

## ABSTRACT

Title of Document:                   ROLE OF EPIGENETIC MODIFICATIONS  
AND DEXRAS1 IN GLUCOCORTICOID  
REGULATION OF GROWTH HORMONE  
EXPRESSION

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Glucocorticoid induction of growth hormone in anterior pituitary cells of embryonic chickens is an indirect effect. A genome-wide microarray screen conducted on embryonic day 11 chickens identified Dexas1 as a novel candidate gene involved in mediating the glucocorticoid effects on GH mRNA expression. The following studies were aimed at characterizing glucocorticoid regulation of chicken Dexas1 and identifying potential involvement of Dexas1 in mediating glucocorticoid effects on GH expression. We determined that glucocorticoid induction of Dexas1 requires glucocorticoid receptor and both MEK1/2 and Ras cell signalling pathways. Overexpression of Dexas1 vector had no effect on GH reporter activity. Transfection of 10ng or 100ng of Dexas1 expression plasmid stimulated basal GH mRNA levels,

whereas transfection of 1000ng of Dexras1 expression plasmid significantly inhibited levels of glucocorticoid-induced GH mRNA. YFP-tagged Dexras1 protein was predominantly found in the cytoplasm and glucocorticoid treatment did not cause Dexras1 to translocate to the plasma membrane. Downstream targets of Dexras1 are not known. Additionally, glucocorticoid-regulated histone modifications within 2kb of the GH gene were investigated using chromatin immunoprecipitation assays. The effects of glucocorticoid (1.5 h or 6 h) on histone H3 modifications, RNA Polymerase II (Pol II) recruitment, and association of GR, Pit-1, and Ets1 with the chicken GH gene were examined. We found increased H3 acetylation and tri-methylation of lysine 4 at both Pit-1 sites and the transcription start site (TSS) in response to 1.5 h glucocorticoid treatment. Furthermore, 1.5 h glucocorticoid treatment significantly increased recruitment of Pit-1 and Pol II to the proximal Pit-1 site and the TSS, respectively. GR was recruited to the glucocorticoid-responsive region (GCRR) and the distal Pit-1 site in response to 1.5 h glucocorticoid treatment, while 6 h glucocorticoid treatment resulted in Ets1 dissociation from the GCRR. Collectively, these results indicate that glucocorticoid induces dynamic changes in histone modifications and transcription factor recruitment within the 5'-flanking region of the chicken GH gene. We conclude that glucocorticoid induction of GH gene expression in chickens is achieved by a combination of genomic and nongenomic pathways. Our studies provide novel mechanisms of GH regulation in chickens, some of which may be found in other vertebrate species as well.

ROLE OF EPIGENETIC MODIFICATIONS AND DEXRAS1 IN  
GLUCOCORTICOID REGULATION OF GROWTH HORMONE EXPRESSION

By

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## Dedication

To my  
amma and appa

## ACKNOWLEDGEMENTS

First, I would like to express my sincere thanks to my advisor, Dr. Tom Porter, who has been an inspiration throughout my graduate school. He has been an important force in molding my scientific thinking. The numerous interactions with him have continuously challenged and encouraged me to become a better person. I owe him for his constant support, guidance, and encouragement that have helped me reach this stage. I hope that I will be able to do justice to everything he has taught me over the last 5 years.

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## LIST OF ABBREVIATIONS

11 $\beta$ -HSD	11 $\beta$ -hydroxysteroid dehydrogenase
$\alpha$ -MSH	melanocyte stimulating hormone
ACTH	adrenocorticotrophic hormone
AF-1	activation function-1
AF-2	activation function-2
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumen
CBG	corticosteroid-binding globulin
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CORT	corticosterone
CRH	corticotropin-releasing hormone
Ct	cycle threshold
d	day
Da	Dalton
DBD	DNA-binding domain
Dexas1	dexamethasone-induced ras-related protein 1
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	DMEM:Ham's nutrient mixture F12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTPs	deoxynucleoside triphosphates
DTT	dithiothreitol
e	embryonic day
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid

EMSA	electrophoretic mobility shift assay
Elk-1	Ets-like gene 1
ERK	extracellular signal-related kinase
FGF	fibroblast growth factor
FITC	Fluorescein isothiocyanate
FKBP5	FK506-binding protein 5
FSH	follicle-stimulating hormone
FSH- $\beta$	follicle-stimulating hormone $\beta$ -subunit
g	gravitational force
GAP	GTPase activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEF	GTP exchange factor
GFP	green-fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRH-R	growth hormone-releasing hormone receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMT	histone methyltransferase
HSP	heat shock protein
IgG	immunoglobulin G
IGF-I	insulin-like growth factor-I
JAK	Janus kinase
JNK	c-jun-N-terminal kinase
kb	kilobase
LBD	ligand-binding domain
LH	luteinizing hormone
LH- $\beta$	luteinizing hormone $\beta$ -subunit
LMH	leghorn male hepatoma

LSM	least-squares mean
min	minutes
MAPK	mitogen-activated protein kinase
MEK1/2	MAPK/ERK kinase 1/2
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
ng	nanograms
NF- $\kappa$ B	nuclear factor- $\kappa$ B
nGRE	negative glucocorticoid response element
NGS	normal goat serum
NLS	nuclear localization signal
NMDA	N-Methyl-D-aspartic acid
NTD	N-terminal domain
Opti-MEM I	optimized Eagle's minimal essential medium
p38MAPK	p38 mitogen-activated protein kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p-Elk1	phosphorylated Elk1
PI3-K	phosphatidylinositol 3-kinase
Pit-1	pituitary-specific transcription factor 1
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
Pol II	RNA polymerase II
POMC	pro-opiomelanocortin
PRL	prolactin
Prop-1	prophet of Pit-1
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate



SDS-PAGE	SDS-polyacrylamide gel electrophoresis
s	seconds
SEM	standard error of the mean
SF-1	steroidogenic factor-1
Shh	sonic hedgehog
Sport6.1	pCMV-Sport6.1
SSTR1	somatostatin receptor type 1
STAT	signal-transducer and activator of transcription
TBS/T	tris-buffered saline with tween-20
TRH	thyrotropin-releasing hormone
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid-stimulating hormone
TSH- $\beta$	thyroid-stimulating hormone $\beta$ -subunit
TSS	Transcription start site
WGA	Wheat germ agglutinin
YFP	yellow-fluorescent protein

# **CHAPTER 1**

## **Literature Review**

## **Introduction**

The proper differentiation of the anterior pituitary gland into five distinct hormone-producing cell types and the establishment of the hypothalamus-pituitary axis is a fundamental process that is necessary for proper development of vertebrate organisms. The hypothalamus-pituitary structure and the process of pituitary development are highly conserved among birds, rodents, and other vertebrate species. In humans, developmental defects of the pituitary gland result in one or more hormone deficiencies, further leading to other abnormalities (2).

Glucocorticoids are important steroid hormones produced by the adrenal cortex that have several physiological roles including the regulation of metabolism, immune functions, and the stress response. Glucocorticoids have long been identified to be required for the maintenance of secretory functions of certain endocrine and exocrine organs. In addition, glucocorticoids play a crucial role in the terminal differentiation of several tissues including liver, lung, small intestine, and pancreas. In the anterior pituitary, glucocorticoids have been shown to be important for the production of growth hormone (GH).

Remarkable progress has been made in understanding the molecular mechanisms controlling pituitary development. This process involves a complex network of transcription factors and epigenetic modifiers that are tightly regulated to achieve spatio-temporal differentiation of the five hormone-producing cell types. Despite this progress, the factors necessary for the cell-type specification and the molecular details of the pathways leading to the expression of these hormones remain unknown.

## **Pituitary development and differentiation**

The pituitary gland, or hypophysis, is an endocrine gland located underneath the hypothalamus at the base of the brain. It is composed of two lobes: the anterior pituitary (or adenohypophysis) and the posterior pituitary (or neurohypophysis) (3). The hypothalamus is connected to the pituitary gland through the infundibular stem (or pituitary stalk). The pituitary gland is controlled by the hypothalamus. The axonal projections originating from the supraoptic and paraventricular nuclei of the hypothalamus terminate in the posterior pituitary, and hypothalamic stimulations by way of these neurons control the secretion of hormones from the posterior pituitary (4). The anterior pituitary is connected to the hypothalamus by an elaborate vascular pathway called the hypothalamic-hypophyseal portal veins. The hypothalamic factors synthesized in the parvocellular neurons of the hypothalamus are transported through the portal system to the anterior pituitary, and these factors regulate the secretion of the anterior pituitary hormones (3-5).

### *Anterior pituitary development*

The pituitary structure and development are highly conserved among vertebrates. The anterior and posterior pituitary tissues have distinct embryological origins (6). During development, a finger-like upward projection of the oral ectoderm forms Rathke's pouch. This pouch develops into the anterior pituitary (7, 8) (Fig 1). A second finger-like projection extends ventrally from the diencephalon, and this develops into the posterior pituitary. Cell-cell contact between the ventral diencephalon and Rathke's pouch is important for pituitary development (9).

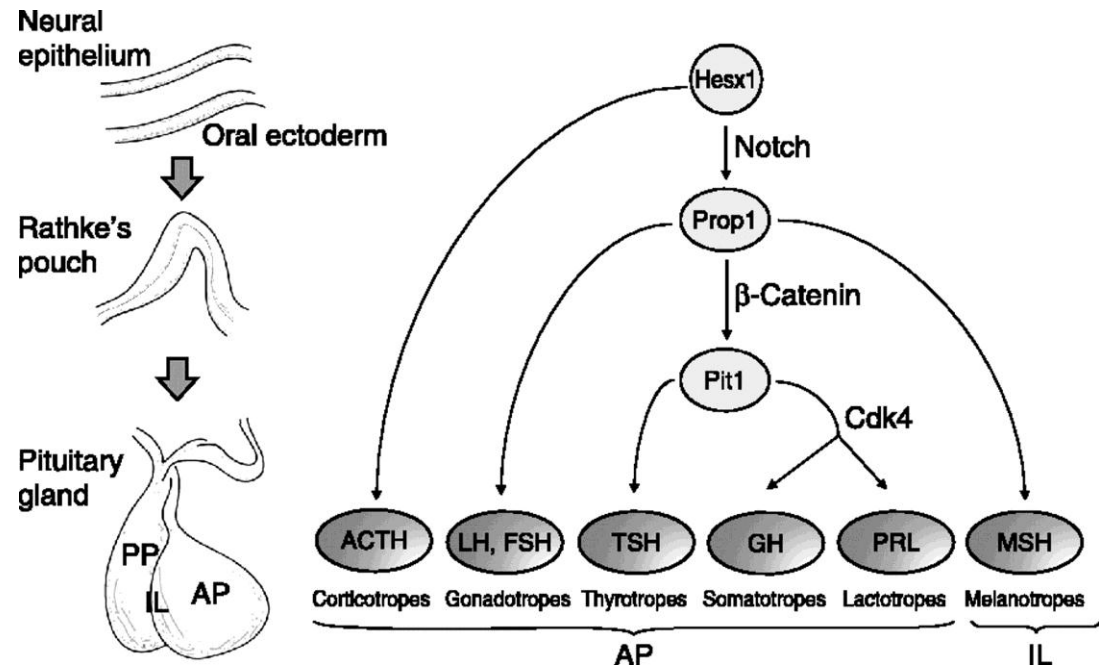


Figure 1. Pituitary development from Rathke's pouch and differentiation of the hormone-producing cell types in the anterior pituitary and intermediary lobe. Also shown are some of the transcription factors that are involved in the differentiation process. Figure taken from Querada, et al. (10).

The opposing signalling cascade formed by the diencephalon cells producing bone morphogenetic protein 4(BMP4), wingless-type MMTV integration site family, member 5A (WNT5a), and several fibroblast growth factors (FGFs) and the oral ectoderm cells secreting sonic hedgehog (Shh) is essential for the positional determination of the pituitary cell types (9, 11). Expression of FGF8 in the diencephalon and Shh in the oral ectoderm leads to the expression of transcription factor LIM-

homeobox 3 (Lhx3) (12). Lhx3 is required for determination and differentiation of pituitary cell lineages.

The anterior pituitary differentiates into five distinct hormone-secreting cell types, each of which is biochemically distinct from each other (13) (Fig 1). Corticotrophs produce the adrenocorticotrophic hormone (ACTH), which stimulates the synthesis of glucocorticoids in the adrenal cortex. Somatotrophs produce GH, which is important for long bone development and muscle growth. Lactotrophs produce prolactin (PRL) that regulates lactation: Thyrotrophs produce thyroid-stimulating hormone (TSH) that stimulates the thyroid gland to produce thyroid hormone, and gonadotrophs produce luteinizing hormone (LH) (14) and follicle-stimulating hormone (FSH), which are essential for reproduction. These hormone-secreting cell types appear sequentially during development in the anterior pituitary. Corticotrophs are the first cell type to appear, followed by gonadotrophs, thyrotrophs, somatotrophs, and finally lactotrophs (4, 15, 16). The different cell types also have distinct regional distribution within the anterior pituitary. For instance, in birds, most of the somatotrophs are localized in the caudal lobe of the anterior pituitary, whereas corticotrophs largely appear in the cephalic lobe of the anterior pituitary (17).

The development and differentiation of anterior pituitary cell types involves the pituitary-specific paired-like homeodomain factor prophet of Pit-1 (Prop-1) (18). Prop-1 is necessary for Pit-1 gene expression. Ames dwarf mice with a Prop-1 mutation do not express Pit-1, and consequently fail to produce Pit-1 lineage cell types (19). Pit-1 mutant Snell and Jackson dwarf mice fail to generate somatotrophs, lactotrophs, and thyrotrophs.

Prop-1, in humans, is also believed to be required for the development of gonadotrophs (20).

Factors that establish the different hormone-producing cell types during early anterior pituitary development are not entirely known. It is believed that the pattern and combination in which certain factors are expressed influence the expression of hormone-producing genes, and in turn define the hormone-producing cell types (15, 21). Studies on proopiomelanocortin (POMC; ACTH precursor), have reported binding sites for several factors, including Nur77 (21). A number of other transcription factors like paired-like homeodomain 1 (Pitx-1), neurogenic differentiation 1 (NeuroD1), and T-box factor (Tpit) have been reported to be involved in transcription of the POMC gene. Tpit is specifically expressed only in corticotrophs and melanotrophs ( $\alpha$ -MSH producing cells) (22). NeuroD1 is transiently expressed in fetal corticotrophs (23). GATA-binding protein-2 (GATA-2) expression is found in both gonadotrophs and thyrotrophs. Gonadotroph development also requires steroidogenic factor 1 (SF-1), early growth response-1 (Egr-1), and Pitx1 (24). SF-1 is expressed prior to the onset of expression of the gonadotropin beta subunits. In gonadotrophs, GATA-2 expression together with SF-1, and importantly the absence of Pit-1 results in gonadotroph specific differentiation (25). On the other hand, in thyrotrophs, Pit-1 expression inhibits SF-1 expression, and GATA-2 and Pit-1 combinatorial expression leads to TSH $\beta$  expression (26). Pit-1 expression is essential for three cell types: somatotrophs, thyrotrophs, and lactotrophs. The factors required for the terminal differentiation of these cell types are still not completely known. Pit-1 interactions with additional factors, regulation of Pit-1 activity or differential expression of Pit-1 isoforms may be involved in the final cell-type determination step (21).

Lactotroph restricted prolactin expression depends on Pit-1, v-ets erythroblastosis virus E26 oncogene homolog (Ets1), and Ptx factors (27). Besides Pit-1, somatotroph differentiation requires several other factors, including thyroid hormone (28) and Zn-15 (29). In chickens, thyroid hormone treatment was shown to increase somatotrophs prematurely, and thyroid hormone synthesis inhibitor, methimazole, inhibited somatotroph differentiation in vivo, supporting the involvement of thyroid hormone synthesis in somatotroph differentiation (28). In rats, Zn-15 was identified to be involved in somatotroph differentiation. The rat GH promoter was thoroughly analyzed to identify a highly conserved sequence located between two Pit-1 sites. The importance of this site for GH expression was confirmed by mutational analysis. The factor binding to this site was subsequently identified by screening expression libraries from rat pituitary and was confirmed to be Zn-15 (29).

#### *Anterior pituitary somatotroph cells*

Somatotroph cells are responsible for GH synthesis and secretion. They are the most abundant cell type in the mature male pituitary (3, 30). They are acidophilic. They may be round, oval, or triangular. They have a large nucleus, well-developed Golgi complexes, granular endoplasmic reticulum, and large secretory granules. The number and size of somatotrophs and their secretory granules varies through ontogeny and maturation state (30)

#### *Somatotroph ontogeny*

During rat embryogenesis, expression of GH mRNA is first noticeable around e17.5, as measured by in situ hybridization (31-34). GH mRNA as well as protein levels



increase considerably between embryonic day (e) 18 and e19. During development in mice, GH mRNA is first detected on e15.5, and increases markedly between e16.5 and e17.5 (35, 36). In chickens, somatotrophs first emerge around e12, and like in rats and mice, the number of somatotrophs significantly increase between e14 and e16 during development (16, 17, 37-41).

### *Growth hormone*

Anterior pituitary somatotrophs secrete GH in an episodic manner (30), and secretion varies during growth and maturation. GH is responsible for several physiological functions, including growth and metabolism. It increases protein synthesis, increases fat metabolism, and is involved in maintaining blood glucose level. GH deficiency in humans, especially children, results in growth defects, short stature, and decreased muscle mass (30, 42, 43). Excess GH leads to acromegaly or gigantism. GH secretion is tightly regulated by two hypothalamic factors: Growth Hormone Releasing Hormone (GHRH) and somatostatin. GHRH stimulates GH secretion, and somatostatin inhibits secretion (44). Upon secretion, GH binds to its receptor (GH-Receptor, GH-R) in the target tissues (liver, muscle), and activates signalling pathways (30). In liver, GH stimulates the Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway to produce insulin-like growth factor 1 (IGF-1) (45). IGF-1 stimulates muscle and bone growth. IGF-1 exerts feedback inhibition on GH secretion through regulating hypothalamic factors (GHRH and somatostatin), GH and IGF-1 itself. In the hypothalamus, GH decreases GHRH mRNA and increases somatostatin mRNA, in turn decreasing GH secretion from the pituitary (43, 45). GH can also regulate GHRH receptor expression levels in the anterior pituitary (46-48). Additionally, in birds, GH

release is also under negative regulation by thyroid hormones, which affect thyrotropin-releasing hormone (TRH)-stimulated GH release (49, 50).

#### *Somatotroph differentiation and GH expression*

The chicken GH gene is about 3.5kb, and is present on the chromosome 27. It contains five exons, four introns, and 3' and 5' untranslated regions. The gene is similar in structure to other mammalian GH genes (51, 52). In humans, the GH gene produces two different mRNA products through alternative splicing at exon 3 (53, 54). In chickens, an alternatively spliced form of GH mRNA has only been reported in the eye, where a second GH mRNA is transcribed from the middle of intron 3 (55). Other than alternative splicing, GH protein can undergo post-translational modifications, including glycosylation and phosphorylation (30). GH gene abnormalities result in GH deficiency, which leads to dwarfism, as seen in GH deficiency type IA (56).

GH gene transcription is regulated by cis-DNA elements that bind trans-acting regulatory factors (58, 59). Even though the chicken GH promoter sequence has few similarities with that of mammalian GH genes, it has binding sites for the essential factors, including Pit-1 (Fig 2) (60-62). It contains two putative Pit-1 binding sites at -113/-104 (proximal site) and -541/-533 (distal site). Additionally, a potential Thyroid hormone response element (TRE) is also found at -137/-128. The TRE is hypothesized to mediate suppression of GH promoter activity by the thyroid hormone. There are two TATA boxes (at -149 and -24) and a predicted translational start site at position 56 (60).

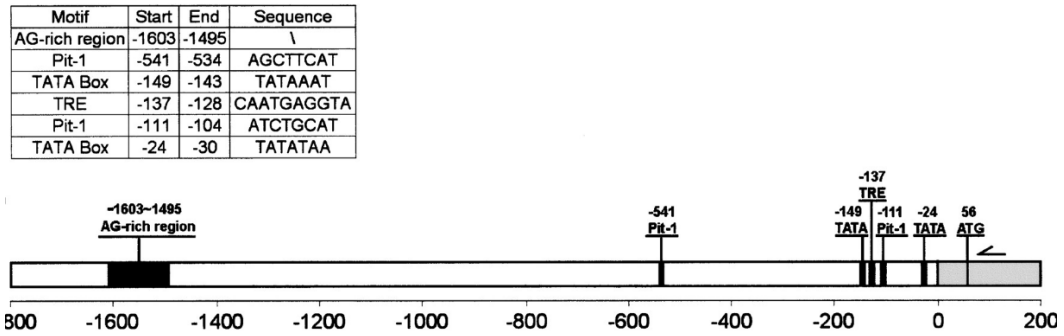


Figure 2. Schematic diagram of the GH gene in chickens. Shown are the putative binding sites for the pituitary-specific transcription factor Pit-1, TRE, and the TATA box within 1800bp of the chicken GH gene. Figure taken from Leung, et al. (57).

Pit-1 is essential but not sufficient for the complete activation of the GH gene promoter (61, 63). A mutation or absence of Pit-1 results in complete loss of functional somatotrophs. During development, Pit-1 expression precedes differentiation of somatotrophs (64). In rats, Pit-1 is first detected in the e15.5 pituitary, and in chickens, the mRNA for Pit-1 is first noted in e5 embryos (65, 66). Pit-1 binding sites are found on many genes, including GH, PRL, TSH, GHRH-receptor, and somatostatin receptor type 1 (sst1) (3). In the human fetus, somatotroph differentiation and proliferation is stimulated by glucocorticoids and thyroid hormones (67-69), and is suppressed by insulin and glucagon (70). In the fetal rat, treatment with thyroid hormones also increases somatotroph proliferation, and this action requires the presence of glucocorticoids (71-74). GHRH stimulates GH expression through binding to GHRH-R, and activates protein kinase A (PKA) signalling (75), which phosphorylates CREB-binding protein (CBP). Pit-1 activation of the GH promoter is suggested to occur by displacing the nucleosome, and

exposing the glucocorticoid and thyroid response elements. In humans, Pit-1 interacts with CBP, and histone acetyl transferase (HAT) activity of CBP allows activation of the GH promoter (76).

## **Glucocorticoids**

Glucocorticoids are a class of steroid hormones (77). They are synthesized in the zona fasciculata cells of the adrenal cortex and released in a circadian manner. The primary glucocorticoid in humans is cortisol, while corticosterone (CORT) is the primary glucocorticoid found in many animals, including rodents and birds. Glucocorticoids have a wide range of functions. They stimulate gluconeogenesis by increasing expression of enzymes involved in gluconeogenesis, inhibit glucose uptake in muscle and fat tissues, and stimulate fat metabolism (77, 78). They suppress immune responses, and inhibit swelling and inflammation by regulating anti- and pro-inflammatory proteins. They also play important roles in fetal development. For instance, glucocorticoids are involved in the transcriptional regulation of surfactant protein A, a major protein required for proper development of the lungs (79, 80). Glucocorticoids are also referred to as the “stress hormones” (78). Under stress conditions, the hypothalamus increases release of Corticotropin Releasing Hormone (CRH), which in turn increases ACTH secretion from the anterior pituitary. ACTH stimulates the synthesis of glucocorticoids in the adrenal cortex, eventually leading to increased glucose availability and increased blood flow.

Availability of glucocorticoids is maintained by two enzymes inside the cell:  $11\beta$ -hydroxysteroid dehydrogenase type II ( $11\beta$ -HSD2) and  $11\beta$ -HSD1. The  $11\beta$ -HSD2

converts active cortisol into inactive cortisone, and  $11\beta$ -HSD1 converts cortisone into an active glucocorticoid (81). Corticosteroid binding globulin (CBG) or transcortin is the major glucocorticoid transport protein in the plasma (82). The free or unbound glucocorticoids, due to their hydrophobic nature, cannot pass freely across membranes. CBGs bind glucocorticoids reversibly. CBGs have high affinity and limited capacity for glucocorticoids (83, 84). It is not clear as to how CBG transports the glucocorticoids across cell membranes, but some reports suggest that CBG may be able to bind to different membranes. CBG also regulates the availability of glucocorticoids to the different tissues. Additionally, the amount of CBG available to bind to glucocorticoids is also regulated. A decrease in CBG level, increases levels of unbound cortisol, as in the case of Cushing's syndrome (85). Stress also decreases the half-life of CBG, which leads to increased free glucocorticoids. Long-term exposure to high levels of glucocorticoids causes damage to muscle tissues, increased blood pressure, diabetes, and weakens the immune system (78).

### *Glucocorticoid receptor*

Glucocorticoids function through glucocorticoid receptor (GR). GR belongs to the nuclear hormone receptor superfamily and functions as a ligand-dependent transcription factor. Other members of this family of receptors include mineralocorticoid receptor (MR), androgen receptor (AR), and progesterone receptor (PR) (86) (87). GR is widely expressed in all tissues, including brain and pituitary, in both mammals and birds. Complete knockout of GR is lethal. Mice lacking GR die shortly after birth, due to improper lung development and respiratory failure (88).

