ABSTRACT

Title of Dissertation: Characterization of Glucocorticoid-Induced Changes in Gene
                         Expression in the Embryonic Pituitary Gland

                         Sultan Ali Jenkins, Doctor of Philosophy, 2006

Dissertation directed by: Professor Tom E. Porter
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    We have previously shown that corticosterone (CORT) can induce premature

differentiation of somatotrophs in the chicken embryo. CORT induction of GH mRNA is

indirect, in that protein synthesis inhibition blocks its inducing effect. In this study, we

used a custom chicken microarray to analyze global gene expression in the embryonic

pituitary gland and to identify potential direct targets of CORT that may regulate its

induction of somatotroph differentiation. Dispersed embryonic (e) day 11 pituitary cells

were pretreated with cycloheximide then with CORT or treated with CORT alone for 24

hrs. Amplified RNA from these samples was then used on our microarray to analyze

gene expression and in quantitative real-time PCR (qRT-PCR) analysis to determine

relative gene expression levels. qRT-PCR from these samples showed that GH was

induced within 1.5 hrs and continued to significantly increase until 3 hrs. Our microarray

analysis revealed 25 direct early targets of CORT. From these 25 we chose 3 genes,

Dexras1, Ras-dva, and Prostaglandin-D Synthase to transfect into e11 pituitary cells and

measure their effect on GH mRNA. Neither of these genes had a direct effect on GH

mRNA; however Dexras1 acted synergistically with CORT to induce GH mRNA.

Dexras1 was discovered in murine AtT-20 corticotroph cells because its expression was

rapidly induced in response to glucocorticoids. We report here a chicken Dexras1 cDNA that is 1631 bp in length and encodes a protein of 278 amino acid residues. Comparison of the consensus chicken Dexras1 amino and nucleic acid sequence with those of human, mouse, and rat Dexras1 showed high homology among the species. Expression of Dexras1 mRNA was detected in various tissues by Northern analysis, but was highest in the pituitary. RT-PCR analysis showed expression of Dexras1 only in the pituitary. The precise role of DexRas1 is unidentified at the present time; however, its distribution in a range of tissues suggests a possible role in glucocorticoid action within a variety of systems.

Characterization of Glucocorticoid-Induced Changes in Gene Expression in the
Embryonic Pituitary Gland

by

Sultan Ali Jenkins

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
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Advisory Committee:

     Professor Tom E. Porter, Chair
     Professor Thomas Castonguay
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     Dr. John McMurtry
     Dr. Mark Richards

never thought I could admire a man who is a Vikings fan, but I suppose that nobody is perfect!!

I would also like to thank my committee members: Dr. Thomas Castonguay, Dr. Mark Richards, Dr. Steve Mount, and Dr. John McMurtry. I realize that your schedules are extremely hectic, and I am extremely grateful that you were able to make some time to be on my committee.

I must also thank Mr. Mike Muchow. Your help to me has been more valuable to me than I could ever tell you. Besides, I couldn't have been able to order my kits and primers without you. THANKS!!

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CHAPTER 1: Glucocorticoids: How they Regulate Gene Transcription

INTRODUCTION

Glucocorticoids are a class of steroid hormones that control a diversity of developmental, immune, metabolic, and growth functions and participate as a central character in basal and stress related homeostasis[1-4]. In addition to their physiologic importance, glucocorticoids are also among the most frequently used drugs, and often prescribed for their anti-inflammatory and immunosuppressive properties. The structures of common glucocorticoids are depicted in Figure 1.1. The vast majority of glucocorticoid activity in most mammals is from cortisol, also known as hydrocortisone. Corticosterone, the major glucocorticoid in rodents and avians, is another glucocorticoid. The hydroxyl group at carbon 11 of the steroid molecule is a definite necessity for glucocorticoid activity[5]. Glucocorticoids are produced and secreted by the adrenal cortex. They originally derived their name from initial studies that demonstrated their involvement in glucose metabolism[6]. Glucocorticoids sustain and raise blood glucose by (1) stimulating gluconeogenesis in the liver; (2) augmenting and extending the effects of epinephrine and glucagons on gluconeogenesis and glycogenolysis; (3) hindering glucose utilization in peripheral tissues; and (4) increasing liver glycogen synthesis, hoarding substrate in preparation for acute responses to glycogenolytic agents such as glucagons and epinephrine[7].

