ABSTRACT

Title of Dissertation: ONTOGENIC AND GLUCOCORTICOID-REGULATED GENE EXPRESSION IN THE DEVELOPING NEUROENDOCRINE SYSTEM

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The neuroendocrine system is a critical regulator of vertebrate homeostasis that includes five hypothalamic-pituitary axes which develop during embryogenesis. Adrenal glucocorticoids play an important role in functional maturation of the anterior pituitary through initiation of growth hormone (GH) production. These studies were aimed at characterizing ontogenic and glucocorticoid-regulated changes in gene expression during neuroendocrine system development in the chick. First, to ascertain timing of initiation and establishment of each neuroendocrine axis, we measured mRNA levels of hypothalamic regulatory factors, their pituitary receptors, and pituitary hormones from embryonic day (e) 10 through post-hatch day (d) 7. We found that the adrenocorticotropic axis is the first to be established (e12), followed by establishment of the thyrotropic (e18), somatotropic (e20), lactotropic (d1), and gonadotropic (d5) axes. Next, we examined in detail mechanisms through which glucocorticoids initiate pituitary GH expression during embryogenesis. We determined that glucocorticoids elevate GH

mRNA levels on e11 by increasing transcriptional activity of the GH gene rather than enhancing mRNA stability, and protein synthesis, histone deacetylase activity, ras signaling, and ERK1/2 signaling are required for this activation. Conversely, sustained activation of ERK1/2 and p38MAPK pathways reduced glucocorticoid stimulation of GH expression, indicating the requirement for ERK1/2 activity is transitory. Finally, we identified ras-dva as a novel Pit-1 and glucocorticoid-regulated gene in the chicken embryonic pituitary gland. Pituitary ras-dva mRNA levels increased between e10 and e18, decreased just prior to hatch, and remained low or undetectable post-hatch. Ras-dva expression was highly enriched within the pituitary gland on e18, and glucocorticoids rapidly induced ras-dva mRNA in cultured pituitary cells through a mechanism involving transcriptional activation. Potential regulatory elements within the 5'-flanking region of chicken ras-dva responsible for pituitary-specific expression were identified, as was a 2 kb fragment necessary for its glucocorticoid induction in embryonic pituitary cells. These results enhance our understanding of neuroendocrine system development and establishment during embryogenesis, reveal mechanisms underlying glucocorticoid initiation of GH expression in somatotrophs, and identify a new Pit-1 and glucocorticoid target gene that may play an important role in pituitary development.

ONTOGENIC AND GLUCOCORTICOID-REGULATED GENE EXPRESSION IN THE DEVELOPING NEUROENDOCRINE SYSTEM

by

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In loving memory of

Eric Sese

October 21, 1973 – August 20, 2008

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11β-HSD	11β-hydroxysteroid dehydrogenase
2D	two-dimensional
AA	amino acid
Ac-H3	acetylated histone 3
ACTB	beta-actin
ActD	actinomycin D
ACTH	adrenocorticotropic hormone
AF-1	activation function-1
Anf-1	anterior neural fold protein-1
Ani	anisomycin
AP-1	activator protein-1
ATCC	American Type Culture Collection
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumen
CA	constitutively active
caMEK1	constitutively active MAPK/ERK kinase 1
caMKK3	constitutively active MAPK kinase 3
caMKK6	constitutively active MAPK kinase 6
CBG	corticosteroid-binding globulin
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CORT	corticosterone
CRH	corticotropin-releasing hormone
CRH-R1	corticotropin-releasing hormone receptor 1
CRH-R2	corticotropin-releasing hormone receptor 2
Ct	cycle threshold
d	post-hatch day
DBD	DNA-binding domain
Dexras1	dexamethasone-induced ras-related protein 1
DMEM	Dulbecco's modified Eagle’s medium
DMEM/F12	DMEM:Ham's nutrient mixture F12
DMSO	dimethyl sulfoxide
DN	dominant negative
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dnERK1	dominant negative ERK1
dnERK2	dominant negative ERK2
dnMEK1	dominant negative MAPK/ERK kinase 1
dnMKK3	dominant negative MAPK kinase 3
dnMKK6	dominant negative MAPK kinase 6
dnp38α	dominant negative p38MAPKα

dnp38β	dominant negative p38MAPKβ
dNTPs	deoxynucleoside triphosphates
DTT	dithiothreitol
e	embryonic day
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
Elk-1	Ets-like gene 1
ERK	extracellular signal-related kinase
FGF	fibroblast growth factor
FKBP5	FK506-binding protein 5
FSH	follicle-stimulating hormone
FSH-β	follicle-stimulating hormone β-subunit
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green-fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRH-LPR	growth hormone-releasing hormone-like peptide receptor
GHRH-R	growth hormone-releasing hormone receptor
GHRH-R2	growth hormone-releasing hormone receptor 2
GHS-R	growth hormone secretagogue receptor
GnIH	gonadotropin-inhibitory hormone
GnIH-R1	gonadotropin-inhibitory hormone receptor 1
GnIH-R2	gonadotropin-inhibitory hormone receptor 2
GnRH1	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEK-293	human embryonic kidney-293
Hesx1	homeobox expressed in ES cells 1
HSP	heat shock protein
ICC	immunocytochemistry
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-β	luteinizing hormone β-subunit
LMH	leghorn male hepatoma
LSmean	least-squares mean
LY	LY294002

Man	manumycin A
MAPK	mitogen-activated protein kinase
MEK1/2	MAPK/ERK kinase 1/2
MEKK2	MAPK/ERK kinase kinase 2
MKK4/7	MAPK kinase 4/7
MLK3	mixed-lineage kinase 3
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-κ B
nGRE	negative glucocorticoid response element
NGS	normal goat serum
Nkx2.1	NK2 homeobox 1
NTD	N-terminal domain
Opti-MEM I	optimized Eagle's minimal essentail medium
p38MAPK	p38 mitogen-activated protein kinase
PACAP	pituitary adenylate cyclase-activating polypeptide
PACAP/GHRH-like	pituitary adenylate cyclase-activating polypeptide/growth hormone-releasing hormone like
PACAP-R1	pituitary adenylate cyclase-activating polypeptide receptor 1
p-ATF2	phosphorylated ATF2
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	PD98059
PDK1	3'-phosphoinositide-dependent kinase
p-Elk1	phosphorylated Elk1
p-ERK1/2	phosphorylated ERK1/2
PGK1	phosphoglycerate kinase 1
PI3-K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate
Pit-1	pituitary-specific transcription factor 1
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
POMC	pro-opiomelanocortin
p-p38MAPK	phosphorylated p38MAPK
PRL	prolactin
Prop-1	prophet of Pit-1
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
RACE	rapid amplification of cDNA ends
ras-dva	ras dorsal-ventral anterior

RNA	ribonucleic acid
RT	reverse transcription
SB	SB203580
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SF-1	steroidogenic factor-1
Shh	sonic hedgehog
SP	SP600125
Sport6.1	pCMV-Sport6.1
SST	somatostatin
SSTR2	somatostatin receptor type 2
STAT5	signal-transducer and activator of transcription 5
$t_{1/2}$	half-life
TBS/T	tris-buffered saline with tween-20
TESS	Transcription Element Search System
TRH	thyrotropin-releasing hormone
TRH-R	thyrotropin-releasing hormone receptor
Tris	tris(hydroxymethyl)aminomethane
TSA	trichostatin A
TSH	thyroid-stimulating hormone
TSH-β	thyroid-stimulating hormone β-subunit
U0	U0126
UTR	untranslated region
VIP	vasoactive intestinal peptide
VIP-R1	vasoactive intestinal peptide receptor 1
VIP-R2	vasoactive intestinal peptide receptor 2
Wort	wortmannin
α-GSU	α-glycoprotein subunit

CHAPTER 1

Literature Review

Introduction

Glucocorticoids are steroid hormones produced by the adrenal cortex that are absolutely essential to life and have effects in virtually every cell in the body except mature erythrocytes. They are important for regulation of several important physiological processes, including metabolism, immune function, the stress response, and development. They have pleiotropic effects that include rapid as well as prolonged cellular changes. Glucocorticoids are involved in functional maturation of several tissues, including adipose, lung, small intestine, and anterior pituitary. Within the anterior pituitary, glucocorticoids play an essential role in initiating hormone expression in somatotroph cells that produce growth hormone (GH).

The hypothalamus and pituitary gland make up the core of the neuroendocrine system. Through integration of internal and external cues, the neuroendocrine system regulates several important physiological processes that include response to stress, metabolic homeostasis, growth, and reproduction. At the central level, the hypothalamus transmits central nervous system signals in the form of releasing and release-inhibiting factors to the anterior pituitary gland, which relays the information to endocrine target organs through secretion of trophic hormones (1). The hypothalamus and pituitary develop during embryonic development in all vertebrates, and considerable progress has been made regarding signaling molecules and transcription factors involved in tissue commitment, organ formation, and cellular differentiation within this system. Despite this progress, molecular mechanisms involved in functional differentiation of specific pituitary cell types, including somatotrophs, are still poorly understood.

Glucocorticoid hormones

Glucocorticoid hormones are produced by the adrenal cortex under control of the hypothalamic-pituitary-adrenal axis. Release of hypothalamic corticotropin-releasing hormone (CRH) triggers adrenocorticotropic hormone (ACTH) production by the anterior pituitary gland, which in turn stimulates synthesis and release of glucocorticoids from the adrenal cortex. The primary circulating glucocorticoid in humans and most mammals is cortisol, while the principal glucocorticoid in birds and rodents is corticosterone (CORT). Glucocorticoid hormones are essential to life and have important immunomodulatory, metabolic, and developmental effects.

Once released into circulation, glucocorticoids are bound by corticosteroid-binding globulin (CBG) and distributed to target tissues throughout the body. Precisely how glucocorticoid hormones are delivered to individual cells within tissues is unknown. Mechanisms that have been proposed include free diffusion across cellular membranes due to their hydrophobic nature, the presence of specific glucocorticoid transporters that mediate active transmembrane transport, and/or binding of the CBG-CORT complex to a membrane receptor which either acts as a transporter or is internalized through endocytosis (2, 3). In any mechanism, CBG would need to be cleaved in order for free CORT to activate intracellular receptors, either the type I mineralocorticoid receptor (MR) or the type II glucocorticoid receptor (GR). The level of glucocorticoid transporters or CBG receptors could influence local delivery of CORT to specific tissues or cells in the last two scenarios. A second level of pre-receptor regulation that can modulate glucocorticoid activity is the presence of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes that catalyze interconversion of active and inactive hormone. 11β-HSD1 in

target tissues facilitates interaction of glucocorticoids with GR, because it increases levels of active, reduced hormone. Conversely, 11β-HSD2 converts active hormone to an inactive, oxidized form unable to bind receptors. Therefore, 11β-HSD2 is expressed mainly in mineralocorticoid target tissues, serving to protect MR from excess levels of glucocorticoids in circulation as compared to the mineralocorticoid aldosterone. These tissues include kidney, colon, and placenta, where 11β-HSD2 prevents fetal exposure to high amounts of maternal glucocorticoids (4).

Glucocorticoid receptor

A member of the nuclear receptor superfamily, GR has the ability to modulate gene expression by acting as a ligand-induced transcription factor. There are three major functional domains of nuclear receptors (5): the N-terminal domain (NTD), a central deoxyribonucleic acid (DNA) binding domain (DBD), and a ligand-binding domain (LBD) at the C-terminus. The NTD contains a ligand-independent transcriptional activation region, activation function-1 (AF-1), which is required for maximal transcriptional enhancement through recruitment of co-activators and association with basal transcriptional machinery. Two conserved zinc fingers within the DBD provide an interface for receptor dimerization or other protein-protein interactions and facilitate binding to glucocorticoid response elements (GREs) located within regulatory regions of direct transcriptional targets of GR. The GRE consensus sequence is an inverted hexameric nucleotide repeat with a 3 base pair (bp) spacer, AGAACAnnnTGTTCT (6). The LBD is responsible for recognition and binding of glucocorticoid ligands, as well as interaction with chaperones that are part of the cytoplasmic multiprotein complex in which GR is held in absence of ligand (see below). In addition, the LBD contains the

nuclear localization signal and ligand-dependent activation domain, activation function-2, which recruits gene-specific co-activators and co-repressors.

Several post-translational modifications can modulate activity of GR. As is the case with many other transcriptional regulators, GR is a phosphoprotein under basal (ligand-free) conditions and additional phosphorylation events occur upon hormone binding (7). Most phosphorylated residues are serines located within AF-1. In human GR, there are 5 serines in this region (S113, S141, S203, S211, S226), and a mutant GR lacking these sites showed substantially reduced phosphorylation levels and transcriptional activity (8). Four of these residues are conserved in chicken GR (9-11) and correspond to S115, S203, S211, and S226. Mitogen-activated protein kinase (MAPK) pathway activity has been implicated in phosphorylation of at least two of these residues. In human GR, S211 may be a target of p38 mitogen-activated protein kinase (p38MAPK) activity (12) and S226 may be a target of c-jun-N-terminal kinase (JNK) (13). Mouse GR S234 (corresponding to human/chicken S226) may be a target of extracellular signal-regulated kinase (ERK) MAPK signaling (14). Phosphorylation of GR at S203 and S211 appears necessary for full transcriptional activity of GR (15), and phosphorylation at S226 has been implicated in decreased GR transcriptional activity (13) and associated with GR nuclear export upon hormone withdrawal (16). It is becoming apparent that the combinatorial code of phosphorylated residues within AF-1 is a mechanism by which gene-specific GR regulation can be achieved. Different GR phospho-isoforms have been shown to selectively occupy certain GR target genes (17), and GR phosphorylation at different residues has been shown to alter recruitment of co-regulators in a gene-specific manner (17).

Activity of GR can be modulated by other post-transcriptional modifications as well. The covalent addition of small ubiquitin-related modifier-1 was shown to increase GR stability and dramatically enhance transcriptional activity (18), and a consensus sumoylation site is present in AF-1. However, this modification only appears to enhance transcriptional activity when multiple GREs are present in a promoter, and the synergy increases as the number of GREs increases. Acetylation of multiple lysine residues within the GR hinge region located between the DBD and LBD has been shown to modulate GR transcriptional activity (19). When GR was acetylated, it exhibited decreased binding to GREs and consequent decreased transcriptional activation.

Mechanism of glucocorticoid action: genomic effects

The classical model for glucocorticoid action involves regulation of gene transcription through interaction with intracellular GR, which then becomes a ligand-activated transcription factor that can induce or repress target gene transcription (20). In the absence of ligand, GR is part of a large cytoplasmic complex that is essential for maintaining it in a state competent to bind glucocorticoid hormones. This multimeric complex includes heat shock protein (HSP) 40, HSP70, HSP90, immunophilins such as FK506-binding protein 5 (FKBP5), and kinases including Src tyrosine kinase (21). Upon ligand binding, GR undergoes a conformational change that allows its dissociation from the chaperone complex and exposes its nuclear localization signal. Following nuclear translocation, GR can interact with regulatory regions of target genes to alter their level of gene expression.

There are four types of GR-binding elements, and in three of these GR directly binds DNA (5, 20) (Figure 1). Binding of GR to simple GREs usually involves

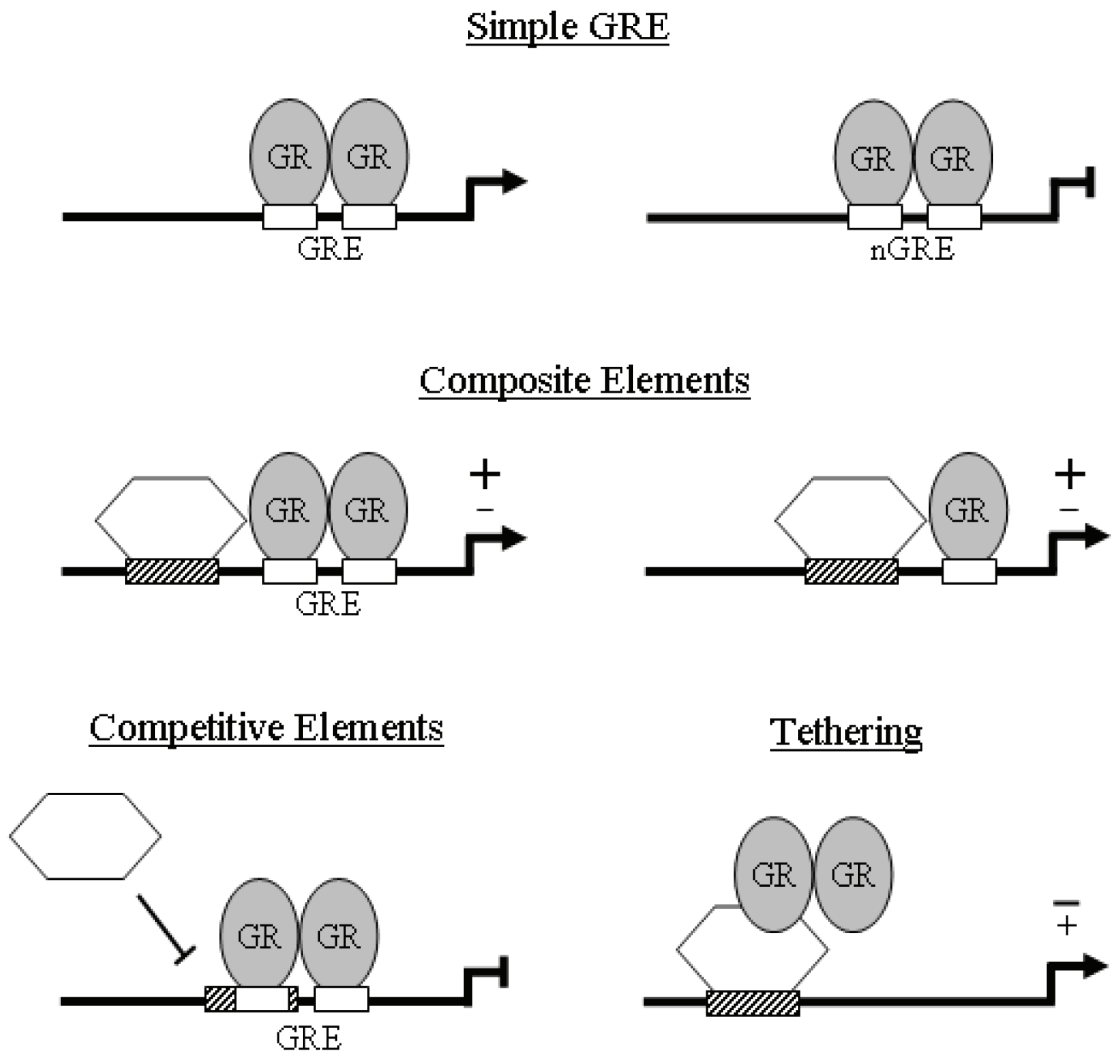


Figure 1. Mechanisms through which GR interacts with its regulatory elements. At simple GREs, GR directly binds DNA as a homodimer and can positively or negatively regulate transcription. Simple elements where GR represses transcription are called negative GREs (nGRE). At composite elements, GR binds in conjunction with another transcription factor as a homodimer or as a monomer. At competitive elements, GR binding prevents another transcription factor from binding. Protein-protein interactions in which GR is tethered to the DNA by another transcription factor also occur.

homodimerization, which leads to recruitment of transcriptional co-regulators and can also include tethering of additional transcription factors to the DNA by GR. Composite elements consist of direct DNA binding by GR and another transcription factor, and can involve GR binding as a homodimer to a GRE or GR binding as a monomer to a half-site. Competitive response elements consist of overlapping, mutually exclusive binding sites for GR and another transcription factor. In the fourth type of element, GR is tethered to the DNA through protein-protein interaction with another transcription factor. Interaction of GR with simple GREs can lead to positive or negative transcriptional regulation. In most cases, composite response elements are involved in positive regulation of transcription by GR and tethering interactions are involved in negative transcriptional regulation by GR (5), although there are exceptions. Tethering of GR to DNA by signal-transducer and activator of transcription 5 (STAT5) is involved in positive regulation of insulin-like growth factor-I (IGF-I) transcription in the liver, and tethering of GR to an activator protein-1 (AP-1) site in the gonadotropin-releasing hormone receptor (GnRH-R) regulatory region is involved in positive regulation of GnRH-R expression in a gonadotroph cell line (14, 22). Competitive response elements are generally associated with transcriptional interference (5, 20). Glucocorticoids can also interfere with transcriptional regulation by other factors through protein-protein interactions that prevent these factors from binding to their response elements. Ultimately, as with other transcription factors, GR is capable of inducing epigenetic changes to chromatin and can recruit co-repressors, co-activators, and basal transcriptional machinery in order to regulate the expression level of target genes.

Mechanism of glucocorticoid action: rapid, "non-genomic" effects

In addition to the classic transcriptional mechanism of action described above, glucocorticoids are capable of eliciting rapid effects that occur within seconds or minutes and involve direct actions on membrane lipids, membrane proteins, and cytosolic proteins. These effects are often termed "non-genomic", although in most cases the ultimate result at the cellular level involves transcriptional changes that may be secondary to rapid glucocorticoid effects. It is more useful to consider these as actions which do not initially affect gene expression but instead stimulate more rapid changes involving generation of second messengers and stimulation of intracellular signaling cascades (23). In many cases, it is hypothesized that these two modes of action are functionally related, and rapid effects lead to immediate changes that are necessary until slower genomic effects can lead to more persistent cellular changes (3). Several criteria are involved when classifying cellular responses as "non-genomic," which include some or all of the following: rapid response time; resistance to transcriptional and translational inhibition; resistance to inhibition of GR/MR; stimulation by membrane-impermeable forms of glucocorticoids; and action in non-nucleated cells (3, 24).

Interaction of steroids, including glucocorticoids, directly with cellular membranes has been shown to influence membrane fluidity and affect ion permeability and adenosine triphosphate (ATP) utilization (3, 25). These effects primarily occur in doses exceeding even pharmacological levels, and therefore their physiological relevance is questionable. Rapid glucocorticoid action also occurs through interaction with membrane proteins, and these effects are implicated in a wide variety of physiological processes such as calcium trafficking, neurotransmission, and stimulation of intracellular

signaling cascades, including protein kinase A (PKA), protein kinase C (PKC), MAPK, and phosphatidylinositol 3-kinase (PI3-K) (3, 12, 24, 26-30). The identity of the membrane associated receptor is unknown, but several lines of evidence implicate involvement of G-protein coupled receptor activity in membrane-initiated signaling events (26). Both low and high affinity glucocorticoid binding sites have been localized to plasma membrane, endoplasmic reticulum, endomembrane vesicles, and mitochondria (3), although their functional involvement in mediating rapid glucocorticoid action is not known. Rapid effects of glucocorticoids have also been shown to be mediated by classical intracellular receptors and/or components of the chaperone complex such as HSP70, HSP90, and Src (3, 31).

Functional anatomy of the neuroendocrine system

The pituitary gland, or hypophysis, hangs immediately beneath the hypothalamus at the base of the brain. The two are connected by the infundibular stalk surrounding the hypothalamic-hypophyseal portal veins that form the vascular connection between the two organs. The hypophysis is made up of two anatomically and functionally distinct parts: the posterior pituitary gland, or neurohypophysis; and the anterior pituitary gland, or adenohypophysis (32). The neurohypophysis is an extension of the hypothalamus, containing axons projecting from magnocellular neurons in the hypothalamus that release arginine vasopressin and oxytocin directly into the general circulation. The endocrine hypothalamus also contains parvocellular neurons that control production and release of hormones from the adenohypophysis. The parvocellular neurosecretory cell bodies are located in distinct clusters, or nuclei, and they release their hypophysiotropic factors from axonal projections in the median eminence at the inferior hypothalamic boundary (33,

34). From here, these factors enter the hypophyseal portal system and influence trophic hormone secretion from five distinct pituitary cell types characterized by the hormones they produce (Figure 2).

Neurosecretory cell bodies that release CRH and thyrotropin-releasing hormone (TRH), which primarily control ACTH and thyroid-stimulating hormone (TSH) secretion from corticotrophs and thyrotrophs, respectively, are localized to the paraventricular hypothalamus (35, 36). Pituitary gonadotrophs produce and secrete two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These are positively regulated by gonadotropin-releasing hormone 1 (GnRH1) released from cells located in the hypothalamic preoptic area and negatively influenced by gonadotropin-inhibitory hormone (GnIH) released from neurons contained in the paraventricular nucleus (37). GH secretion from pituitary somatotrophs is primarily regulated in a positive manner by growth hormone-releasing hormone (GHRH) released from neurons located in the arcuate nucleus of the hypothalamus and in a negative manner by somatostatin (SST) secreted by neurons in the periventricular hypothalamic area (38). GH can also be positively regulated by ghrelin and pituitary adenylate cyclase-activating polypeptide (PACAP), which directly stimulate GH release from the pituitary, enhance GHRH release from the hypothalamus, and potentiate GHRH signaling cascade (39, 40). The primary source of ghrelin in circulation is from the gastric mucosa, although it is also found in the hypothalamic arcuate nucleus and several additional internuclear areas (41). Neurosecretory cell bodies containing PACAP have been localized to the paraventricular nucleus, the preoptic area, and the supra-optic nucleus (42). In birds, TRH is also a potent GH secretagogue (43). Production of prolactin (PRL) from pituitary lactotrophs is unique

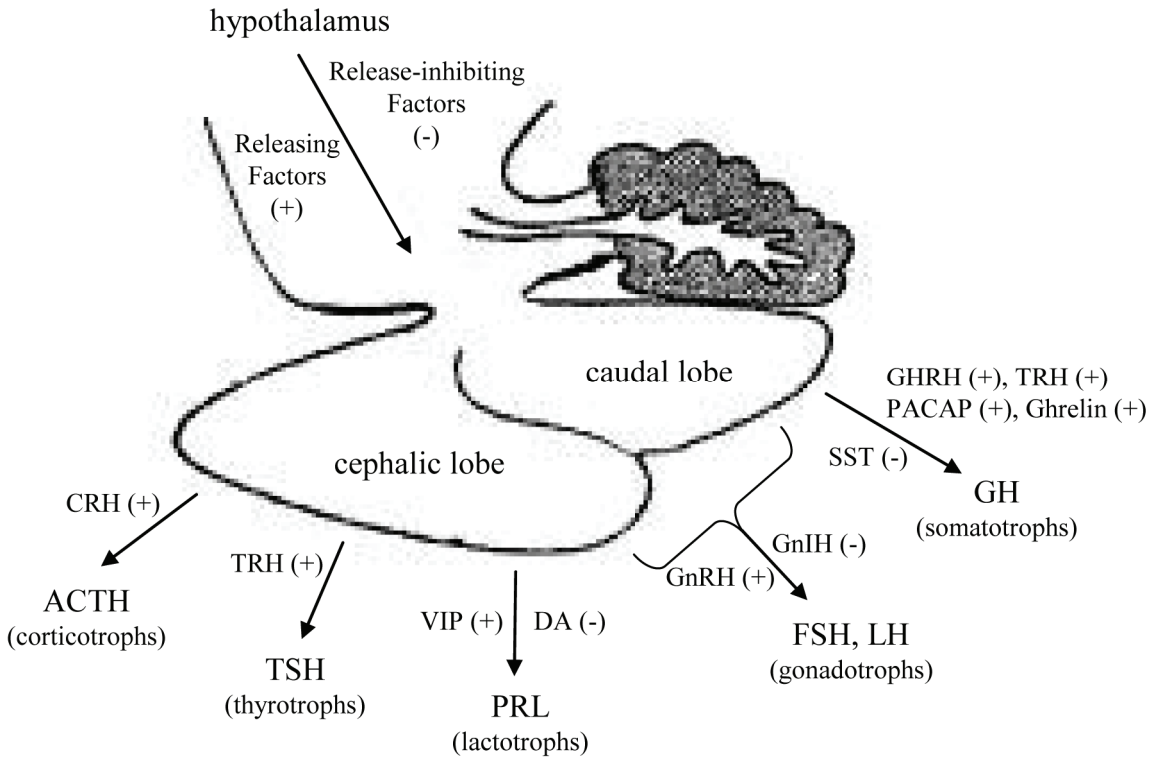


Figure 2. Representation of the avian hypothalamic-pituitary neuroendocrine axis. Hypothalamic releasing and release-inhibiting factors regulate synthesis and secretion of pituitary trophic hormones, which are secreted by distinct cell types. Adapted with permission from reference (44)

in that, in mammals, it is primarily under tonic inhibition from hypothalamic dopaminergic neurons located in the arcuate and periventricular nuclei (45) but can also be positively regulated by vasoactive intestinal peptide (VIP) released from neurons in the paraventricular nucleus in mammals and birds (46).

Anterior pituitary cell types are not randomly distributed throughout the gland; rather, they are organized within spatially restricted zones, such that common cell types tend to be clustered together. Additionally, recent work using two-photon excitation microscopy has revealed that GH-producing somatotrophs are organized into complex three-dimensional networks thought to facilitate cell-to-cell communication and

coordinate pulsatile GH secretion (47). It is likely that other pituitary cell types are organized into these networks as well. Two distinct caudal and cephalic lobes are found in the avian adenohypophysis, with four of the five pituitary cell types unevenly distributed among them. Corticotrophs, thyrotrophs, and lactotrophs are primarily localized to the cephalic lobe of the anterior pituitary gland, while somatotrophs reside in the caudal lobe (48-53). Gonadotrophs are initially present in the caudal lobe but spread throughout the entire gland as the pituitary develops (53, 54). In rodents and humans (55), gonadotrophs are most ventrally located, thyrotrophs are just dorsal to gonadotrophs, somatotrophs and lactotrophs are located in the medial region of the gland and are dorsal relative to thyrotrophs, and corticotrophs are clustered ventrally and rostrally relative to the somatotroph/lactotroph population. In addition to these endocrine cells, agranular folliculostellate cells are distributed throughout the pituitary gland. Folliculostellate cells are important paracrine regulators of anterior pituitary cell function through growth factor and cytokine secretion and are also thought to play an important role in intrapituitary communication between different cell types (56).

Neuroendocrine system development

The hypothalamus and pituitary gland develop in tandem during embryogenesis, and direct contact between the oral ectoderm that will ultimately give rise to the anterior pituitary and the overlying neural epithelium are required for their co-dependent development (57). The majority of what is known about neuroendocrine system development comes from spontaneous and engineered mouse models, and recent work has confirmed that the general principles are highly similar in zebrafish and chicken embryos. The importance of factors identified as having a role in neuroendocrine system

development is underscored by identification of mutations in several of the genes associated with human neuroendocrine pathologies (58). As with the development of many other systems, induction and formation of both the endocrine hypothalamus and anterior pituitary are dependent on signaling gradients leading to a combinatorial code of transcription factor expression in a temporally and spatially restricted manner.

Development of the neuroendocrine hypothalamus

Along the midline, the ventral region of the diencephalon gives rise to the hypothalamus. Ventral sonic hedgehog (Shh) signaling plays a critical inductive and early patterning role in hypothalamic development. Deficiency of Shh leads to hypothalamic loss, while increased activity leads to ectopic expression of hypothalamic markers (59, 60). After induction of the ventral diencephalon is complete, Shh signaling is downregulated by a dorsal gradient of bone morphogenetic proteins (BMPs), so differentiation into hypothalamic progenitor cells can occur (61).

The early Shh ventral-late BMP dorsal signaling gradient is necessary for establishing a correct spatial pattern of transcription factor expression in the developing hypothalamus (34). Complete loss of neurons within the supra-optic, paraventricular, and periventricular nuclei, including CRH-, TRH-, and SST-producing cells, occurs in homozygous mutant mice lacking the basic helix-loop-helix transcription factor single-minded homolog 1 or its dimerization partner aryl hydrocarbon receptor nuclear translocator 2. Loss of the above nuclei, as well as the arcuate nucleus containing GHRH and dopaminergic neurons, occurs in mutants lacking the homeobox gene orthopedia. Mutants for the homeodomain transcripton factor NK2 homeobox 1 (Nkx2.1) also fail to generate the arcuate nucleus and ventromedial nucleus located just dorsal to it. Mutations

in all of the above transcription factors lead to perinatal lethality, but there are other mutations that affect only a single neurosecretory cell type and lead to hypopituitarism. For example, Mash1 has been shown to be indispensable for generation of GHRH neurons (62), and H6 family homeobox 2 and 3 double mutants are deficient in only GHRH neurons within the arcuate nucleus (63).

During neurogenesis, progenitor cells become post-mitotic and differentiate into immature neurons that migrate from their place of "birth" to their final destination, where they develop neuronal projections. Neuronal birthdating studies combining *in utero* BrdU labeling with post-natal dual immunohistochemistry for BrdU and parvocellular peptides has revealed that parvocellular neurons arise concurrently from surrounding neuroepithelium early in the latter half of mammalian embryogenesis, between e12 and e13 in the rat (33, 64). It is generally thought that these neurons migrate inwards, so that the hypothalamus matures laterally to medially (33, 34). It should be noted that GnRH1 neurons are unique in that they arise in the olfactory placode and migrate through the ventral forebrain to the hypothalamus (33, 64). Beyond this, little is known about differentiation of these neuronal populations, particularly regarding the unique sets of transcription factors involved in neurosecretory cell-specific gene expression or timing of their functional differentiation (34). In the somatotropic axis, Gsh1 and Ikaros are required for GHRH expression, and mice with targeted disruption of these genes have almost complete loss of GHRH neurons, hypoplastic pituitary glands with reduced numbers of somatotrophs, and severe growth deficiencies (65-67).

Development of the anterior pituitary gland

The pituitary gland is of dual embryonic origin, with the neurohypophysis generated from a downward evagination of the ventral diencephalon and the adenohypophysis generated from an upward invagination of oral ectoderm known as Rathke's pouch (55). Adenohypophyseal development and differentiation can be divided into three stages (68, 69): gland commitment from primordial tissue; formation of Rathke's pouch; and emergence of terminally differentiated cell types. It is apparent that each stage of development is guided by signaling gradients that coordinate a sequential expression of transcription factors that in turn dictate cell-type specification, precursor proliferation, and terminal differentiation of the five hormone-producing cell types (69).

Inductive signals from the region of the ventral diencephalon that ultimately gives rise to the hypothalamus are required for pituitary gland commitment from primordial tissue, and direct contact between ventral diencephalon and adjacent ectoderm is essential for appearance of pituitary cell types (57, 70). Through an *in vitro* co-culture system and examination of signaling molecule expression in the neuroepithelium and oral ectoderm, it was revealed that BMP4 and fibroblast growth factor (FGF) 8 signaling originating from the ventral diencephalon are essential for pituitary gland commitment from oral ectoderm and Rathke's pouch formation (68). Subsequently, it was determined with Nkx2.1 and BMP4 knockout mice that formation of Rathke's pouch is a two-step process involving at least two sequential inductive signals from the ventral diencephalon (71). BMP4 is necessary for commitment and formation of a rudimentary pouch, and Nkx2.1 is necessary to support FGF8 expression leading to development of the definitive pouch. Shh signaling is also essential for pituitary development. It is initially expressed in both

the ventral diencephalon and throughout the oral ectoderm, but immediately upon formation its expression is restricted from Rathke's pouch (68, 72). Both dorsal FGF8 emanating from the diencephalon and ventral Shh from the oral ectoderm are necessary for induction of Lhx3, a transcription factor essential for proliferation of progenitor cells that allows progression beyond rudimentary pouch formation (68, 71, 72). Shh signaling also appears necessary to support the intrinsic BMP2 signaling that emerges ventrally within the developing pouch (68, 72). These opposing dorsal-to-ventral FGF8 and ventral-to-dorsal BMP2 gradients are ultimately what appear to create the spatial and temporal restriction of transcription factor expression that leads to emergence of pituitary cell types (69, 73). Similar gradients have recently been observed in chicken embryos and zebrafish (53, 74), confirming the highly conserved nature of neuroendocrine development.

During anterior pituitary development, many of the earlier transcription factors induced are involved in proliferation, patterning, and expansion of progenitor cells, while factors expressed later are involved in lineage determination and functional differentiation of cell types. In addition to Lhx3, the homeodomain transcription factors Isl1, Pitx1 and Pitx2 are important for cellular proliferation leading to gland maturation after Rathke's pouch formation (71, 75, 76). In addition to these factors that are important for organogenesis, additional homeodomain transcription factors are necessary for positional commitment of pituitary cell types, including Six1, Six4, and Pax6 (77). Several of these play multiple roles in the pituitary and are involved in regulation of hormone expression in the mature gland in addition to organogenesis. Homeobox expressed in ES cells 1 (Hesx1) is a transcriptional repressor that becomes restricted to

Rathke's pouch shortly after its formation and is downregulated just prior to appearance of terminally differentiated cell types (78). It is thought to play a role in preventing terminal differentiation during proliferation of progenitor cells, and it must be downregulated in order for appearance of all cell types except corticotrophs (77, 79).

The combinatorial pattern of transcription factors necessary for cell type-specific gene expression is slowly being revealed, although by no means are all factors essential for this specificity known. In the pituitary, Tbx19 is restricted to corticotrophs and melanotrophs, and its expression precedes that of pro-opiomelanocortin (POMC), the precursor from which ACTH is proteolytically cleaved. In combination with Pitx1, it is essential for corticotroph and melanotroph gene expression (80). The downregulation of Hesx1 coincides with expression of a closely-related pituitary-specific transcriptional activator, prophet of pit-1 (Prop-1). Hesx1 and Prop-1 have mutually antagonistic activities. Hesx1 can heterodimerize with Prop-1 at consensus DNA binding sites and prevent its activity (81), while Prop-1 can act as a transcriptional repressor of Hesx1 expression in the presence of appropriate cofactors (82). Prolonged expression of Hesx1 during embryogenesis prevents differentiation of thyrotrophs, somatotrophs, lactotrophs, and gonadotrophs through interference with Prop-1 transcriptional activity (81). Prop-1 initially activates expression of pituitary-specific transcription factor 1 (Pit-1) (81), which is essential for differentiation and maintenance of hormone expression in thyrotrophs, somatotrophs, and lactotrophs (83). In thyrotrophs, GATA-2 and Pit-1 are both required for differentiation and maintenance of cell type-specific gene expression (84). Lactotroph-specific PRL expression is known to require estrogen receptor, Pit-1, Ets-1, and Pitx factors (85-87). Steroidogenic factor-1 (SF-1) is required for differentiation of

gonadotrophs, and cell type-specific gene expression requires not only the presence of SF-1, which cooperates with GATA-2, but also the absence of Pit-1 (73, 77). In somatotrophs, Pit-1, retinoid X receptor, retinoic acid receptor, thyroid hormone receptor, Sp-1, and Zn-15 are required for GH expression (77, 88-90). Three closely spaced Pit-1 binding sites located approximately 14.5 kilobases (kb) upstream of the human pituitary GH gene are required for appropriate somatotroph expression (91, 92). This region is hyperacetylated in a pituitary-specific manner, and deletion of two of these Pit-1 sites leads to a decrease in acetylation and reduction in pituitary GH expression (93), indicating that these Pit-1 sites play an important role in epigenetic modifications necessary for transcriptional activation of GH in somatotrophs. It is not known if similar mechanisms are involved in somatotroph-specific GH expression in other species. The above examples are by no means inclusive, but rather represent the best characterized transcription factors involved in specification of pituitary cell type gene expression. In addition, although factors have been identified in regulating gene expression in mature pituitary cell types, there is still a scarcity of information regarding mechanisms and factors specifically involved in initiating cell-type specific gene expression during functional differentiation of the hormone-producing cells.

Despite this, it is well established that pituitary cell types emerge during embryogenesis in a temporally conserved manner in all species. Corticotrophs are the first cell type to appear in rodents and birds (53, 87, 94-96). Gonadotrophs are considered to be the second cell type to appear, despite observations that pituitary FSH and LH levels substantially increase at the end of embryonic developmental or early in post-natal life (53, 54, 87, 94-97). The cell types in the Pit-1 lineage are the final three to appear,

with thyrotrophs emerging first, followed by somatotrophs and, lastly, lactotrophs (51-53, 87, 94-96, 98-106).

Pituitary somatotrophs

Pituitary GH plays a central role in regulating post-natal growth and body composition in all vertebrates. With the exception of lactating mammals and nesting birds, somatotrophs are the most abundant cell type within the mature anterior pituitary gland, comprising up to 50% of all pituitary cells. Additionally, the most common form of congenital hypopituitarism is isolated growth hormone deficiency (58). In humans, deficient GH secretion leads to short stature, decreased muscle and bone mass, and increased fat mass. An excess of GH secretion, generally resulting from pituitary adenomas, causes tissue overgrowth, life-threatening metabolic disorders, and can lead to gigantism if it occurs prior to long bone growth plate closure (107). The effect of GH on overall growth and its contribution to leanness are also important for meat production in agriculturally important animals, such as chickens.

Somatotropic axis and regulation of post-natal growth

Classically, the somatotropic axis is composed of pituitary GH, its chief hypothalamic regulators GHRH and SST, and IGF-I produced in liver and other peripheral tissues. In addition to regulation by hypothalamic factors (GHRH, PACAP, Ghrelin, and SST), pituitary GH is subject to negative feedback from IGF-I and GH itself in the peripheral circulation. Although IGF-I is produced in many tissues, circulating IGF-1 from the liver is primarily responsible for negative regulation of GH (108). IGF-I blocks GH synthesis and release from somatotrophs and can stimulate and inhibit release of hypothalamic SST and GHRH, respectively (109). In the hypothalamus, GH decreases

GHRH messenger ribonucleic acid (mRNA) and increases SST mRNA, and it can decrease GHRH receptor (GHRH-R) mRNA in the anterior pituitary (110). In birds, thyroid hormones negatively feed back on TRH-stimulated GH release (43).

The somatotropic axis is absolutely essential for postnatal growth. This is evident from multiple naturally occurring mutations in the axis that result in decreased somatic growth of mammals and birds. The *little* mouse has a missense mutation in the GHRH-R that disrupts function within the pituitary (111), the *Snell dwarf* is a Pit-1 null mutant (83), and the *Ames dwarf* is a Prop-1 null mutant (81). The *spontaneous dwarf* rat has a point mutation in the GH gene that results in premature translational termination (112). In all these mutations, GH-producing cells are absent or severely reduced. The *sex-linked dwarf* chicken has a mutation in the GH receptor that results in a functional knockout (113). These mutations have relevance in human medicine. Isolated growth hormone deficiency can result from defects in GHRH-R, combined pituitary hormone deficiency can result from mutations in Prop-1 and Pit-1, and Laron dwarf syndrome results from GH insensitivity due to a truncated or inactive receptor (114). Defective GH receptor signaling through Janus kinase (JAK)-STAT can also be a cause of Laron dwarfism. Similarly, deficiencies in IGF-I production, transport and metabolism, and IGF-I receptor levels or signaling can lead to decreased growth (115).

Regulation of postnatal growth by components of the somatotropic axis is referred to as the somatomedin hypothesis (116). According to the current hypothesis, GH stimulates both hepatic IGF-I production that mediates peripheral growth in an endocrine manner and local IGF-I that regulates growth in an autocrine/paracrine fashion. In addition, GH directly affects muscle, bone, and adipose and can have IGF-I independent

effects on growth. The most conclusive evidence for an indispensable role of GH in postnatal growth of mammals and birds comes from ablation/replacement experiments in which pituitary removal results in cessation of growth that can be partially reversed by administration of exogenous GH (117, 118). In addition, transgenic mice overexpressing human GH exhibit accelerated growth relative to normal littermates starting at 3 weeks of age (119).

Somatotroph differentiation during embryogenesis

In both rodents and chickens, somatotrophs functionally differentiate during the second half of embryonic development, and their numbers dramatically increase at the end of this period. During the 21-day pregnancy, GH mRNA and protein expression in rats and mice is first evident on embryonic day (e) 15, and somatotroph numbers substantially increase between e18 and e19 (87, 95, 96, 100, 101, 120-123). Somatotrophs first appear during the chick 21-day incubation period on e12 and become a significant anterior pituitary cell population around e16 (44, 53, 94, 99, 103, 104, 106, 124-127). Ontogeny of somatotrophs is associated with an increase in GH mRNA (101, 124, 126, 128), protein (99, 100, 103, 120), and secretory capacity (103, 129). In chickens, initial somatotrophs are subject to negative regulation by SST and IGF-I and are responsive to GHRH, but they do not become fully responsive to TRH until e20 (103, 130, 131).

Somatotrophs are part of the Pit-1 lineage (83), and the presence of cell types within this lineage is absolutely dependent on pituitary-restricted transcription factors Prop-1 and Pit-1. *Ames* and *Snell* dwarf mutant mice have mutations in Prop-1 and Pit-1, respectively, and have hypoplastic pituitaries with absence (*Snell*) or near-absence

(*Ames*) of thyrotrophs, lactotrophs, and somatotrophs (83, 132). Prop-1 appears to be necessary for Pit-1 lineage-specific proliferation prior to terminal differentiation (132) and for proper initiation of Pit-1 expression (81). Regulation of GHRH-R by Pit-1 is at least partially responsible for the hypoplastic pituitary phenotype in Snell dwarf mice, because GHRH signaling is necessary for proper expansion of the somatotroph lineage during pituitary development (65, 133, 134). Pit-1 expression begins prior to onset of somatotroph appearance in mammals and birds, and Pit-1 mRNA was first apparent in mice on e13.5 (135) and in chickens on e5 (136). Analysis of GH promoter sequences in mammals and birds revealed multiple Pit-1 binding sites (135, 137), and this transcription factor is essential for GH gene expression (135).

Pituitary somatotroph differentiation during embryonic development is modulated by adrenal glucocorticoids and thyroid hormones (44, 121), and appearance of somatotrophs in fetal rats and embryonic chickens coincides with an increase in circulating glucocorticoid and thyroid hormone levels (10, 138-142). Extra-pituitary signals are required for initiation of pituitary GH. In pituitary explants taken from fetal rats prior to the onset of GH appearance, somatotrophs did not autonomously develop in culture (143-145). The involvement of an endocrine signal in initiation of pituitary GH expression is also evident from experiments in which dispersed pituitary cells from e12 chickens did not spontaneously begin producing GH during extended cell culture (through 6 days) but required treatment with serum from e16 chickens (146). Hypothalamic factors that regulate somatotroph function in birds, including GHRH and TRH, were not effective at initiating GH secretion. The blood-borne factor was later identified as CORT (147), the primary circulating glucocorticoid in rodents and birds.

Subsequent experiments further established a role for CORT in somatotroph differentiation in embryonic chickens *in vivo*. When e11 chicken embryos were treated with either serum from e16 chickens or CORT, the number of GH-secreting cells on e13 and e14 was increased (148, 149). Similar results have been observed in mammals. Treatment of pregnant rats with dexamethasone increased the abundance of somatotrophs present in e17 or e18 fetuses (145, 150). Glucocorticoids are also capable of increasing somatotroph differentiation *in vitro*, as they have been shown to increase GH mRNA, GH intracellular protein, and GH secretion in pituitary explants from fetal rats (143, 144, 151, 152) or cultured chick embryonic pituitary cells (129, 147, 153-155). This effect can be enhanced by GHRH. In e12 chicken pituitary cells, GHRH alone did not increase the number of somatotrophs but was shown to augment CORT induction of GH-secreting cells and GH mRNA after at least 3 days in culture (129, 154). Somatotrophs induced prematurely by CORT in embryonic chickens are fully functional and able to respond to the hypothalamic GH-releasing factors GHRH and TRH (129). Aldosterone has also been shown to increase GH production in chicken embryonic pituitary cells (155, 156).

Further evidence that circulating CORT is involved in somatotroph recruitment comes from experiments in rats and chickens where endogenous glucocorticoid levels were manipulated. Suppression of fetal adrenal glucocorticoid synthesis by administration of metyrapone to pregnant rats resulted in a reduction of fetal somatotrophs on e19 (150), and increasing circulating CORT levels in chick embryos through *in ovo* ACTH injection on e11 leads to premature GH cell appearance on e13 (140). Despite the large body of evidence indicating that adrenal glucocorticoids trigger GH expression associated with functional differentiation of somatotrophs, it was recently

reported that GH mRNA levels spontaneously increased in pituitary explants from e11 chickens cultured for 48 h (155). The authors attributed this spontaneous increase to intrapituitary production of corticosteroids, because it was blocked by metyrapone and they detected steroidogenic enzyme transcripts in the embryonic pituitary gland.

Unlike glucocorticoids, thyroid hormones alone cannot increase GH expression in somatotrophs but do appear to modulate effects of corticosteroids. In fetal rat explants and cultured pituitary cells from embryonic chickens, thyroid hormones failed to increase GH expression but did enhance the increase that resulted from glucocorticoid treatment (143, 157). The combined effect of glucocorticoid and thyroid hormone treatment is dependent on hormone dosage. A synergistic effect of thyroid hormones was only apparent at lower glucocorticoid doses in fetal rat explants (143), and higher doses of thyroid hormone blocked CORT stimulation of GH-secreting cells in cultures from embryonic chickens (157). Similar results were obtained when thyroid hormones were administered *in vivo*. Injection of thyroxine into pregnant rats did not induce GH without concomitant administration of dexamethasone (145). In studies using embryonic chickens, *in ovo* injection of thyroid hormones increased the number of GH-secreting cells (141). However, the authors concluded that this was due to the interaction of injected hormones with endogenous glucocorticoids, because the findings were in contrast to the aforementioned *in vitro* results. Suppression of endogenous thyroid hormone levels by methimazole decreased the somatotroph number on e19 in rats and e14 in chickens (141, 145), and the effects were reversed with thyroid hormone injection.

The ability of exogenous glucocorticoids to increase numbers of GH-producing cells *in vivo* is restricted to a narrow period of embryonic development. Oral

administration of dexamethasone to pregnant rats did not induce the number of GH-containing cells on e16, minimally induced them on e17, and led to a maximal increase on e18. On e19, dexamethasone administration led to an increase in the amount of GH protein within somatotrophs but did not increase somatotroph abundance (150). In chickens, *in ovo* CORT injection on e11 and e12 increased the number of GH-secreting cells on e13 and e14, respectively, but injection of CORT on earlier ages did not increase numbers of GH cells (149). However, although CORT injection on e11 increased somatotroph abundance on e13, the increase was not maintained on e16, e19, or post-hatch day (d) 1. Somatotrophs are found in the caudal lobe of the avian pituitary gland (49, 50, 53, 128). The induction of pituitary GH by CORT *in vitro* occurs mainly in the caudal lobe as opposed to the cephalic lobe (129, 155), and the increase resulting from *in ovo* CORT injection was observed to be caudally restricted (149). Taken together, these results imply that glucocorticoids are involved in the final stages of somatotroph differentiation and initiate GH expression in premature somatotrophs already committed to this lineage.