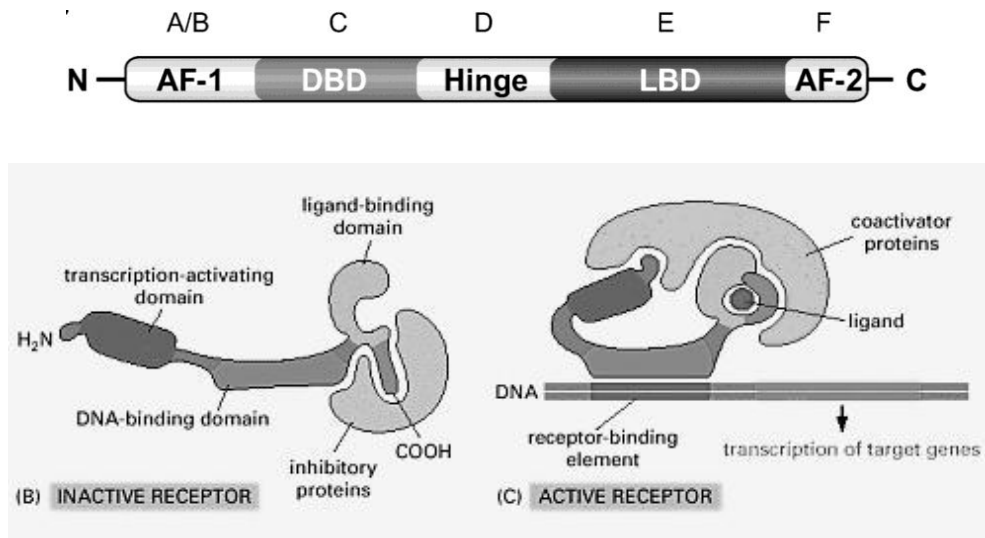
Several isoforms of GR exist, the two main isoforms being GR $\alpha$  and GR $\beta$  (91, 92). The multiple isoforms of GR are generated by alternative splicing and alternative translation initiation (93-95). The full length GR has been cloned and sequenced for several species, including human, rat, mouse, and chickens (96-98).

GR protein contains three major domains: an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Fig 3) (87, 99). The DBD and LBD are separated by a hinge region. There are two nuclear localization signals (NLS) present, one in the hinge region and one in the LBD. The NTD contains transcriptional activation function (AF1), and activates gene transcription through interactions with different coregulators and the basal transcriptional machinery. The DBD contains two zinc-finger motifs that recognize specific DNA sequences on the target genes, and it binds DNA upon activation of the receptor through ligand binding. Additionally, GR also has a P-box that facilitates target site identification and a D-box that mediates receptor dimerization. The LBD binds the glucocorticoid, and contains an additional activation function (AF2) site. The LBD also binds chaperone proteins, such as heat shock protein 90 (hsp90) (Fig 3) (87, 100).

#### *Genomic actions of glucocorticoid receptor*

Genomic actions of GR are a slow process, and occur within hours. In the absence of ligand, GR is found predominantly in the cytoplasm of cells. It resides in the cytoplasm as a multiprotein complex comprised of chaperone proteins (hsp90, hsp70), and FK506 binding proteins (FKBP) (Fig 3) (101, 102). Chaperone proteins and FK506

(A)



(B)

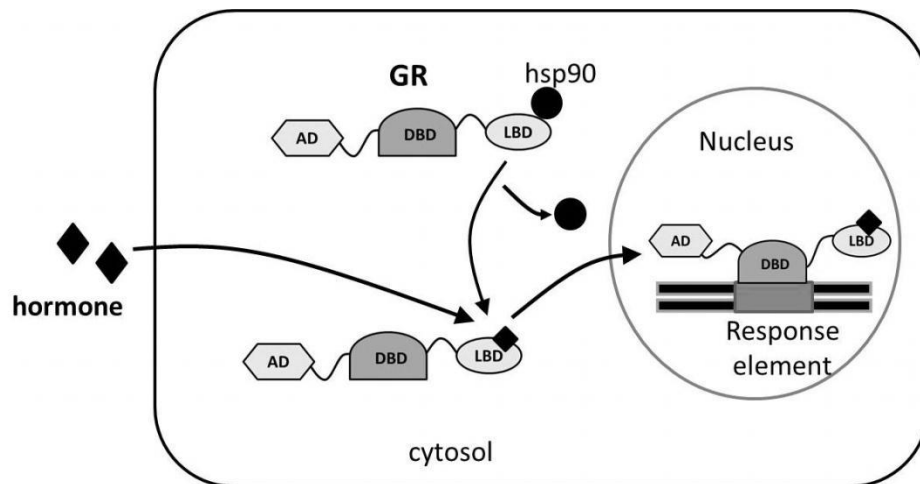


Figure 3. (A) Structural organization of glucocorticoid receptor, including the DNA binding domain (DBD) and ligand binding domain (LBD). Figure taken from Alberts, et al. (89). (B) Functioning of the glucocorticoid receptor. Upon hormone binding, the cytosolic nuclear receptor is activated, and translocated to the nucleus. In the nucleus, the activated receptor binds to the response element on the gene and activates gene transcription. Figure redrawn from Lodish, et al. (90).

facilitate in the proper folding of the GR protein and maintain GR in a transcriptionally inactive state. In the presence of glucocorticoids, GR undergoes a conformational change, which results in the dissociation of hsp90, hsp70 and FKBP (103). The NLS within the LBD is exposed and the glucocorticoid bound GR is translocated to the nucleus. The DBD of GR binds to target sequences on the DNA. Alternatively, GR can directly bind to other transcription factors to regulate gene expression (cis- or trans- regulation respectively) (Fig 4) (87, 105). The specific sequences on DNA that bind GR are called glucocorticoid-responsive elements (GRE) (105). GREs are typically found in the promoter region of a gene. GR binds GRE as a homodimer. A classical GRE sequence is an imperfect palindrome (GGTACAnnnTGTTCT) with two 6-bp half sites that are separated by a 3-bp spacer. GR can also effectively bind a GRE half-site and regulate transcription (106, 107). GR binding to the DNA is a transient process and requires GR dimerization.

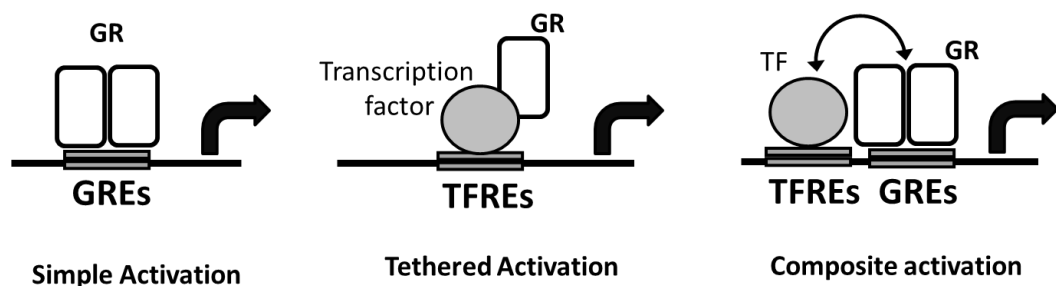


Figure 4. Possible modes of action of GR. GR directly binds GRE on DNA (simple), or tethers to transcription factors that bind to transcription factor response element (TFREs) on DNA (tethered), or binds to DNA as well as interacts with transcription factors bound to TFREs on DNA (composite). Figure redrawn from Miner, et al. (104).



GR can both activate and repress gene expression. In activating gene expression, DNA-bound GR interacts with ATP-dependent chromatin remodelling complexes SWItch/Sucrose NonFermentable (SWI/SNF), factors with histone acetyltransferase (HAT) activity like CBP and p300, and with the basal transcription machinery (108). GR inhibits gene expression by binding to the so-called negative GRE (nGREs), and possibly by mediating the dissociation of positive transcription regulators.

GR can also bind DNA in the form of heterodimers. It has been shown to heterodimerize with the STAT and Ets transcription factors and facilitate recruitment of coactivators or corepressors (109, 110). Coregulators play an essential role in GR-dependent transcriptional regulation. It is important to understand that GR does not repress or activate a certain gene under all conditions. In addition, an activator may function as a repressor of the same gene in a context-dependent manner, and vice versa. Overall, the functioning of GR and its interacting partners is both gene and context dependent (109, 110).

Glucocorticoid signalling is additionally regulated by post-translational modifications. GR can undergo several modifications, including phosphorylation and acetylation. Phosphorylation of GR promotes GR degradation, and regulates the translocation of liganded receptor from cytoplasm to nucleus (111-113). The human GR can be phosphorylated by several kinases, including MAPK and JNK. Five serine (S) residues in hGR may be phosphorylated by kinases: S113, S141, S203, S21, and S226. S203 and S211 phosphorylation by JNK leads to GR activation, while phosphorylation of S226 inhibits GR activity (111-113). Phosphorylation of S211 of GR increases its interaction with coactivator MED14 (mediator complex subunit 14) (111).

GR down-regulation occurs through ubiquitin-proteosomal degradation (114, 115). The PEST motif located upstream of the DBD of GR has been identified as the ubiquitination site. Moreover, regulating the expression of an additional factor, E3 ligase carboxy terminus of Hsc70-interacting protein (CHIP) that is involved in the ubiquitination process, also has the ability to regulate GR signalling. Yet another important post-translational modification is the acetylation of GR at the conserved KXKK motif located near the NLS. GR can undergo both acetylation (by HATs) and deacetylation (by HDACs) (114, 115).

As mentioned earlier, there are two isoforms of GR: GR $\alpha$  and GR $\beta$ . GR $\alpha$  can bind to glucocorticoids, whereas GR $\beta$  cannot (116, 117). Both GR $\alpha$  and GR $\beta$  bind DNA as either homodimers or heterodimers. GR $\beta$  is mainly found in the nucleus and functions independent of GR $\alpha$  (117).

#### *Nongenomic actions of glucocorticoid receptor*

The nongenomic actions of GR play important roles in many physiological processes, including immune and endocrine functions. These actions occur through alterations in membrane protein signalling, changes in cytoplasmic proteins like MAPKs and protein kinases that feed into different signalling pathways (118, 119).

#### *Glucocorticoid regulation of growth hormone gene expression*

Evidence for the involvement of glucocorticoids in GH gene regulation were based on initial studies using fetal rat pituitary cells taken at day 14, prior to the synthesis of GH cells (120, 121). E14 cells cultured in the presence of serum-free medium with

cortisol added were able to differentiate into GH cells, while no GH cells were found in the serum-free medium alone. Similar experiments conducted in chickens, using e10-e14 pituitary cells cultured in the presence of serum from older birds (e12 or e16), pointed to the involvement of a blood-borne factor in initiating somatotroph differentiation (122-124). The appearance of somatotrophs in rodents and chickens correlates with increased levels of glucocorticoids and thyroid hormone in the plasma, suggesting the possible participation of these factors in GH cell differentiation (120-122, 125, 126). Administration of ACTH caused a premature increase in GH cell numbers in chickens (127). Reducing the levels of endogenous glucocorticoids by the administration of metyrapone in pregnant rats resulted in significant reduction in the number of GH cells (128). Treatment with TSH, corticotropin-releasing hormone (CRH),  $\alpha$ -melanocyte-stimulating hormone (MSH), GHRH, and TRH did not show similar effects.

In situ hybridization, northern blot analysis and immunocytochemistry studies of dexamethasone (DEX; a synthetic glucocorticoid) treated rat pituitary cells, revealed an increase in GH mRNA and number of GH cells (120, 125, 126, 128-130). Similar results were also found in chickens, where CORT was able to induce premature differentiation of somatotroph cells both in vitro and in vivo (28, 39). CORT increased GH mRNA and protein levels, and importantly induced expression of GH mRNA in cells that did not express GH mRNA prior to the treatment. GH mRNA expression occurred within 8 h or 3 h CORT treatment in rats and chickens, respectively (28, 39, 122, 125, 128, 129, 131, 132). Also, in response to GHRH and TRH treatment, these cells released more GH, suggesting that the CORT-induced somatotrophs are functional (133). Thyroid hormone treatment increased fetal GH expression, but was able to do so only in the presence of

glucocorticoids (28, 122, 132). Similarly, using reverse hemolytic plaque assays and immunocytochemistry in chickens, it was shown that thyroid hormones can augment CORT effects but had little to no effect on their own (34). Overall, there is sufficient evidence indicating the importance of glucocorticoids and thyroid hormone in GH gene induction, both in rodents and chickens. A working model was proposed summarizing the factors that may be involved in regulating GH expression in chickens (Fig 5).

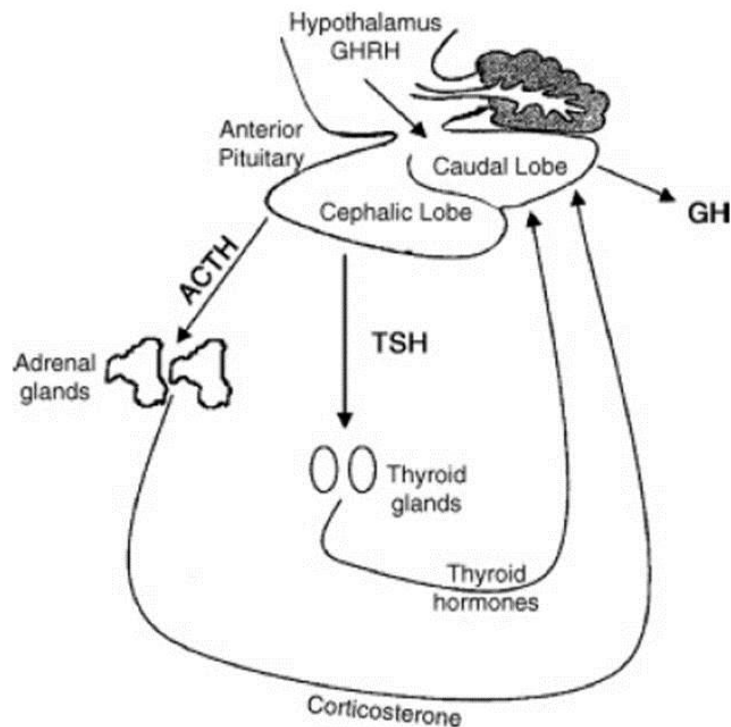


Figure 5. Representation of the factors affecting GH expression in the anterior pituitary. Hypothalamic factors along with glucocorticoids and thyroid hormones regulate the synthesis and secretion of GH from the somatotroph cells of the anterior pituitary. Figure taken from Porter, et al. (28).

*Mechanism involved in glucocorticoid induction of GH gene transcription*

The molecular mechanism involved in glucocorticoid induction of GH is still not completely understood. Studies in rodents and chickens have identified several independent aspects of regulation, but a lot remains unknown. Unlike the human GH gene, a classical GRE has not been found in the rat and chicken GH promoter, suggesting that GR may not necessarily bind to the DNA to mediate glucocorticoid effects (134). Glucocorticoid induction of GH gene expression is believed to be indirect and requires additional factors, both in rodents and chickens (135, 136). In fetal rat pituitaries, the dexamethasone-induced increase in GH mRNA levels was completely abolished in the presence of puromycin, a protein synthesis inhibitor (125). Similarly, pretreatment with cycloheximide (CHX), another protein synthesis inhibitor (135) attenuated the CORT-induced increase in GH mRNA levels in chickens. CORT treatment, both in rats and chickens, did not affect Pit-1 mRNA or protein levels, suggesting that a change in Pit-1 is not the mechanism underlying DEX-induced GH increase (135). Effects of CORT on GHRH-R were found to be species-specific. In fetal rats, CORT treatment increased GHRH-R mRNA levels, whereas in chickens, CORT had no effect on GHRH-R mRNA or protein levels (28, 46, 133, 137). Further, glucocorticoids may stimulate other types of pituitary cells to produce and secrete an unknown regulator, which, in turn may stimulate GH expression. Nevertheless, it can be concluded that CORT induction of GH gene expression requires an intermediary factor.

CORT can induce GH expression by binding to either GR or MR. GR expression is detected in the pituitary gland by e13 in mice and by e15 in rats (138). In mouse, MR is detected by e13.5. In chickens, GR and MR mRNA are detected as early as e5 and

increase between e10 and e14 (139, 140). Evidence in rodents and chickens indicates that CORT regulation of GH requires GR and MR signalling (139). In chickens, blocking GR signalling alone by ZK98299, significantly reduced CORT-induced GH mRNA levels (141). Blocking MR alone with spironolactone had similar effects on CORT induction of GH mRNA. By blocking GR and MR together with both ZK98299 and spironolactone, CORT induction of GH mRNA was completely abolished.

CORT induction of GH gene expression does not seem to involve the two predominant GHRH-R signalling pathways – protein kinase C (PKC) and protein kinase A (PKA) pathways (133, 135). Neither activation nor inhibition of these pathways had any affect on the CORT-induced increase in GH mRNA levels. In contrast, inhibition of ras signalling by manumycin A suppressed CORT induction of GH mRNA, indicating the involvement of ras signalling or a ras-like factor in the process. Subsequently, using pharmacological inhibitors, it has also been determined that ERK1/2 may be the downstream ras effector required for GH induction by CORT (142).

As mentioned earlier, sequence analysis of human, rat and chicken GH gene reveals the presence of half-GRE sites, but no full length GREs. Leung and co-workers reported that a 1727bp sequence of the chicken GH promoter is responsive to dexamethasone treatment (60). In their experiment with rat pituitary cells, they showed that a reporter construct driven by a 1727bp sequence of the chicken GH promoter was able to activate luciferase expression in response to dexamethasone. Similar results were obtained in experiments conducted using chicken pituitary cells. E11 chicken pituitary cells were transfected with either pGL3-Basic empty vector or -1727/+48bp fragment containing vector, followed by CORT treatment. Cells that received pGL3-1727 showed

a two-fold increased luciferase activity. Moreover, CORT induction of -1727/+48bp GH promoter requires ongoing protein synthesis and mimics the endogenous GH gene induction by CORT (141). Further characterization of the -1727/+47bp chicken GH promoter fragment by deletion mutation analysis revealed the presence of a glucocorticoid-responsive region (GCRR) between -1054 and -954. The GCRR can function both in forward and reverse orientations. Sequence analysis of the GCRR fragment for putative transcription binding sites revealed the presence of binding sites for several factors, including Ets1(141). Further, binding of Ets1 and GR to the GCRR was also confirmed by electrophoretic mobility shift assay (EMSA). Further support for the importance of the Ets1 and GRE half-site within the GCRR site for CORT induction of the GH gene came from site-directed mutation studies, wherein mutation of these sites abolished CORT responsiveness (141).

### **Histone Modifications**

The main subunit of chromatin is the nucleosome. The nucleosome contains ~200bp of DNA coiled around a protein core called histone. The histone (H) core is an octamer that consists of two copies each of H2A, H2B, H3 and H4 (143). Series of nucleosomes are tightly packed into chromosomes. The less densely packed regions of the chromatin are called euchromatin, whereas the tightly packed regions are known as heterochromatin. Each core histone is made of a central protein and a flexible N-terminal tail. The N-terminal tail sequences are highly conserved and are important in regulating chromatin structure. Certain amino acids present on the N-terminal tail undergo

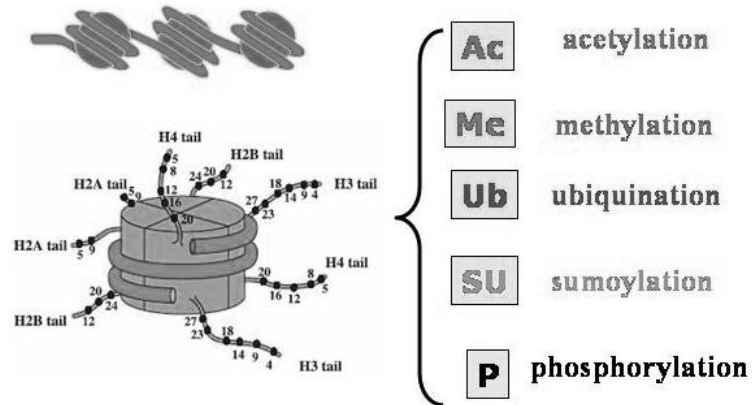
modifications. The patterns of modifications (sometimes referred to as a histone code) are suggestive of a particular read-out and lead to a certain biological outcome (143, 145). Overall, the patterns of histone modifications influence transcription initiation by regulating chromatin accessibility.

### *Types of histone modifications*

Histones can undergo several covalent modifications, including acetylation, methylation and phosphorylation (Fig 6) (145). Each of these modifications is carried out by specific enzymes and can be reversed by a set of specific enzyme. Acetylation was the first identified post-translational modification of histone (145, 146). It is catalyzed by histone acetyltransferases (HATs), which add an acetyl group to the amino side chains of lysine residues (147). Several lysine residues have been identified as acetylation targets, including lysine 9, 14, 18, and 23 on H3. Acetylation by HATs results in the reduced affinity between histones and DNA, and this consequently leads to a more open chromatin that allows the RNA polymerase II and transcription factors to access the promoter region. A highly active gene is usually marked by high levels of histone acetylation (146, 148). Acetylation is reversed (deacetylation) by histone deacetylases (HDACs). HDACs remove the acetyl groups and support gene repression through closed chromatin conformation (147-150). HATs are considered transcriptional coactivators, while HDACs are referred to as corepressors. HATs and HDACs are recruited as a part of a larger complex, and in response to specific biological stimulations, they either induce or suppress gene expression. A second kind of modification is histone methylation, and this is achieved by histone methyltransferase (HMT) enzymes (151). HMT catalyzes the



(A)



(B)

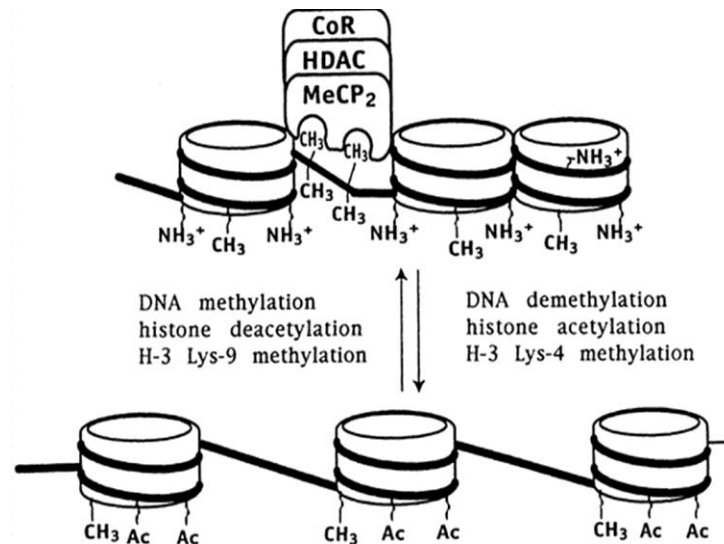


Figure 6. (A) The nucleosome with the histone proteins. A schematic representation of histone modifications involved in regulation of gene transcription. (B) Effect of histone acetylation and DNA methylation on remodelling chromatin structure. The repressed closed state is deacetylated. Acetylation of histone as well as DNA demethylation imparts an open chromatin structure facilitating activation of gene transcription. Figure taken from Momparler, et al. (144).

addition of one, two, or three methyl groups (mono, di, or trimethylated, respectively) to lysine or arginine residues in histones (145, 151). The effect of methylation on gene transcription depends on which lysine residue is modified, as well as the type of methylation (number of methyl groups added). Mono-methylation of lysine 4, 9 or 27 is associated with gene activation, di-methylation is linked to repression, tri-methylation of lysine 4 (H3me3K4) is linked to gene activation, and tri-methylation of lysine 9 or 27 (H3me3K9 or K27) is related to gene repression (145, 151-155).

Histones can be phosphorylated at selected serine and threonine residues. Of all the histones, Histone H1 is the most phosphorylated. Although the role of H1 phosphorylation remains elusive, it is hypothesized that it plays an important role in formation of higher-order chromatin structures (156, 157). Histone H3 phosphorylation occurs on serine residues. H3 phosphorylation of serine 10 (H3S10) is widespread in cells entering mitosis and in cells stimulated by growth factors. Interestingly, increased H3S10 phosphorylation occurs in genes activated by growth factors (156). Kinases responsible for histone phosphorylation are not well known. Other than acetylation, methylation, and phosphorylation, histones can be modified by attachment of ADP-ribose moieties or ubiquitinated (156-159).

Apart from the above-mentioned covalent modifications of histones (by HATs, HDACs, and HMTs), non-covalent modifications also play important roles in gene transcription. The non-covalent modifications are introduced by chromatin remodelling complexes that utilize ATP to bring about changes in the chromatin (160). All the ATP-dependent chromatin-remodelling complexes have an ATPase subunit. Most of the well-known remodelling complexes belong to one of the three groups: SWI2/SNF2, SWI, or

the Snf2 deacetylase (160, 162). The mechanism of action of ATP-dependent remodelling complexes is not well known. In general, it involves recruitment of these factors to the DNA-nucleosome, followed by ATP-dependent disruption or alteration of nucleosome structure. Interestingly, it was found that HATs and HDACs associate with remodelling complexes and seem to work in concert to regulate gene transcription (159, 160, 162-165).

#### *Effects of glucocorticoids on histone modifications*

Histone acetylation by HATs relaxes the DNA and gives chromatin a configuration that allows access to transcription factors, and thus acetylation is linked to transcriptional activation. Some coactivator proteins have intrinsic HAT activity, for example, p300 and CBP (108). CBP and p300 are known GR interacting proteins (Fig 7) (108, 166, 167). Interaction of ligand-bound GR with HATs, including CBP and p300 plays an important role in transcription of glucocorticoid-regulated genes. GR has also been shown to interact with other HAT activity-containing proteins like steroid receptor coactivator-1 (SRC-1) and glucocorticoid receptor-interacting protein 1 (GRIP-1) (167-169). It is known that glucocorticoid-mediated histone modifications play important roles in gene regulation. Glucocorticoids inhibit expression of surfactant protein A (SP-A) through the recruitment of HDACs in the human lungs (79, 170, 171). Glucocorticoids also regulate H3 phosphorylation and methylation of histones associated with SP-A gene. In mice, glucocorticoids induce H3 acetylation on the  $\beta$ -casein gene (166, 171).

