All of the hormones produced by the pituitary are controlled by hormones produced in the hypothalamus. GH is positively regulated by growth hormone-releasing hormone (GHRH) and negatively regulated by somatostatin (SRIF), both of which are produced in nuclei within the hypothalamus (Anderson et al., 2004).

The pituitary is a classical neuroendocrine organ of both neural and ectoderm origin necessary for the maintenance of homeostasis, metabolism, reproduction, growth and lactation. The anterior pituitary is composed of five distinct cell types: corticotrophs, gonadotrophs, thyrotrophs, somatotrophs and lactotrophs. The cell types are classified based on the primary hormone that each one produces. Corticotrophs secrete adrenocorticotropin (ACTH), gonadotrophs secrete luteinizing hormone and follicle-stimulating hormone, thyrotrophs secrete thyroid-stimulating hormone, and lactotrophs

secrete prolactin. Each of these cell types arise from a single primordial cell and is classified based on the type of hormone that is produced by the cell (Griffen & Ojeda, 2004).The pituitary originates as a structure known as Rathke's pouch. It is composed of two distinct parts: the neurohypophysis and the adenohypophysis or the posterior and anterior pituitary, respectively (Griffen & Ojeda, 2004). Discussion of the pituitary will be limited to only the anterior pituitary.

Formation of Rathke's pouch begins as the cells of the forebrain (ventral diencephalon) grow and displace cells of the anterior neural ridge (ANR). Simultaneously, an evagination from the roof of the pharynx (oral ectoderm) pushes dorsally into the displaced cells of the ANR. The cells of the ANR then thicken and invaginate to form Rathke's pouch (Figure 2A) (Griffen & Ojeda, 2004). Pituitary organogenesis can be divided into four phases: formation of Rathke's pouch, cell proliferation and zone partitioning, cell fate determination and terminal differentiation (Figure 2B).

Cell proliferation and zone partitioning are regulated by both extrinsic and intrinsic signals. Extrinsic signals regulating organogenesis include fibroblast growth factors (FGF), bone morphogenetic proteins (BMP), members of the *Wnt* gene family, and Sonic hedgehog (Shh) (Zhu et al., 2003). The former three originate from the ventral diencephalon, whereas Shh emanates from the oral ectoderm (Figure 2C). It seems that FGF8 and BMP4 are required for initial organ commitment, proliferation and progression (Treier et al., 1998). FGF8 induces Lhx3/P-LIM, a LIM homeodomain transcription factor, so that development of the pituitary will continue past the formation of Rathke's pouch. A knockout mouse model of the genes encoding either FGF8 or the FGF receptor

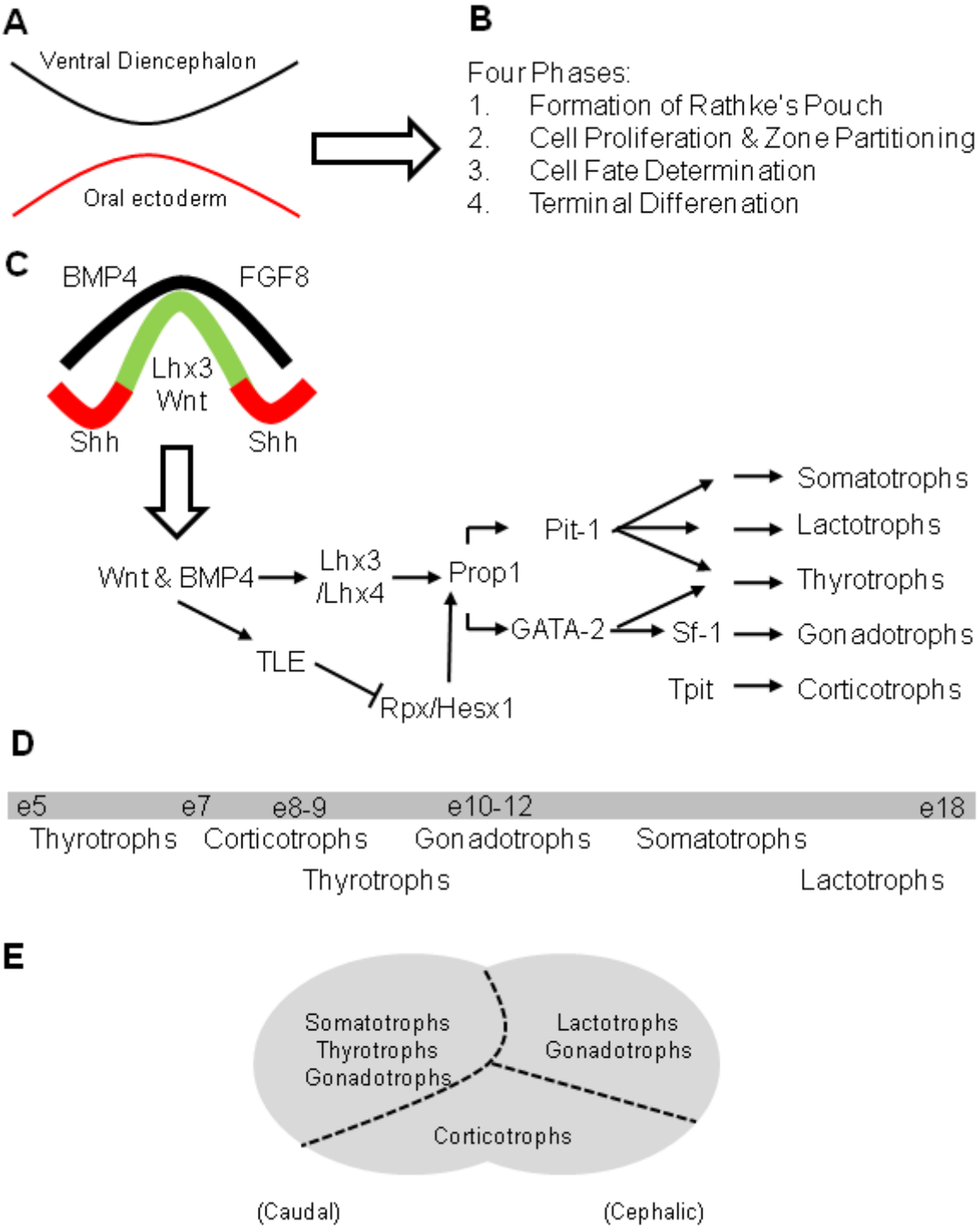


Figure 2: The anterior pituitary gland is formed from Rathke's pouch. A) Rathke's pouch is derived from the oral ectoderm and the ventral diencephalon. B) There are four phases of development of the anterior pituitary gland. C) The differentiation of cell types requires the activation of specific transcription factors in a spatially and temporally regulated manner. D) Ontogeny of the cell types in the chicken anterior pituitary gland during embryonic development. E) Localization of the cell types in the anterior pituitary gland of the adult chicken.

type II results in failure of the organ to proliferate and causes rapid apoptosis immediately following the formation of Rathke's pouch. Deletion of BMP4 causes embryonic death at roughly E10 and failure of invagination of Rathke's pouch (Olson et al., 2003). Shh localizes throughout the oral ectoderm but is entirely absent from the invaginating Rathke's pouch. Shh is required for pituitary proliferation and patterning after E10 and works with FGF8 to sustain ventral expression of Lhx3 (Treier et al., 1998). This cooperativity is similar in limb and neural tube development. Intrinsic signaling or signals originating from Rathke's pouch include BMP2 and Wnt4. Inhibition of BMP2 causes loss of the thyrotrophs, somatotrophs, and lactotrophs, but not corticotrophs. Attenuation of the BMP signal is also required for terminal differentiation of the cell types. Cell types are arranged positionally within the gland's proliferation zones, due to gradient signaling patterns of FGF8, BMP, Wnt and Shh. Thus, somatotrophs and lactotrophs arise caudo-medially, gonadotrophs are positioned more rostro-ventrally and corticotrophs are ventrally positioned in the mouse (Olson et al., 2003).

The gradient signaling patterns described above result in the induction of transcription factors in spatially overlapping patterns. The transcription factors are generally from the homeodomain class, such as the LIM, paired-like, bicoid-like and sine-oculus families. Multiple members of the LIM family: Lhx3, Lhx4, Lhx2 and Isl-1, are expressed throughout Rathke's pouch during pituitary development, however, a specific causal relationship between the LIM family and cell lineage has yet to be established (Zhu et al., 2007). Paired-like homeodomain factors exert opposing activator/repressor functions, as each binds to similar DNA sequence elements and can

heterodimerize with its cognate paired factor. Rpx/Hesx1 is expressed early in pituitary development and appears to be necessary for early proliferation and progression of the gland. It can heterodimerize with *Prophet of Pit-1* (Prop-1) and can inhibit the activation properties of the Prop-1 gene (Zhu et al., 2007). Prop-1 is necessary for the development of three cell types: thyrotrophs, somatotrophs, and lactotrophs. Down regulation of Rpx/Hesx1 occurs when it interacts with and is sequestered by a member of the groucho/transducin-like enhancer of split family (TLE). Down regulation of Rpx/Hesx1 is necessary to allow Prop-1 to exert its effects on cell lineage and proliferation (Olson et al., 2003). *Pax6*, another homeodomain regulator, is necessary to modulate ventral/dorsal determination. *Pax6* mutant mice have an increased number of ventral thyrotrophs and gonadotrophs and a decreased number of dorsal somatotrophs and lactotrophs. Pitx1 and Pitx2, bicoid-related homeodomain factors, are necessary for the development of lung asymmetry, cardiac positioning and tooth morphogenesis, besides the pituitary (Lin et al., 1999). Deletion of Pitx1 results in the diminished expression of the gonadotrophs and thyrotrophs and increased expression of the POMC gene (Zhu et al., 2007). Pitx2 knockout mice exhibit pituitary arrest at e10.5, as well as defects in tooth development, ventral body wall closure and right lung isomerism (Olson et al., 2003). Pitx1 and Pitx2 are necessary for the development of multiple organs and processes and are not specific to pituitary organogenesis.

The aforementioned factors are required for cell proliferation and partitioning of the pituitary organ; but what regulates the differentiation of the distinct cell types, and further, what regulates hormone production, namely GH, within the cell type? It is important to study the transcription factors necessary for the development of the pituitary

gland, in order to determine if these same transcription factors or different factors are necessary for the terminal differentiation of hormone-secreting cells. Some distinct transcription factors that are necessary for terminal differentiation of distinct cell types have already been discovered. Pit-1 (Pituitary specific factor-1) is responsible for the terminal differentiation of somatotrophs and lactotrophs. Pit-1 and GATA2 are the necessary factors for thyrotrophs. GATA2, SF-1 and Egr-1 are necessary for differentiation of gonadotrophs, while T-pit and possibly STAT3 are involved in the differentiation of corticotrophs (Lamolet et al., 2001 & Pulchino et al., 2003). Clearly, distinct combinations of transcription factors are necessary for the terminal differentiation of hormone-secreting cell types. The GH gene may require a unique combination of transcription factors for activation.

In rats, αGSU producing cells first appear on e11.5, ACTH-producing cells first appear on e14.5, followed by TSH-producing cells on e15, LH-producing cells on e16.5, and FSH-producing cells on e17 (Simmons et al., 1990). GH-producing cells begin to appear on e18, with a moderate increase on e20. GH is detected in circulation on e19 (Setalo & Nakane, 1976; Chatelain et al., 1979; Watanabe & Daikoku, 1979). In the chicken, a pit-1 independent population of thyrotrophs appears on e5, corticotrophs are the next cell type to emerge on e7. A pit-1 dependent population of thyrotrophs emerge on e9 followed by gonadotrophs between e10-e12. Then somatotrophs differentiate around e12 and become a significant population of the gland between e14 and e16 (Porter et al, 1997). Last, lactotrophs differentiate around e18 (Parkinson et al., 2010) (Figure 2D). In the mouse, thyrotrophs first appear on e12.5 in the rostral tip of the gland, although this cell population is Pit-1 independent. Pit-1-dependent populations of

thyrotrophs emerge at e14.5 in the caudomedial region (Lin et al, 1994). In mice, GH-producing cells first appear on e15 and the population increases rapidly on e16 (Dole et al., 1990). In the chicken anterior pituitary gland, corticotrophs are localized ventrally throughout. Gonadotrophs are localized throughout the entire gland, while lactotrophs are restricted to the cephalic lobe. In contrast, somatotrophs and thyrotrophs are localized to the caudal lobe (Figure 2E) (Parkinson et al., 2010).

The Role of Pit-1 in Anterior Pituitary Development and Somatotroph Differentiation

Differentiation of a pituitary cell type is complete once production of its hormone is initiated. Many known and unknown factors are necessary for the complete differentiation of hormone-secreting pituitary cells, and somatotrophs in particular. Pit-1, also known as GHF-1, is necessary for the differentiation of a lineage of cell types: thyrotrophs, somatotrophs and lactotrophs (Anderson & Rosenfeld, 1994). But how does one gene/one protein induce the expression of a specific hormone in one cell type and exclude the same expression in other cell types? For example, GH is secreted in somatotrophs and although the gene is present in lactotrophs and thyrotrophs, it is not secreted.

Pit-1 belongs to the POU family of transcription factors that includes Pit-1, Oct-1 and Oct-2, and Unc-86 (Van As et al., 2000). These highly conserved transcription factors each have a homeodomain that is linked to another domain known as the POU-specific domain. Most other family members are highly expressed in the nervous system. Pit-1 was first discovered as the transcription factor that is necessary for the expression of growth hormone and prolactin, and this definition was later extended to include thyroid

stimulating hormone β, or TSHβ (Nelson et al., 1988). The proximal 320 bp of the human GH gene promoter is necessary and sufficient for selective expression of the GH gene in mouse pituitary. This region contains multiple Pit-1 sites and a thyroid hormone response element. Prolactin has a similar promoter region of 3 kb with multiple Pit-1 sites and an estrogen-regulated Pit-1 dependent enhancer as well (Crenshaw et al 1989; Lira et al, 1988). Although Pit-1 is required for normal somatotroph differentiation, it is not the final differentiating factor because it is expressed before GH expression and in two other distinct hormone-secreting cell types. In the rat, Pit-1 containing cells begin to emerge on e15.5 and become significant at e16.5 (Simmons et al., 1990). In the mouse, Pit-1 containing cells emerge on e13.5 (DiMattia et al., 1997). In the chicken, Pit-1 is expressed on e8 (Tanaka et al., 1999).

Pit-1 contains a bipartite DNA binding domain that resembles the classic helix-turn-helix motif that is necessary to bind in the double helix major groove. Further, both the homeodomain and POU specific domain are required for high affinity binding to DNA sequences. The first published Pit-1 binding site was TATNCAT, and it requires an AT-rich sequence immediately 5' to the consensus (Fox et al., 1990). A more recent publication denotes this consensus sequence: 5'-(T/A)NCTNCAT-3' (Ohkubo et al, 1996). The highly conserved Pit-1 sites in the human GH gene are -127/-107 (AGCTTCTAAATTATCCATTA) and -87/-72 (CCATGCATAAATGTA) (Nelson et al, 1988). In the chicken, a Pit-1 site was discovered at -113/-104 with a sequence of ATCTGCAT (Ip et al. 2004). However, the teleost/avian consensus sequence is 5'-(T/A)NCTNNCAT-3'. Interestingly, the spacing between the two sites, consensus and AT-rich, determines the outcome of expression for the corresponding gene. Depending

on the binding site, Pit-1 will induce expression of GH and suppress prolactin in somatotrophs, while the opposite is observed in lactotrophs (Nelson et al., 1998).

Pit-1 is necessary for the terminal differentiation of thyrotrophs. Thyrotrophs produce thyroid-stimulating hormone (TSH), which is composed of a common subunit (αGSU) shared by gonadotropins and a β subunit unique to the thyrotroph cell type. Pit-1 and another factor, GATA-2, bind to specific sequence elements upstream of thyrotroph-specific genes in order for activation (Umeoka et al., 2002). Simultaneously, GATA-2 and Pit-1 can heterodimerize so that GATA-2 is unable to bind to upstream sequence elements of gonadotroph-specific genes, thus ensuring that gonadotroph-specific genes are not activated in cells destined to the thyrotroph cell fate. Conversely, GATA-2 binding upstream of gonadotroph-specific genes without Pit-1 blocking allows for the terminal differentiation of gonadotrophs and the repression of thyrotroph-specific genes (Gordon et al., 1997).

The question remains, what is the final differentiation factor in somatotroph development? It is most likely not Pit-1, since microarray analysis of chicken embryonic pituitary glands during development shows no change in Pit-1 expression between e10 and e17 (Ellestad et al, 2005), and Pit-1 mRNA and protein are not increased by treatment with CORT (Fu et al., 2004).

Growth Hormone: Mechanisms, Actions and Implications

Growth Hormone (GH)

In the chicken, GH is a protein hormone composed of 221 amino acids; *gh* gene has five exons and it is located on chromosome 27 (Tuggle & Trenkle, 1996). In most mammals, GH is a single gene (Page et al., 1981); however, in primates, GH is a gene

locus with six genes (Tuggle & Trenkle, 1996). Pituitary GH is regulated by GH-releasing hormone, a 44-amino acid peptide produced in the hypothalamus, and somatostatin, a 14-amino acid peptide also produced in the hypothalamus (Griffen & Ojeda, 2004). The two peptides regulate GH through their cognate G-protein coupled receptors (GPCR). Regulation also occurs in a classical negative feedback manner via hepatic insulin-like growth factor I (IGF-I). Basal expression of GH is controlled by specificity protein 1 (Sp1), NF-1/AP-2, USF and zinc finger 15 (ZN-15). Factors that stimulate GH secretion include thyroid hormone, glucocorticoids, retinoic acid, and cAMP (Tuggle & Trenkle, 1996). GH is most notably known for directly increasing long bone growth and muscle accretion during development (Tuggle & Trenkle, 1996). In the embryonic chicken, plasma GH can be detected at e17 (Harvey et al, 1979). The receptor for GH is a single-transmembrane-domain, membrane-anchored receptor belonging to the cytokine receptor super-family (Griffen & Ojeda, 2004).

Regulation of Transcription of the Growth Hormone Gene: Promoter Analysis

Growth hormone is predominantly expressed in the anterior pituitary gland. It is also expressed in the mammary gland, lymphoid cells, eye, lung, and testes (Harvey et al., 1996; 2001). A different set of genes is activated in each of these tissues, resulting in a different protein milieu. In the pituitary gland, GH expression is predominantly stimulated by glucocorticoids, while in the mammary gland GH expression is predominantly stimulated by progestins (Mol et al., 2000).

The first studies elucidating transcription factors directing GH gene expression involved mapping DNaseI hypersensitive sites (HS) also known as DNA footprinting.

DNase I is an enzyme that degrades DNA. DNA can only be degraded if it is exposed or not covered by proteins. By treating chromatin (DNA bound with proteins) with DNase I, base pairs that are covered or protected by proteins can be mapped by sequencing. The human GH gene has four DNase I hypersensitive sites (Jones et al., 1995). HS sites I and II were later found to be necessary for GH gene expression (Bennani-Baiti et al., 1998). FPIII contains binding sites for two factors, one of which was found to be upstream stimulating factor (Lemaigne et al., 1989). The protein that caused FPIV was later found to be Sp1. The binding activities of Sp1 and Pit-1 to the distal site were mutually exclusive and cooperative (Lemaigne et al., 1990). However, Sp1 binding to its site alone could not direct transcription of the GH gene.

Analysis of the canine GH promoter showed several shared binding sites with the human, mouse and rat GH genes, including binding sites for Pit-1, progesterone receptor (PR), glucocorticoid receptor (GR), Sp1 and ETS1, as well as lymphoid-specific transcription factors, such as Ikaros and Pax5 (Lantinga-van Leeuwen et al., 2002). Likewise, the mouse GH promoter showed high homology with the rat promoter but conservation with pig, cow, and human of only the proximal promoter (Das et al., 1995). Comparison of the proximal promoters of the GH gene of the chicken, rat, mouse, and human show remarkable similarities (Figure 3).

Initial studies using transgenics demonstrated a minimal promoter necessary to direct GH gene expression to the anterior pituitary gland. This was done mostly in mouse oocytes using the human and rat GH genes (Lira et al., 1988). Using the upstream region of the rat GH gene fused to the human GH coding sequence injected into fertilized mouse oocytes, it was shown that the sequence corresponding to -181 to -45 was necessary to

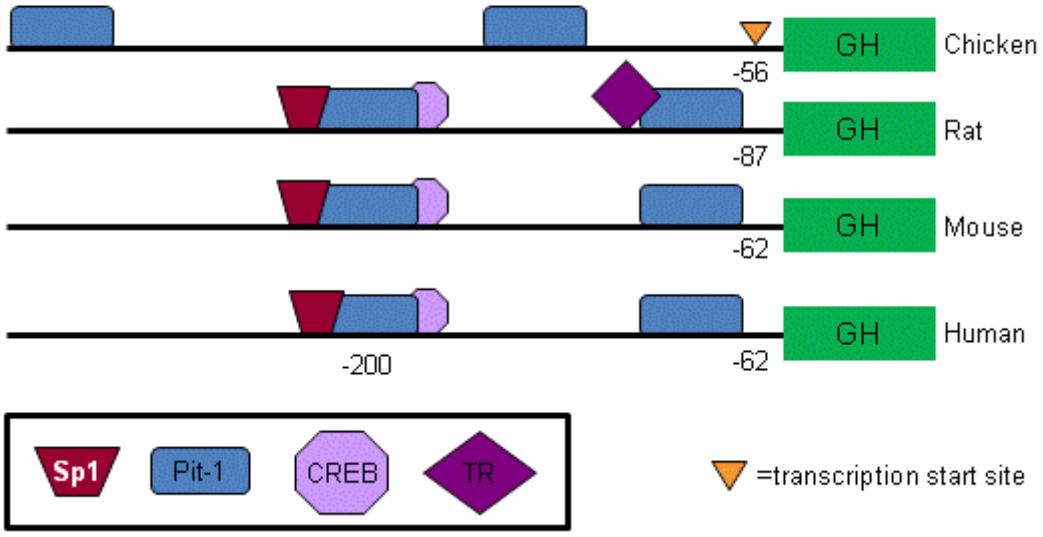


Figure 3: Schematic of the proximal promoter of the GH gene from four different species. The proximal promoter is relatively conserved, with respect to three transcription factors, Sp1, Pit-1 and CREB.

direct expression to the pituitary gland. Using immunohistochemistry, the cells expressing the transgene also expressed predominantly GH, but to a lesser extent, thyrotropin and prolactin (Lira et., 1988). This site was later found to be the Pit-1 proximal binding site.

How, then, does one begin to distinguish between potential transcription factor binding sites in the 5' flanking region of the chicken GH gene and determine the availability of transcription factor proteins present in anterior pituitary cells during the mid-stage of embryonic development? More specifically, what is the differentiating factor for initial GH expression in the anterior pituitary gland of the developing chicken embryo? What regulates GH transcription?

Even though it is well known that Pit-1 directs expression of three cell lineages in the pituitary, many studies are centered on potential Pit-1 interacting partners or nearby promoter elements in order to discover the cell-specific terminal differentiation factors. For example, expression of the Chinook salmon GH gene is controlled by two cAMP response elements (CRE) and Pit-1 (Wong et al., 1996). The yellowtail GH gene was also cloned, and the promoter was analyzed. A sequence similar, but not identical, to the mammalian Pit-1 binding site was found to reside at -128 to -90, which is the same location as the mammalian Pit-1 binding site (Ohkubo et al., 1996). Further analysis showed that this is the conserved Pit-1 binding site found in avian and teleost species. Analysis of evolutionary conserved sequences identified a conserved element located between the proximal and distal Pit-1 binding sites. This element was shown to bind a zinc finger protein, Zn-15 (Lipkin et al., 1993). Mutation of the Zn-15 binding site decreased hGH expression more than 100 fold. (Lipkin et al., 1993). Therefore,

inspection of sequence flanking the Pit-1 site in the chicken GH gene could provide clues to the trans-activating factor required in the CORT induction of GH.

Pit-1 is a homeodomain transcription factor, but there are many other classes of transcription factors. One such class is the nuclear hormone receptor which can be considered as ligand-activated transcription factors. Nuclear hormone receptors bind ligands such as thyroid hormone, glucocorticoid, mineralocorticoid, retinoic acid, estrogens, progesterone and androgen (Griffen & Ojeda, 2004). Each of these nuclear hormone receptors has their own specific response element. Most studies focus on identifying such response elements in the 5' flanking region of the gene; lower eukaryotes have conserved regulatory elements in the 3' or downstream flanking region. Rarely, do regulatory sequences occur within the coding region of a gene. However, a GRE has been found in intron 1 of the human GH gene (Slater et al., 1985). Almost 3 kb of the human GH gene, including 500 bp each of the 5'flanking region and the 3' flanking region was analyzed for CORT-regulated protein binding. It was found that the GH gene contains two glucocorticoid response elements (GRE): one in the upstream region and one in the first intron. Further, only the GRE in the first intron produced a "footprint" or a DNase I hypersensitive site (Slater et al., 1985). Identification of the factor involved in the CORT induction of the chicken GH gene could be accomplished by exploring regulatory sequences for potential response elements.

Analysis and comparison of promoters across species is necessary to determine trans-acting factors. A comparison of the GH gene across species allowed for some insight into speciation and divergence, adding support to the theory that sequence variation is the basis for functional complexity (Buggiotti & Primmer, 2005). This theory

more appropriately fits the description of a gene promoter: complexity and variation allows for tissue- and time-specific expression of a particular gene.

Long Range Control: the Human GH gene and its Locus Control Region

Classical molecular biological and genetic studies typically focus on the immediate flanking DNA regions of a gene of interest. There is also evidence that genes are under long distance control. A region of DNA located as far as 30 kb away from the transcription start site of the gene of interest has been shown to regulate the gene. This region is known as a "locus control region" or LCR.

DNase I hypersensitive site mapping also revealed the presence of a LCR for the human GH gene (Jones et al., 1995). The LCR is located between -14 kb and -30 kb upstream of the transcription start site of the GH gene. It is thought that this region exists because the human GH gene locus actually contains a cluster of five related GH genes (hGH-N, hGH-V, hCS-A, hCS-B and hCS-L). The LCR was originally discovered in the β-globin set of genes in the human (Grosveld et al., 1987). The GH gene is predominantly expressed in the pituitary, while the chorionic somatomammotropin (CS) gene is expressed in the placenta (Jones et al., 1995). The LCR is necessary for human GH expression in mouse pituitary. The minimal essential region of the LCR is between -14.6 kb to -16.2 kb and is necessary to direct human GH expression in the anterior pituitary of the embryonic mouse at the appropriate developmental times (Bennani-Baiti et al., 1998). Pit-1 is essential for the coordinated transcriptional control of the hGH gene cluster (Shewchuk et al., 1999). Pit-1 binding in the HS I and HS II sites is necessary and sufficient to direct regulated tissue-specific expression, more so than binding at the promoter of the hGH-N gene (Shewchuk et al., 2002). Control of the specified set of

genes via the LCR is coordinated through the formation of domains of histone modifications (hyperacetylation or methylation) and chromatin looping (Kimura et al., 2004, Yoo et al., 2006 & Ho et al., 2008). There is also evidence that ongoing non-coding transcription of the CD79b gene is essential for regulation by the LCR (Ho et al., 2006). The continuous non-coding transcription of the nearby gene, CD79b, allows for the constant presence of RNA polymerase II (RNA pol II) and an open chromatin conformation. The regulated transcription of the growth hormone gene cluster begins with Pit-1 binding at HS-I and in the promoter of the hGH-N gene which then triggers an interaction between the two regions accomplished by looping. The looping of the chromatin allows for the juxtaposition of the hGH-N promoter into a region of high transcriptional activity, the LCR/CD79b transcriptional domain that is dense with RNA pol II (Ho et al., 2008). To this date, a similar LCR has not been identified in the GH gene of chicken, mouse or rat, although only one GH gene exists in these species. Rather, an LCR is used to direct expression of a group of related genes and non-primates have a single GH gene.

The Connection between Signaling Pathways and Transcription Factors

Characterizing gene regulation by searching for transcription factor binding sites is only half of the story. Transcription factors can also be the end points of cytoplasmic signaling pathways, such as kinase cascades. One example is the luteinizing hormone receptor (LHR) gene. The LHR gene is induced by the PI3K/PKC signaling pathway and Sp1 (Liao et al., 2008). Phorbol myristyl acetate (PMA) activates PKCα and enhances Sp1 phosphorylation. Sp1 is also a downstream target of ERK. Once this signaling cascade is activated, the HDAC/Sin3A repressor complex is released from the Sp1 site

upstream from the LHR gene, histone H3 is acetylated, Sp1 binds to its response element and TATA binding protein (TBP) and RNA polymerase II are recruited (Liao et al., 2008). Elucidating transcriptional regulation should conclude by analyzing whether a specific activated transcription factor recruits TBP and RNA polymerase II.

Analysis of Mutant Phenotypes is Necessary for Understanding

Dwarfism, which usually results from low or no levels of circulating GH or a mutation in the GH receptor, has been linked to defects in four other genes to date: Pit-1, GHRH-receptor, CREB, and lysine demethylase 1 (LSD1).

Defects in the Pit-1 gene result in the absence of three hormones (GH, TSH, and PRL) and this condition has been characterized as the Snell dwarf mouse (Li et al, 1990). Similarly in humans, defects in the Pit-1 gene result in congenital hypothyroidism, dwarfism and prolactin deficiency. The *little* mouse has been found to have a mutation in the GHRH-receptor (GHRH-R), thus demonstrating that the receptor for the hypothalamic regulatory hormone, GHRH, also plays a role in somatotroph differentiation (Godfrey et al, 1993). Dwarf phenotypes in chickens have also been linked to mutations in the GH receptor gene (Burnside et al., 1991 & 1992).

Another study showed that cAMP response element-binding protein (CREB) is essential for normal growth. CREB was one of the first transcription factors identified that regulates GH gene expression. Over-expression of a dominant negative form of CREB in the anterior pituitary resulted in the loss of somatotrophs, low GH levels and dwarfism (Struthers et al., 1991). A follow-up study showed that loss of CREB in the

brain but not the pituitary also resulted in the same dwarf phenotype (Mantamadiotis et al., 2006).

LSD1 knockout is embryonic lethal. But pituitary-specific LSD1 knockout using Cre Pitx1 mice resulted in normal pituitary morphogenesis. However, there was complete loss of GH and TSHβ cells and a marked decrease of LHβ and ACTH containing cells as well, indicating that LSD1 is necessary for the differentiation of all five hormone secreting cell types in the pituitary (Wang et al., 2007).

Essential Glucocorticoids, the Receptor and the Mechanism of Action

Essential Glucocorticoids

Glucocorticoids (GC) are produced in the adrenal cortex and are known as the essential permissive hormone. They are necessary for life; however, they do not initiate most processes, rather they allow the process to proceed. GCs regulate in intermediary nutrient metabolism, the cardiovascular and central nervous system, the inflammatory and immune responses, and development. The hallmark role of GCs is to increase glucose availability for the central nervous system (CNS) by decreasing glucose utilization in the rest of the body (Griffen & Ojeda, 2004). Developmentally, GCs are necessary for the fetal development of several organs, including the lung and intestine.