The working model of functional differentiation of pituitary somatotrophs involves the interplay of endocrine factors from adrenal and thyroid glands, the hypothalamus, and the pituitary gland itself (Figure 3). The effects of circulating glucocorticoids are influenced by thyroid hormones and at least one hypothalamic factor, GHRH. The increase in circulating adrenal glucocorticoids and thyroid hormones most likely results from increased ACTH and TSH output from the pituitary toward the end of embryonic development, which may be a result of hypothalamic CRH and TRH stimulation.

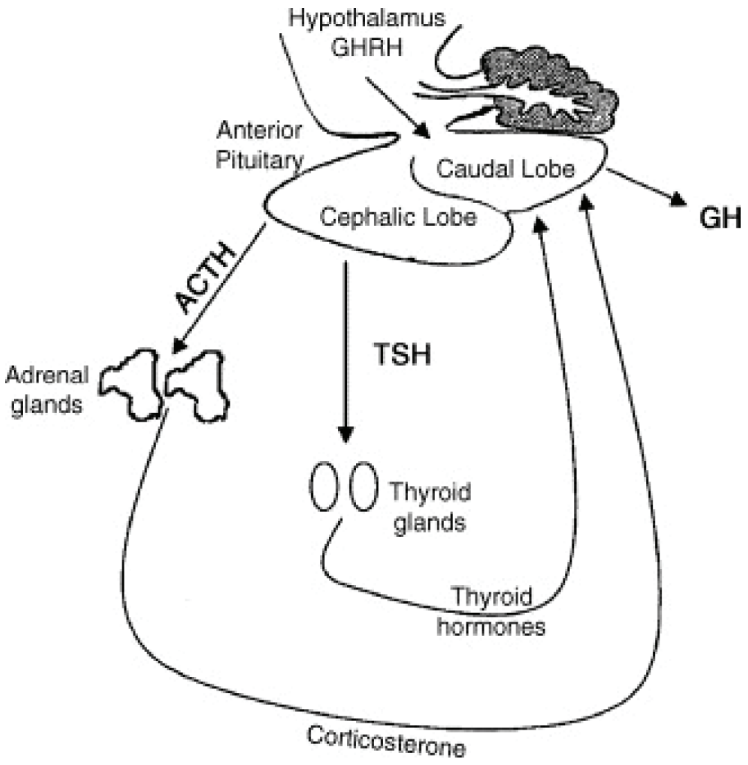


Figure 3. Model for the interplay of endocrine factors involved in pituitary somatotroph differentiation during embryonic development. Reproduced with permission from reference (44).

Mechanisms involved in glucocorticoid induction of pituitary GH expression

Molecular mechanisms underlying glucocorticoid recruitment of somatotrophs during pituitary development are largely unknown. In embryonic chickens, the CORT-stimulated increase in GH-secreting cells was not observed to involve proliferation of existing somatotrophs (147). This is consistent with *in vivo* observations that the majority of initial GH-containing cells that emerge in fetal rats around e18 were not labeled with BrdU, but somatotroph expansion that occurred perinatally did involve mitotic events (123). Glucocorticoid initiation of GH expression in the mammalian and avian pituitary gland appears to occur in an indirect manner requiring synthesis of an intermediary

factor(s). Stimulation of pituitary GH mRNA by dexamethasone in fetal rat pituitaries required at least 8 h exposure (151), and CORT induction of GH mRNA in cultured chicken embryonic pituitary cells also required treatment over 4 h (153). In both cases, the increase in GH mRNA was blocked by protein synthesis inhibitors.

The hypothesis that glucocorticoids are indirectly inducing GH production in anterior pituitary cells through stimulation of an intermediary protein(s) is supported by evidence implicating involvement of nuclear receptors that traditionally act as ligand-induced transcription factors. Glucocorticoids are capable of binding to and activating both GR and MR. In the fetal rat pituitary, GR mRNA is detected by e15, well before somatotroph appearance, and GR protein increased between e17 and e18 (158). Transcripts for MR and GR are detected as early as e5 in the chicken embryonic anterior pituitary gland (155). Quantitative measurements indicate that both MR and GR increase between e10 and e14 (10, 124, 159), when somatotrophs first begin to appear and around the time somatotrophs can be induced by *in ovo* glucocorticoid injection. In the chicken embryonic pituitary gland, expression of GR protein was detected as early as e8 and levels slightly increased through e12. On e12, GR was detected in virtually all pituitary cells. Conversely, MR protein was not detected until e12, when it was present in approximately 40% of all pituitary cells and 90% of somatotrophs induced *in vitro* by CORT. Experiments investigating involvement of these receptors in CORT stimulation of GH in the chicken embryonic pituitary gland indicate that both are likely involved in the response. In one study, the presence of either a GR-specific antagonist (ZK98299) or a MR-specific antagonist (spironolactone) partially repressed CORT increases in GH mRNA or numbers of GH-protein containing cells, but inclusion of both antagonists

completely abolished CORT induction of GH (156). In another study, antagonists to either GR or MR were sufficient to block CORT stimulation of GH mRNA (155). In a third study, antagonism of GR alone completely blocked the CORT-induced increase of GH secretion, while MR antagonism was only partially effective (159). Further evidence for involvement of traditional receptors comes from a report that CORT stimulation of intracellular GH protein content is blocked by geldanamycin, a compound that prevents proper assembly of GR and MR complexes necessary for steroid binding (153).

The identity of the key intermediary factor(s) has yet to be elucidated, although evidence suggests that they are not likely to be Pit-1 or GHRH-R. In fetal rats, dexamethasone treatment of explanted e18 pituitary glands significantly increased GH mRNA and protein but did not increase Pit-1 mRNA or protein (151). Similarly, *in vitro* treatment of e13 chicken anterior pituitary cells did not increase the number of Pit-1 containing cells or the level of Pit-1 protein in the cells (160). Pituitary GHRH-R mRNA was initially detected in fetal rat pituitaries on e19, coincident with appearance of somatotrophs. Likewise, in the chicken embryonic pituitary gland, GHRH-R mRNA increased between e10 and e14 (124), around the time of somatotroph appearance. Despite observations that dexamethasone is capable of increasing GHRH-R mRNA in fetal rat pituitary explants (161), experiments in chicken embryonic pituitary cells do not provide evidence that GHRH-R is involved in CORT induction of GH. Treatment with CORT did not elevate GHRH-R mRNA or protein levels in chick embryonic pituitary cells, nor did it increase GHRH binding to the cells (Ellestad and Porter, unpublished; Bossis and Porter, unpublished). Further, pharmacological inhibition and activation of PKA or PKC, the primary routes for GHRH-R signaling (162), did not block CORT

stimulation of GH mRNA or increase GH mRNA in the absence of CORT. Alternatively, inhibition of ras signaling with manumycin A did suppress CORT induction of GH (153).

Enhancement of CORT-stimulated GH expression by GHRH does appear to involve GHRH-R signaling through the PKA pathway. Pharmacological activation of PKA mimicked the effect of GHRH on CORT induction of GH mRNA, and inhibition of PKA blocked the effect. Manipulation of PKC signaling did not influence GH mRNA levels, either under basal or CORT-treated conditions (153). Nonetheless, while GHRH-R induced PKA signaling may be necessary for synergistic effects of GHRH and CORT on GH mRNA induction, GHRH-R does not appear to mediate the indirect effect of glucocorticoid treatment. Results with manumycin A imply that the intermediary factor may be a ras-like protein, a factor that activates ras signaling, or a target of ras-induced signaling cascades. Of particular interest regarding glucocorticoid initiation of pituitary GH during somatotroph functional differentiation is glucocorticoid stimulation of intracellular signaling pathways that are known effectors of ras signaling, since the ras inhibitor manumycin A repressed CORT induction of GH mRNA in the developing pituitary gland (153). Four pathways that are known be effectors of ras and have been demonstrated to be activated by glucocorticoids are PI3-K (30) and three major MAPK pathways, ERK1/2, p38MAPK, and JNK (12, 27-29). A simplified scheme of well-characterized cascades within these pathways is shown in Figure 4.

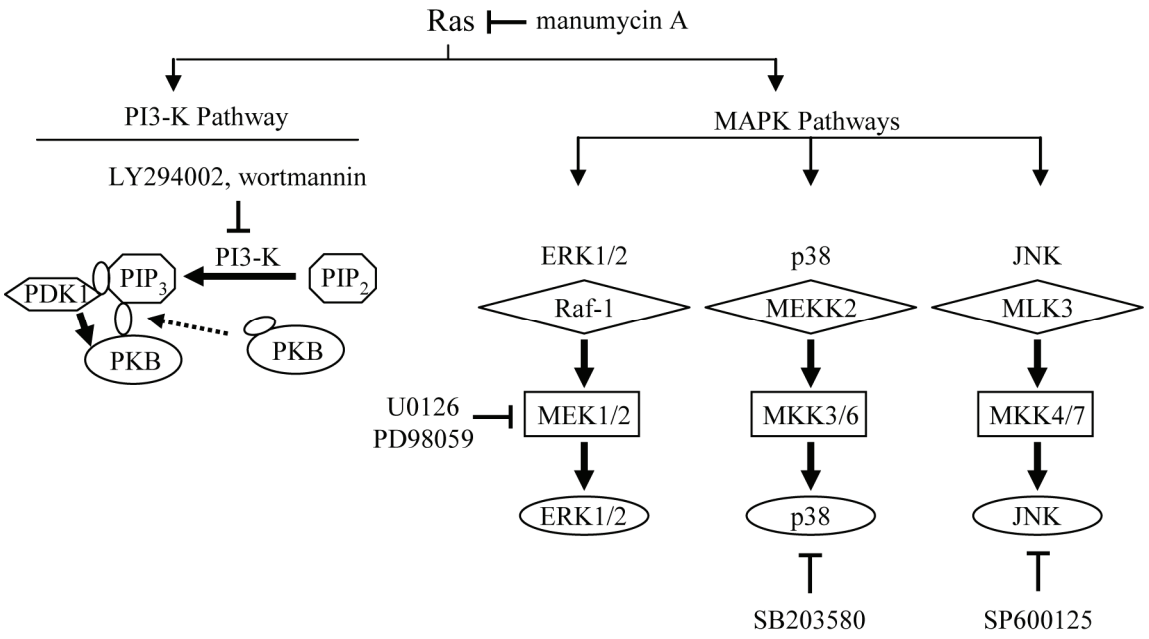


Figure 4. Potential ras-induced signaling cascades involved in glucocorticoid initiation of pituitary GH expression. PI3-K enzymes are intracellular lipid kinases that phosphorylate phosphatidylinositol phosphates (PIPs), creating docking sites for 3'-phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). PDK1 activates PKB by phosphorylation, and PKB can in turn phosphorylate multiple cellular targets. MAPK signaling cascades consist of three core kinases that act in a sequential manner to transmit extracellular signals to elicit cellular responses. Examples of well characterized components are shown. Ras interacts with and activates MAPK kinase kinases (Raf-1, MEKK2, MLK3), which in turn activate MAPK kinases through serine/threonine phosphorylation (MEK1/2, MKK3/6, MKK4/7). These in turn activate MAPKs (ERK1/2, p38MAPK, and JNK) through phosphorylation on tyrosine and threonine residues. Activated MAPKs phosphorylate both cytosolic and nuclear substrates, including kinases, transcription factors, and histones. Sites of action for pharmacological inhibitors used in studies investigating signaling mechanisms involved in CORT initiation of GH are shown.

Avian embryo as a model for neuroendocrine system development

The development, control, and function of the neuroendocrine system are generally similar in mammals and birds. The embryonic/early post-hatch chicken offers unique advantages over mammalian systems for studying endocrine gland development. Accessibility of the embryo readily allows *in ovo* manipulation of individual embryos, and the chicken embryo develops in the absence of interactions with maternal endocrine systems, permitting manipulation of embryonic endocrine systems without maternal

interference (163). Importantly, fertilized chicken eggs are inexpensive and can be developmentally synchronized simply by placing them in an incubator, and chicken embryos are large in size relative to traditional rodent models at comparable developmental stages. This allows collection of adequate tissue for analysis and also facilitates *in vitro* investigation of intracellular mechanisms underlying important developmental endocrine processes in a defined cell culture system. In addition, the neuroectoderm and Rathke's pouch are accessible to several techniques that could enhance understanding of the role that signaling molecules and transcription factors play in hypothalamic and pituitary development, including introduction of retroviral vectors and *in ovo* electroporation of expression plasmids and antisense oligonucleotides. The recent acquisition of a large expressed sequence tag collection and the completion of the genome sequence have confirmed the chicken's status as an excellent developmental model and will allow it to be used in large-scale screens assessing gene function during embryonic development (164-167).

Rationale and Objectives

Although the importance of the neuroendocrine system in regulating several key vertebrate physiological processes is widely recognized, no comprehensive studies investigating gene expression changes during hypothalamic and pituitary development have been conducted. Understanding relationships among components of the five neuroendocrine axes should significantly enhance our comprehension of neuroendocrine system development. The essential nature of the somatropic axis in regulating post-natal growth is apparent from naturally occurring genetic mutations at several levels of the axis in mammals and birds. Despite this, molecular mechanisms underlying appearance of pituitary somatotrophs that secrete GH, a central component of this axis, are poorly understood. Glucocorticoid hormones have important developmental effects in several tissues, including the anterior pituitary gland. Understanding their role in initiating pituitary GH expression and investigating regulation of novel glucocorticoid targets in the anterior pituitary should provide insights into glucocorticoid action in other tissues. Therefore, this research project had three primary objectives:

1. To characterize mRNA levels of hypothalamic releasing and release-inhibiting factors, their pituitary receptors, and pituitary hormones in the major neuroendocrine axes during development;
2. To investigate molecular mechanisms through which glucocorticoid hormones initiate GH expression during somatotroph development;
3. To characterize glucocorticoid and pituitary regulation of a novel gene, ras dorsal-ventral anterior (ras-dva).

CHAPTER 2

Ontogenic characterization of gene expression in the developing neuroendocrine system of the chick

Abstract

The neuroendocrine system consists of five major hypothalamic-pituitary hormone axes that regulate several important metabolic processes, and it develops in all vertebrates during embryogenesis. Despite their importance, a comprehensive analysis of the ontogeny of all axes simultaneously has not been performed in any species. In order to define initiation and establishment of these five axes in the developing chicken, mRNA expression profiles of hypothalamic releasing and release-inhibiting factors, their pituitary receptors, and pituitary hormones were characterized during the second half of embryogenesis and the first week post-hatch in the chick. Axis initiation was defined as the age when pituitary hormone mRNA levels began to increase substantially, and establishment was defined as the age when mRNA for all components had reached maximum expression levels. The adrenocorticotropic axis appears established by e12, as there were no major increases in gene expression after that age. Hypothalamic TRH and pituitary TSH β-subunit (TSH-β) increased between e10 and e18, indicating establishment of the thyrotropic axis during this period. Pituitary GH substantially increased on e16, and hypothalamic GHRH did not increase until e20, indicating that somatotropic axis activity is established late in embryonic development. Lactotropic axis initiation is evident just prior to hatch, as pituitary PRL and vasoactive intestinal peptide receptor 1 (VIP-R1) did not increase until e18 and e20, respectively. Hypothalamic GnRH1 increased after hatch, and pituitary LH β-subunit (LH-β) expression remained low until d3, indicating the gonadotropic axis is not fully functional until after hatching. This study is the first to characterize major hypothalamic and pituitary components of all

five axes and considerably increases our understanding of neuroendocrine system establishment during development.

Introduction

There are five major hypothalamic-pituitary axes, each consisting of releasing and inhibiting hormones produced by the hypothalamus that control pituitary hormone production by acting through receptors on specific cell types within the adenohypophysis. In corticotrophs, ACTH is produced through proteolytic cleavage of the POMC precursor. Thyrotrophs secrete TSH, one of the three pituitary glycoprotein hormones that are dimers of a common α-glycoprotein subunit (α-GSU) and a hormone-specific β-subunit (TSH-β). The two remaining glycoprotein hormones, LH and FSH, are produced by gonadotrophs and consist of α-GSU and LH-β or FSH-β, respectively. Somatotrophs secrete GH, and PRL is produced in lactotrophs.

It has been well established in all vertebrate classes that the endocrine hypothalamus and pituitary gland develop concurrently during embryogenesis, and inductive signals from the neural tissue that will ultimately give rise to the hypothalamus and neurohypophysis are required for anterior pituitary development (53, 57, 77). Terminally differentiated, hormone-secreting cell types of the anterior pituitary do not appear simultaneously; rather, they arise in a temporally distinct manner during mid- to late-embryogenesis that is similar in mammals and birds (53, 77, 87, 95). In contrast, hypothalamic parvocellular neurons appear to be generated simultaneously early in the second half of mammalian embryonic development (64). Based on initiation of neuronal gene expression, however, it seems that there are differences in the timing of functional differentiation of these neuronal populations (34). Around the time that hypothalamic

neurons appear and terminally differentiated cell types begin to emerge in the anterior pituitary, the anatomical and functional linkage between the endocrine hypothalamus and the anterior pituitary occurs. In mammals and birds, the hypothalamo-hypophyseal portal vessels are intact and can be visualized by infusion with India ink, siliconized rubber, or, more recently, fluorescently-conjugated gelatin by mid- to late embryonic development (168-170).

There have been a large number of studies examining developmental changes of a single or small number of hormones and/or receptors in multiple tissues [e.g. (171, 172)], ontogeny of hormone gene expression within one neuroendocrine tissue [e.g. (53, 87, 95, 124)], or development of one or a few neuroendocrine axes [e.g. (126, 173)]. However, a thorough characterization of the establishment of all five hypothalamo-pituitary axes within a single *in vivo* system has not been conducted in any species. Therefore, the objective of this study was to characterize gene expression profiles of major components in each hypothalamic-pituitary axis, including hypophysiotropic factors, their receptors in the anterior pituitary gland, and pituitary hormones, during the second half of chicken embryogenesis and the first week of neonatal life.

There is a high degree of similarity in development and control of the neuroendocrine axes between mammals and birds, and any information obtained regarding ontogenic changes in gene expression within the avian hypothalamus and pituitary should provide insight into mammalian development as well. The chicken embryo offers several advantages over traditional rodent models in examining this process, including accessibility, large size relative to rodent embryos of a comparable stage, and the ability to developmentally synchronize large numbers of embryos to allow

collection of adequate amounts of tissue for analysis. The ages used in this study were chosen based on previous observations that the functionally differentiated cell types arise within the chicken anterior pituitary during the latter half of embryogenesis and early post-hatch (51-54, 94, 96, 97, 103, 105). In addition, the portal vasculature connecting the hypothalamus and the anterior pituitary is present (170) and the earliest pituitary cell types to arise (corticotrophs and thyrotrophs) are thought to come under hypothalamic control around e12 (174). In the current experiment, hypothalami and anterior pituitary glands were collected every two days between e10 and d7. Changes in mRNA levels of the hypothalamic releasing and inhibiting factors, the receptors for these factors in the pituitary gland, and the pituitary hormones were examined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The results presented here significantly increase our understanding of neuroendocrine system establishment during development.

Materials and Methods

Animals and tissue collection

Embryonated broiler strain chicken eggs used for the experiment were obtained from Allen's Hatchery (Seaford, DE). On e0, eggs were placed in a 37.5 C humidified incubator and removed on the appropriate day of the 21-day incubation period or allowed to hatch. Chicks that were allowed to hatch were fed *ad libitum* a commercially available starter diet (Chick Start-N-Grow, CM-25-236007; Cooperative Milling, Gettysburg, PA) that was formulated to meet or exceed all known nutrient recommendations for poultry (175) and included the anti-coccidial drug Amprolium at 0.0125%. Hypothalami and anterior pituitary glands were collected from e10, e12, e14, e16, e18, e20, d1, d3, d5, and

d7 birds with the aid of a dissecting microscope. Two hypothalami from each age were pooled for each replicate sample. Pituitaries from 3 e10, 2 e12, 2 e14, and 2 e16 embryos were pooled for each replicate sample to yield sufficient total ribonucleic acid (RNA) from each developmental age, and one pituitary per replicate was used for the older ages. Four replicate samples were collected for each age (n=4). We did not determine sex of the embryonic birds used. After hatch, sex was determined and we collected two replicate samples of tissue from female birds and two replicate samples from male birds. Tissues were immediately frozen in liquid N_2 and stored at -80 C until RNA extraction. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland.

qRT-PCR

Total RNA was isolated from hypothalami and anterior pituitary glands with RNeasy Midi or Mini kits (Qiagen, Valencia, CA), respectively, which included an on-column deoxyribonclease (DNase) digestion step. Samples were quantified with Quant-iT RiboGreen RNA Quantitation Reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions (20 µl) were performed on 500 ng total RNA with SuperScript III (Invitrogen) and an oligo-dT primer (5'-CGGAATTCTTTTTTTTTTTTTTTTTTTV-3'). As a control for genomic DNA contamination, a pool of total RNA was made and the reaction conducted as the others except reverse transcriptase was not added. Reactions were diluted to 100 µl prior to polymerase chain reaction (PCR) analysis.

Levels of Pit-1 isoform mRNA, as well as hypothalamic and pituitary mRNA levels of the primary genes in each of the five major neuroendocrine axes, were quantified by qRT-PCR. Primers (Sigma-Aldrich, St. Louis, MO) used in the PCR

Table 1. Primers used for qRT-PCR in the neuroendocrine axis development study.

Gene	ID[1]	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
Adrenocorticotropic Axis			
CRH	25026	CATCTCCCTGGACCTGACTT	CCATCAGTTTCCTGTTGCTG
CRH-R1	00503	TTCTCATGACCAAGCTCCGAG	AGGTGATTCCCAGCAAGGG
CRH-R2	09078	CCTGGTGAAGATGATATTTCCCA	CCATCTTTTCCTGGCAGCAG
POMC	36767	CGCTACGGCGGCTTCA	TCTTGTAGGCGCTTTTGACGAT
Thyrotropic Axis			
TRH	13835	TGGATGACATCCTGCAGAGATC	GGAAAGCCATTGTGGCAGA
TRH-R	25942	GGCTCAACAAAACAAGACTGTGAA	TCGATAGGGCATCCACAGAAA
TSH-β	04024	ACTGCCTGGCCATCAACAC	ACACGTTTTGAGACAGAGCACTTTT
α-GSU	37154	CTTTCCCAGATGGAGAATTTCTCA	ACCTGTTCTCCCCTAGCTTGC
Somatotropic Axis			
GHRH	06098	AGGAGAAGGGGTGCACAA	CTCCCAAGAAGTCCCTCAGT
PACAP/GHRH	23977	CATAGACGGCATCTTCACGGA	GCTACTCGGCGTCCTTTGTTT
Ghrelin	13700	GCTCTGGCTGGCTCTAGTTTTTT	TCTGTGCCTCGGCGATGTA
SST	11903	TCCTGCGGAGCTGTGTTACAT	CCGACTCCAGAGCTTCATTTTC
GHRH-R	08374	CCTTGGCATTCGGCTTTATTTAG	TCAGGAAACAGTAGAGGAGTGCTACA
GHRH-R2	00361	ATCCCCAGACATCAGCAAAAACTA	GGAAGAACGCAAACACCACATAG
PACAP-R1	08391	ATTGGGACTGGGATCTTTCCA	GACTTTCCAGCTCCTCCATTTTC
GHRH-LPR	08424	CTTCTATATTGCTTCTCAAATGCGG	CAGTCCCCTGTTTGTGTGTGAA
GHS-R	14964	GAGGAAGAACATCGGTCCGAG	AAGGCAACCAGCAGAGTATGAAA
SSTR2	38508	CTGCTGGCTCCCCTTCTACA	TGGGCACGATCAGGACAGA
GH	00328	CACCTCAGACAGAGTGTTTGAGAAA	CAGGTGGATGTCGAACTTATCGT
Lactotropic Axis			
VIP	22132	TTCGAAAGCAAATGGCTGTG	AGGTTCAAGAATTTCTGCTTCACC
VIP-R1	08443	GGGAAAAATAAATGCCCAACCTAC	CCTGCTCATGTTCTTCCTGTCC
VIP-R2	10623	GATTGATTGTTGCAGTCCTGTATTG	AATTGAGCTGTGCAGTCTGTAGTCTC
PRL	20680	AGGAATGGAGAAAATAGTTGGGC	TCATTTCCAGCATCACCAGAAT
Gonadotropic Axis			
GnRH1	00366	ACACTGGTCTTATGGCCTGCA	ATTCAGCCTTCTGCCCTTCTC
GnIH	37842	GCATGGTATGTGCCTAGATGAACTAAT	TCCTCTGCTTTTCCTCCAAGATA
GnRH-R	33784	TCCGAATCATTTGGGAGATCAG	TGGCAACAATCACAATGGTCA
GnIH-R1	07077	GGGCATCTTCTGCATGCCTA	AAAACAGAGGCGGAGACAGACA
GnIH-R2	18978	AAGGAATCTCTGTCTCTGCCTCC	GACAGCAGTTGAAGTGGTCAGCT
FSH-β	19832	AGCAGTGGAAAGAGAAGAATGTGA	TGTTTCATACACAACCTCCTTGAAG
LH-β[2]		GGATGCCCCCAATGTATGG	TCCCGCGTCCTGCAGTA
Others			
Pit-1α	30398	ACCAATGTTGTCTCCACAGGACT	GAGACTTGCTGATAGCATCTCTGG
Pit-1β	24989	CAATGTTGTCTCCACAGTCCCA	GACAGGAGGGCACAGAGTAGTGTAG
Pit1-γ	36836	TCCTCATGCATTTTCTTACCAGTC	TCTCTGGAGTTGCAGGCTTGA
Total Pit-1		AAATCAATACTGTCCAAGTGGCTG	GTGGTTCTGCGCTTCCTCTT
GAPDH	37122	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT
PGK-1	12893	CTACATGCTGTGCGAAGTGGAA	GCCAGGAAGAACCTTACCCTCTAG

[1]ID is the transcript identification from Ensembl chicken genome assembly (http://www.ensembl.org/Gallus_gallus/Info/Index) and is preceded by ENSGALT000000 in all cases.

[2]The sequence for LH-β is not on the assembled chicken genome and primers were designed based on the sequence from reference (176).

reactions are listed in Table 1 and were designed with Primer Express Software (Applied Biosystems, Foster City, CA) to have a melting temperature (T_m) = 58 – 60 C (optimal 59 C), %GC content = 40 – 60%, length = 18 – 30 nucleotides, and yield an amplicon length

= 100 – 150 bp (optimal 125 bp). Whenever possible, primers were also designed to span an intron within the 3'-end of the transcript. In the adrenocorticotropic axis, mRNA levels of hypothalamic CRH, pituitary CRH receptors 1 and 2 (CRH-R1 and CRH-R2), and pituitary POMC were measured. Hypothalamic TRH and pituitary TRH receptor (TRH-R), TSH-β, and α-GSU mRNA expression levels were measured in the thyrotropic axis. For the somatotropic axis, hypothalamic expression profiles of GHRH, pituitary adenylate cyclase-activating polypeptide/growth hormone-releasing hormone-like peptide (PACAP/GHRH-like), ghrelin, and SST mRNA were determined. Pituitary mRNA levels of GHRH-R, GHRH receptor 2 (GHRH-R2), PACAP receptor 1 (PACAP-R1), GHRH-like peptide receptor (GHRH-LPR), growth hormone secretagogue receptor (GHS-R), SST receptor type 2 (SSTR2), and GH were also quantified. In the lactotropic axis, mRNA levels for hypothalamic VIP, pituitary VIP receptors 1 and 2 (VIP-R1 and VIP-R2, respectively), and pituitary PRL were determined. Hypothalamic expression of GnRH1 and GnIH mRNA were determined for the gonadotropic axis, as were pituitary levels mRNAs for GnRH receptor (GnRH-R), GnIH receptors 1 and 2 (GnIH-R1 and GnIH-R2), FSH-β, and LH-β. For normalization purposes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1) were quantified in each sample.

PCR reactions (15 µl) contained 1 µl diluted complementary deoxyribonucleic acid (cDNA), 400 nM each primer, PCR buffer (50 mM KCl, 10 mM Tris (tris(hydroxymethyl)aminomethane)-HCl, 0.1% Triton-X-100), 0.12 U/µl Taq Polymerase, 200 nM deoxynucleoside triphosphates (dNTPs), 40 nM fluorescein (Invitrogen), and SYBR Green I Nucleic Acid Gel Stain (Invitrogen) diluted 1:10,000

and were carried out in the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR cycling conditions were as follows: initial denaturation at 95 C for 3 m followed by 40 cycles of 95 C for 15 s, 60 C for 30 s, and 72 C for 30 s. Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reverse transcription (RT) reaction and was absent from control reactions containing no enzyme.

The amount of each target gene in the hypothalamus was normalized to the level of GAPDH mRNA, and each target gene in the pituitary gland was normalized to the level of PGK1 mRNA. The genes chosen for normalization in each tissue were based on the observation that GAPDH mRNA levels did not change between e10 and e17 in the hypothalamus but tended to decrease with age in the pituitary samples, and PGK1 mRNA levels were constant between e10 and e17 in the pituitary samples but tended to increase with age in the hypothalamus. The following equation was used for transformation and normalization of each target gene: mRNA level = $(2^{\Delta Ct})_{target}/(2^{\Delta Ct})_{GAPDH\ or\ PGK}$, where $\Delta Ct = Ct_{no\ RT} - Ct_{sample}$. The cycle threshold (Ct) value is the threshold cycle when the amount of amplified product reaches a fixed threshold for fluorescence due to binding of SYBR green to the double-stranded PCR product. The mRNA level of each target gene in each sample was then divided by the mean of the mRNA level in the age with the highest expression level for that gene, such that data are expressed relative to the age with the highest expression level (equal to 100%).

Data analysis

The data, expressed relative to the age with the highest expression level for a given target gene, were log_2-transformed prior to statistical analysis. All data were

analyzed by one-way analysis of variance using the MIXED models procedure of SAS (SAS Institute, Cary, NC), and differences between groups were determined using the test of least significant difference (PDIFF; SAS). The data are presented as backtransformed least-squares mean (LSmean) and positive pooled standard error of the mean (SEM), which were calculated with the following equations: backtransformed LSmean = 2^{LSmean}; backtransformed positive SEM = $2^{(LSmean + SEM)} - 2^{(LSmean)}$.

Results

Pit-1 Isoforms

Pit-1 is necessary for functional differentiation of thyrotrophs, somatotrophs, and lactotrophs. In the chicken, there are three Pit-1 isoforms, Pit-1α, Pit-1β, and Pit-1γ (136). These isoforms have a conserved C-terminal DNA binding domain but differ in the N-terminal transactivation domain, allowing the use of isoform-specific primers within the 5'-end of the transcript to distinguish each variant and the use of primers within the conserved 3'-end to quantify total Pit-1 levels. We measured ontogeny of the three Pit-1 variants, as well as total levels of Pit-1 transcript (Figure 5). Each transcript variant was detectable on all ages examined, and each was expressed at higher levels during mid-embryonic development than during late embryogenesis and early post-hatch. Both Pit1α and Pit1γ mRNA levels increased after e10 and were highest around e14 ($P<0.05$, n=4). Pit1β was highest on the earlier embryonic ages, decreased between e16 and e20, and remained low after hatch ($P<0.05$, n=4). The expression patterns for each isoform are consistent with the appearance and expansion of cell types in the Pit-1 lineage, which occurs between e10 and e17 in the chicken (51, 53, 103, 105, 106, 139).

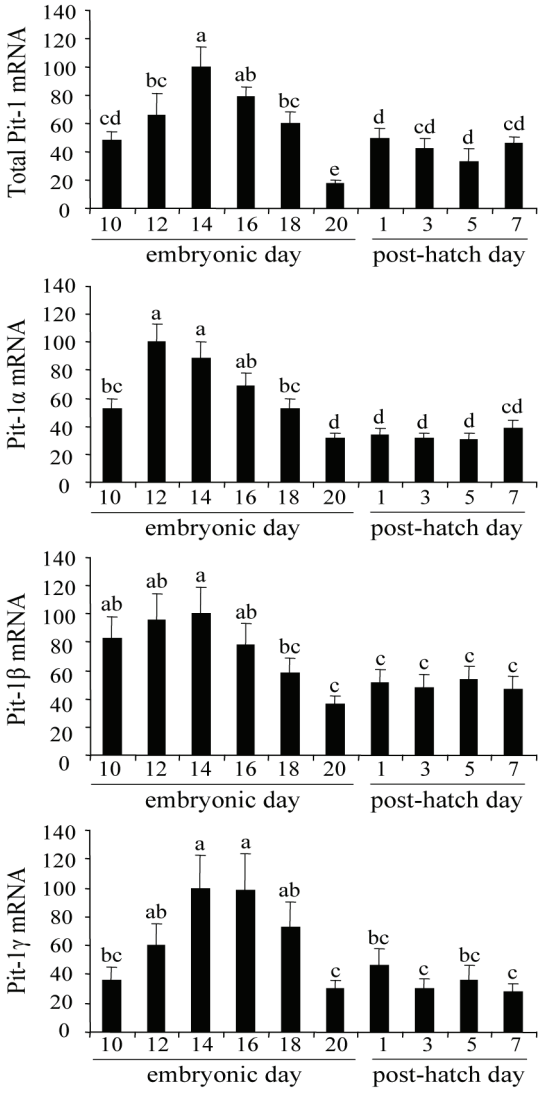


Figure 5. Developmental ontogeny of Pit-1 isoforms in the chiken anterior pituitary gland. Total RNA isolated from embryonic day 10 through post-hatch day 7 pituitaries (n=4) was analyzed by qRT-PCR to measure mRNA expression of Pit-1α, Pit-1β, Pit-1γ, and total Pit-1. Levels of mRNA for each gene were normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different ($P<0.05$).

Adrenocorticotropic Axis

In the neuroendocrine adrenocorticotropic axis, hypothalamic CRH stimulates production of pituitary ACTH from corticotrophs through CRH-R1 (177). Additionally, CRH can act as a TSH secretagogue in non-mammalian vertebrates, and this action is mediated by CRH-R2 localized on pituitary thyrotrophs (178). Levels of hypothalamic CRH mRNA increased between e10 and e12 (Figure 6; $P<0.05$, $n=4$) and remained constant throughout the remainder of the study. CRH-R2 mRNA increased to maximal levels between e10 and e12, but then decreased again between e16 and e20 before reaching intermediary levels after hatch (Figure 6; $P<0.05$, $n=4$). Neither CRH-R1 nor POMC mRNA changed significantly during the developmental period analyzed in this study (Figure 6; $P>0.05$, $n=4$), although both tended to increase between mid- and late-embryonic development. The apparent increase in POMC mRNA is consistent with an earlier report demonstrating an increase in pituitary POMC between e10 and e17 in developing chickens (124). Based on these findings, initial adrenocorticotropic axis activity occurs before e10 and it appears to be established by e12, as the mRNA for all genes measured in this axis have reached their maximum level by this age.

Thyrotropic Axis

Synthesis and release of TSH from pituitary thyrotrophs in birds is controlled by several hypothalamic factors, including TRH acting through the TRH-R, CRH acting through the CRH-R2, and somatostatin acting through the SSTR2 (177, 178). Hypothalamic TRH mRNA remained low until e14, at which point levels increased through e20 and remained elevated for the duration of the ages examined

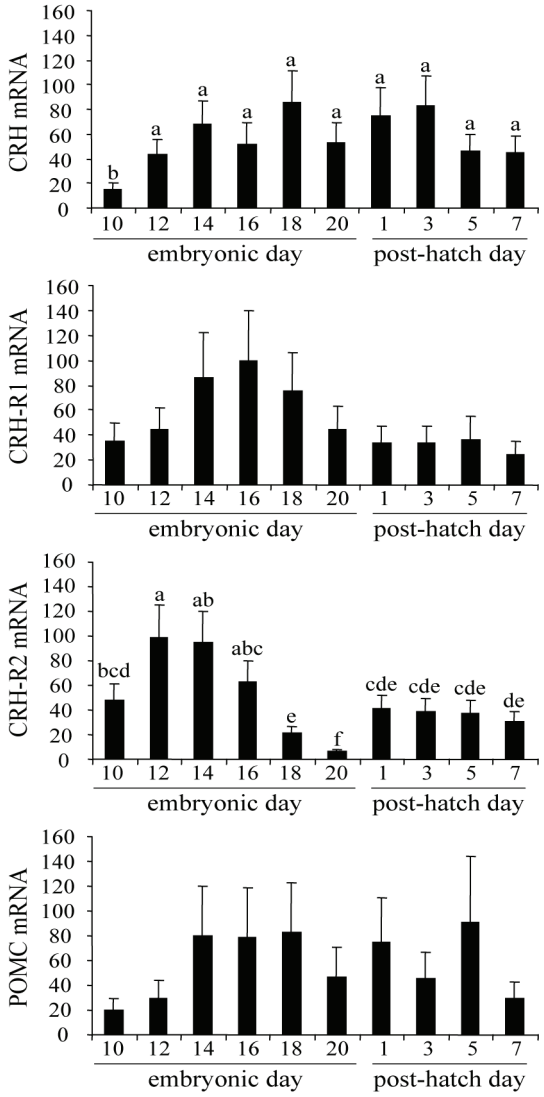


Figure 6. Gene expression profiles of components of the adrenocorticotropic axis in the developing neuroendocrine system of the chick. Total RNA samples isolated from embryonic day 10 through post-hatch day 7 hypothalami and pituitaries (n=4 per tissue) were analyzed by qRT-PCR to measure expression of hypothalamic CRH, pituitary CRH-R1 and CRH-R2, and pituitary POMC mRNA levels. Hypothalamic genes are normalized to levels of GAPDH mRNA, and pituitary mRNA levels are normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level for that gene (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different ($P<0.05$).

(Figure 7; $P<0.05$, n=4). Conversely, levels of TRH-R mRNA in the anterior pituitary gland increased to a maximum on e12, decreased to a minimum on e20, and remained low after hatch (Figure 7; $P<0.05$, n=4). As has been demonstrated previously in chickens (124, 126, 138, 139), we observed an increase in pituitary TSH-β mRNA between e10 and e18 that was followed by a sharp decline in expression just prior to hatch (Figure 7; $P<0.05$, n=4). This pattern correlated with the increase in pituitary α-GSU mRNA that occurred between e10 and e18, as well as the decrease observed on e20 (Figure 7; $P<0.05$, n=4). In contrast to TSH-β mRNA levels, however, expression of α-GSU mRNA then increased steadily through d7 (Figure 7; $P<0.05$, n=4). Taken together, these results indicate that thyrotropic axis activity is initiated around e12, and apparent activity increases throughout embryogenesis until establishment occurs around e18.

Somatotropic Axis

Production and release of GH from pituitary somatotrophs is under the control of both stimulatory and inhibitory hypothalamic factors working through their pituitary receptors. As in mammals, the primary stimulatory factor is GHRH and the main inhibitory factor is SST. In addition, GH is stimulated by TRH in chickens and, to a lesser extent, by PACAP/GHRH-like and ghrelin [reviewed in (40)]. Hypothalamic GHRH and PACAP/GHRH-like mRNA showed profiles similar to GH, with an increase in expression toward the end of embryonic development that persisted after hatch (Figure 8A and B; $P<0.05$, n=4). Pituitary receptors for chicken GHRH include GHRH-R and the recently identified GHRH-R2 (179-181). While GHRH-R mRNA in the pituitary increased between e10 and e12 (Figure 8A; $P<0.05$, n=4), levels changed relatively little for the remainder of the ages investigated. On the other hand, pituitary GHRH-R2 mRNA

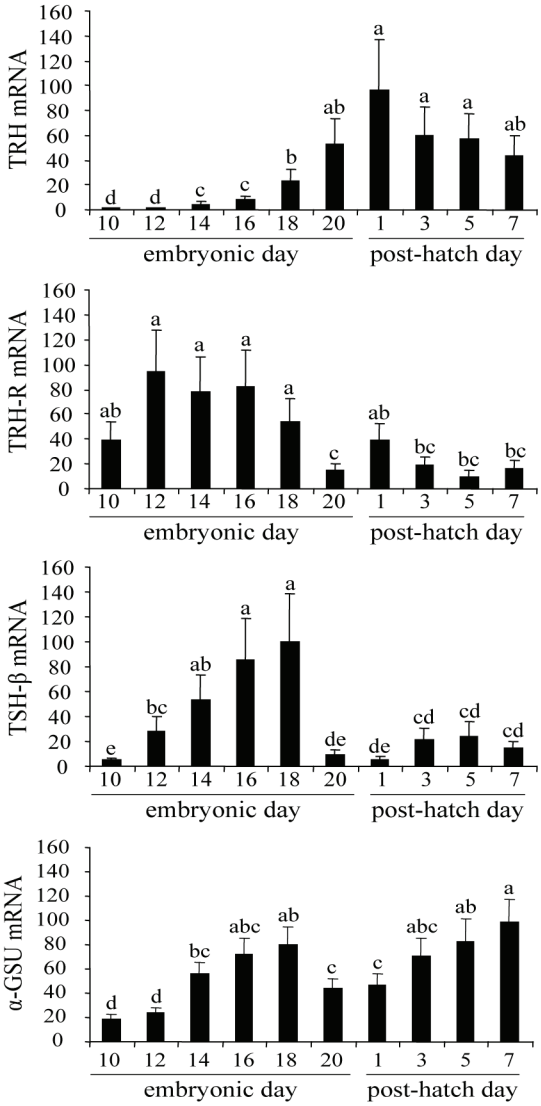
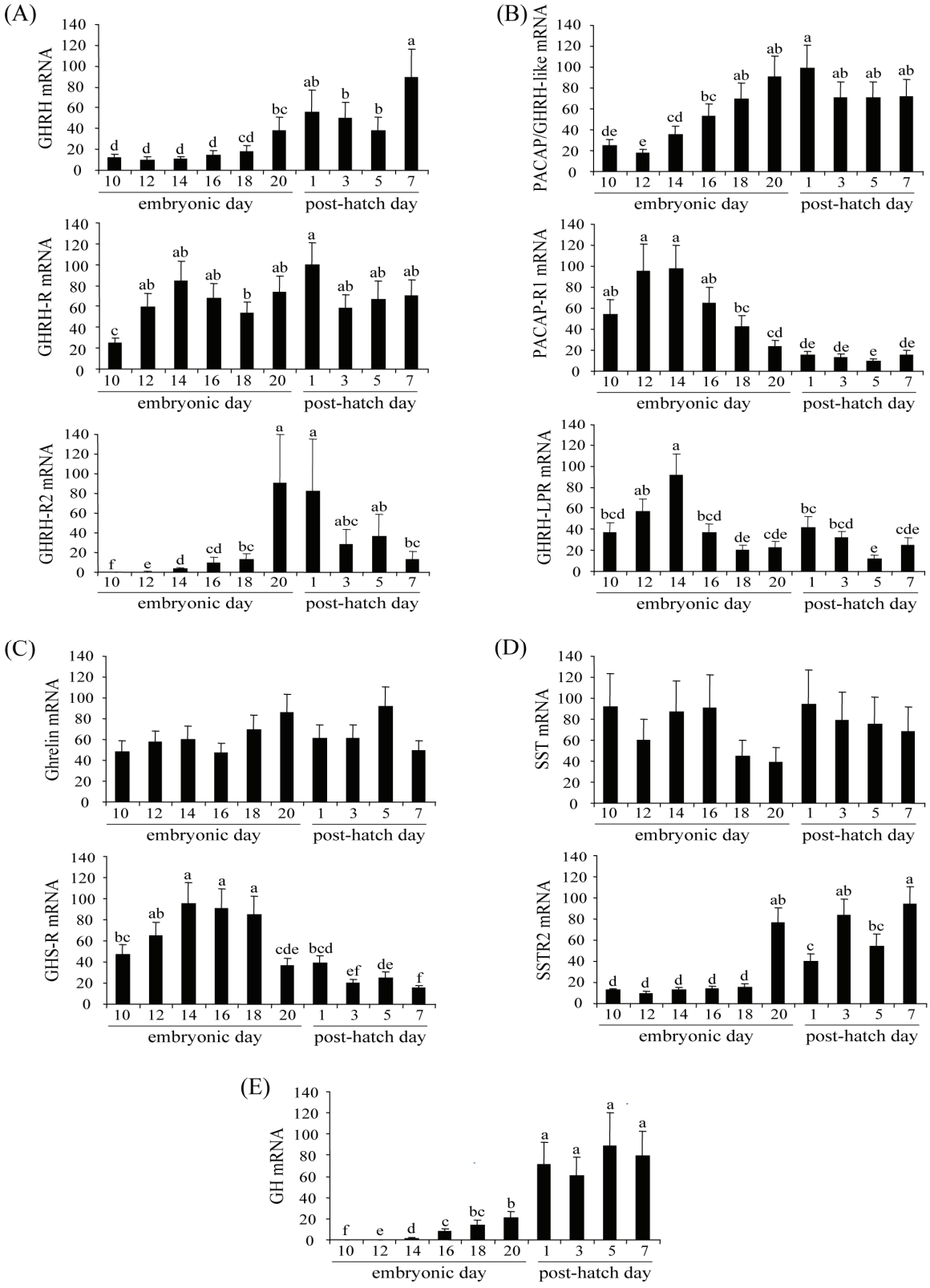


Figure 7. Developmental expression profiles for genes of the thyrotropic axis in the neuroendocrine system of the chick. Samples of total RNA isolated from embryonic day 10 through post-hatch day 7 hypothalami and pituitaries (n=4 per tissue) were analyzed by qRT-PCR to measure expression of hypothalamic TRH and pituitary TRH-R, TSH-β, and α-GSU mRNA. Hypothalamic genes are normalized to levels of GAPDH mRNA, and pituitary mRNA levels are normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level for that gene (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different ($P<0.05$).

Figure 8. Ontogenic characterization of the somatotropic axis in the developing neuroendocrine system of the chick. Total RNA samples isolated from embryonic day 10 through post-hatch day 7 hypothalami and pituitaries (n=4 per tissue) were analyzed by qRT-PCR to measure mRNA expression of: (A) hypothalamic GHRH, and pituitary GHRH-R and GHRH-R2 levels; (B) hypothalamic PACAP/GHRH-like, and PACAP-R1 and GHRH-LPR in the pituitary; (C) hypothalamic ghrelin and pituitary GHS-R; (D) hypothalamic SST and SSTR2 in the pituitary; and (E) pituitary GH. Hypothalamic genes are normalized to levels of GAPDH mRNA, and pituitary mRNA levels are normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level for that gene (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different ($P<0.05$).


(A)
GHRH mRNA
GHRH-R mRNA
GHRH-R2 mRNA
(B)
PACAP/GHRH-like mRNA
PACAP-R1 mRNA
GHRH-LPR mRNA
(C)
Ghrelin mRNA
GHS-R mRNA
(D)
SST mRNA
SSTR2 mRNA
(E)
GH mRNA
embryonic day
post-hatch day

dramatically increased throughout embryonic development to a peak perihatch, and then decreased again by d7 (Figure 8A; $P<0.05$, n=4). In the chicken, hypothalamic PACAP and GHRH-like peptide can activate signaling events from GHRH-R, PACAP-R1, and GHRH-LPR (181) to potentially regulate GH expression. Expression of PACAP-R1 mRNA exhibited a reciprocal expression pattern to PACAP/GHRH-like mRNA, decreasing during the final third of embryonic development and remaining low after hatch (Figure 8B; $P<0.05$, n=4). Levels of mRNA for GHRH-LPR increased between e10 and e14 but then decreased again on e16 and remained at a similar level for the duration of the study (Figure 8B; $P<0.05$, n=4). Unlike GHRH and PACAP/GHRH-like mRNA, the other two hypothalamic regulators of pituitary GH, ghrelin and SST, did not fluctuate during the developmental ages examined (Figure 8C and D; $P>0.05$, n=4). However, levels of mRNA for pituitary receptors of these factors did change. Pituitary GHS-R mRNA increased between e10 and e14, remained elevated through e18, and decreased just prior to hatch on e20. After hatch, there was a slight decrease in GHS-R mRNA expression that occurred during the first week of life (Figure 8C; $P<0.05$, n=4). In chickens, SSTR2 is the primary SST receptor subtype responsible for mediating inhibitory effects of SST on GH release (182). Embryonic expression of SSTR2 mRNA remained low from e10 through e18 then increased on e20. Immediately following hatch, there was a decrease in SSTR2 mRNA expression on d1 before levels increased again on d3 and remained high through d7 (Figure 8D; $P<0.05$, n=4). Consistent with the appearance of pituitary somatotrophs in rodents and birds (101, 103), we found that pituitary GH mRNA levels increased significantly between e10 and e20, continued to further increase after hatch, and remained elevated throughout the first week of post-

hatch life (Figure 8E; $P<0.05$, n=4). Based on expression patterns of pituitary GH, its major hypothalamic regulators, and their pituitary receptors, activity of the somatotropic axis begins to occur around e16 and increases until its establishment during early post-hatch development.

Lactotropic Axis

The neuroendocrine lactotropic axis in mammals and birds consists of hypothalamic VIP, which acts through VIP-R1 and VIP-R2 located on pituitary lactotrophs, to stimulate synthesis and release of PRL (183, 184). Hypothalamic VIP mRNA increased significantly on e14 and remained elevated through d7 (Figure 9; $P<0.05$, n=4). Pituitary VIP-R1 mRNA increased on e20 and remained highest after hatch (Figure 9; $P<0.05$, n=4), and, while VIP-R2 mRNA did not change significantly throughout the course of the study, levels tended to be highest between e14 and e20 before decreasing after hatch. Consistent with the appearance of pituitary lactotrophs in the chicken around e17 (105, 124, 128), PRL mRNA levels increased dramatically between e16 and e20 and remained high during the first week of neonatal life (Figure 9; $P<0.05$, n=4). Although initiation of lactotropic axis activity begins to occur around e18, there are major increases in pituitary VIP-R1 and PRL mRNA perihatch, indicating that full lactotropic axis functionality does not occur until between e20 and d1.