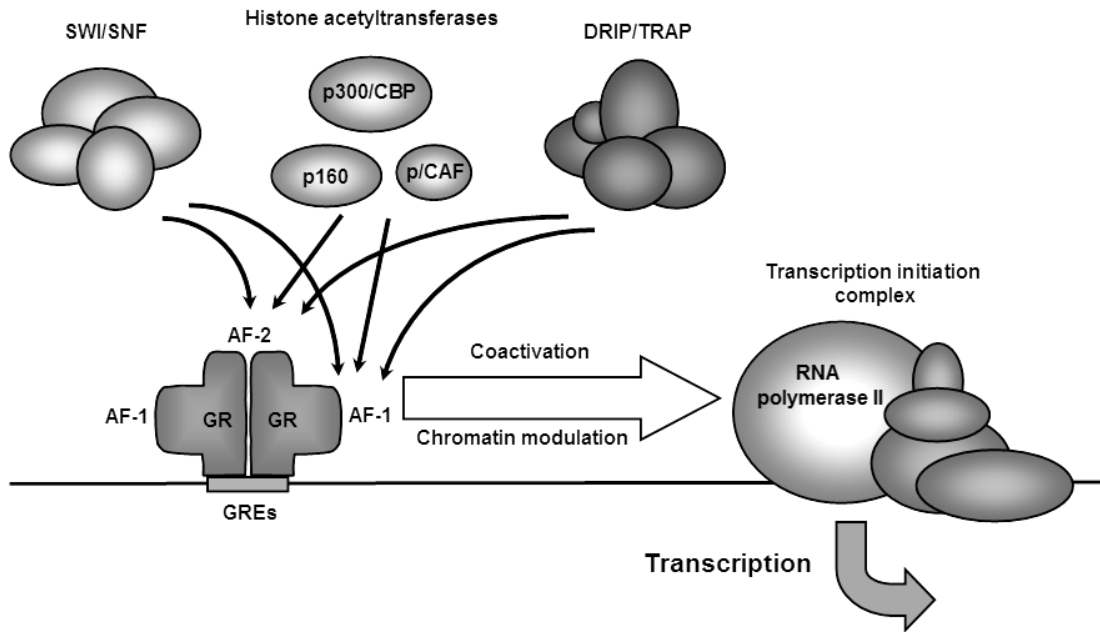


Figure 7. Model representation of factors involved in nuclear receptor-mediated gene transcription. Upon activation, the GR binds to the GREs on the target gene. GR interaction with chromatin remodellers (SWI/SNF) and histone modifiers (p300/CBP) helps chromatin attain a more favorable conformation that facilitates the binding of basal transcription machinery leading to gene activation. Figure taken from Charmandari, et al. (161).

### *Epigenetic regulation associated with GH gene expression*

Pituitary development and cell-lineage determination involve epigenetic modifications of chromatin. Histone lysine demethylase LSD1 is part of a multiprotein corepressor complex, and LSD1 has been shown to be important for the differentiation of somatotroph cells during pituitary development (1, 172). Pituitary-specific knockout of LSD1 in mice resulted in a complete loss of GH and TSH cells (1). In the LSD1 knockout mice, recruitment of Pit-1 to the GH promoter was not affected. Furthermore, co-immunoprecipitation experiments showed interaction between LSD1 and Pit-1, strongly suggesting the requirement of histone modifications in GH gene expression (1, 172-174). Moreover, the expression of Zinc finger E-box-binding homeobox 1 (ZEB1) repressor in lactotroph cells suppresses expression of the GH gene, and ZEB1 was shown to interact with LSD1 as part of a large repressor complex (Fig 8). Taken together, there is clear evidence for involvement of histone modifications in both activation and repression of the GH gene (174).

### **Dexamethasone-Induced Ras1 (Dexas1)**

In addition to studies on epigenetic effects, the current research assessed the regulation and role of Dexas1 in controlling pituitary GH expression. Dexamethasone-induced Ras protein1 (Dexas1) is a member of the Ras superfamily of monomeric G proteins. It is a GTP-binding protein. The Dexas1 gene in chickens is located on chromosome 14. It contains two exons and is 1638bp in length. The chicken Dexas1 cDNA was isolated from a library of cDNAs expressed in the chicken neuroendocrine system (175).

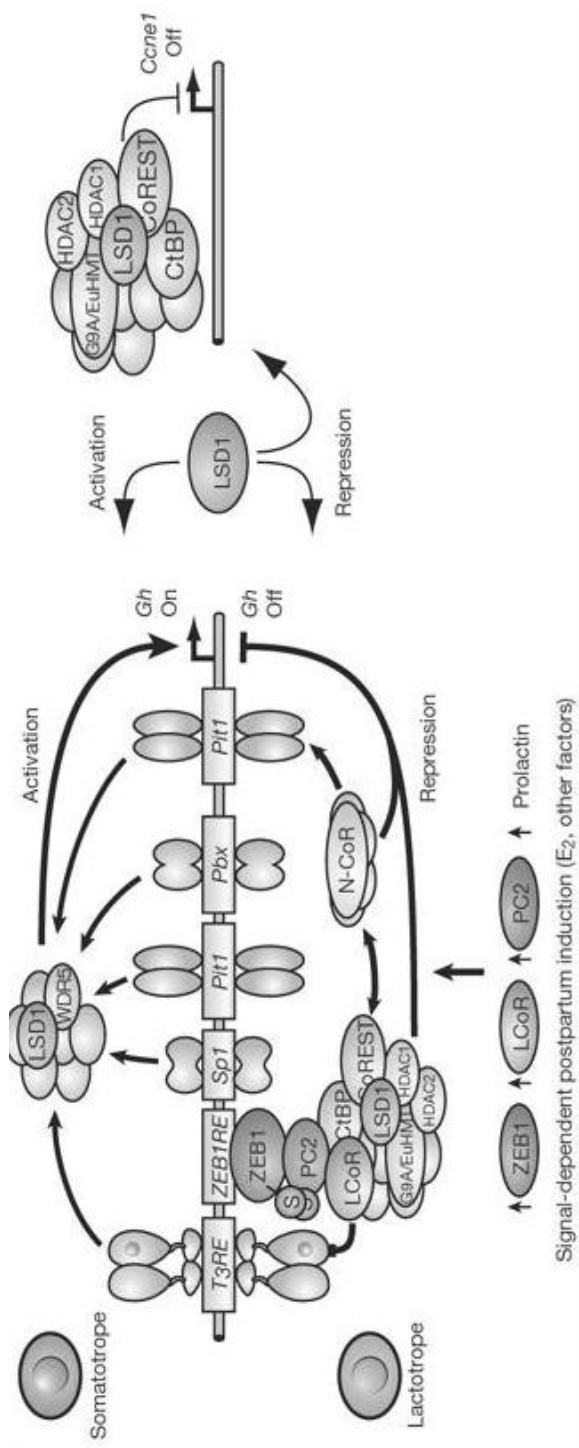


Figure 8. Model representing epigenetic modifications involving histone deacetylase, LSD1. LSD1 differentially regulates the expression of GH in somatotrophs while suppressing GH expression in lactotrophs. This differential regulation is partly achieved through the interacting partners of LSD1. LSD1 forms a complex with co-activators or co-repressors, and switches the gene GH gene transcription "ON" or "OFF". Figure taken from Wang, et al. (1)

The library was generated from the hypothalamus, anterior pituitary gland, and pineal gland of chickens ranging in age from e12 to d3. The Dexras1 cDNA clone has an 837bp open reading frame and encodes for a 278 amino acid protein (176). The predicted Dexras1 protein has a molecular mass of approximately 31.5 kDa. Dexras1 has also been isolated from other species including rat, mouse, and human (177-181). The human homolog of Dexras1 is also referred to as the activator of G-protein signalling-1 (AGS-1).

Dexras1 was first identified in the AtT-20 murine corticotroph cell line (177). In these cells, glucocorticoid treatment significantly stimulated Dexras1 mRNA expression, and this characterized Dexras1 as a novel glucocorticoid-target gene (177, 182). Dexras1 is highly conserved among species. The human and mouse isoforms share approximately 98% protein homology. Chicken Dexras1 is 85% similar to that of its rat and human orthologs. Chicken Dexras1 has characteristic features of proteins belonging to the ras superfamily (176). It contains all the important conserved domains and motifs that are required for ras functions like the guanine nucleotide-binding motif, the C-terminal membrane targeting farnesylation CAAX site, the P-loop, and the EXSAK site (183, 184).

#### *Ras proteins and ras signalling pathway*

Ras proteins function as important molecular switches that regulate cytoplasmic signalling networks. Their activity depends on binding to either GTP or GDP (184). The GTP-bound ras state is considered as “active” or “ON”, while the GDP-bound state is believed to be “inactive” or “OFF”. The guanine nucleotide-binding domain of the ras

protein undergoes conformational changes depending on whether a GTP or a GDP molecule is associated with the ras protein. Ras proteins have low intrinsic GTP hydrolysis and GTP/GDP exchange capabilities, and they require additional regulatory factors for proper functioning. GEFs (guanine nucleotide exchange factors) function as positive regulators, while GAPs (GTPase activating proteins) act as negative regulators (Fig 9). GEFs promote dissociation of GDP and the binding of GTP. GAPs, on the other hand, increase GTP hydrolysis and stimulate formation of inactive GDP-bound ras. The GTP-bound conformation of ras proteins presents a favorable interaction with GAPs, while the GDP form supports binding to a GEF. It is known that under physiological

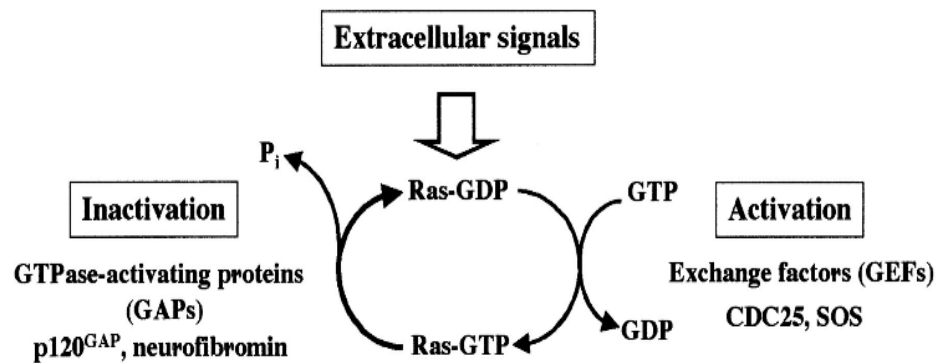


Figure 9. The Ras protein, a small G protein can bind GDP or GTP. Shown here are the two forms of ras protein. GTP-bound form is regarded as the active form, while the GDP-bound ras protein is the inactive form. The GEFs and GAPs are the positive and negative regulators. Figure taken from Reuter, et al. (185).



conditions there is an excess of GTP in the cell. Consequently, it has been found that any mutation within the ras gene that causes a decrease in the affinity for guanine nucleotides produces a constitutively active phenotype. A single nucleotide substitute mutation in the G4 domain of the Dexas1 guanine nucleotide-binding pocket (A178V) was shown to have an increased Dexas1 function (180, 182, 187, 188). The mutant had lowered affinity to GTP than the wild type in vitro. In contrast, in vivo studies showed the opposite, where the mutant Dexas1A178V bound to twice as much GTP as wild type protein (188). Upon activation, ras proteins interact with several downstream effectors leading to control of gene expression, cell proliferation, differentiation, and apoptosis (178, 182, 187, 189). Some of the well-known ras effector molecules include Raf, phosphatidylinositol 3-kinase (PI3-K), and phospholipase-C (PLC $\epsilon$ ). Raf phosphorylation leads to activation of MAPK pathways and phosphorylation of ERK (Fig 10) (190). Activated ERK translocates to the nucleus and phosphorylates Ets-family transcription factors (Elk-1). The ras pathways are complex networks of proteins and present an even more complicated regulatory network. Ras proteins can also be regulated through modifications such as phosphorylation and acetylation (191).

Ras proteins are synthesized as cytosolic precursors with a CAAX motif at the carboxy-terminal (191). The CAAX motif undergoes enzymatic post-translational modification (prenylation or farnesylation), which promotes the translocation of the ras protein to the plasma membrane. The covalent modification is critical for ras membrane association. There are contradicting views about whether all ras proteins need to be membrane-associated for their function. Nevertheless, in many cases the membrane

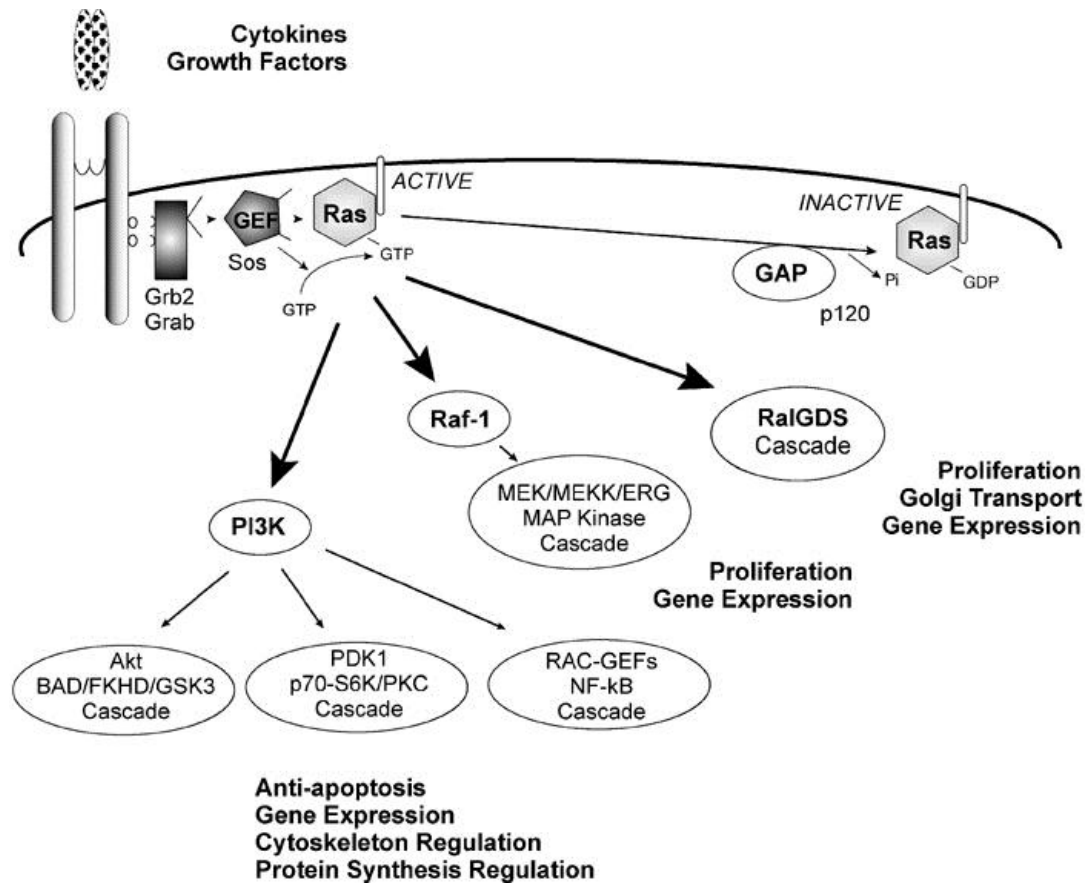


Figure 10. Activation of ras protein and ras signalling pathway. Extracellular stimuli lead to receptor activation, which leads to the activation of ras proteins positioned close to the receptor. Ras proteins are attached to the membrane. Ras signalling activation could then activate any one of the above-mentioned pathways and effect several different functions. Figure taken from Perentesis, et al. (186).

association is a limiting factor for the proper function, and disruption of translocation or farnesylation by pharmacological inhibitors like manumycin or mutation to the CAAX domain has been shown to abrogate ras signalling (178, 190-192). Ras signalling and GPCR (G protein-coupled receptor) signalling are also interconnected (182, 193).

Reports in mammalian cells show that the  $\beta\gamma$  subunits of heterotrimeric G proteins connect GPCRs with the MAPK pathways through small GTP-binding proteins (194).

### *Functions of Dexas1*

Dexas1 mRNA is rapidly induced by glucocorticoids in a dose-dependent manner in AtT-20 cells (177). The amount of time required for glucocorticoid induction of Dexas1 was consistent with the time required for glucocorticoid inhibition of stimulated ACTH secretion, suggesting that Dexas1 may be involved in the glucocorticoid-mediated negative feedback inhibition in the corticotrophs cells (177, 192, 195). The role of Dexas1 in ACTH secretion from pituitary cells remains to be confirmed. In the same study, it was also demonstrated that Dexas1 could regulate cAMP-stimulated secretion of co-transfected hGH (180). In AtT-20 corticotroph cells and COS-7 fibroblast cells, the expression of a constitutively active Dexas1 mutant (Dexas1A1788V) decreased the cAMP-stimulated secretion of transfected hGH by 86% (192). The inhibition was reversed when a CAAX-mutant Dexas1 was transfected, suggesting the need for prenylation in this function. The significance and mechanism underlying this decreased GH secretion is not yet clear. Nevertheless, this study does raise a possible role of Dexas1 in secretory or endocrine functions.

In mouse pituitary cells, Dexas1 expression can be stimulated by glucocorticoids, thyroid hormone, and  $17\beta$ -estradiol ( $E_2$ ) (196, 197). The fact that Dexas1 can be regulated by multiple factors in pituitary cells also points to the possibility that Dexas1 may be expressed in more than one pituitary cell type. Additionally, Dexas1 may have cell-specific functions.

Recent investigations show that Dexas1 has distinct roles in a ligand-independent and ligand-dependent manner (187, 189, 190, 193). An important feature in both cases is that Dexas1 seems to communicate between  $G_i$ -mediated signal transduction and the ERK-1/2 MAP kinase pathway (190). In the absence of receptor activation (ligand-independent), Dexas1 favors the GTP-bound state of  $G_i\alpha$ , which stimulates the dissociation of  $G\beta\gamma$ . This results in increased  $G\beta\gamma$ -dependent activation of the Ras/Raf/MEK/ERK cascade, which leads to an increase in Elk-1 transactivation (187, 190, 194). On the other hand, in the case of ligand-dependent receptor activation, Dexas1 interferes with receptor activation of  $G_i$  by interfering with the ADP-ribosylation of  $G_i\alpha$  (188, 189, 193, 198, 199). Although, it is not completely clear as to how Dexas1 selectively differentiates between the two scenarios. Dexas1 has also been shown to block  $G\beta\gamma$ -regulated inwardly rectifying  $K^+$  channel (GIRK), possibly by interfering with GPCR signalling (200). In addition, Dexas1 has also been found to negatively modulate adenylyl cyclase 2 (AC2) signalling indirectly through the inhibition of both  $G\beta\gamma$ - and PKC-stimulated AC2 activity (188). Further, Dexas1 has been implicated in the maintenance of the circadian clock through integration of photic/nonphotic inputs (201). Dexas1 was shown to affect the photic sensitivity of mice. Behavioral studies conducted using Dexas1 knockout mice ( $Dexas1^{-/-}$ ) showed that  $Dexas1^{-/-}$  mice have lowered responses to photic stimuli and enhanced responses to nonphotic stimuli. Phosphorylated ERK (p-ERK) levels were compared between wild type and mutant mice after both dark and light stimuli (86, 202-204). Light-induced p-ERK expression was significantly attenuated in the  $Dexas1^{-/-}$  mice, indicating that Dexas1 is modulating the response to photic stimulus and regulating the sensitivity of the light-induced activation of the MAPK

pathway. Further, this role of Dexras1 was shown to involve the N-Methyl-D-aspartic acid (NMDA) receptor (205, 206). Other studies have shown that Dexras1 can be activated by the NMDA receptor, which ultimately leads to MAPK activation.

In the primary cortical neurons of mice, activation of Dexras1 by NMDA receptors results in increased iron uptake. In these cells, Dexras1 regulates iron import transporter, divalent metal ion transporter 1 (DMT1), through its interaction with the peripheral benzodiazepine receptor-associated protein (PAP7) (205).

An interesting novel function of Dexras1 as a suppressor of gene transcription was reported in FE65- gamma-secretase-derived amyloid precursor protein (APP)-mediated transcription (207). Dexras1 was shown to be able to bind to the PTB2 domain of FE65 protein and suppresses transcription. Importantly, this study revealed that Dexras1 may also be found in the nucleus, and may be able to interact with other transcription factors to regulate gene transcription.

Other than the fact that glucocorticoids and estradiol regulate Dexras1 expression, the mechanism, and molecular details of Dexras1 regulation have not received much attention. A recent study reported that Dexras1 can also be induced by amphetamine (psychostimulant) in the rat prefrontal cortex, and that D2 dopamine and glucocorticoid receptors regulate these effects (208). The Dexras1 gene can also be silenced through DNA methylation (209). Such an epigenetic inactivation has been suggested to play an important role in the development of glucocorticoid-resistance, especially in the case of multiple myeloma.

### *Chicken Dexas1: Ontogeny and tissue distribution*

The major sites of Dexas1 expression in human and mice include heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (180). Interestingly, Dexas1 expression was found to be limited to the pituitary tissues of 3-weeks-old juvenile chickens (176). Northern blot analysis on other tissues including brain, liver, lung, and pancreas did not reveal Dexas1 expression. Indeed, this does not rule out the possibility that Dexas1 may be expressed in other tissues at a later stage in development or in adult chickens.

Ontogenic profile of Dexas1 expression in the anterior pituitary during development in chicken embryos was determined by microarray and qRT-PCR. Dexas1 expression was detectable at e10 and increased between e12 and e17(142, 176). Dexas1 expression in earlier developmental stages has not been tested. The increase in Dexas1 expression was correlated with an increase in serum CORT levels as well as GH expression in the anterior pituitary(140, 176).

### **Rationale and Objectives**

Glucocorticoids (such as CORT) are involved in GH expression, and glucocorticoid-mediated GH expression is achieved through the combined effects of genomic and non-genomic factors. CORT induction of GH mRNA expression in chickens requires ongoing protein synthesis, suggesting the involvement of unknown intermediary factor(s). Additionally, ras signalling is essential for CORT-mediated GH mRNA expression. The fact that ras signalling is required for CORT-induced GH expression indicates that some

of these unknown intermediary factors are conceivably ras-like factors, or relay signals through the ras pathway. Dexras1, a ras-protein, exhibits a similar expression profile to that of CORT and GH during anterior pituitary development in chickens. Importantly, Dexras1 is a CORT-induced factor, and in e11 cultured chicken pituitary cells, the time required for CORT induction of Dexras1 is comparable to that of CORT induction of GH. Both inductions occur within 3 h of CORT treatment. These facts taken together suggest that Dexras1 could be the unknown CORT-induced factor that mediates CORT effects on GH expression. In addition to the involvement of CORT-induced intermediary factors, GH mRNA expression is also known to involve epigenetic modifications and recruitment of important transcription factors to the GH promoter. Previous studies have never addressed the effect of CORT on epigenetic modifications and transcription factor recruitment associated with the GH promoter. Towards a better understanding of molecular mechanisms involved in CORT induction of GH mRNA, including both genomic and non-genomic factors, the following objectives were addressed in this study.

A) Characterization of glucocorticoid regulation of Dexras1

- Role of GR and MR.
- Role of intracellular cell signalling pathways involved.
- Identification of Dexras1 expression in anterior pituitary cell types
- Effect of CORT on subcellular localization of Dexras1

B) Role of Dexras1 in regulating CORT-induced GH expression

- Effect of overexpression of Dexras1 on GH promoter activity
- Effect of overexpression of Dexras1 on GH mRNA expression

C) Downstream targets of Dexras1 possibly involved in GH expression.

- D) Effect of CORT on histone H3 acetylation, trimethylation of lysine 4, and trimethylation of lysine 27
- E) Effect of CORT on recruitment of GR, Ets-1, and Pit1 to the GH promoter
- F) Effect of CORT on RNA Pol II recruitment



## **CHAPTER 2**

### **Glucocorticoid-regulated histone modifications, and recruitment of GR, Ets1, and Pit-1 to the chicken GH gene**

## **Abstract**

Epigenetic regulation by glucocorticoids through histone modifications plays an important role in gene expression. However, glucocorticoid-regulated modifications of histones associated with the growth hormone (GH) gene remain unknown. In chickens and rodents, glucocorticoids (e.g. corticosterone; CORT) induce GH mRNA expression in the anterior pituitary during embryonic development. 1727bp of the 5'-flanking sequence of the chicken GH gene are sufficient for CORT induction of GH promoter activity. The 1727bp region contains two predicted Pit-1 sites, a glucocorticoid-responsive region (GCRR), and other putative transcription factor binding sites. Using chicken embryonic pituitary cells, we tested the effect of CORT (1.5 h or 6 h) on histone H3 modifications, RNA Polymerase II (Pol II) recruitment, binding of the glucocorticoid receptor (GR) and transcription factors Pit-1 and Ets1 to the 5'-flanking region of the GH gene, using chromatin immunoprecipitation (ChIP) assays followed by quantitative real time PCR. We found increased H3 acetylation and tri-methylation of lysine 4 at both Pit-1 sites and the TSS (transcription start site) in response to 1.5 h CORT treatment. Furthermore, 1.5 h CORT treatment significantly increased recruitment of Pit-1 and Pol II to the proximal Pit-1 site and the TSS, respectively. GR was recruited to the GCRR and the distal Pit-1 site in response to 1.5 h CORT treatment, while 6 h CORT treatment resulted in Ets1 dissociation from the GCRR. Collectively, these results indicate that CORT results in dynamic changes in histone modifications and transcription factor recruitment within the 5'-flanking region of chicken GH gene.

## Introduction

Pituitary growth hormone (GH) expression in chickens can be prematurely stimulated by the adrenal glucocorticoid, corticosterone (CORT). GH expression normally appears around embryonic day (e) 12 in chickens, but e11 chicken pituitary cells treated with CORT show an increase in GH mRNA and protein levels both *in vivo* and *in vitro* (210). In addition, in rodents as well as in chickens, the glucocorticoid-mediated increase in GH gene expression is an indirect effect and involves additional factors (125, 135). Indeed, a role for CORT in regulating GH gene expression in pituitary cells is well established in rodents and chickens (28, 73, 125, 133, 211, 212). Most of the work on glucocorticoid regulation of GH gene transcription has focused on the involvement of transcription factors such as Pit-1 and glucocorticoid receptor (GR). In addition to the transcription factors, the MAPK/ERK pathway is also essential (142). GH expression in mice is known to involve epigenetic modifications (1, 213). However, the function of histone modifications in CORT-mediated GH gene expression is essentially unknown. There have been reports presenting the importance of glucocorticoid-induced histone modifications in regulation of other genes. For example, glucocorticoids inhibit expression of surfactant protein A (SP-A) through the recruitment of histone deacetylases (HDACs) in the human lung (79). Glucocorticoids also regulate H3 phosphorylation and methylation of histones associated with the SP-A gene (80, 170). In mice, glucocorticoids induce H3 acetylation on the  $\beta$ -casein gene (214). In mice, lysine-specific demethylase-1 (LSD1) is essential for pituitary somatotroph and thyrotroph differentiation (1). Successful expression of GH and thyroid-stimulating hormone  $\beta$  (TSH $\beta$ ) depends on the expression of LSD1. Pituitary-specific knockdown of LSD1 results in complete loss of

somatotrophs and thyrotrophs in mice. Although loss of LSD1 did not inhibit Pit-1 recruitment to the GH promoter in these mice, LSD1 was shown to interact with Pit-1, establishing a connection between histone demethylation and pituitary-specific GH expression (1, 174). Moreover, suppression of GH expression in lactotrophs depends on an additional repressor, zinc finger E-box binding homeobox 1 (ZEB1). Interestingly, ZEB1 is known to interact with LSD1 as a part of a repressor complex in the REST (RE1-silencing transcription factor) gene. The role of glucocorticoids in the epigenetic regulation of GH expression was not addressed in this previous study (1).