GCs' more well known and practical mechanism of action is the anti-inflammatory response and gene repression. Inflammation is a natural response to intracellular damage or an extracellular challenger. Proinflammatory signals include lipopolysaccharide (LPS; found in the cell wall of bacteria), viruses, IL-1 β and tumor necrosis factor (TNF) (Griffen & Ojeda, 2004). GCs are used pharmacologically to

reduce inflammation. The review here will primarily focus on GCs role in activation of transcription.

Structure of the Glucocorticoid Receptor

GR is evolutionarily conserved from *Xenopus* and *Teleost* to rat and human. One GR gene has been identified with multiple promoters and multiple splice variants and multiple translational isoforms (Yudl & Cidlowski, 2002). Chicken GR shows 86-98% amino acid homology with human, pig, rat, mouse, tilapia and frog (Yudl & Cidlowski, 2002). Phylogenetically, it clusters more closely with human and rat as opposed to frog and tilapia. GR is composed of three major domains: a ligand binding domain (LBD), a DNA binding domain (DBD), and the transactivation domain (TAD) (Beck et al., 2010). These three domains are highly conserved (Beck at al., 2010). The TAD is responsible for transcriptional activation and contains a sub-domain known as activator function 1 (AF-1). Basal transcription cofactors bind in the TAD (Bodwell et al., 1993). The DBD binds to the DNA double helix and is responsible for dimerization with other nuclear receptors. The DBD contains two zinc fingers that intercalate into the DNA double helix. The LBD contains a pocket for the ligand, protein/chaperone/cofactor binding sites, and a second transactivation domain (AF-2). Two nuclear localization signals exist between the DBD and the LBD; one is ligand-dependent and one is ligand-independent (Beck et al., 2010).

Regulation of GR

GRα is the primary isoform, and GRβ was found to be the dominant negative isoform as it lacks the LBD (Oakley et al., 1997). GRβ can regulate GRα by sequestering

coactivator complexes (Charmandari et al., 2006). GR can be post-translationally modified via phosphorylation, acetylation, nitrosylation, redox regulation, ubiquitination, and SUMOylation (Beck et al., 2010). The GR gene is negatively regulated by GCs due to the presence of a negative GRE. It is also regulated by AP-1, NF-kB, and CREB binding sites located in the promoter (Beck et al., 2010).

Mechanism of Action of the Glucocorticoid Receptor

GR is held inactive in the cytoplasm by a multimeric complex. Within this complex is heat shock protein 90 (Hsp90). Hsp90 physically interacts with GR's ligand binding domain, and it stabilizes the ligand binding pocket of GR indirectly (Figure 4). High affinity binding of the ligand to GR requires Hsp90 (Ricketson et al, 2007). Other proteins in this complex include Hsp70, p23 (binds to Hsp90), Hop (an Hsp-organizing protein), FK506-binding protein 51 and 52, cyclophilin 40, Hsp70 interacting protein, and PP5. The other major function of this complex is to keep the nuclear localization (NLS) inactive (Beck et al., 2010). Hsp90 also promotes the stability of other steroid hormone receptors and some kinases (Kovacs et al., 2005). The activity of Hsp90 is regulated by acetylation by the deacetylase, HDAC6. Inactivation of HDAC6 leads to Hsp90 hyperacetylation, dissociation from an essential co-chaperone, p23, and loss of chaperone activity. Interestingly, in HDAC6 deficient cells, GR activity is compromised (Kovacs et al., 2005). Further investigation of the HDAC6 knockdown phenotype resulted in the discovery that GR itself is not affected; rather the interaction between hsp90 and GR is destabilized. The destabilization is due to a hypoacetylated state of hsp90 (Murphy, et al., 2005). When ligand enters the cell, Hsp90 dissociates, and the ligand pocket is available for binding. Upon ligand binding, GR undergoes a

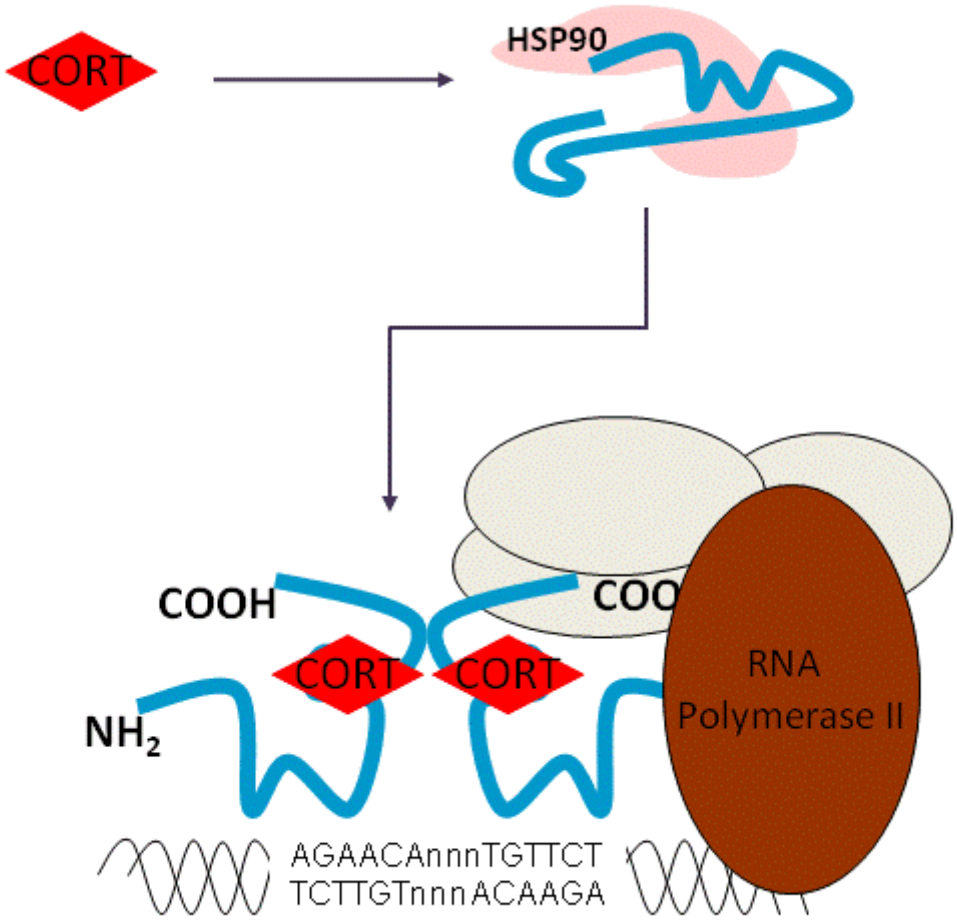


Figure 4: The mechanism of action of the glucocorticoid receptor (GR). CORT enters the cell and binds to GR. GR is held inactive in the cytoplasm by a complex of proteins, including heat-shock protein 90 (hsp90). Upon CORT binding, the receptor undergoes a conformational change, dissociates from the complex and translocates to the nucleus. Once in the nucleus, CORT-bound GR homodimerizes with another GR receptor and the dimer binds to a classical response element. Then coactivator complexes and accessory factors are recruited. Last, RNA polymerase II is recruited and gene transcription ensues.

conformational change and the nuclear localization signal is exposed. GR is chaperoned to the nucleus with the help of importin α and Nup62 (Savory et al., 1998 & Echeverria et al., 2009).

There are numerous conflicting reports regarding the location of GR. Classically, GR is held in the cytoplasm unless ligand-activated and then it is translocated to the nucleus. Although, a snapshot of a cell will reveal that the majority of unliganded GR is in the cytoplasm and liganded GR is in the nucleus, there is constitutive shuttling of the receptor, such that at any given time, unliganded GR will be found in the nucleus at much lower levels (Beck et al., 2010). Importin α and β along with the NLS signals are responsible for the import of GR into the nucleus, ligand bound or not; while CRM1/exportin1 is responsible for nuclear export. A GR nuclear retention signal was also found recently (Carrigan et al., 2007). Another study focused on receptor location and ligand selection. GR distribution between the cytoplasm and the nucleus was random when bound to cortisol, but it was nonrandom when bound to the synthetic agonist, triamcinolone acetonide (Schaaf et al., 2005). A closer look revealed that ligands that induced a nonrandom distribution of the receptor between the cytoplasm and the nucleus actually slowed migration of the receptor, whereas ligands that induced a random distribution did not impede the mobility of the receptor. This same study showed that receptor mobility and distribution are dependent upon the type of ligand, and ultimately the effect was a change in gene transcription. However, more sophisticated methodologies can reveal a snapshot of the living cell, such as using RNA fluorescence in situ hybridization (FISH) to label the MMTV promoter and a GFP-GR fusion protein. GR occupancy on the DNA was studied in individual cells. This showed that even within

a relatively homogenous cell population, the occupancy, duration and frequency of dissociation of receptor from DNA varied widely (Voss et al., 2006).

Once glucocorticoids diffuse freely into the cell and bind to the GR, the complex dissociates, exposing the NLS, and GR moves along cytoskeletal tracts to enter the nucleus. The protein, doublecortin-like (DCL), is associated with microtubules. Using over expression and knockdown studies in COS-7 cells, it was found that DCL controls GR translocation to the nucleus (Fitzsimons et al., 2008).

Regulation of Transcription by GR

GR can positively regulate transcription three different ways: it can bind as a homodimer to a glucocorticoid response element (GRE) to activate transcription, it can bind directly to DNA and either displace or prevent a different transcription factor from binding to its cognate binding site; and it can couple with other transcription factors and bind to a composite element (Necela & Cidlowski, 2004) (Figure 4). GR can also negatively regulate transcription as seen in its anti-inflammatory role. GR can bind as a single receptor to a negative GRE; sequester a transcription factor away from its response element; bind to a negative composite element with another transcription factor; or prevent another transcription factor from binding to the DNA element (Beck et al., 2010).

GR's natural ligand can be cortisol or CORT, depending on the predominant circulating glucocorticoid of the species. Upon ligand binding, GR translocates to the nucleus and can bind to DNA. Ligand/GR complexes are continuously disassembled off of the DNA response element by an active process. The LBD is essential for this phenomenon. However, the dissociation of GR from GREs is faster in the absence of ligand, and the receptor exhibits ligand-specific exchange rates. Plus, ligand dissociation

is not required for receptor dissociation from GREs (Meijsing et al, 2007). These studies have elegantly shown that the response resulting from GR binding to its response element is actually a culmination of many different minute mechanisms tailored at the ligand/receptor and receptor/response element levels.

GR physically interacts most notably with AP-1, NFκB/RelA, and STAT3/5; although this interaction usually results in transcriptional repression of the gene. The anti-inflammatory response mediated by AP-1 and NFκB/RelA is repressed by GR. GR's DBD interacts with either AP-1 or NFκB to repress gene transcription (De Bosscher et al. 2003). The binding of GR to a transcription factor results in the sequestration of that factor away from the DNA response element. GR interacts with members of the Orphan Nuclear Receptors, such as Nurr77 in a similar manner as the GR/AP-1 interaction (Martens et al., 2005). A protein-DNA array was made to determine which transcription factors are regulated by interaction with GR (Jiang et al., 2004). The group overexpressed human GR in COS-7 cells, crosslinked proteins to DNA, immunoprecipitated with a human GR antibody, and hybridized the immunoprecipitated proteins to a DNA array. They validated the study using luciferase transactivation assays and supershift experiments. Additional transcription factors found to interact with GR include CTCF/E47, COUP-TF, and IRF. Pit-1 was on the array, but was not found to interact with GR (Jiang et al., 2004). However, it should be noted the experiments were performed in COS-7 cells, a green monkey kidney cell line, which does not express endogenous GR.

To activate transcription, GR binds to a glucocorticoid response element (GRE). The classic GRE is an imperfect palindrome of six base-pair half sites with a three base-

pair spacer in between (Strahle U et al., 1987). The consensus GRE is 5' GGT ACA nnn TGT TCT. Dimerized GR binds in the major groove of the DNA via its two zinc fingers (Beck et al., 2010). Inspection of a large number of genes found that "glucocorticoid regulated genes" conspicuously lack a classic GRE, implying that the "classic" GRE is not the most common, but rather one of the first identified. A recent study using ChIP-chip technology in the A549 lung carcinoma cell line identified genes regulated by GR occupancy (So et al., 2007). Using free source software, Weblogo, they identified a more relaxed glucocorticoid response element. This glucocorticoid binding region (GBR) is still 15 base pairs long but from that only 5 base pairs and the spacing are highly conserved. The conserved GBR identified is RGNACARRRWGTNCN, where R is a purine, N is any nucleotide, and W is an A or a T. Further, there is an enrichment of particular motifs occurring in close proximity, such as AP-1, HNF4, C/EBP, ETS family, and SP1 (So et al., 2007). Another group identified glucocorticoid response units (GRU) in acute lymphoblastic leukemia cell lines. The GRU closely resembles a degenerate GRE half site (Geng et al., 2008). In close proximity to each GRU was either a c-myb or ETS-1 motif, demonstrating again the possibility of gene regulation by hetero-complexes binding to composite response elements. Another approach to finding and analyzing GC regulated genes involved four treatment groups: with or with GC and with or without cycloheximide to identify direct GC targets and indirect targets (Chen et al., 2003). The RNA was extracted and hybrized to the Affymetrix oligonucleotide array. 44 genes were found to be directly regulated by GC. Analysis of 8 kb of flanking sequence from eight of the GC-regulated genes revealed a consensus sequence of ACAnnnTGTnCT. Although there is some degeneracy in the consensus sequence, there are definitely conserved

nucleotides and spacing. Variability in GREs has been an extensive area of study recently. The positive prediction of functional GREs will be essential in advancing this field, since GCs are pharmacologically important and necessary for life.

Cofactors and their role in transcriptional regulation

Transcription factors that frequently work in concert with GR include Sp1, STAT1/3/5, C/EBP, Ets1, Egr-1, AP-2, AP-1, and NF-κB (Beck et al., 2010). GRIP1, GR-interacting protein 1, was originally discovered as a corepressor of GR during tethering to AP-1 (Beck et al., 2010). The phosphorylation state of GR regulates its association with cofactors (Hsu et al., 1992).

A highly studied gene that is induced by cAMP and interleukin-1 and inhibited by GR and glucocorticoids is surfactant protein A (SP-A) (Islam et al., 2008). Surfactant protein aids in the lubrication of the delicate lung tissues that allows for the transition from fluid filled lungs to air filled lungs that occurs at birth. SP-A expression is further mediated by thyroid transcription factor 1 (TTF-1) and nuclear factor B (NF-κB). GR interacts with TTF-1 and NF-κB at the response element. Knockdown of GR results in increased SP-A expression. Treatment with DEX increased recruitment of endogenous GR and histone deacetylase (HDAC)-1 and -2 and blocked binding of conserved helix-loop-helix ubiquitous kinase (CHUK or IKKα). Inspection of the chromatin environment revealed that DEX treatment blocked acetylation and phosphorylation of histone H3 and mediated the dimethylation of H3 K9 at the SP-A promoter (Beck et al., 2010). GR is a complex and dynamic protein; it interacts with DNA alone or in conjunction with other transcription factors and can aid in changes to the local chromatin environment through histone modifications. This well studied gene demonstrates regulation at the level of the

promoter/response element, transcription factors and their binding partners, and the local chromatin environment. Regulated gene expression is typically the culmination of these three levels of control.

Accessory factors also facilitate the binding of GR to its response element. GC regulate the phosphoenolpyruvate carboxykinase (PEPCK) gene. Using nuclear extracts and the consensus GRE or a degenerate GRE; it was found that GR bound longer and with more frequency to the consensus GRE. Binding was further enhanced with the presence of COUP-TF/HNF4 and HNF3 (Stafford et al., 2001).

A growing class of proteins known as nuclear receptor coregulators modulates the transcriptional activity of steroid receptors. GCs suppress corticotropin-releasing hormone (CRH) in a classic negative feedback loop. Regulation of this gene by GC, GR and nuclear receptor regulators was studied in AtT-20 cells. Overexpression of steroid receptor coactivator 1a (SRC1a) increased efficacy and potency of the GC-mediated repression of the CRH gene (van der Laan et al., 2008). However, nuclear receptor corepressor (NCoR) and silencing mediator of the retinoid and thyroid hormone receptor (SMRT) had no effect on the GC-regulation of the gene, but did modulate the forskolin-induced increase in CRH activity (van der Laan et al., 2008).

Coregulators have been described for all of the steroid and nuclear receptors. They can enhance or repress gene expression, depending on the cell type and signaling milieu. RNA pol II transcribes genes into complementary RNA. The enzyme is part of a large complex with ~30 additional proteins which constitute the basal transcriptional machinery. Coregulators were initially described as bridging complexes that connect basal transcriptional machinery to more specific DNA-binding transcription factors

(Lonard et al., 2007). An important distinction regarding coregulators is their function. They possess enzymatic activity, aid in transcriptional elongation, RNA splicing, and mRNA transport. The enzymatic activities demonstrated thus far include acetyltransferase, methyltransferase, phosphokinase, ubiquitin ligase, and ATPase. As coregulators possess different enzymatic activities, they are also regulated by different posttranslational modifications, such as phosphorylation, acetylation, and methylation. The first nuclear receptor coregulators cloned were steroid receptor coactivator 1 (SRC-1), SMRT, and N-CoR. Almost 300 different coregulators have been described in the literature. To date, research has not demonstrated that coregulators exhibit NR specificity. Knockout studies of coregulators reveal that many result in embryonic lethality (Lonard et al., 2007).

The SRC family consists of three members: SRC-1, -2, and -3. SRC-2 is also known as TIF2 or GRIP1. SRC-3 is also known as AIB1, RAC3, ACTR, pCIP, and TRAM-1 (Lonard et al., 2010). SRC family members have been assigned the role of integrating hormone and growth factor signaling. SRC family knockouts do not result in embryonic lethality, suggesting redundancy in function at the very least, but there are lasting effects on reproduction, growth and energy metabolism (Lonard et al., 2010). Most genes are regulated at some level by a SRC family member or a coregulator. The GnRH-R gene is responsive to GCs and GnRH via an AP-1 site (Kotitschke et al., 2009). GR is required for this response, as demonstrated by siRNA, as is the interaction between GR and SRC-1.

GR and the Local Chromatin Environment

Evidence that GR directs changes to the local chromatin environment is shown in the study of GR interacting with HMG1 (Agresti et al., 2005). Most nuclear proteins reside on a specific chromatin site for only seconds or less. There are two models regarding this: the "hit-and-run" model where transcription factors form complexes in a random fashion from freely diffusible proteins, or the stepwise model where factors assemble in an orderly fashion to form stable holocomplexes. High mobility group 1 (HMG1) interacts with GR only in close proximity to chromatin and not in the nucleoplasm. GR and HMG1 decrease each other's mobility and the assembled complex is stable. Disassembly is affected by active, ATP-consuming processes (Agresti et al., 2005).

HMG1 binds linear DNA with moderate affinity and no sequence specificity (Calogero et al., 1999). It bends the double helix significantly by binding in the minor groove. But it binds sharply bent DNA with high affinity. Sharply bent DNA would be linker DNA that is located at the entry and exit of nucleasomes. It is recruited to DNA by interactions with proteins that are required for basal and regulated transcription. Interestingly, Hmg1-/- mice are born alive but die within 24 hrs of birth due to hypoglycemia. Cell lines lacking Hmg1 grow normally, but genes that are regulated by GR are not expressed or expressed at low levels (Calogero et al., 1999).

A research tool to study GR is the murine mammary tumor virus (MMTV). The promoter of the MMTV genome has multiple consensus GREs. GR-mediated transactivation of the MMTV promoter requires chromatin remodelers (Trotter & Archer, 2004). BRG1 is a chromatin remodeler in the SWI/SNF family. This family of proteins

was originally discovered in yeast (SWI/SNF=mating-type switching/sucrose fermentation) (Winston & Carlson, 1992). Arsenic represses GR-mediated chromatin remodeling (Barr et al., 2009). The presence of activated GR on the MMTV promoter can result in a change in histone modifications.

Glucocorticoid Regulation of Chicken GH During Embryonic Development

Overview

In chickens, GH mRNA in the caudal lobe of the anterior pituitary increases from e16 and reaches a maximum on e20 (Kansaku *et al*., 1994). After embryonic development, GH levels do not vary in birds during different reproduction stages: pre-laying, laying, 1-week incubation, 3-week incubation and brooding. During these reproduction stages, caudal lobe GH mRNA is significantly higher than that in the cephalic lobe, although both lobes show no life-stage effects (Kansaku *et al*., 1994).

Glucocorticoids from the adrenal gland stimulate the differentiation of somatotrophs in the anterior pituitary gland. Our group has previously shown that somatotrophs become a significant population of chicken anterior pituitary cells between embryonic day 14 and embryonic day 16 (e14-e16) (Porter *et al*., 1995a), although a few appear as early as e12. Somatotrophs in culture do not differentiate autonomously. Further, cell division is not necessary for induction of somatotroph differentiation (Porter *et al*., 1995b). Elucidation of this signal began with culturing pre-somatotroph populations of pituitary cells with serum obtained from e12 embryos and e16 embryos. The serum from e12 embryos did not induce cultured pituitary cells to secrete GH, but serum from e16 embryos did induce GH secretion. Finally, pre-absorption of e16 serum with an antibody against CORT abolished the serum-induced secretion of GH. Thus, the

blood-borne signal was determined to be CORT (Morpurgo *et al*., 1997). The most effective dose of CORT to induce somatotroph differentiation was found to be 2.5 nM, a physiologically relevant concentration. Further studies have demonstrated that CORT increases GH mRNA and protein and increases the number of cells that secrete GH. CORT seems to be the extra-pituitary signal causing GH secretion in previously non-hormone secreting pituitary cells. However, the question remains, what is the underlying mechanism of the CORT induction of the chicken GH gene during embryonic development?

Coincidentally with the ontogeny of pituitary somatotrophs, serum levels of CORT rise significantly between e12 and e16 in the embryonic chicken. In developing embryos, CORT increased significantly between e14 and e15 from 11.3 ng/mL to 20.4 ng/mL as measured by RIA, and CORT remained at this concentration until e20, when a significant decrease was observed (Kalliecharan & Hall, 1974). Other adrenal steroids, such as cortisol, peaked at e15 in the chicken at 22.3 ng/mL, while cortisone peaked at e17 at 25.7 ng/mL. Nonadrenal steroids, such as progesterone, exhibited a steady increase from e9 to e20 with a peak at e20 at 31.2 ng/mL, which is different from the profile for glucocorticoids. An alternative study found that serum CORT levels in the embryonic chicken were 3.6 ng/mL on e10 and three times as much (9.0 ng/mL) on e20, determined by RIA (Scott *et al*., 1981). CORT steadily rose during the last half of incubation. Serum levels of CORT on e10 through e16 were statistically different from the previous day. However, serum levels on e16 through e20 were not statistically different from the previous day (Scott *et al*., 1981). Thus, multiple studies have indicated that adrenal glucocorticoid secretion increases around the time of somatotroph differentiation.

Treatment of cultured e11 chicken embryonic pituitary cells with the GR-specific antagonist, ZK98-299, did not result in the blocking of the CORT induced increase in GH mRNA and protein levels, suggesting that CORT is able to bind to an alternative receptor. The CORT induction of GH mRNA was only blocked when pretreatment with ZK98-299 also included spironolactone, a MR specific antagonist.

The effects of CORT in chickens are also seen at the whole embryo level by treating embryos *in ovo* on e11, a few days before normal somatotroph differentiation (Bossis & Porter, 2000). The somatotroph population increased significantly by e13 with a treatment dose of 0.2 μg and 2 μg. However, the observed increase in the number of somatotrophs did not persist later in development (e16) through post-hatch day (d) 1. The same doses administered on e8 and e9 and then examined two days later did not result in an increase in the number of somatotrophs. Clearly, there is a predetermined timeline of when somatotroph precursor cells are responsive to CORT, between e11 and e12. GH secretion from cultured chicken embryonic pituitary cells was measured using an ELISA during the second half of development, under basal conditions and in response to CORT. Pituitary cells were responsive to CORT until e16. The cells were nonresponsive on e18 and e20. The age at which the cells become nonresponsive was mathematically determined to be e16.4 (Heuck et al., 2009). Administration of CORT too early in development results in embryonic death and when given later in development, the cells are already partially differentiated and do not respond to CORT.

CORT is known to induce chicken somatotroph differentiation *in vitro*, and GHRH induces the cultured GH cells to release their stored GH (Dean *et al*., 1997). Sensitivity to GHRH increases in an age-dependent manner between e16 and e20. The

combination of CORT and GHRH significantly increased the amount of GH secreted by e12 anterior pituitary cells into the cell culture medium after two and six days, while neither agent alone had any measurable effect (Dean & Porter, 1999). As stated previously and reconfirmed with these results, CORT induces somatotroph differentiation and increases the number of somatotrophs in culture, while GHRH induces the cells to release their stored GH into the surrounding environment.

CORT increases GH mRNA, but the induction of GH gene expression is delayed and requires protein synthesis (Bossis & Porter, 2003). However, the identity of the protein(s) required is not known. Induction of GH gene expression may involve one or several signal transduction cascades. Through the use of signaling cascade enzyme inhibitors, it was found that the PKA and PKC signal transduction cascades do not mediate the effects of CORT. On the other hand, a RAS GTPase is involved in this process (Bossis & Porter, 2003). Inhibition of the protein Ras with manumycin did block the CORT induced increase in GH mRNA, suggesting that a Ras/MAPK mechanism may be involved.

Since co-treatment of CORT and GHRH increases GH mRNA, the GHRH receptor should be more thoroughly explored as the potential terminal differentiation factor. GHRH binds to a G-protein coupled receptor, a seven transmembrane domain protein, the GHRH receptor. Upon binding, the G_s is activated and the α subunit dissociates from the membrane bound protein and activates adenylate cyclase, causing an increase of cAMP, and activation of PKA. CREB is an immediate downstream target of PKA. First, glucocorticoids increase GHRH-R mRNA in rat pituitary cell lines and fetal rat pituitary glands in culture, but not so in chickens. Concomitant treatment with CORT

and forskolin (FSK) or IBMX, which increase cAMP accumulation and activate PKA, increases GH mRNA over that of CORT alone; however, treatment of FSK or IBMX alone did not have an effect on GH mRNA (Bossis & Porter, 2003). This suggests that GHRH can regulate GH mRNA, but it is not the terminal differentiation factor. Blocking either PKA or PKC with inhibitors did not block the CORT induced increase in GH mRNA. Although there is evidence that thyroid hormones and GHRH modulate the CORT regulation of GH; these regulatory hormones are not the differentiating factor for GH.

In a cDNA microarray experiment to determine possible genes that are involved in somatotroph differentiation, pituitaries dissected from chickens of embryonic age e10, e12, e14 and e17 were assayed for genes expressed during this crucial time. Specifically, genes that exhibited the same expression profile or clustered with GH using self-organizing maps (SOM) analysis were identified as potential candidates involved in GH gene induction (Ellestad et al, 2005). GH gene expression steadily increased between e12 and e17, in accordance with previous findings. Genes that also increased steadily between these ages were identified and highlights include glucocorticoid-induced leucine zipper (GILZ), RAS-DVA, dexamethasone-induced ras-related protein 1 (Dexras1), stress-induced phosphoprotein 1 (STIP1) and FK506-binding protein 51 (FKBP51). Two other genes, mineralocorticoid receptor (MCCR) and JUN, an oncogene that either decreased steadily or decreased dramatically on e17, are also possible candidates (Ellestad et al, 2005). Are these genes involved in somatotroph differentiation? Possibly, but definitive evidence is lacking because this microarray experiment only shows association and not causality.

In a follow-up microarray experiment, e11 CEP cells were pretreated with cycloheximide for 1.5 h and then treated with CORT at the 1.5 h, 3 h, 6 h, 12 h, and 24 h time points. The cells were collected and the total RNA was extracted and amplified for hybridization to the cDNA microarray. This study identified genes that are dramatically up-regulated or down-regulated in the presence of cycloheximide and coincides with the increase of GH mRNA by treatment with CORT (unpublished data). A list of genes was generated, including Dexras1, RAS-DVA, and FK506BP-51.

The effects of CORT on somatotrophs are not only seen in chickens, but also in mammals. Glucocorticoids induced somatotroph differentiation in fetal rats *in vitro* using explants of fetal pituitary primordia obtained on day 14 of gestation (Hemming, 1988). In cultured rat pituitary cells obtained from 13.5 day-old fetal rat pups and maintained in culture for 8 days, cortisol stimulated the differentiation of GH-cells (Sato & Watanabe, 1998). Similar effects were seen in 16.5 day-old fetal pituitary cells that were maintained in culture for 5 days. Dexamethasone (DEX) is a synthetic glucocorticoid and is similar to CORT in structure and action. Pregnant rats were given DEX in their drinking water for 40 hrs before sacrifice (Nogami *et al*., 1993). This treatment increased GH cells in the anterior pituitary gland of the fetus on fetal day 18. The observed effect was decreased earlier in development, as seen in the diminished effect observed on day 17 and the nonexistent effect on day 16. Finally, spontaneous autonomous differentiation of pituitary cells in culture into GH-secreting cells is not observed (Nogami & Hisano, 2008).

Administration of DEX to the pregnant mother in her drinking water elicited an induction of GH-cell differentiation in the fetal rat pups. Normally, somatotroph

differentiation is not observed until e18, and under these conditions, somatotrophs differentiated on e17. Administration of DEX and T_4 augmented the previously observed increase in somatotroph differentiation (Nogami *et al*., 1995). DEX increases GH mRNA-containing cells obtained from e18 rat pituitary cells; however, the increase observed never exceeded that of intact e19 rat pituitary cells (Nogami *et al*., 1997). The same treatment on e16 did not show this, implying that somatotrophs are responsive to glucocorticoids at distinct time points during development. The DEX-induced increase in GH-containing cells was not observed after 5 or 10 h and was only observed after a full 24 h. As in chickens, the addition of a protein synthesis inhibitor completely abolished the DEX-induced increase in GH positive cells.

GR mRNA is first detected on e15 in the pituitary in the rat. GR co-localized in ACTH producing cells and not in GH producing cells. The number of cells expressing GR, but not ACTH increased on e18. A significant population of GH cells on e19 also expressed GR (Nogami & Hisano, 2008).

Differences between the Chicken and Human Genomes and the Implications

In 2004, the chicken genomic sequence was released with 6.6x coverage (Wallis et al., 2004). The chicken is touted as the modern descendant of dinosaurs and it was the first non-mammalian amniote to have its genome sequenced. Additionally, the chicken itself is a major agricultural food product and is at the center of a billion dollar industry. The chicken genome is roughly 1 billion base pairs, and it contains 20 to 23 thousand genes. The human genome is 3 billion base pairs coding for 30 thousand genes. The human genome is about three times the size of the chicken genome, and this is due to the

substantial expansion in interspersed repeat content, pseudogenes and segmental duplications. There are, however, long blocks of segmented alignments, and this corresponds to conserved synteny between the chicken and human genomes. Protein-coding genes have higher synteny than non-coding RNA genes. The independent evolution of birds and mammals can be partially attributable to stark differences in multigene families. Further, there are very few pseudogenes and short interspersed elements (SINEs) in the chicken (Wallis et al., 2004). Comparison of the chicken and the human at the genome level can provide clues as to the differences between genetic regulation of these divergent species.