Gonadotropic Axis

The gonadotropins, FSH and LH, are positively regulated by GnRH1 and negatively regulated by GnIH. These hypothalamic factors act through GnRH-R, GnIH-R1, and GnIH-R2 located on pituitary gonadotrophs to regulate LH and FSH synthesis and release (185). While pituitary GnRH-R mRNA did not change between e10 and d7,

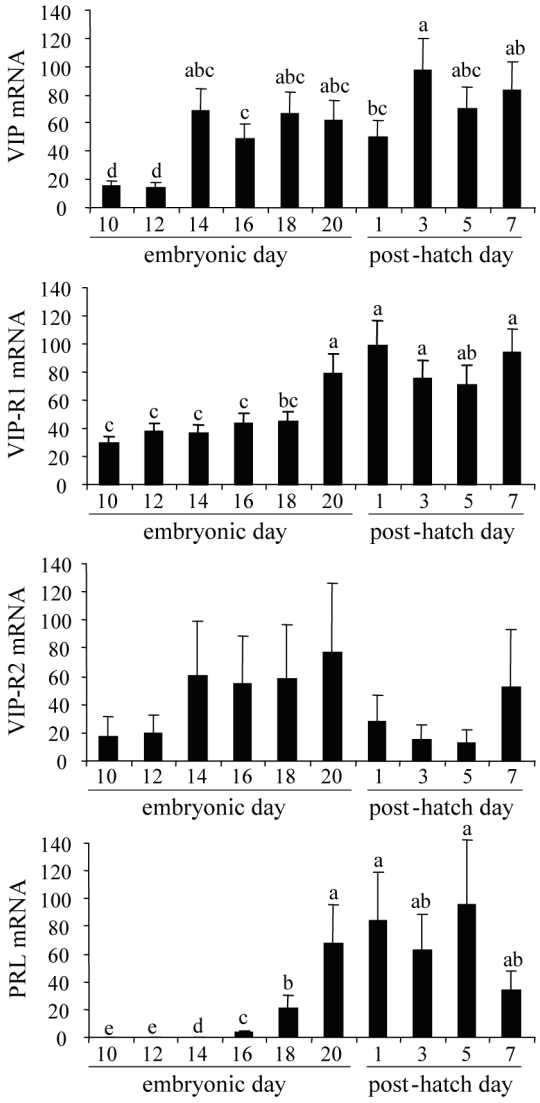


Figure 9. Gene expression profiles of components of the lactotropic axis in the developing neuroendocrine system of the chick. Total RNA samples isolated from embryonic day 10 through post-hatch day 7 hypothalami and pituitaries (n=4 per tissue) were analyzed by qRT-PCR to measure expression of hypothalamic VIP, pituitary VIP-R1 and VIP-R2, and pituitary PRL mRNA levels. Hypothalamic genes are normalized to levels of GAPDH mRNA, and pituitary mRNA levels are normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level for that gene (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different ($P<0.05$).

hypothalamic GnRH1 increased slightly between e10 and e20, further increased just after hatch, and remained high through the first week of post-hatch life (Figure 10A; $P<0.05$, n=4). Hypothalamic GnIH mRNA levels increased from e12 to a maximum on e14, decreased through the last week of incubation, and remained low post-hatch, with the exception of d3 (Figure 10B; $P<0.05$; n=4). Pituitary GnIH-R1 levels were highest between e10 and e16, decreased prior to hatch, and remained low through d7 (Figure 10B; $P<0.05$, n=4). The developmental profile for GnIH-R2 was similar to that of GnIH-R1, although the biggest decrease in expression was observed post-hatch, on d3 (Figure 10B; $P<0.05$, n=4). Pituitary gonadotrophs are immunoreactive as early as e8 in chicken embryos (54, 97). Consistent with this, we were able to detect mRNA for both FSH-β and LH-β at all ages examined, though FSH-β mRNA did not change significantly through the course of the study (Figure 10C; $P>0.05$, n=4). LH-β mRNA remained relatively low with a slight increase throughout embryonic development, and further increased through the first week post-hatch to maximum levels on d7 (Figure 10C; $P<0.05$, n=4). Initiation of gonadotropic axis activity appears to have begun by mid-embryogenesis. However, the changes in hormone and receptor gene expression that occur between d1 and d7 indicate that apparent activity of this axis increases during the first week of post-hatch life, and it is not fully established until d7.

Discussion

This investigation is the first comprehensive study of ontogenic expression profiles of the major genes within all primary neuroendocrine axes in a single *in vivo* system. The results, including overall initiation, establishment, and relative changes in mRNA expression for each axis, are summarized in Figure 11. Initiation of each axis was

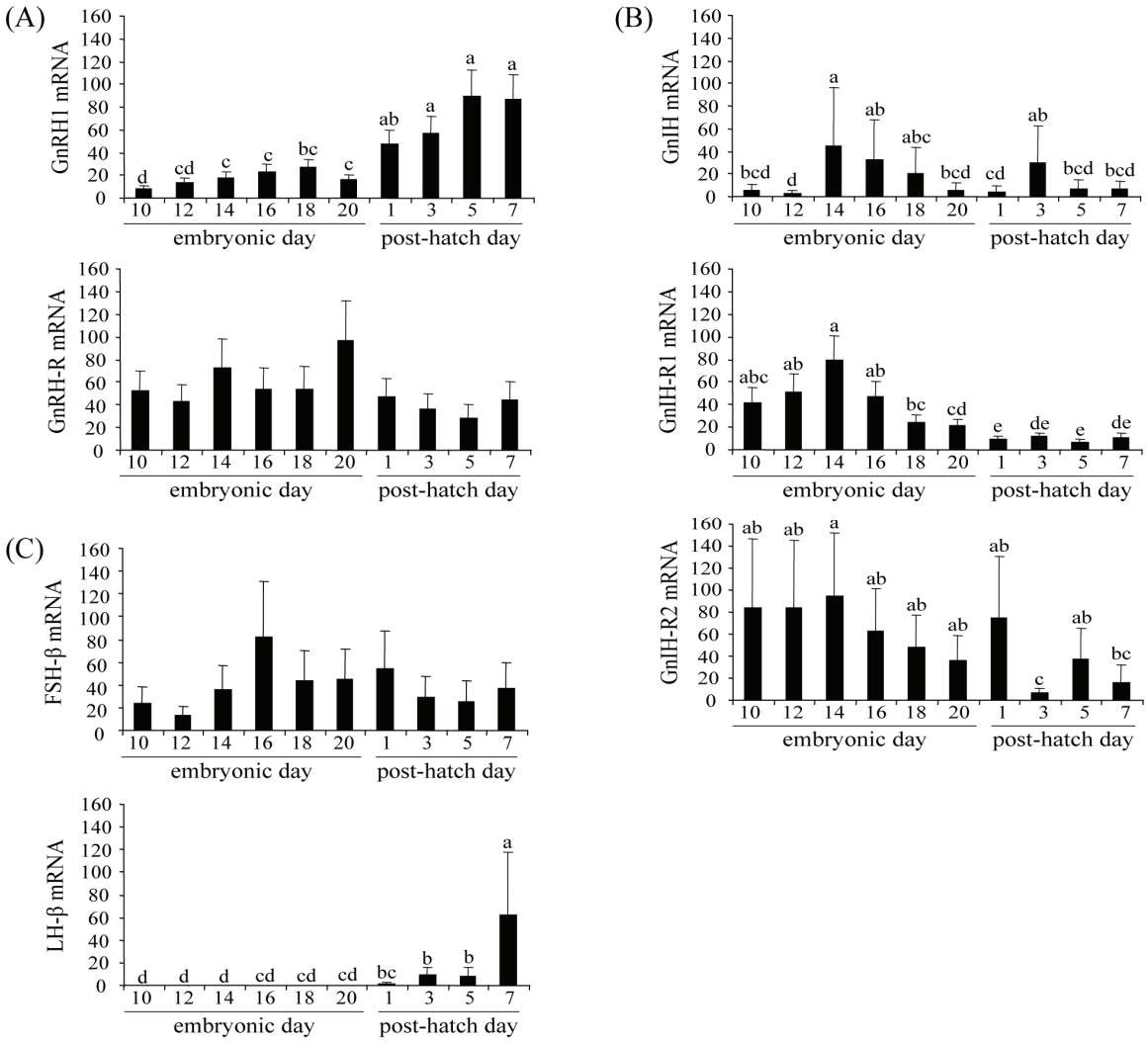


Figure 10. Developmental expression patterns of genes in the gonadotropic axis in the neuroendocrine system of the chick. Samples of total RNA isolated from embryonic day 10 through post-hatch day 7 hypothalami and pituitaries (n=4 for each tissue) were analyzed by qRT-PCR to measure mRNA expression of (A) hypothalamic GnRH1 and pituitary GnRH-R, (B) hypothalamic GnIH, and pituitary GnIH-R1 and GnIH-R2, and (C) pituitary levels of FSH-β and LH-β. Hypothalamic genes are normalized to levels of GAPDH mRNA, and pituitary mRNA levels are normalized to levels of PGK1 mRNA. All genes are expressed relative to the age with the highest expression level for that gene (set to 100% prior to $\log_2$-transformation for statistical analysis). Values (backtransformed mean + pooled SEM) within each graph without a common letter are significantly different from one another ($P<0.05$).

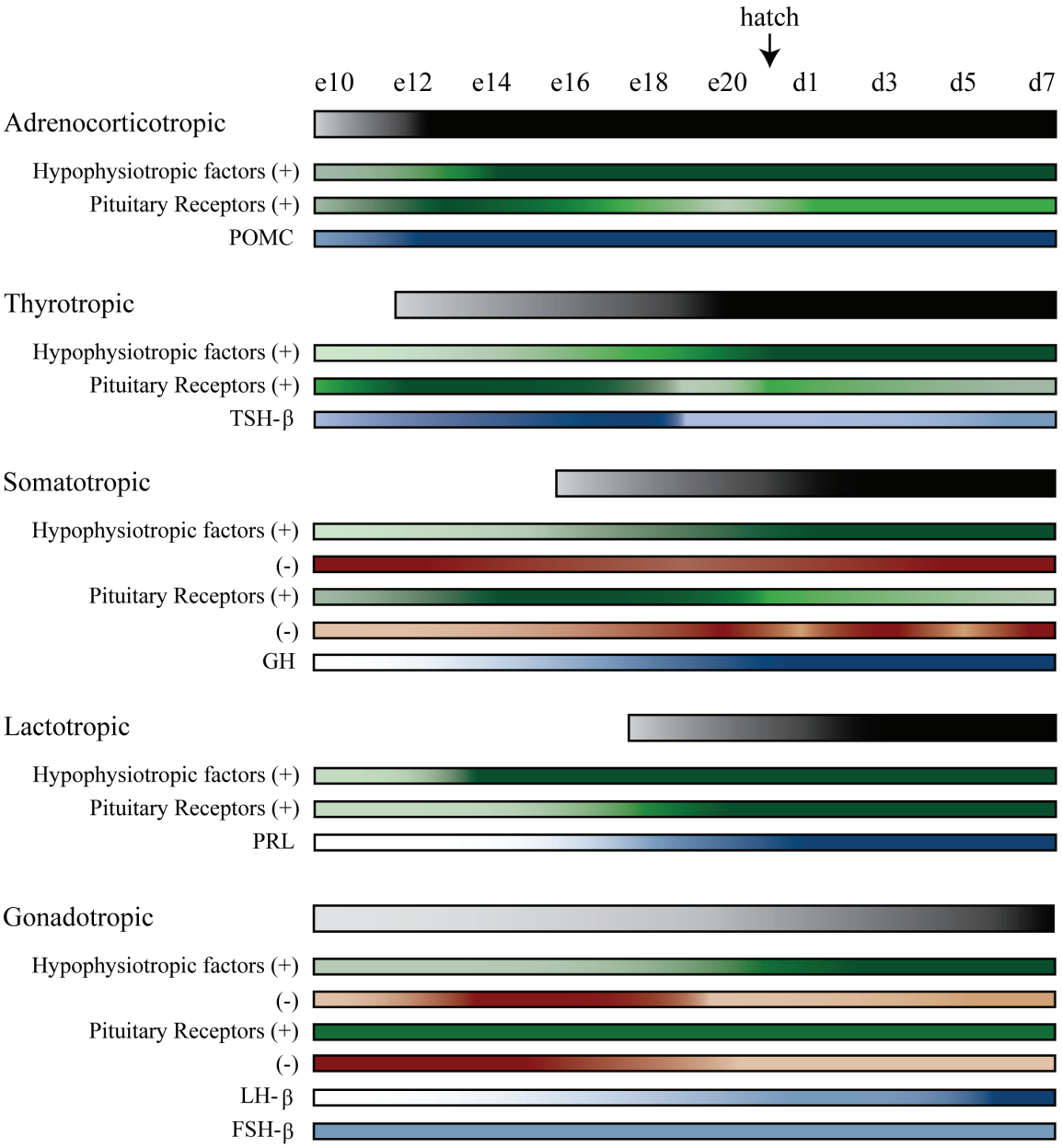


Figure 11. A summary of the results depicting ontogenic changes in hypothalamic and anterior pituitary gene expression during initiation and establishment of the five major neuroendocrine axes in the developing chick. This process occurs during the second half of embryogenesis (embryonic day (e) 10 through e20) and early post-hatch (post-hatch day (d) 1 through d7). For each axis, the thick bar at the top depicts the age at which activity in the axis is initiated (light grey) and the age when activity is fully established (black). Initiation of each axis occurs when mRNA levels of the pituitary hormone begin to significantly and substantially increase, and each axis was determined to be established when mRNA levels of all major components have reached maximum expression levels. The thin bars underneath each axis represent the significant and substantial changes measured for hypothalamic releasing (green) and release-inhibiting (red) factors; pituitary receptors for each of the releasing (green) and release-inhibiting (red) factors; and pituitary hormones (blue). The shade of each line indicates the predominant pattern of relative expression for each gene or genes within a given tissue, with the lightest color representing the lowest expression level and the darkest color representing the highest level of expression.

defined to occur when mRNA levels of the pituitary hormone began to significantly and substantially increase, and each axis was defined to be established when mRNA levels of all major components had reached maximum expression. It should be noted that production and secretion of pituitary hormones was not measured in this study, and the conclusions drawn from the data on neuroendocrine axis activity are based solely on changes in mRNA levels we observed for each component. Detectable levels of mRNA were measured for all genes we investigated at each age, indicating that hypothalamic and pituitary expression of the major neuroendocrine components has begun by mid-embryogenesis in the chicken.

In combination with additional cell-type specific transcription factors, the anterior pituitary-specific transcription factor Pit-1 is essential for expression of pituitary hormones in thyrotrophs, somatotrophs, and lactotrophs (83). As in mammals (186), the avian pituitary gland contains multiple Pit-1 variants that are generated by alternative splicing and differing transcription start sites (136, 187, 188), and Pit-1α is the most abundant variant (188). We designed four sets of primers in order to distinguish each of the isoforms and quantify levels of total Pit-1 mRNA. Although a strict comparison between different mRNA variants in the current study is not possible because of potential differences in efficiency of the PCR reaction with each primer pair, the Ct values for Pit-1α were closer to those of the highly-expressed housekeeping gene (PGK1) and, therefore, Pit-1α was most likely the most abundant isoform detected in this study. In the embryonic chicken anterior pituitary, Pit-1 mRNA is first detectable by RT-PCR beginning on e5 and expression is maintained throughout embryogenesis (106, 136). Consistent with this, we detected expression of all Pit-1 isoforms between e10 and d7.

The isoforms showed a similar ontogenic profile, increasing between e10 and e14 before decreasing toward the end of embryogenesis and remaining relatively low post-hatch (Figure 5). Pit-1 mRNA is increased in cultured chicken primary pituitary cells treated with TRH and is thought to play a role in mediating TRH-stimulated GH and TSH-β expression (189). The observed rise in Pit-1 mRNA between e10 and e14 may be partly due to the increase in hypothalamic releasing factors that regulate hormone production in cells of the Pit-1 lineage that also increased on e14 in the current study, including TRH, PACAP/GHRH-like, and VIP (Figures 7-9).

Levels of Pit-1 mRNA and protein are greater in the fetal human pituitary gland than levels in the adult gland (190). We also found that Pit-1 mRNA levels are highest during mid-embryogenesis and lower during the first week of neonatal life. This expression pattern is likely reflective of the established role for mammalian Pit-1 in terminal differentiation and proliferation of thyrotrophs, lactotrophs, and somatotrophs (77), which occurs during vertebrate embryonic development. In chickens as well as mammals, the appearance of Pit-1 precedes expression of TSH-β, GH, and PRL (87, 106, 190). We observed slightly different ontogenic expression patterns for each isoform, with Pit-1β being highest on e10 and Pit-1α and Pit-1γ increasing after e10 to the highest levels on e12 or e14, respectively. This may indicate differential involvement of the isoforms in appearance of different cell types within the Pit-1 lineage, suggesting that Pit-1β may play a role in thyrotroph differentiation, while Pit-1α and Pit-1γ may play a role in somatotroph and/or lactotroph differentiation. A functional requirement for Pit-1 in thyrotroph, somatotroph, and lactotroph hormone expression has not been demonstrated in the avian adenohypophysis. However, the increase in Pit-1 mRNA during mid-

embryogenesis is concurrent with (TSH-β) or just prior to (GH and PRL) the increase in hormone mRNA levels observed in this study and is consistent with a role for Pit-1 in functional differentiation of thyrotrophs, somatotrophs, and lactotrophs in the avian pituitary gland.

The first pituitary hormone transcript that is expressed in the developing mammalian (87, 95) and avian (53, 191) pituitary is α-GSU. However, the earliest hormone-producing cell type to emerge in these species are ACTH-secreting corticotrophs (53, 87, 94-96). Based on the hypothalamic and pituitary mRNA ontogeny determined in this study, the adrenocorticoptropic axis is the first of the neuroendocrine axes to become established (Figure 11). Initial activity for this axis occurs before e10, and it is established by e12. Levels of mRNA for hypothalamic CRH, pituitary CRH-R1 and CRH-R2, and pituitary POMC were readily detectable on e10, and they had all reached maximum levels by e12 (Figure 6). In this study, hypothalamic CRH mRNA increased between e10 and e12, after which no significant changes in expression occurred. This is consistent with a previous report that CRH mRNA in the hypothalamus changes very little after e14 in two breeds of broiler chickens (126). On the contrary, an earlier study reported a slight, yet significant, decrease in CRH mRNA between e18 and e20 in the chicken diencephalon (192), similar to the perinatal decrease in hypothalamic CRH mRNA observed in fetal rats and mice (193, 194).

In the chicken pituitary gland, CRH-R1 is confined primarily to corticotrophs and is thought to mediate CRH-stimulated ACTH release, and CRH-R2 is expressed on thyrotrophs and is thought to mediate CRH-stimulated TSH release (177, 178). The decrease in CRH-R2 mRNA we observed during the last week of embryogenesis is

identical to that reported previously in chickens (138) and may be reflective of its role in mediating CRH-induced TSH release from pituitary thyrotrophs, as both CRH-R2 and TSH-β mRNA sharply declined in the pituitary between e18 and e20. Pituitary expression of CRH-R2 and TSH-β mRNA in this study is negatively correlated with circulating thyroid hormones, which dramatically increase in the developing chick around e20 (138, 139), and further support a role for pituitary CRH-R2 in mediating thyrotropic effects of CRH rather than adrenocorticotropic effects. The apparent rise in CRH-R1 and POMC mRNA observed in this study between e12 and e14 is consistent with the idea that hypothalamic CRH, which increased significantly on e12, may be stimulating POMC expression on e14 via CRH-R1 located on corticotrophs. The subsequent increase in ACTH production will stimulate adrenal glucocorticoid production, accounting for the substantial increase in circulating CORT that occurs after e14 in developing chick embryos (10, 138, 140, 141).

The ontogenic pattern we observed for hypothalamic CRH mRNA, pituitary CRH-R1 mRNA, and pituitary POMC mRNA support the hypothesis that adrenal CORT secretion comes under hypothalamic and pituitary regulation in the developing chick embryo around e14 (174) and feedback regulation begins to be established around e16. Mammalian neonates undergo a stress hyporesponsive period, and one mechanism partially responsible for this is thought to be a decrease in pituitary CRH-R1 toward the end of embryonic development (195). CRH-R1 is known to be decreased by both CRH and glucocorticoids (195-197) as a result of negative feedback to control the stress response. We observed a seeming decline in CRH-R1 mRNA between e16 and d1, which may reflect both the rise in circulating CORT that begins just prior to this time (10, 138,

140, 141) and the elevated hypothalamic CRH mRNA we observed on these ages. This provides further evidence that CRH-R1 mediates adrenocorticotropic effects of CRH and indicates that birds may undergo a perihatch stress hyporesponsive period similar to that seen in perinatal mammals.

Pituitary thyrotrophs are the second hormone-secreting cell type to emerge during embryogenesis in mammals and birds (53, 87, 95, 96). Pituitary TSH-β mRNA substantially increased on e12 and rose steadily through e18, after which time a sharp decline occurred. This rise from mid- to late-embryogenesis, followed by a decrease just prior to hatch, has been reported previously (126, 138, 139) and may be a result of decreased pituitary sensitivity to TRH or CRH late in embryogenesis. This idea is supported by the current results that decreased levels of pituitary TRH-R and CRH-R2 mRNA were observed on e18 and e20, despite the maintenance of elevated hypothalamic CRH and TRH mRNA during late embryogenesis. The decreased expression of TRH-R, CRH-R2, and TSH-β mRNA we observed also reflects the establishment of negative feedback from increased thyroid hormone levels in late embryogenesis (138, 139), and supports other data indicating that this process occurs in birds just prior to hatch (51, 198). Hypothalamic TRH mRNA did not begin to substantially increase until the 3 days prior to hatch, and it remained elevated through d7. This is consistent with previous ontogenic reports of hypothalamic TRH mRNA (142) and protein (171, 199) in the chicken and is similar to the developmental pattern observed in the rat (200). Both TRH and CRH are capable of increasing TSH-β mRNA and stimulating TSH secretion in embryonic pituitary cells (139, 201). Thus, the increase in hypothalamic TRH toward the end of embryonic development, in addition to the observed rise in hypothalamic CRH,

appears to be driving the increase in TSH-β expression that occurred between e10 and e18. Based on the ontogenic profiles determined for components in the thyrotropic axis (Figure 7), its activity is initiated around e12 and apparent activity increases toward the end of embryonic development, but full establishment does not occur until between e18 and e20 (Figure 11).

As thyrotroph and gonadotroph populations expand in the developing mammalian and avian pituitary gland, α-GSU expression increases (53, 87, 95, 191) due to increasing levels of pituitary glycoproteins (TSH, FSH, and LH). In this study, we detected an increase in α-GSU mRNA between e10 and e18, a decrease perihatch, and then a subsequent increase through d7 (Figure 7). This pattern may be a manifestation of differential regulation of β-subunit gene expression in thyrotrophs and gonadotrophs, as TSH-β mRNA levels were highest just prior to hatching while LH-β mRNA levels increased after hatch between d3 and d7.

It is well established that pituitary somatotrophs become a significant cell population during the final third of embryonic development in both chickens and rodents, and pituitary GH mRNA and protein content increase considerably during this time and even further on the first day of neonatal life (95, 99, 101, 103, 106, 124, 126). Consistent with these reports, we detected a substantial increase in pituitary GH mRNA between e14 and e16, suggesting that initiation of somatotropic axis activity occurs around this time. Pituitary mRNA levels for all hypothalamic GH releasing factor receptors we measured were upregulated by e14, just prior to the rise in pituitary GH. This supports initiation of somatotropic activity at this time and suggests that these releasing factor receptors contribute to somatotroph maturation. It has been shown that initial GH-containing cells

to appear in rat (120, 202) and chicken (103, 129, 130) are responsive to GHRH and TRH, which may be in part due to an increase in receptor level as reflected by the increase in secretagogue receptor mRNA we measured. The observation that mRNA for several critical hypothalamic regulators of GH and pituitary receptors did not reach maximum levels until e20 or d1, in combination with the large increase in pituitary GH mRNA that was measured just after hatch, indicates that somatotropic axis activity increases throughout embryonic development but does not fully mature until late-embryogenesis or early post-hatch (Figures 8 and 11). It is known that hypothalamic GHRH is important for proper development and function of GH-producing cells in the fetal rat (203). Based on the rise in hypothalamic mRNA levels for GHRH, PACAP/GHRH-like, and TRH we observed during late embryogenesis, these factors likely also contribute to somatotropic axis maturation in the chicken and are responsible for the increase in pituitary GH mRNA observed at the same time.

Endogenous ligands for chicken GHRH-R were recently identified (204), as well as a novel receptor for GHRH (GHRH-R2) and the receptor for GHRH-like peptide (GHRH-LPR) (181). Both GHRH-R and GHRH-R2 are highly specific for chicken GHRH (181) and are developmentally regulated in the anterior pituitary gland during chicken embryogenesis. Developmental expression for GHRH-R has been reported previously (126, 172) and was similar to what was observed in this study. GHRH-R mRNA levels increased between e10 and e12 and remained elevated throughout the remainder of development. This study is the first to characterize ontogeny of GHRH-R2 and GHRH-LPR. In contrast to mRNA levels for GHRH-R, GHRH-LPR, PACAP-R1, and GHS-R, which were shown to increase during mid-embryogenesis and then decrease

just prior to hatch, the greatest increase in GHRH-R2 did not occur until late embryonic development, and it remained elevated through d7. The differential patterns of pituitary receptor mRNA levels indicate that GHRH-R, GHRH-LPR, PACAP-R1, and GHS-R may play a role in initial somatotroph responsiveness to secretagogues, and GHRH-R2 may be important in more mature cells and act as the primary mediator for the large increase that occurs in GH expression after hatch. In support of this, GHRH-LPR and PACAP-R1 were barely detected in pituitary glands of adult chickens (181).

In chickens, pituitary GH is negatively regulated by hypothalamic SST acting primarily through SSTR2 (182). While SST mRNA levels did not change in this study, there was a dramatic increase in pituitary mRNA levels of SSTR2 on e20, and these remained elevated through d7. This increase in pituitary SSTR2 mRNA just prior to hatch has been reported previously (138) and, in combination with the decline in mRNA levels for PACAP-R1, GHRH-LPR, GHS-R, and TRH-R observed around the same time, may be reflective of the onset of negative feedback in the somatotropic axis from circulating GH itself or GH-stimulated IGF-I, both of which are substantially elevated after hatch (142, 199). Gene expression for hypothalamic GHRH and pituitary GHRH receptors appears to be resistant to this negative feedback, as mRNA levels for these components were maintained at elevated levels through d7. This resistance may be conveyed, in part, by elevated circulating CORT and thyroid hormones in the chick embryo at this time (10, 138-141). Thyroid hormones and glucocorticoids are capable of stimulating GHRH-R mRNA in the rat fetal pituitary gland (205). Both pituitary TSH-β [Figure 7 and (126, 138, 139)] and circulating TSH levels decrease just prior to hatch (199), and the rise in SSTR2 mRNA that occurred on e20 may reflect the inhibition of pituitary TSH synthesis

and release by SST (201), which is thought to be mediated by SSTR2 localized on pituitary thyrotrophs (178).

In this study, elevation of pituitary PRL mRNA began around e16, substantially increased on e18, continued to rise on e20, and remained elevated after hatch. This is consistent with the appearance of pituitary lactotrophs in the developing pituitary gland shortly after GH-producing cells, just prior to birth in rodents (100, 102) and hatch in birds (53, 98, 105, 106). Pituitary mRNA for PRL has previously been shown to increase just prior to and immediately following the end of embryogenesis in mammals and birds (53, 87, 95, 98, 101, 124, 128), correlating with the rise in circulating PRL that occurs at the same time (98). The considerable increase in pituitary PRL mRNA that occurred on e18 indicates that lactotropic axis activity is initiated around this age. However, maximum expression of VIP mRNA in the hypothalamus, as well as VIP-R1 and PRL mRNA in the pituitary, did not occur until e20, indicating that activity increases perihatch and establishment of this axis occurs between e20 and d3 (Figures 9 and 11).

In both mammals and birds, VIP is a major PRL-releasing factor (183, 184). We observed an increase in hypothalamic VIP mRNA on e14, just prior to the age at which PRL mRNA began to increase in the pituitary, as well as an apparent increase in pituitary VIP-R2 mRNA at the same age and an increase in VIP-R1 mRNA toward the end of embryonic development. In addition to stimulating PRL release, VIP can trigger premature lactotroph differentiation in cultures of e13 primary chicken pituitary cells *in vitro* (105) and increase PRL gene expression through transcriptional and post-transcriptional mechanisms (206). Therefore, cumulative increases in hypothalamic VIP and pituitary VIP-R1 and VIP-R2 may be contributing to the induction of PRL gene

expression in late embryonic development. Both VIP-R1 and VIP-R2 are potently activated by chicken VIP and, therefore, are thought to function as true VIP receptors (181). Interestingly, the same group measured high levels of VIP-R1 expression in adult chicken pituitary glands, but was barely able to detect VIP-R2 mRNA in the same samples. In our current study, VIP-R2 mRNA tended to increase on e14, remained elevated through e20, and then declined after hatch. On the contrary, VIP-R1 did not begin to increase until e20 and remained elevated after hatch. Taken together, the results of our study and those published by Wang *et al.* (181) indicate that VIP-R2 may function to initiate PRL gene expression during lactotroph functional differentiation, while VIP-R1 acts as the primary receptor mediating VIP-induced PRL expression and release in late embryogenesis and after hatch.

Immunoreactive gonadotrophs appear in the pituitary of chick embryos by e8 or e9, and increase in number toward the end of embryonic development (53, 54, 97, 191). Consistent with these reports, the ontogenic profiles we determined for components in the gonadotropic axis (Figure 10) indicate that activity is initiated prior to e10, based on the observation that both gonadotropin-β subunits are already expressed and FSH-β mRNA did not further increase during the period investigated. However, hypothalamic GnRH1 mRNA and pituitary LH-β mRNA do not reach maximum levels until d5 or d7, respectively, which implies that apparent activity of the reproductive axis dramatically increases during the first week of neonatal life and establishment does not occur until well after hatching (Figure 11). It has been shown previously that FSH-β (53) and LH-β mRNA (54) in the pituitary are detectable from e4 in the chick, and that neither change much after e10, the earliest age we investigated. Similarly, we found no significant

increases in levels of either pituitary gonadotropin-β subunit mRNA during embryogenesis. Results from a recent study suggest that there is an increase in both LH-β and FSH-β mRNA between e11, e17, and d1 in female, but not male, chick embryos (173). We did not sex the birds used in our study until after hatch, at which time we used 2 females and 2 males for each age. This sexual dimorphism may explain the lack of an increase in mRNA levels for these genes that we observed, and also the higher level of variability we obtained when analyzing genes in this axis as compared to the others. In fetal rodents, FSH-β and LH-β mRNA are detected at low levels a few days before birth, and their expression dramatically increases in the early postnatal pituitary (87, 95), supporting our conclusion that this axis is initiated during embryogenesis but is not fully developed until neonatal life.

In mammals and birds, pituitary gonadotropins are under dual hypothalamic control, with synthesis and release stimulated and inhibited by GnRH1 and GnIH, respectively (207-210). We observed an increase in hypothalamic GnRH1 beginning around e14 that continued through post-hatch d7, which is consistent with the reported increase in GnRH1 brain content that occurs at the end of embryogenesis in rats and chickens (211, 212). An inverse ontogenic profile was measured for GnIH mRNA, which tended to decrease between late embryonic development and early post hatch. A similar pattern for GnIH mRNA was detected in quail perihatch (213). These changes clearly precede the increase in pituitary LH-β mRNA we observed, suggesting that both GnRH1 and GnIH are important factors in determining the onset of gonadotropin synthesis.

GnIH-R immunoreactive cells were recently found to colocalize with LH-β and FSH-β mRNA (214), indicating that it is directly mediating the effect of GnIH on LH and

FSH expression and secretion. It has also been shown that pituitary GnIH-R mRNA fluctuates during the reproductive cycle in the chicken and is higher in the pituitary of reproductively immature birds (214, 215). Further, stimulation of GnIH-R can decrease GnRH-induced signaling in a dose-dependent manner (215). The authors proposed that, during avian sexual maturation, a down-regulation of GnIH-R on pituitary gonadotrophs leads to an increased GnRH-R:GnIH-R ratio. This mechanism allows for a switch in pituitary sensitivity to hypothalamic factors from inhibitory to stimulatory, which in turn promotes synthesis and release of gonadotropins (185, 214, 215). A similar mechanism may be occurring during gonadotroph development and reproductive axis maturation. We did not detect any changes in pituitary GnRH-R mRNA during the current study. However, the decrease in GnIH-R1 mRNA that clearly occurred between mid-embryogenesis and d7 would cause an increase in the GnRH-R:GnIH-R1 ratio during development and shift pituitary sensitivity from inhibitory to stimulatory. Likewise, increased GnRH1 mRNA, coupled with the trend for decreased GnIH mRNA, suggest hypothalamic influence also switches from inhibitory to stimulatory.

In summary, we have clearly established the timing of initiation and maturation of gene expression within the five major hypothalamic-pituitary axes during neuroendocrine system development in the chick by quantifying gene expression for their major components. Initiation of all five axes occurs during embryonic development, but several are not fully established until after hatch. The first axis established is the adrenocorticotropic axis, followed by the thyrotropic, somatotropic, and lactotropic axes. Although initiation of gonadotropic activity appears to occur rather early in embryonic development, the reproductive axis is the final one to be fully established. These results

are the first comprehensive study to reveal ontogenic relationships among major components of each of the five neuroendocrine axes.

CHAPTER 3

Mechanisms involved in glucocorticoid induction of pituitary growth hormone expression during embryonic development

Abstract

Glucocorticoid hormones are involved in functional differentiation of somatotrophs, the GH-producing cells in the anterior pituitary gland. Glucocorticoid treatment prematurely induces GH expression in mammals and birds in a process that requires both protein synthesis and ras signaling. The objective of this study was to further investigate mechanisms through which glucocorticoids initiate GH expression during embryogenesis. Presently, we determined that stimulation of GH expression occured through transcriptional activation of the GH gene, rather than through enhancement of mRNA stability, and this process requires histone deacetylase (HDAC) activity. Further, through pharmacological inhibition, we identified the ERK1/2 pathway as a possible downstream ras-effector necessary for CORT stimulation of GH. Additional evidence indicated that CORT increased ERK1/2 activity in cultured embryonic pituitary cells after 3 h. However, we also found that chronic activation of ERK1/2 and p38MAPK activity with constitutively active mutants or stimulatory ligands reduced initiation of GH expression by CORT. Therefore, we conclude that the requirement for kinase signaling is transitory and that these pathways must be subsequently down-regulated during CORT treatment for maximal glucocorticoid induction of GH to occur. These results are the first in any species to demonstrate that ras- and ERK1/2-mediated transcriptional events requiring HDAC activity are involved in glucocorticoid induction of pituitary GH during embryonic development.

Introduction

Somatotrophs are one of the most abundant cell types in the mature anterior pituitary gland, and these GH-producing cells functionally differentiate during the second half of mammalian and avian fetal and embryonic development, respectively. Expression of GH in rats and mice is first apparent during the 21-day gestation around e15, and the number of somatotrophs substantially increases between e18 and e19 (87, 95, 96, 100, 101, 120-123). During the chick 21-day incubation period, somatotrophs first begin to appear on e12 and become a significant population in the anterior pituitary gland around e16 (44, 53, 94, 99, 103, 104, 106, 124-127).

Glucocorticoids can prematurely induce somatotroph appearance during fetal rat and embryonic chick development both *in vivo* and *in vitro* (44, 121, 143-150, 152-155, 159). In pregnant rats, treatment with dexamethasone increases somatotroph abundance in e17 or e18 fetuses (145, 150), and glucocorticoids can stimulate somatotroph differentiation *in vitro* (143, 144, 152). Similarly, treatment of chick embryos *in ovo* (148, 149) or chicken anterior pituitary cells *in vitro* with CORT, the primary circulating glucocorticoid in rodents and birds, increases the number of somatotrophs (147, 153-155, 159). In addition to the stimulation of GH expression in mammalian and avian embryos as a result of treatment with exogenous glucocorticoids, evidence exists for involvement of endogenous glucocorticoids in functional differentiation of somatotrophs. A decrease in the level of circulating CORT by administration of metyrapone to pregnant rats leads to reduction in the number of fetal somatotrophs on e19 (150), and an increase in circulating CORT in chick embryos as a result of *in ovo* ACTH injection leads to

premature GH cell appearance (140). These prematurely induced somatotrophs are fully functional, as they are responsive to GH-secretagogues (129).

Although the precise details of somatotroph recruitment by glucocorticoids are unknown, an indirect mechanism influencing the final stages of somatotroph differentiation appears involved. In both rats and chicks, glucocorticoid induction of GH mRNA does not occur until after several hours of treatment, and the induction is blocked by inhibitors of protein synthesis (151, 153). The absence of a classical GRE within the region of the chicken GH gene that is at least partially responsible for mediating induction by CORT (137, 216) further implies that glucocorticoids increase expression of an intermediary factor necessary for stimulation of GH. Induction of GH-producing cells by glucocorticoids *in vivo* is restricted to a narrow period of development, between e17-e18 in fetal rats (150) and e11-e13 in embryonic chickens (149), and earlier or later administration of exogenous corticosteroids has no effect. Further, in chicken embryos, induction of GH expression by CORT is greater in the caudal lobe of the anterior pituitary, where somatotrophs normally reside (49, 50, 53, 128), as compared to the cephalic lobe (129, 149, 155). Taken together, this evidence suggests that circulating glucocorticoids are involved in the functional differentiation and enhancement of GH expression in cells already committed to the somatotroph lineage.

We previously reported that both type I (MR) and type II (GR) receptors mediate glucocorticoid induction of somatotrophs, as antagonists to both are necessary to abolish the CORT response (156, 159). Investigation into possible signaling events involved in CORT regulation of GH expression during embryonic development revealed that pharmacological activation or inhibition of PKA and PKC does not affect glucocorticoid

induction of GH mRNA. However, ras signaling appears to play a role in the induction of GH mRNA, because the ras inhibitor manumycin A suppresses the response to CORT (153, 217). Beyond this, little is known regarding possible signaling mechanisms or the contribution of transcriptional and post-transcriptional events to glucocorticoid initiation of pituitary GH expression during embryogenesis in any species. Therefore, the objective of this study was to examine in detail mechanisms through which glucocorticoids induce GH expression during pituitary development, including determining whether the process involves transcriptional activation of the GH gene, an increase in GH mRNA stability, histone deacetylation, and known ras-induced signaling pathways.

Materials and Methods

Animals and pituitary dispersions

Broiler strain chicken embryos were incubated as described in the Materials and Methods section of Chapter 2, and pituitary glands were isolated using a dissecting microscope from embryos removed from the incubator on e11. For each replicate trial of a given experiment, anterior pituitaries from e11 chickens (50-60 embryos) or e15-e16 mice (25-30 fetuses) were isolated and pooled. On average, an e11 chicken anterior pituitary gland yields $3.5x10^5$ cells, while an anterior pituitary from an e15-e16 mouse yields only $7.5x10^4$ cells. Anterior pituitary glands were dissociated into individual cells using trypsin digestion in combination with mechanical agitation as previously described (103). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland.

Cell culture

Unless otherwise stated, hormones and other chemicals were obtained from Sigma-Aldrich, and all cell culture reagents were obtained from Invitrogen. Cells were maintained in a 37.5 C, 5% CO_2 atmosphere. With the exception of experiments involving transfection (see below), dispersed pituitary cells were allowed to attach overnight in poly-L-lysine coated cell culture plates (Corning Life Sciences, Lowell, MA) 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 μg/ml human insulin, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate prior to addition of any inhibitors or treatment. Leghorn male hepatoma (LMH) cells [American Type Culture Collection (ATCC), Manassas, VA], a chicken hepatocellular carcinoma epithelial cell line, were maintained in Waymouth's medium (Sigma) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX) in 75-cm^2 flasks (Corning Life Sciences) coated with 0.1% gelatin (Sigma). Human embryonic kidney-293 (HEK-293) cells (ATCC) were maintained in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

The protein synthesis inhibitor cycloheximide (CHX) was dissolved in media and used at a final concentration of 10 μg/ml, a concentration that has previously been shown to block the increase in GH mRNA in response to CORT (153). Cells ($1x10^6$/well for chicken and $2.5x10^6$/well for mouse; 24-well plate format) were pretreated with vehicle (media) or CHX for 1.5 h prior to CORT (1 nM) treatment for the indicated times. All remaining inhibitors were dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO in cell culture wells was less than 1%. Actinomycin D (ActD), a

transcriptional inhibitor, was used at a final concentration of 5 μg/ml. The HDAC inhibitors, HC toxin and trichostatin A (TSA), were used at final concentrations of 100 nM or 200 nM, respectively. Intracellular signaling proteins were inhibited using the following compounds: PI3-K with LY294002 (50 μM) and wortmannin (100 nM); ERK1/2 with U0126 (10 μM) and PD98059 (50 μM); p38MAPK with SB203580 (40 μM); JNK with SP600125; and ras with manumycin A (1 μM). Concentrations for HC toxin, TSA, LY294002, wortmannin, U0126, PD98059, SB203580, and SP600125 are based on published reports (218-223). The concentration of manumycin A is the highest non-lethal dose determined in preliminary experiments (Malkiewicz and Porter, unpublished) and is lower than that used previously in chickens (153). Cells ($1x10^6$/well in a 24-well plate format) were pretreated with vehicle (DMSO) or inhibitors for 1 h prior to addition of CORT (1 nM) for the indicated times. In experiments where both GH mRNA and protein expression were examined, cells were harvested by retrypsinization and an aliquot ($2.5x10^5$ cells) was washed and re-plated for detection of intracellular GH protein by immunocytochemistry (ICC). To determine efficacy of U0126 and SB203580, cells ($5x10^6$/well in a 12-well format) were cultured in the absence or presence of CORT (1 nM), epidermal growth factor (EGF; 100 ng/ml), or anisomycin (10 μM) for 3 h, following pretreatment with DMSO or inhibitors for 1 h.

To determine the effect of CORT treatment on GH mRNA stability during embryonic development, pituitary cells ($1x10^6$/dish) were cultured in 35-mm dishes (BD Biosciences, San Jose, CA) in the absence or presence of CORT (1 nM) for 6 h. Subsequently, cell culture medium in all dishes was replaced with medium containing ActD alone (Basal cells) or ActD plus CORT (CORT-treated cells), and cells were

collected at 0, 2, 4, 8, 12, 16, 20, and 24 h after medium replacement for total cellular RNA extraction and analysis by qRT-PCR.

In experiments evaluating the effect of ERK1/2 and p38MAPK pathway activation on CORT induction of GH mRNA expression, cells ($1x10^6$/well in a 24-well plate format) were left untreated or treated with EGF (100 ng/ml) or anisomycin (100 nM) for 30 m, after which time stimuli were washed out and cells were cultured in the absence or presence of CORT (1 nM) for another 6 h. Additional cells were left untreated or treated with CORT in the absence or presence of EGF or anisomycin for 6 h. Cells were also treated alone with EGF (100 ng/ml) or anisomycin at indicated doses for 6 h to assess the effect of longer treatment times on MAPK phosphorylation status.

In experiments evaluating levels of phosphorylated MAPK and kinase activity under basal and CORT-treated conditions, cells ($5x10^6$/well in a 12-well plate format) were left untreated or treated with CORT (1 nM) for the final 5 m, 30 m, 3 h, or 6 h of culture. For each time point, medium was replaced in both basal and CORT-treated wells to control for any effect the media change might have on MAPK pathways. Additional cells were stimulated for 30 m with epidermal growth factor (EGF; 100 ng/ml) or anisomycin (10 μM), as positive controls for ERK1/2 and p38MAPK activation, respectively.

Plasmids and transfection

The reporter construct containing -1,727 to +48 of the chicken GH gene driving firefly luciferase (pGL3-1727) has previously been demonstrated to be responsive to dexamethasone in a rat pituitary cell line (137) and was kindly provided by Dr. F. Leung (University of Hong Kong). 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).

Mouse constitutively active MAPK/ERK kinase (MEK) 1 (caMEK1) and dominant negative MEK 1 (dnMEK1) (224) were kindly provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO) and Dr. Paul Shapiro (University of Maryland School of Pharmacy, Baltimore, MD). The caMEK1 mutant is missing the nuclear export signal at the N-terminus (ΔN32-51) and has negatively charged amino acids (glutamic and aspartic acids) substituted at the serine residues that are phosphorylated in the wild-type enzyme (S218E/S222D). The dnMEK1 mutant is a catalytically inactive ATP-binding mutant with a lysine to methionine substitution in the active site (K97M). Human dominant-negative ERK1 (dnERK1) and rat dominant negative ERK2 (dnERK2) were generously provided by Dr. Melanie Cobb (University of Texas Southwestern Medical Center, Dallas, TX). Both dnERK1 (K71R) and dnERK2 (K52R) are catalytically inactive ATP-binding mutants with a lysine to arginine mutation in the active site (225). Mouse constitutively active mitogen-activated protein kinase kinase (MKK) 3 and MKK6 (caMKK3, caMKK6), dominant negative MKK3 and MKK6 (dnMKK3, dnMKK6), and dominant negative p38MAPKα and p38MAPKβ (dnp38α, dnp38β) were kindly provided by Dr. Jiahuia Han (Scripps Research Institute, La Jolla, CA). The caMKK3 and dnMKK3 mutants were created by replacing the serine residues that are phosphorylated in wild-type MKK3 with glutamic acid (S189E/S193E) or alanine (S189A/S193A), respectively. Similar mutations were made to create caMKK6 (S207E/S211E) and dnMKK6 (S207A/S211A) (226). Mutations in threonine and tyrosine residues that are phosphorylated in activated wild-type p38MAPK to alanine and phenylalanine,

respectively, were used to create the dnp38α (T180A/Y182F) and dnp38β (T188A/Y190F) mutants (227).

Since mutant enzymes were obtained from multiple sources and were in several different expression vectors, each was non-directionally subcloned into the CMV-promoter driven pCMV-Sport6.1 expression vector (Sport6.1; Invitrogen) to allow for a high level of constitutive expression. Primers (Sigma-Aldrich) used for PCR amplification of the coding region of each mutant off the original plasmid template are listed in Table 2 and include restriction enzyme sites at the 5'-end. Amplification was conducted using AccuPrime Pfx SuperMix (Invitrogen) according to the manufacture's protocol, and reactions contained 600 nM each primer. PCR cycling parameters were as follows: 95 C for 5 m; 35 cycles of 95 C for 15 s, 53 C for 45 s, and 68 C for 2 m; and a final extension at 68 C for 5 m. The following restriction enzymes were used to create expression constructs in Sport6.1: HindIII was used to subclone caMEK1, dnMEK1, dnERK2, and dnp38α; EcoRI was used to subclone caMKK3, dnMKK3, and dnp38β; KpnI was used to subclone caMKK6 and dnMKK6; and MluI was used to subclone dnERK1. Clones were screened by PCR for directionality of the insert using Sport6.1 SP6 or T7 primers (Table 2) in combination with forward and reverse cloning primers, and vectors from a clone containing the insert in the forward orientation were purified using the NucleoBond PC 500 plasmid purification kit (Macherey-Nagel, Inc., Bethlehem, PA) according to the manufacturer's directions. All clones were sequenced in their entirety using Sport6.1, cloning, and real-time primers listed in Table 2. Sequencing was conducted at the University of Maryland's Center for Biosystems Research DNA

Table 2. Primers used to investigate mechanisms through which glucocorticoids induce growth hormone expression in the embryonic anterior pituitary gland.

Gene	ID[1]	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
qRT-PCR			
Chicken			
Mature GH	00328	CACCTCAGACAGAGTGTTTGAGAAA	CAGGTGGATGTCGAACTTATCGT
Nascent GH	00328	GGATGTCTCCACAGGAACGC	GACAACTTACTGACTTCTGCTGGG
GHRH-R2	00361	ATCCCCAGACATCAGCAAAAACTA	GGAAGAACGCAAACACCACATAG
FKBP5	01397	TGCCAAGTTTGCTGAGAGGG	CCTCTGTCTCTTTGCCTTCATCA
ACTB[2]		CAGGATGCAGAAGGAGATCACA	TAGAGCCTCCAATCCAGACAGAGTA
GAPDH	37122	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT
Other Species			
Mouse GH	103071	CTCGGACCGTGTCTATGAGAAACT	GCTTGAGGATCTGCCCAACA
Mouse ACTB	031564	GATTACTGCTCTGGCTCCTAGCAC	GACAGTGAGGCCAGGATGGA
Human MEK1	307102	TCCTGAAGAAAGCTGGAAGAATTC	GCTTGACATCTCTGTGCATGATCT
Human Erk1	263025	ATATCTGCTACTTCCTCTACCAGATCCT	TCGCAGGTGGTGTTGATGAG
Rat Erk2	002533	AATGTTCTGCACCGTGACCTC	TCTGGATCTGCAACACGGG
Human MKK3	342679	CTGCATGGAGCTCATGGACA	ACACAGCAATCTCCCCAAGG
Human MKK6	359094	CTCATCAATGCTCTCGGTCAAG	TGTATGGTTTGCAACCTGCATC
Mouse p38MAPKα	062694	TAATTCACAGGGACCTAAAGCCC	CATCTCATCATCAGTGTGCCG
Human p38MAPKβ	330651	CTGAAGTACATCCACTCGGCC	AGCCCGAAATCCAGGATCC
Subcloning ERK1/2 and p38MAPK Pathway Mutants			
Human MEK1	307102	CCCAAGCTTGGGGCCACCATGCCCAAGAAGAAGCCGACGC	CCCAAGCTTGGGTTAGACGCCAGCAGCATGGGTTG
Human ERK1	263025	CGACGCGTCGGCCACCATGGCGGCGGCGGCGG	CGACGCGTCGCTAGGGGGCCTCCAGCACTC
Rat ERK2	002533	CCCAAGCTTGGGGCCACCATGGCGGCGGCGGCGG	CCCAAGCTTGGGTTAAGATCTGTATCCTGGCTGGAATCGA
Human MKK3	342679	CCGGAATTCCGCCACCATGGAGTCGCCCGCCTCGAG	CCGGAATTCCCTATGAGTCTTCTCCCAGGATCTCC
Human MKK6	359094	CGGGGTACCCCGGCCACCATGTCTCAGTCGAAAGGCAAGAAGCG	CGGGGTACCCCGTTAGTCTCCAAGAATCAGTTTTACAAAAGATGC
Mouse p38MAPKα	062694	CCCAAGCTTGGGGCCACCATGTCGCAGGAGAGGCCCACG	CCCAAGCTTGGGTCAGGACTCCATTTCTTCTTGGTCAAGG
Human p38MAPKβ	330651	CCGGAATTCCGCCACCATGTCGGGCCCTCGCGCC	CCGGAATTCCTCACTGCTCAATCTCCAGGCTGC
SPORT6.1[3]		SP6: GGCCTATTTAGGTGACACTATAG	T7: GCTTATAATACGACTCACTATAGGG

[1]ID is the transcript identification from Ensembl genome assembly (http://www.ensembl.org/) and in all cases is preceded by ENSGALT000000 (chicken transcripts), ENSMUST00000 (mouse transcripts), ENST00000 (human transcripts), or ENSRNOT00000 (rat transcripts).