The main aim of the present study was to determine whether CORT induces histone modifications at the GH promoter. We hypothesized that gene-activating histone modifications, specifically histone H3 acetylation and tri-methylation of lysine 4, are stimulated at the GH promoter by CORT. We further hypothesized that these modifications in response to glucocorticoids are associated with recruitment of additional transcription factors. Pit-1 is known to be essential for GH expression. In addition to Pit-1, we also investigated recruitment of GR and v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) to the GH promoter by CORT. A 1727bp sequence of the chicken GH promoter has been shown to be responsive to CORT in e11 chicken pituitary cells (60, 141). E11 chicken pituitary cells transfected with -1727/+48bp of the GH promoter showed five-fold increased luciferase activity of the reporter construct in response to CORT (141). Moreover, CORT induction of -1727/+48bp GH promoter also required ongoing protein synthesis, thereby mimicking induction of the endogenous GH gene by CORT (141). The 1727bp sequence was subsequently characterized and shown to contain two Pit-1 sites, and a GCRR consisting of a non-canonical glucocorticoid response

element (GRE) half-site, and an Ets1 binding site (60, 141). The proximal Pit-1 site has been identified to be important in the expression of GH (60). The GCRR and its Ets1 and GR-binding sites were shown to be important for CORT-responsiveness of the reporter through mutational analysis (141).

Using ChIP on e11 embryonic chicken pituitary cells, we provide evidence for glucocorticoid regulation of GH gene expression through histone modifications and transcription factor recruitment. Specifically, we have identified sites on the GH promoter that undergo histone H3 acetylation and tri-methylation of lysine 4, both of which are accepted markers of gene activation. We also describe occupancy and recruitment of RNA Pol II, GR, Pit-1, and Ets1 to the different elements in a CORT-dependent manner. Overall, we provide a detailed understanding of both temporal and spatial changes in histone modifications and transcription factor recruitment that occur on the GH promoter in response to CORT.

## **Materials and Methods**

### *Reagents and antibodies*

Cell culture media were purchased from Invitrogen (Carlsbad, CA). Corticosterone was obtained from Sigma Chemical Company (St. Louis, MO). Antibodies used were as follows: acetylated H3 (Millipore #06-599), tri-methylated H3 lysine 4 (Abcam #ab8580), tri-methylation lysine 27 (Abcam #ab6002), RNA Pol II (Santa Cruz Biotechnology #sc-899), Pit-1 (rabbit anti-rat Pit-1 antiserum previously validated) (215), GR (mouse monoclonal anti-rat GR antibody that recognizes chicken

GR) (98), Ets1 (Santa Cruz Biotechnology #sc-112), total H3 (Active Motif #39163), and normal rabbit serum (Calbiochem #869019).

#### *Chicken pituitary dissection and dispersion*

All chicken embryo protocols were approved by the Institutional Animal Care and Use Committee on the campus. Broiler chicken fertilized eggs were incubated in a 60% humidified, 37 C incubator. The first day of incubation was marked as embryonic day (e) 0. Pituitaries were dissected from e11 chicken embryos under a light microscope. An e11 chicken pituitary yields approximately 300,000 cells. Approximately 220 e11 pituitary glands were isolated for each replicate experiment. Anterior pituitary tissues were trypsin digested to obtain a single cell suspension as previously described (39). Cells were counted using the trypan blue exclusion method, and the viability rate was above 95% in all experiments.

#### *Cell culture and chromatin preparation*

Approximately  $5 \times 10^6$  e11 pituitary cells per immunoprecipitation were used. Cells were resuspended in DMEM/F12 cell culture medium with supplements of 0.1% BSA (bovine serum albumen), 100 U/ml penicillin, and 5 $\mu$ g/ml human insulin and plated in 100mm x 20mm Corning<sup>®</sup> cell culture petri dishes. Cells were plated on three petri dishes, one for each time point. Each petri dish containing cells was resuspended in 6ml of culture medium and maintained at 37 C. The cells were allowed to recover overnight, and the cells were then treated with either vehicle (0 h) or CORT (1nM) for 1.5 h or 6 h. Following treatment, 200 $\mu$ l of 37% formaldehyde was added drop-wise while mixing the medium. The petri dishes were then incubated on a rotator for 10 min at

room temperature. Next, 625 $\mu$ l of 1M glycine was added for 5 min to quench the formaldehyde. The petri dishes were then transferred to ice, and the cells were scraped into 50ml conical tubes. The petri dishes were washed twice with 2ml of ice-cold PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). The cells were collected at 3000xg, and the pellet was washed once with PBS containing 1mM PMSF. The cells were resuspended in swelling buffer [25mM Hepes pH 7.8, 1.5mM MgCl<sub>2</sub>, 10mM KCl, 0.1% NP-40, 1mM dithiothreitol (DTT), 0.5mM PMSF, and 1x Halt Protease Inhibitor Cocktail (Thermoscientific, Rockford, IL)] and incubated on ice for 10 min. The cell suspension was homogenized with a Dounce homogenizer with 15 up and down strokes using a loose fitting pestle. The preparations were transferred to 15ml conical tubes and centrifuged at 1800xg for 5 min. The nuclear pellets were resuspended in 2ml of Mononuclease (MNase) Buffer (0.32 M sucrose, 50mM Tris-HCl pH 7.4, 4mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.1mM PMSF, 1% sodium dodecyl sulfate (SDS)) and homogenized with a Polytron PT 1200C (Kinematica, Bohemia, NY) for 30 s on ice. A small volume (15 $\mu$ l) of the preparation was removed at this time and stored as the “non-sheared control”. Next, 10 $\mu$ l of BSA and 7.5 $\mu$ l of mononuclease (MNase) enzyme (New England Bio Labs, Ipswich, MA) were added to each sample and incubated at 37 C for 15 min, with tubes inverted every 5 min. 100 $\mu$ l of 0.5M ethylenediaminetetraacetic acid (EDTA) were added to stop the reaction. At this stage, the samples were stored overnight at -80 C. The samples were then sonicated using a Branson Sonifier 250. The tubes were placed in an ice-ethanol bath to keep the samples cold during sonication. Sonication settings were as follows: 5 cycles, each cycle of 20 s with 30 s rest, continuous pulse, output at 60%, power at 6.

After sonication, the samples were centrifuged at 14,000xg at 4 C for 10 min, and the supernatants were transferred to fresh tubes. Shearing (500-700 bp fragments) was confirmed by separating a small amount of each sample in an agarose gel. Chromatin was quantified using Quant-it Picogreen DNA Quantification Kit (Invitrogen, Carlsbad, CA). The quantified chromatin was aliquoted and stored at -80 C.

#### *Chromatin immunoprecipitation*

Sheared chromatin (10 $\mu$ g) was pre-cleared twice for a total of 24 h at 4 C with rotation with prewashed protein G magnetic beads (New England Bio Labs, Ipswich, MA) and 0.25mg/ml normal rabbit serum. The pre-cleared chromatin was transferred to new tubes and incubated overnight at 4 C with appropriate antibodies. Before addition of antibody, a control aliquot (2% input) was removed. Remaining samples were incubated at 4C overnight with antibodies against total H3, acetyl H3, tri-methyl H3 lysine 4, tri-methyl H3 lysine 27, RNA Pol II, Pit-1, GR, Ets1 or normal rabbit serum (NRS). 2 $\mu$ g of each antibody was incubated with 10 $\mu$ g of pre-cleared chromatin in a 750 $\mu$ l reaction volume. Complexes were precipitated by incubating samples with protein G magnetic beads (New England Biolabs, Ipswich, MA) for 5 h at 4 C with rotation. The beads were washed 2x each with low salt wash buffer (50mM Hepes pH 7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5mM PMSF, 1x Protease Inhibitor Cocktail), high salt wash buffer (50mM Hepes pH 7.9, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5mM PMSF, 1x Protease Inhibitor Cocktail), lithium chloride (LiCl) wash buffer (20mM Tris pH 8.0, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 0.5mM PMSF, 1x Protease Inhibitor Cocktail), and Tris-EDTA (TE) buffer wash (10mM Tris pH 8.0, 1mM EDTA).



The immunoprecipitated chromatin was eluted from the beads with elution buffer (50mM Tris pH 8.0, 1mM EDTA, 1% SDS, 50mM NaHCO<sub>3</sub>) at 65 C with rotation for 30 min. The supernatant was transferred to new tubes containing 15µl of 5M NaCl and 25ng RNaseA and incubated at 37 C for 30 min to reverse crosslinking. Next, 250ng of Proteinase K (Sigma Aldrich, St. Louis, MO) was added, and the samples were incubated at 65 C with rotation overnight. Samples were purified using the Wizard SV PCR clean up kit according to the manufacturer's instructions (Promega, Madison, WI) with the following modification: the final product was eluted twice with 40µl of water.

#### *Quantitative real time PCR (qPCR)*

Real time PCR was performed in 96-well plates on a Bio-Rad iCycler. The following reaction mixture was used: 0.1% Triton X-100, 10mM Tris-HCl, 50mM KCl, 1.9mM MgCl<sub>2</sub>, 2U Taq, 10mM each dNTP, 80nM each primer, 20nM fluorescein, and SYBRgreen II. 5ul of each cleaned immunoprecipitated DNA or input sample was used in 30µl reactions in duplicates. A list of primers used is given in Table 1. A three-step qPCR with the following reaction conditions was used: 40 cycles of 95 C for 15 s, 60 C for 30 s, and 72 C for 30 s. Data are presented as percentage of input. For each sample, a NRS antibody control Ct value was subtracted from the sample Ct value to account for general background. The Ct values were transformed ( $2^{Ct}$ ), and the sample Ct values were adjusted to % of input.

### *Statistical analysis*

Data presented are the means of three to six independent replicates. Using the SAS statistical program (SAS Inc; Cary, NC), statistically significant differences among treatments or groups was determined employing a mixed model ANOVA. 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, different letters denote significance at  $p \leq 0.05$ .

Table 1. List of real time primers used in ChIP

Name		Sequence (5' → 3')
-143/+6	Forward	GGCGAACACATCTGCATTTA
	Reverse	GTTGCTCAGGTGTTGCTTGA
-177 / -69	Forward	CTCCCCAACCTTTCCATCT
	Reverse	CGTGATCACTGCCACCTCT
-607 / - 478	Forward	GCAGCTGACTGCCAGTTTGT
	Reverse	CTTGGATCCACTGCAGAACC
-1065 / -859	Forward	TTTAAACACGACCTGGAGCAGAAAAA
	Reverse	ATTTCCAAGAGCAGCATCATCAC
-1436 / -1299	Forward	ATGATCCTTTGGGTTGCACA
	Reverse	GTTTTGTTTCCCCTGCTTGC
-2187 / -2037	Forward	AGGGGAAACTGTGAGCAAAC
	Reverse	GAAGGCTGAAATGCTCTCCT
GAPDH promoter	Forward	GTCACGTCCCAGGAGCAG
	Reverse	AGGACCGTGCTAATGAGGAA
+456/+573 (nascent transcript)	Forward	GGATGTCTCCACAGGAACGC
	Reverse	GACAACCTTACTGACTTCTGCTGGG

## Results

The effects of CORT on covalent modifications of GH-associated histone H3 were evaluated using ChIP. Using the same assay, CORT-regulated recruitment of transcription factors (GR, Pit-1, and Ets1) to their putative binding sites on the GH promoter was also assessed. To characterize the temporal and spatial modifications of histone H3 and recruitment of the above mentioned transcription factors, ChIP analysis was performed on e11 chicken primary pituitary cells treated with CORT for different time points (0 h, 1.5 h and 6 h). The time points reflect CORT induction of GH mRNA expression. As reported previously, in response to CORT, GH mRNA levels in cultured primary pituitary cells begin to increase around 1.5 h, and reach the maximum peak at 6 h (135). The shorter time point was included to allow detection of histone modifications that may be transient. The 0 h time point served as the control. About 2kb of the 5'-flanking region of the GH promoter was analyzed by ChIP. Six real time primer pairs were designed to screen a distance of 2kb upstream of the GH transcription start site, including the GCRR and the two Pit-1 binding sites, as shown in Fig 11.

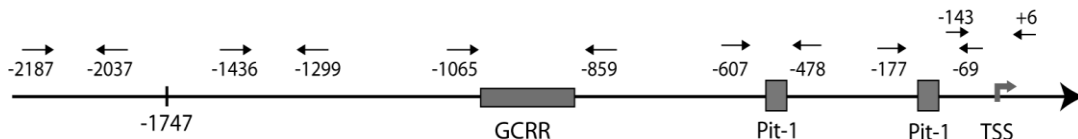


Figure 11. Schematic representation of the chicken GH gene promoter showing primer pairs used in quantitative real time PCR. Also indicated are the positions of the GCRR, two Pit-1 sites, and the TSS.

Primers to glyceraldehyde 3- phosphate dehydrogenase (GAPDH) were used as a control for histone modifications. GAPDH also served as a negative control for transcription factor recruitment and binding. The real time primers were designed at approximately 300-350bp intervals.

*CORT increases histone H3 acetylation at both proximal and distal Pit-1 sites and the TSS*

It is widely accepted that histone modifications influence chromatin remodelling and promoter activation. Histone H3 acetylation at lysine residues 9 or 14 (K9 or K14) by histone acetyltransferases (HATs) is often linked to transcriptional activation. We hypothesized that CORT increases GH mRNA levels by increasing transcriptional activity at the GH promoter through an increase in H3 acetylation. To explore this hypothesis, ChIP experiments were performed on e11 chicken primary pituitary cells cultured in the presence or absence of CORT for 0 h, 1.5 h, or 6 h, using antibody directed against H3-K9/14 acetylation. The results are summarized in Fig 12(a). Effect of CORT on H3 acetylation was compared to the basal (0 h time point) within a single region. We found that treatment with CORT (1nM) for 1.5 h significantly enriched histone H3 acetylation signal at the distal Pit-1 site (-607/-478), proximal Pit-1 site (-177/-69), and the TSS (-143/+6) (n=6, p<0.05). There was no effect of CORT treatment on histone H3 acetylation in regions further upstream from the TSS, including the GCRR region (p>0.05). Interestingly, after 6 h of CORT treatment, H3 acetylation at both Pit-1

sites and the TSS returned to basal levels. Overall, 1.5 h CORT treatment increased histone H3 acetylation at important sites of the GH promoter.

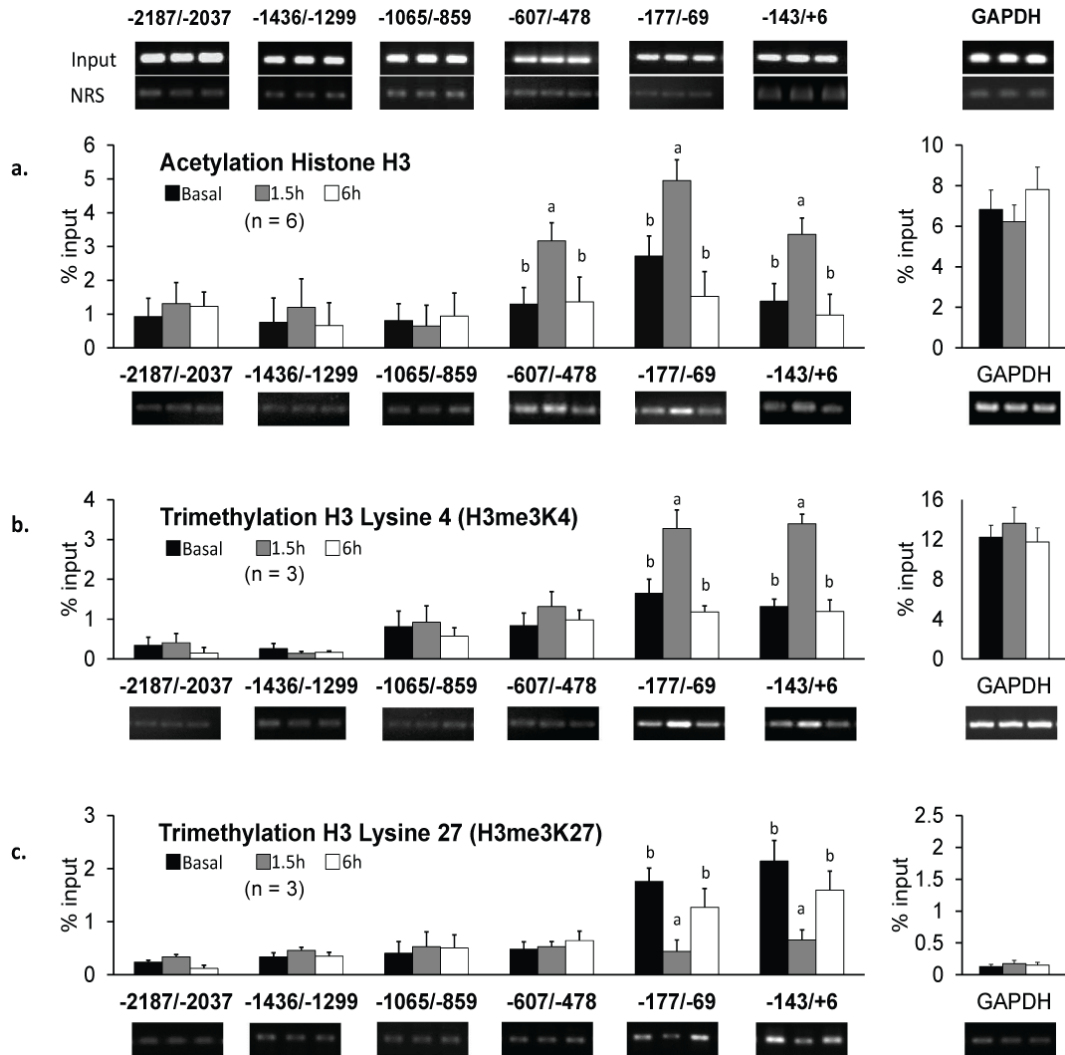


Figure 12. Effects of CORT treatment (0 h, 1.5 h, and 6 h) on histone acetylation (a), trimethylation of lysine 4 (b), and tri-methylation of lysine 27 (c). For each, real time PCR results are presented as percent input. Values with different letters indicate a significant difference (a; n=6, b; n=3, c; n=3 independent experiments) ( $p < 0.05$ ). Real time PCR products after 40 cycles from one replicate were visualized in an agarose gel.

### *CORT-dependent increase in H3me3K4 and reduction in H3me3K27*

Next, we examined a second histone H3 modification associated with gene activation, tri-methylation of lysine 4 (H3me3K4; Fig 12b). ChIP assay was performed using antibodies specific for H3me3K4 (Fig 12b). We found significantly enhanced H3me3K4 at the proximal Pit-1 site and the TSS in response to 1.5 h CORT treatment (n=3, p<0.05). 1.5 h CORT treatment caused ~ 3- to 3.5- fold increase in H3me3K4 at both sites. H3me3K27 states were also examined (Fig 12c). H3me3K27 is generally associated with gene repression. 1.5 h CORT treatment resulted in reduced H3me3K27 at the proximal Pit-1 and TSS sites. The CORT-induced increase in H3me3K4 at the proximal Pit-1 and TSS sites was consistent with the CORT-induced decrease in H3me3K27 signals (n=3, p<0.05). No differences in H3me3K4 or H3me3K27 states were observed at any other sites.

### *Recruitment of RNA Pol II*

Next, we investigated if there was a correlation between recruitment of RNA polymerase II and gene activating histone H3 modifications. As shown in Fig 13, 1.5 h CORT treatment increased recruitment of RNA Pol II to the TSS (n=3, p<0.05). The histone modifications and recruitment of RNA Pol II results had similar profiles.

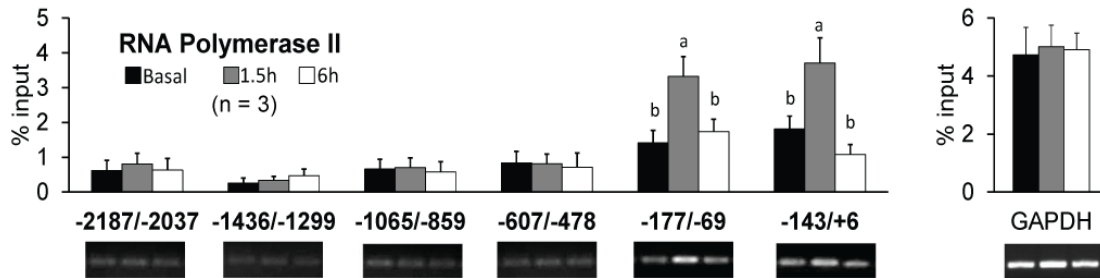
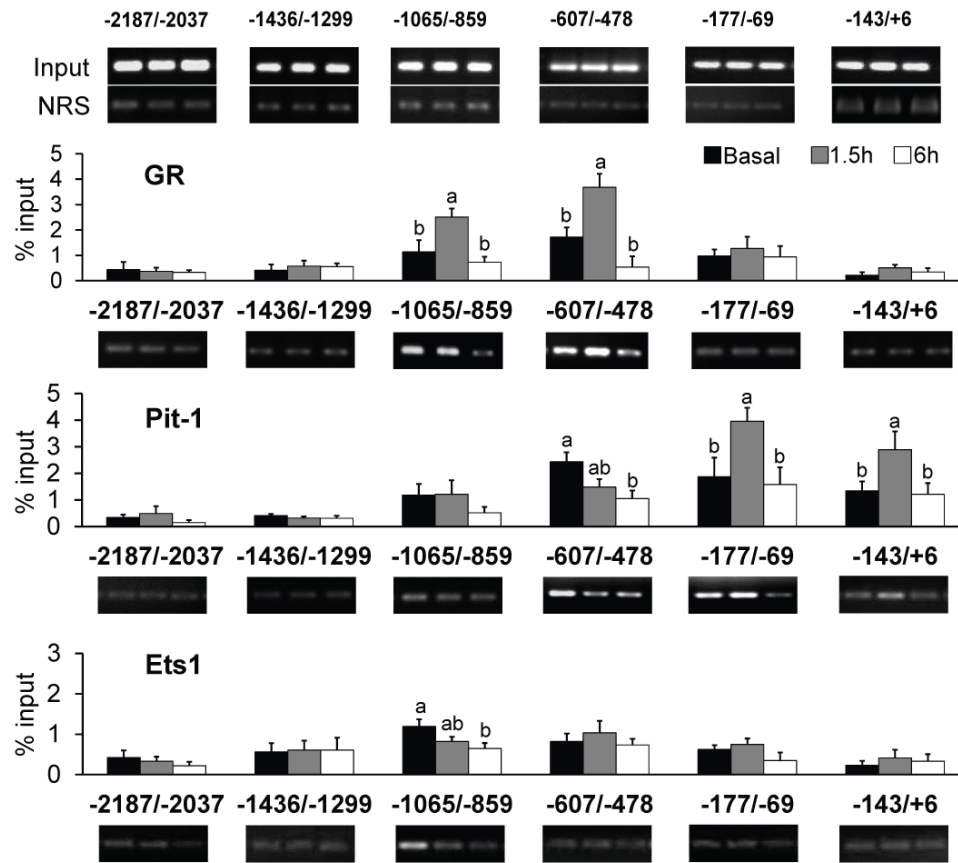


Figure 13. RNA polymerase recruitment to the chicken GH promoter in response to CORT treatment for different time points (0 h, 1.5 h, and 6 h). For each, real time PCR results are presented as percent input. Values with different letters indicate a significant difference (n=3 independent experiments; p<0.05). Real time PCR products after 40 cycles from one replicate were visualized in an agarose gel.

#### *CORT regulates GR, Ets1, and Pit-1 association with the GH promoter*

Knowing that Pit-1 sites and the GCRR site with its GRE-half site and Ets1 site are important for GH gene expression, we tested whether GR, Ets1, and Pit1 are recruited to their respective binding sites in response to CORT. Results are summarized in Fig 14a. Under basal conditions, Pit-1 was associated with the distal Pit-1 site. In response to 1.5 h CORT treatment, we detected increased Pit-1 binding to the proximal Pit-1 site, and decreased Pit-1 binding to the distal Pit-1 site (n=5, p<0.05). Further, under basal conditions, Ets-1 and GR were associated with the GCRR of the GH gene, and 1.5 h CORT treatment increased GR recruitment to the GCRR (n=5, p<0.05). Interestingly, 1.5 h CORT treatment also increased GR binding to the distal Pit-1 site (n=5, p<0.05). Surprisingly, 6 h CORT treatment resulted in dissociation of Ets1 from the GCRR site and significantly reduced Pit-1 at the distal Pit-1 site (n=3, p<0.05).

a)



b)

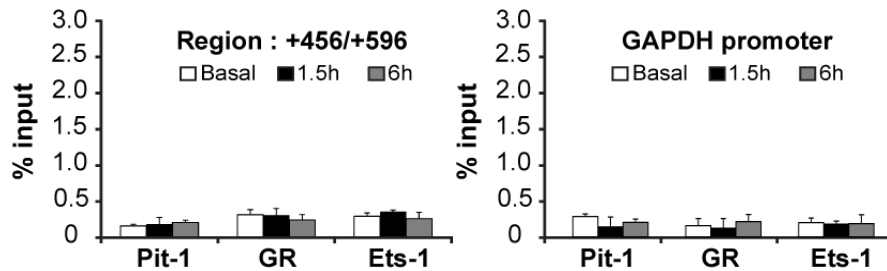


Figure 14. Recruitment of transcription factors to the chicken GH promoter in response to CORT treatment. (a) GR, Pit-1, and Ets1 (b) Real time PCR results using primers against nascent GH transcript (+456/+596) and GAPDH. ChIP on e11 chicken pituitary cells looking at recruitment of transcription factors in response to CORT treatment for 0 h, 1.5 h, or 6 h. Real Time PCR results presented as percent input. Values with different letters indicate a significant difference (n=5 independent experiments; p<0.05). Real time PCR products after 40 cycles from one replicate were visualized in an agarose gel.