The Embryonic Chicken as a Model for Endocrine Cell Differentiation

The embryonic chicken is an ideal model for both endocrine studies and cell differentiation studies. Since the chicken develops *in ovo*, outside of the hen's body, there is little to no interference by maternal hormones. The mammalian placenta is a large reservoir for 11β-HSD2. This enzyme converts GCs into their inactive form. This is a protective mechanism for mammalian species in that excess GCs during gestational development are highly deleterious to the fetus. Since the chicken develops outside of the maternal body, circulating GCs are not a problem. Another benefit of the chicken model is accurately timed development. With mice, pregnancy is typically determined by evidence of a copulation plug in the female mouse. This plug is usually only found the following morning after placing the mating pair together the previous evening. Based on this, developmental age could be poorly estimated by as much as twelve hours. In contrast, chicken embryonic development can be accurately timed and coordinated by placing them in a 37 ^{o}C incubator. This allows for the simultaneous collection of

hundreds of embryos at the same age. Eggs can easily be manipulated for injection of treatments and dissection of tissues from the embryo. Likewise, organs and tissues can quickly be collected and immediately frozen or the cells can be dispersed and cultured in as little as two hours.

Conclusions and Future Directions

The GH gene is controlled by Pit-1 binding to consensus binding sites upstream of the GH gene. Pit-1 is necessary for GH expression, but it is not sufficient. GCs are necessary to induce GH *in vitro* and *in vivo*, but studies have shown that they do not act alone. There is evidence for the involvement of thyroid hormone, retinoic acid, GHRH, C/EBPα, and CREB. Other transcription factors involved include Sp1 and Zn-15. Regulation of the GH gene in the human occurs at the LCR because the human GH gene is in a cluster of five other closely related genes that must be expressed in different tissues. Other species have only one GH gene and, therefore, an LCR does not exist. The GH promoter has been studied extensively, aiming to identify the trans-activating factors necessary for regulated GH induction. The purpose of the present research was to investigate the role of *cis*-acting elements in the 5' flanking region in the GC regulation of the GH gene during chicken embryonic development.

Chapter 2: Characterization of the Chicken Growth Hormone Gene

Introduction

Growth hormone (GH) is produced in the anterior pituitary gland by cells known as somatotrophs and regulates long bone growth, muscle accretion, lipolysis, and nutrient utilization (Griffen & Ojeda, 2004). GH contributes to the lean phenotype of the modern broiler chicken that is becoming a staple in the American diet. Exogenous GH has either no effect or negative effects on post-hatch growth of chickens. Broilers have a higher baseline level of GH, which may account for the lack of exogenous GH. Thus, distinct differences regarding the regulation of growth exist in the domesticated chicken due to selective breeding. However, the mechanism of action initiating GH secretion is still unknown.

Current research in our laboratory centers on the onset of GH secretion, differentiation of somatotrophs, and the extra-pituitary signals that regulate this phenomenon using the chicken as a model. The chicken is a unique model for studies of pituitary development because the head is relatively large compared to mammalian species, thus providing more cells; there is little maternal endocrine interaction as in mammals; and the egg allows for easy manipulation of the embryo for a multitude of experiments. Furthermore, it is more feasible to obtain the large number of embryonic pituitary cells necessary for cell culture experiments than from common mammalian models, because embryonic development can be precisely timed and controlled by artificially incubating the eggs.

The anterior pituitary contains five distinct cell types, corticotrophs,

gonadotrophs, thyrotrophs, somatotrophs and lactotrophs, which differentiate sequentially in that order and are spatially restricted. Gonadotrophs secrete luteinizing hormone and follicle-stimulating hormone, thyrotrophs secrete thyroid-stimulating hormone (TSH), somatotrophs secrete growth hormone, and lactotrophs secrete prolactin. Corticotrophs differentiate first around embryonic day (e) 7 and begin to secrete adrenocorticotrophic hormone (ACTH) that then maturizes the adrenal gland (Woods et al., 1971). Differentiation of somatotrophs naturally occurs between embryonic day (e) 14 and 16 (Porter et al., 1995a). Plasma corticosterone (CORT), originating from the adrenal gland, and ACTH dramatically increase between e11 and e17 (Jenkins et al., 2007), mimicking the pattern of somatotroph abundance and preceding the increase in plasma GH levels. GH secretion can be induced earlier (e11 and e12) both *in ovo* and *in vitro* through administration of CORT (Dean & Porter, 1999). CORT is the extra-pituitary signal that induces final somatotroph differentiation in embryonic chickens (Morpurgo et al., 1997). Further evidence using pharmacological inhibitors shows that CORT-induced somatotroph differentiation involves both the type I (mineralocorticoid, MR) glucocorticoid receptor and the type II (glucocorticoid, GR) corticosteroid receptors and requires the chaperone protein Hsp90 (Bossis et al., 2004).

GR protein is detected in pituitary extracts as early as e8, while MR wasn't detectable until e12 (Bossis et al., 2004). GR was expressed in about 95% of all pituitary cells, while MR was expressed in about 40% of pituitary cells. Co-localization studies revealed that most GH containing cells expressed both MR and GR (Bossis et al., 2004). GR and MR mRNA levels peak at e14, concomitant with the normal differentiation of GH cells (Heuck et al., 2009).

Induction of GH mRNA by CORT can be blocked *in vitro* by pre-treating anterior pituitary cells with cycloheximide (CHX), a protein synthesis inhibitor (Bossis & Porter, 2003). This suggests that either one or more proteins must be synthesized first for CORT induction of GH cell differentiation or that on-going protein synthesis is necessary. Plus, examination of 10 kilo-bases (kb) upstream of the GH gene and 5 kb downstream reveals no full-length consensus glucocorticoid responsive element (GRE). Therefore, it seems that CORT induction of GH is an indirect effect. This leads to the formation of two hypotheses: 1) the product of an unknown glucocorticoid-responsive gene induces the GH gene; 2) ligand bound GR and an unknown protein bind to an unknown composite element in the 5' flanking region of the GH gene and induce the GH gene together (Figure 5).

The promoters of the GH gene of many other species have been characterized to date, including mouse, human, rat, dog, cow, grass carp and chicken. The studies have focused on the necessary transcription factors involved in the induction of GH. Pit-1 is the most studied, tissue-specific, required transcription factor for the GH gene. Pit-1 is a required transcription factor for somatotrophs, lactotrophs, and thyrotrophs (Zhu et al., 2007). It is necessary, but not sufficient, for full expression of these three cell types (Nelson et al., 1988). Pit-1 has a conserved binding site located less than 200 bp upstream of the transcription start site of all of the GH genes studied. Most of the promoters also contain a functional, distal Pit-1 binding site. Within the same proximal region of the Pit-1 binding site of the rat GH gene, lie putative binding sites for thyroid hormone receptor (TR), Sp1, and a zinc finger protein ZN-15. Different combinations of the four different

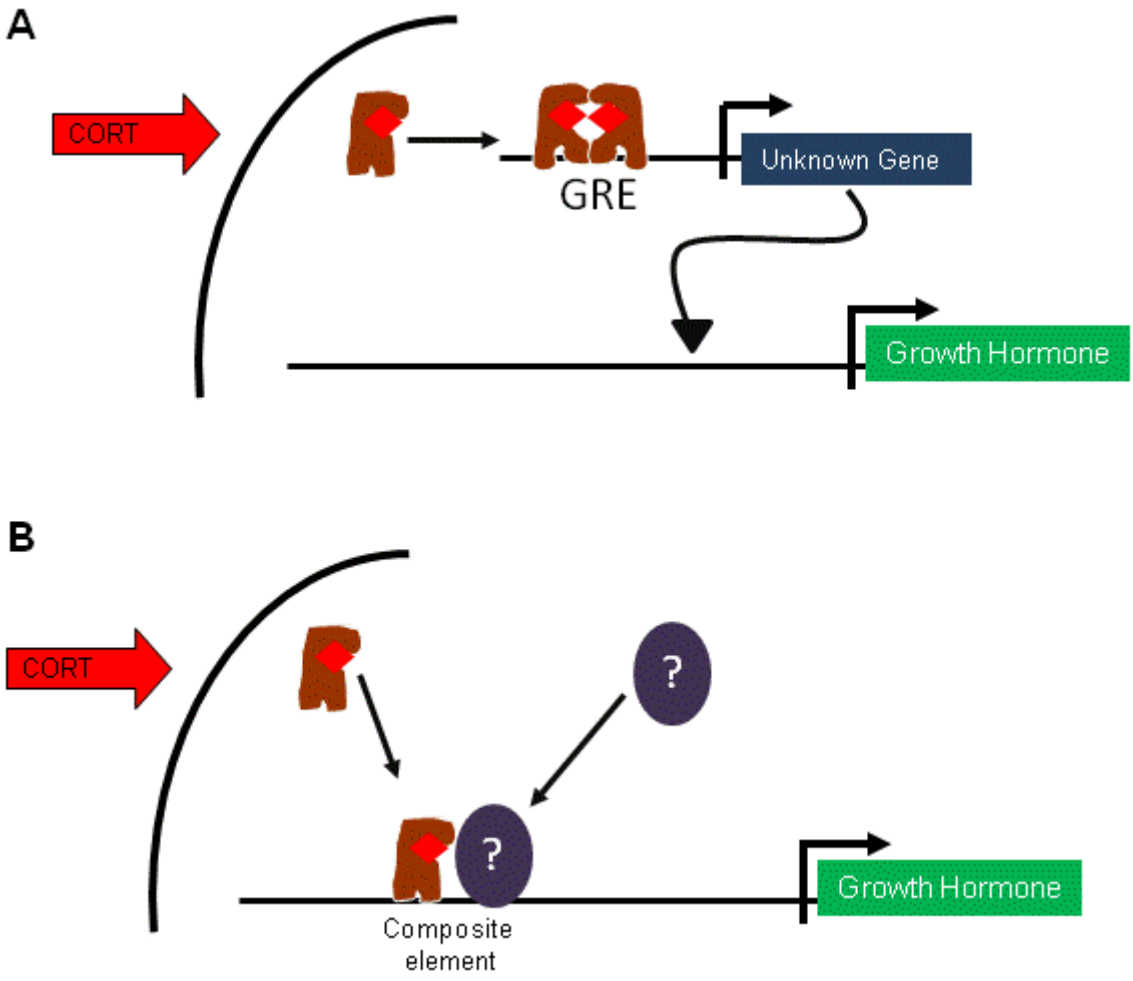


Figure 5: Two hypotheses: A) the product of an unknown glucocorticoid-responsive gene induces the GH gene; B) ligand bound GR and an unknown protein bind to an unknown composite element in the 5' flanking region of the GH gene and induce the GH gene together.

transcription factors, plus other unknown transcription factors, can direct expression of GH, prolactin and TSH (Lira et al., 1988).

Pit-1 is necessary for pituitary-restricted expression of GH; however, other molecules are definitely involved in the regulation of GH gene expression. Thyroid hormone (T3) and retinoic acid (RA) are known regulators of GH (Dean et al., 1997). Thyroid hormone response elements (TRE) were identified and found to negatively regulate the chicken GH gene promoter, antagonistic to Pit-1 (Ip et al., 2004). The hypothalamic factor, GHRH, signals through its receptor and activates cAMP and the PKA pathway. The downstream target of this pathway is cAMP response element binding protein (CREB). CREB and Pit-1 physically interact and synergize to activate the rat GH gene (Xu et al., 1998). A potential candidate critical for the Pit-1-dependent expression of GH is a cysteine/histidine zinc finger, Zn-15 (Lipkin et al., 1993). Zn-15 was found to synergize with Pit-1 and be necessary for full expression of the human GH gene in a heterologous cell type.

Many of the studies to date have attempted to characterize the GH gene in a heterologous system, e.g. human GH gene in mouse and chicken GH gene in a rat cell line. The present study will characterize regulation of the GH gene in primary chicken embryonic pituitary cells. A transcription factor search using MatInspector was conducted on the proximal promoter of the GH genes of human, rat, mouse, and dog. Nine identical sites were found: a TATA box, MEF3, Pit-1, WTI, Oct-1, CEBP, IRF2, PRE, and STAF (Lantinga-van Leeuwen et al., 2002). There is some conservation across GH promoters and involvement of potential transcription factors, but the differentiating factor of the GH gene induction remains to be elucidated in all species.

There are multiple levels of regulation of the GH gene. The necessity for ongoing protein synthesis during CORT induction of the GH gene suggests involvement of other proteins. CORT induction of the chicken GH gene can be blocked by inclusion of manumycin, a Ras GTPase inhibitor (Bossis & Porter, 2003). However, pharmacological inhibitors to PKA, PKC, and MAPK did not block CORT induction of the chicken GH gene.

An area of intense research involves the regulation of genes at the chromatin and histone level. The human GH gene is actually in a cluster of five related GH genes, where one is pituitary specific, GH-N, and the rest are placental specific. The cluster of genes are located on chromosome 17 and are regulated by a locus control region (LCR) located 15 kb upstream of the gene cluster. Recent evidence has implicated histone lysine demethylase (LSD1) as a necessary player in the regulation of GH gene expression (Wang et al., 2007). An LSD1 knockout mouse results in early embryonic lethality, but a pituitary-specific LSD1 knockout resulted in the loss of all Pit-1 lineage cells, while maintaining a normal overall morphology of the pituitary gland. Additionally, a histone deacetylase (HDAC) is necessary for CORT induction of the chicken GH gene. Pre-treatment with the HDAC inhibitors, trichostatin A or HC toxin, blocked the CORT response of the GH gene (unpublished data).

In our lab, two fragments of the chicken GH gene were separately cloned into a luciferase reporter plasmid: -488 to +1 and -488 to +1004. Each of these constructs, as well as empty vector, was transfected into e11 chicken embryonic pituitary cells. The cells were treated with CORT and assayed for luciferase activity. Neither of the constructs were CORT responsive (Liu, dissertation; 2003).

In another study, the rat somatotroph cell line, GH_4C_1, was transfected with a luciferase reporter construct containing 1775 base pairs, spanning -1727 to +48, of the 5'-flanking region of the chicken GH gene (Ip et al., 2004). The cells were treated with dexamethasone, a synthetic glucocorticoid, and luciferase activity was measured. Dexamethasone produced a two-fold increase in promoter activity over empty vector. Deletion of the -1727 bp insert to -1467 bp ablated the dexamethasone induction of promoter activity (Ip et al. 2004). From this, it is possible that a glucocorticoid responsive the region exists in the -1727 to -1467 bp region.

Therefore, the objectives of this study were 1) to confirm that the -1727/+48 reporter construct responds to glucocorticoids in chicken embryonic pituitary (CEP) cells; 2) to determine if the response is specific to glucocorticoids; 3) to define the glucocorticoid responsive region through deletion/mutation analysis of the luciferase constructs; 4) to determine if activation of the -1727/+48 luciferase construct requires ongoing protein synthesis; 5) to identify the transcription start site of the chicken GH gene in e11 chicken pituitary cells; 6) to determine whether the glucocorticoid responsive region binds nuclear proteins using an electrophoretic mobility shift assay (EMSA); and 7) to test for binding of specific proteins to this region using EMSA and chromatin immunoprecipitation (ChIP). Through characterization of the 5' flanking region of the chicken GH gene and by defining the *cis*-acting elements of the chicken GH gene, it is possible to identify *trans*-acting candidate proteins involved in glucocorticoid induction of the GH gene. Uncovering the glucocorticoid-inducible element in the GH gene will greatly aid in understanding the mechanisms regulating somatotroph differentiation and growth in vertebrates.

Methods

Reagents and Materials

Cell culture media and additives and transfection reagents (OptiMEM and Lipofectamine 2000) were purchased from Invitrogen (Carlsbad, CA). Hormones and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Pituitary dissection, dispersion, cell culture and transfection

Ross x Ross fertilized eggs were set in a 60% humidified, 37.5 ^{o}C incubator such that the first day was denoted as embryonic day (e) 0. Pituitaries were pooled from e11 dissected embryos and dispersed with trypsin as previously described (Porter et al., 1995a). Cells were counted using the trypan blue exclusion method, and the viability rate was above 95% in all experiments. Dispersed cells were plated at a density of $1x10^{6}$ cells/well in poly-L-lysine coated 24-well plates and were allowed to attach for one h. Plasmids to be transfected were diluted in sterile filtered OptiMEM (Invitrogen, Carlsbad, CA) to a concentration of 1 µg per well for all pGL3-Basic vector constructs and 10 ng per well for renilla luciferase. Diluted plasmids were combined with Lipofectamine according to manufacturer's instructions. Plated cells were transfected with the OptiMEM/Lipofectamine/Plasmid mixture for 4 hours. The medium was replaced with DMEM/F12 supplemented with 0.1% BSA, 100 U/ml penicillin and 100 µg/ml streptomycin, and 5 µg/ml human insulin, and cultured for an additional 20 hours. CORT (100nM final concentration in well) was added to appropriate wells for an additional 20 hours and then the cells were lysed with Passive Lysis Buffer (Promega,

Madison, WI) according to manufacturer's instructions in the Dual luciferase Reporter Assay Kit (Promega, Madison, WI).

Generation of DeletionLluciferase Reporter Constructs

To make nondirectionally cloned deletion constructs, the original -1727 plasmid was used as the template in PCR amplification of the other deletion constructs. *Pfx* DNA polymerase, a recombinant DNA polymerase with 3'→5' exonuclease activity was used to generate all of the deletion constructs (Invitrogen, Carlsbad, CA). The primers used are listed in Table 1. The PCR product of each of the inserts and the Basic vector was incubated for 4 h at 37 ^{o}C and then overnight at 4^{o}C with the restriction enzyme *HindIII* or *KpnI* (Invitrogen, Carlsbad, CA) and its appropriate buffer. The next day, shrimp alkaline phophatase (SAP) (Invitrogen, Carlsbad, CA) was added to the Basic vector and incubated at 37^{o}C for 1 h, followed by phosphatase inactivation at 65 ^{o}C for 1 h. All inserts and the digested, phosphatase-treated Basic vector were then gel purified, vacuum dried, and resuspended in 20 µL nuclease free water. Inserts and vectors were quantified by both gel electrophoresis using the molecular weight ladder, DNA Ladder I (Gene Choice), and absorbance reading at 260 nm. Appropriate concentrations of vector and insert were placed in tubes in a ratio of 1:1 and 1:3 vector to insert, heated to 65 ^{o}C, then allowed to slowly cool to 37 ^{o}C when buffer and T4 DNA ligase were added. The reaction was incubated at 16 ^{o}C for 8 h and then at 4^{o}C overnight. DH5α Max Efficiency Competent cells were transformed according to manufacturer's instructions with the newly ligated plasmids and then ampicillin-selected colonies were counted the next day. Selected colonies were grown overnight in 2 mL of Terrific Broth with ampicillin (100 µg/mL) for plasmid purification and qualification. Colony PCR was used to confirm the

Table 1: Primer names and sequences used for cloning

Name	Sequence
XhoI_1727F	TTACCTCGAGCCTGGTTTGTATCCCACC
XhoI_1544F	TTACCTCGAGAAGGGAAGGGGGAAAGAG
HindIII_1496F	TACCAAGCTTTTCCCTCTTTCAAATACAAG
HindIII_1477F	TTACAAGCTTATTTTGGAGGTTACTGAG
HindIII_1467F	TTCACAAGCTTGTTACTGAGCGTCATGC
HindIII_1462F	TACCAAGCTTTGAGCGTCATGCTGCTT
HindII_1398F	TACCAAGCTTAGAATGCCAAGCTGATAT
HindIII_1430F	TACCAAGCTTTTGGGTTGCACACGTGGGCA
HindIII_1201F	TACCAAGCTTTTTACTGACAGAGCTGCAGG
Hindlll_1045F	TTACAAGCTTACACCCCCAGATGTTGCT
KpnI_1045F	TTACGGTACCACACCCCCAGATGTTGCT
KpnI_1045R	TTACGGTACCAGCAACATCTGGGGGTGTGGT
HindIII_954F	TACCAAGCTTCAAACACCGCGGAGCTTCTC
KpnI_954R	TTACGGTACCGAGAAGCTCCGCGGTGTTTG
HindIII_807F	TACCAAGCTTCTATGGGGAGTGAAAGCCCT
KpnI_650F	TACCGGTACCCACGTCAAGCAAAGAGCAGAAGGCTC
HindIII_382F	TACCAAGCTTTGGCAGCCCTGTTAACCGTG
KpnI_382F	TTACGGTACCCACGGTTAACAGGGCTGCCA
HindIII_+48R	TACCAAGCTTGGAGAGTTGCTCAGGTGT
HindIII_newExon2GH_R	TACCAAGCTTGAGAGGAGAAAACCACGAGCC
HindIII_TSHbetaF_1272	TACAAGCTTTTAGGACACAAAGTTACATGCAGC
HindIII_TSHbetaR_1272	TACAAGCTTTCTTCCCTATTACAGGATCC

correct size and orientation of the insert. Plasmids containing the correct size and orientation of the desired insert were sequenced fully by the dye terminator method in both directions to confirm the sequence and Nucleobond Maxi Plasmid Purification columns (Clontech, Mountain View, CA) were used to purify a large quantity of the plasmid for transfection.

Generation of Mutant Luciferase Reporter Constructs

Mutant constructs were generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's protocol. Briefly, the -1045/+48 plasmid was used as the template in the PCR reaction with the following primers: ETS1_muta_fwd cagatgttgctggctattgggtgaaattctacctgatagctgcaggacccact, ETS1_muta_rev agtgggtcctgcagctatcaggtagaatttcacccaatagccagcaacatctg; GREF_muta_fwd ggtgaccggatacctgatagctgcaggaaaaaaggggccctctcctctggggactgaca, GREF_muta_rev tgtcagtccccagaggagagggccccttttttcctgcagctatcaggtatccggtcacc. After PCR, the reaction was incubated with *DpnI* enzyme for 30 m in 37 ^{o}C to digest the original template. XL1-Blue Supercompetent cells were immediately transformed with 1 µL of the digested PCR reaction. The transformed bacteria were spread on TB/Agar/Ampicillin plates containing 80 ug/mL X-gal and 20 mM IPTG. Clones containing the correct mutations were verified by dideoxy dye terminator sequencing, and the selected clones were sequenced in both directions with 4x coverage. Sequencing reactions were performed by the University of Maryland's Center for Biosystems Research DNA Sequencing Facility with AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP in an Applied Biosystems DNA Sequencer (Model 3100; Foster City, CA, USA).

Analysis of Luciferase mRNA in transfected cells

To determine the effect of protein synthesis inhibition on the activity of the 5' flanking region of the chicken GH gene, e11 chicken embryonic pituitary cells were dispersed, plated at a density of $4x10^6$ cells per well, and transfected with pGL3_ -1727/+48 GH construct (1.45 µg), renilla luciferase (0.01 µg), and a Golgi-targeted green fluorescent protein (GFP) expression plasmid (1.45 µg) (Pecot & Malhotra, 2004). The transfection reagents were replaced with fresh medium at 6 h post-transfection. At 22.5 h post-transfection, the cells were treated with cycloheximide (10 µg/mL final concentration in well) for 90 min and then CORT [1 nM] was added at 24 h post-transfection. The cells were incubated for another 21 h and then collected using trypsin digestion and washed one time each with SMEM, DMEM, and PBS. The cells were sorted based on GFP fluorescence as previously described (Ellestad et al., 2009) and collected into Buffer RLT from the RNeasy Mini Kit (QIAGEN, Valencia, CA). The RNA was extracted immediately following the manufacturer's protocol with the optional on-column DNA digestion and quantified using the Ribogreen Quantitation Kit (Invitrogen, Carlsbad, CA). 20 ng of total RNA was reverse transcribed into cDNA using Super Script III according to manufacturer's instructions (Invitrogen, Carlsbad, CA). A volume of 2 µL of the diluted cDNA sample was used for analysis. Three-step qRT-PCR was used to quantify cDNA levels using a Bio-Rad iCycler. The final concentration of the reaction mixture was 0.1% Triton X-100, 10mM Tris–HCl, 50mM KCl, 1.9mM $MgCl_2$, 2U Taq, 10mM each dNTP, 1µM each primer, 20nM fluorescein, and SYBR® green II (Bio Rad, Hercules, CA). The cycling parameters were 40 cycles of 95 ^oC for 15 s, 60 ^oC for 30 s, and 72 ^oC for 30 s. All primers used to quantify cDNA are listed in

Table 2. Primers were designed to span an intron where appropriate to ensure amplification of cDNA and not genomic DNA. Relative cDNA levels for each sample were calculated using the relative Ct method (level = $2^{(\text{Ct of the no RT}-\text{control}-\text{Ct of the sample})}$), as previously described (Ellestad et al., 2005).

5' Rapid Amplification of cDNA Ends (RACE)

Anterior pituitaries were dissected and pooled from e11 chicken embryos. The pituitaries were dispersed as described previously and plated at a cell density of $1x10^6$ cells per well in a 24-well plate. The cells were allowed to recover overnight and were treated with CORT [100 nM] for 24 hours before retrypsinization and snap freezing of the cell pellet. RNA was extracted from the frozen cell pellet using the RNeasy Mini Kit (Qiagen) and the samples were quantified using ribogreen dye. One µL of RNA was used in the 5'RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The GH gene specific primer 1 (GSP1) used was 5'-CACGGGGGTGAGCCAGGACT-3' and the GH gene specific primer 2 (GSP2) used was 5'- TCCCCGTGGGAGCTGGGATG-3'. The primers were tested on cDNA first to verify correct specificity. The single PCR product generated was sequenced in both directions using the dideoxy dye terminator method with the primers: GHGSP2 and the Universal Abridged Adaptor Primer (UAAP) included in the kit.

Preparation of Nuclear Extracts

Twenty million cells per treatment were cultured for 6 h in the presence or absence of CORT ($1x10^{-9}$ M). Cells were scraped on ice, collected into 15 mL centrifuge tubes and rinsed once with PBS. The nuclear and cytoplasmic fractions were isolated

Table 2: Primers used for quantitative Real Time PCR

Primer	Sequence
GH fwd	CACCTCAGACAGAGTGTTTGAGAAA
GH rev	CAGGTGGATGTCGAACTTATCGT
Actin Beta fwd	CAGGATGCAGAAGGAGATCACA
Actin Beta rev	TAGAGCCTCCAATCCAGACAGAGTA
ETS-1 fwd	GCCGGCTACACAGGCAGTGG
ETS-1 rev	ACCGCCTGGCCACCTCATCT
Renilla Luc fwd	AGGTGGTAAACCTGACGTTGTACA
Renilla Luc rev	ATCCTGGGTCCGATTCAATAAAC
Firefly Luc fwd	TTGGAATCCATCTTGCTCCAA
Firefly Luc rev	TCCGTGCTCCAAAACAACAA

according to the method as described previously with modifications (Dignam et al., 1993). The cells were washed once in 1.5 mL of hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl ,1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT), collected and resuspended in 1 mL of hypotonic buffer, and then incubated on ice for 10 min. The cells were transferred to a 1 mL size Dounce homogenizer fitted with a tight pestle. The cells were homogenized with 40 up-and-down strokes. An aliquot of cells were checked under the microscope for loss of cell membrane and retention of nuclei with trypan blue. The cells were transferred to a microcentrifuge tube and centrifuged at 3300 g for 15 min. The cytoplasmic fraction was removed and saved. In the cold room (4 ^{o}C), the nuclear pellet was rapidly resuspended in 120 µL of low salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.02 PMSF and 0.05 mM DTT). The same volume of high salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.02 PMSF and 0.05 mM DTT) was added drop-wise while mixing the tube in between drops. The sample was placed on a rotator (end to end) at 4 ^{o}C. The sample was checked to ensure that the liquid sample was moving inside of the tube and incubated for 30 min. The sample was then centrifuged for 30 min at maximum speed. The buffer was replaced in the nuclear extract using Microcon Centrifugal Filter Devices (YM-10) (Millipore) following manufacturer's instructions. The sample was quantified using Coomassie Plus: The Better Bradford Reagent (Pierce) with absorbance at 560 nm.

Electrophorectic Mobility Gel Shift Assays (EMSA)

EMSA probes were designed for the proximal (-1042/-956) and distal GCRR (-1496/-1465), proximal Pit-1 binding site (-133/-103), and exon 3 (+207/+237of the

chicken GH gene. 5' infrared labeled oligonucleotides (0.1 pmol/μL) were annealed at 100 ^{o}C for 5 min and then allowed to cool slowly (Integrated DNA Technologies, Coralville IA). One μL of probe and 2.5 μg of nuclear extract were added to the binding reaction. The binding reaction [buffer (10 mM Tris, 50 mM NaCl, 1 mM DTT, pH 7.5), 50 ng sheared salmon sperm DNA, 5 mM $MgCl_2$, 2.5 mM DTT and 0.25% Tween 20, 0.2% NP-40] was incubated for 30 min on ice in the dark. For super-shift experiments, nuclear protein and the appropriate antibody were incubated in the binding reaction mixture overnight at 4^{o}C. The next morning, the infrared labeled probe was added for 30 min before loading the entire reaction onto the gel. The gel was pre-run for 30 min at 70 V in 0.5 X TBE. Orange loading dye (LI-COR, #927-10100) was added to each sample and loaded into the gel. The gels were electrophoresed for 3 h at 70 V and imaged on a Li-COR Odyssey Infrared Imaging System using intensity level 8 (Li-COR Biosciences, Lincoln, NE).

Western Blot for ETS-1

A western blot for ETS-1 was performed in order to determine specificity of the ETS-1 antibody (catalog # sc-112; Santa Cruz Biotechnology, Santa Cruz, CA). Total protein was obtained from embryonic chicken liver and adult rat liver, as previously described (Proszkowiecz-Weglarz & Porter, 2010). Nuclear protein was obtained from e20 chicken embryonic pituitary cells and GH_4C_1 cells, a rat somatotroph cell line. Equal protein amounts (12 μg) were subjected to SDS-PAGE under reducing conditions on 7.5% gel. The transfer and blotting procedure was performed as described previously (Proszkowiecz-Weglarz & Porter, 2010). The ETS-1 antibody was used at 1:1000. The

secondary antibody was a horseradish peroxidase-conjugated donkey anti-goat IgG (1:5000; GE Healthcare Life Sciences, Piscataway, NJ).