[2]The sequence for ACTB is not on the assembled chicken genome and primers were designed based on the sequence in GenBank (accession no. X00182).

[3]The Sport6.1 vector (Invitrogen) was the expression vector that the ERK1/2 and p38MAPK pathway mutants were cloned into for the over-expression studies. The SP6 primer is on the 5'-end of the multiple cloning site, and the T7 promoter primer is on the 3'-end of the multiple cloning site relative to the CMV promoter.

Sequencing Facility with AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP in an Applied Biosystems DNA Sequencer (Model 3100). Overlapping sequences were assembled into the full-length cDNA sequence using the ContigExpress feature of Vector NTI 9.0 software (Invitrogen). The AlignX tool of the same software was used for sequence comparisons to ensure that clones contained the appropriate mutations and had not gained any additional mutations during the subcloning process.

All cells were transfected in supplement-free optimized modified Eagle's minimal essential medium (Opti-MEM I) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In experiments investigating CORT induction of GH promoter activity in the absence or presence of MAPK pathway inhibitors, e11 pituitary cells ($5x10^5$/well in a 24-well format) were allowed to attach the cell culture plate for 2 h in supplement-free Opti-MEM I medium prior to transient transfection with Lipofectamine 2000 according to the manufacturer's protocol. Plating medium was then replaced with transfection medium, consisting of Opti-MEM I containing 1 µg pGL3-1727 or 1 µg pGL3-Basic together with 20 ng pRL-SV40 and 2 µl Lipofectamine. After 6 h, transfection medium was replaced with cell culture medium, and cells were allowed to recover for 18 hours. Subsequently, cells were pretreated for 1 h with DMSO or MAPK inhibitors prior to addition of CORT (100 nM) to appropriate wells for 20 h.

In experiments evaluating effects of constitutively active mutants on GH promoter activity under basal and CORT-treated conditions, cells were plated and transfected as described above, with the following exceptions: cells received 1 µg Sport6.1, 1 µg caMEK1, or 0.5 µg MKK3 and 0.5 µg MKK6 in addition to reporter and normalization

plasmids; and the medium contained 4 µl Lipofectamine. After recovery, cells were left untreated or treated for 20 h with CORT (100 nM).

In experiments determining effects of constitutively active mutants on GH mRNA expression, e11 pituitary cells ($3x10^6$/well in a 12-well format) were co-transfected for 6 h with 1 µg golgi-targeted green-fluorescent protein (GFP) expression vector (228) and either 1 µg Sport6.1, 0.5 µg Sport6.1 and 0.5 µg caMEK1, or 0.5 µg caMKK3 and 0.5 µg caMKK6 in Opti-MEM I containing 4 µl Lipofectamine. To enhance transfection efficiency, cells were transfected in suspension for 2 h with gentle inversion every 30 m and then plated into 12-well culture plates for the remaining 4 h. GFP was used to assess transfection efficiency and as a marker to isolate transfected cells using flow cytometric cell sorting. Subsequently, transfection medium was replaced with culture medium, and cells were allowed to recover for 18 h prior to addition of CORT (1 nM) to appropriate wells for 20 h. Cells were collected by retrypsinization, washed one time with ice-cold phosphate-buffered saline (PBS; 2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4), resuspended in 0.25 ml ice-cold PBS, and held on ice until flow cytometric analysis and sorting.

When LMH and HEK-293 cells were used in transfection experiments, cells were recovered from culture flasks using retrypsinization in the presence of 0.03% ethylenediamine tetraacetic acid (EDTA) and re-plated ($1x10^6$/well) in their respective growth media in 12-well plates coated with 0.1% gelatin (Sigma). Cells were grown for approximately 24 h, until they reached ~90% confluence, after which time they were transfected with 2 µg Sport6.1, 1 µg Sport6.1 and 1 µg caMEK1, 1 µg Sport6.1 and 1 µg dnMEK1, 1 µg dnERK1 and 1 µg dnERK2, 1 µg caMKK3 and 1 µg caMKK6, 1 µg

dnMKK3 and 1 μg dnMKK6, or 1 μg dnp38α and 1 μg dnp38β in Opti-MEM I containing 4 μl Lipofectamine. After 6 h transfection, medium was replaced with serum-free growth medium supplemented with 0.1% BSA, and cells were allowed to recover for 24 h prior to treatment. To investigate ERK1/2 MAPK activity and functionality of ERK1/2 pathway mutants, cells were left untreated or stimulated with EGF (100 ng/ml) for 30 m. To investigate p38MAPK activity and functionality of pathway mutants, cells were left untreated or stimulated with anisomycin (100 nM) for 30 m.

Analysis of promoter activity

After removal of culture medium, cells were gently rinsed one time with PBS and lysed using 100 μl passive lysis buffer (Promega) with gentle agitation. Lysates were stored at -20 C until luciferase reporter activity was measured. Cell lysates (20 μl) were analyzed for firefly (reporter gene) and renilla (normalization gene) enzyme activities with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. For each culture well, firefly luciferase activity was divided by renilla luciferase activity to normalize for variations in transfection efficiency. Promoter activity (mean + SEM) is expressed as fold induction over basal cells transfected with pGL3-Basic and, where appropriate, Sport6.1.

Flow cytometry

Successfully transfected cells in constitutively active ERK1/2 and p38MAPK pathway mutant overexpression studies were identified by flow cytometric detection of GFP (fluorescence detection 530/30 nm) at the University of Maryland, Department of Veterinary Medicine's Flow Cytometry Core Facility. Cells were passed through a cell-strainer cap containing 35 μm nylon mesh (BD Biosciences) to remove clumps, and GFP-

positive cells were sorted using a high-speed benchtop flow cytometer and cell sorter (FACSAria II; BD Biosciences) equipped with a fixed-alignment 488 nm laser (Sapphire; Coherent, Inc., Santa Clara, CA). Data acquisition and analysis were performed with FACSDiva software (version 6.1.2; BD Biosciences). Sorting of the GFP-positive population (13.8 ± 0.9% of cells; n=3 replicate experiments) resulted in collection of approximately 50,000 GFP-positive cells per group. GFP-negative cells were also collected. GFP-positive cells were collected directly into 0.5 ml cell lysis buffer RLT of the RNeasy Mini Kit (Qiagen) containing no β-mercaptoethanol, vortexed several times, and held at room temperature approximately 1 h until all samples were collected. Final volumes were measured and adjusted using nuclease-free water or buffer RLT so the RLT:sample ratio was 3.5:1, β-mercaptoethanol was added to each sample (1 µl per 100 µl RLT buffer), and total RNA was extracted immediately, as described below.

qRT-PCR

Cells were harvested at the completion of each experiment by retrypsinization, immediately frozen in liquid nitrogen, and stored at -80 C until RNA extraction. Total RNA was extracted from cultured cells with the RNeasy Mini Kit (Qiagen) and included an on-column DNase digestion. Quantification of RNA, RT reactions using 500 ng total RNA, and real-time PCR reactions were all performed as described in the Materials and Methods section of Chapter 2, with the following exceptions. RT reactions were conducted using random primers (Invitrogen) in the experiment investigating effect of CORT treatment on GH mRNA stability. For the experiment involving detection of nascent GH mRNA transcript, RT reactions were conducted on 350 ng total RNA using random primers (Invitrogen) and were diluted to 70 µl prior to PCR analysis. In addition,

reactions containing no reverse transcriptase enzyme were conducted for each sample to ensure that the transcript detected in this experiment was truly nascent mRNA and not contaminating genomic DNA. The resulting PCR product was sequenced to confirm the presence of intronic sequence, which distinguished the product from mature GH mRNA species. RT reactions contained 25 ng total RNA and were left undiluted prior to PCR analysis for experiments involving overexpression of ERK1/2 and p38MAPK constitutively active mutants. For the experiment using cultured fetal mouse anterior pituitaries, RT reactions were also used undiluted for the PCR, and contained 100 ng total RNA.

Primers (Table 2; Sigma-Aldrich) were designed as described in the methods of Chapter 2 to detect chicken nascent and mature GH mRNA, GHRH-R2 mRNA, FKBP5 mRNA, beta-actin (ACTB) mRNA, and GAPDH mRNA; mouse GH mRNA, ACTB mRNA, and p38MAPKα mRNA; human MEK1 mRNA, ERK1 mRNA, MKK3 mRNA, MKK6 mRNA, and p38MAPKβ mRNA; and rat ERK2 mRNA. In all experiments except the one evaluating the effect of CORT treatment on GH mRNA stability, the amount of target mRNA was normalized to the amount of ACTB mRNA as previously described (229) using the following equation: $\Delta Ct = (Ct_{no\ RT} - Ct_{sample})_{target} - (Ct_{no\ RT} - Ct_{sample})_{ACTB}$, where Ct is the cycle number when the amount of amplified product reaches a fixed threshold for fluorescence due to binding of SYBR green to the double-stranded PCR product. Data were then transformed using the equation $2^{\Delta Ct}$, and the transformed value for each sample was divided by the mean of the transformed value for basal cells receiving no CORT, EGF, anisomycin, or inhibitors and, where appropriate, transfected with Sport6.1. Data for each gene (mean + SEM) are presented as fold induction over

basal mRNA levels for each experiment. In the experiment investigating the effect of CORT treatment on mRNA stability, mRNA levels of GH, ACTB, and GAPDH for each time point were expressed as a percentage of levels at time of ActD addition (0 h) under basal and CORT-treated conditions for each replicate (set to 100% for each condition). The half-life ($t_{1/2}$; h) of each gene under basal and CORT-treated conditions was then calculated for each replicate by plotting $\log_2$-transformed percentage versus time after ActD addition (h) and calculating the slope of the resulting line with linear regression, which was then used to determine half-life for each mRNA species using the equation: $t_{1/2}$ (h) = -1/slope. Data (mean + SEM) for this experiment are presented in two ways, as fold induction relative to levels in basal cells receiving no CORT for the first 6 h and as a percentage of levels at 0 h after ActD addition for each condition (set to 100% with no variance for both basal and CORT-treated conditions).

Immunocytochemistry

After being gently washed one time with PBS, plated cells were fixed with 3.7% formaldehyde in PBS for 20 m. Cells were then washed with PBS (3x5 m), permeabilized with 0.1% Triton-X-100/0.1% Tween-20 in PBS for 8 m, and quenched with 0.3% H_20_2 in PBS for 5 m after washing twice with PBS. After blocking with 2% normal goat serum (NGS) in PBS for 1 h, cells were incubated overnight at 4 C with a previously validated rabbit anti-chicken GH primary antibody (1:4000 in 1% NGS) (103). Cells were then thoroughly washed with PBS and analyzed for intracellular GH protein content using a rabbit Avidin/Biotin-Complex kit followed by development with VIP peroxidase substrate according to the manufacturer's protocols (Vector Laboratories, Burlingame,

CA). GH-containing cells were visualized with an inverted light microscope, and results (mean + SEM) expressed as a percentage of all pituitary cells present.

Western blotting

To verify expression of constitutively active and dominant negative pathway mutants in chicken cells, LMH cells were left untransfected or transfected with Sport6.1, caMEK1, dnMEK1, dnERK1 and dnERK2, caMKK3 and caMKK6, dnMKK3 and dnMKK6, or dnp38MAPKα and dnp38MAPKβ. Following 24 h of culture, cells were placed on ice, gently rinsed one time with ice-cold PBS, and incubated on ice with rocking for 5 m in non-denaturing cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF; added immediately before use)]. Cells were scraped, transferred to a 1.7-ml microcentrifuge tube, and sonicated on ice (3x10 s with 30 s break in between each round) with the sonicator (Model W350; Heat Systems-Ultrasonics, Inc., Plainview, NY) on the continuous setting at output level 4. Subsequently, samples were centrifuged at 14,000xg for 10 m at 4 C, and the supernatant was stored at -80 C until analysis.

Protein levels in each sample were quantified with the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Extracts (15 µg total cellular protein) were boiled in Laemmli sample buffer [60 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue] for 5 m, resolved on a 12% gel by SDS- polyacrylamide gel electrophoresis (SDS-PAGE) with Tris-glycine buffer (25 mM Tris, 192 mM glycine) containing 0.1% SDS, and transferred to a

polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA) for 30 m at 16 V and 1 h at 25 V using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) in Tris-glycine buffer containing 20% methanol. Membranes were washed for 15 m with Tris-buffered saline containing Tween-20 (TBS/T; 20 mM Tris-HCl, 136 mM NaCl, 0.1% Tween-20, pH 7.6), blocked for 2 h at room temperature in TBS/T containing 5% nonfat dry milk, and incubated overnight at 4 C with rabbit polyclonal antibodies (1:1000; Cell Signaling Technology, Inc.) against MEK1, ERK1/2, MKK3, MKK6, p38MAPKα/β/γ, or a mouse monoclonal antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against α-tubulin diluted in TBS/T containing 1% nonfat dry milk. Following thorough washing with TBS/T, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000; Cell Signaling Technologies) or anti-mouse IgG (1:5000; Amersham Biosciences; Piscataway, NJ) diluted in TBS/T containing 5% nonfat milk. Immunoreactive bands were detected using enhanced chemiluminescent detection reagents (LumiGLO; Cell Signaling Technologies, Inc.) and the ChemiDoc XRS system equipped with Quantity One software (version 4.5.2; Bio-Rad).

Samples used for determining efficacy of SB203580 were analyzed for levels of endogenous phosphorylated activating transcription factor 2 (p-ATF2), a downstream target of p38MAPK, as described above, except total cellular protein (10 μg) was resolved using SDS-PAGE on an 8% gel, and the membrane was incubated with a rabbit polyclonal antibody (1:1000) against p-ATF2 (Thr71; Cell Signaling Technology, Inc.). Following detection of p-ATF2, membranes were stripped for 30 m at 55 C in buffer containing 62.5 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol, and 2% SDS and re-

probed for α-tubulin as described above. Bands were quantified using Quantity One Software (Bio-Rad) and data [(mean + standard deviation (SD)] are presented as average band intensity for p-ATF2 divided by average band intensity for α-tubulin.

MAPK enzyme activity assays

Cells were lysed and quantified as described above, and protein extracts (80 μg for e11 primary cells and 100 μg for cell lines) were analyzed for MAPK enzyme activities using nonradioactive p44/42 MAP Kinase (Thr202/Tyr204) and p38 MAP Kinase (Thr180/Tyr182) Assay Kits (Cell Signaling Technology, Inc.). Briefly, total cellular protein was immunoprecipitated overnight by gentle rotation at 4 C with immobilized monoclonal antibodies specific for phosphorylated ERK1/2 (p-ERK1/2 ;1:15) or phosphorylated p38MAPK (p-p38MAPK; 1:10). Immunoprecipitates were washed twice with cell lysis buffer and twice with kinase assay buffer [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 5 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate] prior to incubation for 1.5 h at 30 C in kinase assay buffer supplemented with 200 μM ATP and 1 μg of the appropriate substrate. During kinase assay incubation, samples were flicked gently every 15-20 m to prevent beads from settling. Substrates used in the assays are fusion proteins of well-established downstream targets for ERK1/2 activity [(Ets-like gene 1 (Elk-1)] and p38MAPK activity (ATF2). Reactions were stored at -20 C overnight until analyzed for levels of phosphorylated Elk-1 (p-Elk1) and p-ATF2. Samples were resolved on a 12% gel by SDS-PAGE and levels of p-Elk1 or p-ATF2 were detected by western blotting (1:1000 primary antibody dilution) as described above. Where indicated, bands were quantified using Quantity One Software (Bio-Rad) and data (mean + SD) are presented as average band intensity.

Detection of phosphorylated MAPKs

Cells were lysed and total cellular protein was quantified as described above. Protein levels in each sample were adjusted to 0.5 µg/µl with cell lysis buffer prior to analysis using PathScan Phospho-p44/42 MAPK (Thr202/Tyr204) and PathScan Phospho-p38 MAPK (Thr180/Tyr182) Sandwich ELISA Kits (Cell Signaling Technology, Inc., Danvers, MA) to assess levels of p-ERK1/2 and p-p38MAPK. In order to express results in a semi-quantitative manner, standard curves were generated for each assay by analyzing two-fold dilutions of LMH cell lysates stimulated with EGF or sodium arsenite, a strong inducer of p38MAPK activity in these cells (230). Data (mean + SEM) are expressed as equivalents p-ERK1/2 or p-p38MAPK per mg protein, where one equivalent is equal to the amount of p-ERK1/2 or p-p38MAPK in 1 µg total cellular protein extracted from LMH cells stimulated with EGF or sodium arsenite, respectively.

Data analysis

Promoter activity and qRT-PCR data (expressed as fold induction over basal cells receiving vehicle and transfected with empty reporter and expression vectors, as appropriate), were $\log_2$-transformed prior to statistical analysis. To correct for non-homogeneity of variance in comparisons of somatotroph percentages, the ICC percentage data were transformed by taking the $\log_{10}$ of the arcsin of the percentage prior to statistical analysis. All data were analyzed using SAS software (SAS Institute). Data were analyzed using the t-test procedure (two-tailed) for the experiment evaluating mRNA half-life, and all remaining data were analyzed by analysis of variance using the MIXED models procedure, with differences between groups determined by the test of least significant difference (PDIFF).

Results

Glucocorticoids initiate GH gene transcription during embryogenesis

Although it is well-established that corticosteroid treatment elevates GH mRNA levels in the embryonic anterior pituitary gland (44, 121, 129, 145, 149-153, 155, 156, 159), it is not known whether this is due to an increase in gene transcription, enhanced mRNA stability, or both. The transcriptional inhibitor ActD completely abolished the rise in GH mRNA resulting from CORT treatment of e11 anterior pituitary cells (Figure 12A; $P<0.05$, n=3 replicate trials), indicating this increase requires active gene transcription. The observation that CORT stimulated promoter activity from a reporter construct driven by 1,727 bp of the chicken GH gene 5'-flanking region (pGL3-1727; Figure 12B; $P<0.05$, n=3 replicate trials) suggests that at least part of the response is a result of an increase in transcription of the GH gene itself. In order to definitively confirm that CORT increases transcription of GH in embryonic pituitary cells, we designed primers to span exon 3 of the GH gene that would partially bind intronic sequence and, therefore, only amplify newly synthesized transcript that had not yet been spliced. The increase in nascent GH mRNA resulting from treatment of e11 pituitary cells with CORT is similar in magnitude to the observed increase in spliced, mature GH mRNA (Figure 12C; n=3 replicate trials) and clearly indicates that glucocorticoids initiate transcription of GH during embryogenesis. In order to ensure that the detected nascent transcript was not actually contaminating genomic DNA, we performed control RT reactions for each sample that did not contain any reverse transcriptase enzyme. Amplified PCR product of the predicted size was detected only in samples from reactions containing enzyme (Figure 12D), and the presence of intronic sequence was confirmed in the product.

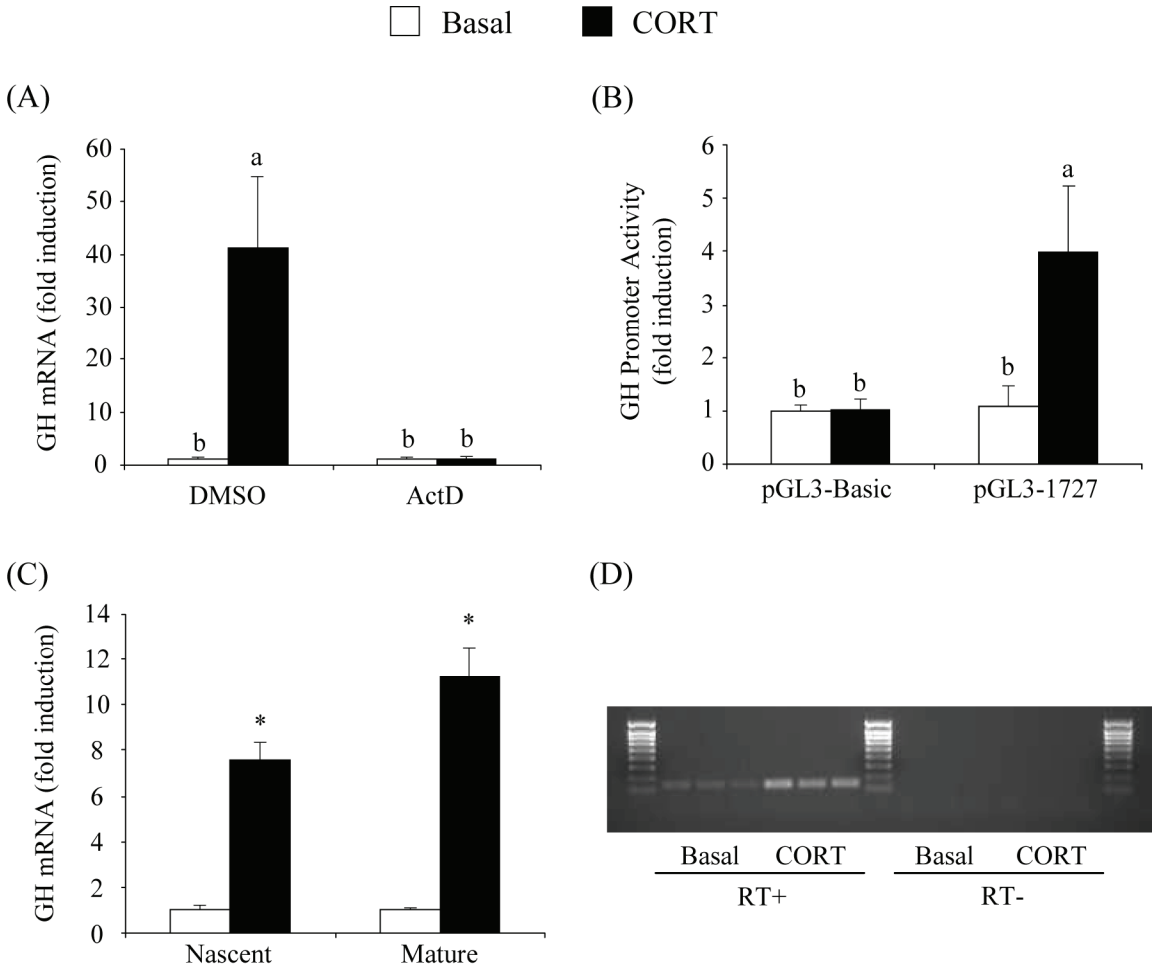


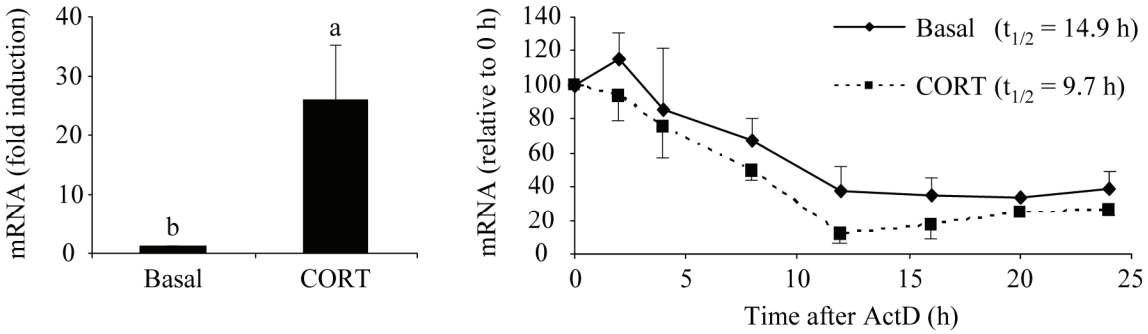
Figure 12. Glucocorticoids induce pituitary GH mRNA during chicken embryonic development through transcriptional activation of the GH gene. (A) After pretreatment for 1 h with DMSO or the transcriptional inhibitor ActD (5 µg/ml), e11 pituitary cells (n=3) were cultured in the absence or presence of CORT (1 nm) and DMSO or ActD for 6 h. Levels of GH mRNA, analyzed by qRT-PCR and normalized to ACTB mRNA levels, are expressed as fold induction over levels in basal cells receiving DMSO. (B) Anterior pituitary cells (n=3) from e11 chickens were cultured in the absence or presence of CORT (100 nM) for 20 h following transfection with a firefly luciferase reporter construct containing 1,727 bp of the chicken GH 5'-flanking region (pGL3-1727) or an empty reporter vector (pGL3-Basic) in combination with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic. (C) E11 anterior pituitary cells (n=3) were cultured in the absence or presence of CORT (1 nM) for 6 h. Levels of GH mRNA, analyzed by qRT-PCR and normalized to ACTB mRNA levels, are expressed as fold induction over levels in basal cells. Nascent mRNA was distinguished from mature, spliced mRNA through the use of primers that span the intron/exon junctions on either side of exon 3 of the GH transcript. (D) An agarose gel picture depicting nascent GH mRNA PCR product in each of the 3 basal and CORT treated samples from reactions containing reverse transcriptase (RT+) or reactions without reverse transcriptase (RT-), which served as a control for genomic DNA contamination. (A,B) Values (mean + SEM) without a common letter are statistically different ($P<0.05$). (C) Values (mean + SEM) denoted with an asterisk (*) indicate a significant increase in GH nascent or mature mRNA levels over basal cells ($P<0.05$).

An increase in GH transcription does not preclude the possibility that glucocorticoids may also increase GH mRNA stability. In order to evaluate this, anterior pituitary cells (n=4 replicate trials) were cultured in the absence and presence of CORT for 6 h prior to addition of ActD to inhibit new gene transcription for various time points. As expected, treatment with CORT increased GH mRNA levels (Figure 13A; $P<0.05$) but did not affect levels of ACTB (Figure 13B) or GAPDH (data not shown) mRNA. The half-life ($t_{1/2}$) was determined for each gene as a measure of mRNA stability (Figure 13 and data not shown). For GH mRNA, half-lives under basal and CORT-treated conditions did not differ ($P=0.36$) and were $t_{1/2}=14.9\pm6.2$ h and $t_{1/2}=9.7\pm2.3$ h, respectively, indicating that stabilizing mRNA is not a mechanism by which CORT enhances GH expression. Likewise, half-lives for ACTB mRNA (Basal $t_{1/2}=10.0\pm0.9$, CORT $t_{1/2}=9.5\pm0.4$ h; $P=0.62$) and GAPDH mRNA (Basal $t_{1/2}=29.7\pm6.9$, CORT $t_{1/2}=26.3\pm3.9$ h; $P=0.74$) did not differ. Taken together, these results indicate that glucocorticoids initiate GH expression in differentiating somatotrophs primarily through stimulating transcription of the GH gene rather than enhancing GH mRNA stability.

Glucocorticoid induction of GH requires ongoing protein synthesis and HDAC activity

Stimulation of pituitary GH mRNA by dexamethasone in fetal rat pituitaries requires at least 8 h exposure and is suppressed by the protein synthesis inhibitor puromycin (151). Similarly, CORT induction of GH mRNA during chicken embryonic development, as assessed by an in situ hybridization plate assay, requires continual protein synthesis and long-term exposure (153). In order to confirm these results using an alternative technique and extend them to another rodent model, anterior pituitary cells from e11 chickens and e15-e16 fetal mice were left untreated or treated with CORT in

(A) Growth Hormone



(B) Beta-actin

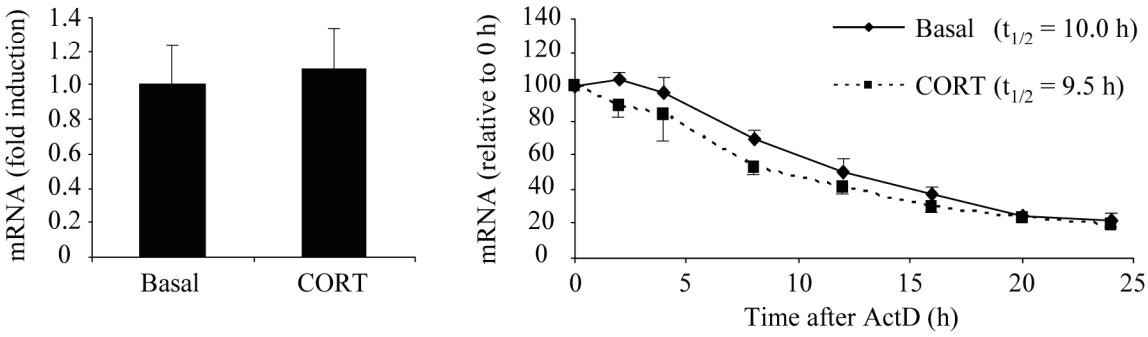


Figure 13. Glucocorticoids do not stimulate pituitary GH expression by increasing GH mRNA stability. E11 chicken anterior pituitary cells (n=4) were left untreated or treated with CORT (1 nM) for 6 h, after which time ActD (5 µg/ml) was added to the culture. (A) GH and (B) ACTB mRNA levels were measured using qRT-PCR after 6 h of CORT treatment (left panel and time 0 h in right panel) and in cells collected at 2, 4, 8, 12, 16, 20, and 24 h after ActD D addition. In the left panel, mRNA levels are expressed as fold induction relative to levels in basal cells receiving no CORT for the first 6 h of culture prior to addition of ActD. In the right panel, levels of mRNA in basal and CORT-treated cells are expressed relative to levels at 0 h after ActD addition for each condition (equivalent to the respective levels depicted in the left panel). Values (mean + SEM) without a common letter are statistically different ($P<0.05$).

the absence or presence of the protein synthesis inhibitor CHX (Figure 14). In mouse cells, no increase in GH mRNA was detected after 1.5 h (Figure 14B) or 6 h (data not shown) CORT treatment ($P>0.05$). Consistent with previous data (151, 153), the dramatic increase in GH mRNA levels observed in both species as a result of CORT treatment (6h, chicken; 24 h, mouse) was suppressed or completely abolished by inclusion of CHX ($P<0.05$, n=3 replicate trials).

One mechanism by which glucocorticoids may initiate pituitary GH expression during embryogenesis is through transcriptional de-repression. This phenomenon often involves an increase in histone acetylation state around transcriptionally active genes through removal of HDAC proteins from their regulatory region, and may also involve a decrease in expression of repressor proteins through recruitment of HDAC enzymes to their regulatory regions (231, 232). We evaluated involvement of HDAC activity in CORT induction of GH expression by treating e11 pituitary cells (n=4 replicate trials) with or without CORT in the absence or presence of two HDAC inhibitors, HC toxin and TSA, for 16 h. HDAC inhibition did not affect basal GH mRNA levels, but induction of GH mRNA and protein was completely abolished in the presence of either inhibitor (Figure 15A and B; $P<0.05$). Recently, it has been shown that hyperacetylated GR has reduced transcriptional activity in certain contexts (19), and that HDAC activity is necessary for proper assembly of the GR chaperone complex and optimal signaling through the receptor (233, 234). To verify that these HDAC inhibitors were not globally decreasing GR transcriptional activity, we analyzed mRNA levels of two additional genes which are known to be directly upregulated by CORT, FKBP5 (235, 236) and GHRH-R2 (Ellestad and Porter, unpublished observation). Both genes were still induced by CORT

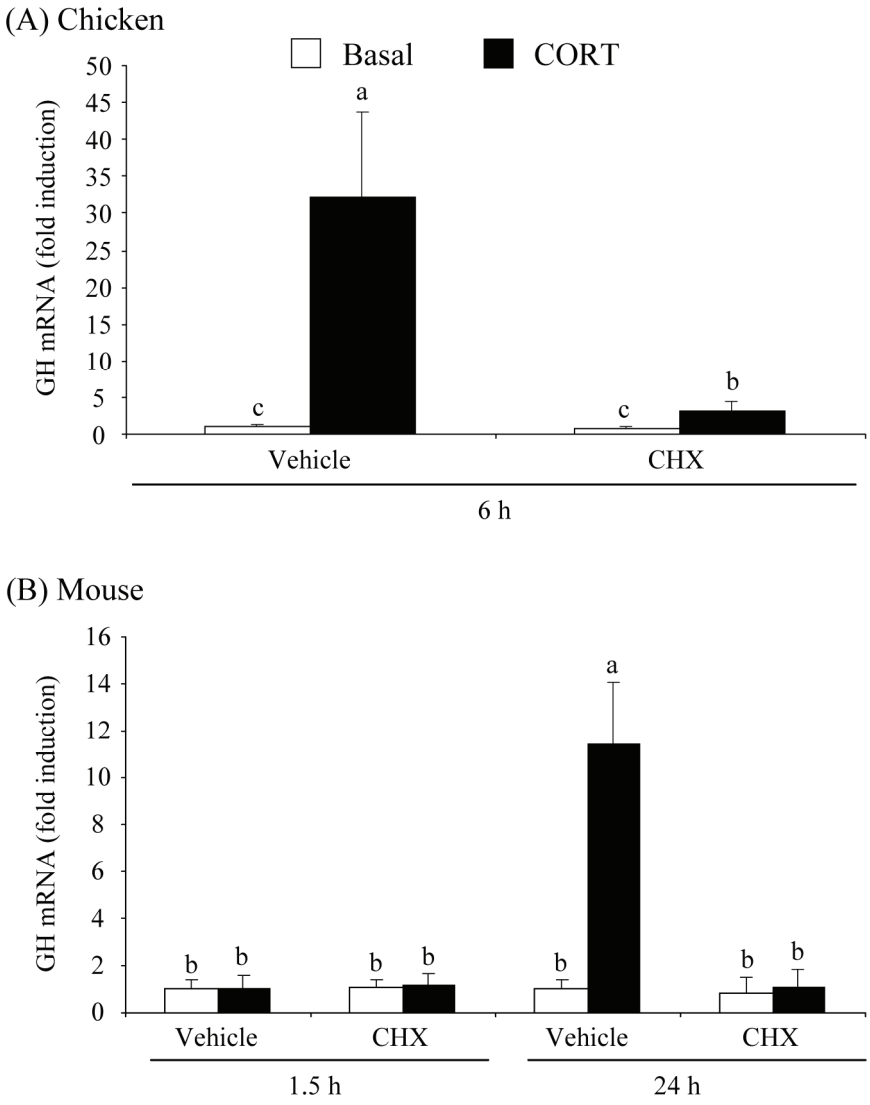


Figure 14. Glucocorticoid induction of pituitary GH mRNA is suppressed by the protein synthesis inhibitor cycloheximide in both (A) embryonic chickens and (B) fetal mice. Anterior pituitary cells (n=3) from (A) e11 chickens or (B) e15-e16 mice were cultured in the absence or presence of CORT (1 nM) with or without CHX (10 μg/ml) for the indicated times. Cells receiving CHX were pretreated for 1.5 h prior to addition of CORT. Levels of GH mRNA were determined using qRT-PCR and normalized to ACTB mRNA levels. GH mRNA levels are expressed as fold induction over basal cells receiving no CHX for that time point. Values (mean + SEM) without a common letter are statistically different ($P<0.05$).

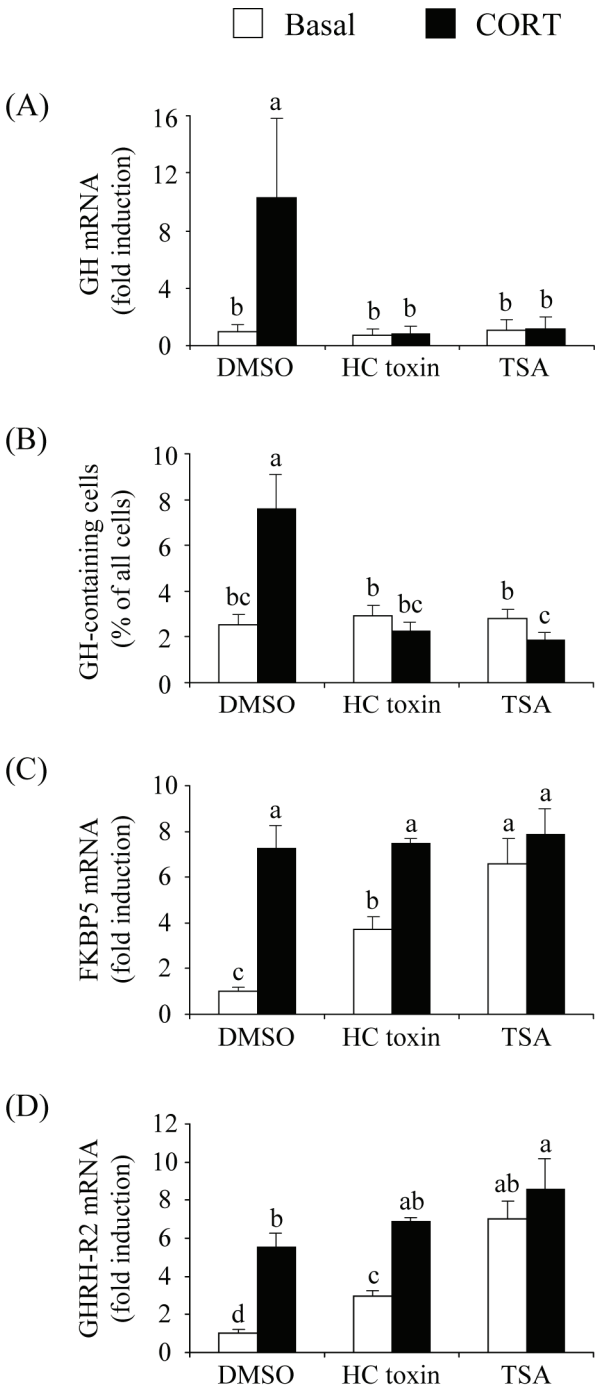


Figure 15. Glucocorticoid upregulation of GH expression during chicken embryonic development is blocked by the histone deacetylase inhibitors HC toxin and TSA. E11 anterior pituitary cells (n=4) were pretreated for 1 h with DMSO, HC toxin (100 nM), or TSA (200 nM) prior to addition of no treatment or CORT (1 nM) for 16 h. (A-C) Levels of GH, FKBP5, and GHRH-R2 mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells receiving DMSO. Levels of mRNA for each gene were normalized to ACTB mRNA levels. (D) The number of GH-positive cells was determined with ICC in fixed cells. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

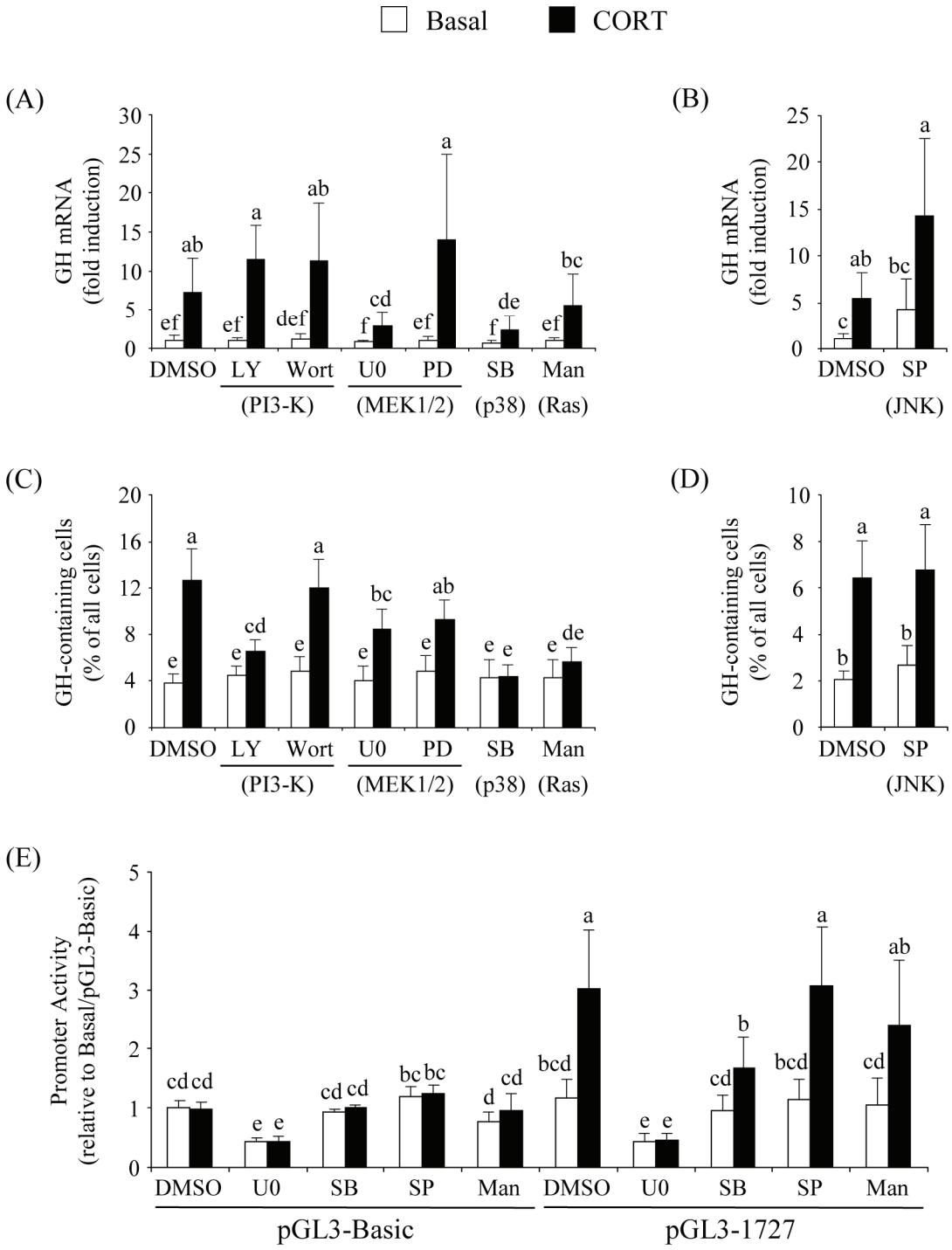
in the presence of HC toxin, although basal levels of each were increased two- to three-fold by HC toxin (Figure 15C and D; $P<0.05$). Treatment of the cells with TSA alone led to a further increase in FKBP5 and GHRH-R2 expression, such that mRNA levels in basal and CORT-treated cells were not different in the presence of this HDAC inhibitor (Figure 15C and D; $P>0.05$). Nonetheless, it appears that CORT induction of GH expression does require HDAC activity, and our findings with HC toxin indicate that this effect is not due to an overall repression of GR signaling.

Ras and ERK1/2 signaling are necessary for glucocorticoid initiation of GH expression

Previous results from our laboratory implicate involvement of ras or a ras-like protein in the mechanism by which CORT induces GH mRNA during somatotroph differentiation (153, 217). Pharmacological inhibitors of several known ras-induced signaling cascades were used to evaluate which, if any, of these pathways are involved in initiating GH expression in somatotrophs. Anterior pituitary cells (n=4 replicate trials) from e11 chickens were cultured for 12 h in the presence or absence of CORT and DMSO (vehicle) or inhibitors of ras (manumycin A), PI3-K (LY294002 and wortmannin), MEK1/2 (U0126 and PD98059), p38MAPK (SB203580), and JNK (SP600125). As expected from previous results (129, 143, 144, 152, 153, 155-157, 159, 217), CORT treatment increased GH mRNA levels (Figure 16A and B; $P<0.05$) and the abundance of GH protein-containing cells (Figure 16C and D; $P<0.05$). Inhibition of ras using manumycin A abolished the increase in percentage of GH-containing cells resulting from stimulation with CORT (Figure 16C; $P<0.0.5$), and it slightly reduced the increase in GH mRNA levels, although this effect was not significant (Figure 16A; $P>0.05$). Despite the fact that the two PI3-K inhibitors yielded differing results in their effect on

intracellular GH protein levels [LY294002 significantly suppressed CORT induction of somatotrophs (Figure 16C; $P<0.05$) while wortmannin had no effect], neither influenced induction of GH mRNA by CORT (Figure 16A; $P>0.05$). Likewise, the two MEK1/2 inhibitors had differential effects, particularly in their ability to interfere with CORT stimulation of GH mRNA. Levels of GH mRNA in the presence of U0126 were lower than those in the presence of CORT alone (Figure 16A; $P<0.05$), but inclusion of PD98059 did not suppress stimulation of GH mRNA. Both MEK1/2 inhibitors also partially repressed the increase in percentage of GH-containing cells that resulted from CORT treatment (Figure 16C; $P<0.05$). Of the compounds used, the p38MAPK inhibitor, SB203580, was most effective at suppressing induction of both GH mRNA and protein by CORT (Figure 16A and C; $P<0.05$). Inhibition of JNK with SP600125 did not influence CORT regulation of GH expression (Figure 16B and D; $P>0.05$), although GH mRNA in both basal and CORT-treated cells had a tendency to be higher in the presence of the inhibitor.

Figure 16. Pharmacological inhibitors of ERK1/2, p38MAPK, and ras signaling suppress glucocorticoid induction of GH expression in chicken embryonic anterior pituitary cells. E11 pituitary cells (n=4) were pretreated for 1 h with DMSO or inhibitors for PI3-K [LY294002 (LY; 50 μM) and wortmannin (WORT; 100 nM)], MEK1/2 [U0126 (U0; 10 μM) and PD98059 (PD; 50 μM)], p38MAPK [SB203580 (SB; 40 μM)], Ras [manumycin A (Man; 1 μM)], or JNK [SP600125 (SP; 10 μM)] prior to addition of no treatment or CORT. (A-D) Cells were treated with CORT (1 nM) for 12 h. (A,B) Levels of GH mRNA, analyzed using qRT-PCR and normalized to ACTB mRNA levels, are expressed as fold induction over basal cells receiving DMSO. (C,D) Cells were fixed and the number of GH-positive cells was determined using ICC. (E) Cells were pretreated and treated with CORT (100 nM) for 20 h following transfection with a firefly luciferase reporter construct containing 1,727 bp of the chicken GH 5'-flanking region (pGL3-1727) or an empty reporter vector (pGL3-Basic) in combination with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells cultured in the presence of DMSO and transfected with pGL3-Basic. Values (mean + SEM) without a common letter are statistically different ($P<0.05$).


Basal
CORT
(A)
GH mRNA (fold induction)
DMSO
LY
Wort
U0
PD
SB
Man
(PI3-K)
(MEK1/2)
(p38)
(Ras)
(B)
GH mRNA (fold induction)
DMSO
SP
(JNK)
(C)
GH-containing cells (% of all cells)
DMSO
LY
Wort
U0
PD
SB
Man
(PI3-K)
(MEK1/2)
(p38)
(Ras)
(D)
GH-containing cells (% of all cells)
DMSO
SP
(JNK)
(E)
Promoter Activity (relative to Basal/pGL3-Basic)
DMSO
U0
SB
SP
Man
DMSO
U0
SB
SP
Man
pGL3-Basic
pGL3-1727

Treatment with U0126 and SB203580 had the most consistent and significant effects on glucocorticoid induction of GH expression, making ERK1/2 and p38MAPK the most likely candidates to be downstream effectors of ras signaling in this process. Therefore, we determined whether inhibiting these pathways had any effect on CORT stimulation of the pGL3-1727 reporter construct. In addition, we included SP600125 because of the slight increase in GH mRNA observed when JNK was inhibited. Pituitary cells (n=4 replicate trials) were transfected with the empty reporter (pGL3-Basic) or pGL3-1727 and treated with CORT for 20 h in the presence of DMSO, U0126, SB203580, SP600125, or manumycin A (Figure 16E). It should be noted that the decrease in normalized luciferase activity observed in U0126-treated cells was primarily due to an increase in renilla luciferase activity (internal transfection control) and not a decrease in firefly luciferase activity (data not shown). Treatment with CORT induced a 3-fold increase in GH promoter activity ($P<0.05$), and this induction was suppressed by U0126 and SB203580 ($P<0.05$). Inhibiting JNK with SP600125 did not decrease CORT-stimulated GH promoter activity, and surprisingly, neither did manumycin A ($P>0.05$).

In the current experiments, manumycin A completely suppressed the increase in numbers of GH-positive cells resulting from treatment with CORT (Figure 16C) but was only slightly effective at reducing CORT-stimulated GH promoter activity and mRNA expression. One possibility is that manumycin A initially blocked the increase in GH mRNA resulting from CORT treatment, which led to a decrease in GH protein accumulation, but lost its effect the longer the cells were in culture. In this case, GH mRNA may have begun to increase again before a detectable increase in GH protein occurred. To address this and further confirm ERK1/2 and p38MAPK involvement, a

time course experiment was conducted in which e11 pituitary cells (n=4 replicate experiments) were treated with and without CORT in the presence of U0126, SB203580, and manumycin A for the final 3, 6, or 12 h of a 24 h culture (Figure 17). Since none of the inhibitors affect GH expression under basal conditions (Figure 16A and B), cells that were treated with DMSO alone for the final 13 h of culture served as a control. Treatment with CORT stimulated GH mRNA at all time points (Figure 17A), and inclusion of manumycin A completely blocked induction at 3 h and substantially repressed the response at 6 and 12 h ($P<0.05$). Neither U0126 nor SB203580 had any effect on CORT induction of GH mRNA at 3 h ($P>0.05$), but both were equally effective at attenuating the response at 6 h and further reducing CORT induction after 12 h ($P<0.05$). In order to verify that inhibiting these signaling pathways in embryonic anterior pituitary cells was not globally affecting their ability to respond to CORT, mRNA levels for FKBP5 and GHRH-R2 were also measured in this experiment (Figure 17B and C). Both genes were substantially upregulated at all time points ($P<0.05$) and, though there were effects of the inhibitors on their induction at the earlier time points [e.g. U0126 and manumycin A suppressed induction of FKBP5, and all inhibitors suppressed induction of GHRH-R2 and at 3 h ($P<0.05$)], none of the inhibitors suppressed induction of either gene at 12 h ($P>0.05$). Therefore, it appears that these compounds are not perturbing the general response to CORT, and the effect is somewhat specific to regulation of GH expression. Representative pictures of ICC results from e11 cells treated with DMSO (vehicle), DMSO plus CORT, U0126 plus CORT, SB203580 plus CORT, and manumycin A plus CORT are shown in Figure 17D and clearly demonstrate the reduction in intracellular GH protein accumulation resulting from inhibitor treatment.