Primers to the 3' region of the nascent GH transcript (region +456/+573) and GAPDH promoter were used as negative controls (Fig 14b). None of the factors bound to either of these regions. Overall, these results indicate that CORT regulates Pit-1, Ets1 and GR association with the GH promoter elements.

## **Discussion**

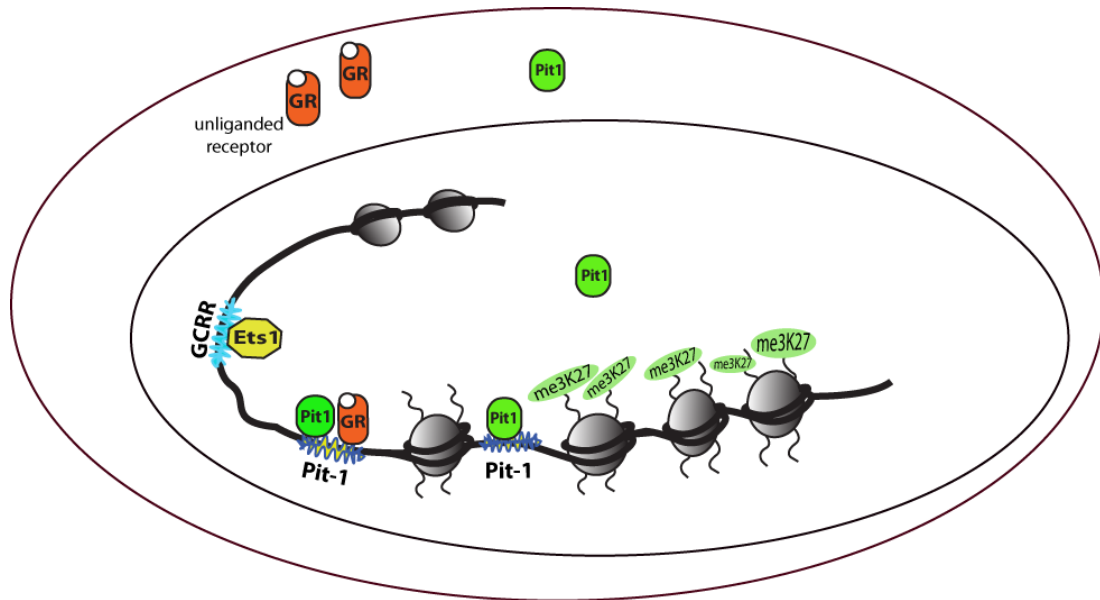
The aim of this study was to determine if CORT-induced increase in GH transcription involves histone modifications and recruitment of specific transcription factors to the GH promoter. Previous work has shown indirect involvement of epigenetic modifications in somatotroph differentiation (1, 213). To the best of our knowledge, there have been no studies conducted investigating the epigenetic modifications with respect to glucocorticoid regulation of the GH gene in any species. Using ChIP on cultured embryonic chicken pituitary cells, we evaluated CORT-dependent histone modifications as well as transcription factor recruitment to the 5'-flanking region of the GH gene. Importantly, we demonstrate for the first time changes that occur on the endogenous chicken GH gene in response to CORT. We believe that glucocorticoid-stimulated histone modifications together with recruitment of transcription factors and RNA Pol II are important events leading to GH mRNA expression.

Histone modifications play an important role in regulating gene transcription (146, 149, 150, 216). Histone H3 tails undergo several different modifications, including acetylation, tri-methylation and phosphorylation, and the state of chromatin is controlled by the combined effects of the different modifications. Histone acetylation and tri-

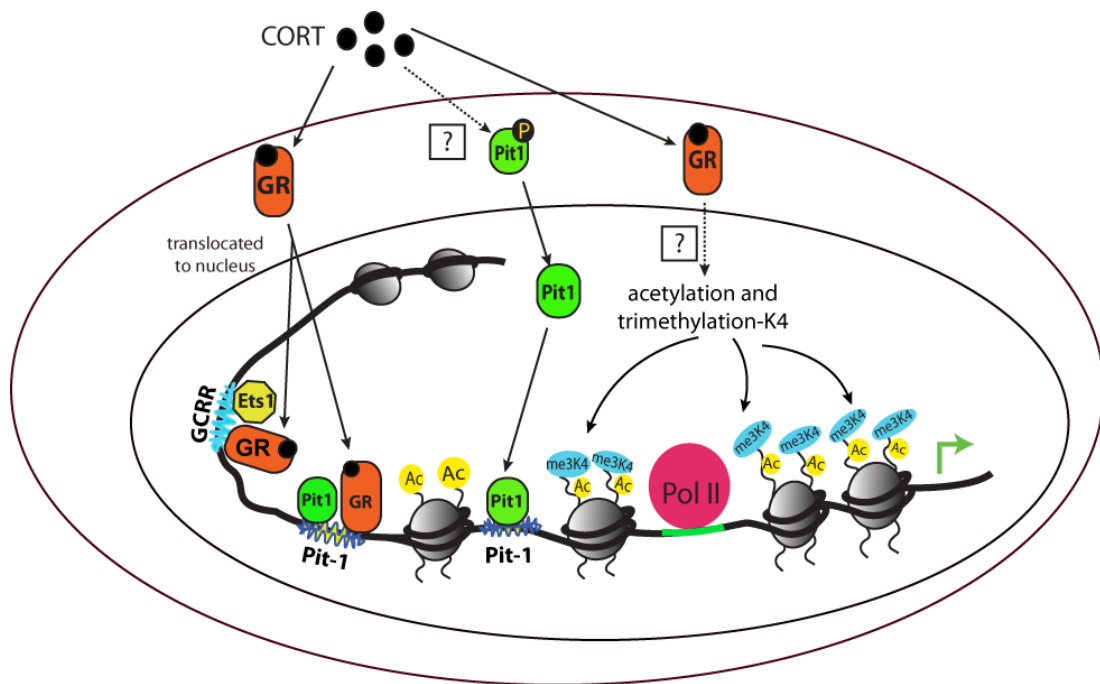
methylation are two well-established modifications associated with activation of gene transcription (217). Consistent with the idea that CORT increases GH gene transcription through histone modifications, our results show that CORT treatment indeed increases histone acetylation at the TSS and proximal Pit-1 site of the 5'-flanking region of the GH gene. Like acetylation of histone H3, tri-methylation of H3 lysine 4 is also associated with actively transcribed genes (218-221), while tri-methylation of H3 lysine 27 marks gene silencing or a repressed state (222, 223). We demonstrate that CORT increases tri-methylation of lysine 4 at the GH TSS. The pattern of lysine 27 tri-methylation was opposite to that of tri-methylation of lysine 4. The histone H3 trimethylation of lysine 4 and acetylation results taken together support a role for these modifications in active transcription of GH gene in response to CORT treatment. The acetylation and tri-methylation results correlate with the observed RNA Pol II recruitment, which further supports the idea that CORT enhances GH gene transcription through dynamic changes in chromatin structure through histone modifications.

GR, Pit-1, and Ets1 are important transcription factors involved in GH gene expression, and each has putative binding sites in the 5'-flanking region of the GH gene. Deletion of the GCRR or mutation of its Ets1 or GR-binding sites significantly attenuates CORT induction of GH promoter activity (141). Further, Ets1 and GR association with their putative binding sites within the GCRR have also been confirmed by EMSA, further supporting our ChIP results (141). Our overall results can be summarized into three states as illustrated in Fig 15.

A) Basal :



(B) 1.5 hour CORT



(C) 6 hour CORT

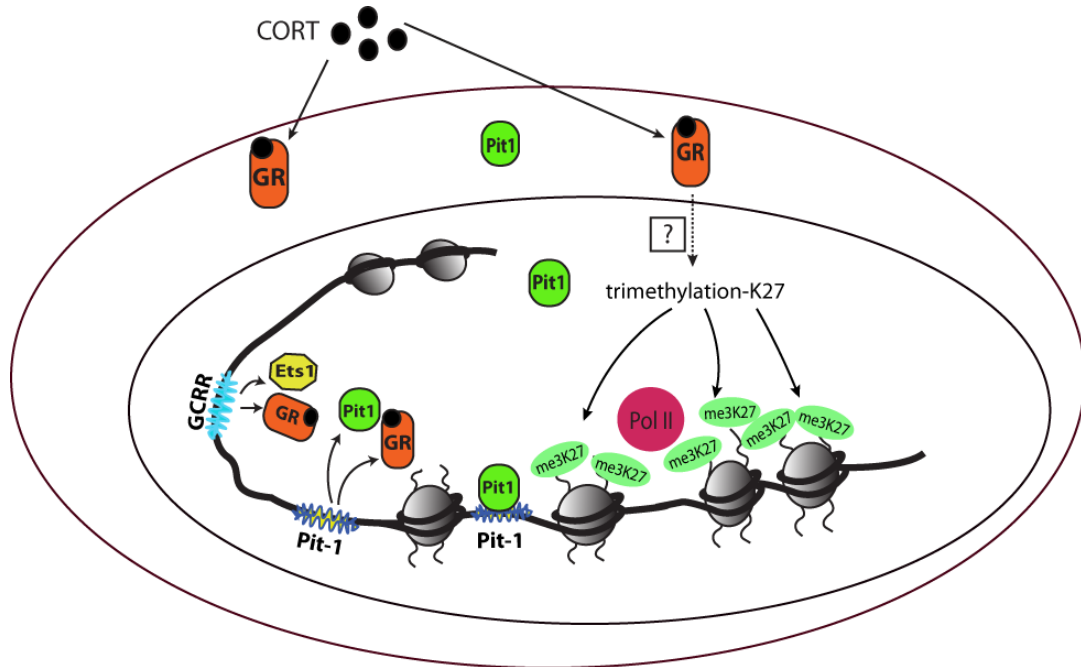


Figure 15. Proposed model for histone modifications and transcription factor recruitment to the 5'-flanking region of the GH gene in response to CORT. (A) Basal: Chromatin is highly trimethylated on lysine 27 of H3, a marker for repressed or inactive state. The TSS is not accessible. GR is found in the cytoplasm. Pit-1 is bound to distal Pit-1 site, and Ets1 is associated with the GCRR. (B) 1.5 h CORT: GR translocates to the nucleus and binds to the GCRR. In addition, CORT increases Pit-1 recruitment to the proximal Pit-1 site. CORT-induced histone acetylation and tri-methylation of lysine 4 opens up the chromatin. RNA Pol II is recruited to the TSS, and switches 'ON' gene transcription. (C) 6 h CORT: Ets1 dissociates from the GCRR. Transcription factors GR and Pit-1 also dissociate, as does RNA Pol II. Increased chromatin deacetylation along with increased tri-methylation of lysine 27 returns chromatin to the closed repressed state.

Under basal conditions, Pit-1 is bound to the proximal and distal Pit-1 sites, but Pit-1 alone may not be sufficient for GH expression. The TSS is likely inaccessible with histone H3 highly trimethylated at lysine 27. This keeps the chromatin in a closed inactive state. Ets1 is bound to the GCRR, and we hypothesize that Ets1 may have a role in maintaining a repressed state of GH gene transcription. The unliganded GR stays in the cytosol, although under basal conditions some GR is bound to the GCRR and the distal Pit-1 sites. Even though some studies have suggested that Pit-1 is predominantly found in the nucleus, the mechanism of CORT regulation of Pit-1 remains largely unknown. Nevertheless, we have shown here that some Pit-1 is bound to both distal and proximal Pit-1 sites in the GH promoter, even under basal conditions. In the presence of CORT, CORT binds to GR and activates it. Liganded GR translocates to the nucleus, and binds to the GCRR. Ets1 may be displaced, but our ChIP results suggest it is still bound to the GCRR region. There is a substantial increase in histone acetylation and histone trimethylation of lysine 4 at both the proximal Pit-1 and the TSS sites, and trimethylation of lysine 27 decreases, conferring an open and more active chromatin state. There is concomitant increased Pit-1 binding to the proximal Pit-1 site consistent with the idea that Pit-1 is required for GH expression. Further, there is a correlated increase in RNA Pol II recruitment, again in accordance with the idea of increased transcription. GR recruitment to the distal Pit-1 site and Pit-1 recruitment to the proximal Pit-1 site raise several interesting propositions. The obvious question being: Is there an interaction between GR, Ets1, and Pit-1, and is this crosstalk important for GH gene regulation? This question begs to be asked and further investigated.

The mechanism by which CORT induces histone modifications near the TSS of GH gene remains to be answered. In e11 pituitary cells, treatment with histone deacetylase inhibitors HC toxin or TSA completely blocked CORT induction of GH mRNA, consistent with our results (142). It is important to note that in our studies acetylation of histone H3 was decreased at 6 h, which provides additional support for involvement of HDACs in the process. It is known from previous studies that GR interacts with histone modifiers (HATs and HDACs) and may indirectly regulate gene transcription in a tethered manner. The CREB binding protein (CBP) and p300 are important GR modulators and interacting partners with intrinsic HAT activity (108). The p300/CBP associated factor (P/CAF) is the other HAT that is known to interact with GR (224). Other than the HATs, GR-mediated activation of genes also involves coactivator-associated arginine methyltransferase 1 (CARM1), a histone H3 methyltransferase (225-227). In mice, the lysine demethylase LSD1 is suggested to interact with WD repeat domain 5 (WDR5) factor, a component of the myeloid/lymphoid or mixed-lineage leukemia (MLL1) coactivator complex in the GH promoter. The MLL1 coactivator complex consists of four elements: MLL1 (a histone methyltransferase), RbBP5 (retinoblastoma binding protein 5), Ash2L (ash2 (absent, small, or homeotic)-like), and WDR5, and this complex catalyzes histone H3 methylation (1, 154, 174). Overall, GR-mediated histone modifications have been demonstrated in other genes, and our findings indicate that similar GR-mediated factors may be involved in regulating the GH gene in chicken embryos. Further studies are required to characterize the detailed molecular mechanisms of these actions. Based on our results, GR is found bound to the GCRR and in response to CORT treatment we noted an increase in GR recruitment to the GCRR

along with increased Pit-1 binding to the proximal Pit-1 site. From previous work reported by others about GR-interacting partners (224), we can speculate that GR may be recruited or bound to the GH promoter as part of a large complex consisting of factors with intrinsic HAT activity such as CBP/p300, etc. In response to CORT, a cascade of events is initiated, including additional recruitment of GR to the GCRR site and Pit-1 to the proximal Pit-1 site. This is followed by RNA Pol II recruitment and HAT- and histone methyltransferases-dependent histone H3 modifications that result in increased histone H3 acetylation and trimethylation. These events finally lead to the recruitment of basal transcriptional factors and GH gene transcription.

This study is the first of its kind in characterizing histone modifications in association with transcription factor recruitment to the GH promoter. The direct interacting partners involved in CORT-mediated regulation of GH transcription are yet to be identified, but this study does provide the basis to investigate possible protein-protein interactions on the GH promoter. Protein-protein interactions not limited to GR, Pit-1, and Ets1 also need to be investigated. In summary, our results demonstrate CORT-mediated histone modifications and transcription factor recruitment to the chicken GH gene.

## **CHAPTER 3**

### **Characterization of chicken Dexras1 and its potential involvement in regulation of pituitary growth hormone gene expression**



## **Abstract**

Glucocorticoid (CORT) induction of growth hormone (GH) in the anterior pituitary of embryonic chickens requires ongoing protein synthesis. Microarray screening conducted on 14,053 unique chicken cDNAs identified Dexas1 as a novel candidate possibly involved in regulating GH expression in response to glucocorticoids. The aim of this project was to characterize chicken Dexas1 and its effects on CORT-mediated GH regulation. Dexas1 was only recently identified and is not well characterized in any species, and importantly Dexas1 has not been previously studied in the context of pituitary functions. We have confirmed expression of endogenous Dexas1 mRNA in both caudal and cephalic lobes of the chicken anterior pituitary, specifically within somatotroph and corticotroph pituitary cell-types. Further, CORT induction of Dexas1 involves glucocorticoid receptor (GR) and requires both MEK1/2 and Ras cell signalling pathways. The effect of overexpression of Dexas1 on GH promoter activity as well as GH mRNA levels was examined. Dexas1 (1 $\mu$ g of vector) alone did not influence the activity of a 1727bp-GH reporter. In contrast, overexpression of Dexas1 (1 $\mu$ g of vector) significantly inhibited CORT-induced GH mRNA levels, while lower doses of Dexas1 plasmid (10ng and 100ng) stimulated basal GH expression. This effect was specific to GH and did not include mRNA levels for other hormones or CORT-regulated genes. Downstream targets of Dexas1 in this GH effect are not clear. Knowing that ERK1/2 signalling is required for CORT induction of GH, effects of Dexas1 expression on a well-established ERK1/2 effector, Elk-1 were investigated. Dexas1 overexpression did not affect phosphorylation of Elk-1 as examined in LMH (Leghorn male hepatoma) and GH<sub>4</sub>C<sub>1</sub> cells. We conclude that Dexas1 is a novel ras protein expressed in the

somatotroph cells of the anterior pituitary in chickens that exhibits a potential role in regulating GH expression. The molecular mechanism of GH mRNA regulation by Dexras1 needs further investigation.

## **Introduction**

Regulation of growth hormone (GH) expression by corticosterone (CORT) during development in embryonic chicken pituitary cells is an indirect effect requiring ongoing protein synthesis (135). The GH secreting somatotroph cells in the anterior pituitary appear around embryonic day (e) 12 in chickens (39), and somatotroph differentiation can be prematurely induced by CORT treatment (124). Ontogeny of GH-producing cells correlates with an increase in serum CORT levels in chickens, further supporting the importance of CORT in GH regulation (28, 228). In cultured embryonic chicken pituitary cells, GH mRNA levels are significantly increased within 6 h of CORT treatment (135). In a microarray study conducted to identify possible genes mediating the effects of CORT on GH expression, Dexras1 was selected as a potential candidate (176). Dexras1 was selected, because it is directly regulated by CORT and because Dexras1 mRNA levels were significantly enhanced within 6 h of CORT treatment. In addition, the ontogeny of Dexras1 correlates with that of GH and CORT in developing chicken embryos (140, 175, 176). Furthermore, CORT induction of GH mRNA expression was completely blocked in the presence of manumycin, a ras inhibitor, suggesting that ras signalling or a ras-like factor is required for CORT induction of GH expression (135). The requirement for a ras-like factor made Dexras1 an even more interesting candidate to be pursued.

Dexas1 is a guanine triphosphate (GTP)-binding protein first identified in AtT-20 corticotroph cells as a CORT-inducible gene (177). Subsequently, Dexas1 has been identified in several other species, including humans (180, 229), rodents (181, 230), and chickens (175, 176). The mechanism of CORT induction of Dexas1 and the downstream signalling targets of Dexas1 are largely unknown. The regulation of Dexas1 by CORT appears to be at the gene transcription level and occurs in a dose-dependent manner (177). Although a glucocorticoid response element (GRE) has been identified in the 3'-flanking region of the human Dexas1 gene (229), sequence analysis of 5'- and 3'-flanking regions of chicken Dexas1 did not reveal a conserved GRE (176). Dexas1 belongs to the family of activators of G protein signalling (AGS) and is structurally similar to that of other ras proteins, except that it contains an extended carboxyl terminus (177, 180, 195, 197). The role of Dexas1 appears to be very complicated, and it has been shown to exhibit different ligand-dependent and ligand-independent (or basal) signalling (86, 188, 190, 192, 198, 200). Dexas1 has been proposed to act as a guanine exchange factor (GEF) and promote ligand-independent activation of the G $\beta\gamma$  signalling effector and extracellular signal-regulated kinases 1/2 (ERK1/2), while at the same time suppressing the receptor-dependent activation of the same signalling pathway.

Some reports suggest a role of Dexas1 in pituitary hormone secretion (195). In pituitary corticotrophs, the negative feedback regulation of ACTH secretion by CORT may involve Dexas1. Further, a constitutively active form of Dexas1 in AtT-20 cells suppressed cAMP-stimulated secretion of transiently overexpressed GH (195). Chicken Dexas1 is highly similar to known species like human, mouse, and rat. It contains all important ras motifs, including the P-loop, the guanine base binding loops, and the C-

terminal CAAX domain. Dexras1 mRNA in juvenile chickens was only found in the pituitary (176).

The aim of this work was to identify potential roles of Dexras1 in regulating chicken GH expression. In addition, we examined whether Dexras1 may play a role in regulating expression of other pituitary hormones. We confirmed expression of Dexras1 in chicken pituitary cells. Glucocorticoid regulation of Dexras1 mRNA expression was also addressed. We tested the involvement of some of the known cell signalling pathways in glucocorticoid regulation of Dexras1 expression, and defined the role of GR and mineralocorticoid receptor (MR) in this process. Dexras1, like other ras proteins, has been shown to undergo post-translational modifications such as prenylation. Prenylation in ras proteins results in the translocation of the ras protein factor to the plasma membrane (182). Although, functioning of Dexras1 does not necessarily always depend on its translocation to the plasma membrane in all systems, we tested whether CORT treatment results in Dexras1 translocation or causes a change in its cellular localization. Our study provides evidence for Dexras1 mRNA expression in chicken pituitary cell types, an understanding of the processes that regulate Dexras1, and identifies a novel potential role for Dexras1 in regulating GH expression.

## **Materials and Methods**

### *Chicken pituitary dissection and dispersion*

All chicken embryo protocols were approved by the Institutional Animal Care and Use Committee on the campus. Broiler chicken fertilized eggs were incubated in a

60% humidified, 37 C incubator. The first day of incubation was defined as embryonic day (e) 0. Pituitaries were collected from e11, e17 or e18 (depending on the experiment) embryos. An e11 chicken pituitary yields approximately 300,000 cells, while an e17-e18 pituitary yields about 850,000 cells. Anterior pituitary tissues were trypsin digested to obtain a single cell suspension as previously described (39). Cells were counted using the trypan blue exclusion method, and the viability rate was above 95% in all experiments. To define *Dexas1* expression within the anterior pituitary cells, caudal and cephalic pituitary lobes from e18 chicken embryos were dissected under light microscopy. The lobes were snap frozen with liquid nitrogen and stored at -80 C for total RNA extraction.

#### *Cell culture*

Primary chicken pituitary cells (e11, e17, or e18) after trypsin digestion were plated on poly-L-lysine coated 12-well or 24-well plates (Corning Life Sciences, Lowell, MA). In the case of experiments that did not involve transfection, dispersed pituitary cells were plated and allowed to attach for 1 h in a 37 C incubator in serum-free Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12) supplemented with 0.1% bovine serum albumen (BSA), 5 $\mu$ g/ml human insulin, 100 U/ml penicillin G, and 100 $\mu$ g/ml streptomycin sulfate, before treatment with any hormones, inhibitors or activators. GH<sub>4</sub>C<sub>1</sub> cells, a rat pituitary cell line, were maintained in DMEM/F12 medium supplemented with 12.5% horse serum and 2.5% fetal bovine serum in 75-cm<sup>2</sup> flasks. LMH cells, a chicken hepatocellular carcinoma epithelial cell line, were maintained in Waymouth's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) in 0.1% gelatin coated 75-cm<sup>2</sup> flasks.

For GR and MR studies, intracellular GR and MR signalling were inhibited using antagonists, ZK98299 (kindly supplied by Schering AG, Berlin, Germany) and spironolactone (Sigma), respectively. Both inhibitors were used at 100nM. Cells were pretreated with antagonists for 1 h followed by CORT (1nM) treatment, as indicated.

For intracellular signalling pathway studies, the following inhibitors and activators were used: LY294002 (PI3-K inhibitor, 50 $\mu$ M), U0126 (ERK1/2 inhibitor; 10 $\mu$ M), manumycin A (ras inhibitor; 1 $\mu$ M), anisomycin (p38/JNK MAPK activator; 10 $\mu$ M), EGF (receptor tyrosine kinase activator; 100ng/ml), forskolin-IBMX (PKA activator; 1 $\mu$ M), and phorbol-12-myristate-13-acetate (PMA; PKC activator; 1 $\mu$ M). Cells (1x10<sup>6</sup>/well in a 24-well plate) were pretreated with vehicle (DMSO) or inhibitors for 1h before addition of CORT (1nM) for 3 h. In the case of activators, no pretreatment was performed. CORT and activators were added together for 3 h. In experiments evaluating levels of phosphorylated Elk-1, 24 h after transfection, LMH or GH<sub>4</sub>C<sub>1</sub> cells (5x10<sup>6</sup>/well in a 12-well plate) were left untreated (basal) or treated with CORT (1nM) or EGF (100ng/ml) for 3 h.

#### *Plasmids and transfection*

The reporter construct used to analyse GH promoter activity was kindly provided by Dr. F. Leung (University of Hong Kong). The -1727 to +48 bp sequence of the chicken GH gene driving firefly luciferase (pGL3-1727) has previously been demonstrated to be responsive to dexamethasone in GH<sub>4</sub>C<sub>1</sub> cells (137) and to CORT in chicken embryonic pituitary cells (141). The empty reporter construct (pGL3-Basic) and

the normalization plasmid constitutively expressing renilla luciferase (pRL-SV40) used in promoter analysis studies were purchased from Promega (Madison, WI).