Cell Culture and Chromatin Preparation

E11 chicken embryonic pituitary cells were dissected and dispersed as previously described. Twenty-five million cells per treatment were plated with 6 mL of DMEM/F12 supplemented with 0.1% BSA, 100 U/ml penicillin and 100 µg/ml streptomycin, and 5 µg/ml human insulin in 4 100 mm x 20 mm Corning® cell culture petri dishes and allowed to recover overnight. The next morning, the cells were treated with either vehicle or CORT [1 nM] for six h. At that time, 200 µL of 37% formaldehyde was added drop-wise while swirling to each dish. The dishes were placed on a rotator for 10 m at RT. Then 625 µL of 1 M glycine was added for 5 m to quench the formaldehyde. The plates were placed on ice and the cells were scraped into 50 mL conical tubes. The dishes were washed twice with 2 mL of ice-cold PBS containing 1 mM PMSF. The cells were collected at 3000 g and the pellet was washed once with PBS containing 1 mM PMSF. The cells were resuspended in swelling buffer [25 mM Hepes pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, and 1x Halt Protease Inhibitor Cocktail (Thermoscientific, Rockford, IL)] and incubated on ice for 10 m. The cell suspension was homogenized with a Dounce homogenizer with 15 up and down strokes using a loose fitting pestle. The cells were transferred to 15 mL conical tubes and centrifuged at 1800 g for 5 m. The cells were resuspended in 2 mL of Mononuclease (MNase) Buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 1% SDS) and homogenized with a Polytron PT 1200 C (Kinematica, Bohemia, NY) for 30 s on ice. Fifteen µL of the cell solution was removed at this time

and stored as the "non-sheared control." Ten µL of BSA and 7.5 µL of mononuclease (MNase) enzyme (New England Bio Labs, Ipswich, MA) were added to each sample and incubated at 37 ^{o}C for 15 m. 100 µL of 0.5 M EDTA was added to stop the reaction. The samples were then stored overnight at -80 ^{o}C. The next day, the samples were sonicated in an ice-ethanol bath for 5 cycles (20 s continuous pulse, output at 60%, power at "6") using a Branson Sonifier 250. The samples were centrifuged at 14,000 rpm for 10 m, and the supernatant was collected into a new tube and quantified using Quant-it Picogreen DNA Quantification Kit (Invitrogen, Carlsbad, CA). The quantified chromatin was aliquoted and stored at -80 ^{o}C.

Chromatin Immunoprecipitation

For each immunoprecipitation (IP), 10 µg of sheared chromatin was precleared 2x for a total of 24 h at 4 ^{o}C with rotation with prewashed protein A magnetic beads (New England Bio Labs, Ipswich, MA), 0.25 mg/mL normal rabbit serum. The next day, the cleared chromatin was transferred to a new tube, and 5 µL of the appropriate antibody was added and incubated overnight at 4 ^{o}C with rotation. The next morning, the chromatin was incubated with prewashed protein A magnetic beads (New England Biolabs, Ipswich, MA) for 5 h at 4 ^{o}C with rotation. The beads were washed 2x each with low salt wash buffer (50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1x Protease Inhibitor Cocktail), high salt wash buffer (50 mM Hepes pH 7.9, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1x Protease Inhibitor Cocktail), LiCl wash buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 0.5 mM PMSF, 1x Protease Inhibitor Cocktail), and TE buffer wash

(10 mM Tris pH 8.0, 1 mM EDTA). The immunoprecipitated chromatin was eluted from the beads with elution buffer (50 mM Tris pH 8.0, 1 mM EDTA, 1% SDS, 50 mM $NaHCO_3$) at 65 ^oC with rotation for 30 m. The supernatant was transferred to new tubes containing 15 µL of 5 M NaCl and 25 ng RNase A and incubated at 37 ^oC for 30 m. Next, 250 ng of Proteinase K (Sigma Aldrich, St. Louis, MO) was added, and the samples were incubated at 65 ^oC with rotation overnight. The next day, the samples were purified using the Wizard SV PCR clean up kit according to manufacturer's instructions (Promega, Madison, WI) with the following modification: the final product was eluted twice with 40 µL of water.

Real Time PCR of Immunoprecipitated Chromatin

Three-step qRT-PCR was used to quantify immunoprecipitated DNA levels using a Bio-Rad iCycler. The final concentration of the reaction mixture was 0.1% Triton X-100, 10mM Tris–HCl, 50mM KCl, 1.9mM $MgCl_2$, 2U Taq, 10mM each dNTP, 80 nM each primer, 20nM fluorescein, and SYBRgreen II. 4 uL of the immunoprecipitated DNA was used in each 30 µL reaction. The primers used are listed in Table 3. The cycling parameters were 40 cycles of 95 ^oC for 15 s, 60 ^oC for 30 s, and 72 ^oC for 30 s. Before immunoprecipitation, 2% of the sheared chromatin sample was removed and saved as the "input". The starting input fraction was 2%; therefore a dilution factor (DF) of 50 or 5.644 cycles (i.e., log2 of 50) was subtracted from the Ct value of the diluted input. The input Ct values were adjusted to 100% efficiency by subtracting 5.64 from each. All Ct values were adjusted to % of input using the equation $100*2^{(\text{adjusted input Ct-sample Ct})}$ (Haring et al., 2007).

Table 3: Primer name, start site and sequence used for Real time PCR of immunoprecipitated chromatin

Name	Start site	Sequence
GCRR fwd	-1065	TTTAAACACGACCTGGAGCAGAAAAA
GCRR rev	-859	ATTTCCAAGAGCAGCATCATCAC
distal control fwd	-1436	ATGATCCTTTGGGTTGCA
distal control rev	-1299	GTTTTGTTTCCCCTGCTTGC

Statistical Analysis

Each experiment was replicated 3 to 5 times, as indicated. Using the SAS statistical program (SAS Inc; Cary, NC), statistically significant differences among treatments or groups were determined employing a mixed model ANOVA, where replicate experiment was a random effect in the model. An *a priori* test of least significant differences (LSD) with a Tukey method of adjustment to control the experiment-wise error rate was used to determine significant differences between groups. Differences were considered significant at $p \leq 0.05$. In figures, an asterisk or different letters denote significance at $p \leq 0.05$.

Firefly luciferase was normalized to renilla luciferase, and then relative luciferase data were transformed into log scale to correct for heterogeneity of variances between groups and non-normality. The least squared means (LSMeans) were back-transformed and then normalized to the -1727/+48 plasmid, or a different plasmid as indicated, for graphical representation.

Real Time qRT-PCR values for firefly luciferase or GH were normalized to renilla luciferase or β-actin, respectively. The delta delta Ct value was then log transformed to correct for variance heterogeneity and non-normality and for significance testing. The log transformed data was back-transformed for graphical representation.

Results

Luciferase Activity of the -1727/+48 Insert in Response to CORT

In order to confirm CORT responsiveness of the -1727/+48 GH luciferase construct, we used GH_4C_1 cells, a rat somatotroph cell line. The day of the transfection, the cells were washed twice to remove any traces of glucocorticoids from the fetal bovine

serum. The cells were transfected with 1 µg of either the -1727/+48, -1467/+48, or pGL3-Basic plasmid and 20 ng of the renilla luciferase plasmid per well for 4 h. The next day, CORT was added to appropriate wells and then the cells were lysed for determination of luciferase activity. The addition of the -1727/+48 GH fragment resulted in an eight-fold increase in basal luciferase activity over the empty vector (Figure 6A). Alternatively, the addition of the -1467/+48 GH fragment resulted in only a four-fold increase in basal luciferase activity over the empty vector. CORT treatment did not affect activity of the empty vector, pGL3-Basic. CORT treatment of the -1727/+48 plasmid resulted in a significant five-fold increase in luciferase activity, while CORT treatment resulted in a slight increase of luciferase activity from the -1467/+48 plasmid, although this response was not significant ($n=3$; $p<0.05$) (Figure 6A). Values denoted by different letters are significantly different at $p<0.05$. Thus, the -1727/+48 GH construct was CORT responsive in GH_4C_1 cells, and this response was lost with deletion down to -1467/+48.

The -1727/+48 Luciferase Construct is CORT-inducible in Embryonic Chicken Pituitary Cells.

The initial experiment was replicated using chicken embryonic primary pituitary cells. E11 pituitary cells were dispersed and transfected immediately for 4 h with the plasmids: basic, -1727/+48, or -1467/+48, and renilla luciferase. 24 h after the conclusion of transfection, the cells were treated with vehicle or 100 nM CORT. 21 h later, the cells were lysed and assayed for luciferase activity. Insertion of the -1727/+48 GH fragment into the pGL3-Basic vector resulted in a two-fold increase in basal luciferase activity (Figure 6B). Insertion of the -1467/+48 GH fragment into the pGL3-Basic vector resulted

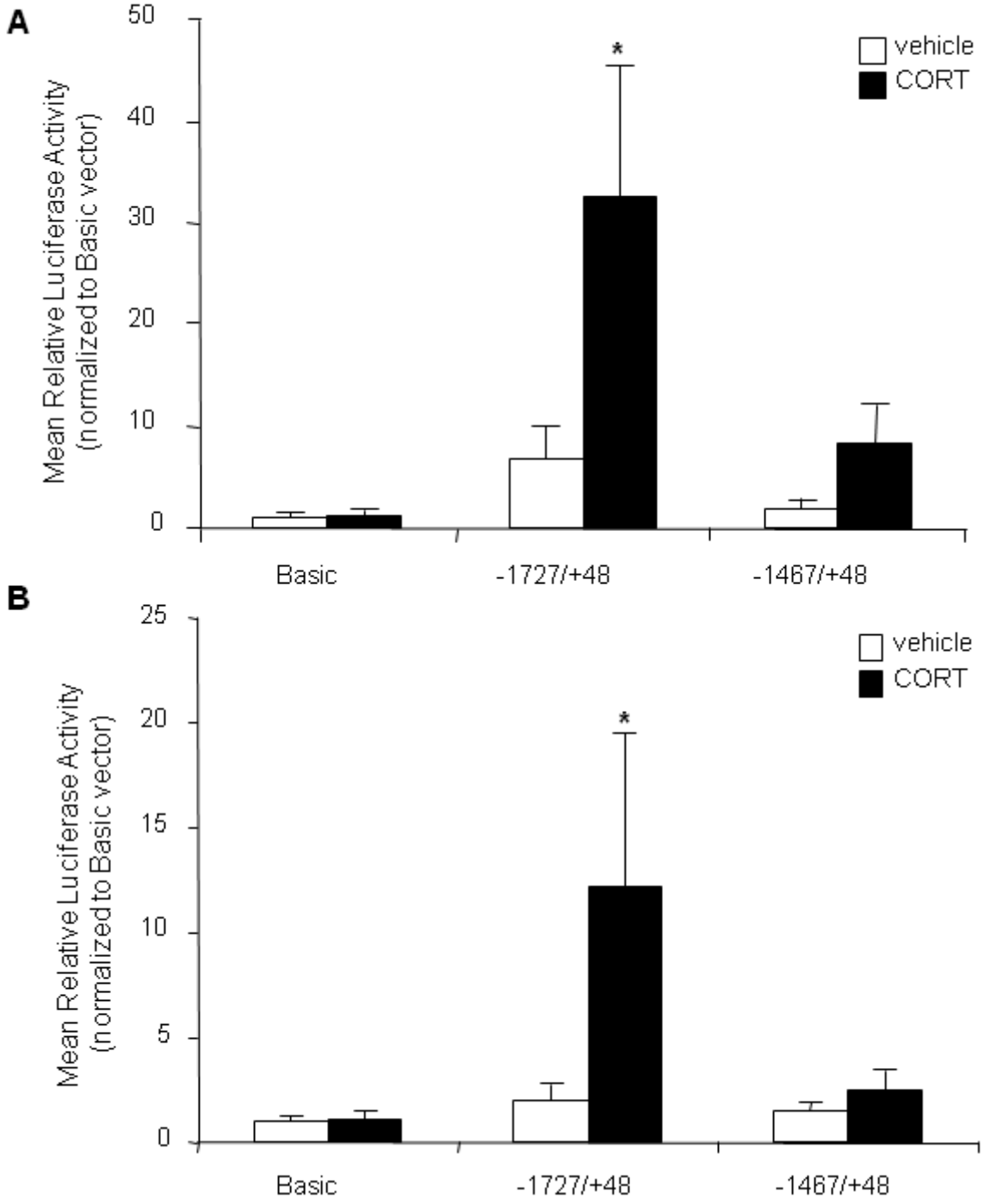


Figure 6: Mean Relative Luciferase Activity of the GH Constructs in Response to CORT. 1 x 107 cells were transfected with either the Basic Luciferase empty vector, -1727/+48 GH Luciferase or -1467/+48 GH Luciferase and allowed to recover overnight. The cells were treated with CORT (100 nM) the next day and assayed for Luciferase activity 24 h later. A) GH4C1 rat somatotroph cell line, B) e11 chicken embryonic pituitary cells. *, significant difference between vehicle and CORT-treated cultures (n=3; $p<0.05$).

in a 50% increase in basal luciferase activity. CORT treatment did not affect luciferase activity of the empty vector. CORT (100 nM) treatment significantly increased luciferase activity of the -1727/+48 plasmid an average of six-fold over vehicle; however, the -1467/+48 was not CORT responsive ($n=3$; $p<0.05$) (Figure 6B). Values denoted by different letters are significantly different at $p<0.05$. Therefore, the -1727/+48 GH construct was CORT responsive in chicken embryonic pituitary cells, and this response was lost with deletion down to -1467/+48.

The -1727/+48 Luciferase Construct is Responsive to Corticosterone and Progesterone.

E11 pituitary cells were dispersed, transfected with either the -1727/+48 plasmid or basic vector and allowed to recover overnight. 24 h after the conclusion of transfection the cells were treated with vehicle or various steroids: CORT, aldosterone, testosterone, estradiol and progesterone (100 nM) to define the specificity of the response to CORT. The cells were lysed and assayed for luciferase activity 21 h after addition of the treatments. Addition of CORT resulted in a significant 10-fold induction of the -1727/+48 luciferase plasmid as compared to vehicle ($n=3$; $p<0.05$) (Fig. 7), whereas treatment with aldosterone, testosterone, or estradiol did not produce a significant increase. Progesterone treatment resulted in a partial increase in luciferase activity that was not significantly different from basal or CORT. Values denoted by different letters are significantly different at $p<0.05$. Therefore, the -1727/+48 GH construct was responsive to CORT and partially responsive to progesterone.

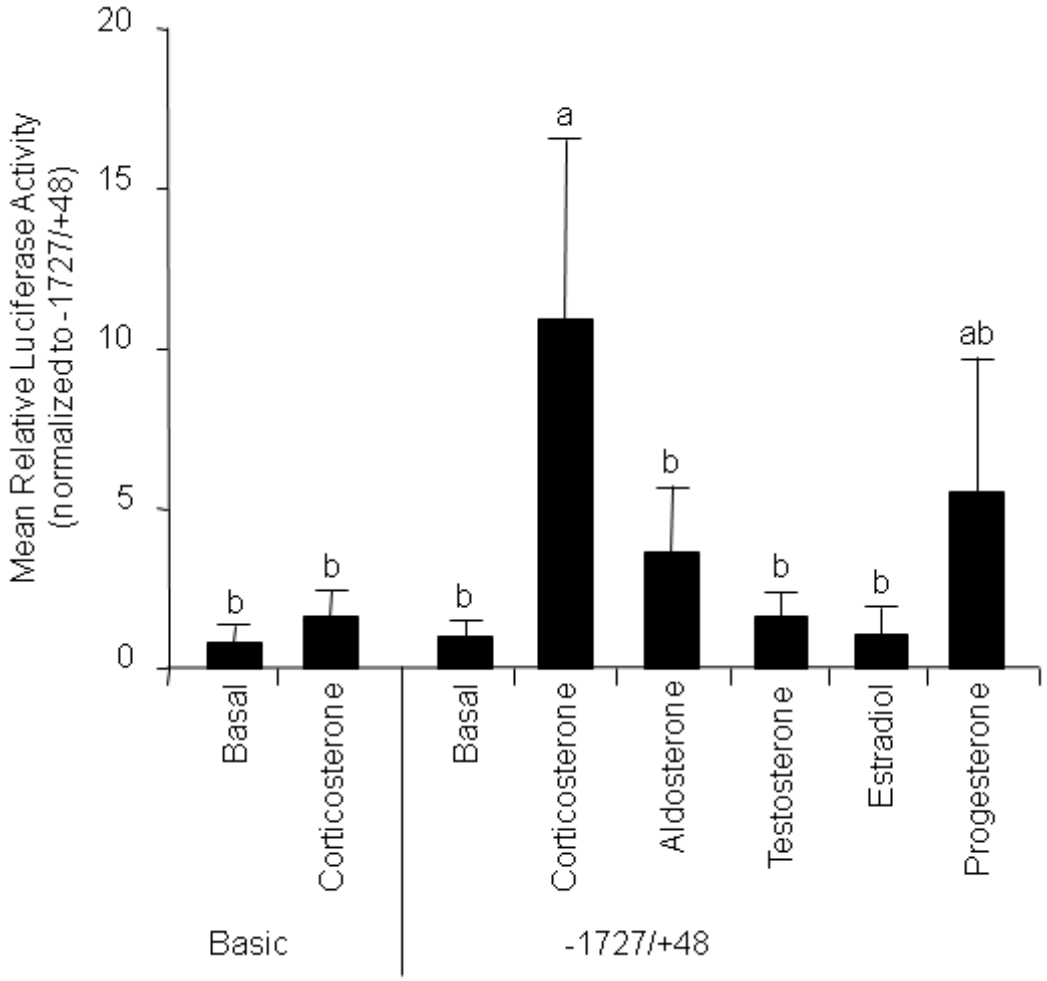


Figure 7: Mean Relative Luciferase Activity in Response to Steroid Hormones. 1 x 10^7 cells were transfected with either the Basic Luciferase empty vector or the -1727/+48 GH Luciferase and allowed to recover overnight. The cells were treated with different steroid hormones the next day and assayed for Luciferase activity 24 h later. Values denoted by different letters are significantly different (n=3; $p<0.05$).

The -1727/+48 Luciferase Construct Is an Appropriate Model for CORT Induction of the Endogenous GH Gene.

The chicken GH gene is induced by glucocorticoids, and this effect can be blocked with the addition of cycloheximide (CHX), a protein synthesis inhibitor. This implicates an unknown protein in the CORT induction of GH. Therefore, it is important that the luciferase activity driven by the -1727/+48 insert of the 5' flanking region of the chicken GH gene is induced by glucocorticoids and that the induced activity is blocked by cycloheximide. In other words, does this artificial system recapitulate the activity of the endogenous chicken GH gene?

To this end, mRNA levels of firefly luciferase and renilla luciferase in response to CORT and CHX alone and in combination were quantified using qRT-PCR. E11 chicken embryonic pituitary cells were dispersed and transfected in suspension with a GFP expression vector, renilla luciferase and either the -1727/+48 firefly luciferase plasmid or Basic firefly luciferase plasmid for 2 h. The transfected cells were plated at a density of 4 x 10^6 cells per well in a 12-well plate and incubated for an additional 4 h before the transfection reagents were replaced with fresh medium. At 22.5 h post-transfection, the cells were treated with cycloheximide (10 µg/mL final concentration in well) for 90 mins and then CORT was added at 24 h post-transfection. The cells were incubated for another 21 h and then collected using trypsin digestion and washed twice. Positively transfected cells were sorted, based on GFP expression. The sorted cells were collected, and RNA was immediately extracted including an on-column DNA digestion step. Total RNA was reverse-transcribed into cDNA and used in qRT- PCR ($n=4$; $p<0.05$). Firefly luciferase mRNA was normalized to renilla luciferase mRNA as a control for transfection

efficiency (Fig 8A). GH mRNA, normalized to β-actin, was also quantified in the same samples (Fig 8B). Values denoted by different letters are significantly different at $p<0.05$. Firefly luciferase mRNA transcribed off the -1727/+48 construct increased about 80 fold in response to CORT, and this response to CORT was blocked by pre-treatment with CHX. CORT treatment induced firefly luciferase mRNA from the -1727/+48 insert four-fold compared to CORT-treated Basic plasmid. CORT induced GH mRNA regardless of the transfected plasmid (-1727/+48 vs. Basic), and the CORT induction of GH mRNA was blocked by pretreatment with CHX. Thus, on-going protein synthesis was required for CORT induction of the -1727/+48 GH construct and the endogenous GH gene.

The Transcription Start Site of the Chicken GH Gene is the Same as the -1727/+48 Luciferase Construct.

In order to define the transcriptional start site of the endogenous GH gene, CORT-treated chicken e11 pituitary cells were cultured overnight and then treated with CORT [100 nM] or vehicle for 6 h. The cells were collected using trypsin, snap frozen and RNA was extracted. The transcriptional start site of the GH gene was determined using the 5' RACE system for rapid amplification of cDNA ends kit (Invitrogen, Carlsbad, CA). A single product was observed from the nested PCR reaction (Fig.9A). The gel band was excised, purified and sequenced. The resulting chromatogram and sequence are shown (Fig. 9B). The start codon, known 5' UTR, and the new 5' end of the chicken GH gene are highlighted. The sequence of the 5' RACE product was aligned with the chicken genomic sequence from the Ensembl website (www.ensembl.org), which is based on the Washington University assembly (Wallis et al., 2004) and the 3' end of the -1727/+48

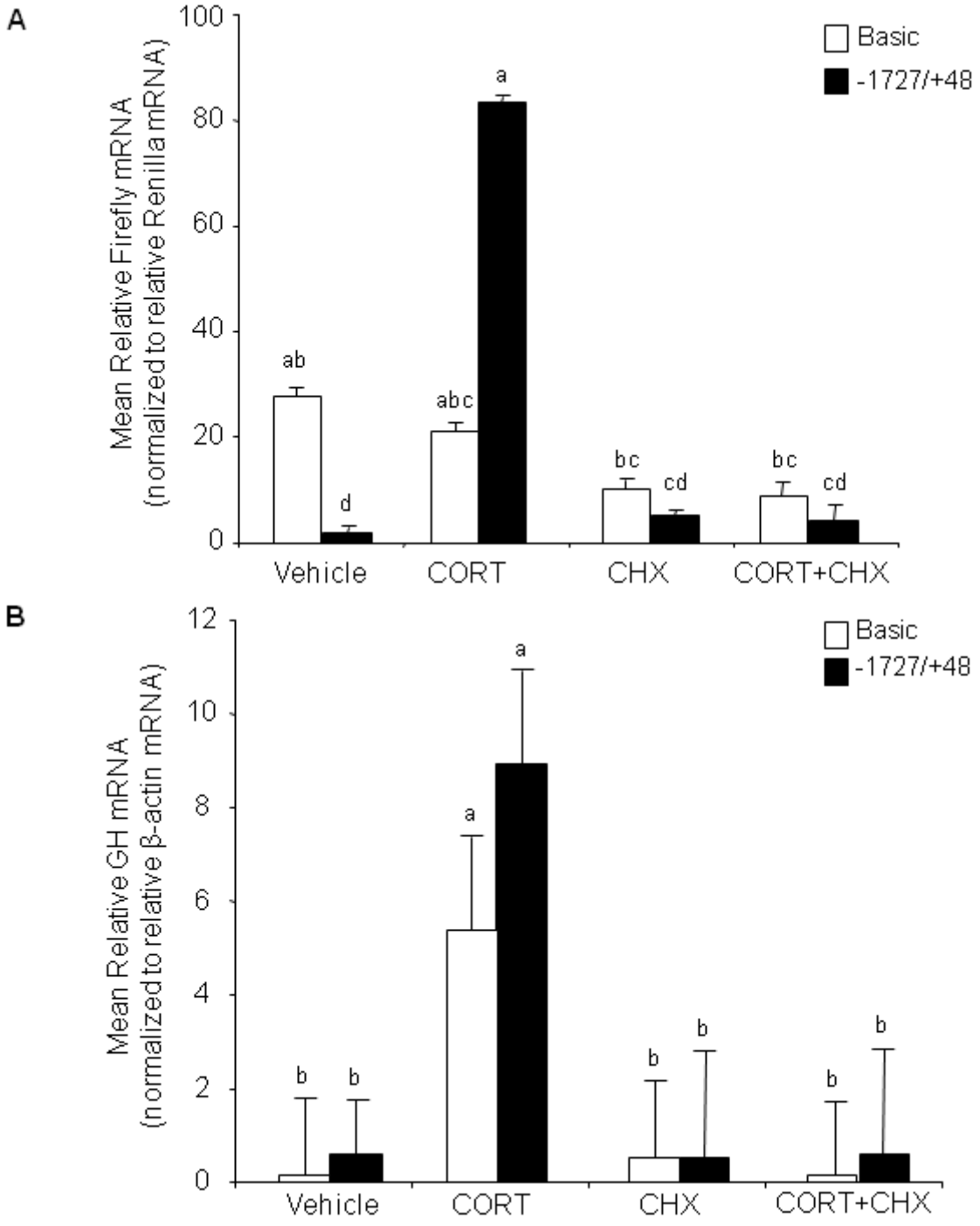


Figure 8: Mean Relative mRNA levels of Firefly Luciferase and GH in Response to CORT. 4 x 10^7 cells were transfected with Basic empty vector or the -1727/+48 GH Luciferase plasmid and the Renilla Luciferase plasmid and a GFP expression plasmid (3 µg total). Cells were treated with cylcoheximide for 90 m and then CORT for 20 h and then sorted based on GFP expression. RNA was extracted immediately, reverse transcribed into cDNA and quantified with qRT-PCR. A) Firefly mRNA normalized to Renilla mRNA, B) GH mRNA normalized to β-actin mRNA. Values denoted by different letters are significantly different (n=3; $p<0.05$).

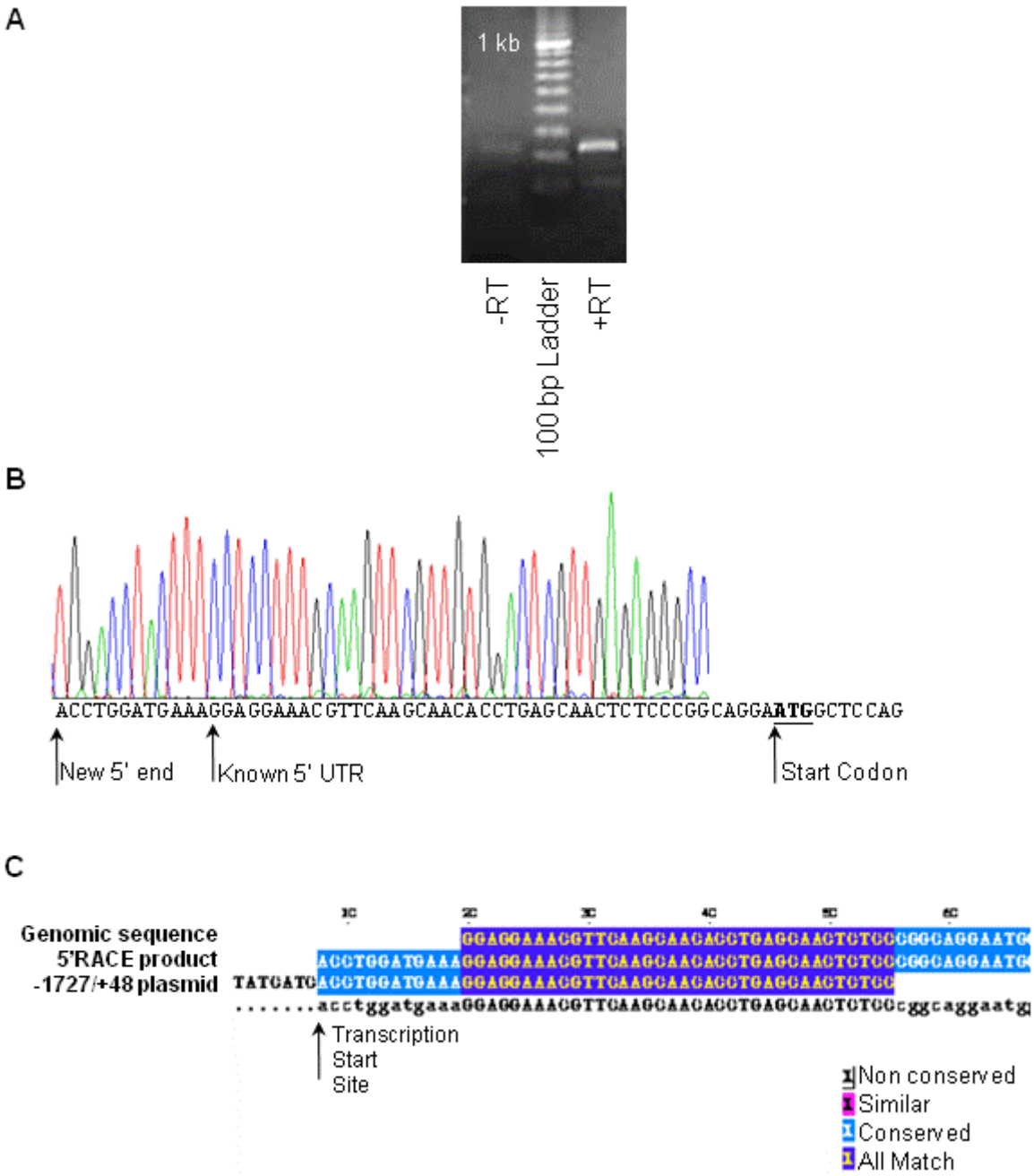


Figure 9: 5' Rapid Amplification of cDNA Ends (5' RACE) of the Chicken GH Gene.A) PCR results of tailed cDNA reaction, Left: "no reverse transcriptase" reaction, Center: 100 bp ladder, Right: "reverse transcriptase" reaction. B) chromatogram and sequencing results of 5' RACE product. C) alignment of genomic sequence, 5' RACE product, and 3' end of the -1727/+48 Luciferase plasmid.

chicken GH gene plasmid (Fig. 9C). Sequence analysis of the 5' RACE product indicated that the transcription start site resides at -56 bp of the GH gene.

Deletion Constructs of the -1727/+48 Luciferase Construct Reveal Two Potential Glucocorticoid Responsive Regions.

The -1727/+48 luciferase plasmid was used as the template to generate progressively shorter deletion constructs of the 5' flanking region of the chicken GH gene, in order to identify the glucocorticoid responsive region. Nine additional deletion constructs were cloned into the luciferase plasmid and tested for CORT responsiveness (Fig. 10 & 11A) by transfection into e11 pituitary cells as previously described. The -1467/+48 firefly luciferase plasmid remained unresponsive to CORT. The shorter -1430/+48, -1398/+48, and -1201/+48 constructs were CORT responsive, while the -954/+48, -807/+48, and -382/+48 constructs were not CORT responsive ($p<0.05$; $n=3$). Values denoted by different letters are significantly different at $p<0.05$. One additional deletion construct was made, -1045/+48, tested and was found to be CORT-responsive ($p<0.05$; $n=3$) (Fig. 11B). Values denoted by different letters were significantly different at $p<0.05$. Therefore, a glucocorticoid inhibitory region (GC-IR) was identified between -1477 and -1430 and a proximal GCRR was identified between -1045 and -954 of the chicken GH gene. Further experiments are warranted for characterize the GC-IR and its role in glucocorticoid induction of the GH gene during chicken embryonic development.