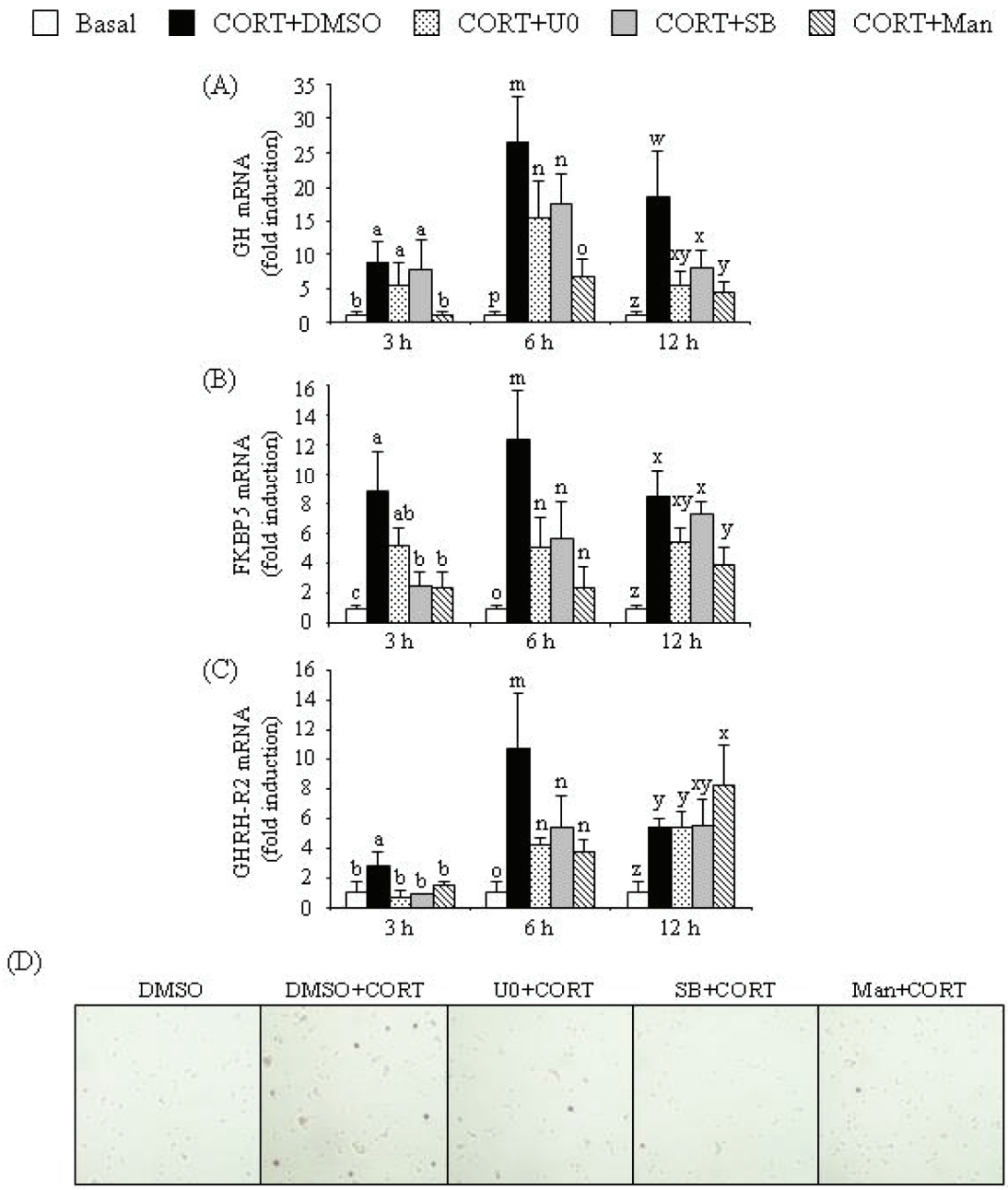


Figure 17. Glucocorticoid stimulation of GH mRNA and protein expression during chicken embryonic development is decreased in the presence of ERK1/2, p38MAPK, and ras signaling inhibitors. E11 pituitary cells (n=4) were pretreated for 1 h with DMSO or inhibitors for MEK1/2 [U0126 (U0; 10 μM)], p38MAPK [SB203580 (SB; 40 μM)] and Ras [manumycin A (Man; 1 μM)] prior to addition of no treatment or CORT (1 nM) for (A-C) 3 h, 6 h, 12 h, or (D) 24 h. (A-C) Levels of GH, FKBP5, and GHRH-R2 mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells receiving DMSO. Levels of mRNA for each gene were normalized to ACTB mRNA levels. (D) Cells were fixed and the number of GH-positive cells was determined using ICC. Cells with detectable intracellular GH protein are purple. Values (mean + SEM) denoted with different letters within each time point are significantly different from one another ($P<0.05$).

An experiment was conducted to confirm efficacy of the two MAPK pathway inhibitors that consistently reduced CORT induction of GH expression in embryonic anterior pituitary cells. The ERK1/2 pathway inhibitor, U0126, inhibits MEK1/2 activity and, therefore, prevents phosphorylation and subsequent activation of ERK1/2 (Figure 4). We verified that U0126 was reducing ERK1/2 pathway signaling in our cell culture system on two levels, by determining the amount of p-ERK1/2 in the presence and absence of the inhibitor using an ELISA and by assessing ERK1/2 kinase activity under the same conditions. Anterior pituitary cells from e11 chickens (n=2 replicate trials) were left untreated or treated for 3 h with CORT or EGF in the presence of DMSO or U0126. ERK1/2 kinase activity in the cells was assessed by incubating immunoprecipitated active, p-ERK1/2 with Elk-1 substrate *in vitro* and determining the level of p-Elk1 by immunoblotting. The inhibitor clearly reduced ERK1/2 kinase activity under basal, CORT-treated, or EGF-stimulated conditions (Figure 18A). As expected, levels of p-ERK1/2, as determined by an ELISA, were substantially reduced by U0126 under all conditions as well (data not shown). A similar experiment was conducted to determine efficacy of the p38MAPK pathway inhibitor, although in this case the only way to verify this was to measure p38MAPK activity itself, since SB203580 inhibits p38MAPK activity but does not affect phosphorylation status of the enzyme (Figure 4). Cells (n=2 replicate trials) were cultured under basal, CORT-treated, or anisomycin-stimulated conditions for 3 h in the presence of DMSO or SB203580. Kinase activity for p38MAPK was determined in a similar manner to ERK1/2, except the amount of p-ATF2 was assessed by western blotting after incubating the p-p38MAPK immunoprecipitate with ATF2. Surprisingly, this inhibitor was completely ineffective at blocking p38MAPK

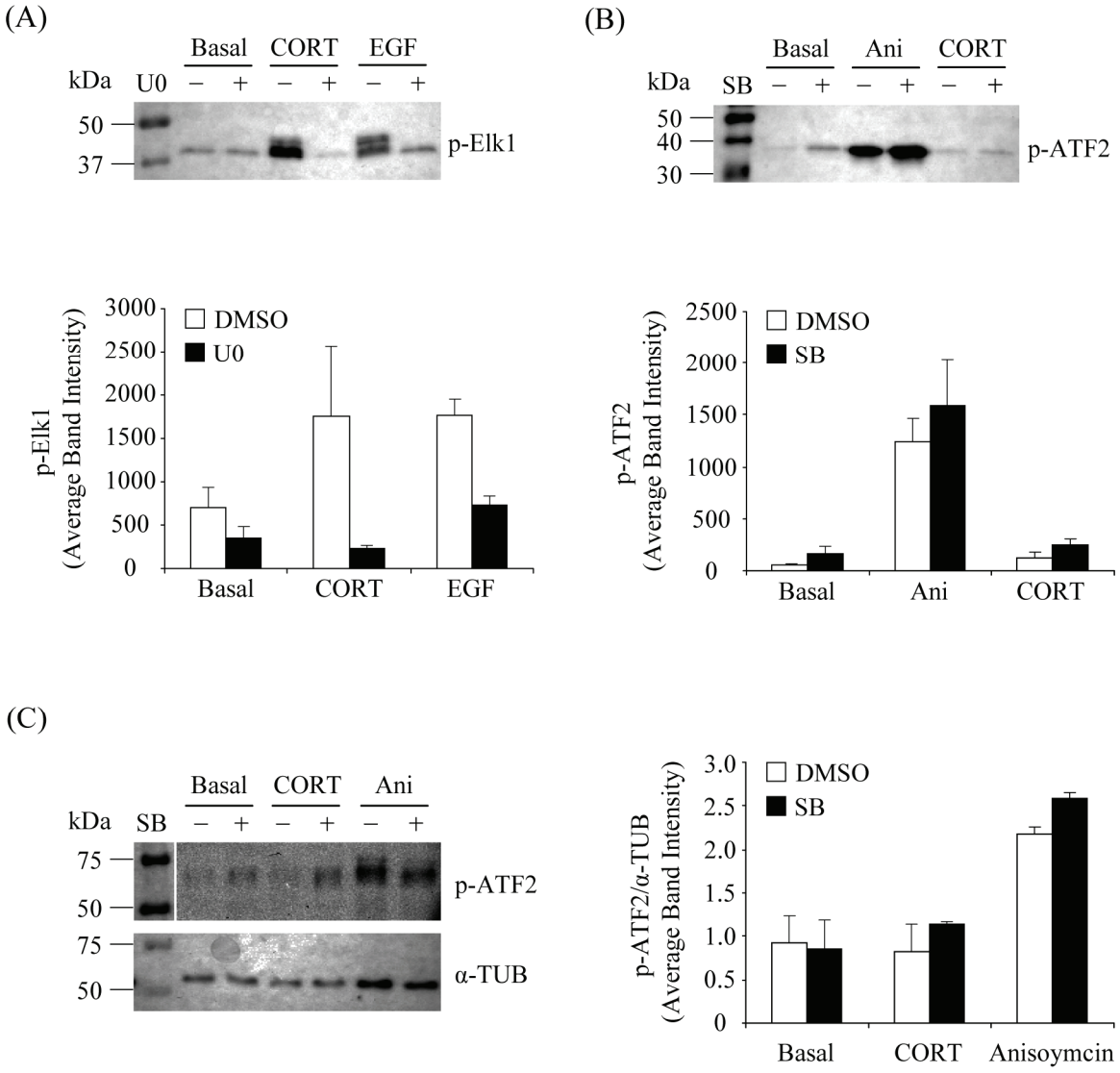


Figure 18. The pharmacological inhibitor of MEK1/2, U0126, is effective at reducing ERK1/2 MAPK pathway activity in chicken embryonic anterior pituitary cells, but SB203580 does not block p38MAPK activity. E11 pituitary cells (n=2) were pretreated for 1 h with DMSO or inhibitors for (A) MEK1/2 [U0126 (U0; 10 μM)] or (B,C) p38MAPK [SB203580 (SB; 40 μM)] prior to addition of no treatment, CORT (1 nM), (A) EGF (100 ng/ml), or (B,C) anisomycin (Ani; 10 μM) for 3 h. (A) ERK1/2 and (B) p38MAPK kinase activity were measured with non-radioactive MAPK assays in which *in vitro* phosphorylated substrate was detected by Western blotting. In each panel, the image is a representative blot depicting levels of phosphorylated (A) Elk-1 (p-Elk1) or (B) ATF2 (p-ATF2), and the graph represents the quantification (mean + SD) of 2 independent trials. (C) Levels of endogenous p-ATF2 and α-tubulin (α-TUB) were determined by Western blotting. The image on the left is a representative blot. The graph on the right depicts quantification (mean + SD) of 2 independent replicate trials in which levels of p-ATF2 were normalized to α-TUB.

activity and even appeared to enhance it in one replicate trial (Figure 18B). In order to ensure that the lack of an effect was not due to an artifact of the *in vitro* assay, endogenous levels of p-ATF2 were determined in the cells by western blotting (Figure 18C). Again, the inhibitor was completely ineffective at reducing basal or stimulated levels of p-ATF2 and appeared to increase it in one replicate trial. Thus, it appears that the inhibitory effect of U0126 on CORT-stimulated GH expression is indeed due to inhibition of ERK1/2 signaling, but the suppressive effect of SB203580 is not a result of p38MAPK inhibition in these cells and may implicate involvement of another kinase. It should be noted that SB203580 had no effect on ERK1/2 pathway activity in these experiments (data no shown), indicating that the suppressive effects of SB203580 are not due to inhibition of ERK1/2 signaling. Taken together, our results indicate that ras- and ERK1/2-mediated transcriptional events are involved in glucocorticoid initiation of pituitary GH during development.

Concurrent MAPK activation during glucocorticoid treatment suppresses GH induction

In an attempt to delineate the role of ERK1/2 signaling in this process, as well as evaluate involvement of p38MAPK signaling with an alternative approach, we obtained dominant negative and constitutively active elements of these pathways to further assess their involvement in CORT stimulation of GH expression during development. In addition, this approach should alleviate concerns regarding specificity and stability of pharmacological inhibitors. It was hoped that results observed using pharmacological inhibitors would be complemented by this genetic approach. We hypothesized that dominant negative mutants would interfere with GH induction, while constitutively active mutants would augment induction or recapitulate it all together. Although these are

mutant forms of mammalian kinases, the proteins are highly conserved across species. Chicken MEK1 and 2, ERK2, MKK3, MKK6, and p38MAPK are 88-99% identical to mammalian counterparts, as determined by Basic Local Alignment Search Tool (BLAST) alignments. Further, mammalian mutant enzymes have been successfully utilized in chickens (230, 237, 238).

First, it was confirmed that each mutant was successfully expressed at the protein level in chicken cells. Western blot analysis was performed on total cellular protein extracted from chicken LMH cells left untransfected or transfected with Sport6.1 (empty expression vector), caMKK3 and caMKK6, caMEK1, dnMKK3 and dnMKK6, dnp38α and dnp38β, dnMEK1, or dnERK1 and dnERK2. Each mutant was readily detected in appropriate lysates at approximately the same level (Figure 19), indicating that these proteins are efficiently expressed in chicken cells. Next, functionality of each mutant was determined in both chicken and mammalian cell lines by assessing levels of phosphorylated MAPK and enzyme activity for each pathway in LMH and HEK-293 cells transfected as described above and left untreated (Basal) or stimulated with EGF or anisomycin to assess ERK1/2 and p38MAPK mutants, respectively. Functionality of all mutants was tested using the *in vitro* kinase assay for the appropriate pathway, and functionality dnMEK1, caMEK1, dnMKK3, dnMKK6, caMKK3, and caMKK6 were further evaluated using an ELISA to measure intracellular p-ERK1/2 or p-p38MAPK levels. As expected, treatment with EGF and anisomycin stimulated an increase in detectable p-ERK1/2 and p-p38MAPK (Figure 20A and B; $P<0.05$, n=4 replicate trials), respectively, as well as an increase in ERK1/2 and p38MAPK kinase activity (Figure 20C and D), respectively, in both cell lines. Unexpectedly, however, dominant negative

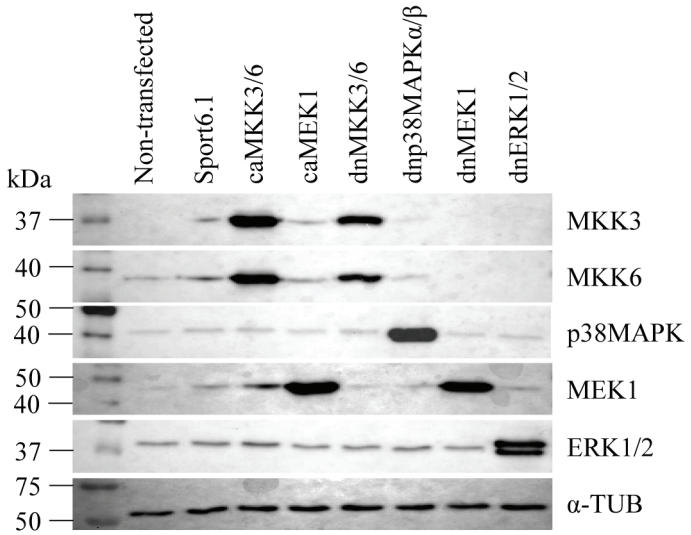


Figure 19. Constitutively active and dominant negative mutants of mammalian ERK1/2 and p38MAPK pathways are efficiently expressed in chicken cells. LMH cells, a chicken hepatoma cell line, were left untransfected, transfected with an empty expression vector (Sport6.1) or expression vectors for constitutively active MKK3/6 (caMKK3/6), constitutively active MEK1 (caMEK1), dominant negative MKK3/6 (dnMKK3/6), dominant negative p38MAPKα/β (dnp38MAPKα/β), dominant negative MEK1 (dnMEK1), or dominant negative ERK1/2 (dnERK1/2). Total cellular protein was extracted and analyzed by Western blotting using antibodies for MKK3, MKK6, p38MAPK, MEK1, ERK1/2, or α-tubulin (α-TUB) to verify that proteins were successfully expressed at comparable levels for each mutant.

mutants of each pathway did not prevent the increase in either phosphorylated MAPK status (Figure 20A and B; $P>0.05$) or MAPK activity. This is clearly not an issue of using mammalian proteins in chicken cells, as the mutants were not functional in HEK-293 cells either, and is not related to expression level. At this point, it is unclear why the dominant negative enzymes did not function in our transient transfection system. On the other hand, in both cell types, caMEK1 expression was able to elevate p-ERK1/2 to levels that were not different from those in EGF-stimulated cells (Figure 20A; $P<0.05$), and expression of caMKK3 and caMKK6 resulted in levels of p-p38MAPK that were equal to (HEK-293) or greater than (LMH) those in anisomycin-treated cells (Figure 20B;

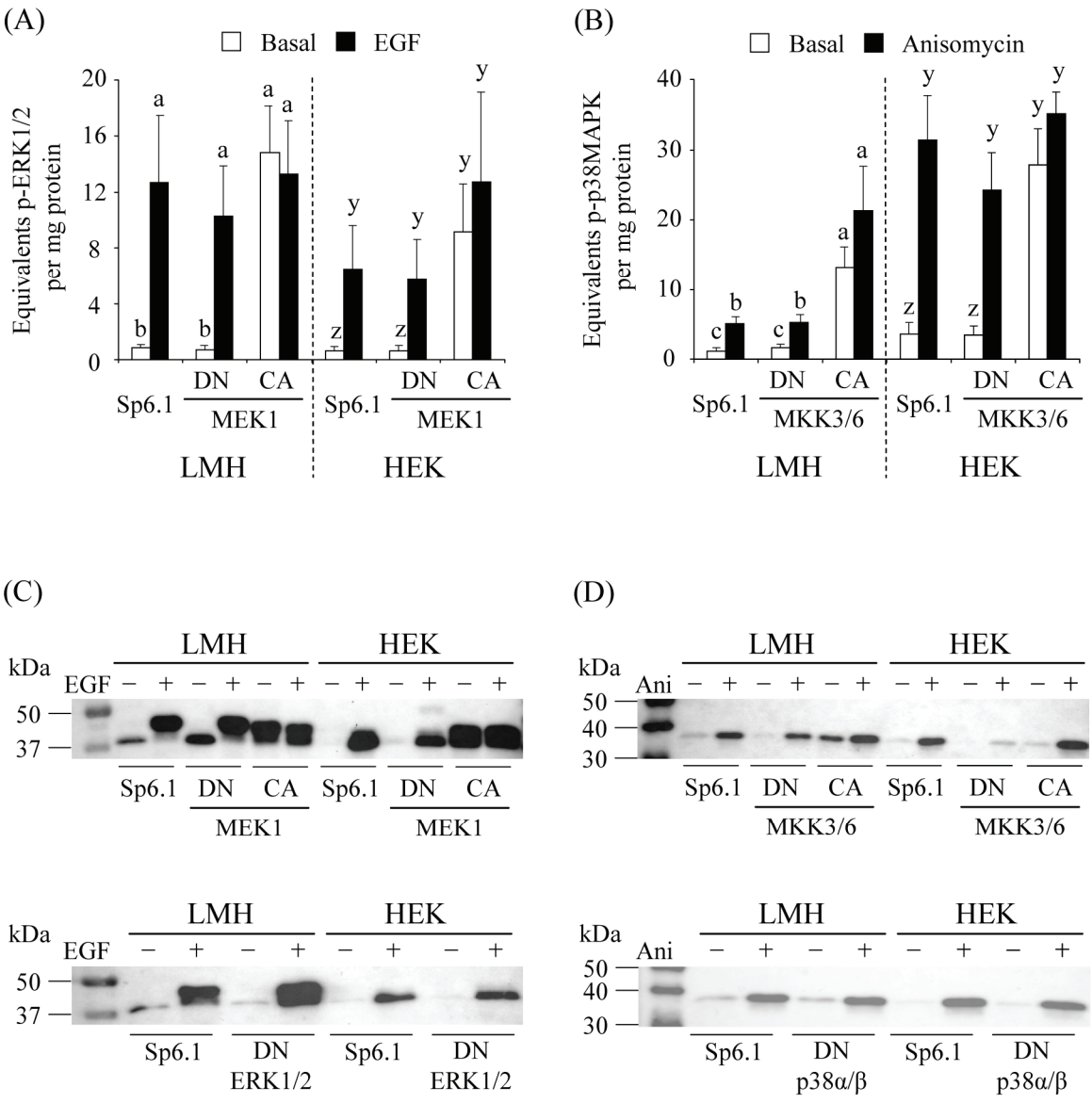


Figure 20. Constitutively active (CA) mutants of (A,C) ERK1/2 and (B,D) p38MAPK pathways are highly effective at increasing MAPK signaling, but dominant negative (DN) mutants of each pathway are non-functional in both chicken and human cells. Total cellular protein was extracted from chicken LMH cells and human HEK-293 (HEK) cells transfected with the empty expression vector Sport6.1 (Sp6.1) or expression vectors for (A,C) DN MEK1, CA MEK1, and DN ERK1/2 or (B,D) DN MKK3/6, CA MKK3/6, and DN p38MAPKα/β (p38α/β). Cells were left untreated (-) or stimulated (+) with (A,C) EGF (100 ng/ml) or (B,D) anisomycin (100 nM) for 30 m. (A,B) Levels of phosphorylated (A) ERK1/2 and (B) p38MAPK were determined using a sandwich ELISA. One equivalent is equal to the amount of phosphorylated ERK1/2 or p38MAPK in 1 µg total cellular protein extracted from LMH cells stimulated with EGF or anisomycin, respectively. Values (mean + SEM) denoted with different letters for each cell line are significantly different from one another ($P<0.05$). (C) ERK1/2 and (D) p38MAPK kinase activity were measured using non-radioactive MAPK assays in which *in vitro* phosphorylated (C) Elk1 or (D) ATF2, substrates for ERK1/2 and p38MAPK, respectively, were detected by Western blotting.

P<0.05). Similar results were obtained when transfected cell lysates were analyzed with the kinase assays (Figure 20C and D), confirming functionality of constitutively active pathway mutants. As a result, only these were used in subsequent experiments.

A series of experiments was conducted in order to determine the consequence of ERK1/2 and p38MAPK activation on GH expression in the embryonic anterior pituitary gland. First, effects of overexpressing constitutively active mutants within each pathway on CORT regulation of GH promoter activity and mRNA expression were determined. Anterior pituitary cells (n=3 replicate trials) from e11 chickens were transfected with pGL3-Basic or the GH promoter-driven pGL3-1727 reporter construct in combination with Sport6.1, caMEK1, or caMKK3 and caMKK6. Surprisingly, although overexpression of constitutively active mutants in both pathways had no effect on promoter activity in basal cells, they completely blocked the CORT-stimulated increase in GH promoter activity (Figure 21A and C; P<0.05). Cells were also transfected with the constitutively active mutants or Sport6.1 along with a Golgi-targeted GFP expression vector, used for sorting of successfully transfected (GFP-positive) cells, and GH mRNA levels were determined in cells cultured under basal and CORT-treated conditions for 20 h. The presence of high levels of caMEK1, caMKK3, and caMKK6 in appropriately transfected cells was confirmed by qRT-PCR (data not shown). Consistent with the effect of constitutively active mutant overexpression on CORT induction of GH promoter activity, levels of GH mRNA in CORT treated cells transfected with either caMEK1 or caMKK3 and caMKK6 were approximately 3-fold lower than those in CORT-treated cells transfected with Sport6.1 (Figure 21B and D; P<0.05, n=3 replicate trials), although there was still an induction in the presence of the mutants (P<0.05).

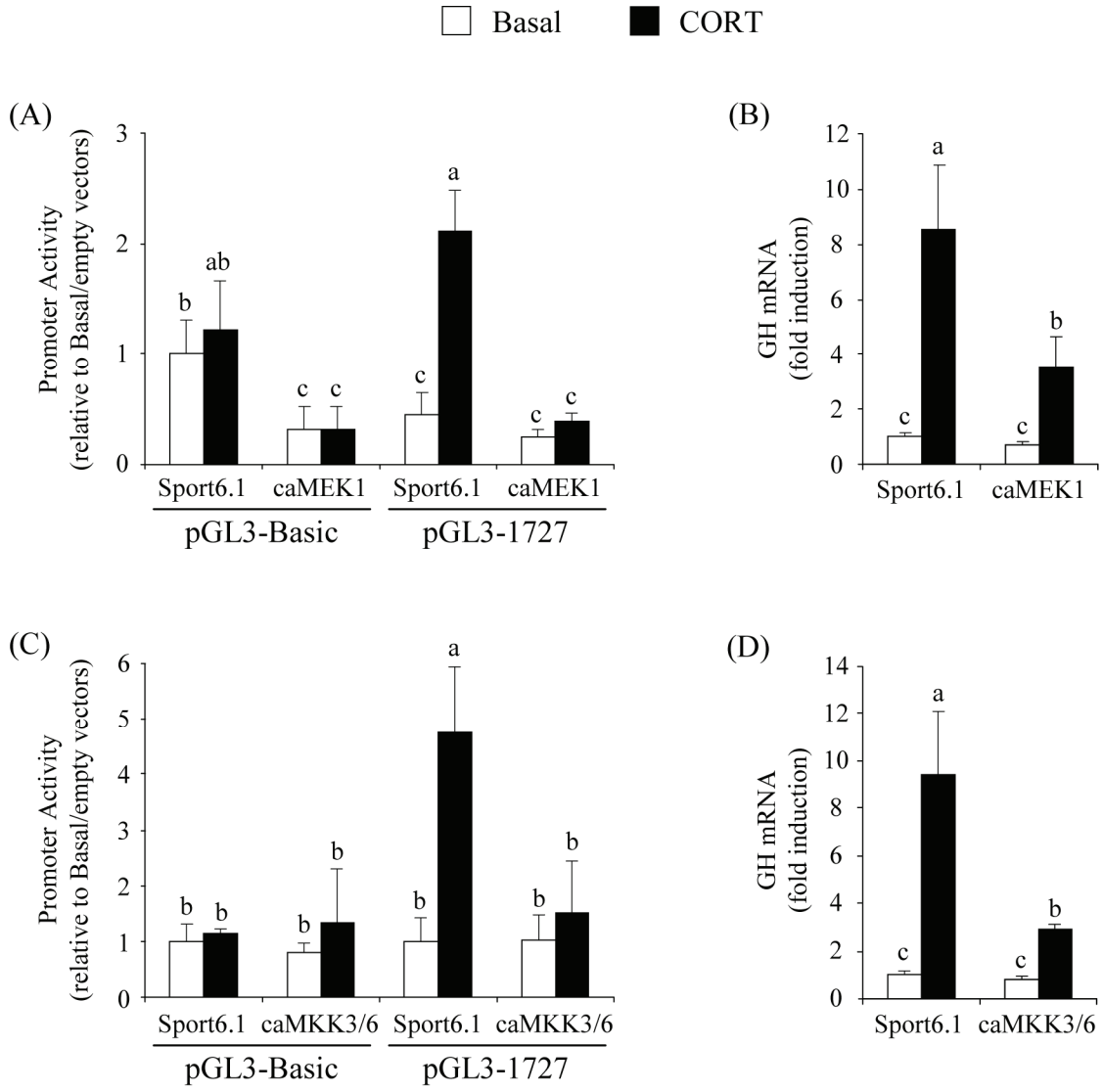


Figure 21. Glucocorticoid induction of GH expression in the chicken embryonic pituitary gland is suppressed by overexpression of constitutively active mutants in the ERK1/2 and p38MAPK signaling pathways. Anterior pituitary cells (n=3) from e11 chickens were transfected with (A,C) a firefly luciferase reporter construct containing 1,727 bp of the chicken GH 5'-flanking region (pGL3-1727) or an empty reporter vector (pGL3-Basic) in combination with a renilla luciferase expression construct and expression vectors for constitutively active MEK1 (caMEK1), constitutively active MKK3/6 (caMKK3/6), or an empty expression vector (Sport6.1) or (B,D) Sport6.1, caMEK1, or caMKK3/6 expression vectors alone. (A,C) Following addition of no treatment or CORT (1 nM) for 20 h, promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic and Sport6.1. (B,D) Cells were left untreated or treated with CORT (1 nM) for 20 h, and levels of GH mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells transfected with Sport6.1. Levels of GH mRNA were normalized to ACTB mRNA levels. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

We next took an alternative approach to confirm the observed inhibitory effect of ERK1/2 and p38MAPK pathway activation on CORT stimulation of GH expression. We examined if co-treatment of the cells with CORT and EGF or anisomycin would influence the GH response to glucocorticoids, as well as the effect of "priming" each pathway by exposing the cells to EGF or anisomycin and washing it out prior to CORT treatment. In one experiment, e11 anterior pituitary cells (n=3 replicate trials) were left untreated or stimulated with EGF for 30 m. Subsequently, EGF was washed out and cells were cultured for an additional 6 h in the presence and absence of CORT. Additional cells were treated for 6 h with EGF alone or EGF and CORT. Pretreatment with EGF for 30 m did not have any effect on CORT stimulation of GH mRNA ($P>0.05$); however, co-stimulation of the cells with CORT and EGF reduced induction of GH by about 50% (Figure 22A; $P<0.05$). We also measured the effect of EGF treatments on FKBP5 and GHRH-R2 mRNA levels, to determine specificity of the inhibition. CORT stimulation of FKBP5 and GHRH-R2 mRNA in these cells was 12- and 4-fold, respectively (Figure 22B and C; $P<0.05$), and these responses were not influenced by EGF pre- or co-treatment. There was a tendency for the level of GHRH-R2 mRNA in basal and CORT-treated cells that were exposed to EGF for both short and long-term treatments to be higher than that in cells receiving no EGF, although only in the case of cells treated with EGF alone for 6 h was the difference significant (Figure 22C; $P<0.05$). An increase in the level of p-ERK1/2 was maintained after 6 h exposure of the cells to EGF (Figure 22D; $P<0.05$), indicating that the inhibitory effect of chronic stimulation does not appear to be due to a downregulation of ERK1/2 activity resulting from negative feedback.

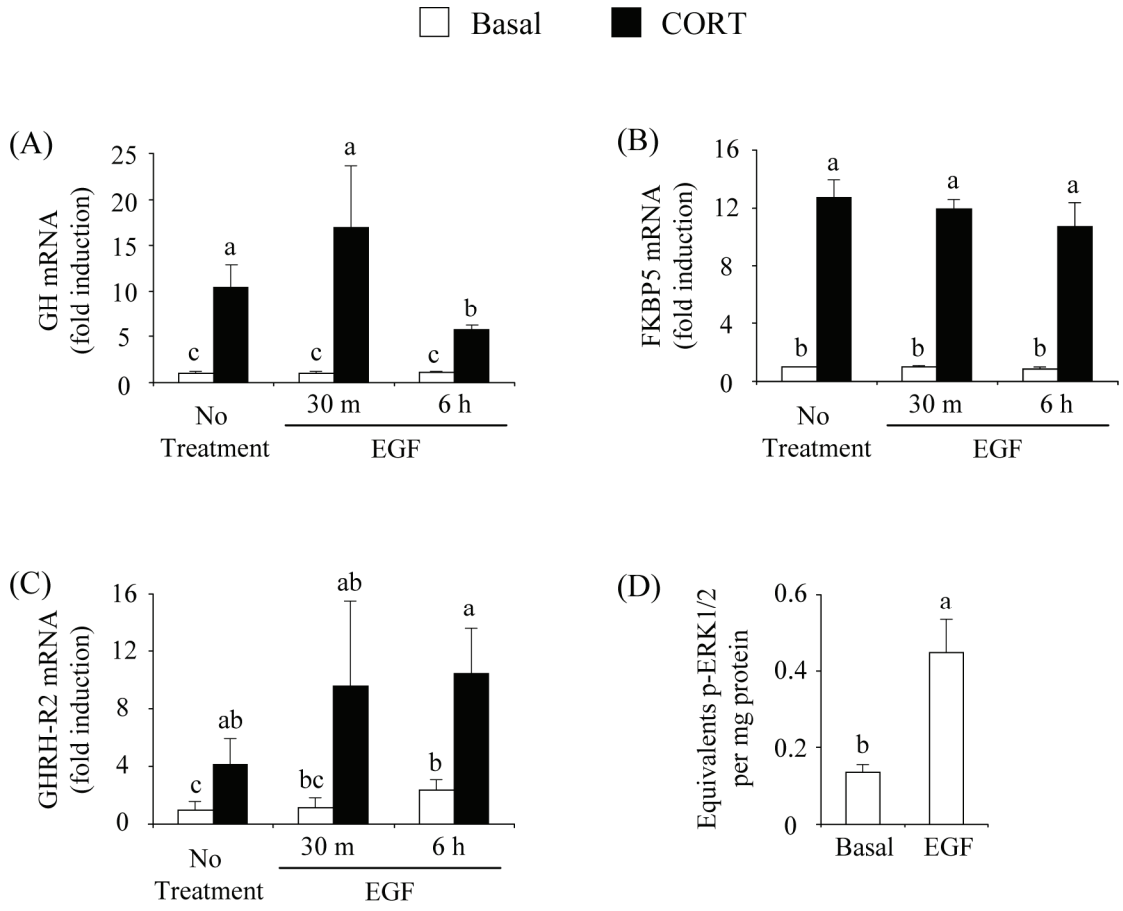


Figure 22. Co-stimulation of ERK1/2 signaling with EGF during glucocorticoid treatment reduces induction of GH mRNA in the chicken embryonic anterior pituitary gland. Pituitary cells (n=3) from e11 chickens were left untreated or treated with EGF (100 ng/ml) for 30 m, after which time EGF was washed out and cells were cultured in the absence or presence of CORT (1 nM) for 6 h. Additional cells were left untreated or treated with CORT in the absence and presence of EGF for 6 h. (A-C) Levels of GH, FKBP5, and GHRH-R2 mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells receiving no EGF. Levels of mRNA for each gene were normalized to ACTB mRNA levels. (D) Levels of phosphorylated ERK1/2 were determined using a sandwich ELISA in cells stimulated for 6 h with EGF alone. One equivalent is equal to the amount of phosphorylated ERK1/2 in 1 µg total cellular protein extracted from LMH cells stimulated with EGF. Values (mean + SEM) without a common letter are statistically different ($P<0.05$).

Anisomycin is a translational inhibitor similar to CHX at higher concentrations (e.g. 10 μM), but at lower concentrations (e.g. 100 nM) can stimulate p38MAPK activity without inhibiting protein synthesis (239). Therefore, we first conducted a trial to determine the least effective dose of anisomycin that would suppress CORT stimulation of GH mRNA, allowing us to distinguish any inhibitory effects of p38MAPK activation from those due to inhibition of protein synthesis, which is known to be necessary for this process (151, 153). Cells (n=3 replicate trials) were left untreated or treated for 6 h with CORT in the absence and presence of anisomycin at 3 concentrations: 10 μM, 100 nM, and 20 nM. The highest dose most strongly increased the amount of p-p38MAPK in the cells and substantially decreased both basal and CORT-stimulated GH mRNA levels (Figure 23A and B; $P<0.05$). Co-treatment with 100 nM anisomycin suppressed CORT stimulation of GH mRNA expression, and this dose increased levels of p-p38MAPK above those in basal cells, but less than those in cells stimulated with 10 μM anisomycin (Figure 23A and B; $P<0.05$). The lowest dose had no effect on GH mRNA levels and did not stimulate a detectable increase in p-p38MAPK (Figure 23A and B; $P>0.05$). Based on these results, we conducted an experiment identical to the one described above for ERK1/2 pathway activation, except we used 100 nM anisomycin as the stimulus. Pretreatment and subsequent removal of anisomycin had no influence on basal or CORT-treated mRNA levels, but concurrent treatment with CORT and anisomycin for 6 h suppressed induction of GH mRNA (Figure 23C; $P<0.05$, n=3 replicate trials). An increase in p-p38MAPK levels in these cells following exposure to 100 nM anisomycin for 6 h was verified (Figure 23F; $P<0.05$). Again, the inhibitory effect of anisomycin treatment on CORT-induced gene expression is not a global one, as FKBP5 and GHRH-

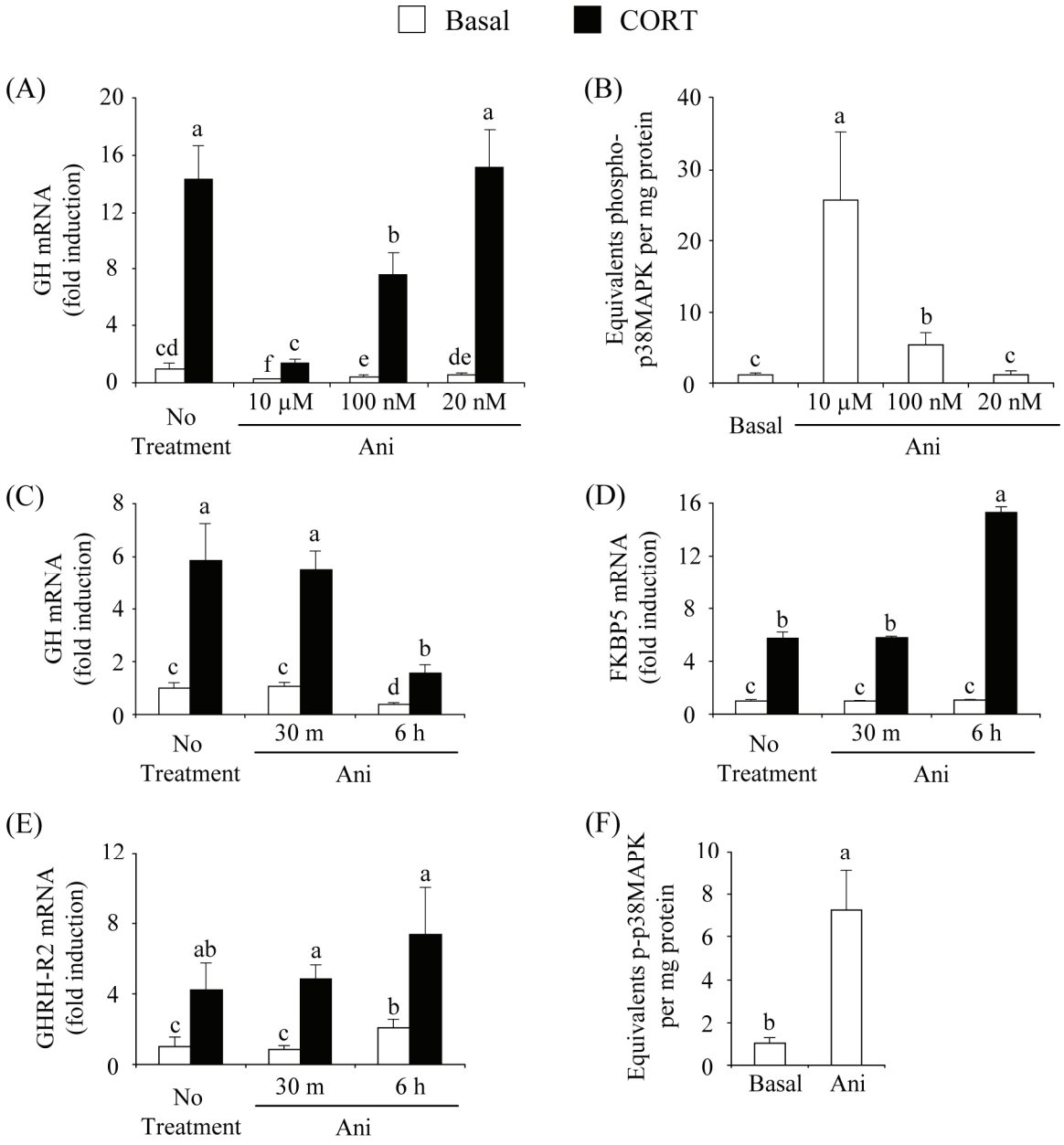


Figure 23. Concurrent activation of p38MAPK signaling with anisomycin during glucocorticoid treatment suppresses stimulation of GH mRNA in the chicken anterior pituitary gland during embryogenesis. (A) E11 pituitary cells (n=3) were left untreated or cultured with CORT (1 nM) for 6 h in the absence and presence of anisomycin (Ani) at the indicated doses. (C-E) Pituitary cells (n=3) from e11 chickens were left untreated or treated with Ani (100 nM) for 30 m, after which time Ani was washed out and cells were cultured in the absence or presence of CORT (1 nM) for 6 h. Additional cells were left untreated or treated with CORT in the absence or presence of Ani for 6 h. (A, C-E) Levels of GH, FKBP5, and GHRH-R2 mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells receiving no Ani. Levels of mRNA for each gene were normalized to ACTB mRNA levels. (B,F) Levels of phosphorylated p38MAPK were determined using a sandwich ELISA in cells stimulated for 6 h with anisomycin (100 nM unless otherwise indicated) alone. One equivalent is equal to the amount of phosphorylated p38MAPK in 1 µg total cellular protein extracted from LMH cells stimulated with Ani. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

R2 mRNA stimulation were not reduced (Figure 23D and E; $P>0.05$). In fact, co-stimulation with anisomycin for 6 h substantially increased CORT induction of FKBP5 (Figure 23D; $P<0.05$). Thus, although somewhat contradictory to what was expected based on our results from the pharmacological inhibition experiments, it appears that chronic activation of both ERK1/2 and p38MAPK interferes with glucocorticoid regulation of GH expression during somatotroph development.

Glucocorticoids increase ERK1/2 kinase activity in embryonic pituitary cells

Glucocorticoids have been shown to both initiate and prevent MAPK signaling in several systems (12, 27-29, 240), and the results described above provide evidence that CORT induction of pituitary GH requires active MAPK signaling but is prevented by chronic stimulation (Figures 16, 17, 21, 22, and 23). In order to determine if glucocorticoid treatment affects either ERK1/2 or p38MAPK signaling in the embryonic anterior pituitary gland, cells (n=3 replicate trials) were cultured in the absence or presence of CORT for 30 m, 3 h, or 6 h, and levels of phosphorylated MAPK were determined. Although we were able to demonstrate an increase in p-ERK1/2 and p-p38MAPK levels in response to EGF and anisomycin stimulation, respectively (Figure 24; $P<0.05$), we did not detect any differences in phosphorylated MAPK levels in response to CORT treatment ($P>0.05$). Since active MAPK enzymes are phosphorylated, this implies that glucocorticoids are not stimulating or inhibiting ERK1/2 and p38MAPK signaling in e11 anterior pituitary cells. However, we did observe an increase in ERK1/2 kinase activity after 3 h CORT treatment in one of the two trials we conducted to determine efficacy of the MEK1/2 inhibitor, U0126 (Figure 18A). Therefore, we examined ERK1/2 kinase activity in protein extracts from e11 anterior pituitary cells

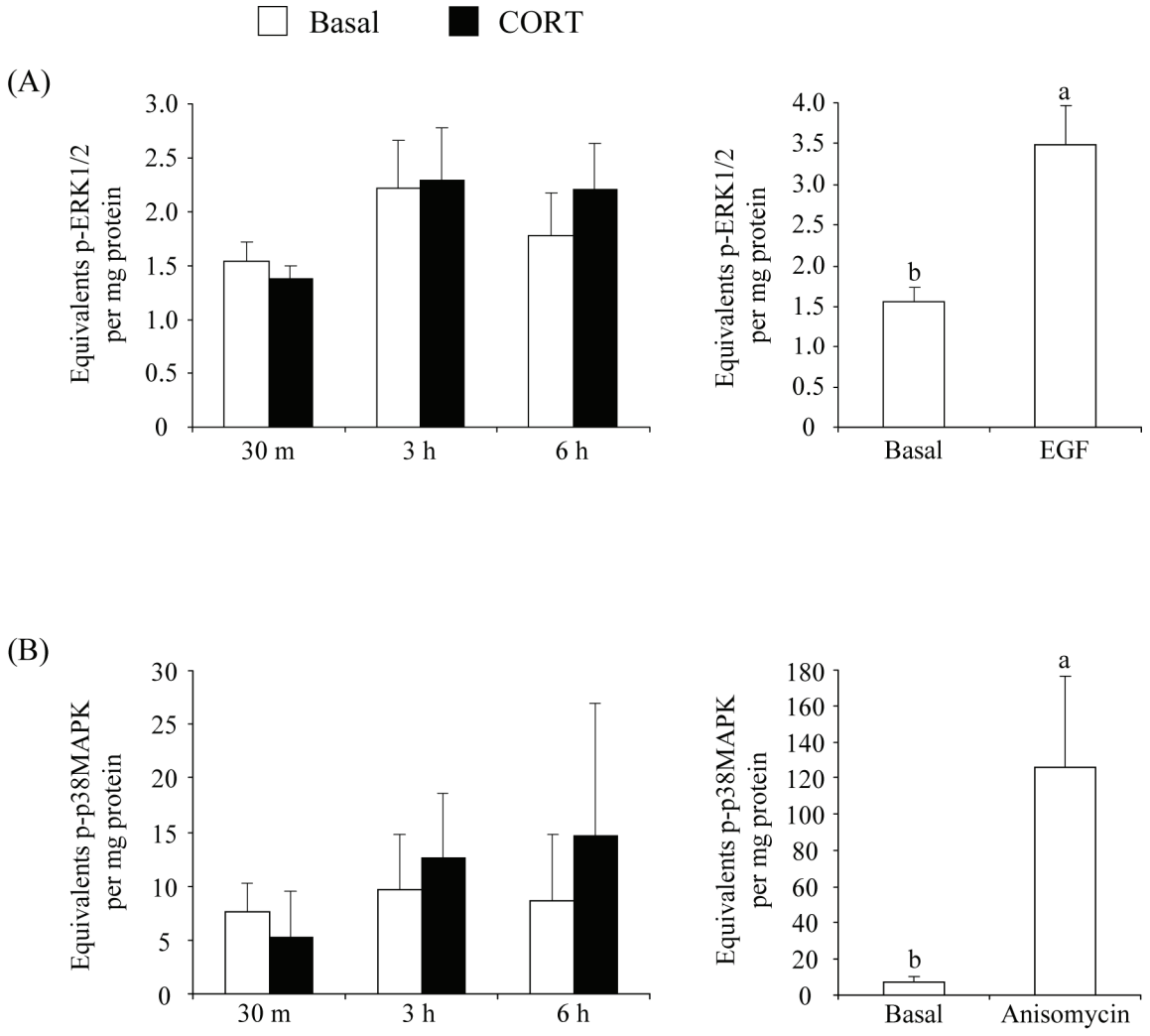


Figure 24. Glucocorticoids do not affect (A) ERK1/2 or (B) p38MAPK phosphorylation in chicken embryonic anterior pituitary cells. Pituitary cells (n=3) from e11 chickens were left untreated or treated with CORT (1 nM) for 30 m, 3 h, or 6 h. Levels of phosphorylated (A) ERK1/2 and (B) p38MAPK were determined using a sandwich ELISA. To serve as positive controls for the ability to detect increased MAPK phosphorylation, cells were also treated with EGF (100 ng/ml) or anisomycin (10 μM) for 30 m to activate ERK1/2 and p38MAPK pathway activity, respectively. One equivalent is equal to the amount of phosphorylated ERK1/2 or p38MAPK in 1 μg total cellular protein extracted from LMH cells stimulated with EGF or anisomycin, respectively. Values (mean + SEM) denoted with different letters are significantly different from one another ($P<0.05$).

treated with CORT for short and long time periods (Figure 25). This was not a completely balanced experiment, and only one replicate trial was analyzed for 5 m and 30 m treatments, while 3 replicate trials were analyzed for 3 h and 6 h treatments. Treatment with CORT for 3 h increased ERK1/2 activity in all 3 replicate trials, and statistical analysis demonstrated this increase to be significant ($P<0.05$; n=3 replicate trials).