Dexas1 cDNA was non-directionally subcloned into the CMV promoter driven pCMV-Sport6.1 expression vector (Sport6.1; Invitrogen) or into pEYFP-C1 expression vector (for N-terminal YFP tagging, kindly provided by Dr. Iqbal Hamza, University of Maryland). Primers (Sigma Aldrich) used for PCR amplification of the coding region are given in Table 2. PCR amplification to generate insert was conducted using AccuPrime Pfx SuperMix (Invitrogen) according to the manufacturer's protocol. PCR cycling parameters were as follows: 95 C for 5 min; 35 cycles of 95 C for 15 s, 58 C for 45 s, and 68 C for 2 min; and a final extension at 68 C for 5 min. Products were digested using EcoRI restriction enzyme. Clones were screened using PCR for directionality of the insert using vector specific and insert specific cloning primers. A clone with the appropriate insert in the correct orientation was purified using the NucleoBond PC 500 plasmid purification kit (Macherey-Nagel, Inc., Bethlehem, PA). All clones were sequenced in their entirety by the sequencing center at the University of Maryland's Center for Biosystems Research.

All transfections were performed in supplement-free optimized modified Eagle's minimal essential medium (OptiMEM I) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For studies investigating the effect of Dexas1 overexpression on GH promoter activity,  $5 \times 10^5$  e11 pituitary cells per well in a 12-well plate were allowed to attach for 1h in supplement-free OptiMEM I medium, followed by transient transfection with Lipofectamine 2000. The transfection medium was formulated in OptiMEM I, and contained 1 $\mu$ g of expression plasmid (pSport6.1 or Dexas1), 1 $\mu$ g of

reporter plasmid (pGL3-1727 or pGL3-Basic), 20ng pRL-SV40 and 4 $\mu$ l Lipofectamine. Cells were transfected for 6 h, and after 6 h, the medium was replaced with DMEM/F12 cell culture medium. Cells were allowed to recover for at least 16 h, followed by CORT (100nM) treatment for 20 h.

For experiments investigating effects of Dexras1 on GH mRNA expression, e11 pituitary cells ( $3 \times 10^6$  cells in a 12-well plate) were co-transfected with 1 $\mu$ g of golgi-targeted green-fluorescent protein (GFP) expression vector and 1 $\mu$ g Sport6.1 or Dexras1 expression plasmid in transfection medium as before (OptiMEM I with 4 $\mu$ l Lipofectamine). Cells were transfected in suspension for 2 h, followed by 4 h of transfection in the plate (total 6 h of transfection). At the end of 6 h of transfection, medium was removed and replaced with DMEM/F12 cell culture medium. After 16 h of recovery, cells were treated with CORT (1nM) for 20 h. GFP was used as a marker to collect positively transfected cells by fluorescence activated cell sorting (FACS). After 20 h of CORT treatment, cells were retransfected and washed once with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 0.25ml of ice-cold PBS until FACS sorting.

Transfection of LMH and GH<sub>4</sub>C<sub>1</sub> cells were similarly conducted using Lipofectamine2000 in plates. For confocal microscopy, LMH or GH<sub>4</sub>C<sub>1</sub> cells were plated on coverslips placed on the bottom of a 6-well plate, and transfected with 2 $\mu$ g of EYFP-Dexras1 expression plasmid for 6 h in OptiMEM-I with 8 $\mu$ l of Lipofectamine. After 6 h of transfection, medium was replaced with cell culture medium, and cells were allowed to recover and express protein for 36 h after transfection.



To investigate the effects of Dexras1 on phosphorylation of p-Elk-1, LMH and GH<sub>4</sub>C<sub>1</sub> cells were plated (12-well plate) overnight, and transfected when 90% confluent with 1µg of pSport6.1 or Dexras1 expression plasmid for 6 h in OptiMEM I with 4ul of Lipofectamine. After 6 h of transfection, medium was replaced with appropriate cell culture medium (Waymouth for LMH or DMEM/F12 for GH<sub>4</sub>C<sub>1</sub> with supplements). Cells were allowed to recover for at least 16 h. Cells were then left untreated (basal) or treated with EGF (100 ng/ml) or CORT (1nM) for 3 h followed by cell lysis for protein analysis.

#### *Intracellular staining of hormone cell types*

Following dispersion, e17 pituitary cells ( $5 \times 10^6$  cells per treatment) were subjected to intracellular staining using the BD Cytotfix/Cytoperm™ Kit (#554714) with the following modifications. After dispersion, cells were resuspended in 250µl of cytotfix/cytoperm buffer (3.7% formaldehyde), and incubated for 20 min at 4 C. Cells were washed twice in 250µl of Perm/Wash buffer, using centrifugation at 1000xg for 5min for each wash. Primary unconjugated antibodies were diluted in the perm/wash buffer, and cells were resuspended in the primary antibody containing perm/wash buffer and incubated for 1 h at 4 C. All the hormone-specific primary antibodies have been previously characterized. Dilution of different antibodies used were as follows: rabbit anti-chicken GH (1:2000), rabbit anti-chicken PRL (1:1000), rabbit anti-rat TSHβ (1:1500), mouse monoclonal anti-chicken ACTH (1:1000), and mouse monoclonal anti-chicken LHβ (1:1000). Cells were washed thrice (each time with 500µl of buffer) with perm/wash buffer, followed by incubation for 30 min (4 C in dark) with FITC-conjugated

anti-rabbit (1:100) or anti-mouse IgG (1:400) diluted in perm/wash buffer. Negative control samples were treated exactly the same, except they received only secondary antibody and no primary antibody. Samples were maintained in the dark from this point onwards. Finally, cells were washed thrice with perm/wash buffer, with 5 min centrifugation at 1000xg. Cells were resuspended in 200µl of perm/wash buffer and stored on ice until flow cytometric analysis.

### *Confocal microscopy*

After transfection and recovery, LMH or GH<sub>4</sub>C<sub>1</sub> cells were allowed to express protein for 48 h. Cells were kept in the dark. Cells were washed once with 1ml PBS at room temperature. Cells were labeled with 5µg/ml WGA conjugate Alexa Fluor 633 (Molecular probes, #W21404 plasma membrane staining) for 10 min at 37 C, followed by two washes with 1 ml room temperature PBS. Cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, followed by three washes of 5 min (3x5 min) each with PBS. Next, cells were permeabilized with 0.1% Triton-X-100/0.1% Tween-20 in PBS for 8 min, and again washed 3x5 min. Nuclei staining was achieved with DAPI (300nM), staining for 5 min at room temperature followed by 3x5 min washing with PBS. After the final wash, coverslips were mounted on clean microscope slides using VectaShield Mounting Medium, and preparations were visualized using a Leica SP5 X Confocal Microscope in the Imaging Core facility on the University of Maryland campus.

### *Luciferase assay to measure promoter activity*

Cell culture medium was removed, and cells were washed once with PBS followed by cell lysis with 100 $\mu$ l of passive lysis buffer (Promega). Lysates were stored at -20 C until luciferase reporter activity was measured. Cell lysates (20 $\mu$ l) were analyzed for firefly (reporter gene) and renilla (normalization gene) enzyme activities using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to renilla luciferase activity, and promoter activity is represented as fold change over basal (pGL3-Basic, untreated).

### *Flow cytometry*

All flow cytometric analysis and sorting were conducted at the University of Maryland, Department of Veterinary Medicine's Flow Cytometry Core Facility. For Dexras1 overexpression studies, positively transfected cells were sorted based on GFP. For the hormone-based intracellular staining, cells were sorted based on FITC staining. Before sorting, cells were filtered through a 35 $\mu$ M nylon mesh to remove clumps. Cells were analyzed using a high-speed benchtop flow cytometer and cell sorter (FACSAria II; BD Biosciences). Data acquisition and analysis were performed with FACSDiva software. For Dexras1 overexpression experiments, approximately 50,000 GFP-positive cells per group were collected into 0.5ml of RLT buffer (RNeasy Mini Kit, Qiagen). Collected samples were maintained at room temperature until RNA extraction. 1 $\mu$ l of  $\beta$ -mercaptoethanol per 100 $\mu$ l of RLT buffer was added to each sample immediately before RNA extraction using RNeasy Mini Kit as per the manufacturer's protocol. In the case of hormone cell-type sorting based on FITC, both positive and negative cells were sorted

into 0.5ml of buffer PKD from the RNeasy-FFPE-kit (Qiagen) and maintained at room temperature until RNA extraction.

#### *Total RNA extraction, quantification and reverse transcription*

Total RNA was extracted from tissues stored at -80 C or immediately extracted following sorting. Extracted RNA was stored at -80 C. Total RNA extraction was conducted based on the manufacturer's protocol and included on-column DNase digestion. RNA yields for all the experiments were quantified using the Quant-iT Ribogreen RNA Reagent and Kit. Reverse Transcription reactions using total RNA were performed with SuperScript III (Invitrogen) and anchored-dT-one-V primer (Table 3.1). No reverse transcription (RT) controls were included that contained everything except the reverse transcriptase enzyme.

#### *Quantitative Real Time PCR (qRT-PCR)*

Real Time PCR reactions were conducted using the MyiQ Single-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA). Most of the real time PCR reactions were performed in either 15 $\mu$ l or 30 $\mu$ l volume. 1 $\mu$ l of complementary deoxyribonucleic acid (cDNA) was added to the PCR mix (400nM of each primer, PCR buffer: 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton-X-100), 0.12 U/ $\mu$ l Taq Polymerase, 200nM dNTPs, 40nM fluorescein (Invitrogen), and SYBR Green I Nucleic Acid Gel Stain (Invitrogen) diluted 1:10,000), and reactions were performed using the following conditions: denaturation at 95 C for 3 min, 40 cycles of 95 C for 15 s, 60 C for 30 s, and 72 C for 30 s. The melting dissociation curves as well as gel electrophoresis visualization

confirmed single PCR products for all reactions. The negative control (No RT) reactions did not contain any amplified products. The different primers used for the real time PCR reactions are listed in Table 2 given below:

Table 2. List of primers used for cloning, sequencing, reverse transcription, and quantitative real time PCR.

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
<i>Quantitative real time PCR primers</i>		
POMC	CGCTACGGCGGCTTCA	TCTTGTAGGCGCTTTTGACGAT
GH	CACCTCAGACAGAGTGTGAGAAA	CAGGTGGATGTCGAACTTATCGT
PRL	AGGAATGGAGAAAATAGTTGGGC	TCATTTCCAGCATCACCAGAAT
LH- $\beta$	GGATGCCCCAATGTATGG	TCCCGCGTCCTGCAGTA
TSH- $\beta$	ACTGCCTGGCCATCAACAC	ACACGTTTTGAGACAGAGCACTTTT
Beta-actin	CAGGATGCAGAAGGAGATCACA	TAGAGCCTCCAATCCAGACAGAGTA
Dexas1	GATGTTCTCCTTGGTTTTGTTCTT	GTCTACCAGCTCGACATCCTG
GILZ	ACCCAAAGCCGGACTTGAG	CGCATAGACACAGCTACAGAAACAC
FKBP51	TGCCAAGTTTGCTGAGAGGG	CCTCTGTCTCTTTGCCTTCATCA
Ras-DVA	ACACCAGCGGCAGTTACTCCT	GTAGACCAAAGCGAAGGCGTC
<i>Cloning, sequencing and reverse transcription primers</i>		
Anchored-dT-one-V	CGGAATTC	TTTTTTTTTTTTTTTTTTTTTTTTT
Sp6microarray+	GGCCTATTAGGTGACACTATAG	
T7microarray+	GCTTATAATACGACTCACTATAGGG	
EYFP-Nfwd	CTGGTCGAGCTGGACGGCGACG	
EYFP-Nrev	CATGGTCCTGCTGGAGTTCGTG	
Dexas1-EcoR1-Fwd	CCGGAATCCGGGCCACCATGAAACTGGCAGCGATGAT	
Dexas1-EcoR1-Rev	CCGGAATCCGGCTAGCTGATCACACAGCGGTCTT	

Beta-actin was used as a housekeeping gene for normalization of qRT-PCR results. The following equation was used for transformation and normalization of each target gene:  $\text{mRNA level} = (2^{\Delta\text{Ct}})_{\text{target}} / (2^{\Delta\text{Ct}})_{\text{beta-actin}}$ , where  $\Delta\text{Ct} = \text{Ct}_{\text{noRT}} - \text{Ct}_{\text{sample}}$ . For overexpression studies, mRNA levels were normalized to beta-actin mRNA levels, and data are presented as mRNA fold change over basal. Values are presented as means+SEM. For detection of Dexras1 expression in different hormone cell types, mRNA levels were transformed and normalized to beta-actin mRNA levels as described above. Means of the transformed values for each gene from three independent replicates are presented relative to the mRNA level of beta-actin x 1000.

#### *Protein extraction and quantification*

For phosphorylation of Elk-1 studies, total protein was extracted from LMH or GH<sub>4</sub>C<sub>1</sub> cells transfected as described above using 500µl of 1X cell lysis buffer. Cell lysis buffer consisted of 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na<sub>2</sub>EDTA, 1mM EGTA, 1% Triton-X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1µg/ml leupeptin, and 1mM PMSF added just prior to use. Cells were rinsed one time with ice-cold PBS. Total protein was extracted in 250µl of ice-cold cell lysis buffer with agitation, followed by sonication (3x10 s with 30 s rest at output= 4 continuous). Samples were centrifuged for 10 min at 14,000xg at 4 C, and supernatants were transferred into new tubes. Protein extracted was quantified using Pierce MicroBCA Protein Assay according to the manufacturer's protocol.

### *Western blotting*

Protein samples were thawed on ice. 30µg of total cellular protein were boiled in Bio-Rad Laemmli sample buffer and loaded onto a 12% SDS-polyacrylamide gel. Proteins were resolved and transferred to a PVDF membrane (Immobilon-P; Millipore, Billerica, MA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer. Following transfer, membranes were washed for 15 min with Tris-buffered saline containing Tween-20 (TBS/T; 20mM Tris-HCl, 136mM NaCl, 0.1% Tween-20, pH 7.6), and blocked for 2 h at room temperature in TBS/T containing 5% nonfat dry milk. Membranes were washed 3 x 5 min in 1% nonfat dry milk in TBS/T and incubated overnight at 4 C with primary phospho-Elk-1 (Ser383) antibody (1:1000, cell signalling #9181) or a mouse monoclonal antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against  $\alpha$ -tubulin. Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Cell Signalling Technologies) or anti-mouse IgG (1:5000; Amersham Biosciences; Piscataway, NJ) diluted in TBS/T containing 5% nonfat milk. Membranes were developed using enhanced chemiluminescent detection reagents (LumiGLO; Cell Signalling Technologies, Inc.) and the ChemiDoc XRS system.

### *Statistics and data analysis*

For promoter activity and qRT-PCR data for mRNA expression studies, data are presented as fold change over basal, where basal refers to cells either at the 0-time point, untreated cells, or cells treated with vehicle. For the promoter analysis, firefly luciferase

activity was normalized to renilla luciferase activity. For expression studies, the Ct values were log-2 transformed ( $2^{Ct}$ ) and analyzed as indicated for the different studies. All data were analyzed using SAS software (SAS Institute) by analysis of variance using the MIXED models procedure, with differences between groups determined by the test of least significant differences (PDIFF). Differences are considered significant at  $P < 0.05$ .

## **Results**

### *Dexas1 is directly regulated by CORT in chicken embryonic pituitary cells*

We have previously reported that Dexas1 has a similar developmental profile as that of GH in the chicken pituitary cells (175). A microarray study conducted to identify unknown factors involved in CORT regulation of GH, recognized Dexas1 as a potential CORT-induced factor (176). To confirm that Dexas1 is directly regulated by CORT, e11 chicken primary pituitary cells were cultured in the presence or absence of cycloheximide, a protein synthesis inhibitor, and treated with CORT (1nM) for different time points (0 h, 1.5 h, 3 h, and 6 h). The 0 h time point was used as the basal control. Following treatment, total RNA was extracted, and Dexas1 mRNA levels were measured by qRT-PCR. Dexas1 mRNA levels were normalized to both basal and beta-actin. As seen in the Fig 16, CORT treatment significantly increased Dexas1 mRNA levels by 1.5 h, and levels remained increased through 6 h ( $n=3$ ;  $p < 0.05$ ). Dexas1 induction by CORT was not affected by the presence of CHX, at least at 1.5 h and 3 h, indicating that Dexas1 mRNA induction by CORT does not require the synthesis of an intermediary protein.



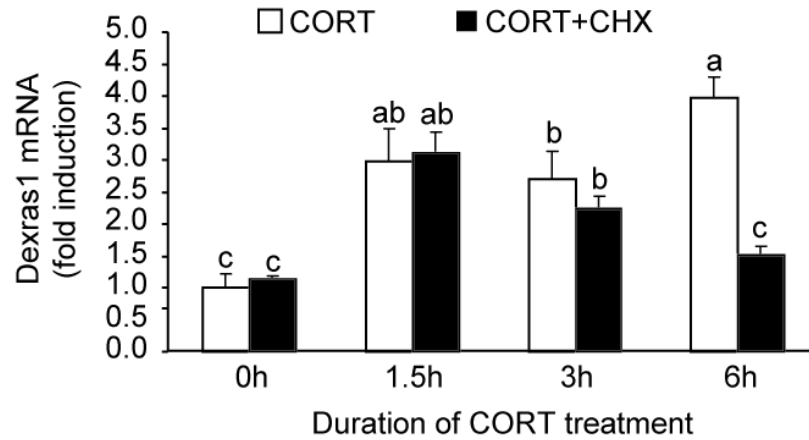


Figure 16. Quantitative real time PCR results confirm CORT induction of Dexas1 mRNA in cultured e11 chicken pituitary cells (n=3). E11 chicken pituitary cells were cultured in the presence or absence of CHX and treated with or without CORT (1nM) for 0, 1.5 h, 3 h or 6 h. Dexas1 mRNA levels were measured and normalized to beta-actin mRNA levels. Values with different letters are significantly different from one another (P<0.05).

#### *CORT induction of Dexas1 mRNA involves GR*

Knowing that chicken Dexas1 is regulated by CORT, we next investigated the involvement of GR or MR in this process. CORT induction of target genes conventionally occurs through binding of CORT to GR or MR, and activated ligand-bound nuclear receptor subsequently binds to its response elements on the target gene.

We tested the requirement for GR and MR in CORT induction of Dexas1 expression using pharmacological inhibitors, spironolactone (MR inhibitor; 100nM) and ZK98299 (GR inhibitor; 100nM). These doses of the inhibitors have been previously used in our system (98). E11 chicken pituitary cells were pretreated for 3 h with each inhibitor alone or in combination. Following pretreatment, cells were treated with CORT (1nM) for 1.5 h or 3 h. Substantial increases in Dexas1 mRNA were only observed with 3 h of CORT treatment, although there was a 2-fold increase in Dexas1 mRNA levels in response to 1.5 h CORT (n=3, p<0.05). Addition of spironolactone significantly increased Dexas1 mRNA levels even under basal conditions, and this was further augmented by the addition of CORT for 3 h (Fig 17). The CORT-induced increase in Dexas1 mRNA levels was significantly reduced by the presence of ZK98299, indicating the involvement of GR (n=3, p<0.05). These results indicate that Dexas1 induction of CORT involves GR. The effects of spironolactone on Dexas1 mRNA induction are not clear but likely involve agonistic effects of this compound (98).

*CORT-induced increases in Dexas1 mRNA are attenuated by inhibitors of Ras signalling and the MEK1/2 pathway*

To define the molecular mechanism of CORT induction of Dexas1 mRNA expression, involvement of different cell signalling pathways in this process was investigated. The MAPK/ERK, PKA, PKC, ras, PI-3 kinase, and protein tyrosine kinase pathways were manipulated using the following activators or inhibitors: LY294002 (PI-3 kinase inhibitor; 50µM), UO126 (MEK1/2 inhibitor; 10µM), manumycin (ras inhibitor;

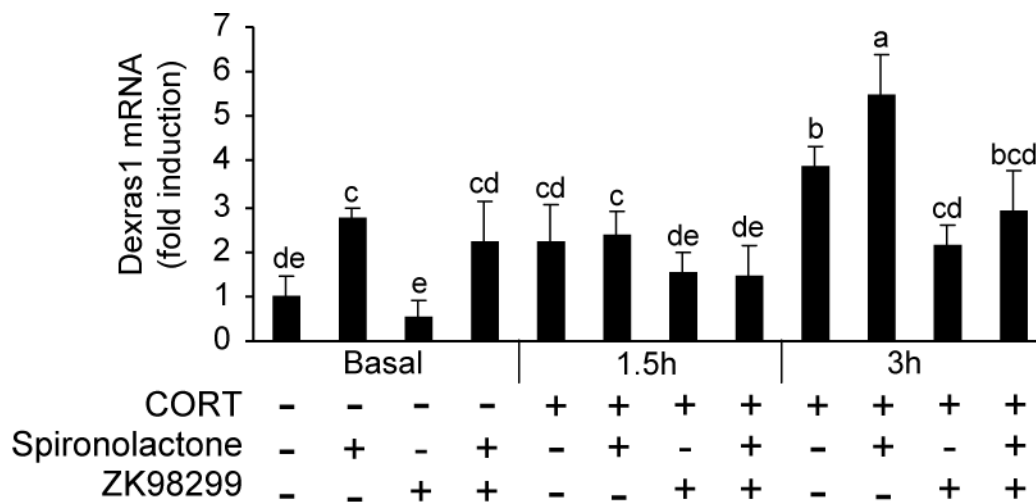


Figure 17. CORT-induced increase in Dexas1 mRNA levels involves GR in e11 chicken pituitary cells. E11 chicken pituitary cells were pretreated for 3 h with 100nM GR or MR inhibitors (ZK98299 and Spironolactone, respectively) as indicated, followed by treatment with or without CORT (1nM) for 0 h, 1.5 h or 3 h. Dexas1 mRNA levels were measured and normalized to beta-actin mRNA levels as well as the basal untreated samples. Values with different letters indicate significant difference from one another (n=3, P<0.05).

1 $\mu$ M), anisomycin (p38/JNK MAPK activator; 10 $\mu$ M), EGF (receptor tyrosine kinase activator; 100ng/ml), forskolin-IBMX (PKA activator; 1mM), and phorbol-12-myristate-13-acetate (PMA, PKC activator; 1 $\mu$ M). E11 chicken pituitary cells were pretreated with the inhibitors for 1h, followed by 3 h of CORT treatment. In the case of activators, there

was no pretreatment. Cells received activators and CORT (1nM) and were treated in combination for a total of 3 h. Data represent mRNA levels normalized to beta-actin and DMSO-treated control samples (Fig 18). None of the pathway activators had any effect on CORT-induced Dexras1 mRNA levels (n=3). Inhibition of PI3-K did not change the CORT-induced increase in Dexras1 mRNA levels ( $p>0.05$ ). Ras pathway inhibition by manumycin or MEK1/2 inhibition by U0126 significantly reduced the CORT-dependent increase in Dexras1 mRNA levels (n=4,  $p<0.05$ ). These results indicate that CORT induction of Dexras1 mRNA in embryonic chicken pituitary cells does not require the PKA, PKC or PI3-K signalling cascades, but may involve ras or a ras-like compound and MEK1/2 signalling.

#### *Cellular distribution of Dexras1 mRNA expression in embryonic chicken pituitary cells*

Pituitary Dexras1 mRNA levels increase concomitantly with expression of GH during chicken embryonic development (175), and CORT indirectly induces GH mRNA (135), while its effects on Dexras1 expression do not require ongoing protein synthesis. If Dexras1 is to play a functional role in regulating expression of hormone genes during cell type differentiation in the pituitary, then expression of Dexras1 in the particular cell type may be considered as a prerequisite. Since chicken Dexras1-specific antibody was unavailable, two indirect methods were employed to examine the cellular distribution of Dexras1. For the following experiments, Dexras1 mRNA expression was tested in e17 or e18 chicken embryos, when expression levels are greatest and all pituitary cell types have differentiated (27, 175, 228). The anterior pituitary in birds has two distinct lobes – caudal and cephalic. Somatotrophs are present in the caudal lobe, while corticotrophs, lactotrophs, and thyrotrophs are primarily found in the cephalic lobe (16, 211, 231).

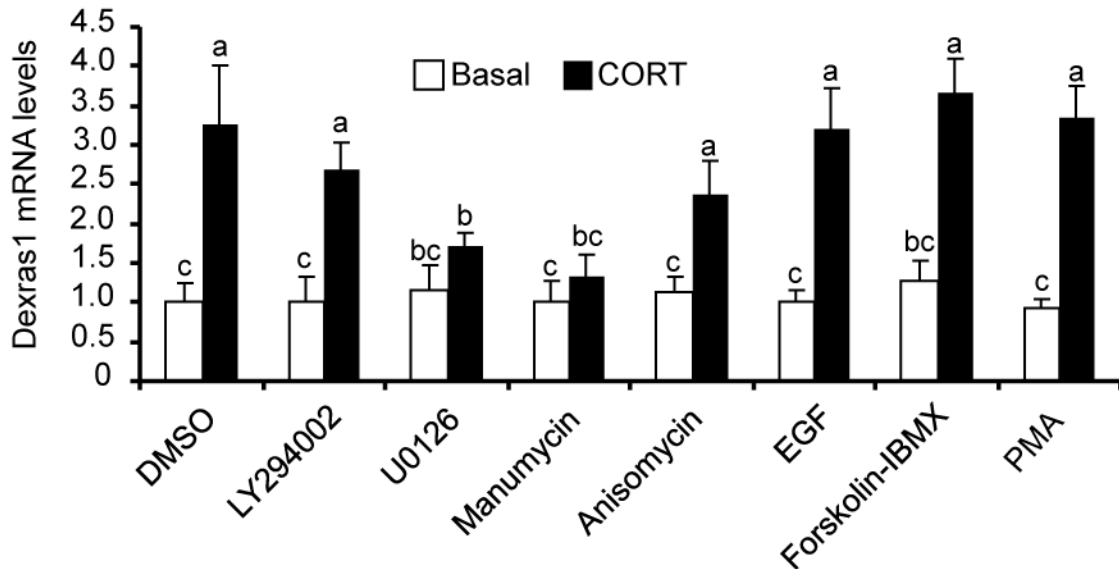


Figure 18. Involvement of endogenous cell signalling pathways in CORT induction of Dexas1 mRNA in cultured chicken e11 pituitary cells. E11 chicken pituitary cells were cultured in the presence or absence of the different activators (anisomycin, EGF, forskolin and PMA; n=3) and inhibitors (LY294002, U0126 and manumycin; n=4) as indicated, followed by CORT treatment for 3 h. Dexas1 mRNA levels were normalized to beta-actin mRNA levels. Data values (Means + SEM) with different letters are significantly different ( $P < 0.05$ ).