Identification of Putative Transcription Factor Binding Sites in the Proximal GCRR

CORT responsiveness of the chicken GH gene was lost when the 5' flanking region was deleted down to -954. Potential transcription factors involved in regulating

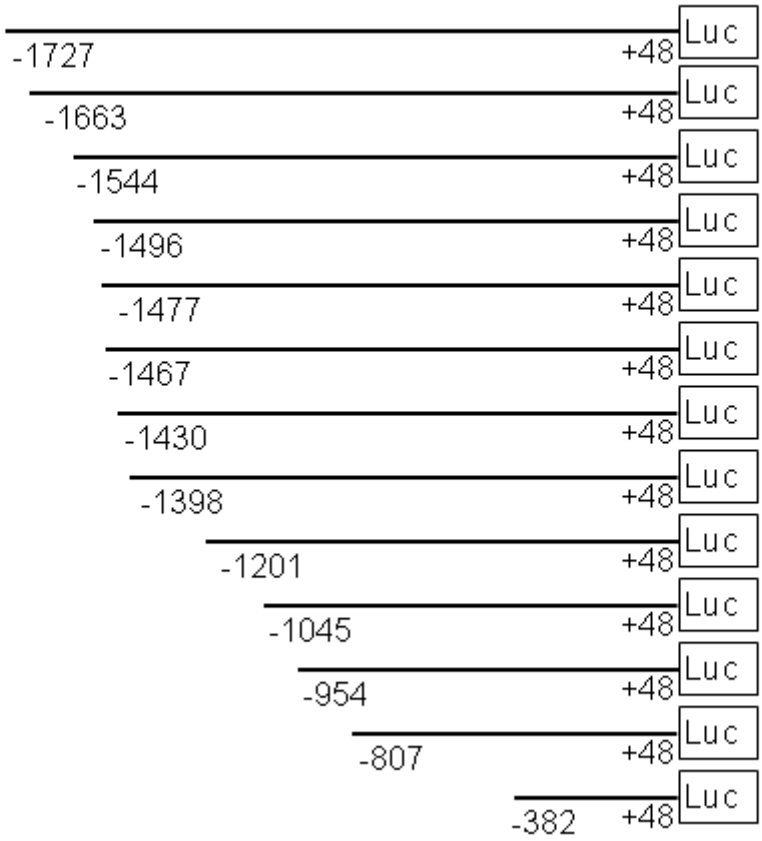


Figure 10: Scaled schematic of the deletion luciferase constructs.

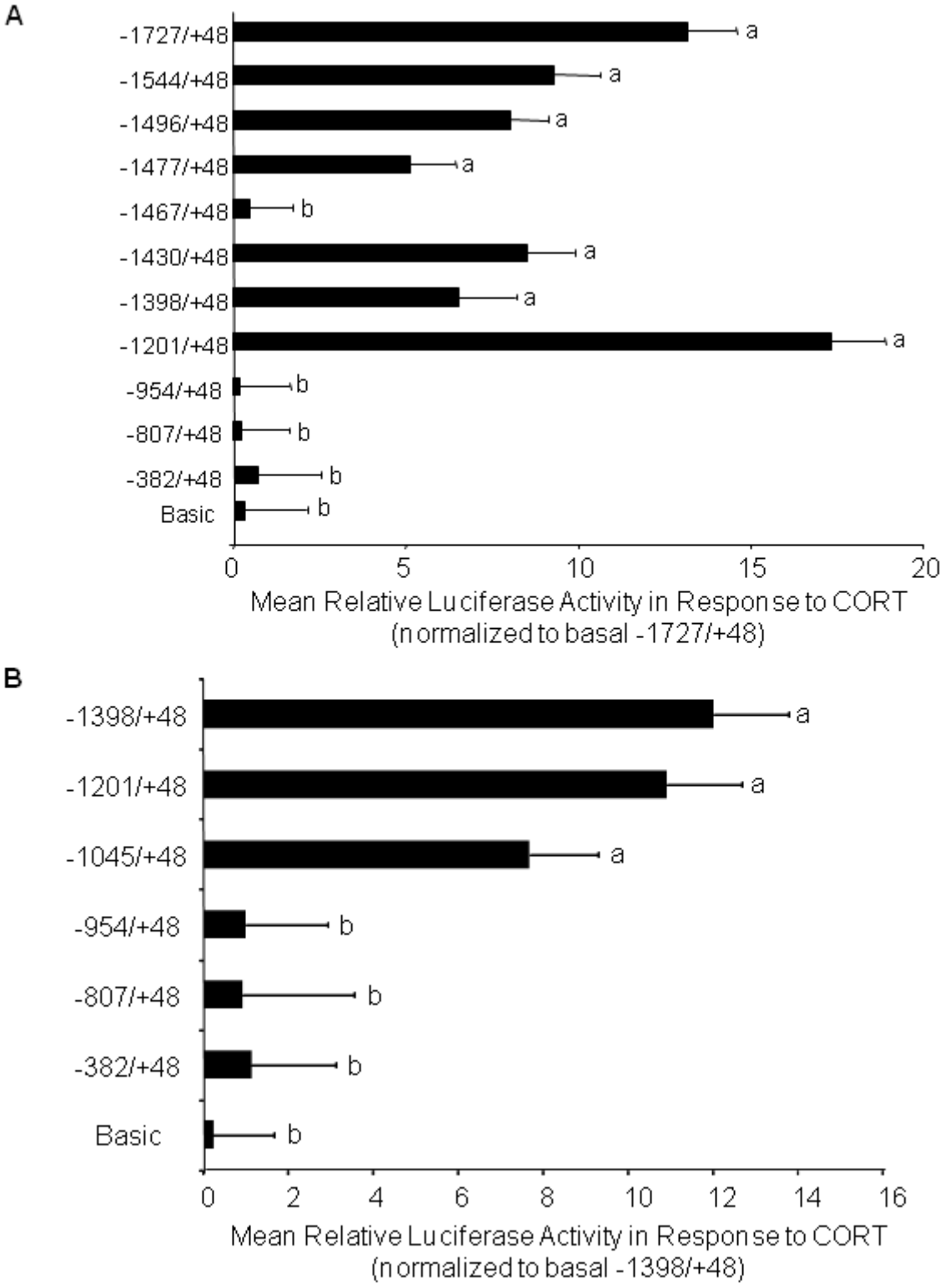
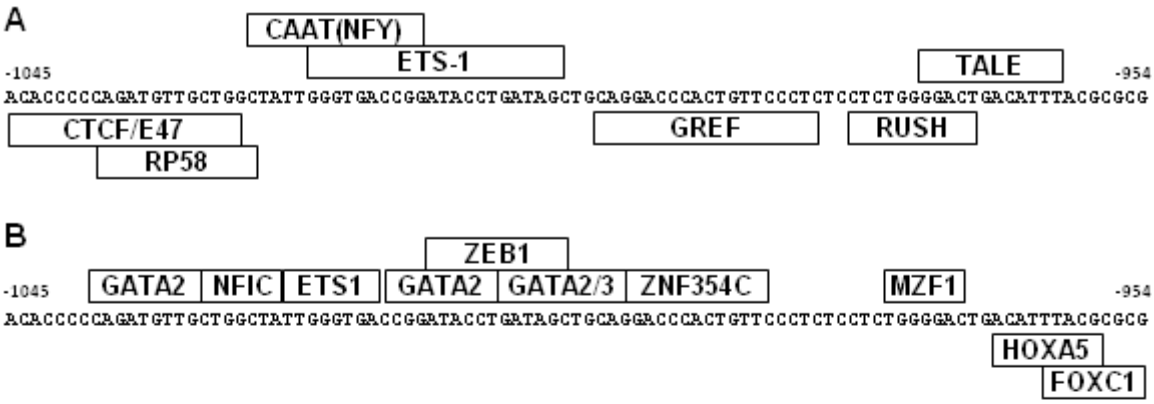


Figure 11: Mean Relative Luciferase Activity of Deletion Constructs in Response to CORT. A) Nine deletion constructs. B) One additional deletion construct (-1045/+48) to narrow down the region to less than 100 base pairs. Values denoted by different letters are significantly different ($p<0.05$), n=3.

GH gene expression can be identified by examining the sequence between -1045 and -954 of the chicken GH gene. Two transcription factor search engines were used to generate this list: MatInspector (Quant et al., 1995) and JASPAR (Sandelin, et al., 2004) (Fig. 12). MatInspector is based on a position weight matrix, a conservation profile and a core region to identify putative transcription factor binding sites. The matrix is the entire transcription factor binding sequence (up to 15 bp). A matrix similarity score is calculated by matching the query sequence to the matrix or pattern. The highest score possible is 1 when the test sequence corresponds to the most conserved nucleotide at each position of the matrix. The core region or core similarity is the set of 4 to 5 nucleotides that is the most conserved in the transcription factor binding site. Thus, two scores can be calculated: matrix similarity and core similarity (Cartharius et al., 2005). Using the vertebrate database in the MatInspector program and a cutoff of 0.9 identity (10% error) to the matrix, putative transcription factor binding sites were identified, including CTCF/E47, RP58, NFY (CAAT-box binding protein), ETS-1, GREF (glucocorticoid responsive and related elements), RUSH (SMARCA3), and TALE (Fig. 12A). The putative GR binding site as identified by MatInspector was classified as a "glucocorticoid responsive and related elements". The algorithm identified the following sequence, aggGAACagtgggtcctgc, where the upper case letters match the conserved core element and the underlined letters match the overall matrix. The core similarity score was 1.0 and the matrix similarity score was 0.94. The putative ETS-1 site as identified by MatInspector was: gggtgaCCGGatacctgatag, where the upper case letters match the conserved core element and the underlined letters match the overall matrix. The core similarity score was 1.0 and the matrix similarity score was 0.93.



C

Trx Factor	Description	Strand	Consensus
RP58	Zinc finger protein	-	AACATCTG
CTCF/E47	CCCTC binding factor; transcriptional repressor; 11-zinc finger protein	-	CCCCTC
CAAT/NFY	Twist subfamily; class B bHLH	+	CAGATG
ETS-1	Human and murine ETS1	+	CCGGA
GREF	Glucocorticoid responsive and related elements	-	GAACA
RUSH	SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	-	TCAGT
TALE	Hox-like; homeodomain of 3 amino-acid loop extension	+	CATTT
GATA2	Endothelial transcription factor; highly conserved	+	AGATG; GGATA; TGATAG
NFIC	Nuclear factor I/C; CCAAT-box-binding transcription factor	+	CTGGCT
ZEB1	Zinc finger E-box binding homeodomain; interacts with SMAD	+	TACCTG
FOXC1	Forkhead family; ocular development	-	CGCGCGTA
HOXA5	Homeobox family; upregulates p53	-	CGTAAATG
ZNF354C	Zinc finger; repressor in brain and kidney	+	ACCCAC
MZF1	Myeloid zinc finger; SCAN-domain, retinoic-acid responsive	+	TGGGGA

Figure 12: Potential Transcription Factors of the -1045 to -954 Region of the Chicken GH Gene. Two different internet based search engines with their associated databases were employed. A) MatInspector results B) JASPAR results C) Transcription factor, description, orientation, and consensus binding site.

JASPAR-CORE is based on the same weight matrix scoring, however, the library is smaller. Using the vertebrate database in the JASPAR-CORE program and a cutoff of 0.9 identity (10% error), different putative transcription factor binding sites were identified (Fig. 12B). The factors include GATA2, GATA2/3, NFIC, ETS1, ZEB2, ZNF354C, MZF1, FOXC1, and HOXA5 (Fig. 12B). Each program generated a list of potential transcription factor binding sites and these two lists were different. The differences are attributed to the different databases; the JASPAR database is much smaller than the MatInspector database. Notably, ETS-1 was the only putative transcription factor binding site identified by both programs. A brief description of each putative transcription factor binding site, the orientation of that site, and the consensus sequence are listed in the table in Fig. 12C.

The GRE Half Site Located in Intron 1 Did Not Increase the CORT Response of the -1727/+48 firefly luciferase Plasmid.

A half site of a canonical GRE is located in intron 1 (+302 to +320) of the chicken GH gene. Another report indicates that a half site GRE located in intron 1 of the human GH gene was functional (Kolb et al., 1998). To address whether or not the purported half site in the chicken GH gene is functional as well, -1727 to +1004 inclusive of intron 1, was cloned into the firefly luciferase plasmid and tested (Fig. 13A & 14A). Previously, two fragments of the GH gene were separately cloned into a luciferase reporter: -488/+1 and -488/+1004. Neither of these constructs was found to be responsive to CORT (Liu, dissertation; 2003). By cloning the fragment containing -1727/+1004 into a Luciferase reporter, it may be possible to answer the question of functionality of the intron 1 half site GRE. To this end, e11 pituitary cells were transfected with either the Basic, -1727/+48 or

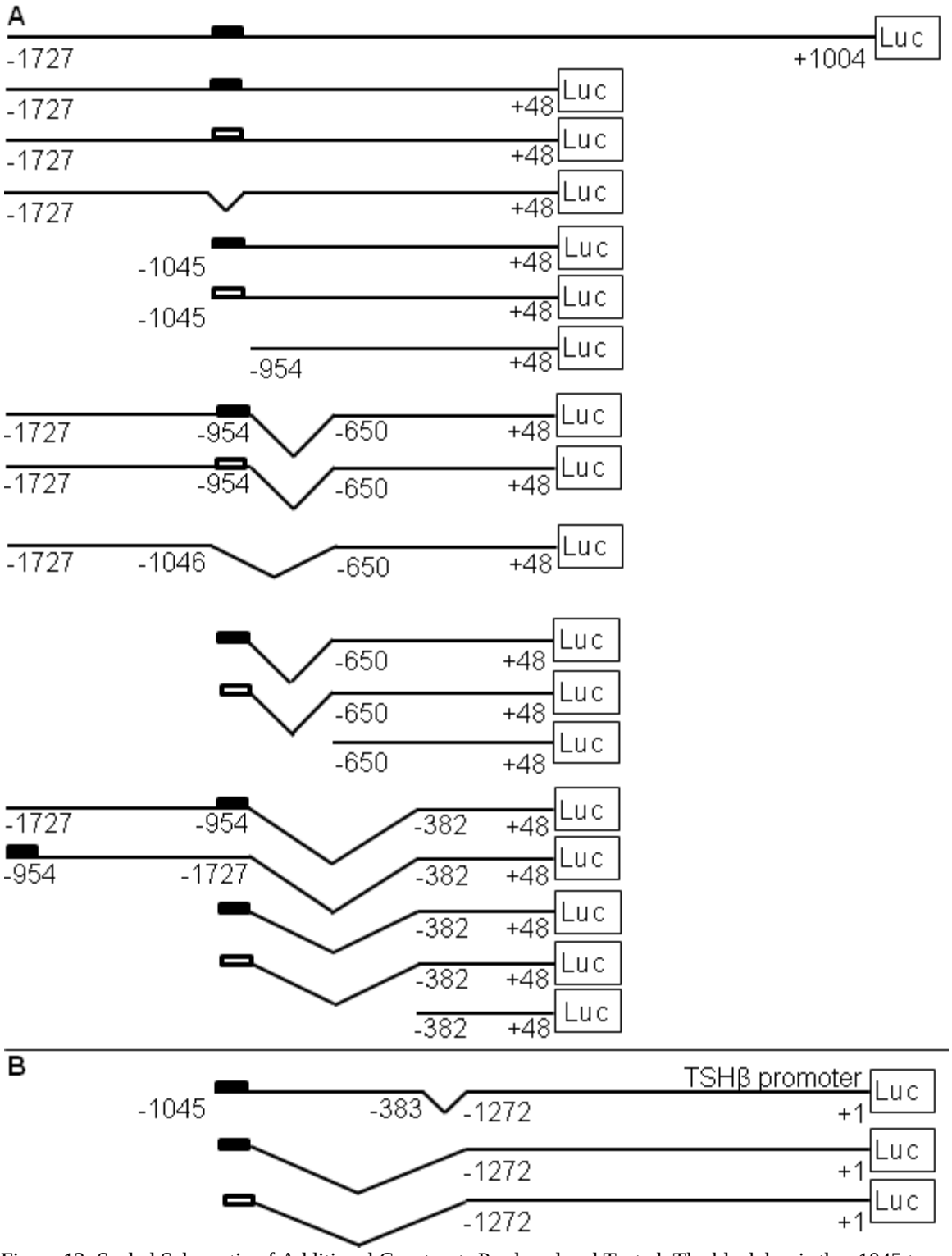


Figure 13: Scaled Schematic of Additional Constructs Produced and Tested. The black bar is the -1045 to -954 region. The white bar is the -1045 to -954 region inserted in the reverse orientation. A) Additional constructs made of the 5' flanking region of the GH gene. B) Additional constructs made using 1272 bp of the 5' flanking region of the chicken TSHβ gene.

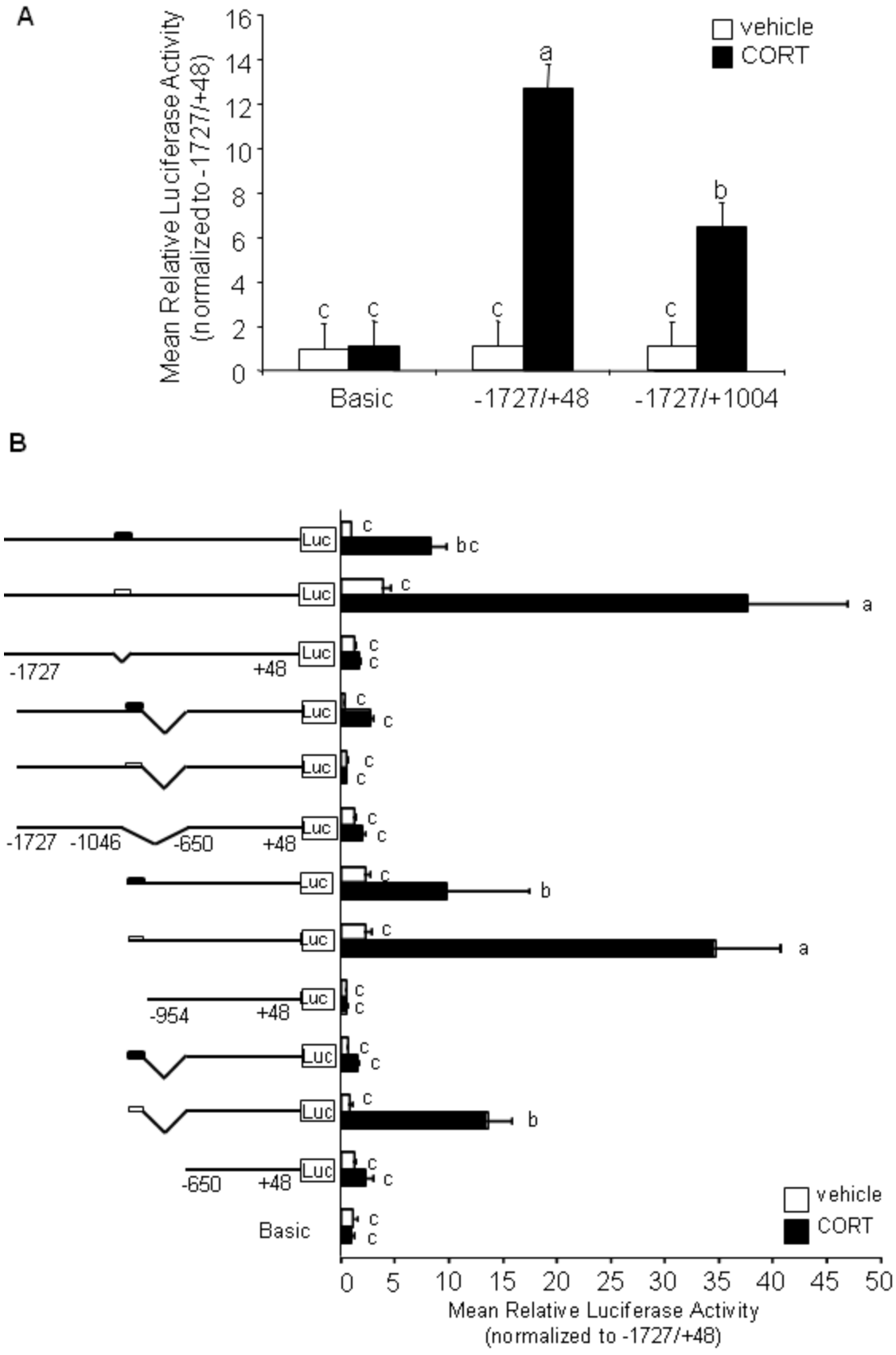

A
Mean Relative Luciferase Activity
(normalized to -1727/+48)
16
14
12
10
8
6
4
2
0
vehicle
CORT
a
b
c
c
c
c
Basic
-1727/+48
-1727/+1004
B
Luc
-1727
+48
-1727
-1046
-650
+48
-954
+48
-650
+48
Basic
c
bc
a
b
a
b
vehicle
CORT
0
5
10
15
20
25
30
35
40
45
50
Mean Relative Luciferase Activity
(normalized to -1727/+48)

Figure 14: Mean Relative Luciferase Activity of Additional Constructs in Response to CORT. A) Comparison of the -1727/+48 GH promoter to the -1727/+1004 GH promoter that includes intron 1. Values denoted by different letters are significantly different ($n=3$; $p<0.05$). B) GCRR orientation in response to CORT was tested in 4 different constructs. Values denoted by different letters are significantly different ($n=4$; $p<0.05$).

-1727/+1004 plasmids, and then treated with vehicle or CORT the next day. The cells were lysed 24 h later for determination of luciferase activity. CORT treatment of the -1727/+48 plasmid resulted in a significant 12-fold increase in luciferase activity. CORT treatment of the -1727/+1004 plasmid resulted in a significant 6-fold increase in luciferase activity. The CORT induced luciferase activity was significantly different between the two -1727 plasmids ($n=4$; $p<0.05$). Values denoted by different letters are significantly different at $p<0.05$. Thus, inclusion of intron 1 and the GRE half site did not significantly increase responsiveness to CORT.

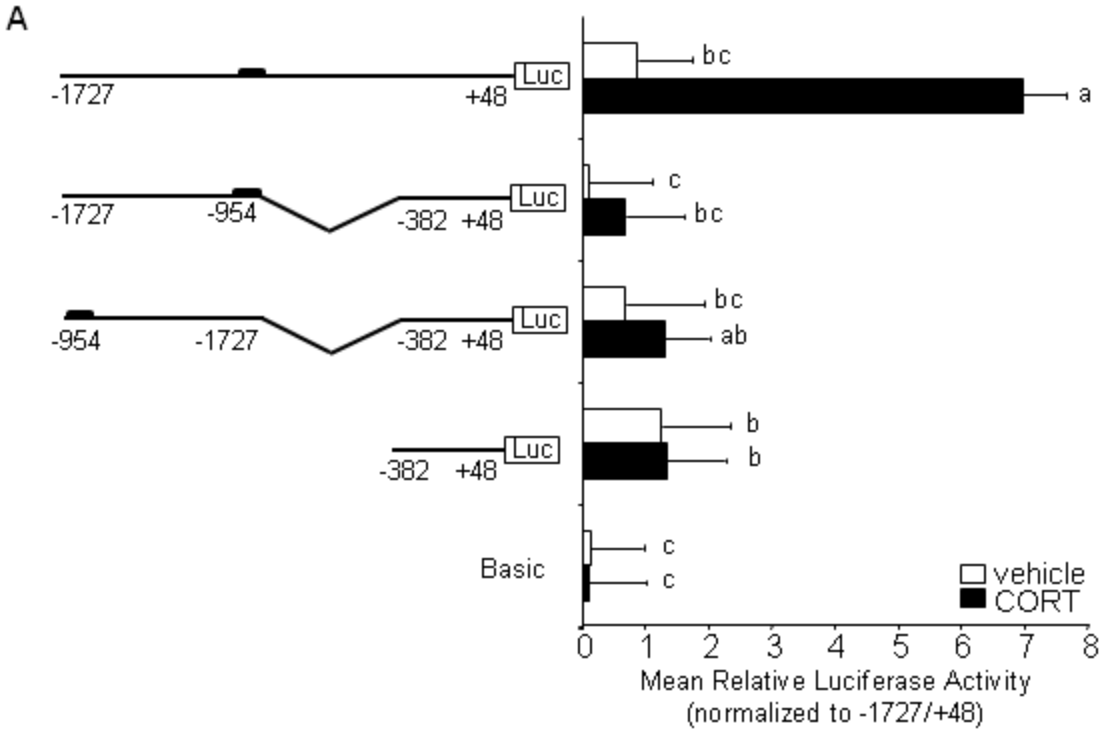
Analysis of Additional Firefly Luciferase Constructs Shows that the GCRR Requires Additional Sequence Elements for CORT Responsiveness.

The -1045/+48 GH construct is the shortest plasmid that retained CORT responsiveness, and the responsiveness was lost with deletion down to -954 of the 5' flanking region of the GH gene. To further characterize the -1045 to -954 GCRR, ten additional constructs were made in order to test whether the proximal GCRR is position-, orientation-, or context-independent (Fig. 13A). The GCRR was placed in the reverse orientation in the -1727/+48 and -1045 plasmids (white bar). The GCRR was placed, in both the forward (black bar) and reverse directions (white bar), and moved closer to the transcription start site by deleting the intervening sequence between -953 and -650 and between -953 and -382. Similarly, in the -1727/+48 plasmid, intervening sequence between -1045 and -650 was deleted, and the GCRR was re-inserted in both orientations. Finally, the segment from -1727 to -954 was placed in front of the -382/+48 region in both orientations.

The GCRR exhibited greater CORT responsiveness when placed in the reverse orientation (n=4; $p<0.05$) (Fig. 14B). Values denoted by different letters are significantly different at $p<0.05$. The addition of the reverse GCRR to the -1727/+48 and -954/+48 constructs resulted in an average 35-fold increase in luciferase activity, but only a 13-fold increase in luciferase activity when placed in front of the -650/+48 construct. The GCRR in the forward orientation linked to the -650/+48 exhibited a two-fold response to CORT, but this was not significant. The luciferase activity of the -1727/+48, with 953 to 650 deleted and the GCRR intact, still responded to CORT about 8-fold, but this was not significant and the overall activity of the construct was significantly reduced compared to the -1727/+48 construct. However, when the GCRR, in either orientation, was linked to -382/+48, CORT induction of luciferase activity was lost (Fig. 15A). This suggests that intervening sequences between -650 and -382, including the distal Pit-1 site (-541/-528), are required for the CORT induction of luciferase activity. Thus, other transcription factor binding sites between -953 and -650 are necessary for CORT responsiveness. Taken together, these results indicate that responsiveness to CORT through the GCRR requires cis-acting elements between -650 and -382 and additional elements between -953 and -650.

The GCRR Cannot Confer CORT-responsiveness to the Thyroid Stimulating Hormone β Promoter.

The -1272/+1 5' flanking region of the thyroid stimulating hormone β (TSHβ) subunit was cloned into the firefly luciferase plasmid. The TSHβ gene also requires Pit-1 for expression, but it has not been shown to be induced by CORT to date. Three constructs using the TSHβ promoter were made (Fig. 13B). The segment from -1045 to -


A
-1727
+48
Luc
bc
a
-1727
-954
-382
+48
Luc
c
bc
-954
-1727
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+48
Luc
bc
ab
-382
+48
Luc
b
b
Basic
c
c
vehicle
CORT
0 1 2 3 4 5 6 7 8
Mean Relative Luciferase Activity
(normalized to -1727/+48)

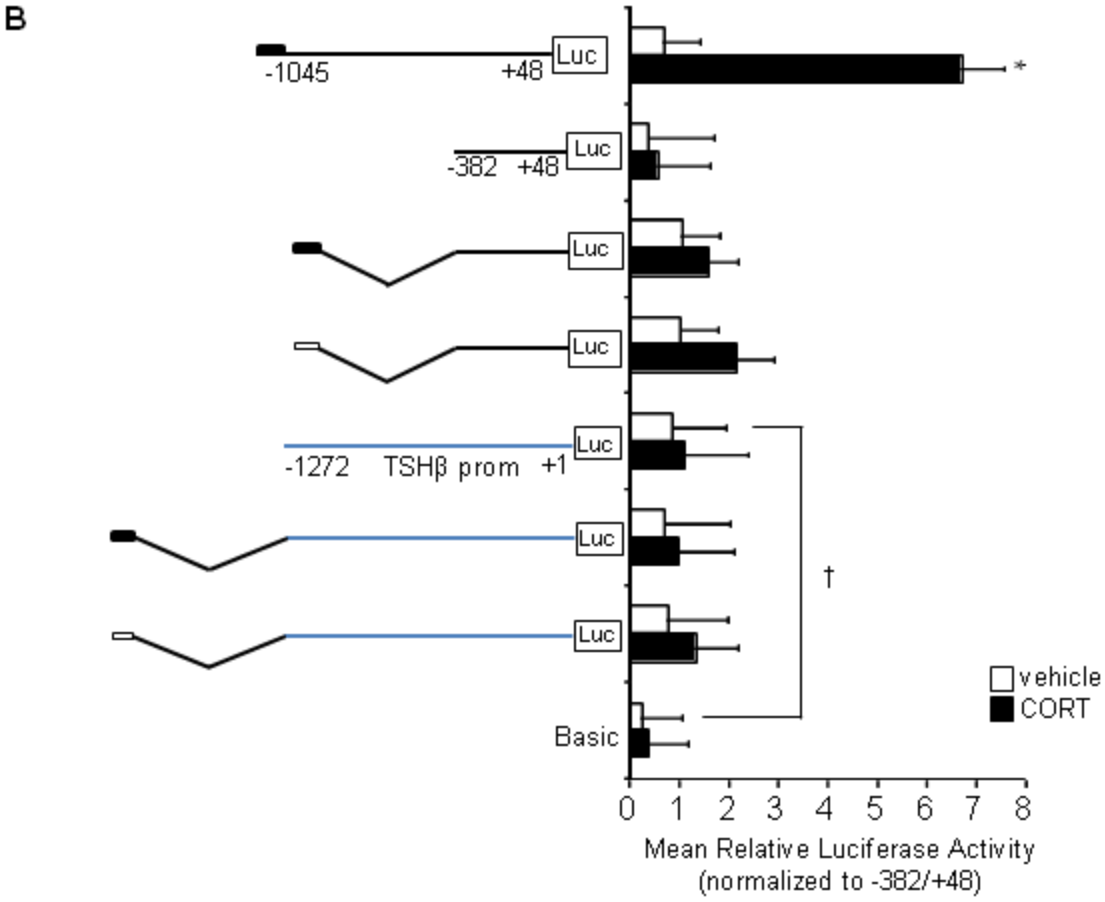

B
-1045
+48
Luc
*
-382
+48
Luc
Luc
Luc
-1272
TSHβ prom
+1
Luc
Luc
Luc
†
Basic
vehicle
CORT
0 1 2 3 4 5 6 7 8
Mean Relative Luciferase Activity
(normalized to -382/+48)

Figure 15: Mean Relative Luciferase Activity of the TSHβ Constructs in Response to CORT. A) Constructs using the GH promoter and the region from -1727 to -954. Values denoted by different letters are significantly different ($p<0.05$), n=3. B) Constructs using the TSHβ promoter and only the GCRR. *, denotes significant difference from vehicle. †, denotes significant difference from Basic. (n=3; $p<0.05$).