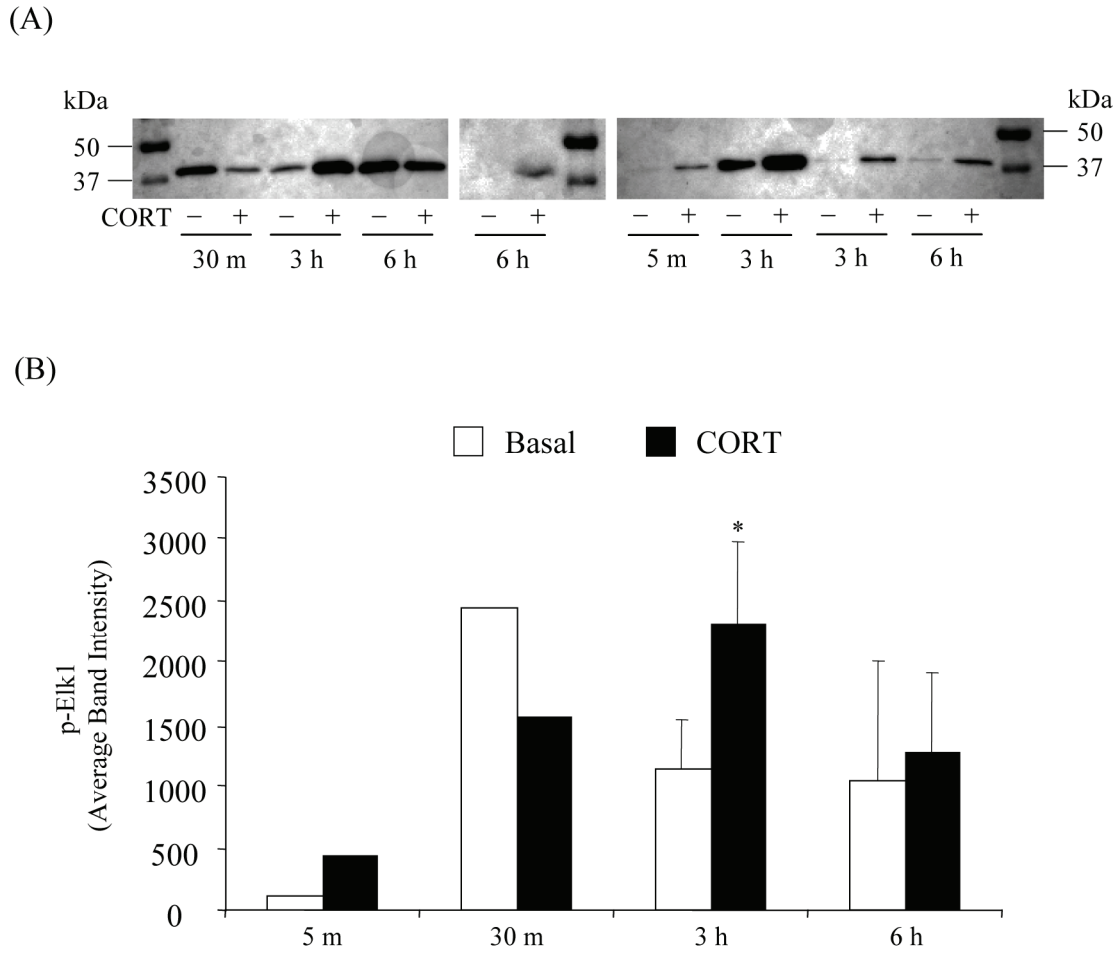


Figure 25. Long-term treatment with glucocorticoids may increase ERK1/2 signaling in chicken embryonic pituitary cells. Anterior pituitary cells from e11 chickens were cultured in the absence or presence of CORT (1 nM) for 5 m (n=1), 30 m (n=1), 3 h (n=3), or 6 h (n=3). The level of ERK1/2 kinase activity was measured with a non-radioactive MAPK assay in which *in vitro* phosphorylated substrate (p-Elk1) was detected by Western blotting. (A) Western blots depicting detection of p-Elk1 in each of the samples. (B) Quantification of the level of p-Elk1 detected by Western blotting. In the case of 3 h and 6 h treatments, the value (mean + SEM) was determined from 3 independent replicate samples. The asterisk (*) denotes a significant increase in p-MEK1 over basal cells ($P<0.05$).

Activity also appeared elevated after 6 h CORT treatment in two of the three replicate trials (Figure 25A), but this effect was not statistically significant ($P>0.05$). Cells exposed to CORT for 5 m demonstrated increased ERK1/2 kinase activity (Figure 25A), although the significance of this cannot be determined due to incomplete replication. Nonetheless, treatment of embryonic anterior pituitary cells with glucocorticoids is capable of stimulating ERK1/2 activity, implicating this pathway in the mechanism by which CORT increases GH expression during development.

Discussion

In this study, we investigated mechanisms through which glucocorticoids induce GH expression in pituitary somatotrophs. We determined that the primary mechanism through which CORT increases GH mRNA levels in the embryonic pituitary gland is through transcriptional initiation. We confirmed published reports (151, 153) demonstrating a requirement for ongoing protein synthesis in this process and extended these to another rodent model, reiterating the high level of conservation in processes governing pituitary development among vertebrates. In addition, we determined that CORT stimulation of GH expression requires HDAC activity. We previously reported that inhibition of ras with manumycin A partially prevents the CORT-stimulated increase of GH mRNA in embryonic pituitary somatotrophs (153, 217). Here, we confirmed this finding and extended the investigation to implicate ERK1/2 as a potential downstream effector of ras signaling in this process, as well as demonstrated that CORT treatment may increase ERK1/2 kinase activity in the anterior pituitary gland. Despite this, we also found the surprising result suggesting that chronic stimulation of ERK1/2 and p38MAPK pathways suppresses CORT induction of GH.

Treatment with CORT leads to an accumulation of GH mRNA in embryonic pituitary cells during a stage when these cells normally contain very low or undetectable levels (44, 121, 129, 149, 151, 152), and this increase was also evident in the current experiments. Although one group has reported that dexamethasone did not stimulate reporter activity from a construct driven by 1.7 kb of the rat GH 5'-flanking region in the rat MtT/S somatotroph cell line (241), other evidence indicates that glucocorticoids are capable of transcriptionally activating the GH gene in other pituitary cell lines (242-246). In most cases the transcriptional effect is very small, and the increase in GH mRNA expression stimulated by glucocorticoids is thought to occur primarily as a result of enhanced mRNA stability (121, 245-247). In primary pituitary cells from embryonic chickens or fetal mice removed just prior to normal onset of GH expression *in vivo*, the induction of GH mRNA is at least 10-fold, and often observed to be 20- to 30-fold, when measured by qRT-PCR [(159) and Figures 12-17 and 21-23]. Thus, it is highly unlikely that during this stage in pituitary development, the principal mechanism through which CORT increases GH expression is through mRNA stabilization. The results presented here provide no evidence of CORT stabilizing GH mRNA in e11 chicken primary pituitary cells (Figure 13); rather, based on the induction of GH promoter activity and nascent transcript by CORT (Figure 12), it is clear that the primary mechanism through which glucocorticoids increase GH expression during somatotroph recruitment is transcriptional activation of the GH gene. The agreement between fold induction of nascent GH mRNA transcript and mature GH mRNA transcript also demonstrates that discrepancy between fold induction of GH promoter activity (4-fold) and GH mRNA (10-

to 30-fold) is most likely due to differences in the two techniques, rather than any contribution of mRNA stability.

In both fetal rats and embryonic chickens (151, 153), the requirement for ongoing protein synthesis in glucocorticoid stimulation of GH mRNA implies an indirect mechanism, wherein an intermediary factor is induced that is crucial for the increase in GH transcription resulting from CORT treatment. We confirmed this requirement in the present study, as the inclusion of CHX with CORT abolished induction of GH mRNA as determined by qRT-PCR, in both embryonic chickens and fetal mice (Figure 14). Glucocorticoids do not induce expression of Pit-1 in fetal rats (151) or embryonic chickens (160), making it unlikely that this transcription factor is the required intermediary protein, although it is known to be necessary for somatotroph differentiation and GH expression. The reported increase in Pit-1 mRNA that occurred around the time of somatotroph development in the chicken embryonic pituitary gland (Figure 5) is most likely due to an expansion in somatotroph number, which rapidly occurs after appearance of this cell type (53, 103, 123). However, this intermediary protein may be another transcription factor or co-activator that stimulates expression of the GH gene in combination with Pit-1 via protein-protein and/or protein-DNA interactions. Another attractive candidate as the intermediary factor involved in this process is GHRH-R, whose expression pattern in the embryonic pituitary also reflects a potential role in somatotroph differentiation in mammals and birds [(124, 161) and Figure 8]. Glucocorticoid treatment in vitro increased GHRH-R mRNA in fetal rat pituitaries in a manner that is insensitive to inhibition of protein synthesis (161, 205); however, CORT does not appear to increase GHRH binding to chick embryonic pituitary cells (179) or

increase GHRH-R mRNA or protein levels in the cells (Ellestad and Porter, unpublished; Bossis and Porter, unpublished). The recent discovery of GHRH-R2 as a functional receptor for GHRH in the chicken pituitary (181), in combination with its developmental profile in the embryonic pituitary gland (Figure 8) and ability to be upregulated by CORT in anterior pituitary cells (Figures 17, 22, and 23), identify it as an exciting prospect to investigate further. Notably, it has been demonstrated that CORT stimulation of GHRH-R2 occurs in the presence of CHX (Ellestad and Porter, unpublished), implying it may be a direct target of glucocorticoids in the anterior pituitary.

Inclusion of HDAC inhibitors abolished glucocorticoid induction of GH mRNA and protein expression (Figure 15). Epigenetic modifications, including histone acetylation status, have an important effect on chromatin organization and gene transcription during development (232), and glucocorticoids are known to regulate target genes through influencing their histone acetylation state (231). Further, acetylation of the GH regulatory region is thought to contribute to GH expression level (248, 249). Current research in our laboratory is aimed at identifying CORT-regulated epigenetic changes in the proximal GH promoter, and it has been shown that CORT treatment leads to a short-lived increase in acetylated histone 3 (Ac-H3) levels around the GH transcriptional start site and two Pit-1 sites within the chicken GH 5'-flanking region, one very near the transcriptional start site (-111 to -117) and one more distal (-538 to -546) (Narayana and Porter, unpublished). This increase occurred after 1.5 h of exposure to CORT, but after 6 h CORT treatment Ac-H3 levels were no different than those in untreated cells. The immediate acetylation event may be necessary to recruit appropriate transcription factors to initiate transcription from the GH promoter, and subsequent downregulation in

acetylation status mediated by HDAC enzymes may be necessary for sustained transcription leading to elevated GH mRNA. A recent mapping of genome-wide histone acetyltransferase (HAT) and HDAC chromatin localization revealed both are targeted to transcriptionally active genes, and HDAC activity is necessary to reset chromatin for further activation by removing acetyl groups added by HATs (250). An alternative requirement for HDAC activity may result from a mechanism involving indirect de-repression of GH to allow for initiation of transcription. The HDAC inhibitors used in this study did not alter GH mRNA in cells cultured under basal conditions, implying that the mechanism by which CORT increases histone acetylation after 1.5 h is not simply a direct effect of CORT reducing HDAC activity at the GH regulatory region to increase accessibility for recruitment of the transcriptional machinery. Glucocorticoids are capable of stimulating HDAC expression (251), as well as enhance their recruitment to repressor complexes (252), and an indirect requirement for HDAC activity implies that CORT-mediated deacetylation may be required to reduce expression of an inhibitory factor that represses GH transcription, while CORT-mediated transcriptional activation may induce expression of a gene that stimulates GH transcription. It cannot be ruled out, however, that the abolishment of GH induction by CORT in the presence of HDAC inhibitors was due to hyperacetylation of GR itself, resulting from HDAC inhibition. It was recently demonstrated that the self-oscillating transcription factor CLOCK is capable of acetylating a cluster of lysine residues on GR, and in this hyperacetylated state the receptor has decreased capability of binding GREs and transactivating a subset of glucocorticoid induced genes (19). These potential requirements for HDAC activity in glucocorticoid induction of pituitary GH are by no means mutually exclusive, and the

response may indeed involve a combination of these mechanisms. Nonetheless, stimulation of other CORT-induced genes was not affected by HDAC inhibition, implying that the requirement for GH expression is relatively specific and not due to compromised GR signaling resulting from HSP90 hyperacetylation (233, 234).

Inhibitors of PI3-K inhibitors and JNK did not interfere with the increase in GH mRNA resulting from CORT treatment, indicating that these are not major pathways involved in glucocorticoid induction of GH expression during pituitary development. However, LY294002, but not wortmannin, repressed CORT stimulation of GH protein expression and may be due to the instability of wortmannin in aqueous solution (253) or inhibition of a PI3-K enzyme that is insensitive to wortmannin by LY294002, such as many class IV PI3-Ks (253). The reduction in GH protein levels after CORT treatment in the presence of LY294002 indicates that PI3-K activity is necessary for synthesis of GH protein, although this effect may not be specific to GH, as PI3-K signaling is a major pathway that stimulates initiation of translation through phosphorylation of eukaryotic initiation factor 4E binding proteins (254). The JNK inhibitor, SP600125, did tend to elevate levels of GH mRNA under basal and CORT-stimulated conditions (Figure 16B), indicating that JNK signaling may play a role in repression of GH during this period of pituitary development. The observation that inhibition of JNK did not affect basal or CORT-induced GH promoter activity (Figure 16E) indicates that the repressive effect of JNK on GH mRNA levels is either non-transcriptional or that the responsive region lies outside the -1,727 to +48 fragment of the GH gene contained in the reporter construct.

Glucocorticoid induction of GH promoter activation, mRNA, and protein were strongly repressed by SB203580, a compound traditionally used as a p38MAPK

inhibitor. Although SB203580 is clearly affecting CORT regulation of GH expression, its activity as an inhibitor of p38MAPK signaling could not be confirmed in the chicken embryonic anterior pituitary cells used in these experiments and, at times, it appeared to enhance p38MAPK activity (Figure 18). Although initial studies investigating specificity and mechanism of SB203580 indicated it was highly selective for p38MAPK isoforms (255), it has since been reported to inhibit additional kinases with similar or increased potency (256). As a result, there is no indication of a requirement for p38MAPK activity in CORT regulation of GH during development, although other kinases that are inhibited by SB203580, which include cyclin G-associated kinase, casein kinase, and receptor-interacting protein 2 (256), may play a role in this process.

Manumycin A, a ras farnesyltranferase inhibitor, was partially effective at suppressing glucocorticoid induction of GH mRNA and abolished the increase in intracellular GH protein (Figure 16A and C). Suppression of GH mRNA levels by manumycin A under CORT-treated conditions was more effective the shorter the CORT treatment (Figure 17), and this inhibitor did not reduce CORT-induced GH promoter activity after 20 h exposure (Figure 16E). This indicates that either the inhibitor is losing effectiveness over long term culture conditions or that ras signaling is involved in events leading to initiation of GH transcription but is not required for sustained transcription. Regardless, the reduction in CORT-induced GH mRNA levels in the presence of manumycin A observed here is consistent with previous work, in which GH mRNA stimulation by CORT was partially attenuated in the presence of manumycin A (153). A simple explanation that satisfies the requirements for ras signaling, ERK1/2 activity, and ongoing protein synthesis is that CORT is directly inducing a ras-like protein which then

stimulates ERK1/2 activity, ultimately leading to initiation of GH expression. Although the observation that the ras inhibitor is more effective than U0126 at reducing GH mRNA expression during shorter treatment times may seem inconsistent with this, one explanation is that the hypothetical ras protein independently stimulates multiple signaling cascades with some functional redundancy. Two potential ras proteins that are intriguing candidates in mediating CORT effects on somatotroph GH expression are dexamethasone-induced ras-related protein 1 (Dexras1) and ras-dva. Each has a developmental profile similar to GH in the chick embryonic pituitary gland [(124) and Figure 27] and is upregulated by CORT in the presence of CHX in chicken embryonic pituitary cells [(235) and Figure 31). Dexras1 was identified as a transcript rapidly upregulated by dexamethasone in a mouse corticotroph cell line (257), and the human Dexras1 gene contains a functional GRE in its 3'-flanking region (258), indicating that it is most likely a direct target of the GR. Ras-dva was identified in Xenopus embryos as a novel transcriptional target of anterior neural fold protein-1 (Anf-1) (259), a transcriptional repressor essential for normal pituitary development (58), and is an essential signaling component for anterior neural plate and ectodermal patterning in Xenopus embryos (260).

Of the two ERK1/2 pathway inhibitors utilized, only U0126 effectively reduced CORT stimulation of GH mRNA and protein expression (Figures 16 and 17). Stimulation of GH promoter activity was also completely suppressed by U0126 (Figure 16E). The lack of an effect of PD98059 on GH mRNA levels is similar to previous results from our laboratory (153), and inconsistent effects of these two inhibitors have been observed previously. Induction of nitric oxide synthase in differentiating PC12 cells was blocked

by U0126 but not PD98059, and it was ultimately found that U0126 was a more effective inhibitor of ERK1/2 signaling (261). Our results clearly demonstrate that U0126 decreased ERK1/2 kinase activity under basal, CORT-treated, and EGF-stimulated conditions (Figure 18), implying that the reduction in GH expression in the presence of U0126 is a result of ERK1/2 inhibition. Although an increase of p-ERK1/2 levels after CORT treatment of embryonic pituitary cells was not detected in this study (Figure 24), ERK1/2 kinase activity was increased after 3 h of CORT treatment and may also be elevated after both a 5 m and 6 h treatment (Figure 25). This suggests that ERK1/2 signaling is not only required for GH induction by CORT, but also activated by CORT treatment.

We previously reported that the CORT response in somatotrophs is mediated by classical glucocorticoid nuclear receptors (156). Glucocorticoids may be working through GR and/or MR to increase expression of an intermediary protein that activates the ERK1/2 signaling pathway. Activated GR has previously been shown to induce expression of ras and Raf-1, upstream activators of ERK1/2 signaling cascades (29). Alternatively, CORT may be working through GR and/or MR to activate the ERK1/2 signaling cascade in a non-transcriptional manner. The GR antagonist RU486 blocks dexamethasone-induced rapid phosphorylation of ERK1/2 (27), and this may occur through direct interaction of ligand-bound GR with Raf (262). ERK1/2-mediated phosphorylation of GR promotes efficient nuclear translocation of the receptor (263), and kinase phosphorylation of GR has been implicated as a means to fine-tune glucocorticoid transcriptional regulation (7, 17).

Although the dominant negative mutants of ERK1/2 and p38MAPK pathways obtained to examine their involvement in glucocorticoid initiation of GH expression did not appear to be functional, constitutively active mutants in each pathway highly increased both MAPK phosphorylation and enzyme activity (Figure 20). Based on pharmacological inhibition data, it was hypothesized that activation of these pathways would increase GH expression in the absence of CORT or enhance CORT induction. Unexpectedly, overexpression of caMEK1 or caMKK3 and caMKK6 completely abolished CORT-stimulated promoter activity and substantially repressed CORT stimulation of GH mRNA (Figure 21). The inhibitory effects of elevated ERK1/2 and p38MAPK activity were confirmed using alternative stimulatory ligands. Concurrent treatment with CORT and EGF, which activates ERK1/2 signaling, or anisomycin, which activates p38MAPK, also suppressed stimulation of GH mRNA expression (Figures 22 and 23). On the other hand, pretreatment with and removal of the stimulatory ligands had no effect, indicating the repression truly is due to chronic activation. This implies that down-regulation of MAPK activity is necessary for full induction of GH by CORT, and there are several examples of glucocorticoids decreasing MAPK signaling, often through upregulation of phosphatase enzymes (240). However, there is no evidence that CORT treatment led to a reduction in either p-ERK1/2 or p-p38MAPK levels or their kinase activities (Figures 18, 24, and 25), suggesting that overstimulation of these pathways is responsible for their suppressive effect on CORT stimulation of GH. Recently, it was reported that different GR phospho-isoforms are preferentially recruited to certain GR-induced genes (17), and it is known that phosphorylation status can affect sub-cellular GR localization (7). Chronic stimulation of ERK1/2 or p38MAPK, which is known to

phosphorylate GR (12), may alter the phospho-isoforms present and prevent recruitment to certain target genes whose regulation is required for the CORT response. Another possibility is that a transcription factor involved in initiating GH expression, either through de-repression or activation, is regulated by phosphorylation and, further, that chronic MAPK stimulation leads to an altered phosphorylation state of the protein that perturbs its transcriptional regulatory activity. One such possible transcription factor is Ets-1. Recent research in our laboratory has uncovered a glucocorticoid-responsive region within the chicken GH gene that lies between -985 bp and -1019 bp relative to the transcriptional start site (216). That region contains a putative Ets-1 binding site, and it was demonstrated using chromatin immunoprecipitation that Ets-1 appears to bind near that region under basal conditions but is released upon treatment with CORT for 1.5 h or 6 h [(216) and Narayana and Porter, unpublished]. Ets-1 DNA binding activity is regulated, in part, by phosphorylation status at different domains (264). Activation of Ets-1 through phosphorylation of T38 by ERK1/2 increases DNA binding, and phosphorylation at alternative regions by other kinases inhibits DNA binding. It is possible that Ets-1 is part of a repressor complex that must be released from the GH 5'-flanking region in order for transcriptional activation by CORT and that chronic ERK1/2 activity maintains Ets-1 phosphorylation and prevents its release. Nonetheless, it appears that sustained overstimulation of MAPK signaling interferes with glucocorticoid induction of GH and that the requirement for and/or activation of ERK1/2 activity in this response is transitory.

In conclusion, these results are the first to provide strong evidence that glucocorticoid induction of GH expression during pituitary development occurs through

an indirect mechanism requiring ongoing protein synthesis and HDAC-, ras-, and ERK1/2-mediated transcriptional events. In addition, the requirement for ERK1/2 signaling is transitory, and chronic stimulation of both ERK1/2 and p38MAPK interfere with CORT stimulation of GH expression. Although further studies are needed to precisely define the molecular mechanism underlying glucocorticoid-induced GH gene expression and somatotroph differentiation during development, this report substantially increases our understanding of this process and may provide insight into glucocorticoid-induced developmental changes in other tissues and cell types as well.

CHAPTER 4

Ras-dva is a novel Pit-1 and glucocorticoid-regulated gene in the embryonic anterior pituitary gland

Abstract

Glucocorticoids are involved in the functional differentiation of at least two cell types within the developing anterior pituitary gland. Ras-dva was initially identified as a gene regulated by Anf-1/Hesx1, a transcription factor known to be critical in pituitary development, and has an expression profile in the chicken embryonic pituitary gland that is consistent with *in vivo* regulation by glucocorticoids. However, nothing has been reported regarding the presence or regulation of ras-dva in the neuroendocrine system. Therefore, the objective of this study was to characterize expression and regulation of ras-dva in the developing chicken anterior pituitary gland. Pituitary ras-dva mRNA levels increased during embryogenesis to a maximum on e18, then decreased and remained low or undetectable after hatch, and ras-dva expression was highly enriched in the pituitary gland on e18 relative to other tissues examined. Glucocorticoid treatment of pituitary cells from mid- and late-stage embryos rapidly increased ras-dva mRNA, both in the absence and presence of a protein synthesis inhibitor, suggesting that it may be a direct transcriptional target of glucocorticoids. Ras-dva mRNA stability was unaffected by CORT treatment, further suggesting that it is transcriptionally regulated by glucocorticoids. A reporter construct driven by 4 kb of the chicken ras-dva 5'-flanking region, containing six putative Pit-1 binding sites and two potential GR binding sites, was highly activated in embryonic pituitary cells and upregulated by CORT treatment. Mutagenesis of the most proximal Pit-1 site substantially reduced promoter activity in chicken e11 pituitary cells, and overexpression of Pit-1 in cells lacking endogenous Pit-1 (COS-7) led to significant activation of ras-dva promoter activity. On the other hand, mutagenesis of either putative GR binding site or both sites in combination did not reduce

induction of ras-dva promoter activity by CORT, indicating that additional DNA elements within the 5'-flanking region are responsible for glucocorticoid regulation. In conclusion, we have identified ras-dva as a glucocorticoid-regulated gene that is most likely expressed in cells of the Pit-1 lineage within the developing anterior pituitary gland.

Introduction

In the developing anterior pituitary gland, terminally differentiated hormone-producing cell types arise during the latter half of embryogenesis (53, 77, 87, 95). In both mammals and birds, circulating glucocorticoids increase toward the end of embryonic development (174, 265, 266) and are thought to play a critical role in functional maturation of the pituitary through initiation of hormone production in at least two of these pituitary cell types, GH-producing somatotrophs and PRL-producing lactotrophs (44, 121, 147-150, 152, 155, 160, 267). Glucocorticoids also repress POMC expression during negative feedback on ACTH release from pituitary corticotrophs (268), although it is unclear when this feedback is established.

Ras-dva was identified as a transcript upregulated between mid- and late-embryogenesis in a study investigating global gene expression changes in the chicken embryonic pituitary gland that occur around cellular differentiation of cells in the Pit-1 lineage (TSH-producing thyrotrophs, somatotrophs, and lactotrophs) (124). The observed increase in pituitary ras-dva mRNA between e12 and e17 occurs concurrently with, or just prior to, appearance of pituitary somatotrophs and lactotrophs in the chicken, respectively (53, 94, 103-106, 127, 149). In addition to the correlation of pituitary ras-dva expression with the increase in circulating CORT in the chick embryo that occurs around

the same time (10, 138, 140, 141), ras-dva was recently identified as a gene whose mRNA levels are increased in cultured chicken embryonic pituitary cells treated with CORT (235). Based on its developmental expression pattern and its upregulation by CORT in embryonic pituitary cells, ras-dva may be mediating effects of glucocorticoids in this tissue.

Anf-1, also known as Hesx1, is a homeobox factor that functions as a transcriptional repressor to regulate expression of transcription factors involved in anterior embryo patterning (269). Ras-dva was originally identified in a screen aimed at discovering targets that are downregulated by *Xenopus* Anf-1/Hesx1 in anterior neural ectoderm (259), and subsequently determined to be an essential component in the FGF signaling network required for patterning of the early anterior neural plate and adjacent ectoderm in *Xenopus laevis* embryos (260). Importantly, during embryogenesis, Anf-1/Hesx1 expression eventually becomes restricted to the ventral diencephalon and Rathke's pouch, the pituitary primordium, and it is one of the earliest markers of the anterior pituitary gland (77, 78). In the absence of Anf-1/Hesx1, a small number of embryos lack a pituitary gland altogether but the majority of mice exhibit hypopituitarism that ranges from combined pituitary hormone deficiency to isolated growth hormone deficiency (79).

Thus, not only is ras-dva known to be expressed and developmentally regulated in the anterior pituitary gland (124), it was initially discovered as a target of a transcription factor essential for normal pituitary development (259), although in an extra-pituitary context. Despite this, no studies examining the presence or regulation of ras-dva in the neuroendocrine system have been reported, and there have been no published reports

regarding glucocorticoid regulation of this gene. Therefore, the objectives of this study were four-fold: 1) to determine the expression pattern of chicken ras-dva, both in terms of its ontogeny in the developing pituitary and its tissue distribution in the embryo; 2) to characterize glucocorticoid regulation of ras-dva in embryonic pituitary cells; 3) to identify regions of the ras-dva gene that may be involved in its pituitary and/or glucocorticoid regulation; and 4) to determine whether overexpression of ras-dva can mimic glucocorticoid effects on pituitary hormone expression.

Materials and Methods

Sequencing of chicken ras-dva and comparison with other species

The putative chicken ras-dva clone was part of a neuroendocrine cDNA library used to construct a custom cDNA microarray and was initially identified through random sequencing of the library in combination with BLAST comparisons (270, 271). The plasmid containing chicken ras-dva (pgp2n.pk003.j19; GenBank accession no. BM492047) was purified according to a standard protocol (272), and the clone was sequenced in its entirety in both directions so that there was sequence from at least two reactions covering each region of the assembled full-length cDNA. Primers (Sigma-Aldrich) used for sequencing were Sport6.1 SP6, Sense462, and Sense1145 for the forward reactions, and Sport6.1 T7, Antisense734, and qRT-PCR reverse primer for the reverse reactions (Tables 2 and 3). Sequencing reactions and assembly of overlapping sequences into full-length cDNA were performed as described in the Materials and Methods section of Chapter 3.

BLAST searches of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ensembl (http://www.ensembl.org/index.html) databases were conducted using the putative coding

sequence in order to identify potential ras-dva homologs in other species, and any BLAST scores ≥200 were considered positive. The predicted amino acid sequence for chicken ras-dva was compared with those of other species using the AlignX tool of VectorNTI 9.0 (Invitrogen). The chicken sequence was compared with zebra finch (*Taeniopygia guttata*), green anole (*Anolis carolinensis*), common platanna frog (*Xenopus laevis)*, western clawed frog (*Xenopus tropicalis*), fugu puffer (*Takifugu rubripes*), stickleback (*Gasterosteus aculeatus*), rainbow trout (*Oncorhynchus mykiss*), green spotted puffer (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), sea lamprey (*Petromyzon marinus*), and opossum (*Monodelphis domestica*) sequences. Zebra finch and opossum sequences were predicted by automated computational analysis and derived from genomic sequence, and sequences from remaining species were from cloned cDNAs. Biology Workbench (http://workbench.sdsc.edu/) was used to create the alignment (CLUSTALW tool), color-coding (BOXSHADE tool), and unrooted phylogenetic tree diagrams. The chicken ras-dva cDNA sequence was compared with the chicken genome sequence using Ensemble (http://www.ensembl.org/Gallus_gallus/Info/Index). Analysis of the 5'- and 3'-flanking regions of the chicken ras-dva gene for predicted transcription factor binding sites was performed using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess). Default search parameters were used, except stringency was increased by changing the minimum log-likelihood ratio score from 12 to 18 to decrease the total number of putative sites identified and, therefore, reduce the likelihood of false prediction.

Animals, tissue collection, and pituitary dispersions

Broiler strain chicken embryos were incubated and removed on the appropriate day or allowed to hatch as described in the Materials and Methods section of Chapter 2. Pituitary samples described in Chapter 2 were used for the ontogeny experiment. In a separate experiment, anterior pituitary glands from d7, d21, d35, and d48 chickens were also collected (n=4 replicate samples per age). For the tissue distribution experiment, pituitary, hypothalamus, whole brain, lung, kidney, spleen, liver, heart, breast muscle, and stomach (proventriculus) were collected from e18 chickens. Four replicate samples were collected for each tissue (n=4). Two hypothalami were pooled for each replicate, and one bird per replicate was used for the remainder of the tissues. Caudal and cephalic lobes (n=4 replicate samples each) of the anterior pituitary from e18 chickens were collected to examine ras-dva distribution within the pituitary gland. Tissues were immediately frozen in liquid N_2 and stored at -80 C until RNA extraction. For cell culture experiments, pituitaries were isolated and dispersed from e11 and e18 chickens as described in the Materials and Methods section of Chapter 3. For each replicate trial of a given experiment, anterior pituitaries from e11 (50-60 embryos) or e18 (10 embryos) embryos were isolated and pooled. On average, an e18 chicken anterior pituitary gland yields $1x10^6$ cells. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland.

Cell culture

Embryonic pituitary cells and cell lines were cultured as described in the Materials and Methods section of Chapter 3. To confirm glucocorticoid-induced upregulation of ras-dva in the anterior pituitary gland, dispersed cells from e11 and e18

chickens were cultured in the absence or presence of CORT (1 nM) for indicated times. To investigate glucocorticoid regulation of pituitary ras-dva, the same samples from the following experiments described in Chapter 3 were also analyzed for ras-dva mRNA levels: e11 cells treated with CORT in the presence or absence of ActD to determine a requirement for transcription (Figure 12); e11 cells pretreated with CORT and cultured for 24 additional hours with ActD to evaluate CORT affects on mRNA stability (Figure 13); and e11 cells cultured with CORT in the presence or absence of CHX, HC toxin, and TSA to assess a requirement for protein synthesis and HDAC activity (Figures 14 and 15). Cells were harvested at the completion of each experiment by retrypsinization, immediately frozen in liquid nitrogen, and stored at -80 C until RNA extraction. The monkey kidney fibroblast cell line, COS-7 was kindly provided by Dr. G. L. Hager (National Cancer Center, Bethesda, MD) and maintained in DMEM supplemented with 10% fetal bovine serum (Equitech-Bio, Inc) in 75-cm^2 flasks (Corning Life Sciences).

Plasmids, transfection, analysis of promoter activity, and flow cytometry

Two fragments of the 5'-flanking region of the ras-dva gene were amplified from chicken genomic DNA extracted from liver using primers listed in Table 3. With the exception of the 5'-2kb_rev primer, which binds to the region in an area that contains an EcoRI site, primers have restriction enzyme sites at their 5'-end. Amplifications were conducted using Phusion High-Fidelity PCR Master Mix (Finnzymes, Inc., Woburn, MA) according to the manufacturer's protocol. Reactions contained 250 ng genomic DNA and 500 nM each primer, and the PCR cycling parameters were as follows: 98 C for 10 s; 35 cycles of 98 C for 1 s, 60 C for 5 s, and 72 C for 1 m; and a final extension at 72 C for 1 m. All fragments are numbered relative to the ATG start codon, rather than the predicted

Table 3. Primers used to characterize chicken ras-dva and investigate its regulation within the embryonic anterior pituitary gland.

Gene	ID[1]	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
qRT-PCR			
Ras-dva	34869	ACACCAGCGGCAGTTACTCCT	GTAGACCAAAGCGAAGGCGTC
GH	00328	CACCTCAGACAGAGTGTTTGAGAAA	CAGGTGGATGTCGAACTTATCGT
PRL	20680	AGGAATGGAGAAAATAGTTGGGC	TCATTTCCAGCATCACCAGAAT
POMC	36767	CGCTACGGCGGCTTCA	TCTTGTAGGCGCTTTTGACGAT
TSH-β	04024	ACTGCCTGGCCATCAACAC	ACACGTTTTGAGACAGAGCACTTTT
GHRH-R	08374	CCTTGGCATTCGGCTTTATTTAG	TCAGGAAACAGTAGAGGAGTGCTACA
SSTR2	38508	CTGCTGGCTCCCCTTCTACA	TGGGCACGATCAGGACAGA
Total Pit-1[2]		AAATCAATACTGTCCAAGTGGCTG	GTGGTTCTGCGCTTCCTCTT
PGK-1	12893	CTACATGCTGTGCGAAGTGGAA	GCCAGGAAGAACCTTACCCTCTAG
ACTB[3]		CAGGATGCAGAAGGAGATCACA	TAGAGCCTCCAATCCAGACAGAGTA
Other Ras-dva primers			
Full-length		CAAACTTTGCAGAACCGGGAGCAG	CCGCTTTGCATCACCGTGTTTATTG
Sense462		AAATTCCCTCCCATCGTGGT	
Sense135		AGGGAAGGAGAAGAGCCACG	
Sense1145		AGGGCAGGATCCCAAAAAGG	
Antisense734			CTGTTGGTTTTGTTCATGGG
Cloning 5'-flanking region			
5'-4kb_fwd		CCGGAATTCCCTCTCTGCCGCTGCTAATCCTGG	
5'-2kb_fwd		CCCAAGCTTGGGGAATTCCTTCCTGATGAAAAGAAATATGCCTATTTC	
5'-flank_rev		CCCAAGCTTGGGGGCTGGGAGCAGAGGGGACG	
5'-2kb_rev		GGAAGGAATTCTGTTTGTGCTTCTTGGTG	
5'-3.2_fwd		GTTACACTGGGGTCATGGGGAGC	
5'-2.4_fwd		GGGCAAGCTCAGCATGAAGTCG	
5'-2.1_fwd		CCGAAATCCTCCGTTGCTTTGAGG	
5'-1.5_fwd		GCCAAAACTGAAGGGTGAAACTGGC	
5'-0.9_fwd		CTCCACAGCCGGTGCCAGG	
5'-0.3_fwd		GGTGCGGTGCTCTCCAGGG	
5'-0.3_rev			CCCTGGAGAGCACCGCACC
5'-0.9_rev			CCTGGCACCGGCTGTGGAG
5'-1.5_rev			GCCAGTTTCACCCTTCAGTTTTGGC
5'-2.4_rev			CGACTTCATGCTGAGCTTGCCC
5'-3.2_rev			GCTCCCCATGACCCCAGTGTAAC
pGL3-Basic_4770[4]		GCTGTCCCCAGTGCAAGTGCAG	
pGL3-Basic_90[4]			CCATGGTGGCTTTACCAACAGTACCG
Site-directed mutagenesis primers			
Prox-Pit1_fwd		GCGCTGCCGGCAGCGGTGAGAGTTCCCCCTGACGTGCGCAACGCAGCGGTGC	
Prox-Pit1_rev		GCACCGCTGCGTTGCGCACGTCAGGGGGAACTCTCACCGCTGCCGGCAGCGC	
Dist-GR_fwd		AGAGATGCTATAGAAACTCCTCTGAGCCTGCTCCACGCGGTGCTAAATCCCTTTAGTCCTAACG AGAGG	
Dist-GR_rev		CCTCTCGTTAGGACTAAAGGGATTTAGCACCGCGTGGAGCAGGCTCAGAGGAGTTTCTATAGC ATCTCT	
Prox-GR_fwd		ACCTTTCAGGCTCTCGGTAACCATAGCCTCCACGCGGTGAAAACGCACAGCAGACAGCTGATG	
Prox-GR_rev		CATCAGCTGTCTGCTGTGCGTTTTCACCGCGTGGAGGCTATGGTTACCGAGAGCCTGAAAGGT	

[1]ID is the transcript identification from Ensembl chicken genome assembly (http://www.ensembl.org/Gallus_gallus/Info/Index) and is preceded by ENSGALT000000 in all cases.

[2]The primers used to measure total Pit-1 mRNA levels detect all 3 transcript variants (ENSGALT000000): Pit-1α (30398); Pit-1β (24989); and Pit-1γ (36836).

[3]The sequence for ACTB is not on the assembled chicken genome and primers were designed based on the sequence for chicken cytoplasmic beta-actin in GenBank (accession no. X00182).

[4]The pGL3-Basic vector (Promega) was firefly luciferase reporter vector used to determine Ras-dva promoter activity. The pGL3-Basic_4770 primer is on the 5'-end of the multiple cloning site and the pGL3-Basic_90 primer is on the 3'-end of the multiple cloning site relative to the luciferase reporter gene.

transcriptional start site. A region from -1 bp to -2,009 bp was amplified using 5'-2kb_fwd and 5'-flank_rev primers, and the resulting fragment was cloned into an empty reporter construct (pGL3-Basic; Promega) using HindIII to generate the pGL3-2kb reporter construct. A region from -1,999 bp to -4,154 bp was amplified using 5'-4kb_fwd and 5'-2kb_rev primers, and the resulting fragment was cloned into the pGL3-2kb plasmid using EcoRI to generate the pGL3-4kb reporter construct. As such, pGL3-2kb contains a luciferase reporter driven by 2,009 bp of the chicken ras-dva 5'-flanking region, and pGL3-4kb contains a luciferase reporter driven by 4,154 bp of the chicken ras-dva 5'-flanking region. Both reporter vectors were sequenced in their entirety with vector- and insert-specific primers listed in Table 3. The QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA) was used according to the manufacturer's directions to mutate the proximal putative Pit-1 site within pGL3-2kb and the distal and proximal putative GR binding sites within pGL3-4kb. Primers (Table 3) for site directed mutagenesis were designed using the QuikChange Primer Design application available from the company's website (http://www.agilent.com/genomics/qcpd). Reactions contained 25 ng plasmid template and 125 ng each primer. The proximal putative Pit-1 site was mutated within pGL3-2kb to generate pGL3-mPit1 using the following cycling parameters: 95 C for 30 s; and 18 cycles of 95 C for 30 s, 55 C for 1 m, and 68 C for 7 m. The parameters for mutating the putative GR binding sites within pGL3-4kb were identical, with the exception that the 68 C extension was conducted for 9 m. The proximal GR binding site was mutated to create pGL3-mpGR, the distal GR binding site was mutated to create pGL3-mdGR, and the proximal GR binding site within pGL3-dGR was mutated to create the double GR binding site mutant, pGL3-mGR. The

proximal putative Pit-1 site was mutated from ACAAATGCAT to CCCCCTGACG, the proximal predicted GR binding site was mutated from AGCACAGATG to CTCCACGCGG, and the distal predicted GR site was mutated from AGAACAGCTG to CTCCACGCGG (underlined nucleotides were mutated). All plasmids were sequenced to confirm presence of the mutations, and sequence from mutated plasmids was re-analyzed using TESS to verify that mutations did not create additional putative transcription factor binding sites. Expression vectors containing the coding region for chicken Pit-1α (Mukherjee and Porter, unpublished) and the coding region for chicken GR (11) cloned into the empty expression vector Sport6.1 were generated by our laboratory. The plasmid from our neuroendocrine cDNA library (124, 270, 271) that contained full-length chicken ras-dva (pgp2n.pk003.j19) in Sport6.1 was used in ras-dva overexpression experiments.

Cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM I as described in the Materials and Methods section of Chapter 3. In experiments investigating ras-dva promoter activity in embryonic pituitary cells, e11 cells ($1x10^6$/well in a 24-well format) were transfected with 1 µg pGL3-Basic, 1 µg pGL3-2kb, or 1 µg pGL3-4kb together with 20 ng pRL-SV40. After 6 h, transfection medium was replaced with cell culture medium, and cells were allowed to recover for 18 h. Subsequently, cells were left untreated or treated with CORT (100 nM) for 6 h or 24 h prior to lysis. In the experiment investigating the effect of mutating the proximal Pit-1 site, e11 pituitary cells were plated and transfected as described above with 1 µg pGL3-Basic, 1 µg pGL3-2kb, or 1 µg pGL3-mPit1 in combination with 20 ng pRL-SV40. Cells were cultured under basal conditions an additional 42 h after transfection prior to lysis. In the experiment investigating the effect of mutating the predicted GR binding sites, e11 pituitary cells

were plated and transfected as described above with 1 μg of pGL3-Basic, pGL3-4kb, pGL3-mdGR, pGL3-mpGR, or pGL3-mGR together with 20 ng of pRL-SV40. Following transfection, cells were allowed to recover for 18 h and left untreated or treated with CORT (100 nM) for 6 h prior to lysis. In the experiment evaluating effects of ras-dva overexpression on GH promoter activity, cells were plated and transfected as described in the Materials and Methods section of Chapter 3 with 1 μg Sport6.1 or 1 μg ras-dva expression vector in addition to pRL-SV40 and pGL3-Basic or the pGL3-1727 reporter driven by the GH 5'-flanking region. After recovery, cells were left untreated or treated for 20 h with CORT (100 nM) prior to lysis.

When COS-7 cells were used in transfection experiments, cells were recovered from culture flasks by retrypsinization in the presence of 0.03% EDTA and re-plated ($2.5x10^5$/well in a 24-well plate format) in growth medium for 24 h. To investigate functionality of the predicted proximal Pit-1 site, COS-7 cells were transfected with 0.5 μg pGL3-Basic or 0.5 μg pGL3-2kb and pRL-SV30 together with 0.5 μg Sport6.1 or 0.5 μg Pit-1 expression vector in Opti-MEM I containing 2 μl Lipofectamine. After 6 h, transfection medium was replaced with serum-free DMEM supplemented with 0.1% BSA, and cells were cultured an additional 42 h under basal conditions prior to lysis. To investigate functionality of the putative GR binding sites, COS-7 cells were transfected and cultured as described above with 0.5 μg pGL3-Basic or 0.5 μg pGL3-4kb and pRL-SV30 together with 0.5 μg Sport6.1 or 0.5 μg GR expression vector. During the final 6 h of culture, CORT (100 nM) was added to some wells prior to lysis. For experiments evaluating ras-dva or GH promoter activity, cells were lysed and reporter gene activity was determined and normalized as described in the Materials and Methods section of

Chapter 3. Promoter activity (mean + SEM) is expressed as fold induction over basal cells transfected with pGL3-Basic and, where appropriate, Sport6.1.

In the experiment investigating a potential role for ras-dva in regulating gene expression in the embryonic anterior pituitary gland, e11 chicken cells ($3x10^6$/well in a 12-well format) were co-transfected for 6 h with 1 µg GFP expression vector (228) and either 1 µg Sport6.1 or 1 µg ras-dva expression vector as described in the Materials and Methods section of Chapter 3. Following transfection, cells were allowed to recover for 18 h prior to addition of CORT (1 nM) to appropriate wells for 20 h. Cells were collected by retrypsinization, and successfully transfected cells were identified using flow cytometric detection as described in the Materials and Methods section of Chapter 3. Sorting of the GFP-positive population (10.0 ± 0.3% of cells; n=3 replicate experiments) resulted in collection of approximately 75,000 GFP-positive cells per group. Cells were collected as described in Chapter 3, and total RNA was immediately extracted as described below.

qRT-PCR

Total RNA was isolated from cultured cells and anterior pituitary, lung, kidney, and spleen tissue with the RNeasy Mini kit (Qiagen). Total RNA was isolated from hypothalamus, whole brain, liver, heart, breast muscle, and stomach tissue using the RNeasy Midi kits (Qiagen). Heart, breast muscle, and stomach tissue were digested with proteinase K (100 µg/ml) for 20 m at 55 C after homogenization, and all RNA extractions included an on-column DNase digestion. Quantification of RNA, RT reactions, and real-time PCR reactions were all performed as described in the Materials and Methods sections of Chapters 2 and 3.

Primers (Table 3) designed as described in the methods of Chapter 2 were used to detect mRNA levels of chicken ras-dva, GH, PRL, POMC, TSH-β, GHRH-R, SSTR2, total Pit-1, PGK1, and ACTB. In the ontogeny experiment, ras-dva mRNA levels in pituitary glands from e10 through d48 chickens were transformed and normalized to PGK1 using the equation described in the Materials and Methods section of Chapter 2. For both experiments (e10 through d7, and d7 through d48), the level of ras-dva mRNA in each sample was divided by the mean of ras-dva mRNA levels on d7, such that data (mean + SEM) are expressed relative to d7 (equal to 100%) for each experiment. In the tissue distribution experiments and all cell culture experiments, the amount of target gene mRNA was normalized to the amount of ACTB mRNA and transformed using the equations described in the Materials and Methods section of Chapter 3. For the tissue distribution experiment, the transformed value for each sample was divided by the mean of the transformed value for anterior pituitary tissue, such that data (mean + SEM) are expressed relative to levels in the pituitary gland on e18 (equal to 100%). For the experiment assessing mRNA levels in caudal and cephalic anterior pituitary lobes, the transformed value for each sample was divided by the mean of the transformed value for the lobe with the highest expression level for a particular gene, such that data (mean + SEM) are expressed relative levels in the caudal or cephalic lobe (equal to 100%). In cell culture experiments, the transformed value for each sample was divided by the mean of the transformed value for basal cells receiving no CORT or inhibitors and, where appropriate, transfected with Sport6.1. Data for each gene (mean + SEM) are presented as fold induction over basal mRNA levels for each experiment. In the experiment investigating the effect of CORT treatment on ras-dva mRNA stability, $t_{1/2}$ (h) calculation

and data (mean + SEM) presentation are as described in the Materials and Methods section of Chapter 3.

RT-PCR and 3'-rapid amplification of cDNA ends (RACE)

A pool of cDNA for each tissue collected on e18 was made from the four replicate samples, and primers (Table 3) which amplify full-length ras-dva cDNA were used as an alternative method to qRT-PCR to assess ras-dva tissue distribution. The no RT control for genomic contamination and a no template control were also analyzed. Reactions were conducted using GoTaq Green Master Mix (Promega) as directed, and contained 500 nM each primer and 1 µl cDNA, no RT, or water template, as appropriate. Cycling parameters were as follows: 95 C for 3 m; 35 cycles of 95 C for 45 s, 52 C for 45 s, and 72 C for 2 m; and a final extension at 72 C for 5 m.

For 3'-RACE, cDNA from e18 pituitary glands and a no RT control were amplified using two consecutive, nested PCR reactions. The first reaction contained undiluted cDNA or no RT template and was amplified using Sense135 (Table 3) and oligo-dT (5'-CGGAATTCTTTTTTTTTTTTTTTTTTTV-3') primers, purified with a spin column-100 (Sigma–Aldrich), and diluted 1:100 for subsequent amplification using Sense462 (Table 3) and oligo-dT primers. Reactions contained 1 µl template, 1 µM sense135 or sense462, and 1.5µM oligo-dT primer and were amplified using GoTaq Green Master Mix (Promega) with the following cycling parameters: 94 C for 3 m; 30 cycles of 94 C for 45 s, 48.2 C for 45 s, and 72 C for 2 m; and a final extension at 72 C for 7 m. Products were visualized using agarose gel (1%) electrophoresis and ethidium bromide staining. No product was visible after the first amplification, and the 816 bp product visible after the second amplification was sequenced to confirm it was ras-dva.

Data analysis

The ras-dva ontogeny qRT-PCR data were analyzed as described in the Materials and Methods section of Chapter 2. Promoter activity and cell culture qRT-PCR data (expressed as fold induction over basal cells receiving vehicle and transfected with empty reporter and expression vectors, as appropriate), were subjected to statistical analysis as described in the Material and Methods section of Chapter 3. Tissue distribution data (expressed as percentage of pituitary levels or percentage of the lobe with the highest levels for a given gene) were log2-transformed prior to statistical analysis using SAS software (SAS Institute). To examine ras-dva mRNA tissue distribution on e18, data were analyzed by analysis of variance using the MIXED models procedure with differences between groups determined by the test of least significant difference (PDIFF). Data were analyzed using the t-test procedure (two-tailed) in the experiment evaluating mRNA levels in caudal and cephalic pituitary lobes.

Results

Chicken ras-dva is highly similar to ras-dva from other vertebrates

Although a homolog for chicken ras-dva (accession no. AY729886) has previously been identified through a bioinformatics approach (260), the submission was based on screening GenBank databases using BLAST searches for *Xenopus laevis* ras-dva homologs and was not verified by direct sequencing. Therefore, we sequenced the insert contained in the ras-dva clone in its entirety in both directions. Chicken ras-dva mRNA (accession no. HQ317880) is 1,276 nucleotides long, and the longest open reading frame encodes a predicted protein consisting of 208 amino acids. Our mRNA sequence contains 122 nucleotides in the 5'-untranslated region (UTR) and 527

nucleotides in the 3'-UTR. It should be noted that the final 156 nucleotides in the 3'-UTR of our sequence did not align very well with that of the chicken ras-dva sequence already in GenBank, so 3'-RACE was performed on a cDNA sample generated from a pool of four e18 chickens to determine if alternative transcript variants exist that differ in their 3'-UTR. The resulting PCR product (Figure 28B) was of the predicted length (816 bp), and sequencing confirmed that a single variant corresponding to our clone insert exists in the embryonic pituitary gland. Importantly, this sequence contains a polyadenylation signal (AAUAAA) just upstream of the 3'-end of our sequence and aligns perfectly with the chicken genome sequence. Based on alignments of our ras-dva insert sequence with the assembled chicken genome, ras-dva is an intronless gene and, therefore, consists of only one exon.