To assess whether GH-producing somatotroph cells express Dexas1, caudal and cephalic lobes from e18 chickens were isolated and analyzed separately (n=3). As expected, GH mRNA levels were highly enhanced in the caudal lobe, and POMC, PRL and TSH $\beta$  mRNA levels were enriched in the cephalic lobe (Fig 19). Dexas1 mRNA was detected in both cephalic and caudal fractions. These results indicate that Dexas1 mRNA is

expressed in more than one cell type in e18 chickens, suggesting it may play a role in regulating multiple cell types.

In a second approach investigating the cell type distribution of Dexas1 mRNA, e17 chicken pituitary cells were FACS sorted based on the characteristic hormone

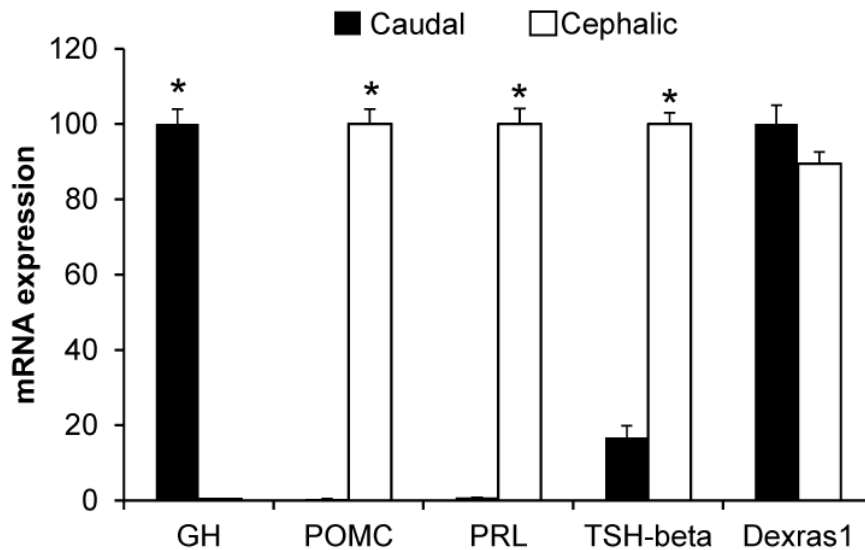


Figure 19. Dexas1 mRNA is expressed in both caudal and cephalic lobes of e18 chicks (n=3). Levels of GH, POMC, PRL, TSH $\beta$ , and Dexas1 mRNA were quantified by quantitative real time PCR for both lobes. Data are presented as a percentage of the lobe with highest expression of that gene. Within each gene, the mRNA level in the lobe with highest expression is fixed at 100%. Asterisk (\*) represents higher expression levels of the gene within that lobe (n=3, P<0.05).

produced by each cell type. E17 chicken pituitaries were dispersed, washed, and stained for a particular hormone (GH, POMC, TSH, LH, and PRL). There were five experimental groups ( $\sim 10^7$  million cells per group), and each group was stained with a primary antibody against a particular hormone followed by FITC-conjugated secondary antibody. The control groups used for background detection received only the secondary FITC-antibody. Each group was sorted separately, and negative and positive cells from each group were collected into tubes for RNA extraction. Within each group, the positive cells represent cells that were positively stained for a particular hormone. For example, positive cells collected from the group stained with POMC represent the corticotrophs. The negative cells collected from the same group will therefore contain all the other cell types. A representative result from FACS sorting is shown in Fig 20. The whole experiment was repeated three times. Dexas1 mRNA level was measured in each collected population. GH, POMC, TSH $\beta$ , LH $\beta$ , and PRL mRNA were also measured for each to confirm the purity of the collected sample. The positive cells were all enriched for the respective hormone for which they were sorted. Cross-contamination between different cell types in the collected samples was very low. Dexas1 mRNA was detected in both somatotroph and corticotroph cells as shown in Fig 21 (n=3, P<0.05).

*Subcellular localization of YFP-tagged Dexas1 in response to CORT treatment in LMH and GH<sub>4</sub>C<sub>1</sub> cells*

To test whether CORT has an effect on the subcellular localization of Dexas1, chicken Dexas1 was N-terminal tagged with YFP. The C-terminal CAAX prenylation

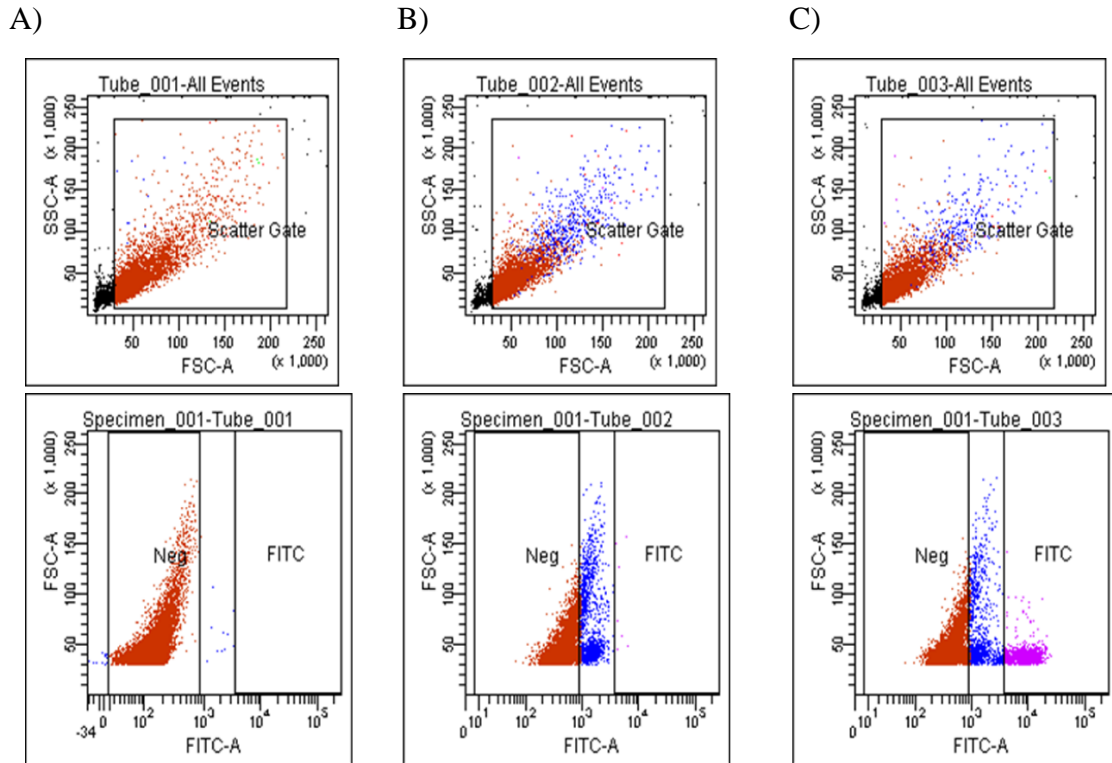


Figure 20. Representative FACS sorting analysis using GH primary antibody in e17 chicken pituitary cells. (A) Control cells with no treatment. (B) Cells with only secondary antibody (C) Cells treated with both primary and secondary antibody. Top panels: shows all the events for samples A, B, and C. Bottom panels: (A) No FITC signal for sample A. (B) No FITC signal for cells receiving only secondary antibody (background). (C) Cells stained with both primary and secondary antibody shows clear FITC signal. For each hormone, the positive cells (within the FITC box) and the negative cells (Neg box) were collected (n =3).



■ Positive □ Negative

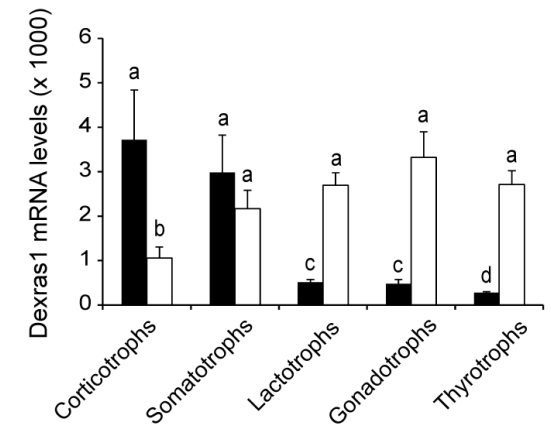
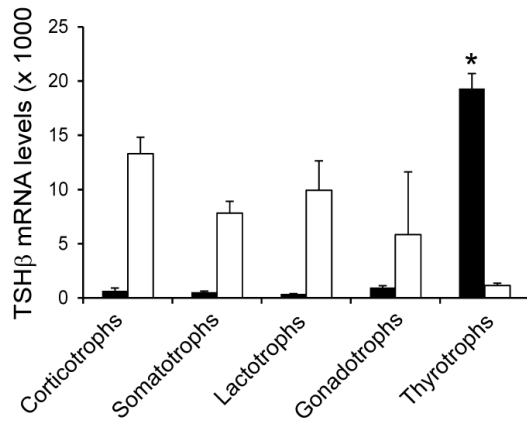
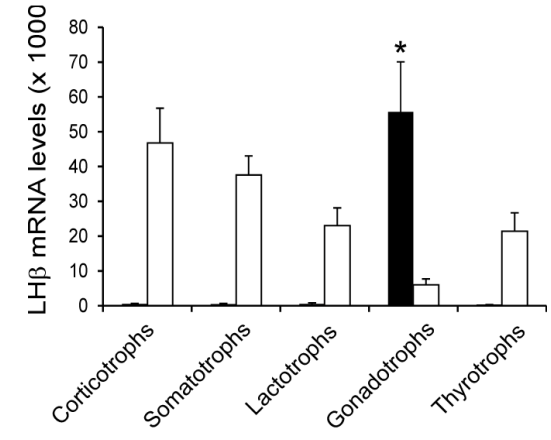
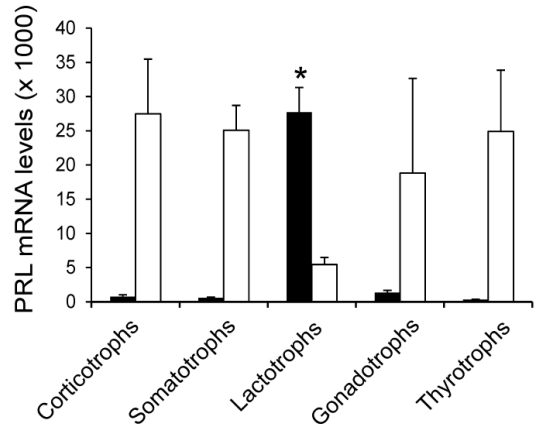
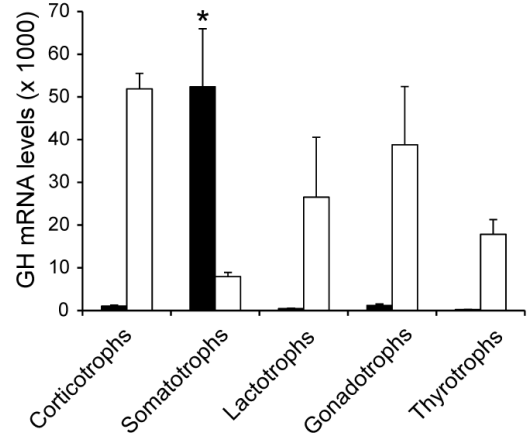
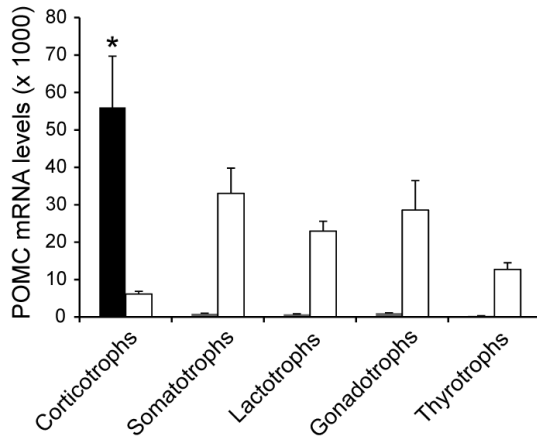


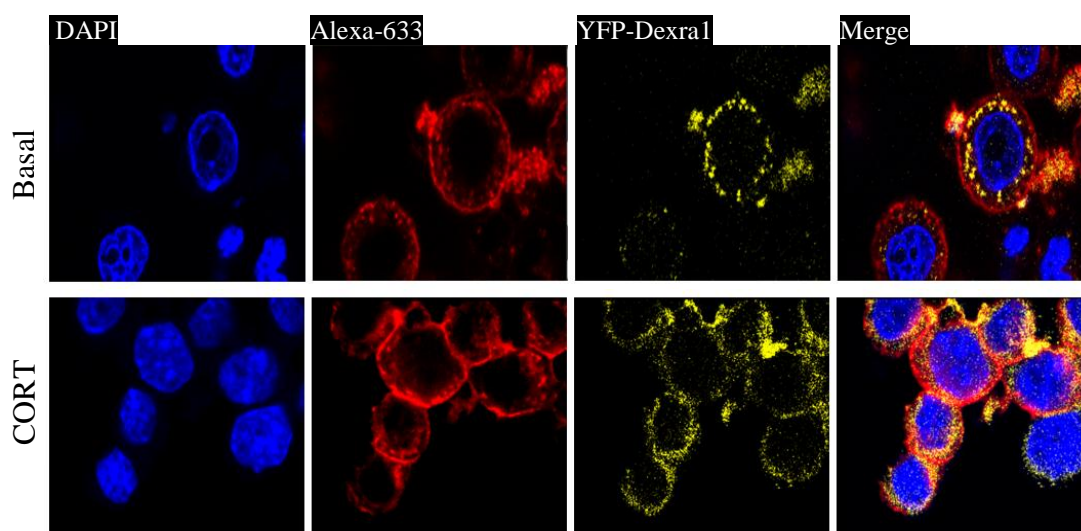
Figure 21. Expression of POMC, GH, PRL, LH $\beta$ , TSH $\beta$ , and Dexras1 mRNA in FACS sorted e17 chicken pituitary cells. E17 chicken pituitary cells were subjected to intracellular staining using antibodies specific for POMC, GH, PRL, LH $\beta$ , and TSH $\beta$  followed by FITC-based FACS sorting. Positive and negative cells from each group were collected. For each group, mRNA levels for POMC, GH, PRL, LH $\beta$ , TSH $\beta$ , and Dexras1 were measured and normalized to beta-actin mRNA levels. Values (Means+SEM) with different letters denote significant difference in Dexras1 mRNA levels. Asterisks (\*) denote a significant difference in hormone mRNA levels for positive and negative cells sorted for that hormone (n=3, P<0.05).

domain was undisturbed. N-terminal YFP-tagged Dexras1 was transfected into LMH and GH4C1 cells, which were then treated with CORT for 3 h. WGA-Alexa-633 fluorophore was used to stain the plasma membrane, and DAPI was used to mark the nucleus. Cells were visualized using a Leica SP5X confocal microscope. Fig 22(A) shows YFP-tagged Dexras1 in GH<sub>4</sub>C<sub>1</sub> cells. Under basal conditions, Dexras1 was found mostly in the cytoplasm close to the plasma membrane. Upon CORT addition, the YFP-Dexras1 signal was still seen bordering the plasma membrane. An apparent change in Dexras1 localization was not detected in response to CORT. In the case of LMH cells (Fig 22 (B)), YFP-tagged Dexras1 gave a more diffuse signal. It was distributed throughout the cell with a few concentrated spots within the nucleus. Upon CORT addition, there was a marked increase in YFP signal in the nucleus, although YFP signal was still found throughout the cell. Overall, in GH<sub>4</sub>C<sub>1</sub> cells, CORT did not have a significant influence on the subcellular distribution of Dexras1, whereas in LMH cells, CORT treatment resulted in an increased nuclear localization of Dexras1.

#### *Effect of overexpression of Dexras1 on growth hormone reporter activity*

To test whether Dexras1 is involved in regulating the GH promoter activity, a reporter construct (pGL3-1727) containing the CORT-responsive -1727 to +48 sequence of the chicken GH gene driving firefly luciferase was co-transfected into e11 chicken pituitary cells along with 1 µg of Dexras1 expression plasmid. The cells were then treated with or without CORT (1nM) for 12 h, and firefly luciferase activity was measured. Results shown in the Fig 23(A) represent means from three independent experiments (n=3). The pGL3-1727 reporter plasmid in response to CORT showed a ~5-fold increase

(A)



(B)

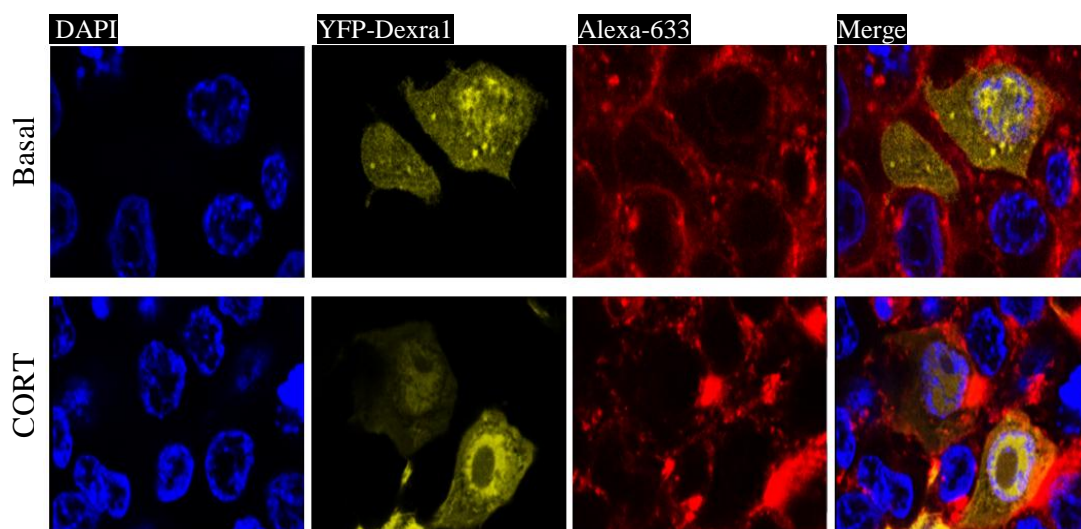
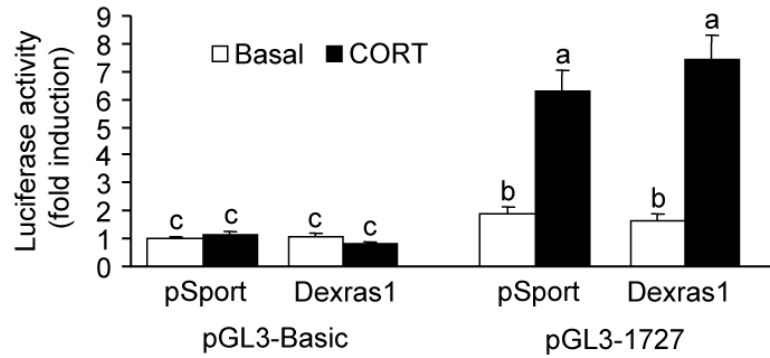


Figure 22. Subcellular distribution of YFP-tagged Dexras1 in GH<sub>4</sub>C<sub>1</sub> (A) and LMH (B) cells. In both A and B, top panels are images of cells under basal conditions, and the bottom panels show cells after 3 h of 1nM CORT treatment.

(A)



(B)

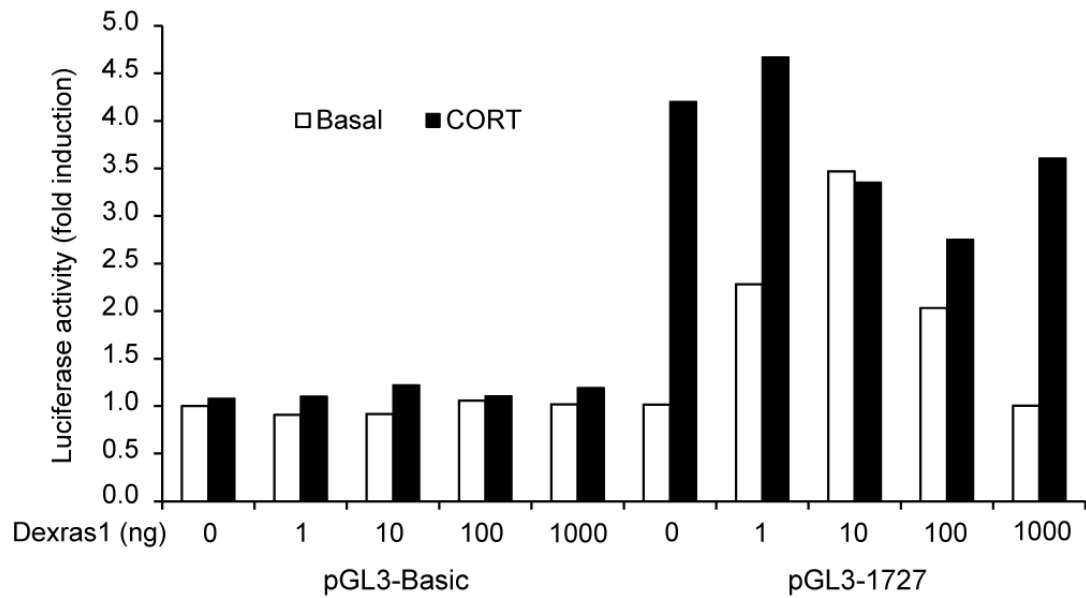


Figure 23. Effect of overexpression of Dexas1 on GH reporter activity. (A) E11 chicken pituitary cells were transfected with pGL3-Basic or -1727bp reporter plasmid with or without 1 $\mu$ g of Dexas1 expression plasmid, followed by treatment with or without CORT(1nM). Values (Means+SEM) with different letters denote significant differences (n=3, p<0.05). (B) Effect of different doses of Dexas1 expression plasmid on reporter activity in GH<sub>4</sub>C<sub>1</sub> cells (n=1).

in promoter activity. Overexpression with 1 $\mu$ g of Dexras1 expression plasmid did not introduce a significant change in the reporter activity ( $P>0.05$ ). It neither enhanced nor attenuated the CORT effect, suggesting that Dexras1 may not be altering the activity of the -1727 to +48bp region of the GH promoter. Since Dexras1 is a ras protein and its activity may depend on the availability of GTP in the cell, effects of different doses of Dexras1 expression plasmid (1ng, 10ng, 100ng and 1 $\mu$ g) were also tested in a similar reporter assay in GH<sub>4</sub>C<sub>1</sub> cells (Fig 23B). Preliminary results (n=1) show that lower doses (1ng and 10ng) of Dexras1 expression plasmid induced luciferase activity (~2- and 3-fold respectively) under basal (in the absence of CORT) conditions, whereas 10ng and 100ng of Dexras1 plasmid decreased reporter activity under CORT- treated conditions.

#### *Overexpression of Dexras1 affects GH mRNA levels*

To investigate the role of Dexras1 in regulating GH gene expression, the effect of overexpression of Dexras1 on GH mRNA levels in transfected e11 chicken pituitary cells was determined. In initial experiments, e11 chicken pituitary cells were co-transfected with 1 $\mu$ g of pSports6.1 empty vector or 1 $\mu$ g of Dexras1 expression plasmid along with 1 $\mu$ g golgi-targeted GFP-expression plasmid. Cells were treated with or without CORT for 20 h post-transfection. Positively transfected cells were collected based on GFP using FACS. Total RNA was extracted from the sorted and collected cells, and levels of mRNA for GH, PRL, POMC, GILZ, FKBP51, and RasDVA were measured by qRT-PCR. As shown in the Fig 24, CORT treatment (1nM) alone increased GH mRNA levels by ~ 40 fold. Dexras1 overexpression significantly decreased the CORT-induced increase in GH mRNA levels (n=3,  $p<0.05$ ). This effect was specific to GH, as Dexras1 overexpression

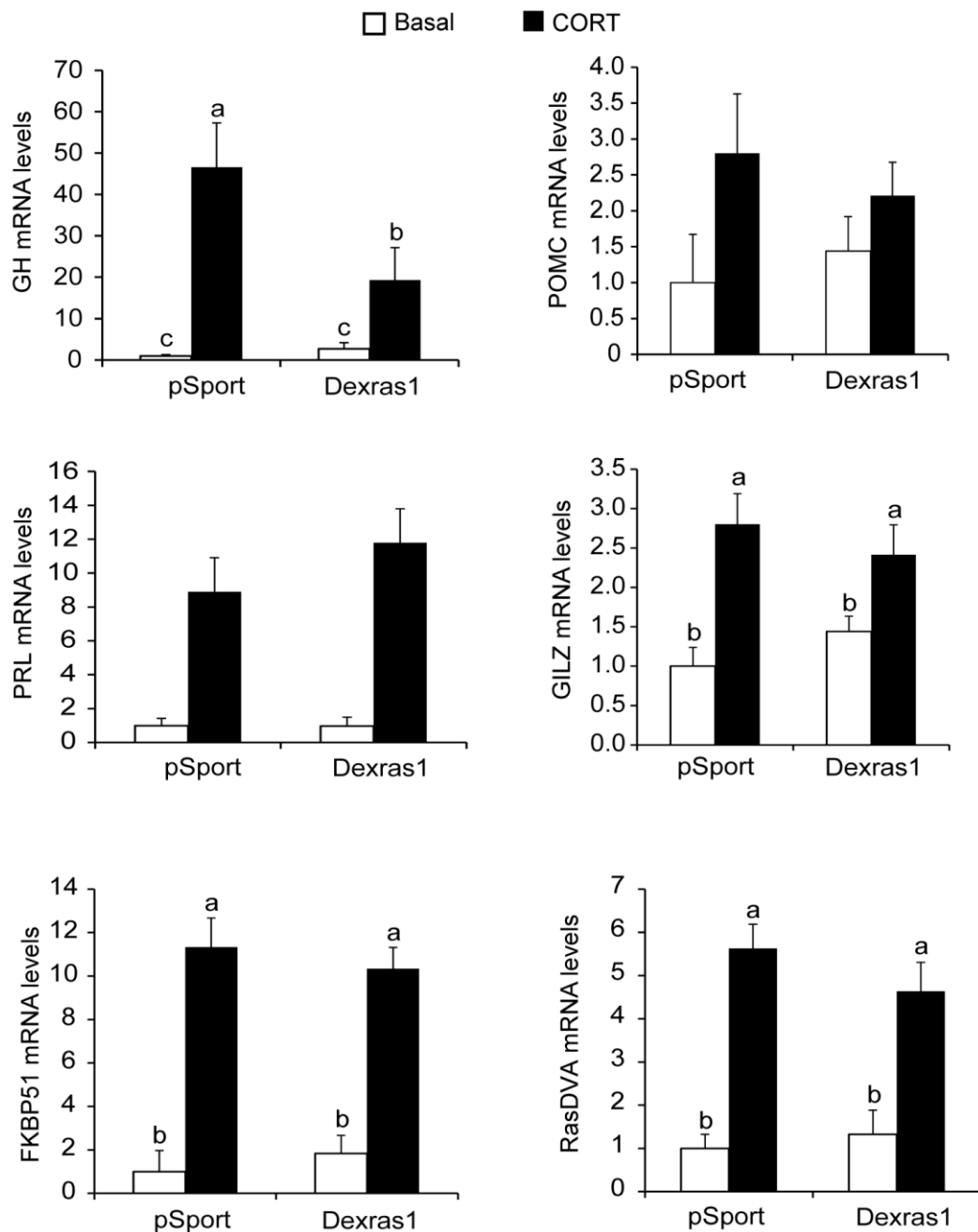


Figure 24. Effect of overexpression of Dexras1 expression plasmid on mRNA levels. E11 chicken pituitary cells were co-transfected with Dexras1 expression plasmid (1 $\mu$ g) or empty vector (pSport) and GFP-plasmid, and treated with or without CORT (1nM). Positively transfected cells were FACS sorted, and RNA was extracted from collected cells. Values (Means+SEM) are represented normalized to beta-actin. Values with different letters denote significant differences (n=3, p<0.05).

did not affect mRNA levels of PRL, POMC or other CORT-regulated genes tested (Fig 24). In a subsequent series of experiments, different doses of Dexras1 expression plasmids were also tested. 1ng to 1 $\mu$ g of Dexras1 expression plasmid were co-transfected (Fig 25). As seen before, 1 $\mu$ g of Dexras1 expression plasmid significantly decreased the CORT-dependent GH mRNA increase (Fig 25A; n=3, p<0.05). There was no effect of any other doses of plasmid (1ng, 10ng and 100ng) on CORT-induced GH mRNA levels (p>0.05). Under non-CORT-treated basal conditions, lower doses of Dexras1 plasmid (10ng and 100ng) significantly stimulated GH mRNA levels (Fig 25B; n=3, p<0.05). However, the effect of Dexras1 overexpression on GH mRNA was not as great as that seen with CORT.