383 was placed upstream of the TSHβ promoter, thus including the distal Pit-1 of the GH gene, the GCRR and all intervening sequence. Additionally, the GCRR (-1045 to -953) alone was placed upstream of the TSHβ promoter in both the forward and reverse orientation. The TSHβ promoter exhibited significantly increased luciferase activity compared to Basic vector (n=3; †, $p<0.05$) (Fig. 15B). Addition of the GCRR in either orientation did not affect CORT-stimulated or unstimulated luciferase activity. Similarly, addition of the -1045 to -383 segment also did not affect CORT-stimulated or unstimulated luciferase activity (n=3; $p<0.05$). Thus, the GCRR of the GH gene could not confer CORT-responsiveness to the TSHβ gene.

Electrophoretic Mobility Shift Assays Show that Nuclear Proteins Bind to the -1045 to -954 GCRR.

Nuclear proteins incubated with the -1045 to -954 proximal GCRR probe produced a noticeable shift of the probe, as indicated by the arrow (Fig 16A). Nuclear proteins incubated with the exon 3 probe or the distal GCRR probe (-1566 to -1467) did not produce an observable shift. Because the -1467/+48 firefly luciferase plasmid was not CORT responsive and the -1566 to -1467 did not bind nuclear proteins in the gel shift assay, additional experiments to further characterize this apparent GC-inhibitive region were conducted (Appendix A). However, we were unable to define the basis for the non-responsiveness of the -1467/+48 construct. Therefore, we focused our attention on the proximal GCRR, which bound nuclear proteins and responded to CORT.The amount of the GCRR probe used in the gel shift assays was serially diluted 1:4 to show that binding as concentration dependent, as indicated by the arrow (Fig. 16B). Likewise, the amount

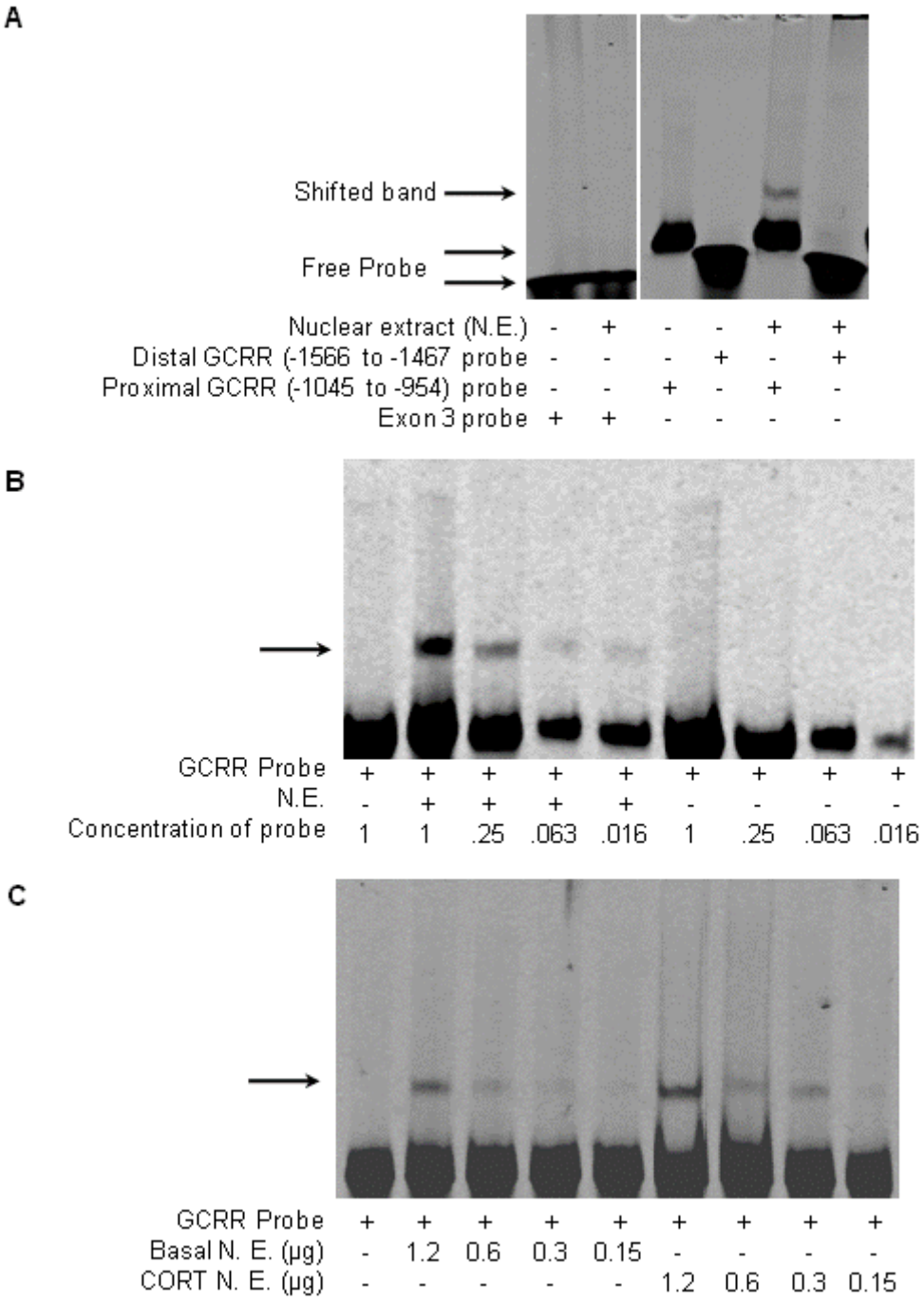


Figure 16: Electrophoretic Mobility Shift Assay (EMSA) with Nuclear Protein Extracts (N.E.) from e11 Pituitary Cells. A) Nuclear extract (2.5 µg) binding to the proximal GCRR probe and not the distal GCRR probe nor the exon 3 probe. The arrow denotes the shifted band. B) Titration of the GCRR probe. Equal amounts of protein were incubated with decreasing amounts of probe. The arrow denotes the shifted band. C) Titration of nuclear extracted proteins. Equal amounts of probe were incubated with decreasing amounts of protein. The arrow denotes the shifted band.

of nuclear protein incubated with the probe was serially diluted 1:1, as indicated by the arrow (Fig. 16C). In both cases, serial dilutions reduced intensity of the shifted band. In conclusion, the proximal GCRR probe binds nuclear proteins.

Nuclear Extract Binding to the GCRR Probe Is Increased by CORT Treatment, and Competitors Show that the Observed Shift is Specific.

The GCRR probe was incubated with CORT- or vehicle-treated nuclear extract proteins (n=4) (Fig. 17A). Two shifts, one high molecular weight and one low molecular weight, were observed upon incubation of nuclear extracts with the GCRR probe. CORT treatment significantly increased binding of nuclear proteins, both high and low molecular weight, to the GCRR probe. The high molecular weight band was not seen in all experiments, so we focused our attention on the lower molecular weight band. The intensity of the lower molecular weight band, as indicated by the arrow, was quantified and normalized to the free probe lane. Quantification of the mean integrated intensity of the shifted band as compared to the free probe lane showed that CORT treatment significantly increased binding of proteins to the GCRR probe ($p<0.05$) (Fig. 17B).

To determine if the observed shift with the proximal GCRR probe was specific, three unlabeled double-stranded DNA competitors were made: one corresponding to the immediate upstream region (-1201 to -1046) of the GH gene, one corresponding to the 5' half of the probe, and one corresponding to the 3' half of the probe (Fig. 17C). Nuclear extracts were pre-incubated with either the 5' competitor, 3' competitor or the upstream competitor in 100-fold molar excess for 30 m prior to addition of the proximal GCRR probe, as indicated by the arrow (Fig. 17D). The nuclear extracted proteins produced an observable shift. Addition of the 5' or 3' competitors resulted in reduced protein binding

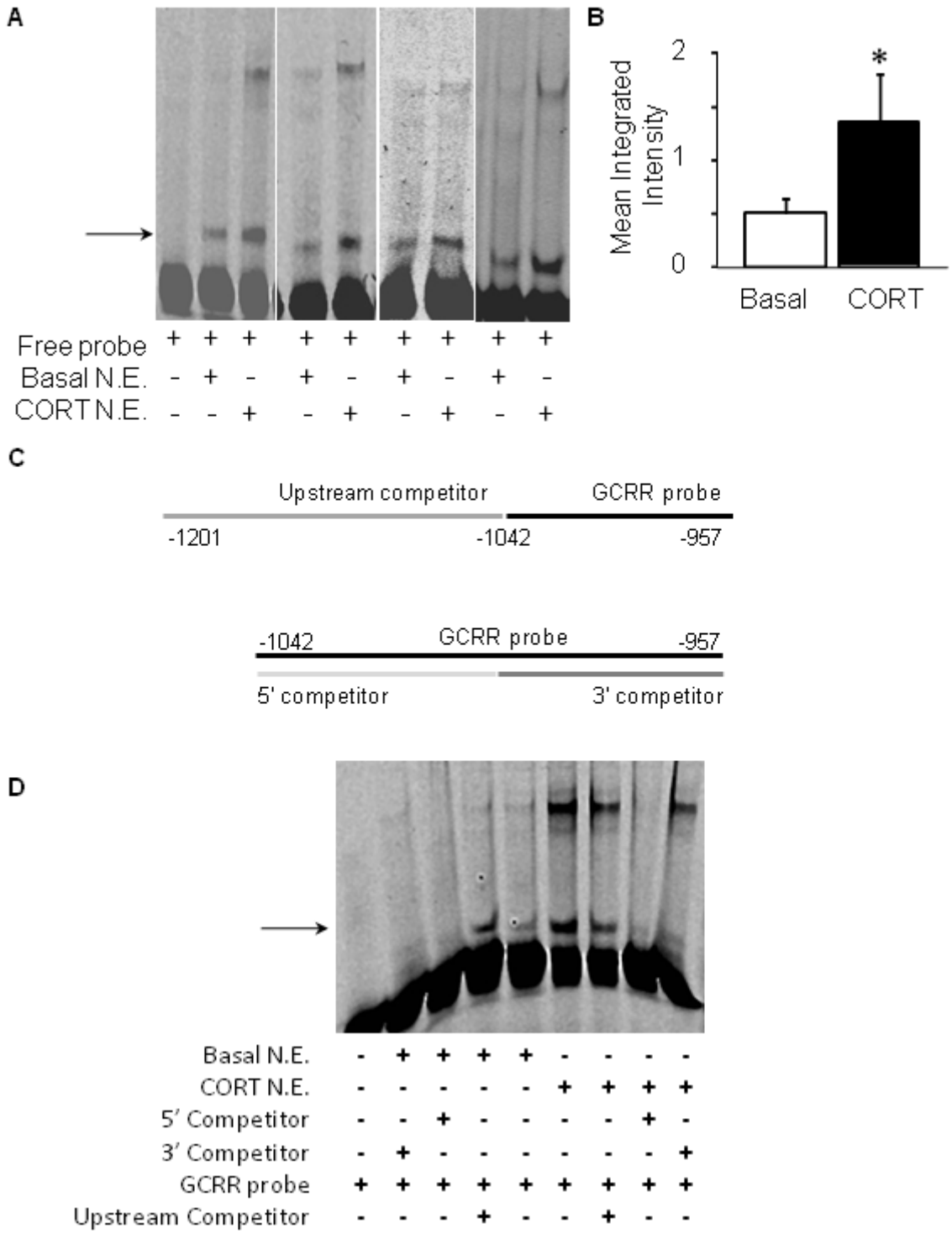


Figure 17: Nuclear Protein Binding is CORT Regulated. A) Nuclear extracts from vehicle and CORT treated cells (n=4). The arrow denotes the shifted bands that were used for intensity quantification. B) Mean integrated intensity of the shifted bands. *, denotes significant difference at $p<0.05$. C) Schematic of the 5', 3' and upstream competitors. D) Binding of nuclear protein to the GCRR probe and competition with unlabeled competitors. The competitors were used in excess (100x) (n=4).

to the probe, while addition of the upstream competitor did not abolish protein binding to the probe (n=4). Thus, nuclear protein binding to the GCRR probe is CORT-regulated, and the binding can be competed off with addition of either a 5' competitor or a 3' competitor. Because the 5' and 3'competitors reduced binding to the probe and did not provide any additional insight into the region bound by proteins, another competitor was made. An unlabeled centered competitor that corresponds to the center 34 bp of the GCRR probe that spans the putative ETS-1 and GREF binding sites as identified by the MatInspector program was made and tested with the nuclear extracts (Fig. 18A). Nuclear extracts were pre-incubated with either the upstream-competitor or the centered-competitor in 100-fold molar excess for 30 min prior to addition of the proximal GCRR probe. Addition of the centered competitor abolished protein binding to the GCRR probe. Addition of the upstream competitor did not affect protein binding to the probe, as indicated by the arrow (n=4) (Fig. 18B). Taken together, regulated protein binding may occur in the central 34 bp of the GCRR probe, which contains putative ETS-1 and GREF binding sites.

A Mutated Probe Results in Decreased Protein Binding.

The proximal GCRR probe binds nuclear proteins in a CORT-regulated manner, and this binding can be competed off through the use of a competitor DNA probe used in excess that corresponds to the central 34 bp and spans the putative ETS-1 and GREF sites. The unlabeled competitor probes were in excess and pre-incubated with the nuclear proteins. This could potentially create an artificial environment conducive to selective binding. To confirm specificity of protein binding to the GCRR, a mutated probe was employed. The central 34 bp of the GCRR probe were scrambled twice and reinserted

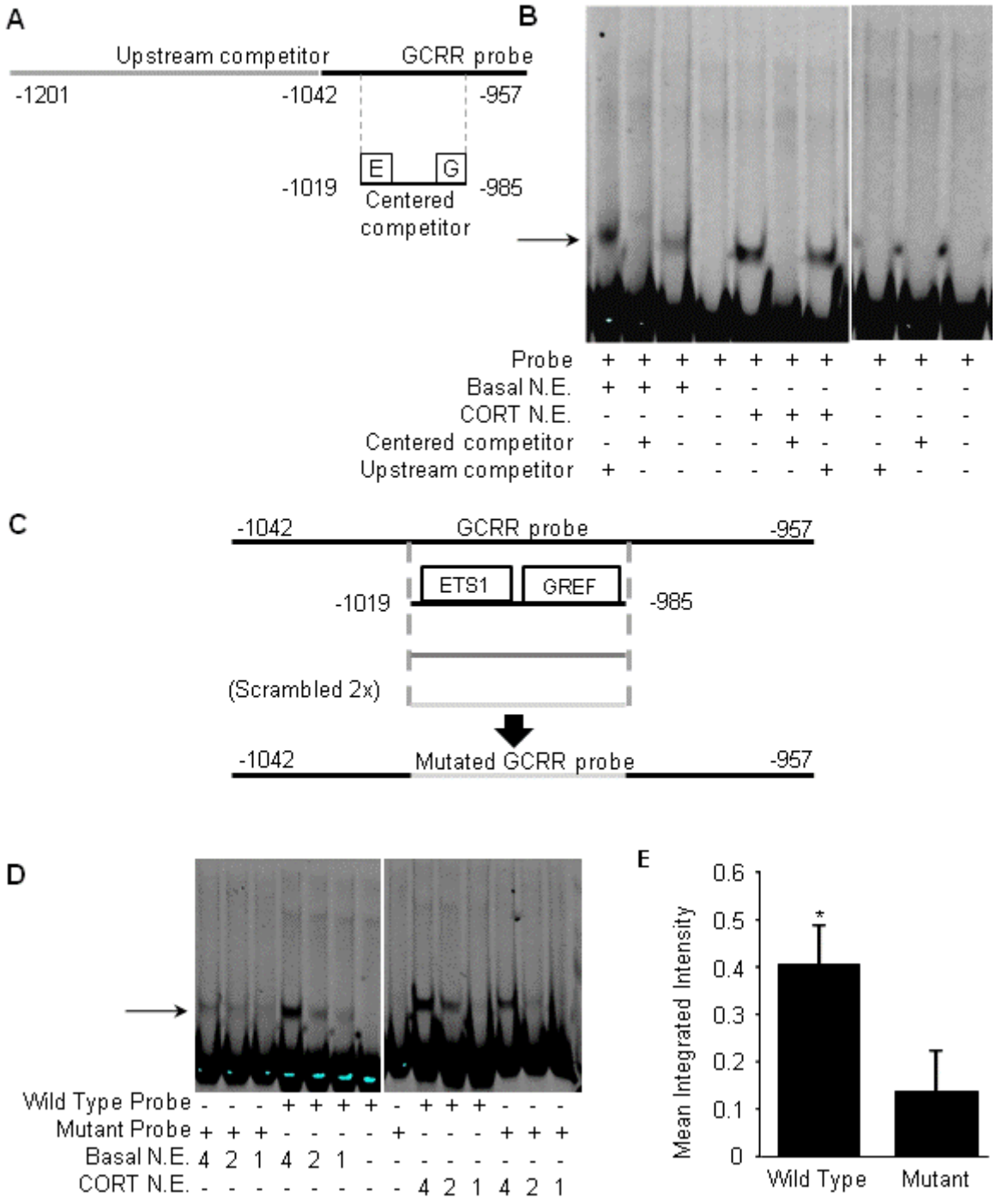


Figure 18: EMSA with a Centered Competitor and a Mutant Probe. A) Schematic of the upstream competitor, the GCRR probe, and the centered competitor. "E" corresponds to the putative ETS-1 binding site. "G" corresponds to the putative GREF binding site. B) Specificity of nuclear extract binding to the GCRR probe through the use of unlabeled competitors in excess (20x). The arrow denotes the shifted band (n=4). C) Design of the mutated probe. D) Binding of nuclear extracts to the wild type probe versus the mutated probe (1, 1.2 ug; 2, 2.4 ug; 4, 4.8 ug) (n=4). E) Mean integrated intensity of the shifted bands. *, denotes significant difference at $p<0.05$.

into the full length GCRR probe sequence, such that the flanking region was conserved, but the central 34 bp, although the same in nucleotide content, was different in sequence (Fig. 18C). The wild type or mutant probe was incubated with increasing amounts (1.2, 2.4, 4.8 µg) of nuclear protein extracts from basal or CORT-treated e11 pituitary cells (Fig. 18D). The shifted band intensity increased with increasing amounts of protein. A shifted band of the same molecular weight was also observed with the mutant probe; however, intensity was significantly reduced. The shifted bands from both the wild type and mutant probe from 3 replicate experiments were quantified in the same manner as previously stated. A 3-way ANOVA (probe, treatment, protein amount) was conducted on the mutant vs. wild type probe data. The main effect of probe was significant; however, there was no main effect of treatment or protein amount, and therefore, these terms were eliminated from the model. The intensity of the shifted band from the mutant probe was significantly decreased compared to the wild type shifted band (Fig. 18E). These results indicate that the central 34 bp of the GCRR are important for nuclear protein binding.

Transcription of ETS-1 Is Not Induced by CORT Treatment, and Super-shift Experiments with Antibodies to ETS-1 and GR Are Inconclusive.

GR mRNA is expressed in e11 chicken pituitaries (Heuck et al., 2009), and it is not responsive to CORT treatment (unpublished data). GR protein is expressed and the antibody has been validated (Proszkoweicz-Weglarz et al., 2010). A western blot for ETS-1 was conducted to test the ETS-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using adult rat liver, adult chicken liver and nuclear extracted proteins from the

GH_4C_1 rat pituitary cell line and e20 primary chicken pituitary cells (Fig. 19A). A single band of the correct size (45 kDa) was observed in the chicken liver and nuclear extracted protein lanes (lanes 2, 3, 4). CORT induces GH mRNA after 6 h of treatment, but it does not affect ETS-1 mRNA (Fig. 19B). Addition of the GR, ETS-1 or rabbit IgG antibody at 10% of the reaction volume (2 µL) in EMSA produced results that could not be interpreted (Fig. 19C; n=6). Addition of any antibody resulted in an upward shift of most of the probe, and there was no observable shifted band in the probe + protein only lanes (lanes 6 & 8). Unusual smearing was also observed throughout the gel. Therefore, less antibody was used, in order to avoid these non-specific effects. Addition of the rabbit IgG, GH, GR, ELK4 (an ETS-1 family member) or ETS-1 antibodies at 1% of the reaction volume did not affect nuclear protein binding to the probe (Fig. 19D). Addition of nuclear protein resulted in an observable shift of the GCRR probe (lanes 6 & 13), and addition of different antibodies did not affect the shifted probe (lanes 2-5 & 14-18) (n=3).

Mutation of the ETS-1 and GREF Potential Binding Sites in the GH Luciferase Construct Results in Ablation of the CORT-Responsiveness.

Because there was significantly reduced binding with the mutant probe compared to the wild type probe in the EMSA assays, the -1045/+48 firefly luciferase plasmid was mutated. The ETS-1 or the GREF site was mutated using site-directed mutagenesis (Fig. 20A). E11 pituitary cells were dispersed and transfected with either the Basic, -1045/+48, ETS-1 mutant or GREF mutant plasmids (Fig. 20B). The cells were treated with CORT the next day and assayed for luciferase activity 24 h after that. CORT significantly induced a five-fold increase in the luciferase activity of the -1045/+48 plasmid, while both the ETS-1 mutant and the GREF mutant were not CORT responsive. This study

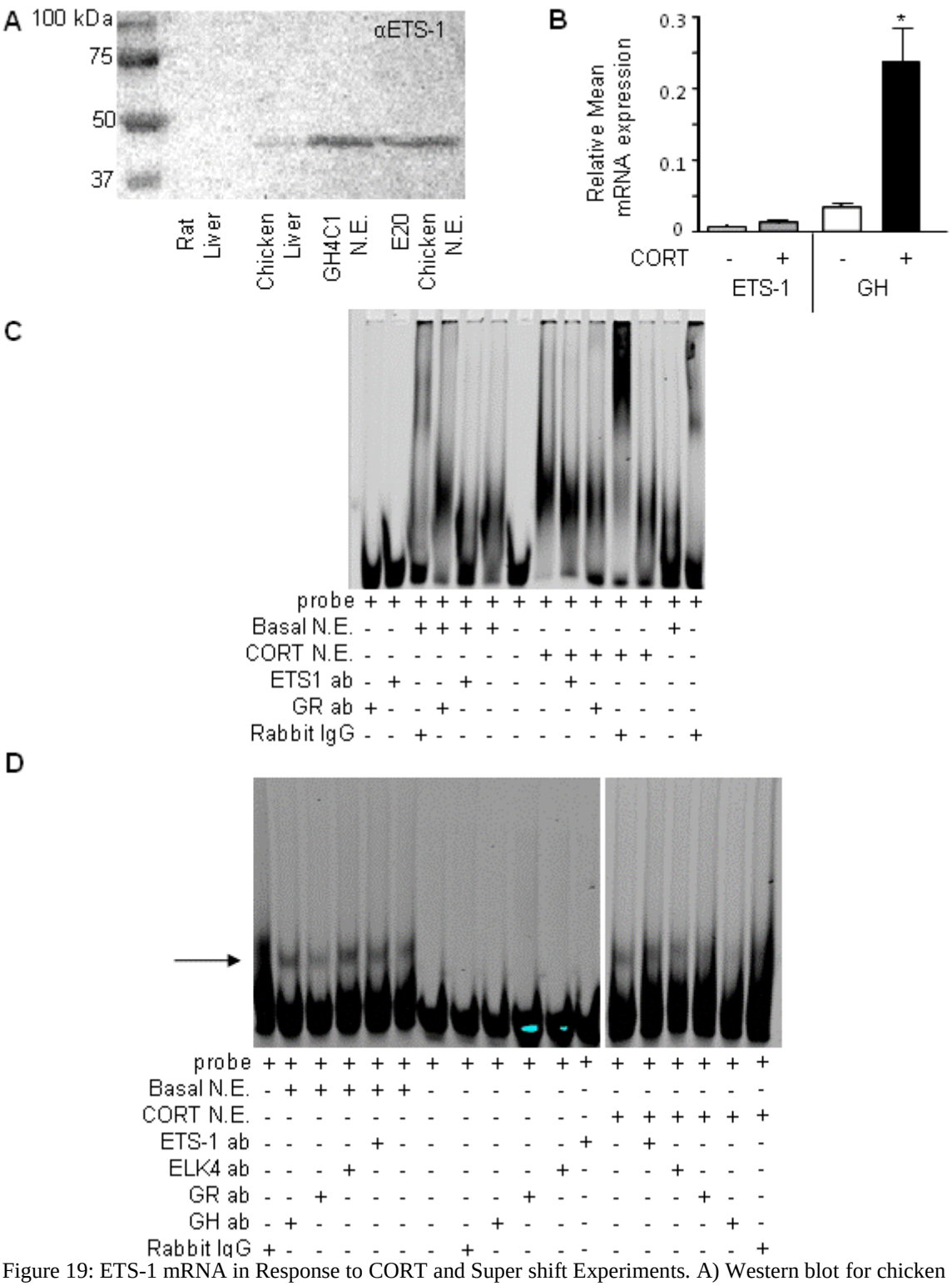


Figure 19: ETS-1 mRNA in Response to CORT and Super shift Experiments. A) Western blot for chicken ETS-1. Nuclear Extract=N.E. B) Mean Relative mRNA expression of ETS-1 and GH in response to 6 h CORT (10 nM) (n=3; $p<0.05$) C)Super-shift experiment using antibodies (ab) at a final concentration of 1:10 (n=3). D) Super-shift experiment using ab at a final concentration of 1:100 (n=3).

A

Wild Type GGGTGACCGGATACCTGATAGCTGCAGGACCCACTGTT

ETS-1 mutant GGGTGAAATTCTACCTGATAGCTGCAGGACCCACTGTT

GREF mutant GGGTGACCGGATACCTGATAGCTGCAGGAAAAAAGGGG

B

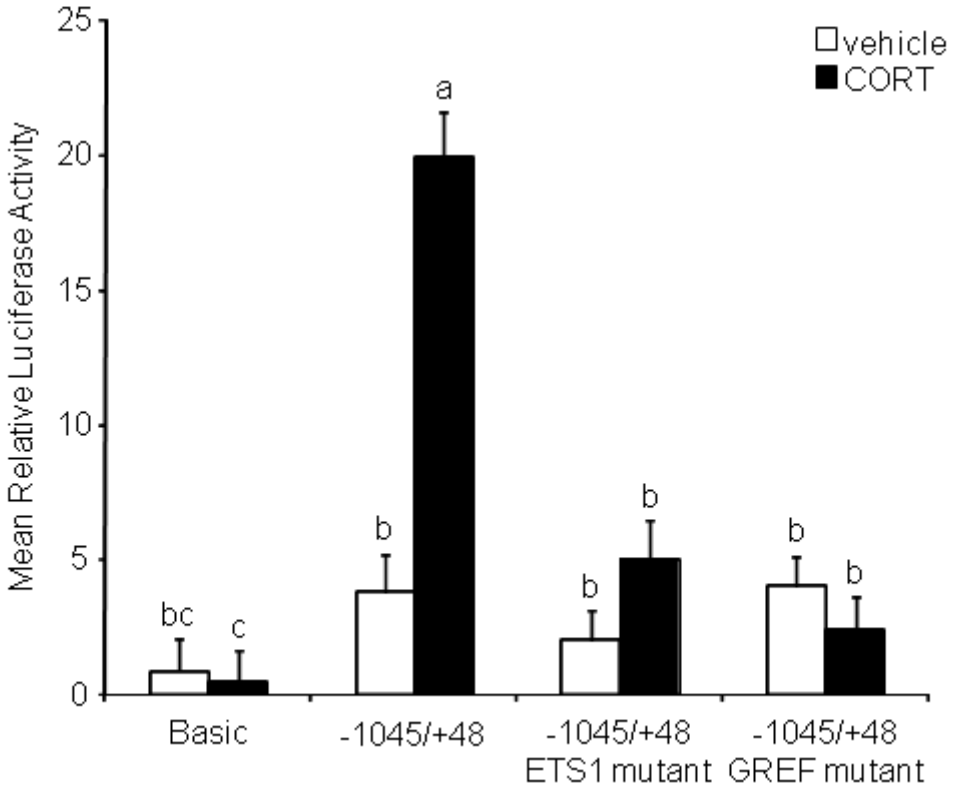


Figure 20: Mean Relative Luciferase Activity of Mutant Constructs in Response to CORT. 1 x 107 cells were transfected with either the Basic Luciferase empty vector, the -1045/+48 GH Luciferase, the ETS-1 mutant or the GREF mutant and allowed to recover overnight. The cells were treated with CORT the next day and assayed for Luciferase activity 24 h later. Values denoted by different letters are significantly different ($p<0.05$), n=3. B) Sequence of the wild type -1045 to -954 region and the ETS-1 and GREF mutations. The mutated base pairs are highlighted in red (ETS-1) or green (GREF).

demonstrates that the putative sites for ETS-1 and GR are indeed necessary for glucocorticoid regulation of the GH gene during chicken embryonic development.

ETS-1 and GR Are Associated with the GCRR and not the Distal Control Region.

Chromatin immunoprecipitation was performed on e11 chicken embryonic pituitary cells that were untreated or treated with CORT [1 nM] for 1.5 h and 6 h. Chromatin was immunoprecipitated with normal rabbit serum and antibodies towards GR (Proszkowiecz-Weglarz & Porter, 2010) and human ETS-1. Real time qRT-PCR was performed on the immunoprecipitated chromatin using primers corresponding to the proximal GCRR (-1065 to -869) and the distal GCRR (-1436 to -1299) (Fig 21). The real time PCR products of the proximal GCRR and the distal GCRR after 40 cycles from one replicate were electrophoresed on an agarose gel to check the product size (Fig 21A & B, respectively). Cycle threshold values from real time PCR of the input sample and the immunoprecipitated samples were used in a three-way ANOVA. There was a significant effect of region (proximal vs. distal), immunoprecipitation (IP), and treatment. The three-way interaction was also significant ($n=3$; $p<0.05$). ETS-1 was associated with the proximal GCRR under both 0 h and 1.5 h CORT treatment. ETS-1 association with the GCRR was significantly decreased at 6 h. Association of GR with the proximal GCRR was increased after 1.5 h, and then decreased after 6 h. Neither GR nor ETS-1 was associated with the distal control region under any condition. Taken together, these results demonstrate that GR and ETS-1 are associated with their putative response elements in the proximal GCRR of the GH gene during embryonic development of the chicken.