BLAST searches of available databases were conducted using the putative coding sequence of chicken ras-dva to identify potential homologs in other species. Orthologs were identified in one mammal, opossum, and several non-mammalian vertebrate species, with frog and several of the fish species having multiple ras-dva paralogs. No orthologs were identified in any other mammalian species or invertebrate organism. Comparison of the predicted amino acid sequence of chicken ras-dva with that of another bird, a lizard, two frog species, several species of fish, and opossum indicates that there is high sequence similarity among these vertebrates (52-92%), especially when positive amino acid substitutions are considered (59-95%). As expected, the species with highest similarity are zebra finch and green anole, while sea lamprey and opossum ras-dva are least similar with chicken ras-dva (Table 4). The predicted amino acid sequence of chicken ras-dva and those of other species were aligned (Figure 26A). In cases where a

species had multiple ras-dva paralogs, the one with the highest identity to chicken ras-dva (Table 4) was used. Black underlined regions depict the G-box motifs necessary for GTP binding and hydrolysis, and consensus sequences for the ras-dva family of small GTPases (260) are given in the legend of Figure 26. The dotted underlined region is the C-terminal prenylation site (-CaaX; where "C" is cysteine; "a" is any aliphatic amino acid; and "X" is cysteine or serine), and the cysteine residue has been demonstrated as essential for

Table 4. Comparison of predicted amino acid (AA) sequence of chicken ras-dva with those of other vertebrate species.

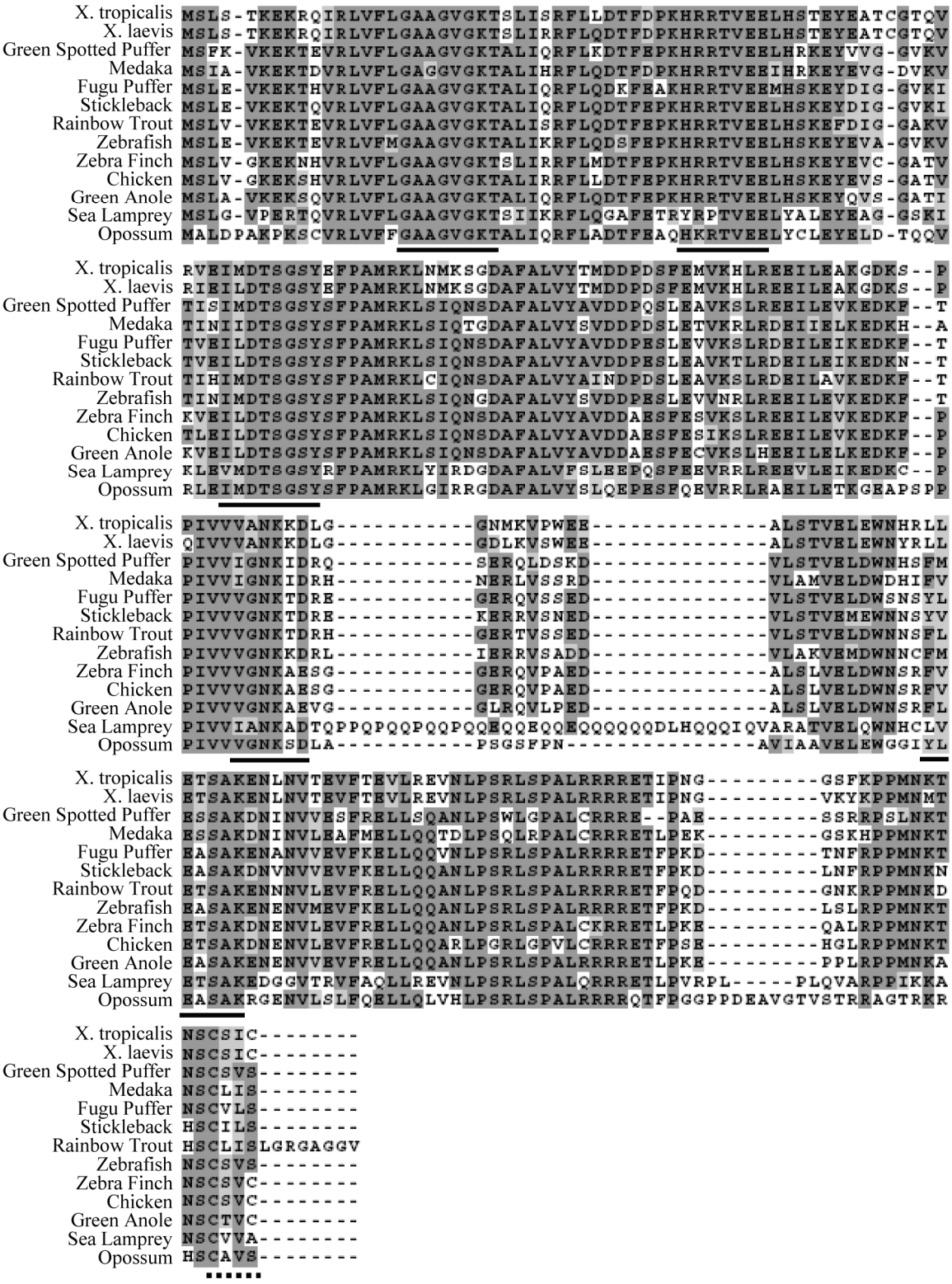
Species		GenBank Accession No.	AA[1]	% Identical AA	% Positive Substitutions
Zebra Finch[2]	Ras-dva	XP_002194543	208	92.3	94.7
Green Anole	Ras-dva	ABY84978	208	84.7	88.0
X. laevis	Ras-dva	NP_001082322	209	46.7	59.4
	Ras-dva2	NP_001165979	209	66.7	75.2
X. tropicalis	Ras-dva	NP_001011503	211	46.7	59.0
	Ras-dva2	NP_001037874	209	67.6	76.2
Fugu Puffer	Ras-dva	ABB84859	212	52.3	63.6
	Ras-dva2	ABB84860	208	74.2	82.3
	Ras-dva3	ABB84861	206	72.2	78.9
Stickleback	Ras-dva3	ABB84862	208	73.2	81.3
Rainbow Trout	Ras-dva3	ABB84863	216	71.9	79.3
Green Spotted Puffer	Ras-dva	CAG02679	205	74.2	80.4
Medaka	Ras-dva3	ABB84864	208	67.5	77.0
Zebrafish	Ras-dva	NP_001073403	211	54.7	65.1
	Ras-dva2	NP_001007782	208	75.1	83.3
Sea Lamprey	Ras-dva	ABY86653	237	49.2	58.8
Opossum[2]	Ras-dva	XP_001377674	219	52.9	62.4

[1]Chicken ras-dva has 208 amino acids.

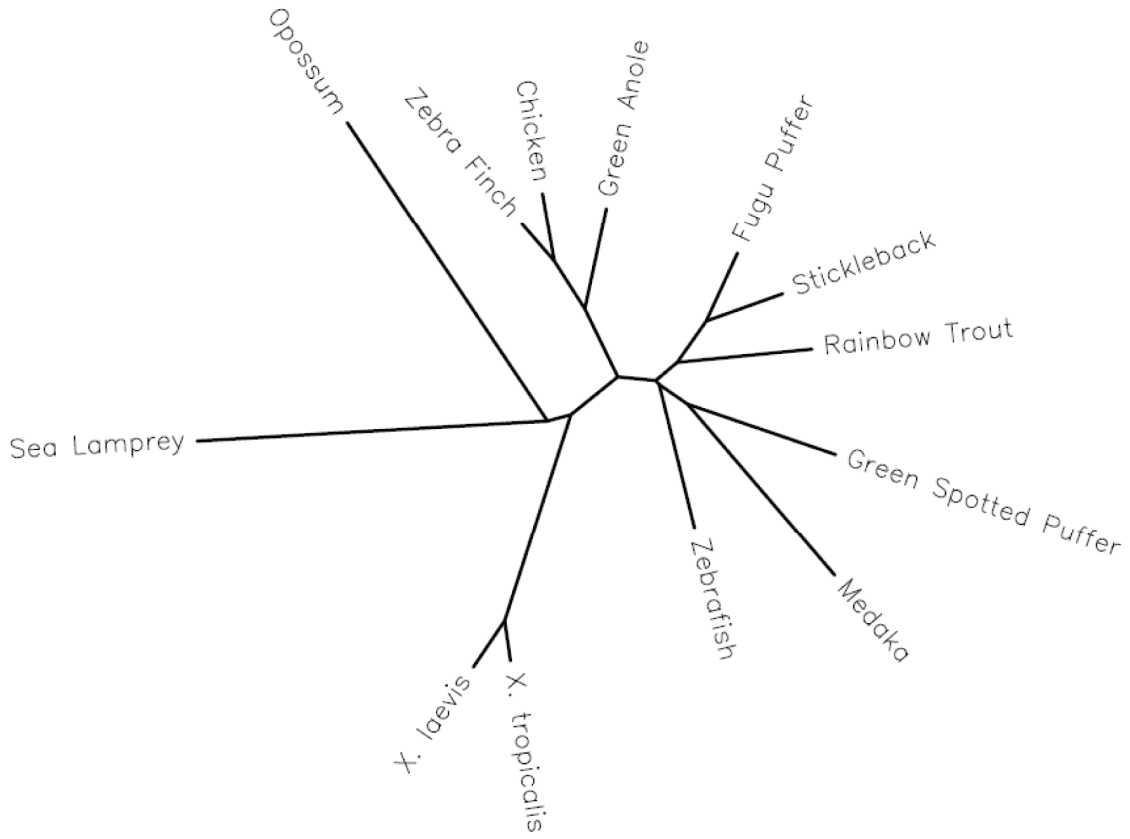
[2]These sequences were predicted by automated computational analysis and are derived from genomic sequence.

Figure 26. Chicken ras-dva is highly similar to ras-dva from other vertebrate species. (A) Predicted amino acid sequences for the indicated species were aligned. The paralog with the highest identity to chicken ras-dva (see Table 4) was used from species with multiple ras-dva genes. Identical residues are denoted in dark gray and similar residues are denoted in light gray. The G-box motifs (G1-G5) necessary for GTP binding and hydrolysis are underlined in black. Consensus for the ras-dva family of small GTPases are as follows: G1 – GAAGVGKT; G2 (switch) – (H/Y)RRTVEE; G3 – I(I/L)DTSGSY; G4 – (V/I)GNKX(D/E); G5 – F(V/L)ESSAK. The C-terminal CaaX box prenylation motif is underlined with dashed line. (B) Unrooted phylogenetic tree of ras-dva amino acid sequences from species listed in panel (A). The length of the lines connecting the species indicates the predicted evolultionary distance.

(A)


X. tropicalis
X. laevis
Green Spotted Puffer
Medaka
Fugu Puffer
Stickleback
Rainbow Trout
Zebrafish
Zebra Finch
Chicken
Green Anole
Sea Lamprey
Opossum

(B)



localization to the plasma membrane and other endomembrane structures (273). Despite the insertions in sea lamprey and opossum ras-dva proteins, the high degree of similarity among the species is apparent. Phylogenetically, chicken ras-dva is closest to zebra finch and green anole ras-dva, furthest from opossum and sea lamprey, and lies between opossum and the other non-mammalian vertebrate species (Figure 26B).

Ras-dva is developmentally regulated in the pituitary gland

The mature anterior pituitary gland consists of five major cell types that emerge during development in a temporally specific manner. Initiation of hormone transcription in these cells occurs during the second half of embryogenesis and continues during early

neonatal life in both mammals and birds (53, 87, 95, 104, 124). We characterized anterior pituitary levels of ras-dva mRNA during this time in order to evaluate if its expression in this tissue is regulated in a manner that indicates it may play a role in development of any pituitary cell type. In addition, we measured ras-dva mRNA in pituitary glands of birds between d7 and d48 (Figure 27). As expected from a previous report (124), ras-dva mRNA steadily increased from mid- to late-embryonic development, between e10 and e18 ($P<0.05$, $n=4$). Pituitary mRNA levels then began to decrease just prior to hatch and were equivalent to levels during mid-embryogenesis through d7. Consistent with the decrease in expression detected between late embryonic development and early post-hatch, pituitary ras-dva mRNA decreased between d7 and d21 and was undetectable in older birds ($P<0.05$, $n=4$). This expression profile indicates that ras-dva may play a developmental role in this tissue, and it is most consistent with a potential role in maturation of somatotrophs and lactotrophs, based on timing of their appearance.

Ras-dva is highly enriched in the pituitary gland during late embryogenesis

Based on the ontogenic profile observed in the developing and mature pituitary gland, tissue distribution of ras-dva mRNA was determined on e18, the age when levels in the anterior pituitary gland were observed to be highest. We examined ras-dva mRNA expression in neuroendocrine tissues as well as tissues that are important glucocorticoid targets, including pituitary, hypothalamus, whole brain, lung, kidney, spleen, liver, heart, breast muscle, and stomach (proventriculus). Standard RT-PCR analysis indicated that ras-dva mRNA was highly enriched in the pituitary gland and also detectable in hypothalamus, brain, kidney, and breast muscle (Figure 28A). The high level of ras-dva mRNA in the anterior pituitary relative to other tissues examined was confirmed with

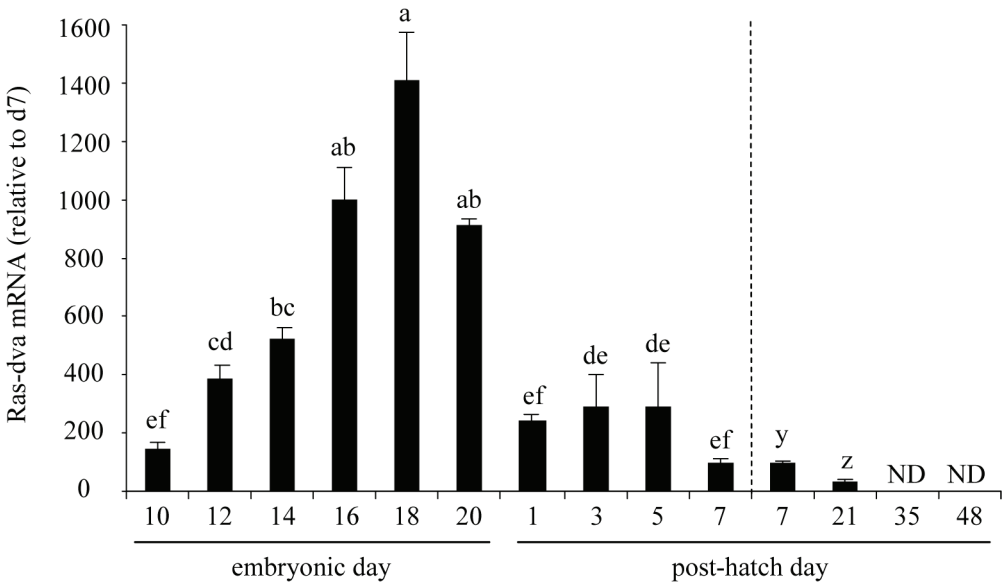


Figure 27. Ontogeny of ras-dva in the developing and mature avian anterior pituitary gland. Total RNA isolated from embryonic day 10 through post-hatch day 48 pituitaries (n=4) was analyzed by qRT-PCR to determine expression levels of ras-dva mRNA, which were normalized to PGK1 mRNA levels. The graph depicts results from two experiments, one profiling mRNA expression from embryonic day 10 through post-hatch day 7 and the other profiling expression from d7 through d48. The data are expressed relative to the level on d7 (set to 100%) for each experiment. Ras-dva mRNA was not detected (ND) on d35 and d48. Values (mean + SEM) denoted with different letters are significantly different from one another ($P<0.05$).

qRT-PCR. Although it was detected everywhere, levels of ras-dva mRNA in the pituitary were over 1,200-fold higher than those in other tissues (Figure 28C; $P<0.05$, n=4). The tissues with the next highest expression level were those where ras-dva mRNA was detected using standard RT-PCR, namely hypothalamus, brain, breast muscle, and kidney. The high level of ras-dva mRNA in the pituitary during late embryogenesis, as well as elevated levels in the hypothalamus and brain, suggests it may play an important role in neuroendocrine system development or function during this time.

Given the degree of pituitary specificity observed for ras-dva on e18, localization within this tissue was further defined in an attempt to implicate a functional role for ras-

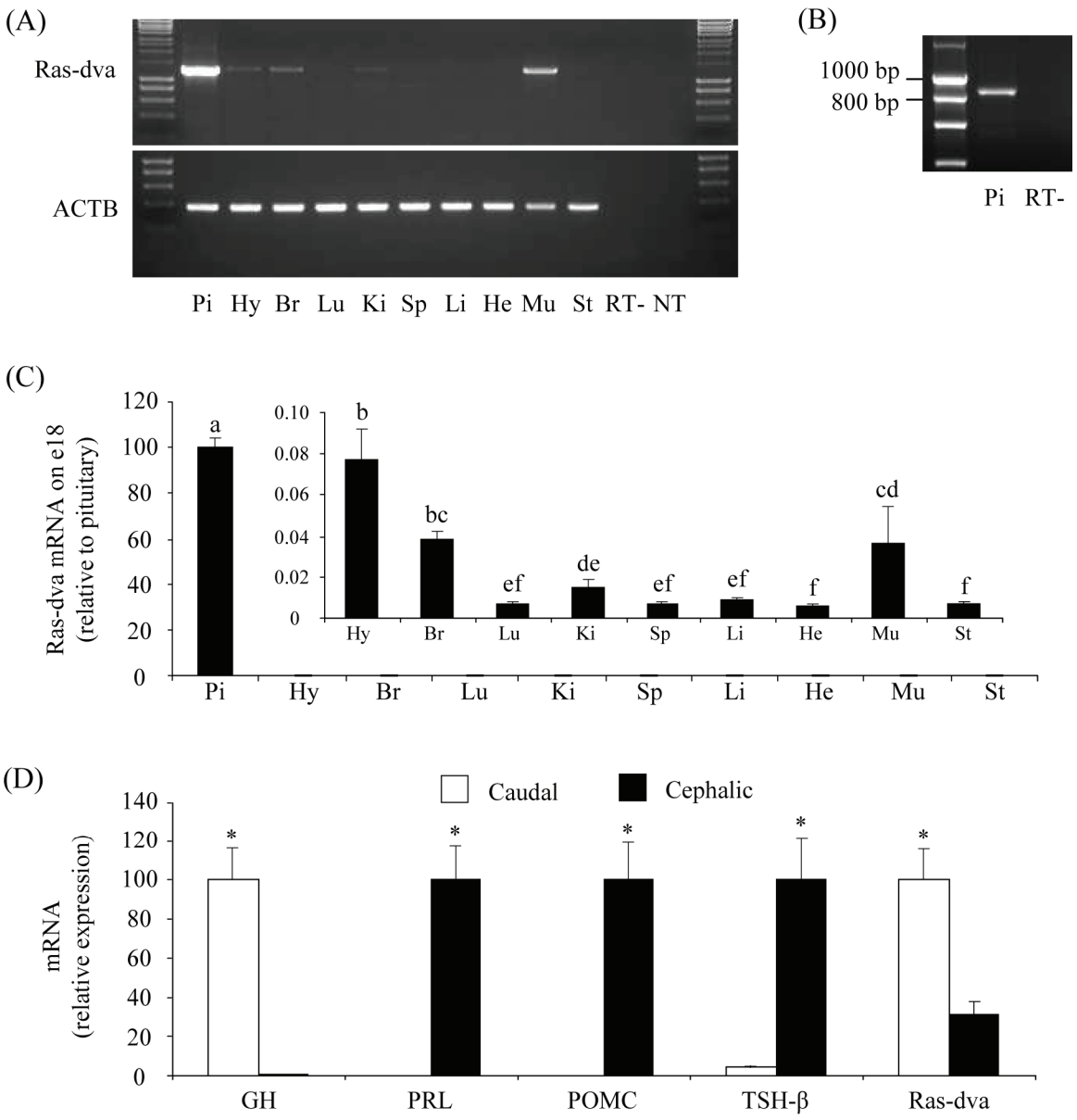


Figure 28. Ras-dva mRNA is highly enriched in the anterior pituitary during late embryogenesis and expressed in both caudal and cephalic lobes of the avian pituitary gland. (A) Agarose gel picture depicting RT-PCR reactions for ras-dva and ACTB from e18 pituitary (Pi), hypothalamus (Hy), whole brain (Br), lung (Lu), kidney (Ki), spleen (Sp), liver (Li), heart (He), breast muscle (Mu), and stomach (St). Control reactions containing no reverse transcriptase (RT-) and no template (NT) were also analyzed. (B) Agarose gel picture of the 3'-RACE PCR product and RT- control conducted on an e18 pituitary sample. (C) Levels of ras-dva mRNA in the indicated e18 tissues (n=4) were determined by qRT-PCR and are expressed relative to the level in the pituitary gland. Levels of ras-dva mRNA were normalized to ACTB mRNA levels. Inset depicts levels in extra-pituitary tissues. Values (mean + SEM) without a common letter are significantly different ($P<0.05$). (D) Levels of GH, PRL, POMC, TSH-β, and ras-dva mRNA were measured with qRT-PCR in caudal and cephalic pituitary lobes (n=3) collected from e18 chicks. Levels of mRNA for each gene are expressed relative to the lobe with the highest expression level for that gene (set to 100%) and were normalized to ACTB mRNA levels. Values (mean + SEM) denoted with an asterisk (*) indicate significantly higher levels in that lobe ($P<0.05$).

dva in a specific cell type(s) during late embryogenesis. The avian anterior pituitary consists of two anatomically distinct caudal and cephalic lobes, and four of the major pituitary cell types are unevenly distributed between the two lobes. GH-producing somatotrophs reside primarily in the caudal lobe, while PRL-producing lactotrophs, TSH-producing thyrotrophs, and ACTH-producing corticotrophs are mainly localized within the cephalic lobe (48-53, 128). Gonadotrophs, which produce LH and FSH, are initially present in the caudal lobe but spread throughout the entire gland as the pituitary develops (53, 54). We measured mRNA levels of GH, PRL, POMC, TSHβ, and ras-dva in caudal and cephalic portions of pituitary glands dissected from e18 chicks. Hormone mRNA levels were highly enriched in the appropriate lobes, as expected, and ras-dva mRNA levels were easily detected in both lobes but approximately 3-fold higher in the caudal lobe than in the cephalic lobe (Figure 28D; $P<0.05$, $n=4$). The distribution of ras-dva mRNA within both lobes of the pituitary gland indicates that it may play a role in multiple cell types that are each specific to different lobes, such as corticotrophs, somatotrophs, and lactotrophs. Alternatively, ras-dva may function in cell types that are more evenly distributed throughout the gland, such as gonadotrophs.

Ras-dva is directly upregulated by glucocorticoids in embryonic pituitary cells

The expression profile of ras-dva in the developing chicken pituitary gland is consistent with *in vivo* regulation by glucocorticoids, as ras-dva mRNA levels increased at the same time during embryogenesis that circulating CORT levels increase and decreased just around hatch similar to serum CORT (10, 138, 140, 141, 266, 274). Further, ras-dva appears to be upregulated by glucocorticoids in e11 chicken embryonic pituitary cells *in vitro* (235). To confirm that CORT can induce ras-dva in embryonic

pituitary cells from birds at an age when both pituitary ras-dva mRNA and circulating glucocorticoid levels are comparably low, and to determine whether CORT can induce ras-dva in cells from embryos at an age when pituitary ras-dva mRNA and circulating glucocorticoids are high, embryonic pituitary cells from e11 (n=4 replicate trials) and e18 (n=3 replicate trials) chickens were left untreated or treated with CORT for the indicated times. On both ages, CORT increased ras-dva mRNA levels in pituitary cells at all time points examined (Figure 29; $P<0.05$). In the case of e18 pituitary cells, CORT treatment for as little as 30 m led to an increase in ras-dva mRNA (Figure 29B; $P<0.05$).

Based on the rapid induction by CORT in both mid- and late-embryogenesis, a set of experiments was conducted to evaluate if ras-dva is a direct transcriptional target of glucocorticoids in the embryonic pituitary gland. In e11 pituitary cells, the transcriptional inhibitor ActD completely blocked induction of ras-dva by CORT (Figure 30A; $P<0.05$, n=3 replicate trials). Promoter activity from a reporter construct driven by 4,154 bp of the 5'-flanking region of the chicken ras-dva gene (pGL3-4kb) was upregulated approximately 4-fold by CORT treatment of e11 cells (Figure 30B; $P<0.05$, n=3 replicate trials), indicating that transcriptional activation of the ras-dva gene occurs upon exposure to glucocorticoids. In order to determine if CORT treatment enhances ras-dva mRNA stability in addition to increasing transcriptional activation of the ras-dva gene, e11 anterior pituitary cells (n=4 replicate trials) were cultured in the absence or presence of CORT for 6 h prior to addition of ActD to inhibit new gene transcription for various time points. Although ras-dva mRNA levels were stimulated by 6 h CORT treatment (Figure 30C; $P<0.05$), half-life under basal conditions ($t_{1/2}=3.7\pm0.4$ h) was the same as that under CORT-treated conditions ($t_{1/2}=3.6\pm0.6$ h; $P=0.98$) and, therefore, CORT does not appear

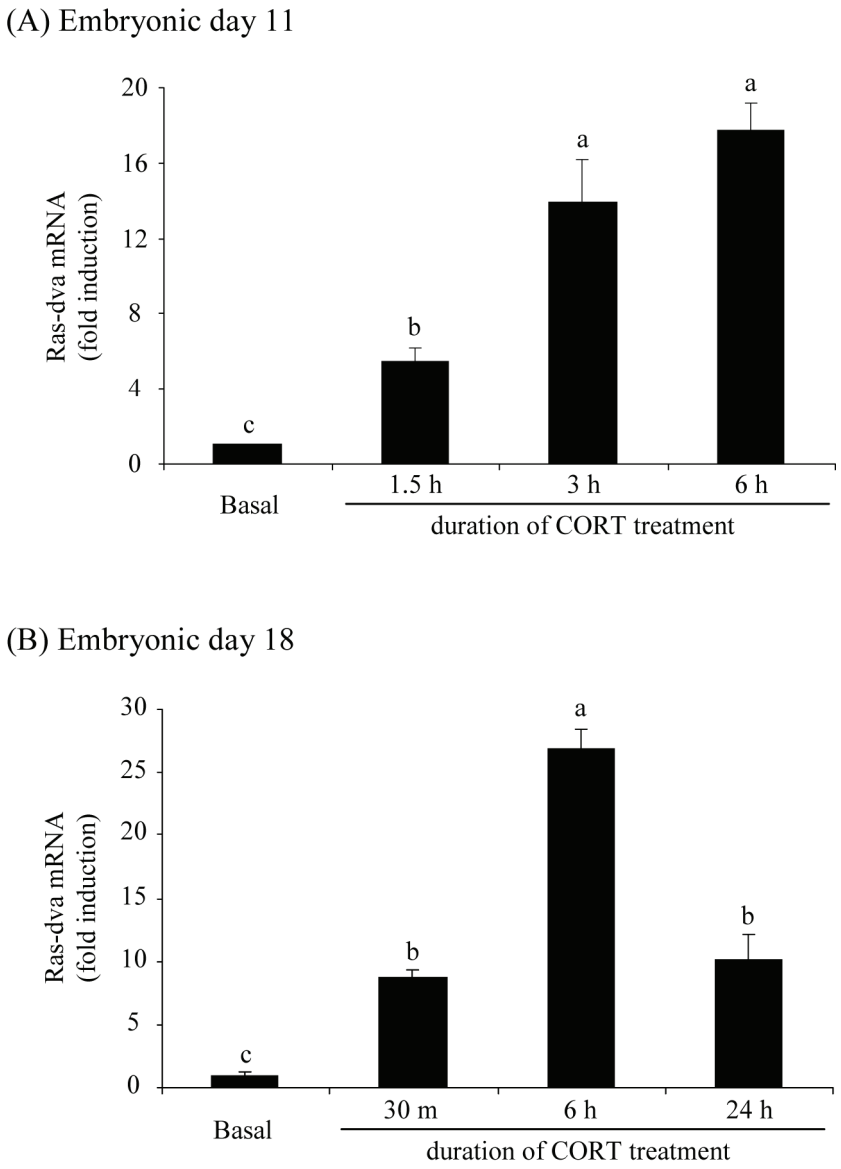


Figure 29. Glucocorticoids induce ras-dva mRNA expression in the chicken anterior pituitary gland during mid- and late-embryogenesis. (A) E11 (n=4) and (B) e18 (n=3) anterior pituitary cells were cultured in the absence or presence of CORT (1 nM) for the indicated times. Levels of ras-dva mRNA, analyzed using qRT-PCR and normalized to ACTB mRNA levels, are expresssed as fold induction over basal cells. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

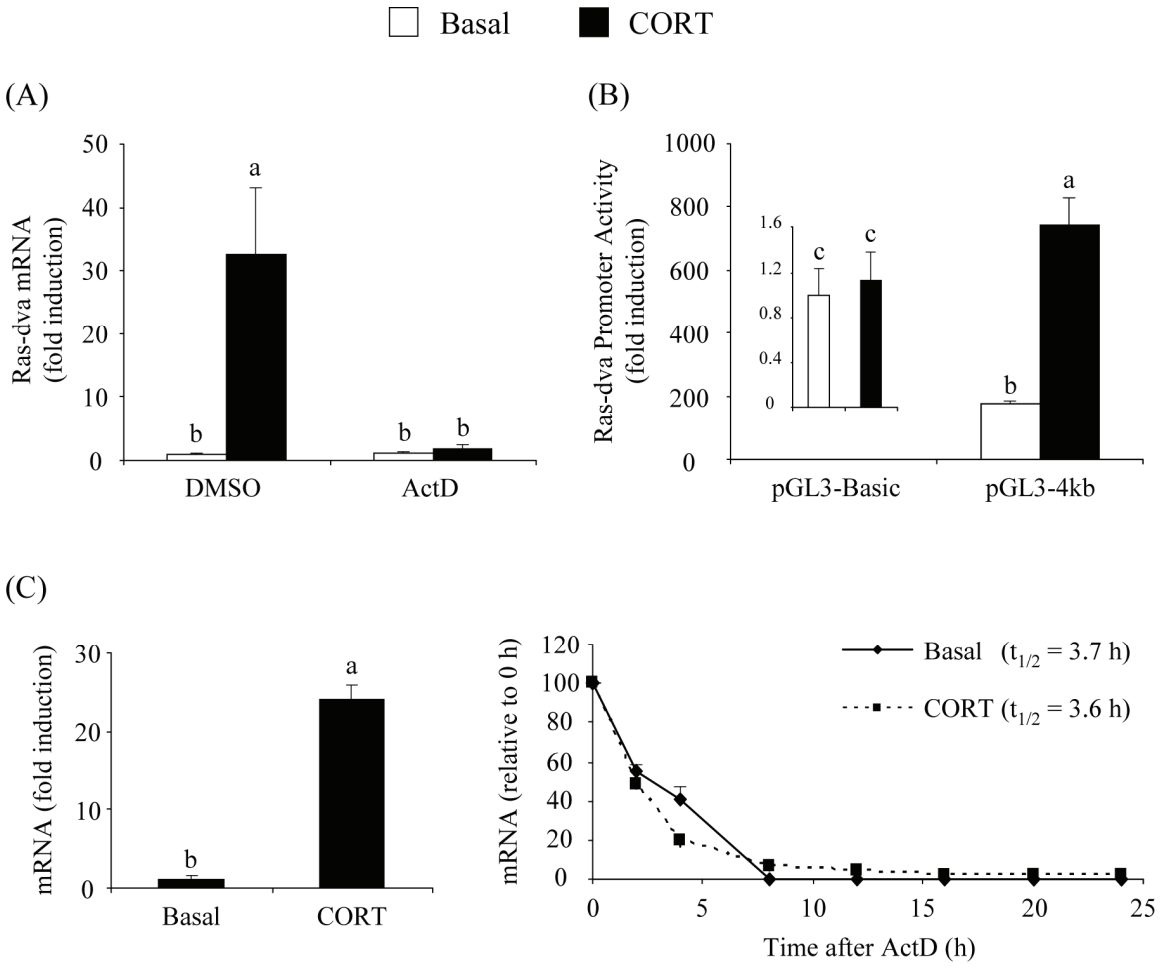


Figure 30. Glucocorticoids induce ras-dva mRNA through transcriptional activation and not through enhancing ras-dva mRNA stability in the embryonic pituitary gland. (A) After pretreatment for 1 h with DMSO or the transcriptional inhibitor ActD (5 μg/ml), e11 chicken anterior pituitary cells (n=3) were cultured in the absence or presence of CORT (1 nM) and DMSO or ActD for 6 h. Levels of Ras-dva mRNA, analyzed by qRT-PCR and normalized to ACTB mRNA levels, are expressed as fold induction over levels in basal cells receiving DMSO. (B) E11 anterior pituitary cells (n=3) were cultured in the absence or presence of CORT (100 nM) for 20 h following transfection with a firefly luciferase reporter construct containing 4,154 bp of the chicken ras-dva 5'-flanking region (pGL3-4kb) or an empty reporter vector (pGL3-Basic) in combination with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic. Inset depicts activity for the pGL3-Basic construct. (C) Anterior pituitary cells (n=4) from e11 chickens were left untreated or treated with CORT (1 nM) for 6 h, after which time ActD was added to the culture. Ras-dva mRNA levels were measured using qRT-PCR after 6 h of CORT treatment (left panel and time 0 h in right panel) and in cells collected at 2, 4, 8, 12, 16, 20, and 24 h after ActD addition. In the left panel, mRNA levels are expressed as fold induction relative to levels in basal cells receiving no CORT for the first 6 h of culture prior to addition of ActD. In the right panel, levels of mRNA in basal and CORT-treated cells are expressed relative to levels at 0 h after ActD addition for each condition (equivalent to the respective levels depicted in the left panel). Values (mean + SEM) without a common letter are statistically different ($P<0.05$).

to increase ras-dva mRNA stability. To determine if glucocorticoid upregulation of pituitary ras-dva is a direct effect, e11 anterior pituitary cells (n=3 replicate trials) were treated with and without CORT for 6 h in the absence or presence of CHX, an inhibitor of protein synthesis. Levels of ras-dva mRNA were induced by CORT, both in the absence and presence of CHX (Figure 31A; $P<0.05$). Finally, neither of the HDAC inhibitors, HC toxin nor TSA, decreased CORT induction of ras-dva mRNA in e11 pituitary cells (Figure 31B; $P<0.05$, n=4 replicate trials). Taken together, these results imply that the rapid induction of ras-dva expression by glucocorticoids during mid- and late-embryogenesis results from direct transcriptional activation of the ras-dva gene.

The ras-dva promoter is highly activated in embryonic pituitary cells

Expression of ras-dva mRNA is highly specific to the anterior pituitary gland during late embryogenesis, and ras-dva appears to be a direct transcriptional target of glucocorticoids in embryonic pituitary cells. Therefore, 5 kb of the 5'-flanking region and 2 kb of the 3'-flanking region of the chicken ras-dva gene were analyzed for putative Pit-1 and GR binding sites using TESS, a web-based search tool for transcription factor binding site prediction. The binding site for Pit-1, a pituitary specific transcription factor, is a rather degenerate AT-rich region with a consensus sequence of Ta/ttAT/aTT/aATT/aCAT, where upper-case letters are more highly conserved (275). Traditional GREs are an inverted repeat of a hexanucleotide motif with a 3 bp spacer, AGAACAnnnTGTTCT (6). Within approximately 4 kb of the ATG start codon, 6 potential Pit-1 binding sites were identified and 2 putative GR binding sites were identified (Table 5). The predicted GR binding sites are actually one copy of the hexanucleotide motif, or a half-site. Based on this, we cloned two fragments of the 5'-

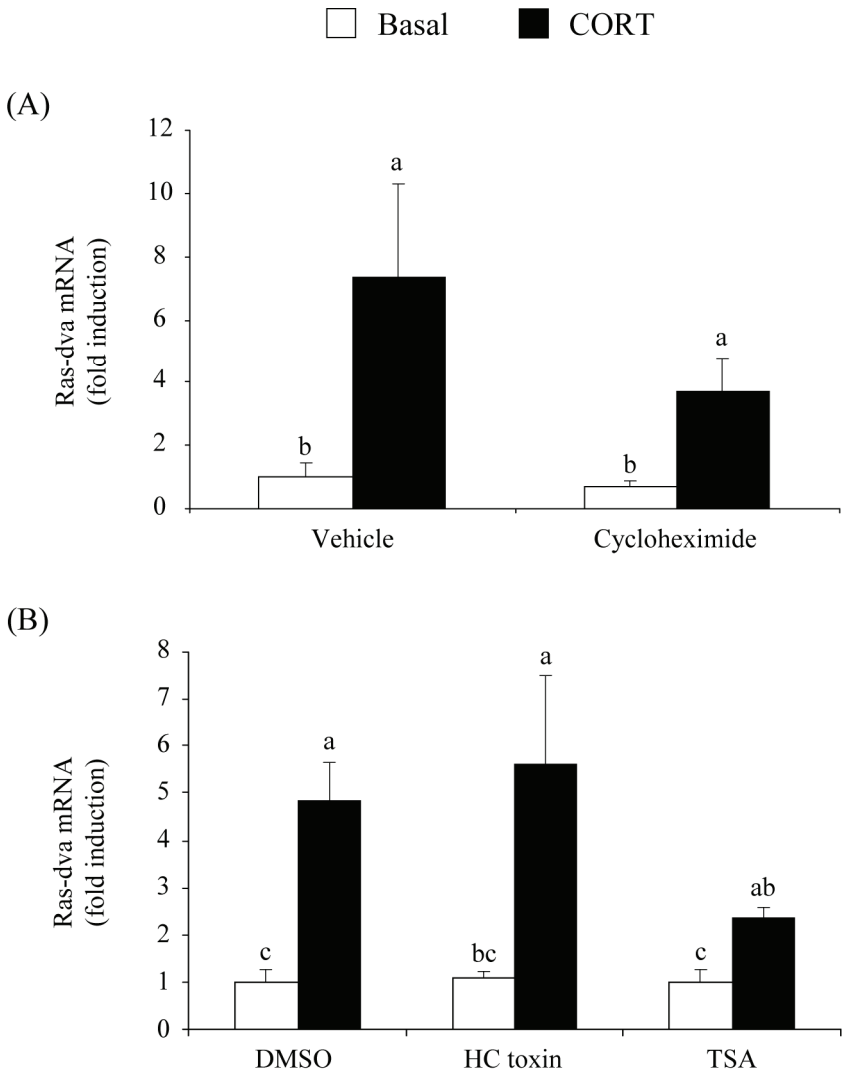


Figure 31. Pituitary ras-dva is upregulated by glucocorticoids in the presence of the protein synthesis inhibitor cycloheximide and the histone deacetylase inhibitors HC toxin and TSA. (A) Anterior pituitary cells (n=3) from e11 chickens were cultured in the absence or presence of CORT (1 nM) with or without cycloheximide (10 µg/ml) for 6 h. Cells receiving cycloheximide were pretreated for 1.5 h prior to addition of CORT. (B) E11 pituitary cells (n=4) were pretreated for 1 h with DMSO, HC toxin (100 nM), or TSA (200 nM) prior to addition of no treatment or CORT (1 nM) for 16 h. (A,B) Levels of ras-dva mRNA, analyzed by qRT-PCR and normalized to ACTB mRNA levels, are expressed as fold induction over basal cells receiving DMSO. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

Table 5. Predicted[1] Pit-1 and GR binding sites located within the 5'-flanking region of chicken ras-dva.

Location[2]	Strand	Sequence (5'→3')[3]
Pit-1		
-341 → -350	+	ACAAATGCAT
-2167 → -2176	+	AAATATTCAT
-2502 → -2514	+	ATGAATTAATCCA
-3200 → -3208	+	AAAATGTAT
-3221 → -3230	-	ATGCATAGAT
-3368 → -3377	+	AATTAATCAC
GR		
-2071 → -2080	+	AGCACAGATG
-4071 → -4062	+	AGAACAGCTG

[1]Pit-1 and GR binding sites were predicted using web-based tool Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess).

[2]Locations of putative binding sites are relative to the ATG start codon.

[3]Underlined nucleotides were changed to create mutated binding sites in pGL3-mPit1, pGL3-mpGR, pGL3-mdGR, and pGL3-mGR.

flanking region into a luciferase reporter construct (Figure 32A). The longer 4,154 bp fragment contains all 8 putative binding sites (pGL3-4kb), and the shorter 2,009 bp fragment contains only the most proximal predicted Pit-1 binding site.

To investigate ras-dva promoter activity under basal and CORT-treated conditions in the embryonic pituitary gland, e11 anterior pituitary cells (n=3 replicate trials) were transfected with the empty reporter pGL3-Basic, pGL3-2kb, or pGL3-4kb and left untreated or treated with CORT for the final 6 h or 24 h of culture. Under basal conditions, pGL3-2kb was activated approximately 40-fold over pGL3-basic, and pGL3-4kb was activated an additional 4-fold above pGL3-2kb, or approximately 160-fold over the empty reporter (Figure 32B, left; $P<0.05$). Furthermore, CORT treatment for either 6 h or 24 h increased promoter activity of pGL3-4kb an additional 4-fold (Figure 32B, right; $P<0.05$), while having no affect on promoter activity of the shorter construct ($P>0.05$). The dramatic increase observed in promoter activity of the pGL3-2kb construct

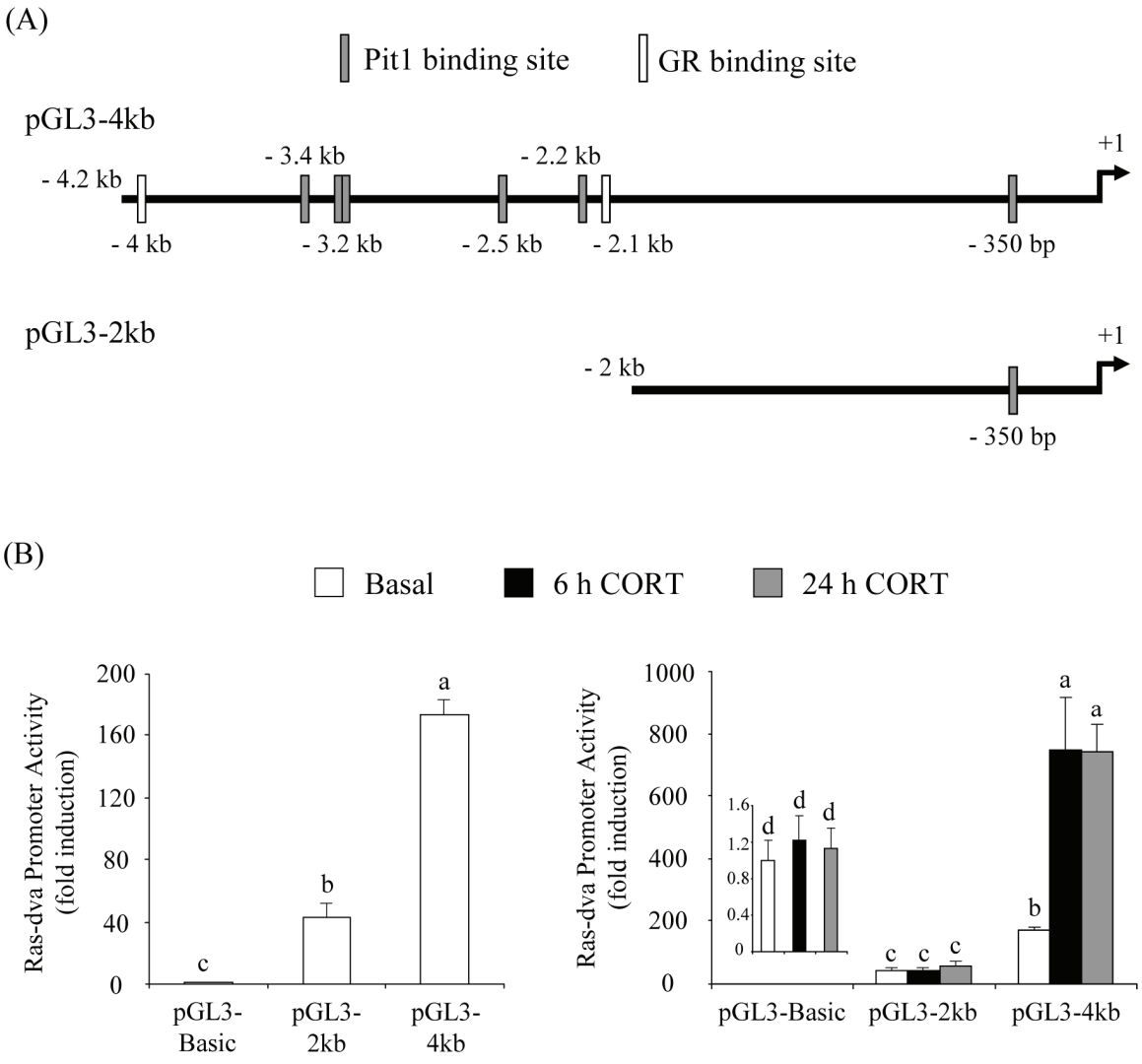


Figure 32. The ras-dva promoter is highly activated in chicken embryonic anterior pituitary cells and can be induced by glucocorticoids. (A) Schematic depicting the two ras-dva promoter-driven firefly luciferase reporter constructs containing 4,154 bp (pGL3-4kb) or 2,009 bp (pGL3-2kb) of the chicken ras-dva 5'-flanking region. The six putative Pit-1 binding sites are denoted with grey boxes, and the two putative GR-binding sites are denoted with white boxes. (B) Anterior pituitary cells (n=3) from e11 chickens were cultured in the absence or presence of CORT (100 nM) for 6 or 24 h following transfection with an empty reporter vector (pGL3-Basic), pGL3-4kb, or pGL3-2kb in combination with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic. The left graph depicts promoter activity under basal conditions only. Inset in the right graph depicts activity for the pGL3-Basic construct. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

over the reporter lacking ras-dva *cis* regulatory elements in pituitary cells suggests that the most proximal putative Pit-1 binding site contained in this construct is involved in the constitutive expression. Likewise, the two predicted GR binding sites contained in pGL3-4kb may play a role in glucocorticoid regulation of ras-dva in embryonic anterior pituitary cells.

Two approaches were taken to investigate functionality of the most proximal predicted Pit-1 binding site and the two putative GR binding sites identified by TESS. First, site-directed mutagenesis was used to mutate critical nucleotides in the sequences to the alternate purine or pyrimidine of their complement (i.e. A↔C, G↔T). The mutated nucleotides are underlined in Table 5. In the second approach, reconstitution of promoter activity through overexpression of Pit-1 or GR in a non-pituitary cell type lacking GR (COS-7) was evaluated. To assess whether the most proximal Pit-1 site is necessary for full ras-dva promoter activation in pituitary cells, e11 cells (n=3 replicate trials) were transfected with pGL3-Basic, pGL3-2kb, or pGL2-mPit1, a reporter construct in which the Pit-1 site was mutated in the context of pGL3-2kb, and cultured under basal conditions. Again, pGL3-2kb reporter activity was dramatically stimulated as compared to the empty reporter construct (Figure 33A; $P<0.05$), and, although pGL3-mPit1 was still activated over pGL3-Basic, it had significantly lower activity than pGL3-2kb ($P<0.05$). Reporter activity in COS-7 cells transfected with pGL3-2kb in either the absence of presence of Pit-1 was higher than activity in cells transfected with pGL3-Basic (Figure 33B; $P<0.05$, n=4), indicating slight activation of the ras-dva promoter

even in non-pituitary cell types. More importantly, ras-dva promoter activity in COS-7 cells co-transfected with pGL3-2kb and an expression vector for Pit-1 was approximately

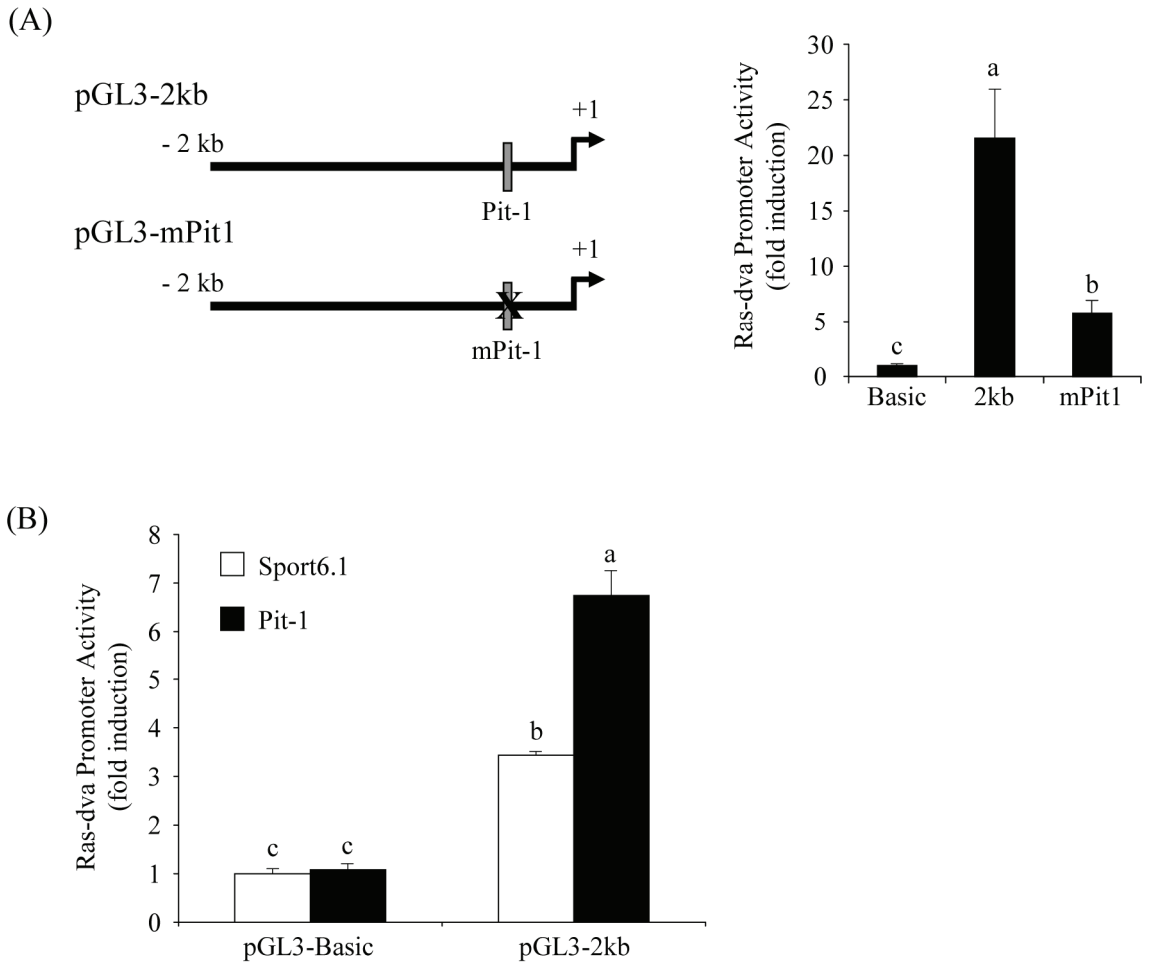


Figure 33. The most proximal Pit-1 binding site located in the chicken ras-dva 5'-flanking region is functional and necessary for full activation of the promoter in embryonic anterior pituitary cells. (A) E11 pituitary cells (n=3) were cultured under basal conditions after tranfection with an empty reporter vector (pGL3-Basic), a reporter construct containing 2,009 bp of the chicken ras-dva 5'-flanking region (pGL3-2kb), or a reporter construct containing the same fragment as pGL3-2kb in which the putative Pit-1 binding site was mutated (pGL3-mPit1) in combination with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over cells transfected with pGL3-Basic. The diagram on the left is a schematic depicting the two Ras-dva promoter-driven firefly luciferase reporter constructs. (B) Monkey kidney-derived COS-7 cells (n=4) were transfected with pGL3-Basic or pGL3-2kb and the empty expression vector Sport6.1 or an expression vector for chicken Pit-1α (Pit-1). Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over cells transfected with Sport6.1 and pGL3-Basic. Values (mean + SEM) without a common letter are statistically different ($P<0.05$).