#### *Downstream targets of Dexras1*

Dexras1 has been implicated in regulating several signalling pathways, including MAPK signalling (190). MAPK signalling is also involved in CORT regulation of Dexras1, as shown in Fig 26. Furthermore, CORT induction of GH mRNA in chicken embryonic pituitary cells is blocked by inhibition of MEK1/2 (142). To identify if MAPK is a downstream target of Dexras1 in chickens, effect of overexpression of Dexras1 on phosphorylation of endogenous Elk-1 was examined in LMH and GH<sub>4</sub>C<sub>1</sub> cells. Cells (GH<sub>4</sub>C<sub>1</sub> and LMH) were transfected with empty pSport6.1 or Dexras1 expression plasmid for 6 h, and treated with CORT for 3 h. EGF treatment was included as a positive control, since EGF is known to increase phosphorylation of Elk-1. As shown in Fig 26 (A and B), phosphorylated Elk-1 was detected in samples treated with EGF in both cell types.



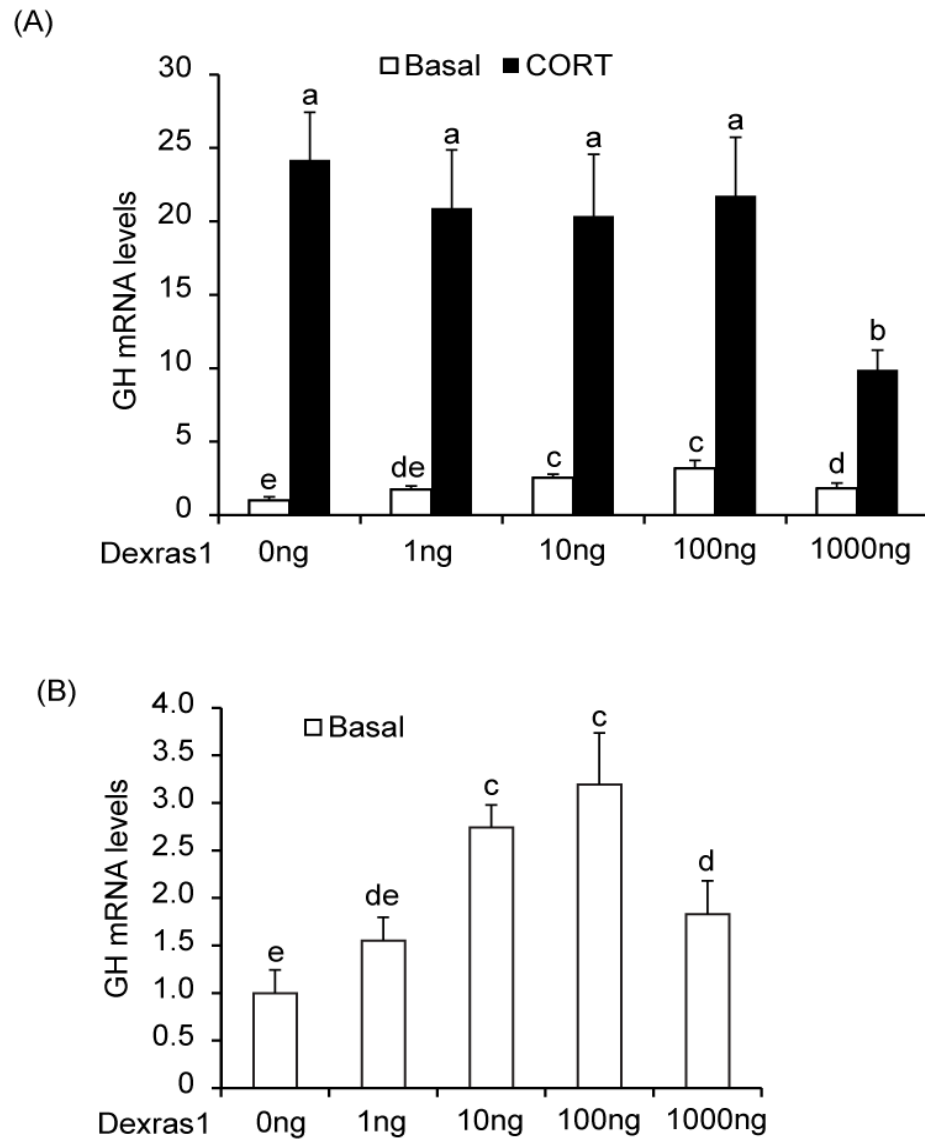


Figure 25. Effect of overexpression of different doses of Dexras1 expression plasmid on GH mRNA levels. E11 chicken pituitary cells were transfected with indicated doses of Dexras1 expression plasmid, and treated with or without CORT (1nM). (A) GH mRNA levels. (B) GH mRNA levels under basal conditions alone. Values (Means+SEM) are presented normalized to beta-actin. Values with different letters denote significant differences (n=3, p<0.05).

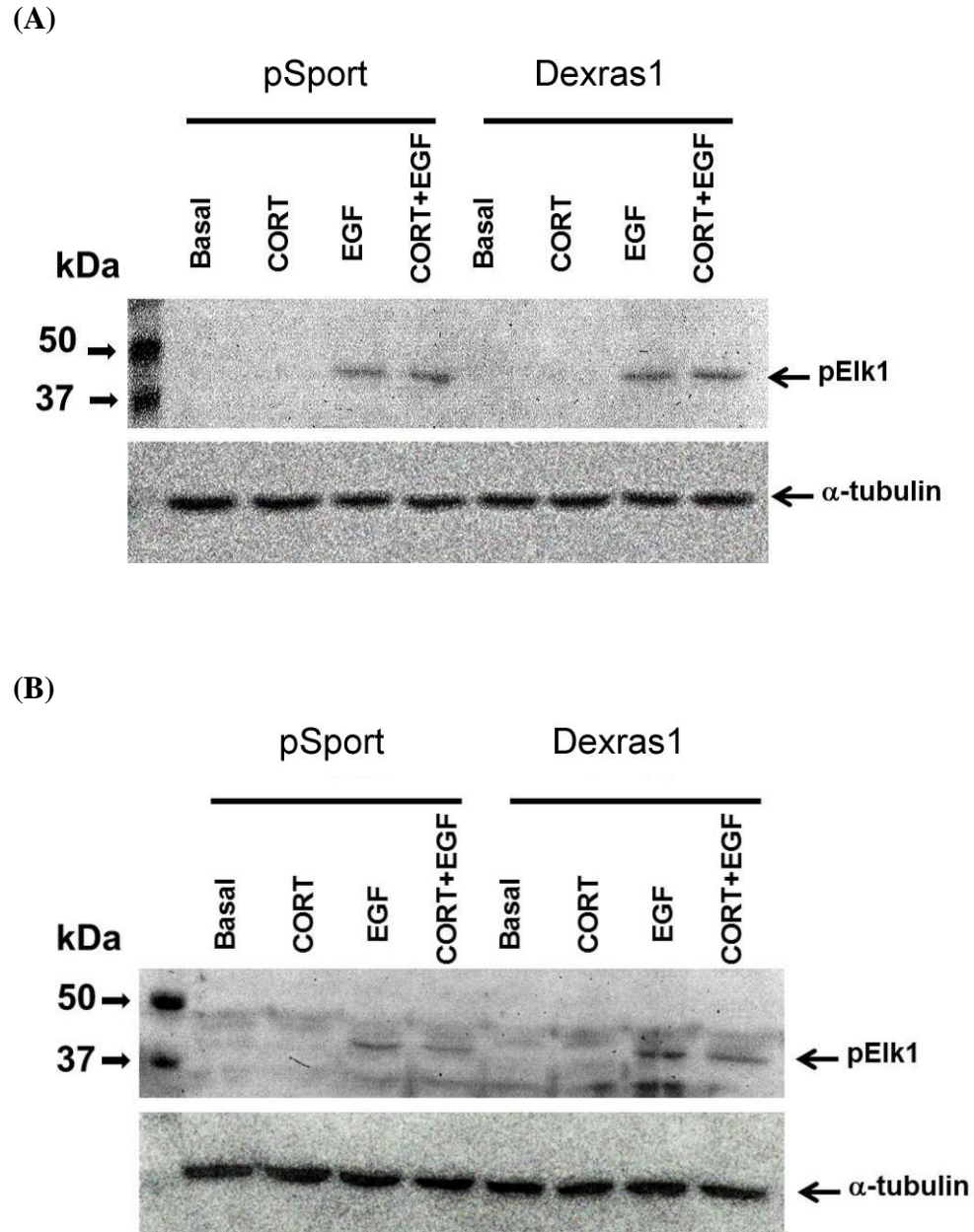


Figure 26. Western blot analysis of effects of overexpression of Dexras1 on phosphorylation of endogenous Elk-1 in (A) GH<sub>4</sub>C<sub>1</sub>, and (B) LMH cells. 1 μg of vector or Dexras1 plasmid was transfected into GH<sub>4</sub>C<sub>1</sub> or LMH cells, which were then left untreated (basal) or treated with CORT, EGF, or both for 3 h.

Overexpression of Dexras1 did not affect phosphorylated Elk-1 levels in basal, EGF-, or CORT- treated samples in either cell type.

## **Discussion**

We have characterized CORT regulation of Dexras1 expression in chicken pituitary cells and have attempted to identify a possible role of Dexras1 in regulating CORT-mediated GH gene expression. CORT regulation of GH in chicken anterior pituitary cells is an indirect effect (135), mediated through additional factors. The same is true for somatotroph differentiation and GH expression in rodents (125). These additional factors have not been identified in any species. A genome-wide microarray study was previously conducted in an effort to identify these factors (176). Dexras1 was identified as a potential candidate gene. In an independent experiment looking at involvement of cell signalling pathways in GH expression, a ras pathway or a ras-factor was shown to be essential (135). Dexras1 made an interesting candidate, considering that it is a ras protein and has a developmental expression profile similar to that of GH and CORT in chickens (140, 175, 176).

If Dexras1 is involved in cell-type specific functions within the anterior pituitary, then expression of Dexras1 in these cells may be considered as a prerequisite. Endogenous Dexras1 expression in pituitary cells was therefore confirmed by two different methods. The FACS-based isolation of different pituitary cell types as well as the caudal-cephalic experiment results are consistent. Both experiments confirmed expression of Dexras1 in somatotroph and corticotroph cells of the e17 chicken pituitary gland. Dexras1 may also be present in other cell types within the pituitary, but this was

not clearly detectable in our experiments. Very low levels of Dexas1 were detected in the thyrotrophs. In any case, it can be said that Dexas1 is expressed in multiple cell types, including somatotrophs and corticotrophs.

The mechanism of CORT regulation of Dexas1 has not been defined in any species. Human Dexas1 has been shown to contain a GRE in its 3'-flanking region, suggestive of a possible CORT-induction through the involvement of GR (229). The chicken Dexas1 gene on the other hand does not have a canonical GRE, but only half sites. In the present study, we investigated involvement of GR and MR in CORT induction of chicken Dexas1. We demonstrated that in embryonic chicken pituitary cells, CORT induction of Dexas1 is significantly reduced by blocking GR using a pharmacological inhibitor, confirming the participation of GR in the process. The MR inhibitor spironolactone had a different effect than the GR inhibitor. It is not clear if MR plays a role in Dexas1 expression. Blocking known signalling pathways indicated the need for a ras pathway or ras-like factors as well as MEK1/2 signalling for Dexas1 induction by CORT, since UO126 and manumycin diminished the CORT effects on Dexas1 mRNA levels. No other pathways investigated had any effect on Dexas1 mRNA levels.

Next, we determined whether Dexas1 could influence CORT regulation of the GH gene. As a first step, we determined whether Dexas1 overexpression could modify promoter activity of the chicken GH gene. The -1727bp region of the chicken GH gene, identified as being CORT-responsive, was used as the reporter to measure whether the presence of Dexas1 can augment or attenuate CORT effects. In our study, Dexas1 overexpression had no effect on basal GH promoter activity when 1 $\mu$ g of the expression

plasmid was tested. To test if overexpression of Dexas1 affects GH mRNA levels, cells transfected with Dexas1 were sorted using FACS, and GH mRNA levels were measured in sorted cells. Contrary to our hypothesis, Dexas1 overexpression inhibited CORT-induced GH gene expression, at least when higher amounts of the expression plasmid were transfected. Interestingly, transfecting lower amounts of Dexas1 expression plasmid had a stimulatory effect on basal GH expression. Experiments were conducted with two doses of CORT, 1nM and 100nM, and both sets of experiments showed similar results. From these results, we can conclude that Dexas1 overexpression (at low levels) by itself increased GH gene expression. Dexas1 overexpression did not influence expression of other pituitary hormone genes (POMC and PRL) investigated, even though Dexas1 expression was found in both somatotrophs and corticotrophs. The mechanism of GH induction by Dexas1 is not yet clear.

Dexas1 is a ras protein, and ras proteins are known to undergo post-translational modifications such as prenylation, which allows them to be translocated and attach to the plasma membrane (195). Although localization to the plasma membrane is not a necessary step for Dexas1 functioning in all cases, we still wanted to determine if CORT affects Dexas1 distribution within a cell. To this end, Dexas1 cellular localization was examined using aYFP-tagged fusion protein in GH<sub>4</sub>C<sub>1</sub> rat pituitary cells and LMH cells. In GH<sub>4</sub>C<sub>1</sub> cells, results suggest that Dexas1 may be predominantly found in the cytoplasm. There was no evident effect of CORT on Dexas1 localization. Functionality of Dexas1 was not tested in our experiments, although others have reported mixed results about localization of Dexas1, and this appears to be cell and function specific. For instance, in HEK293 cells Dexas1-mediated inhibition of adenylate cyclase 2 (AC2)

activity is dependent on Dexras1 localization to the plasma membrane where it interacts with protein kinase C, PKC $\delta$  (188). Dexras1 has also been shown to bind to the amyloid beta precursor protein-binding member, FE65-PTB2 domain and potently suppress FE65-APP (amyloid beta precursor protein)-mediated transcription. In this study, FE65 and Dexras1 were shown to colocalize in HEK cells, and FE65 increased Dexras1 translocation from the cytosol to the nucleus (207). The subcellular localization of Dexras1 appears to be dependent on the cell type and to some extent on the interacting partners. Nevertheless, in LMH and GH<sub>4</sub>C<sub>1</sub> rat pituitary cells, chicken Dexras1 localization was not translocated to the plasma membrane in response to CORT.

Finally, we investigated the downstream effects of Dexras1. Previous studies have reported that CORT stimulation of GH transcription involves ERK1/2 signalling, and CORT increases ERK1/2 activity after 3 h in cultured embryonic pituitary cells (142). We wanted to test if overexpression of Dexras1 could affect ERK1/2 activity, which was measured by the phosphorylation levels of Elk-1, a well-established downstream target of ERK1/2. Dexras1 overexpression did not significantly alter phosphorylation levels of Elk-1. Dexras1 has been shown to affect other signalling pathways (especially G-protein coupled receptor (GPCR) signalling) in other systems, and further experiments will have to be conducted to test downstream targets of Dexras1 in pituitary cells.

Our study presents a novel role for Dexras1 in pituitary function. Dexras1 expression has never been examined in embryonic tissues in other species. Our study demonstrates expression of Dexras1 in chicken embryonic somatotrophs and

corticotrophs and we found that Dexras1 can affect GH promoter activity and GH mRNA levels in these cells.

## **CHAPTER 4**

### **Conclusions and Future directions**



The main aim of this study was to understand glucocorticoid regulation of GH gene expression in developing chickens. Glucocorticoid regulation of the GH gene is a complex process involving both genomic and non-genomic mechanisms. In this study, we addressed the involvement of histone modifications in association with transcription factor recruitment to the chicken GH gene, as well as investigated potential involvement of Dexras1 in mediating glucocorticoid-induced GH expression in chicken embryos.

Epigenetic modifications play an integral part in regulating gene expression. Observations made in LSD1 knockout mice identified involvement of epigenetic processes in GH expression and somatotroph differentiation (1). Therefore, we hypothesized that glucocorticoid regulation of GH expression in chickens may occur through glucocorticoid-induced changes in histone modifications and transcription factor recruitment to the chicken GH promoter. It is also known that glucocorticoid induction of GH expression requires ongoing protein synthesis in chickens (135), which indicates that glucocorticoid induction of GH expression requires one or more additional factors. Pit-1 is an essential transcription factor required for somatotroph differentiation (61), and the chicken GH promoter has two Pit-1 binding sites near the TSS (60). Glucocorticoids activate GR translocation to the nucleus, where GR regulates gene transcription by either directly binding to its GRE or by tethering to other transcriptional factors that have binding sites on DNA (104). In the chicken GH promoter, the GCRR with its Ets1 and GR binding sites is located near the distal Pit-1 binding site, which raises the possibility of GR interacting with Pit-1 and Ets1 factors. GR is known to interact with chromatin modifiers, such as SWI/SNF (160), CBP/p300 (224), etc. that have intrinsic HAT activities. Our CHIP results suggest that under basal conditions the GCRR site may be

occupied by GR and Ets1. Similarly, the distal and proximal Pit-1 sites appear to be occupied by some Pit-1. We observed increased GR recruitment to the GCRR and the distal Pit-1 site in response to glucocorticoid treatment. We can speculate that GR may be recruited to the GCRR and the distal Pit-1 site as a part of a large complex. Factors, such as SWI/SNF, CBP/p300, etc. may be part of this complex. Additionally, Pit-1 and Ets1 may transiently interact with GR at the GCRR or distal Pit-1 sites on DNA. It is also possible that glucocorticoid treatment increases GR recruitment as well as recruitment and association of chromatin modifiers, such as SWI/SNF, with GR, which is already present at the GCRR site. Furthermore, increased GR recruitment may be required for displacing Ets1 from its site in the GCRR, which may be necessary for the binding of additional factors to the chicken GH promoter. The increased GR recruitment to the distal Pit-1 site in response to glucocorticoid treatment may also explain the observed decrease in Pit-1 binding at the distal Pit-1 site.

We believe that glucocorticoid-induced recruitment of GR to the GCRR and distal Pit-1 sites, Pit-1 to the proximal Pit-1 site, and the dissociation of Ets1 from the GCRR site allow recruitment and association of chromatin modifiers with the chicken GH gene. Once recruited, factors such as SWI/SNF, CBP/p300, etc. catalyze histone acetylation and tri-methylation of lysine 4 of histone H3 near the TSS of the chicken GH gene. Increased histone acetylation and tri-methylation of lysine 4 of histone H3 allows RNA Pol II and basal transcription factors to bind the TSS. By 6 h, we observe a decrease in histone acetylation and tri-methylation of lysine 4 at the TSS. We also observed dissociation of RNA Pol II from the TSS, Pit-1 from the proximal and distal Pit-1 sites, GR from the GCRR and the distal Pit-1 sites, and Ets1 from the GCRR site. Our lab has

previously shown that HDAC activity is required for glucocorticoid-induction of GH expression at 6 h (142). The observed decrease in histone acetylation and tri-methylation with 6 h glucocorticoid treatment in our present study agrees with the previous results, and therefore supports the involvement of HDAC activity in glucocorticoid induction of GH expression.

The identity of any HATs, HDACs, and HMTs involved in glucocorticoid induction of GH expression is yet to be established. CBP/p300 interaction with Pit-1 has been investigated in association with Pit-1 activity (232, 233), rendering CBP/p300 a possible candidate to be investigated. As a first step, coimmunoprecipitation studies looking at GR interaction with Pit-1, Ets1, and factors with HAT-activity, such as CBP/p300, need to be conducted. It is possible that these proteins interact with each other to form a large complex only in the presence of DNA. In addition, not all proteins within the complex may bind to DNA. For instance, GR or Pit-1 may be bind to DNA and act as mediators for other proteins. It is possible that GR and Pit-1 will not interact directly but through a larger complex. These questions will need further investigation to be able to understand the molecular details of glucocorticoid induction of GH expression. Reconstitution experiments using recombinant proteins could be conducted to answer some of these questions. Probe with the GCRR site sequence or the distal Pit-1 site sequence or both need to be included in the reconstitution experiments to confirm that these interactions do occur at these sites. Further, mutant GR or Pit-1 may be included in these studies to confirm interactions.

As mentioned earlier, glucocorticoid regulation of GH expression in chickens involves intermediary factors, and Dexras1 was identified as a potential candidate

involved in mediating glucocorticoid induction of GH expression. Dexras1 has only recently been identified as a glucocorticoid-regulated gene and remains to be characterized in context to pituitary functions. In the current study, glucocorticoid regulation of Dexras1 was characterized and a potential role for Dexras1 in regulating glucocorticoid-mediated GH expression in chickens was investigated. Glucocorticoid-regulated Dexras1 expression requires GR and MEK1/2 signalling. Dexras1 mRNA is expressed in somatotroph and corticotroph cells of the anterior pituitary in chickens. Dexras1 was predominantly found in the cytoplasm, and cellular localization of Dexras1 was not affected by glucocorticoid treatment. Transfection of 1 $\mu$ g of Dexras1 vector significantly decreased glucocorticoid-induced GH mRNA levels, while transfection of 10ng and 100ng of Dexras1 vectors stimulated basal GH mRNA levels. These results indicate that Dexras1 has a potential role in glucocorticoid induction of GH expression in chickens. The mechanism of Dexras1 action is although not clearly understood. We attempted to investigate the effect of Dexras1 on GH secretion in chicken pituitary cells using reverse hemolytic plaque assays. However, we were unable to perform the assays due to potential low transfection efficiency of embryonic pituitary cells. In our present study, overexpression of Dexras1 had no effect on the phosphorylation of Elk-1. Dexras1 overexpression also did not attenuate GH promoter activity. The mechanism involved in Dexras1 regulation of GH expression needs further investigation. To confirm involvement of Dexras1 in GH expression, Dexras1 knockdown experiments using siRNA will need to be conducted. Dexras1 may be indirectly regulating phosphorylation of Pit-1 or phosphorylation of GR. It will be interesting to find out effects of knockdown

of Dexras1 on Pit-1 and GR phosphorylation. Effect of Dexras1 on histone modifications and transcription factor recruitment to the chicken GH gene may also be investigated.

Dexras1 may be interacting with other proteins to regulate GH expression. Coimmunoprecipitation experiments to identify factors that interact with Dexras1 will provide insights into downstream targets of Dexras1. Factors known to interact with Dexras1 in other systems include carboxy-terminal PDZ ligand of nNOS (CAPON) (206), FE65-APP (207), etc. Dexras1 has been suggested to regulate secretion of hormone in AtT-20 cells (192). Role of Dexras1 in regulating secretion of GH from chicken pituitary cells needs to be investigated. We have demonstrated that Dexras1 is expressed in somatotroph and corticotroph cells of the anterior pituitary in chickens, and therefore, Dexras1 may be involved in regulating multiple cell types in the anterior pituitary. Effect of Dexras1 on ACTH expression (both promoter activity and mRNA levels) and secretion will need to be further investigated.

Overall, our present study has provided important insights into glucocorticoid-mediated GH expression. It has demonstrated that glucocorticoids mediate dynamic changes in histone modifications and transcription factor binding to the endogenous GH gene. It has identified a potential novel role for Dexras1 in mediating glucocorticoid effects on GH expression in chickens. It provides a basis for future studies for better understanding of glucocorticoid induction of GH expression.

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