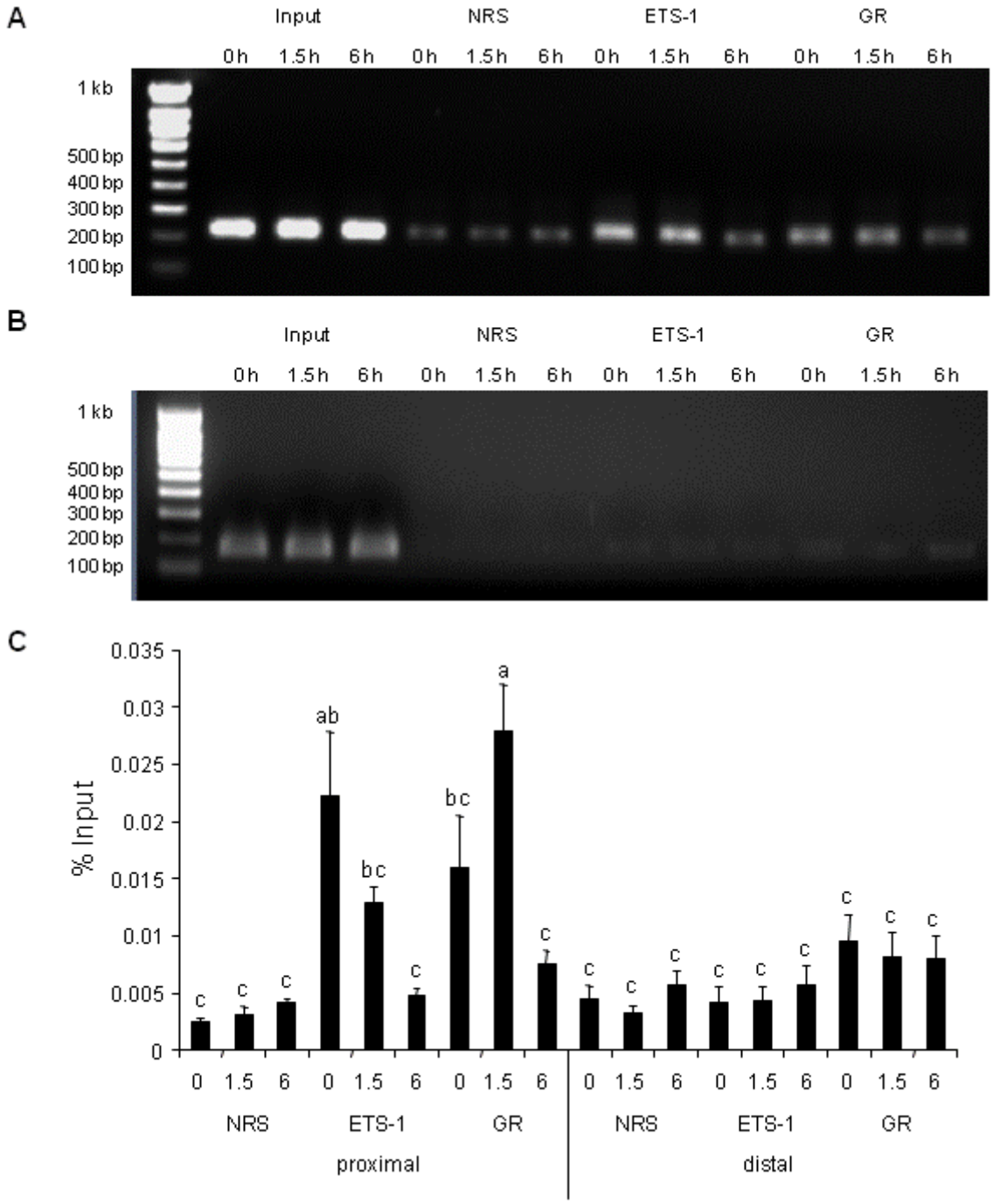


Figure 21: ETS-1 and GR are recruited to the GCRR based on chromatin immunoprecipitation of the proximal and distal GCRR. A) Real time PCR products after 40 cylces of the proximal GCRR primer set as visualized in an agarose gel from one replicate. B) Real time PCR products after 40 cycles of the distal GCRR primer set as visualized in an agarose gel from one replicate. C) Real Time PCR results represented as percent input as calculated from the cycle threshold values. Values with different letters indicate a significant difference ($n=3$; $p<0.05$).

Discussion

The overall objective of this study was to identify *cis*- and *trans*-acting elements and factors underlying glucocorticoid regulation of the GH gene during chicken embryonic development. A luciferase reporter containing -1727/+48 of the 5' flanking region of the chicken GH gene was used to analyze the CORT response of the GH gene. In our studies, it was determined that the -1727/+48 GH construct is an appropriate model for glucocorticoid regulation of the endogenous GH gene, because the response was specific to CORT, it required on-going protein synthesis, and the transcription start site was the same as the endogenous gene. It was also shown that the basal activity of the basic construct compared to the -1727/+48 GH construct was significantly increased. This suggests that the GH gene is under tonic repression until another unknown protein is activated or released. Additional studies are warranted to explore the repression of the GH gene under basal conditions.

The -1727/+48 GH construct responded to CORT treatment and possibly progesterone. Glucocorticoids can induce GH mRNA and protein in chickens (Porter, 2007) and rats (Nogami, 2008). This is the second study to show that the promoter of the chicken GH gene was responsive to glucocorticoids. The -1727/+48 GH construct exhibited a 2-fold increase in response to dexamethasone when transfected into a rat pituitary cell line (Ip et al., 2004). Previously, only 500 bp of the 5' flanking region of the chicken GH gene was cloned, but it was not tested for CORT-responsiveness (Tanaka et al., 1992). It is interesting to note, but not surprising, that the 5' flanking region was partially responsive to progesterone. The glucocorticoid and the progesterone steroid

hormone receptors recognize an imperfect half site of TGTYCY, where Y is a pyrimidine (C or T) (Truss et al., 1991). The canine GH promoter, as analyzed in mammary tissue, is responsive to progestins, contains a conserved PRE, and nuclear proteins bind to a probe corresponding to the aforementioned PRE in gel shift assays. A super-shift with a progesterone receptor antibody was not performed in that study (Lantinga-van Leeuwen et al., 2002). Other studies have shown that aldosterone can induce GH mRNA (Bossis et al., 2004), but the promoter seems to be aldosterone insensitive. In another study, GH secretion could be induced with progesterone, corticosterone, dexamethasone, and to some extent, testosterone and estradiol (Morpurgo et al., 1997). Different results were observed based on the end point tested, i.e. mRNA, secretion of protein, activity of the promoter. Nonetheless, the GH gene responds to CORT at the mRNA, protein and now the promoter level.

The CORT response of the -1727/+48 GH construct requires ongoing protein synthesis. CORT induction of GH mRNA also required ongoing protein synthesis (Bossis & Porter, 2003). This suggests that the underlying mechanism of CORT induction of the GH gene during chicken embryonic development is conserved at the promoter level. It also suggests that the -1727/+48 GH construct recapitulates the native system and may be an appropriate model for use in studies of glucocorticoid regulation of the GH gene during chicken embryonic development. The requirement for ongoing protein synthesis possibly implicates the involvement of an unknown intermediary protein in glucocorticoid induction of the GH gene. Glucocorticoid induction of the GH gene during rat embryonic development also requires on-going protein synthesis (Nogami et al., 1997). It is possible that the unknown inducible protein is conserved between rats and

chickens. Identification of *cis*-acting elements in the upstream regulatory region of the chicken GH gene could aid in the identification of *trans*-acting proteins necessary for GH gene induction.

The transcription start site of the chicken GH gene was found to be 56 bp upstream from the translational start codon. The first study characterizing the chicken GH gene also found the transcription start site to be located 56 bp upstream from the start codon (Tanaka et al., 1992). Our current findings indicate that the same transcription start site is used during embryonic development and in response to CORT. The chicken GH gene, as annotated on the Ensembl website and as based on the sequence of the chicken genome, places the transcription start site 42 bp upstream from the start codon (Wallis et al., 2004). The transcription start site for the mouse GH gene was found to be 62 bp upstream from the start codon (Das et al., 1996). There is some variability, but it seems that the transcription start site is somewhat conserved between chicken, mouse, rat and human. If the transcription start site is conserved, then it is possible that the underlying mechanism of glucocorticoid induction of the GH gene during embryonic development is also conserved. The 5' RACE results did not find an alternative transcription start site further upstream or in the first intron of the chicken GH gene. The transcription start site of the -1727/+48 GH construct was determined by alignment with the chicken genome. The conservation of the transcription start site between the endogenous gene and the -1727/+48 GH construct also provides further evidence that the -1727/+48 GH construct is an appropriate model for the glucocorticoid regulation of the GH gene during chicken embryonic development.

Through deletion analysis of the -1727/+48 GH construct, two glucocorticoid responsive regions (GCRR) were identified: a proximal and a distal. The distal GC-IR was found to be located between -1477 and -1430, and the proximal GCRR was found to be located between -1045 and -954. The previous study characterizing the chicken GH gene concluded that a glucocorticoid response element was located between -1727 and -1467 (Ip et al., 2004). The present study further defined this region and concluded more appropriately that there is a repressor region located at -1477 to -1430 and not a responsive region. However, future studies are necessary to determine the underlying mechanism of the regulation of the distal GC-IR and whether or not it is biologically relevant.

There is a putative half site GRE located at +302/+320 with the sequence of TGTTCT in intron 1 of the chicken GH gene The -1727/+1004 GH construct was CORT responsive, however, the observed response was significantly less than with the -1727/+48 GH luciferase construct. Addition of intron 1 to the GH luciferase construct did not result in an increased synergistic response to CORT treatment. Research on the human GH gene has identified a GRE half site in the first intron that binds the GR complex in an exonuclease III protection/foot printing assay (Slater et al., 1985). Cloning of the intron 1 fragment containing the half site GRE in front of a glucocorticoid responsive gene devoid of its GRE, metallothionein-II_A, resulted in a three-fold increase in activity in response to dexamethasone treatment. The metallothionein-II_A gene devoid of its GRE was non-responsive to dexamethasone treatment. The intact metallothionein-II_A containing its GRE responded six-fold to dexamethasone treatment (Slater et al., 1985). Another study implicated a 123 bp region contained in the first intron of the

human GH gene that was sufficient to direct GH expression from a promoterless luciferase reporter (Kolb et al., 1998). It was not tested for glucocorticoid responsiveness. The putative half site GRE in intron 1 of the chicken GH gene did not result in synergistic activation of the chicken GH gene in the context of a luciferase reporter; however, it is still unknown whether or not this putative half site GRE binds GR in a regulated manner. Nonetheless, we conclude that the putative half site GRE in intron 1 of the chicken GH gene is not necessary for CORT induction of the GH gene during chicken embryonic development.

Additional constructs were made to determine if the CORT response of the -1045/+48 construct was independent of GCRR orientation and context. The CORT response of the reverse orientation of the GCRR was significantly increased compared to the forward orientation of the GCRR. Therefore, responsiveness of the GCRR was not orientation dependent and glucocorticoid regulation of the GH gene during chicken embryonic development is not dependent on the orientation of the GCRR. However, the GCRR was context dependent. The mammalian GH gene contains two functional Pit-1 sites: a distal and a proximal site (Lira et al., 1988 & Jin et al., 1999). The chicken GH gene contains a distal Pit-1 site at -541/-528 and a proximal site at -113/-104 (Ip et al., 2004). This possibly suggests that the CORT response requires both Pit-1 elements or another unknown transcription factor that binds between -650 and -382. A transcription factor search using MatInspector was conducted on the -954 to -382 fragment, with a 95% confidence level. From -650 to -382, besides Pit-1, there are putative binding sites for CREB, STAT5, EVI1, ZNF35, MZF1 and the ETS1 family members, SPI1 and PU1. There are conserved putative binding sites for EVI1 and MZF1 in the dog, mouse, rat and

human GH genes (Lantinga-van Leeuwen et al., 2002). CREB and STAT5 are known to be involved in the regulation of the GH gene (Cohen et al., 1999 & Udy et al., 1997). Glucocorticoid regulation of the GH gene during chicken embryonic development is dependent on sequences contained in the -650/+48 GH construct. Similarly, the GCRR alone cannot confer CORT-responsiveness to the TSHβ promoter. The TSHβ gene requires Pit-1 for normal expression (Haugen et al., 1996). Addition of the GCRR in either orientation did not confer CORT-responsiveness to the TSHβ promoter. Therefore, it seems that other transcription factors, binding to sites outside of the GCRR within the GH 5' flanking region, are required for the CORT response. In an attempt to test this, the -1045 to -382 fragment of the chicken GH gene was cloned 5' of the TSHβ promoter, where the distal Pit-1 site and the GCRR are maintained, and tested for CORT responsiveness. Again, this construct was not CORT-responsive. Transfer of -1045 to -382 of the chicken GH promoter failed to transfer CORT responsiveness to the TSHβ promoter, suggesting that additional sequences of the GH gene are necessary for glucocorticoid regulation of the GH gene during chicken embryonic development. This also suggests that the underlying mechanism of glucocorticoid induction of the GH gene is not shared by the TSHβ gene, even though they both require Pit-1 for activation. Nonetheless, the GCRR was found to be orientation independent and context dependent in mediating glucocorticoid induction of the GH gene during chicken embryonic development.

Incubation of nuclear proteins with a probe corresponding to the proximal GCRR resulted in an upward shift of the probe and inclusion of unlabeled competitor DNA demonstrated that the shift was specific. This suggests that both the 5' and 3' competitors

interacted with protein(s) in the nuclear extract, but binding of the protein to the GCRR occurred along the entire length. This also demonstrates that the observed shift is a result from specific protein binding and not non-specific interference. Glucocorticoid induction of the GH gene during embryonic development involves recruitment of nuclear proteins to the GCRR.

There was a significant increase in binding to the GCRR of nuclear proteins from the CORT-treated cells. This leads to two possibilities: there is increased recruitment of a specific set of proteins from CORT-treated cells or there is recruitment of a different set of proteins from CORT-treated cells compared to non-treated cells. These two possibilities could be tested by co-immunoprecipitation. This would allow for the identification of proteins that interact with either GR or ETS-1. It is already known that GR interacts with a myriad of other proteins in almost all known mechanisms. Another interesting possibility would be to include nuclear extracts from cells that were pretreated with CHX and then treated with CORT. Differences in intensities or molecular weights of the shifted bands could be further explored. Regardless, glucocorticoid regulation of the GH gene during chicken embryonic development involves increased recruitment of proteins to the GCRR.

The centered competitor corresponding to the central 34 bp of the GCRR probe effectively competed off nuclear protein binding. The GCRR probe was also mutated in the center 34 bp, and this significantly decreased protein binding. This portion of the GCRR contains putative binding sites for ETS-1 and GR. The flanking regions of the mutated probe were conserved. The 5’ flanking region contains a putative binding site for E47/CTCF, while the 3’ flanking region contains a putative binding site for

RUSH/SMARCA3. These two proteins could be involved in glucocorticoid regulation of the GH gene during chicken embryonic development. CCCTC-binding factor (CTCF) is a zinc finger protein with almost 100% conservation of the central DNA-binding domain between mouse, chicken, and human. CTCF has been implicated as a transcriptional repressor, activator, and insulator (Phillips & Corces 2009). CTCF's interaction with TR is well characterized (Arnold et al., 1996 & Lutz et al., 2003). CTCF is also known to interact with ER and PR (Chan & Song 2008; D'Arcy et al., 2008) However, to date, there is no evidence that CTCF interacts with GR. ER and GR have similar response elements. TR and GR do not. There is also no evidence that CTCF interacts with ETS-1. The GCRR contains a putative binding site for RUSH/SMARCA3 at its 3' end, in addition to sites for GR and ETS-1. In this study, the chicken GH promoter was responsive to CORT and to a lesser extent progesterone. CORT induction of the chicken GH gene also required the synthesis of an intermediary protein or ongoing protein synthesis. The RUSH/SMARCA3 gene was induced by progesterone in rabbit uterine epithelial cells (Chilton & Hewetson, 2008). The RUSH/SMARCA3 gene was regulated by RUSHα and Sp1. RUSH/SMARCA3 was capable of specific sequence DNA binding and is responsible for ATP-dependent DNA unwinding. Chromosome conformation capture showed that RUSHα interacted with Sp1 to facilitate long range DNA looping necessary for gene activation. It is possible that the putative RUSH/SMARCA3 site in the GCRR in the chicken GH promoter is functional and necessary for DNA unwinding and looping for activation of the chicken GH gene. Additional studies are warranted to implicate RUSH/SMARCA3 in glucocorticoid regulation of the GH gene during chicken embryonic development.

In the -1045/+48 construct, the GREF site was mutated and the CORT-response of the -1045/+48 GH construct was abolished. GREs seem to be degenerate. Two recent studies have been conducted demonstrating functional GR binding to a degenerate GRE and not a full-length classical GRE. The GREF identified in the GCRR, although not a classical GRE, can be classified as a glucocorticoid binding region, GBR. The GBR was identified by performing a ChIP-chip with GR (So et al., 2007). The conserved GBR identified is RGNACARRRWGTNCN, where R is a purine, N is any nucleotide, and W is an A or a T. This exact same sequence was identified on the reverse strand of the GCRR. Only chromatin immunoprecipitation or a positive super-shift experiment could definitively implicate GR binding to this site. A glucocorticoid receptor binding sequence (GBS) was found in the dual specificity phosphatase 1 gene promoter (DUSP1) and this sequence seems to be conserved across a wide range of species, such as armadillo, horse, cow, mouse, rat, dog and human (Tchen et al., 2010). A GBS was defined to be composed of the sequence GNACANNNNG. The GREF found in the GCRR of the chicken GH gene promoter also contains the sequence GNACANNNNG. There was also a good correlation between the glucocorticoid activation of the DUSP1 gene and the recruitment of GR to the GBS as demonstrated using ChIP (Tchen et al., 2010). The present study shows that the GREF site in the GCRR is essential to glucocorticoid regulation of the GH gene during chicken embryonic development.

In the -1045/+48 construct, the ETS-1 site was mutated, and this mutation rendered the -1045/+48 GH construct unresponsive to CORT. The ETS family of transcription factors consists of ten family members that bind to a core sequence of GGAA. Five family members bind to the core sequence of CCGGAA (Thompson et al.,

1994). It was also shown that functional redundant binding sites for all ETS-1 family members were more likely to occur close to the transcription start site of housekeeping type genes, while more specific, non-redundant functional binding sites for individual ETS-1 family members occurred further away from the transcription start site of more specialized genes (Hollenhorst et al., 2007). It is possible that an ETS-1 family member binds to the putative ETS-1 site in the GCRR of the chicken GH gene. ETS-1 has been implicated as the other required factor in the regulation of transcription of the prolactin gene in the rat (Bradford et al., 1997). Pit-1 is necessary, but not sufficient to direct GH expression in the pituitary in the rat, chicken, mouse and human. Pit-1 is also necessary, but not sufficient to direct prolactin expression in the pituitary in the rat. ETS-1 physically interacts with Pit-1 at a composite *cis* element to direct prolactin expression. The phosphorylation state of Pit-1 regulates its interaction with ETS-1 (Augustijn et al., 2002). Further, binding of Pit-1 and ETS-1 to the composite site in the proximal promoter of the prolactin gene is necessary for the Ras-MAPK activation of the prolactin promoter (Duval et al., 2003). ETS-1 has been to be involved in the regulation of the human GH gene. AP-1 and the ETS family member, Elk-1, were found to bind in the HS III and HS IV sites, respectively, of the human GH gene LCR (Jin et al., 2004). Binding of these two transcription factors was associated with hyperacetylation of pituitary chromatin. This was the first study to implicate ETS-1 or an ETS family member in GH induction. In a follow-up study, it was shown that Pit-1 binds to HS I and HS II of the hGH-N LCR, but it cannot bind to HS III alone (Yang et al., 2010). For this, it requires interaction with ETS-1 via Pit-1's POU homeodomain and a composite DNA binding element. ETS-1 and another ETS-1 family member, Elk-1, can be co-immunoprecipitated from human

pituitary extracts with a Pit-1 antibody, and overexpression of Elk-1 or Pit-1 alone or together could increase hGH-N expression in HEK293 cells (Yang et al., 2010). This is the first study demonstrating that Elk-1 could induce the GH gene. Elk-1 is an immediate downstream target of ERK. Pharmacological inhibition of ERK signaling blocks CORT induction of the chicken GH gene (unpublished data). Elk-1 belongs to the ternary complex family (TCF), a subfamily of the Ets family (Buchwalter et al., 2004). Members are downstream targets of the ras-Raf-MAPK kinase signaling pathway. These two studies implicate ETS-1 in the regulation of the hGH-N gene. This potentially implicates the ETS-1 binding site in the GCRR as a necessary and essential element for glucocorticoid regulation of the GH gene during chicken embryonic development.

The ETS-1 site and GREF site in the GCRR of the chicken GH gene are separated by only 17 bp. This begs the question, do ETS-1 and GR physically interact? The rat tyrosine aminotransferase (TAT) gene is induced by glucocorticoids, and it was also found that ETS-1 participates in this response (Espinas et al., 1994). An ETS-1 binding site is located in close proximity to the GR binding site. Inactivation of the ETS-1 binding site results in a two-fold decrease of the TAT gene to GCs. The ETS-1 site is occupied in a GC-independent manner. Combinatorial transcription factor binding sites may allow for the integration of signaling from multiple external stimuli. The promoter of the cytochrome P450 c27 multifunctional enzyme is dexamethasone responsive. The promoter contains a functional GR binding site and an ETS-like site that binds ETS-2 in a GR-dependent manner that appears to synergistically activate the gene (Mullick et al., 2001). GR and ETS-2 were able to co-immunoprecipitate together. The DNA binding domain of GR appeared to mediate the synergistic effect, while the DNA binding domain

of ETS-2 appeared to be a dominant negative. A fusion protein consisting of only the GR DNA binding domain and the ETS-2 transcriptional activation domain recapitulated the activation of the P450 c27 promoter, suggesting a novel synergy between these two proteins (Mullick et al., 2001). In the P450 c27 promoter, the GR binding site is flanked by two ETS like binding sites and a CTCF binding site. The sites span a region of 50 bp; the ETS-1 and GREF putative sites in the chicken GH promoter span a region of 34 bp. Additional studies are necessary to determine if GR and ETS-1 physically interact during CORT induction of the chicken GH gene.

Chromatin immunoprecipitation showed that ETS-1 is associated with the GCRR under basal and 1.5 h CORT-treated conditions. GR is also associated with the GCRR and is recruited to the proximal GCRR after 1.5 h CORT treatment. After 6 h CORT treatment, neither ETS-1 nor GR was associated with the GCRR. Neither of these proteins was associated with the distal GCRR. Chromatin immunoprecipitation is an elegant method to show what proteins are associated with a particular gene region under a set of conditions. However, the method is only successful when the protein and gene region of interest are known. The findings from chromatin immunoprecipitation agree with our previous results. CORT induced GH mRNA after 8 h of treatment, but not after 4 h (Bossis & Porter, 2003). More recent findings from our lab show that CORT treatment induces GH mRNA after 1.5 h, 3 h and 6 h. GH mRNA induction stays high until 24 h (unpublished data). The increase of GR association with the GCRR after only 1.5 h is supported by these findings. In the present study, it was found that ETS-1 was associated with the GCRR under basal conditions as well as after CORT treatment. ETS-1 is a transcription factor that contains a nuclear localization signal (NLS) and a nuclear

export signal (NES) (Boulokos et al., 1989). The protein contains a serine rich region (SRR) that modulates the flexibility of the DNA binding domain (Lee et al., 2008). The affinity for ETS-1 to bind to DNA is regulated by calcium-dependent phosphorylation (Pufall et al., 2005). In the present study, chromatin immunoprecipitation revealed that ETS-1 was associated with the GCRR and CORT treatment recruits GR to the GCRR after 1.5 h. This is the first study to demonstrate that GR and ETS-1 are associated with the promoter of the GH gene during embryonic development in the chicken.

E11 CEP cells were treated with CORT for 1.5 h and 6 h and fixed for later chromatin immunoprecipitation analysis. Likewise, e11 CEP cells were treated with CORT for 6 h and nuclear proteins were extracted for immediate use in EMSA. The 6 h chromatin immunoprecipitation data and the 6 h EMSA data do not agree. Results from EMSA show that protein binding to the proximal GCRR probe is increased after 6 h. Results from ChIP show that GR and ETS-1 association with the proximal GCRR is decreased after 6 h. EMSA is a technique, with which a free DNA probe is incubated with extracted proteins in a cell free system under optimized binding conditions. ChIP is a technique, with which endogenous proteins are crosslinked to the endogenous gene and the sample is probed with antibodies followed by PCR with primers specific to a genomic region to acquire a "snapshot" of the endogenous gene. There are inherent differences between EMSA and ChIP methodology, including the questions that the methods are able to answer. It is possible that another protein or protein complex is necessary to maintain association of ETS-1 and GR with the GCRR during glucocorticoid regulation of the GH gene. Further, glucocorticoid regulation of the GH gene is indirect: inclusion of CHX blocks CORT induction of GH mRNA. It is not known what CHX is acting on. It is

possible that ETS-1 and GR associate with the GCRR of the GH gene and their association is dependent on another protein that is synthesized at a later time in response to another signaling pathway. In our lab, a microarray study was conducted identifying genes that were regulated by glucocorticoids in the presence and absence of CHX (unpublished data). Inspection of the list of genes generated from this microarray may reveal another associated protein. Regardless, the ChIP results do agree with the mutant luciferase construct results. GR and ETS-1, or an ETS family member, are recruited to the proximal GCRR and the sites are necessary for glucocorticoid regulation of the GH gene during chicken embryonic development. Nonetheless, both EMSA and ChIP implicate binding of GR and ETS-1, or an ETS family member, to the GCRR of the GH gene in chickens.

Based on the cumulative results from the EMSA and ChIP studies, it is possible that GR and ETS-1 are recruited to specific sequences located in the GCRR of the endogenous GH gene. Once recruited, GR and ETS-1 then initiate recruitment of nucleosome remodeling enzymes and basal transcriptional machinery including RNA polymerase II. This process most likely involves cofactors and accessory proteins to form a bridging complex. This may also involve DNA looping to allow for the GCRR and its associated proteins to come into close proximity of the transcription start site. It most likely also requires the unwinding of nucleasomes and the formation of an “open” chromatin conformation that is conducive to a high rate of gene transcription. Once gene transcription has moved beyond the initiation phase and into the elongation phase, it is possible that the bridging complex, consisting of GR, ETS-1 and cofactors, falls away

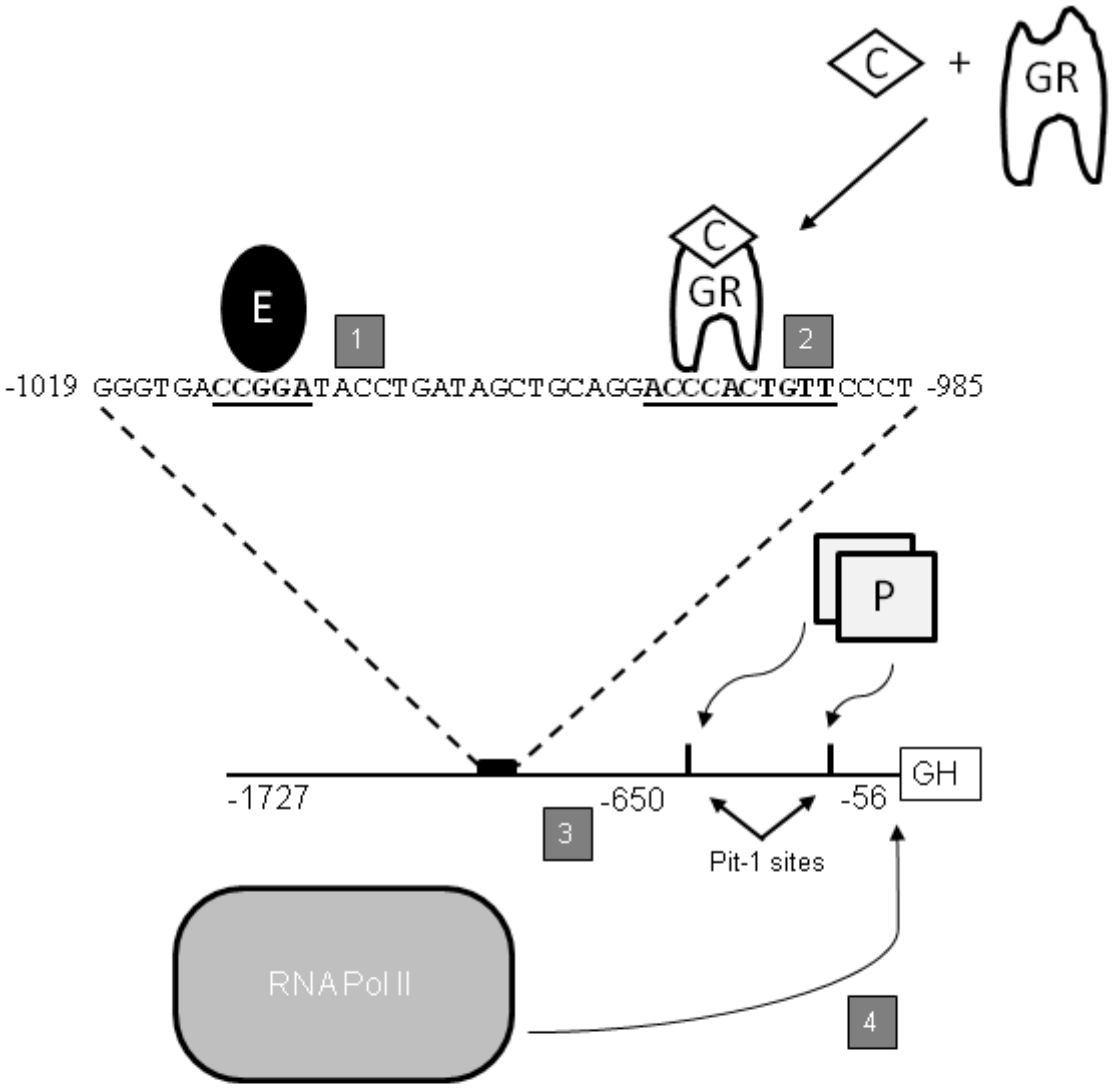
from the gene and maximum transcription can ensue. It is known that GH transcription is maximal at 6 h after CORT treatment.

This project began with two hypotheses: 1) the product of an unknown glucocorticoid-responsive gene induces the GH gene; 2) ligand bound GR and an unknown protein bind to an unknown composite element in the 5' flanking region of the GH gene and induce the GH gene together. The findings of the current study did not outright reject either of the hypotheses, but it did identify two proteins, GR and ETS-1, that are involved in glucocorticoid regulation of the GH gene. We identified a functional degenerate GRE upstream of the GH gene in the chicken. We identified a functional ETS-1 site upstream of the GH gene in the chicken. We showed that each of these proteins bind to their own response element and not a composite element. We showed that ETS-1 is not a glucocorticoid regulated gene. However, it is not known whether ETS-1 is phosphorylated in response to glucocorticoids. Further, it is unknown whether bridging complexes or accessory factors are necessary for glucocorticoid induction of the GH gene. We also do not know the target of CHX. CHX inhibits protein synthesis and we do not know if glucocorticoid induction of the GH gene is dependent on a protein with a high turnover rate. This hypothesis is supported by the CHX data.