2-fold higher than in COS-7 cells receiving pGL3-2kb and the empty expression vector, Sport6.1 (Figure 33B; $P<0.05$), indicating that Pit-1 can regulate ras-dva promoter activity.

In the case of the predicted GR binding sites, 3 mutated constructs in which the distal GR site was mutated (pGL3-mdGR), the proximal GR site was mutated (pGL3-mpGR), or both sites were mutated together (pGL3-mGR) were created in the context of pGL3-4kb. Pituitary cells from e11 chickens (n=3 replicate trials) were transfected with pGL3-Basic, pGL3-4kb, pGL3-mdGR, pGL3-mpGR, and pGL3-mGR and treated with and without CORT for 6 h. As observed previously, activity of pGL3-4kb was increased dramatically by CORT (Figure 34A; $P<0.05$). However, mutation of either putative GR binding site alone or the two in combination had no substantial impact on the CORT response of the ras-dva 5'-flanking region, although there was a slight, but significant, reduction in activation of pGL3-mdGR by CORT as compared to pGL3-4kb (Figure 34A; $P<0.05$). An approximate 50% reduction in promoter activity under basal conditions from constructs in which the distal GR binding site was mutated (pGL3-mdGR and pGL3-mGR) was the largest observed effect in this experiment (Figure 34A; $P<0.05$). COS-7 cells lack functional GR, and only in cells co-transfected with pGL3-4kb and an expression vector for GR was ras-dva promoter activity induced by CORT treatment (Figure 34B; $P<0.05$). However, while this may indicate that GR protein is necessary for the CORT induction of ras-dva promoter activity, the response was marginal at best.

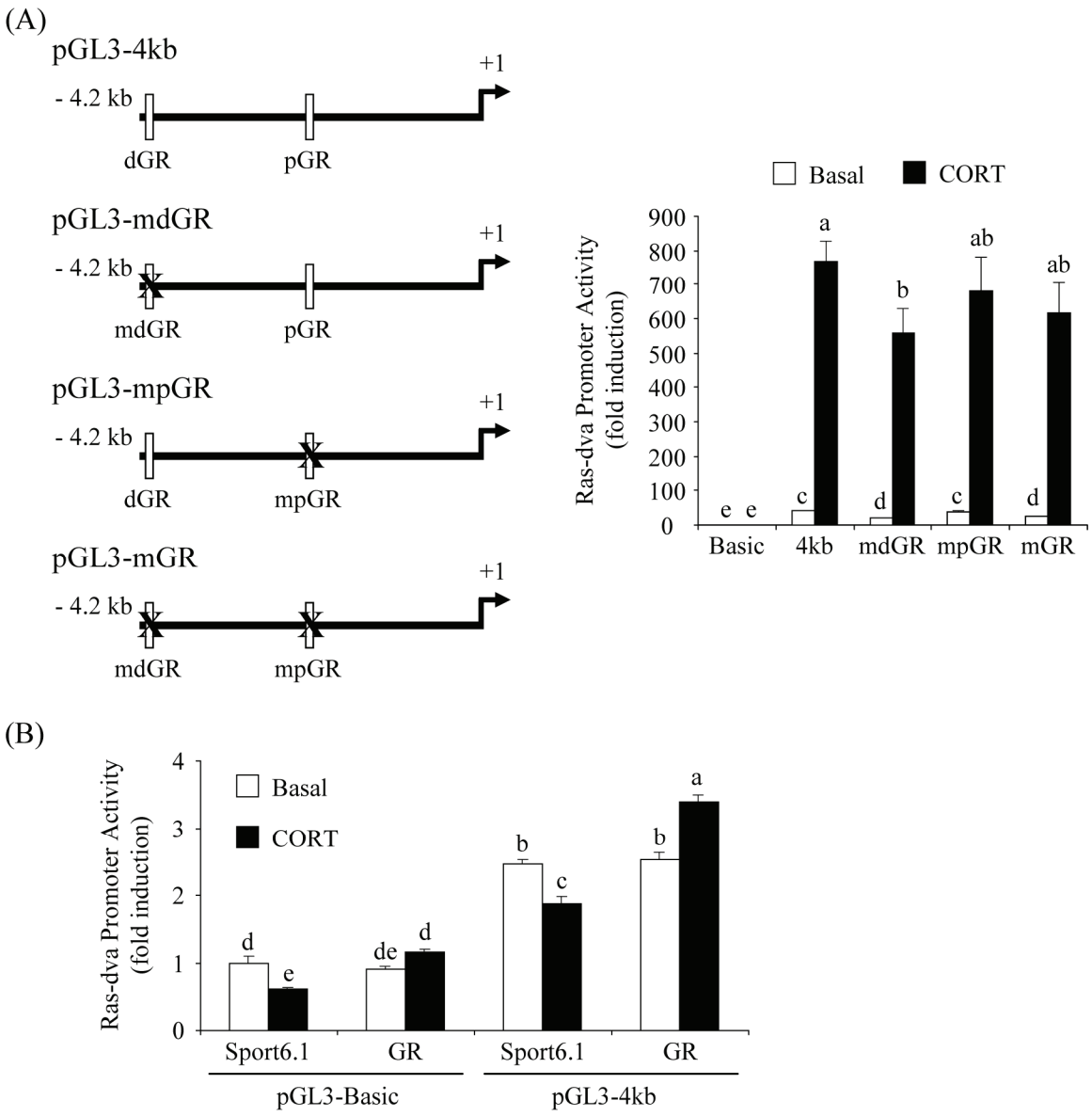


Figure 34. Neither of the predicted GR binding sites located within 4 kb of the chicken ras-dva 5'-flanking region are involved in glucocorticoid-induced expression of ras-dva in the embryonic anterior pituitary gland. (A) E11 pituitary cells (n=3) were cultured in the absence or presence of CORT (100 nM) for 6 h after transfection with an empty reporter vector (pGL3-Basic), a reporter construct containing 4,154 bp of the chicken ras-dva 5'-flanking region (pGL3-4kb), or constructs containing the same fragment as pGL3-4kb in which the distal and proximal putative GR binding sites were mutated singly (pGL3-mdGR and pGL3-mpGR, respectively) or in combination (pGL3-mGR) together with a renilla luciferase expression construct. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic. The diagram on the left is a schematic depicting the four ras-dva promoter-driven firefly luciferase reporter constructs. (B) Monkey kidney-derived COS-7 cells (n=4) were transfected with pGL3-Basic or pGL3-4kb and the empty expression vector Sport6.1 or an expression vector for chicken GR. Promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with Sport6.1 and pGL3-Basic. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).

Ras-dva overexpression does not affect pituitary GH mRNA levels

Pituitary ras-dva expression is developmentally regulated, and it appears to be a direct transcriptional target of glucocorticoids in the embryonic anterior pituitary gland. Moreover, the enrichment of ras-dva in the pituitary during late embryogenesis and apparent functionality of at least one of the Pit-1 binding sites in its 5'-flanking region indicate it may mediate CORT initiation of pituitary hormones in cells of the Pit-1 lineage, namely somatotrophs and lactotrophs (44, 121, 143-150, 152-155, 160). Two experiments were conducted to evaluate whether overexpression of ras-dva in chicken embryonic pituitary cells would alter CORT-regulated expression of pituitary hormones initiated during mid- to late-embryogenesis. Examining the effect of both CORT treatment and ras-dva overexpression on e11, when both circulating glucocorticoids and pituitary ras-dva mRNA levels are low, allowed us to assess whether ras-dva can mimic effects of CORT on pituitary hormone regulation.

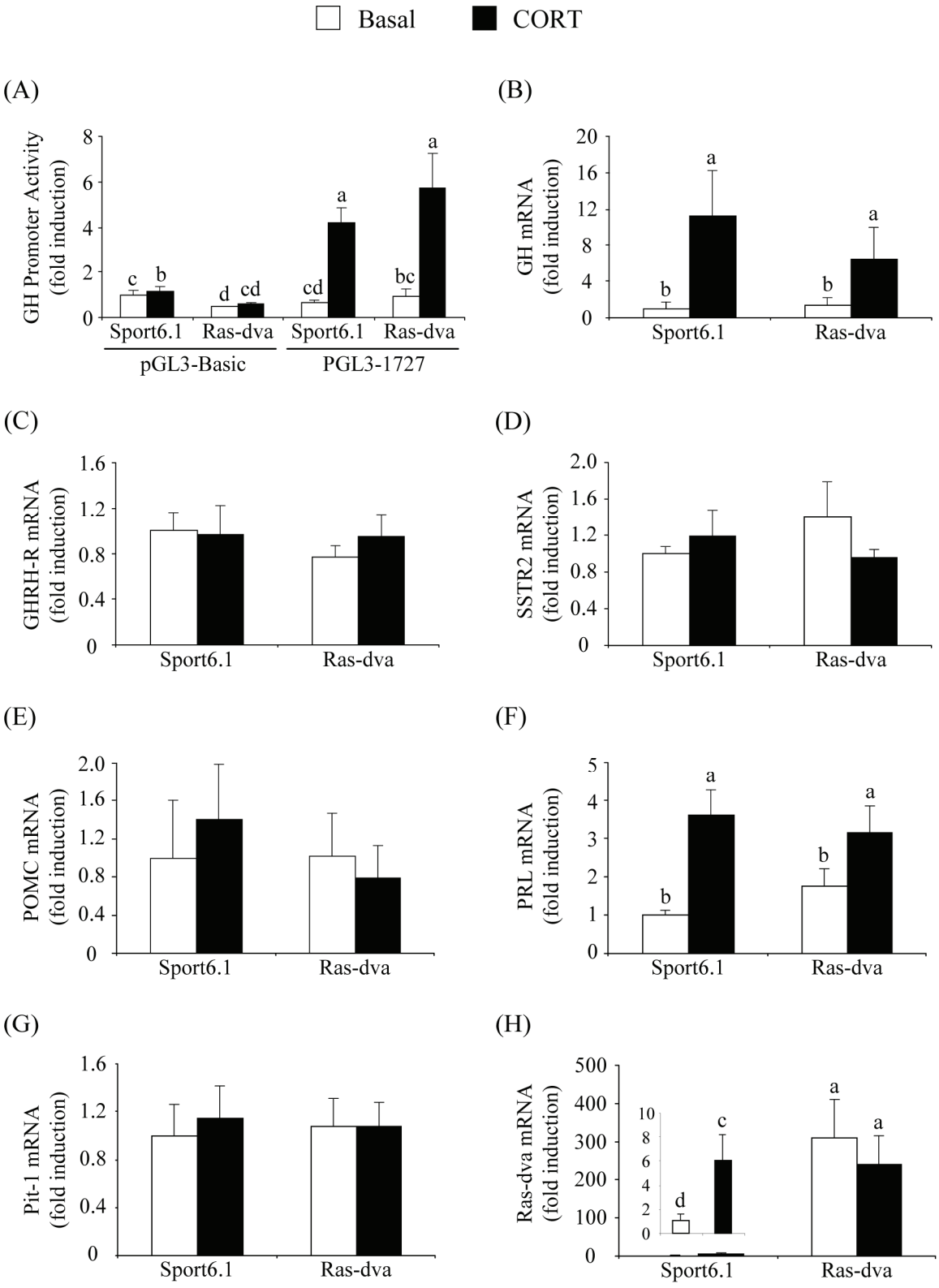
In one experiment, pituitary cells (n=4 replicate trials) transfected with an empty reporter vector (pGL3-Basic) or the reporter construct driven by the GH 5'-flanking region (pGL3-1727) in combination with Sport6.1 or an expression vector for ras-dva were cultured in the absence or presence of CORT for 20 h. CORT treatment increased GH promoter activity approximately 4-fold (Figure 35A; $P<0.05$), as expected, but overexpression of ras-dva did not influence the induction ($P>0.05$). In the second experiment, anterior pituitary cells (n=4 replicate trials) were transfected with Sport6.1 or an expression vector for ras-dva along with the GFP expression vector to allow collection of successfully transfected (GFP-positive) cells. We then analyzed mRNA levels of GH, PRL, and pituitary receptors for the major hypothalamic regulators of GH (40), GHRH-R

and SSTR2. In addition, we determined the effect of ras-dva overexpression on POMC mRNA, which is repressed by glucocorticoids (268), as well as mRNA levels for Pit-1 and TSH-β (data not shown). As previously reported (150, 152, 153, 155, 159, 160, 235) and similar to the results shown in Figures 29-31, CORT treatment induced mRNA levels for ras-dva (6-fold), GH (12-fold), and PRL (3.5-fold) (Figure 35B, F, and H; $P<0.05$). However, CORT did not affect POMC, GHRH-R, SSTR2, or TSH-β expression (Figure 35C- E, G, and data not shown; $P>0.05$). Ras-dva mRNA levels were 250- to 300-fold higher in cells transfected with the ras-dva expression vector as compared to those transfected with Sport6.1 (Figure 35H; $P<0.05$). Despite this, overexpression of ras-dva did not alter mRNA levels for any of the genes examined under basal or CORT-treated conditions (Figure 35B-G and data not shown; $P>0.05$).

Discussion

In this report, which is the first description of ras-dva in the neuroendocrine system of any species, expression and regulation of ras-dva in the chicken pituitary gland was characterized. Pituitary ras-dva mRNA levels increased between e10 and e18, decreased slightly just prior to hatch, and remained low or undetectable during post-natal life. On e18, ras-dva mRNA was highly enriched in the anterior pituitary gland as

Figure 35. Overexpression of ras-dva does not influence glucocorticoid regulation of GH or pituitary receptors for its major hypothalamic regulators. Anterior pituitary cells (n=4) from e11 chickens were transfected with (A) a firefly luciferase reporter construct containing 1,727 bp of the chicken GH 5'-flanking region (pGL3-1727) or an empty reporter vector (pGL3-Basic) in combination with a renilla luciferase expression construct and an empty expression vector (Sport6.1) or an expression vector for ras-dva or (B-H) Sport6.1 or ras-dva expression vectors alone. (A) Following addition of no treatment or CORT (1 nM) for 20 h, promoter activity in each sample was determined by dividing firefly luciferase activity by renilla luciferase activity and is expressed as fold induction over basal cells transfected with pGL3-Basic and Sport6.1. (B-H) Cells were left untreated or treated with CORT (1 nM) for 20 h, and levels of GH, GHRH-R, SSTR2, POMC, PRL, Pit-1 and ras-dva mRNA were measured by qRT-PCR and are expressed as fold induction over basal cells transfected with Sport6.1. Levels of mRNA for all genes were normalized to ACTB mRNA levels. Values (mean + SEM) without a common letter are significantly different ($P<0.05$).


Basal
CORT
(A)
GH Promoter Activity (fold induction)
Sport6.1
Ras-dva
Sport6.1
Ras-dva
pGL3-Basic
PGL3-1727
(B)
GH mRNA (fold induction)
Sport6.1
Ras-dva
(C)
GHRH-R mRNA (fold induction)
Sport6.1
Ras-dva
(D)
SSTR2 mRNA (fold induction)
Sport6.1
Ras-dva
(E)
POMC mRNA (fold induction)
Sport6.1
Ras-dva
(F)
PRL mRNA (fold induction)
Sport6.1
Ras-dva
(G)
Pit-1 mRNA (fold induction)
Sport6.1
Ras-dva
(H)
Ras-dva mRNA (fold induction)
Sport6.1
Ras-dva

compared to other tissues. During both mid- and late-embryogenesis, glucocorticoids rapidly induced ras-dva mRNA expression in cultured anterior pituitary cells, and the mechanism of induction appears to involve a direct effect on transcriptional activation of the ras-dva gene. Potential sites that may mediate pituitary-specific and glucocorticoid-induced ras-dva expression were identified within the regulatory region of the chicken ras-dva gene.

Interestingly, homologs for ras-dva were identified in several different taxa of non-mammalian vertebrates, but only in one mammalian species (Table 4). In all vertebrates, the principles of pituitary gland commitment and development are very similar, although positional location of the differentiated cell types can differ (53, 55, 73, 77). In particular, the major cell types are localized to anatomically discrete areas in a rostral-to-caudal organization in non-mammalian vertebrates (48-54, 276, 277), while cell types with a similar phenotype in the mammalian pituitary are localized to distinct regions in a more ventral-to-dorsal manner (53, 55, 73, 77). It is possible that ras-dva participates in a rostral-to-caudal signaling gradient unique to non-mammalian vertebrates leading to the different pituitary cell type distribution as compared to mammals. A role such as this is consistent with its postulated involvement in the signaling network essential for anterior ectoderm patterning and development of head structures in *Xenopus* embryos (273).

In the chick embryo, serum glucocorticoid concentrations rise from subnanomolar levels on e10 to 40-50 nM on e17, and decrease slightly on e20 (10, 138, 140, 141, 174, 266). Levels remain in the 10-30 nM range through d7 (274). The expression profile of ras-dva mRNA in the developing anterior pituitary during the latter half of

embryogenesis and early post-hatch development observed in this study reflect these levels of serum CORT (Figure 27), indicating that its expression may be regulated by circulating glucocorticoids *in vivo*. Pituitary ras-dva mRNA increased almost 10-fold between e10 and e18, similar to what has been reported previously (124), and decreased slightly just prior to hatch. Beyond d21, ras-dva was no longer detected in the anterior pituitary. Exposure of e11 anterior pituitary cells to levels of CORT reflective of those found in circulation on e14 increased ras-dva mRNA (Figure 29), strongly suggesting that the rise in ras-dva mRNA observed between e10 and e18 is a result of increasing circulating glucocorticoid levels. In the current study, ras-dva mRNA was upregulated by CORT within 30 m, and this upregulation was demonstrated to be sensitive to transcriptional inhibition but insensitive to an inhibitor of protein synthesis. Additionally, CORT treatment increased ras-dva promoter activity but did not influence mRNA half-life (Figures 29-31). Taken together, these observations strongly suggest that ras-dva is directly regulated at the transcriptional level by glucocorticoids.

Examination of the 5'-flanking region of chicken ras-dva revealed the presence of two putative GR binding sites within the region that is responsive to CORT treatment (Figure 32), although mutagenesis of these sites did not substantially affect induction of the promoter (Figure 34). Classically, the mode of action of glucocorticoids involves binding to intracellular GR, which then functions as a ligand-activated transcription factor to regulate gene expression through direct DNA binding or indirect association with DNA through protein-protein interactions. GR can bind to DNA as a homodimer at GREs or in conjunction with other factors at half-sites within composite binding elements (5, 278). The GR half-sites within the ras-dva promoter do not appear to be part of a

composite element, so it is more likely that the mechanism through which GR is transcriptionally activating the ras-dva gene is through tethering of the receptor to DNA via another transcription factor. There are several examples of this in the literature, including AP-1, nuclear factor-κ B (NF-κB), and STAT5 (5, 14, 22, 279). Both an NF-κB site and an AP-1 site were predicted within the CORT-responsive region of the ras-dva promoter. In most cases, the interaction of GR with these two transcription factors involves negative regulation of gene expression (5). Recently, however, it was demonstrated that glucocorticoids can stimulate GnRH-R expression in a pituitary gonadotroph cell line in a mechanism that involves recruitment of GR to the AP-1 site within the GnRH-R regulatory region bound by c-Jun and c-Fos (14). It is possible that a similar mechanism is involved in glucocorticoid stimulation of ras-dva expression in pituitary cells. The predicted AP-1 binding site within the ras-dva regulatory region is adjacent to the most distal putative Pit-1 binding site. It is tempting to speculate that CORT-bound GR is recruited to this region by AP-1, and transcriptional activation within pituitary cells involves its interaction with Pit-1. It is also possible that GR does not interact with *cis*-regulatory elements within the ras-dva 5'-flanking region at all, but rather sequesters a repressor protein and relieves transcriptional inhibition of ras-dva when glucocorticoids are present.

In addition to *in vivo* regulation by glucocorticoids, the observed increase in pituitary ras-dva expression after e10 is consistent with a relief from repression by Anf-1/Hesx1 in this tissue, which was shown to downregulate ras-dva in the anterior neural ectoderm of *Xenopus* embryos (259). In the developing mouse pituitary gland, Anf-1/Hesx1 expression is observed only in progenitor cell populations and is thought to be

important for initial progression of pituitary gland formation and cell proliferation (55, 77, 79, 81, 132). Its subsequent downregulation allows appearance of Prop-1-dependent pituitary cell lineages, which include gonadotrophs as well as thyrotrophs, lactotrophs, and somatotrophs (81, 132). Early expression of Anf-1/Hesx1 in Rathke's pouch and its subsequent downregulation prior to emergence of functionally differentiated cells during chicken pituitary organogenesis has recently been demonstrated in our laboratory (Proszkowiec-Weglarz and Porter, unpublished). Therefore, relief from repression as a result of Anf-1/Hesx1 downregulation by the end of the first half of embryonic development, in combination with activation by circulating glucocorticoids that increase during the latter half of embryonic development, likely contributes to the increase in ras-dva mRNA expression that occurs between e10 and e18.

The ras-dva promoter is highly activated in embryonic anterior pituitary cells, regardless of treatment with glucocorticoids (Figures 32-34). In conjunction with the relative pituitary-specific expression of ras-dva observed on e18 in this study, the high level of activation in pituitary cells strongly suggests that elements within the regulatory region are stimulated by factors enriched in, or specific to, the anterior pituitary gland. Pit-1 is a pituitary-specific transcription factor essential for functional differentiation and expansion of three cell types in the anterior pituitary: thyrotrophs, somatotrophs, and lactotrophs (83). Six potential Pit-1 binding sites were identified within 4 kb of the translational start site, and one of these is located within only -350 bp (Table 5). Mutation of this most proximal Pit-1 site substantially inhibited ras-dva promoter activation in pituitary cells, and actually repressed it to the level observed in COS-7 cells, a non-pituitary cell type (Figure 33), indicating that this is an important site in pituitary

induction of ras-dva expression. Overexpression of Pit-1 in COS-7 cells increased ras-dva promoter activity, although it did not fully restore it. This implies that, while the proximal Pit-1 site appears to be essential for full activation of ras-dva expression in pituitary cells, other cell-type specific factors are also involved. Nonetheless, functionality of the Pit-1 site and the presence of additional predicted Pit-1 binding sites suggest that ras-dva is expressed in cells of the Pit-1 lineage.

On e18, ras-dva mRNA expression was detected within both lobes of the chicken pituitary (Figure 28), indicating that expression is not restricted to any particular cell type. Thyrotrophs and lactotrophs are found in the cephalic lobe, while somatotrophs reside in the caudal lobe (49-53, 128), and ras-dva may be found in multiple Pit-1-expressing cell types. Alternatively, expression of ras-dva may be restricted to one cell type within the Pit-1 lineage and also expressed in cells that are distributed throughout the gland, such as gonadotrophs that secrete FSH and LH or folliculostellate cells that play an important role in autocrine/paracrine regulation of the pituitary (56). Although overexpression of ras-dva in e11 anterior pituitary cells did not influence hormone mRNA expression in cells of the Pit-1 lineage (Figure 35 and data not shown), this does not mean that it may not play a role in regulating other aspects of cell function, including hormone secretion or proliferation. A major source of pituitary growth factors is folliculostellate cells, and pituitary FGF2 is known to regulate proliferation and hormone secretion of multiple pituitary cell types (280, 281). Interestingly, ras-dva was identified as a probable component of the FGF signaling network during anterior ectoderm development (260), and FGF2 signaling has recently been identified as an important autocrine regulator of folliculostellate cell growth (282). Perhaps ras-dva functions to

mediate these autocrine and/or paracrine effects of FGF2 in folliculostellate and/or hormone-producing cells within the pituitary.

Although the developmental profile and upregulation by glucocorticoids are consistent with initiation of hormone expression in pituitary somatotrophs and lactotrophs, our results do not indicate that ras-dva is a major limiting factor in the process of hormone initiation. Interestingly, homologs for ras-dva were identified in several different taxa of non-mammalian vertebrates, but only in one mammalian species (Table 4). In all vertebrates, the principles of pituitary gland commitment and development are very similar, although positional location of the differentiated cell types can differ (53, 55, 73, 77). In particular, the major cell types are localized to anatomically discrete areas in a rostral-to-caudal organization in non-mammalian vertebrates (48-54, 276, 277), while cell types with a similar phenotype in the mammalian pituitary are localized to distinct regions in a more ventral-to-dorsal manner (53, 55, 73, 77). It is possible that ras-dva participates in a rostral-to-caudal signaling gradient unique to non-mammalian vertebrates leading to the different pituitary cell type distribution as compared to mammals. A role such as this is consistent with its postulated involvement in the signaling network essential for anterior ectoderm patterning and development of head structures in *Xenopus* embryos (273).

This is the first report to demonstrate that ras-dva is present and transcriptionally regulated by glucocorticoids in the anterior pituitary gland of any species. The expression profile of pituitary ras-dva in the embryonic, neonatal, and mature gland indicates it may play a critical role in development of this important tissue, although at this time a function has not yet been identified. The presence of several putative Pit-1 binding sites

in the 5'-flanking region of chicken ras-dva, and the demonstration that at least one of these is functional, suggests that ras-dva is expressed in cells of the Pit-1 lineage. In conclusion, ras-dva was identified as a novel Pit-1 and glucocorticoid-regulated gene in the developing anterior pituitary gland.

CHAPTER 5

Perspectives and Future Directions

The overall objective of these studies was to characterize ontogenic and glucocorticoid-regulated gene expression in the developing neuroendocrine system. To this end, three studies were conducted using chickens as the primary developmental model. In the first, we measured mRNA levels of hypothalamic releasing and release-inhibiting factors, pituitary receptors for these factors, and pituitary hormones for adrenocorticotropic, thyrotropic, somatotropic, lactotropic, and gonadotropic neuroendocrine axes during the last half of embryogenesis and the first week of post-hatch life. Next, we investigated mechanisms behind glucocorticoid initiation of pituitary GH expression during somatotroph development. Finally, we characterized pituitary expression and glucocorticoid regulation of ras-dva, a developmentally interesting gene recently identified by our laboratory as a novel CORT-induced target in embryonic anterior pituitary cells (124, 235).

The developmental and functional conservation of the neuroendocrine system highlights its essential role in maintenance of metabolism, growth, body composition, reproduction, and the stress response in vertebrate organisms. Given the importance of this system, it is rather surprising that no comprehensive studies investigating global changes in gene expression during hypothalamic and pituitary development have been conducted. Although there have been reports published investigating a single axis or one tissue (53, 87, 95, 124, 126, 171-173), this study is the first to characterize ontogenic expression profiles for major hormones and receptors in all five hypothalamo-pituitary axes within a single *in vivo* system. From these results, precise timing of initiation and establishment of each axis were determined based on the measured mRNA expression profiles (Figure 11). Initiation occurred when mRNA levels of the pituitary hormone

began to significantly and substantially increase, and each axis was established when mRNA of all major components had reached maximum expression levels. Clearly, a major limitation to our findings is that the conclusions regarding initiation and establishment of neuroendocrine axis activity are based solely on changes in mRNA expression level. In the case of pituitary hormones, the age of axis initiation that was determined in this study is in good agreement with previous reports of pituitary cell type appearance based on immunohistochemical analysis (51-54, 94, 97, 99, 103-106). Therefore, it is not unreasonable to draw inferences regarding protein production based on expression levels of mRNA.

One main function of the neuroendocrine system is secretion of anterior pituitary hormones, which is ultimately controlled by hypothalamic factors. In order to truly assess neuroendocrine activity, one would need to measure release of hypothalamic factors into the portal vasculature, activation of pituitary receptors for those factors, and release of pituitary hormones into circulation as a result of receptor activation. Performing such measurements for each of the five neuroendocrine axes was beyond the scope of the current study, not to mention technical difficulties associated with determining levels of hypothalamic factors in the embryonic/early post-hatch portal vasculature and assessing receptor activation *in vivo*. However, future studies should focus on confirming the mRNA expression profiles determined here by measuring protein levels of those components for which this information has not yet been published. Further, functionality of hypothalamic-pituitary axes as a unit can be evaluated by measuring, at different ages, the ability of anterior pituitary cells to respond to hypothalamic factors in an *in vitro* cell culture system. In regards to this, the results presented here provide valuable information

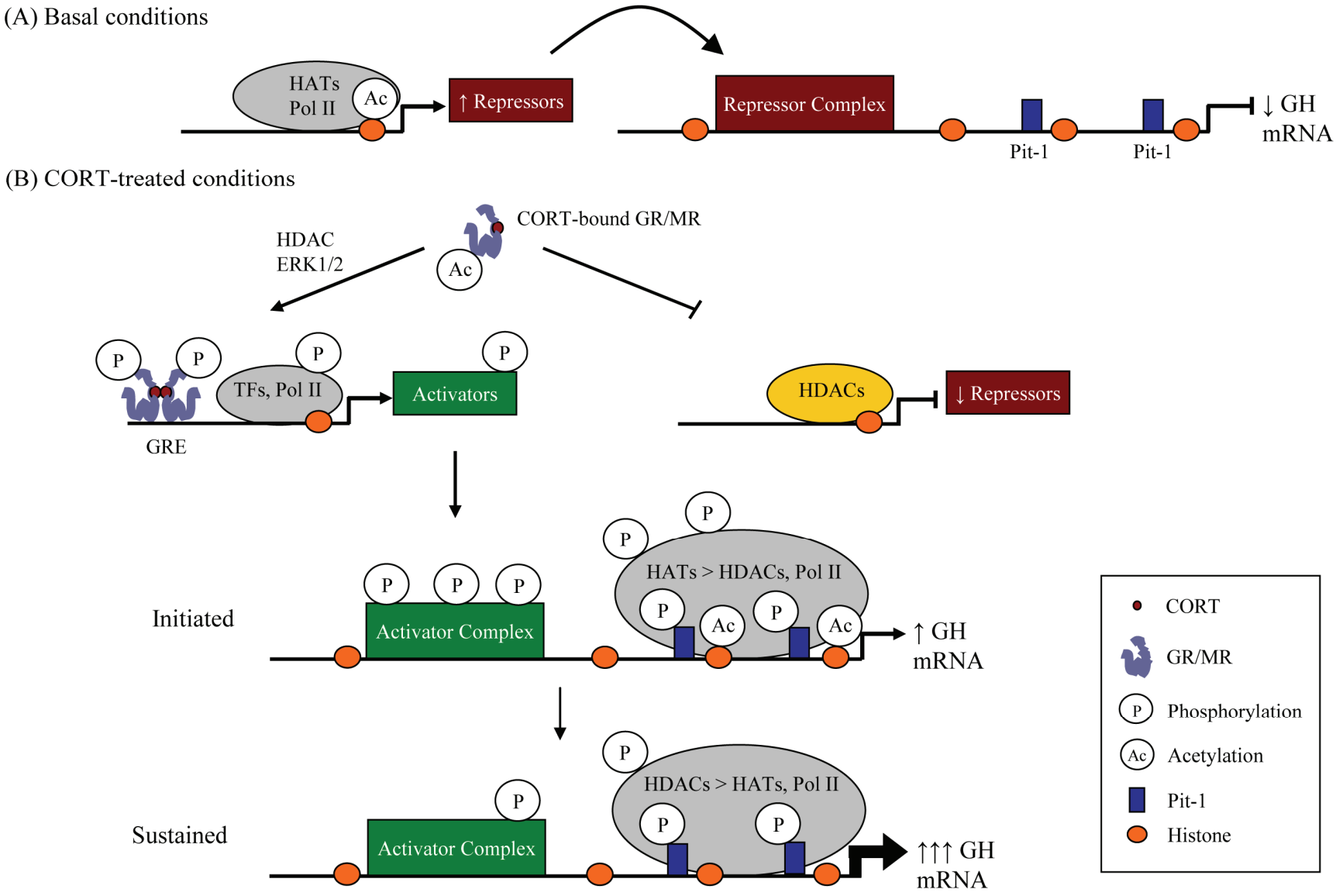
regarding the age of embryos which should be used for such experiments. For example, in assessing functionality of the somatotropic axis, it would make sense to evaluate effects of PACAP on GH production and release between e12 and e16, when PACAP-R1 mRNA levels were highest, while effects of SST would be better determined later in embryonic development, when SSTR2 mRNA levels are highest.

Pituitary GH is essential for normal post-natal growth, and GH-producing somatotrophs are the most abundant cell type within the mature anterior pituitary gland. Adrenal glucocorticoids play a critical role in triggering functional differentiation of pituitary somatotrophs by initiating GH expression in both fetal rats and embryonic chickens (44, 121, 143-150, 152-155, 159). Recently, a group examining GR function in regulation of the hypothalamic-pituitary-adrenal axis generated a conditional knockout mouse lacking GR in the pituitary and parts of the brain (283). The authors reported that knockout mice were indistinguishable in size from control littermates at birth but were 50% smaller by post-natal day 6. The authors determined that loss of GR protein occurred by e14.5, prior to appearance of somatotrophs in mice. Although they did not measure pituitary GH content or somatotroph abundance in the study, it is tempting to speculate that the smaller phenotype is due to GH insufficiency resulting from lack of somatotroph recruitment by glucocorticoids. Despite the importance and relative abundance of pituitary somatotrophs, little is known about underlying mechanisms involved in glucocorticoid induction of GH expression in this cell type. Therefore, a major focus of this project was to investigate mechanisms behind this process.

Our current findings confirm a requirement for ongoing protein synthesis and involvement of a ras protein in glucocorticoid induction of GH during embryonic

development, and extend previous findings to demonstrate that HDAC activity and ERK1/2 signaling are also involved. It was also demonstrated that an increase in transcriptional activity of the GH gene is the primary mechanism by which glucocorticoids initiate hormone production during functional differentiation of this cell type. These findings, as well as other recent results obtained in our laboratory, are incorporated into the working model for glucocorticoid induction of pituitary GH expression during embryogenesis presented in Figure 36. In immature somatotroph precursors, GH mRNA levels are barely detectable, likely resulting from negative regulation of GH transcription by repressor proteins that are expressed in this cell type (Figure 36A). As circulating glucocorticoid levels increase in the embryo, CORT initiates hormone production in a process that may involve both de-repression and activation of GH transcription (Figure 36B). Both HDAC activity and ERK1/2 signaling may function to enhance the ability of CORT-activated nuclear receptors to bind to GREs and transactivate transcription of glucocorticoid target genes through deacetylation and

Figure 36. Model for glucocorticoid induction of pituitary growth hormone expression during embryogenesis. (A) In anterior pituitary cells harvested from e11 chickens or e15 mice, just prior to initiation of GH expression, transcriptional activation of the GH gene is minimal and GH mRNA levels are barely detectable (right). This likely results from negative regulation of GH transcription by repressor proteins that are highly expressed in somatotrophs during this stage of development (left). (B) CORT treatment may downregulate repressor proteins in a mechanism requiring HDAC activity, while simultaneously increasing expression of transcription factors, chromatin modifying enzymes, and/or signaling molecules (activators) that increase GH expression. HDAC activity may be required for deacetylation of GR to facilitate its binding to GREs, and ERK1/2 activity may be required to enhance GR transcriptional activity through phosphorylation (top left). Ultimately, this leads to de-repression and activation of GH transcription in a process that involves a temporary increase in histone acetylation status at the GH proximal 5'-flanking region and transient ERK1/2-mediated phosphorylation events. The activator complex and histone acetyl transferase enzymes (HATs) alter chromatin accessibility and allow for recruitment of RNA polymerase II and other transcriptional machinery (middle). Other potential ERK1/2 targets include transcription factors in the activator complex, chromatin modifying enzymes, and/or Pit-1. HDAC activity may also be required for deacetylation of histones in the proximal 5'-flanking region of the GH gene, which occurs after prolonged exposure to CORT. The transitory nature of both epigenetic modifications and MAPK-mediated phosphorylation events, depicted by reduced phosphorylation and histone acetylation, is required for sustained transcriptional activation of the GH gene, leading to a dramatic rise in GH mRNA during initiation of hormone expression in pituitary somatotrophs (bottom).


(A) Basal conditions
HATs
Pol II
Ac
↑ Repressors
Repressor Complex
Pit-1
Pit-1
↓ GH
mRNA
(B) CORT-treated conditions
HDAC
ERK1/2
CORT-bound GR/MR
Ac
P
P
P
TFs, Pol II
Activators
GRE
HDACs
↓ Repressors
Initiated
P
P
P
Activator Complex
P
HATs > HDACs, Pol II
P
Ac
P
Ac
↑ GH
mRNA
Sustained
P
Activator Complex
P
HDACs > HATs, Pol II
P
P
↑↑↑ GH
mRNA
CORT
GR/MR
P
Phosphorylation
Ac
Acetylation
Pit-1
Histone

phosphorylation of the receptor, respectively. Glucocorticoid-induced HDAC recruitment to target genes may also be necessary for downregulation of proteins which repress GH transcription. Potential activators upregulated by CORT are transcription factors, chromatin modifying enzymes such as HATs and HDACs, and signaling molecules that include ras proteins which activate ERK1/2 pathway activity. Ultimately, GH transcription is induced in a manner that involves a temporary increase in Ac-H3 levels at the proximal 5'-flanking region, as well as transient ERK1/2-mediated phosphorylation events. Potential ERK1/2 targets include transcription factors in the activator complex, chromatin modifying enzymes, and Pit-1. This temporary increase in phosphorylation of transcriptional regulators and Ac-H3 may facilitate recruitment of RNA polymerase II and other transcriptional machinery. After initial transcriptional activation, Ac-H3 levels decrease and ERK1/2 activity must be downregulated for sustained expression of GH to occur, highlighting the dynamic and transitory nature of signaling mechanisms involved in the developmental programming leading to somatotroph maturation.

The results presented in Figures 12 and 13 clearly demonstrated that CORT increases GH mRNA levels in embryonic anterior pituitary cells through transcriptional activation of the GH gene rather than through enhancement of mRNA stability, and provide evidence that HDAC activity is necessary for full induction. The requirement for HDAC activity leads to at least three non-mutually exclusive hypotheses (Figure 36): HDAC activity is necessary for indirect derepression, or glucocorticoid downregulation of proteins that repress GH transcription prior to this stage of development; HDAC activity is required for deacetylation of GR to enhance its transcriptional activity; and/or HDAC activity is required for transient epigenetic changes that appear to occur in the GH

5'-flanking region during glucocorticoid induction. In all cases, the hypotheses are easily testable and the following experiments should be conducted. To investigate involvement of HDAC activity in derepression of GH, a microarray screen could be conducted using e11 embryonic pituitary cells treated with CORT in the presence or absence of HDAC inhibitors. Any transcripts that are repressed by CORT in the absence of HDAC inhibition, but not in their presence, would be candidate repressors. These could then be overexpressed, both alone and in combination, in e11 pituitary cells treated with CORT to examine if they suppress the glucocorticoid induction of GH. Additionally, these candidates could be downregulated through RNA interference to determine if loss of their activity leads to increased GH expression on e11 in the absence of CORT. To examine whether hyperacetylation of GR in the presence of HDAC inhibitors is preventing its ability to transcriptionally regulate critical genes necessary for the response, the acetylation state of GR as a result of CORT treatment in the presence and absence of HDAC inhibitors can be determined. In addition, predicted acetylation sites could be mutated to determine if this affects HDAC suppression of CORT induction of pituitary GH. Finally, to examine if HDAC activity plays a role in the transient nature of the increase in Ac-H3 that occurs at the proximal GH 5'-flanking region, Ac-H3 levels can be measured in that region in e11 pituitary cells treated with CORT for 1.5 h and 6 h in the presence and absence of HDAC inhibitors. In retrospect, the results from the HDAC inhibitor experiment conducted in this study would have been more informative had the cells been treated for 1.5 h in addition to 6 h. In the hypothetical model, initial activation of GH transcription by CORT involves an increase in Ac-H3, while sustained activation leading to maximal induction involves a decrease in Ac-H3, possibly resulting from

HDAC activity. If this is true, short-term inhibition should not block the initial CORT activation of GH, while inhibition for 6 h should prevent full induction.

Experiments investigating mechanisms through which glucocorticoid treatment initiates pituitary GH expression also followed up on previous reports implicating ras signaling in the response (153, 217). We attempted two approaches, pharmacological inhibition and use of genetic mutants. The pharmacological approach was taken to narrow down possible involvement of all known ras-induced signaling cascades previously reported to be activated by glucocorticoids (12, 27-30). In hindsight, efficacy and proper dosing of each inhibitor should have been confirmed in chicken embryonic anterior pituitary cells prior to performing any experiments determining their effects on CORT stimulation of GH. This would have allowed us to more confidently distinguish a lack of inhibitor efficacy from a lack of pathway involvement. For example, neither the PI3-K inhibitors (wortmannin or LY294002) nor the JNK inhibitor (SP600125) affected the CORT increase in GH mRNA levels. This was interpreted as a lack of involvement of these pathways, but without demonstrating efficacy of the inhibitors in these cells, it cannot be ruled out that the lack of an effect was due to lack of pathway inhibition. Additionally, a compound such as BIRB 0796, which is thought to be more specific and potent than SB203580 at inhibiting p38MAPK (256), could have been evaluated. As it is, a requirement for p38MAPK signaling in glucocorticoid induction of GH has not yet been tested. However, stimulation of p38MAPK using caMKK3/6 and anisomycin blocked the response, indicating this pathway may play a role. Similarly, activity of each dominant negative and constitutively active mutant we obtained should have been confirmed in LMH and HEK-293 cell lines prior to subcloning and use in experiments

with embryonic pituitary cells. Again, we may have been able to obtain alternative mutants that truly functioned in a dominant negative capacity to assess involvement of ERK1/2 and p38MAPK pathways. Not only would this have allowed us to confirm the pharmacological inhibition studies, these could be used in combination with EGF and anisomycin to demonstrate more convincingly that the decrease in CORT-stimulated GH mRNA levels in the presence of these compounds was truly due to ERK1/2 and p38MAPK activity.

Our results do conclusively demonstrate that active ERK1/2 signaling is required for glucocorticoid induction of pituitary GH, and further suggest that CORT treatment may stimulate ERK1/2 kinase activity in embryonic pituitary cells. Clearly, more experiments evaluating CORT effects on ERK1/2 kinase activation need to be conducted before it can be definitively concluded that glucocorticoid treatment stimulates ERK1/2 activity. In addition, targets of ERK1/2 phosphorylation should be identified. Potential candidates to test are indicated in the model of glucocorticoid induction of pituitary GH (Figure 36) and include GR, Pit-1, and other yet-to-be identified factors. Phosphorylation state of GR and Pit-1 can be determined with and without CORT treatment in the presence and absence of the ERK1/2 pathway inhibitor, U0126. Identifying unknown targets of ERK1/2 would be somewhat more complicated, although not impossible. A functional proteomics approach involving two-dimensional (2D) gel electrophoresis and mass spectrometry has been successfully used in the past to reveal previously unidentified targets of ERK1/2 signaling (284). Protein extracts from e11 anterior pituitary cells treated with and without CORT could be resolved by a 2D gel, which would not only indicate up- or downregulation by CORT but also provide information

about post-translational modifications such as phosphorylation. Proteins resolved on a duplicate gel could be transferred to a membrane and blotted for phosphorylated serine and threonine residues, and spots with altered phosphorylation state could be identified using mass spectrometry. Ideally, the above experiment would be performed in the absence and presence of U0126 to identify ERK1/2 targets that are phosphorylated after CORT treatment only in the absence of the inhibitor. However, including U0126 treatments may not be possible due to limitations in the number of anterior pituitary cells that can be used in a given experiment and the amount of protein extract needed for 2D analysis. In this case, simply identifying proteins that are phosphorylated in the presence of CORT would allow a more targeted, one-protein-at-a-time approach to be taken, similar to that outlined above for GR and Pit-1.

Given that manumycin A prevents full glucocorticoid induction of GH in embryonic anterior pituitary cells (153, 217), ras-dva was identified as a potential intermediary factor involved in the response when it was reported to be upregulated by CORT in e11 pituitary cells (235). The developmental profile of ras-dva expression in the anterior pituitary gland between e10 and e17 (124), as well as its initial identification as a target of a transcriptional repressor important in pituitary development (259), further enhanced its appeal as a candidate gene mediating initiation of pituitary GH. In e11 anterior pituitary cells, ras-dva mRNA was stimulated by CORT even in the presence of a protein synthesis inhibitor, and treatment of e18 cells with CORT for as little as 30 m increased ras-dva mRNA levels. Taken together, these results imply that it is a direct transcriptional target of GR in embryonic anterior pituitary cells. In retrospect, treatment of e11 cells for 30 m and/or treatment of e18 cells in the presence of CHX would provide

even more compelling evidence that ras-dva is directly regulated in the pituitary at both stages of development. In addition, although levels of ras-dva mRNA were much lower in other tissues on e18, it would have been informative to assess glucocorticoid regulation of ras-dva in other tissues to determine if the effect is pituitary-specific.

At least one GR binding site was predicted within 4 kb of the translational start site in all species with an assembled genome in which a ras-dva homolog was identified. This strongly suggests that it is also a glucocorticoid regulated gene in other species. In the chicken, two putative GR binding sites were identified within the 5'-flanking region, at approximately -2 kb and -4 kb relative to the translational start site. The observation that a reporter construct containing these sights was induced by CORT, while a construct containing a shorter fragment that lacks the putative GR sites was not, further suggested that ras-dva is a direct transcriptional target of GR. Despite this, mutation of the predicted binding sites did not substantially reduce CORT induction of ras-dva promoter activity. This does not mean ras-dva is not directly regulated by glucocorticoids, only that these sites are not fully responsible for the induction. Future experiments should include identification of the *cis*-acting elements necessary for CORT stimulation of ras-dva promoter activity. Given that GR has recently been reported to be recruited to an AP-1 site during dexamethasone-stimulated activation of the GnRH-R promoter (14), an obvious site to test by mutagenesis would be the AP-1 site located at approximately -3.4 kb. An alternative approach would be to test reporter activity from constructs created by serial deletions of the 5'-flanking region between -4 kb and -2 kb.

In addition to GR sites, multiple Pit-1 binding sites were predicted in the 5'-flanking region of ras-dva in all species, suggesting pituitary expression in these species.

Of the six predicted sites in the chicken, one was experimentally tested and determined to be necessary for full activation in embryonic anterior pituitary cells. The remaining sites are located rather distally, in the region that is responsive to glucocorticoids. Future experiments should evaluate functionality of these distal predicted sites, in terms of basal ras-dva promoter activity and CORT-responsiveness. In addition, the prediction that ras-dva mRNA is expressed in cells of the Pit-1 lineage should be tested. To achieve this, e18 pituitary cells can be sorted by flow cytometry on the basis of TSH-β, GH, and PRL expression, and ras-dva mRNA can be measured in positive and negative populations.

An attempt was made to identify a functional role for ras-dva in the developing anterior pituitary gland. We focused on hormones that are known to be regulated by glucocorticoids, both developmentally (GH and PRL) and in the mature gland (ACTH, as measured by POMC mRNA levels). In addition, we evaluated a role for ras-dva in regulating expression of TSH-β, Pit-1, and two pituitary receptors involved in regulating GH release (GHRH-R and SSTR2). We were unable to demonstrate that overexpression of ras-dva influenced basal or CORT-stimulated mRNA levels for any of the genes we measured. This indicates that ras-dva is not a limiting factor involved in regulating these genes under basal conditions and demonstrates that increased ras-dva expression alone is not sufficient to recapitulate glucocorticoid regulation of these genes. However, at this time, it cannot be ruled out that ras-dva is necessary for expression of these genes under basal or CORT-stimulated conditions. In order to test this, an experiment in which mRNA levels are measured in the absence and presence of CORT after knocking down ras-dva in embryonic anterior pituitary cells by RNA interference needs to be conducted. In order to more accurately predict a function for ras-dva in the developing anterior

pituitary gland, gene expression profiling of embryonic pituitary cells in which ras-dva is both overexpressed and knocked down could be conducted under basal and CORT-treated conditions. Based on its developmental profile, regulation by glucocorticoids, and identification as a Pit-1 target, it seems most practical to concentrate efforts on investigating a role for ras-dva in cell types of the Pit-1 lineage. Potential functions to test can include developmental expansion of thyrotrophs, somatotrophs, and lactotrophs, as well as expression and/or secretion of TSH, GH, and PRL from these cells.

In summary, we have determined developmental mRNA expression patterns of important hypothalamic and pituitary genes involved in neuroendocrine system function and delineated mechanisms necessary for GH expression during anterior pituitary development. We have also identified and characterized regulation of a novel gene that has never been investigated within the neuroendocrine system of any species. These results substantially increase our knowledge of neuroendocrine system establishment during embryogenesis and particularly enhance our understanding of mechanisms involved in glucocorticoid initiation of GH expression in somatotrophs. Further, ras-dva was identified as a Pit-1 and glucocorticoid target gene that may play an important role in development of the anterior pituitary. Despite these advances, further studies are clearly needed to truly understand development of the neuroendocrine system as a unit and precisely define mechanisms involved in emergence of terminally differentiated cell types.

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