Glucocorticoids play an important role in the maturation of the lungs, intestine and pituitary. In each of these systems, GR, although an inducing factor, does not act alone. In the lung, glucocorticoids induce surfactant protein with the aid of Hsp90 and p23 (Grad et al., 2006). The present study shows that GR and ETS-1 act together to induce the GH gene. The intestine is similar to the pituitary in that many different cell types secrete specialized enzymes necessary for digestion. Glucocorticoids are necessary

for the differentiation of these cell types (Lebenthal & Lebenthal, 1999). Clearly, glucocorticoids are necessary for life because of its role in the development of many organs.

Collectively, these results suggest that ETS-1 or an ETS family member is associated with its response element located at -1014 to -1009 upstream from the chicken GH gene under basal conditions and CORT treated conditions. GR is also associated with its response element located at -995 to -985, and upon CORT treatment, GR recruitment is increased. Both *cis*-acting elements are required for glucocorticoid induction of the GH gene during chicken embryonic development. Additional elements contained in the -650 to +48 region, such as Pit-1, are necessary for glucocorticoid regulation of the GH gene during chicken embryonic development. Finally, upon CORT treatment, RNA polymerase II (RNA Pol II) is recruited to the transcription start site located at -56 bp. These findings are summarized in a model (Fig. 22). In conclusion, glucocorticoid regulation of the GH gene during chicken embryonic development requires *cis*-acting elements located between -1018 and -985 bp upstream from the transcription start site and the *trans*-activating factors likely include GR and belong to the ETS family.



1. An ETS family member (E) is associated with its response element in the proximal GCRR under basal and 1.5 h CORT treatment conditions.
2. GR association with its response element in the GCRR is increased after 1.5 h CORT treatment.
3. The Pit-1 sites or other *cis*-acting elements contained in the region from -650 to +48 are required for CORT induction of the GH gene.
4. RNA Polymerase II (RNA Pol II) is recruited to -56 of the GH gene.
5. Both the ETS family member and GR are no longer associated with the proximal GCRR after 6 h CORT treatment.

Figure 22: Proposed Model of the Glucocorticoid Regulation of the GH Gene during Chicken Embryonic Development.

Chapter 3: Conclusion & Future Directions

Summary

A region spanning -1727/+48 of the chicken GH gene was cloned into a luciferase reporter construct. This GH construct was responsive to CORT, and inclusion of CHX blocked the CORT induction of luciferase mRNA. The transcription start site of the endogenous GH gene was found to be 56 bp upstream from the start codon. These findings, taken together, indicate that the -1727/+48 GH construct would serve as an appropriate model for future studies of glucocorticoid regulation of the GH gene during chicken embryonic development. Through deletion analysis of the GH construct, a non-classical glucocorticoid responsive region (GCRR) of the chicken GH gene was identified and characterized. The GCRR was 90 base-pairs long and occurs 1 kb upstream of the transcriptional start site. The GCRR contained putative binding sites for ETS-1, GREF, CTCF and RUSH/SMARCA3. The GCRR was found to be orientation-independent, but context-dependent. However, the GCRR cannot confer glucocorticoid responsiveness to the TSHβ promoter in a luciferase assay. The GCRR was able to bind nuclear proteins in an EMSA, as opposed to a probe that corresponds to exon 3 or a distal portion of the GH gene (-1566 to -1467), and in a CORT-regulated manner. The observed shift was competed off with a centered competitor that spans the ETS-1 and GREF sites. Use of a mutated probe, where the central portion of the probe spanning the putative ETS-1 and GREF sites was mutated, resulted in significantly reduced binding. Mutation of the putative ETS-1 site or the GREF site in the -1045/+48 GH construct resulted in

loss of CORT-responsiveness. Through chromatin immunoprecipitation, it was found that ETS-1 was associated with the GCRR in the endogenous GH gene under both basal and CORT treated conditions and GR is recruited to the GCRR after 90 m of CORT treatment. But both of these factors were no longer associated with the endogenous GH gene after 6 h. Collectively, these studies demonstrate that glucocorticoid induction of the GH gene during chicken embryonic development involves GR and ETS-1 binding to a response element approximately 1 kb upstream from the start codon.

Future Directions

The present study has identified *cis*-acting elements required for glucocorticoid regulation of the GH gene during chicken embryonic development. This study has not positively implicated a *trans*-activating factor necessary for the glucocorticoid regulation. Potential candidates include members of the ETS-1 family. Chromatin immunoprecipitation has shown that both ETS-1 and GR are recruited to the GCRR in e11 pituitary cells. Successful knockdown of ETS-1 or an ETS-1 family member would definitively implicate the trans-acting factor in glucocorticoid regulation of the GH gene during chicken embryonic development. This could be accomplished using several techniques.

One way to determine the function of a gene is to investigate the molecular and physiological effects when the gene has been inactivated or "knocked down". This can be accomplished through the use of small interfering RNAs or siRNA (Elbashir et al., 2001). siRNA has been routinely used for the past decade. RNA that is 20 to 22 bp in length is engineered to be specific to the mRNA of interest. The cells are transfected with the siRNA. The siRNA binds to its complementary mRNA. This signals a degradation

pathway to the cell and the target mRNA is degraded. It is typical to use a set of 3 to 5 slightly different siRNAs to accomplish a knockdown of 50% or more. Knockdown of ETS-1 in e11 pituitary cells could be accomplished using commercially available siRNAs and then using FACS for GFP to sort only the transfected cells. Then, GH mRNA could be measured in the sorted cells in the context of knockdown of ETS-1. In our model system, cell sorting is necessary because the transfection efficiency of e11 chicken embryonic pituitary cells is prohibitively low. The transfection efficiency of cell lines is typically in the range of 75% to 95%. In our lab, the observed transfection efficiency of chicken embryonic pituitary cells using a GFP expression plasmid and FACS was in the range of 5 to 20% (Ellestad et al., 2009). A recent study demonstrated that the cellular uptake of siRNA could be enhanced by using cell penetrating peptides (Meade & Dowdy, 2008). The cell membrane is largely impenetrable. This presents an obstacle to effectively delivering siRNA into the cell. Cell penetrating peptides are a class of small cationic peptides 10 to 30 amino acids in length. These charged peptides interact with the anionic cell membrane, are endocytosed and then escape the vesicles into the cytoplasm through an unknown mechanism (Meade & Dowdy, 2008). Packaging of siRNA molecules with the cell penetrating peptides markedly increased the delivery. Knockdown via siRNA could also be accomplished by infecting e11 chicken embryonic pituitary cells with siRNA that is driven by a replication competent virus. Evaluation of the effects of knockdown of ETS-1 or another candidate on the glucocorticoid regulation of the GH gene during chicken embryonic development using siRNA would require cell sorting of transfected cells or infection of the siRNA by a replication competent virus. Conditionally replicating adenoviruses can be engineered to encode short-hairpin RNAs

to knock-down a gene target (Carette et al., 2004). Short hairpin RNAs require a cellular processing step, but act via a similar mechanism as siRNA to knockdown its gene target (Paddison et al., 2002). Conditionally replicating viruses encoding shRNAs allow for a more efficient delivery of the interfering RNA. This approach could circumvent our problem of low transfection efficiency of primary chicken embryonic pituitary cells. Definitive knockdown of ETS-1 could positively implicate ETS-1 as a necessary factor in glucocorticoid regulation of the GH gene during embryonic development in the chicken.

RNA interference is typically used *in vitro* at the cell culture or tissue culture level. To explore the effects of the knockdown of ETS-1 or another candidate involved in glucocorticoid regulation of the GH gene at the organism or whole body level, a transgenic chicken could be engineered. Two approaches using transgenics could be attempted: pituitary-specific deletion of the ETS-1 gene or another suitable candidate or mutation of the GCRR in the GH gene in the pituitary only. Multiple studies have been conducted systematically knocking out members of the ETS family in mice (Bartel et al., 2000). Some ETS family members are necessary for life, as evidenced with the PU.1 knockout and its role in hematopoiesis. The PU.1 knockout is embryonic lethal (Scott et al., 1994) or post-natal lethal (McKercher et al., 1996). But two different ETS-1 knockout mouse models were viable, fertile and exhibited 50% neonatal lethality (Barton et al., 2000) or viable and fertile (Bories et al., 1995). The ETS-1 knockout mouse models had defects in T cell and B cell maturation and responsivity. To date, ETS-1 has not been knocked out specifically in the pituitary. Recent advances have allowed for the engineering of transgenic chickens. An attempt to make a transgenic chicken using a lenti-viral vector targeted to the germ line proved to be quite successful (McGrew et al.,

2004). Ten founder cockerels were able to transmit the lenti-viral vector to 4% to 45% of their offspring. The study also demonstrated successful transmission to the G_2 generation. For the past 20 years, groups have been attempting to make transgenic chickens with varying success. Previous attempts at making a transgenic chicken using avian retro-viruses, replication-competent vectors derived from avian leucosis virus (ALV) resulted in an extremely low transfer frequency: 1% of hatched males were germline transgenic (Salter & Crittendon, 1989, Bosselman et al., 1989, Rapp et al., 2003). The most appealing aspect of the use of lentiviral retroviruses is the fact that they can infect non-dividing cells (Naldini et al., 1996). It is also possible to achieve tissue-specific knock-outs in mammals using the lenti-viral system (Lois et al., 2002). Engineering of a lenti-viral vector for knock-out of ETS-1 in only the pituitary may be possible in the future. This would allow for the investigation of ETS-1 in glucocorticoid regulation of the GH gene during anterior pituitary gland differentiation in the embryonic chicken. Additionally, it might be advantageous to engineer the chicken GH gene to contain a mutated GCRR in the 5' flanking region and explore the ramifications. This would circumvent the necessity for positively identifying the correct trans-activating factor prior to exploring the mutant phenotype. The present study has made great strides in the characterization of glucocorticoid regulation of the GH gene during embryonic development in the chicken, and future studies using more sophisticated methods, such as siRNA and transgenics, in an attempt to knockdown candidate trans-activating factors or alter the GH gene may provide greater insight into glucocorticoid regulation of the GH gene.

Conclusion

Glucocorticoid regulation of the GH gene during chicken embryonic development requires on-going protein synthesis or an intermediary protein. Positive identification of the intermediary protein may be aided by identifying glucocorticoid-regulated *cis*-acting elements. A *cis*-acting element located 1 kb upstream from the transcription start site at -1045 to -954 was found to be necessary in CORT induction of the GH gene during chicken embryonic development. The *cis*-acting element or GCRR contains putative binding sites for ETS-1 and GREF. ETS-1 was associated with the GCRR, and GR was recruited to the GCRR upon glucocorticoid treatment. We conclude that GR and ETS-1 or an ETS family member bind to the GCRR in a glucocorticoid-regulated manner as part of the mechanism underlying glucocorticoid induction of GH gene expression during chicken embryonic development.

Appendix A:

The -1467/+48 FireflyLluciferase Plasmid Is Not Responsive to Other Treatments.

The -1467/+48 GH construct was not CORT-responsive, and a probe corresponding to -1566 to -1467 of the GH gene did not bind nuclear proteins in a gel shift assay. Additional experiments were conducted to investigate why the -1467/+48 GH construct is not CORT-responsive.

Using a transcription factor search engine, a Sp1 site was identified at -1472 to -1467 of the chicken GH gene. WP-631, an inhibitor of Sp1, was used to determine if this site was functional (Appendix A, Fig. 1A). E11 pituitary cells were transfected with the -1496/+48, -1467/+48, -1398/+48 or Basic plasmids, allowed to recover overnight and then treated with vehicle or WP-631 for 1 h. CORT (100 nM) was added to appropriate wells, and then the cells were lysed for luciferase activity 24 h later. The -1496/+48 and -1398/+48 were again CORT-responsive and the -1467/+48 plasmid remained unresponsive. Pre-treatment with the Sp-1 inhibitor, WP-631, had no effect on the CORT-responsiveness and did not affect the basal activity of the -1467/+48 plasmid.

CORT induction of the GH gene can also be blocked by inclusion of histone deacetylase inhibitors, such as trichostatin A (TSA) and HC toxin (HCT) (unpublished results). Therefore, e11 pituitary cells were transfected with either the -1496/+48, -1477/+48, -1467/+48, -1430/+48, -382/+48 or Basic plasmids. The cells were pretreated with TSA or HCT for 90 m before the addition of CORT to appropriate wells (Appendix A, Fig. 1B). The cells were lysed 24 h later and luciferase activity was determined. The -1496/+48, -1477/+48, and -1430/+48 plasmids were CORT responsive. The -1467/+48 and -382/+48 plasmids were not CORT responsive. Treatment with the HDAC inhibitors

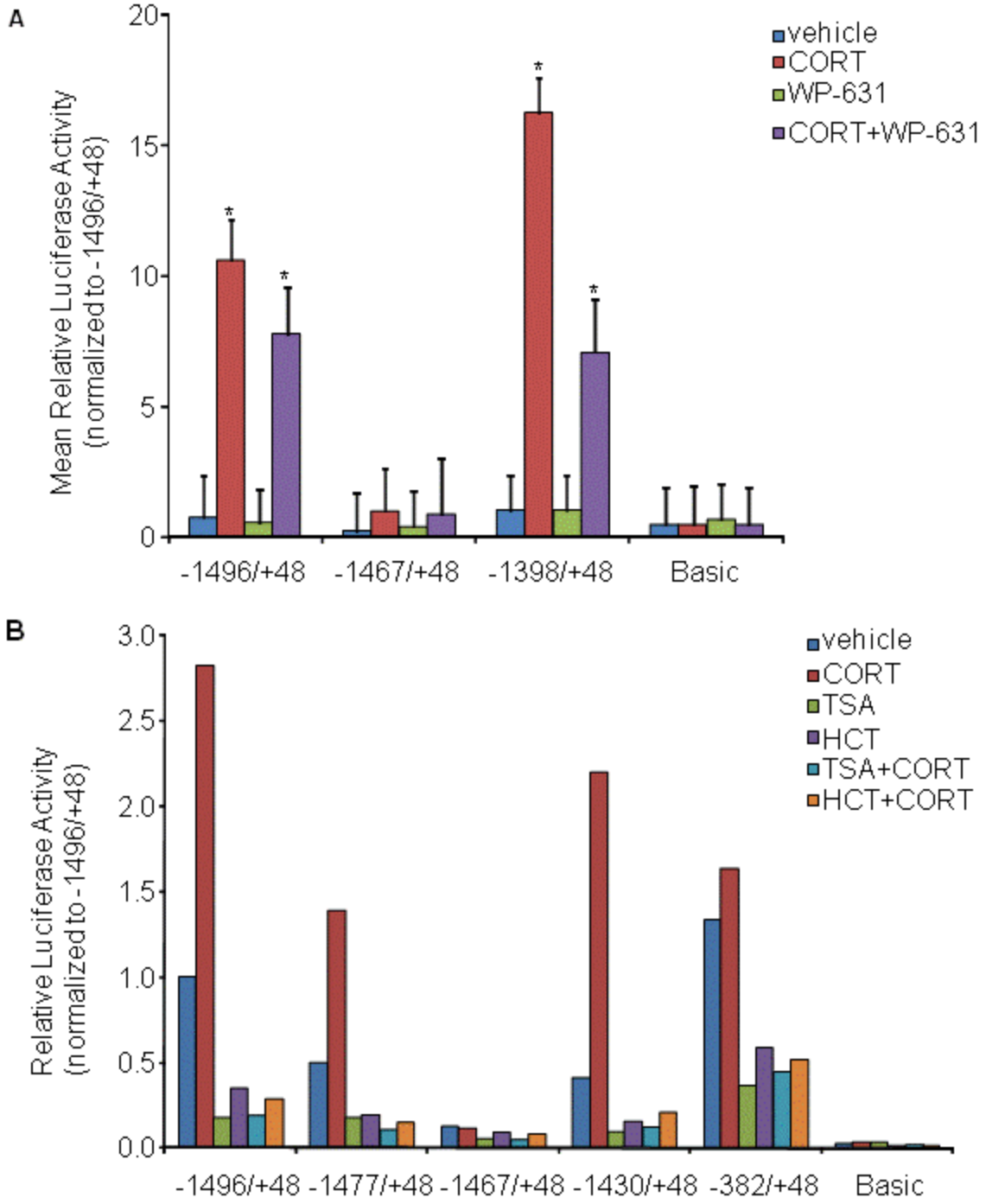


Figure 1: A) Transfected cells were pretreated with the Sp1 inhibitor, WP-631, for 1 h before the addition of CORT (100 nM). The cells were assayed for Luciferase activity 24 h after the completion of transfection. *, denotes significant difference from vehicle (n=3; $p<0.05$). B) Transfected cells were pretreated with Trichostatin A (TSA) or HC Toxin (HCT) alone or in combination for 90 m before CORT (100 nM) addition. The cells were assayed for Luciferase activity 24 h after the completion of transfection (n=1).

resulted in a global repression of luciferase activity of all of the plasmids. The plasmids remained non-responsive to CORT in the presence of the HDAC inhibitors. This experiment warrants replication.

The -1467/+48 plasmid contains a putative CREB (cAMP-responsive element binding protein) site; it is possible that cAMP-regulated pathways need to be activated first in order for the -1467/+48 plasmid to be CORT responsive (Appendix A, Fig. 2A). E11 pituitary cells were transfected with either the -1496/+48, -1467/+48, -1430/+48 or Basic plasmids. The next day, the transfected cells were pretreated with IBMX and forskolin (FSK) for 1 h before the addition of CORT. The cells were lysed for determination of luciferase activity 24 h later. CORT induced the -1496/+48 and -1430/+48 plasmids, regardless of IBMX and FSK pretreatment. IBMX and FSK treatment alone induced a 2-fold increase in luciferase activity from the -1430/+48 plasmid. The -1467/+48 plasmid remained unresponsive to all treatments.

The CORT induction of the GH gene also involves kinase signaling pathways, such as Ras and MAPK (Bossis et al., 2003 & unpublished results). Therefore, e11 pituitary cells were transfected with either the Basic or the -1467/+48 plasmids and then pre-treated with the pathway inhibitors: U00126, PD098059 or manumycin for 90 m prior to CORT treatment (Appendix A, Fig. 2B). Neither the -1467/+48 or Basic plasmids were CORT responsive and the pathway inhibitors had no effect.

Additionally, an experiment was conducted where both circular plasmids and linearized constructs were transfected into e11 pituitary cells and tested for luciferase activity (Appendix A, Fig. 3A). Again, the -1727/+48 plasmid was CORT responsive,

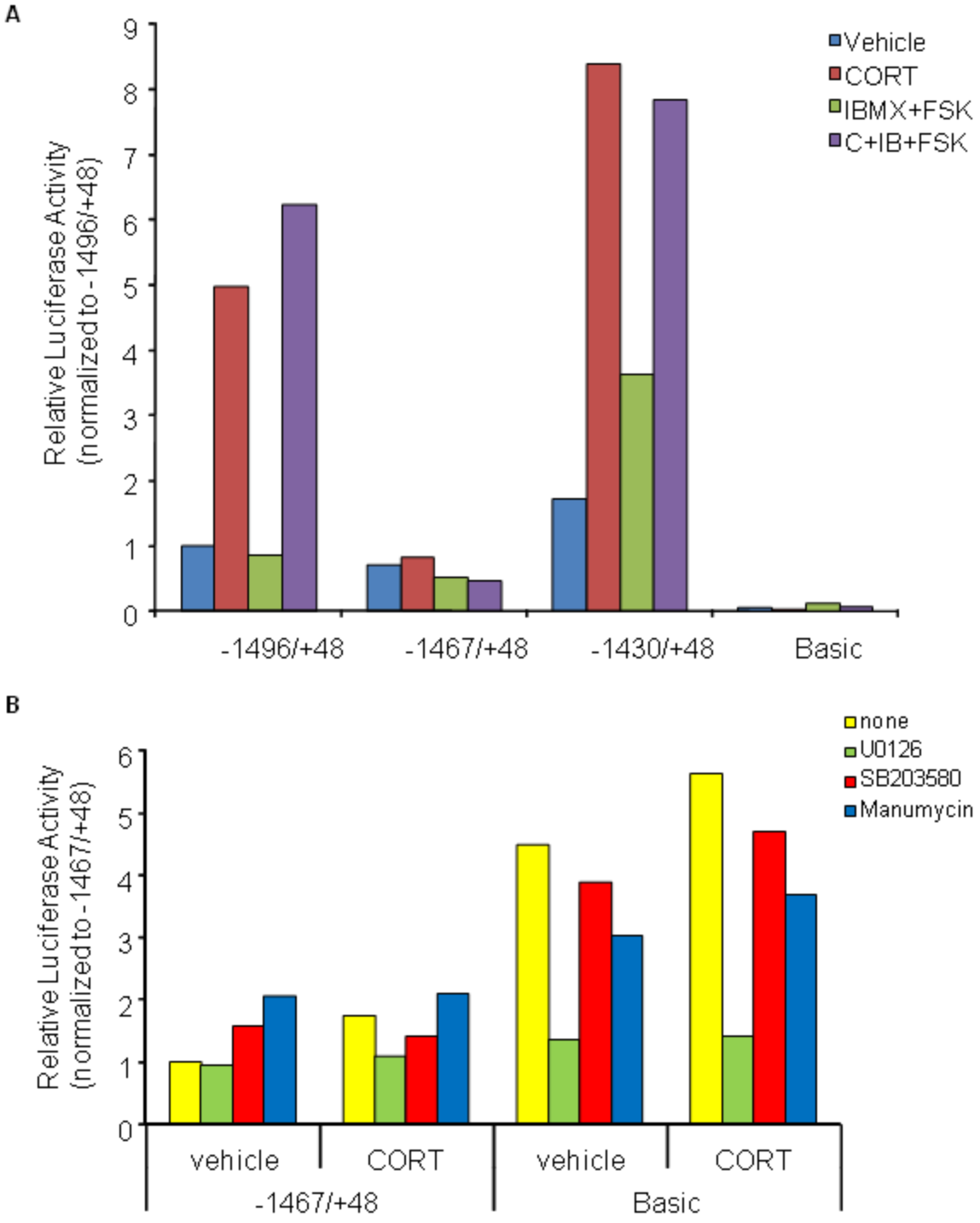


Figure 2: A) Transfected cells (-1496/+48, -1467/+48, -1430/+48 or Basic) were pretreated with IBMX and Forskolin (FSK) alone and in combination for 1 h prior to CORT (100 nM) addition. Cells were lysed 24 h later for Luciferase activity (n=1). B) Transfected cells (-1467/+48 or Basic) were pretreated with the pathway inhibitors for 90 m prior to CORT (100 nM) addition. Cells were lysed 24 h later and assayed for Luciferase activity (n=1).

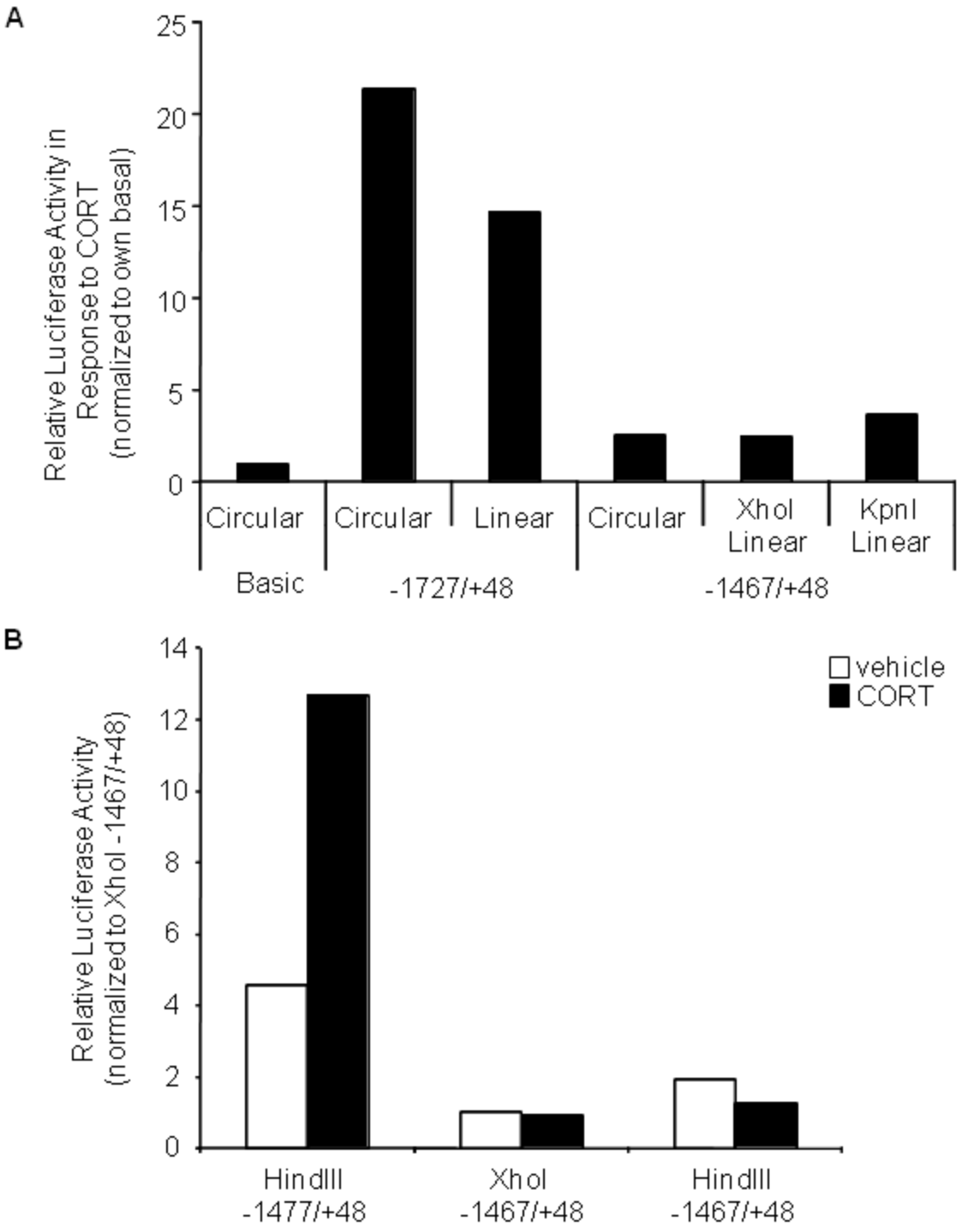


Figure 3: A) Cells transfected with either circular or linearized (linearized by 2 different restriction enzymes) plasmid. The cells were assayed for CORT activity 24 h after the completion of transfection (n=1). B) The -1467/+48 insert was cloned into the Luciferase vector using different restriction enzyme sites.(n=1).

regardless of the configuration of the plasmid. However, the -1467/+48 remained unresponsive to CORT, regardless of the configuration of the plasmid.

Originally, the -1727/+48 and the -1467/+48 constructs were cloned directionally with an XhoI site at the 5' end. All additional constructs were cloned non-directionally using HindIII sites at each end. Therefore, it is possible that the restriction site used in cloning introduced an artifact that interfered with the luciferase activity. The -1467/+48 plasmid was re-cloned non-directionally using the HindIII site (Appendix A, Fig. 3B). However, this did not restore CORT responsiveness.

Attempts at characterizing the -1467/+48 GH construct were unsuccessful. Therefore, one additional deletion construct was made, -1462/+48 and tested in e11 chicken embryonic pituitary cells (Appendix A, Fig 4). All constructs were responsive to CORT treatment except for the -1467/+48 and the -1462/+48 constructs. This suggests that the lack of a CORT response by the -1467/+48 construct may be due to the presence of a repressor element that only functions when elements further upstream are deleted.

In summary, the -1467/+48 GH construct is not responsive to CORT, kinase pathway inhibitors, or cAMP-pathway inhibitors. Inclusion of an inhibitor to Sp1 did not block the CORT response of the -1477/+48 GH construct and did not alter the non-activity of the -1467/+48 GH constructs. Changing the restriction enzyme site for cloning or using a linearized plasmid also did not have an effect. One additional construct, -1462/+48, was made and this, too, was not CORT-responsive. These results suggest that the lack of a CORT response by the -1467/+48 GH construct is due to the presence of a repressor element or a biologically relevant phenomenon.

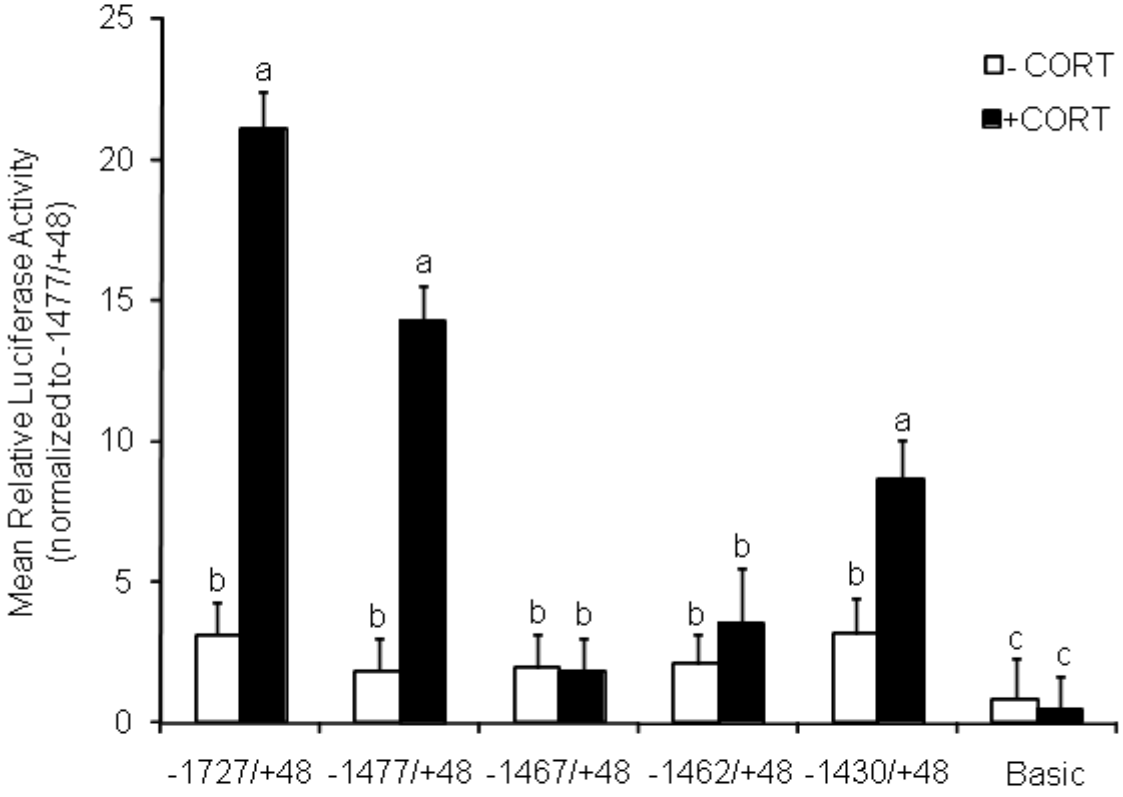


Figure 4: Mean Relative Luciferase Activity in response to CORT. Values are normalized to -1477/+48. One additional construct was made, -1462/+48 to test the CORT response. Values denoted by different letters are significantly different (n=3; $p<0.05$).

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