Synthesis and secretion of glucocorticoids by the adrenal cortex is closely tied to a single stimulator: adrenocorticotropic hormone (ACTH) [8, 9]. ACTH is formed as a

component of a large precursor called pro-opiomelanocortin (POMC) in the corticotrophs of the anterior pituitary[10]. POMC production is stimulated by the hypothalamic peptide corticotropin-releasing factor (CRF) [11] and reduced by glucocorticoids[12].
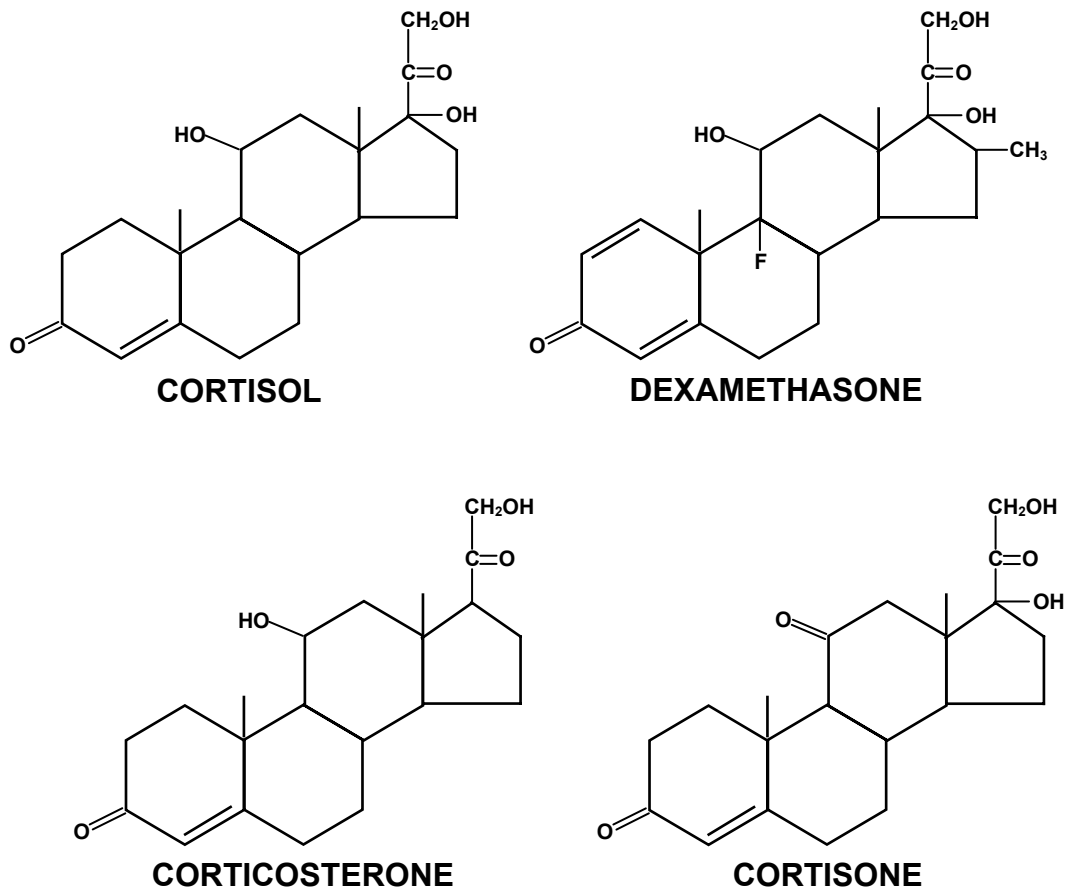
Figure 1.1. The structures of commonly used glucocorticoids.

THE GLUCOCORTICOID RECEPTOR

The majority of the cellular actions that are carried out by glucocorticoids are mediated through the interaction with the established transcription factor known as the glucocorticoid receptor (GR). The gene for GR was first cloned in 1985, and it encodes a protein 777 amino acids in length[13]. It has been shown that GR is ubiquitously expressed and distributed, but is more prevalent in hepatic, nervous system, and muscular tissues. Despite being broadly dispersed, sensitivity of cells to glucocorticoids is very complicated and is dependent on the actions of multiple factors[14]. GR is a member of a rather large family of proteins known as the nuclear receptors (NRs). Thus far, 48 nuclear receptors have been identified in humans. In particular, based on sequence comparison, GR is a member of the steroid hormone receptor (SHR) subset of NRs; this division comprises the progesterone receptor (PR), androgen receptor (AR), the mineralocorticoid receptor (MR), and the estrogen receptor (ER). As shown in Figure 1.2, the GR like most members of the NR protein family is an assemblage of three smaller protein modules: the N-terminal domain (NTD), the DNA binding domain (DBD), and the ligand binding domain (LBD)[15, 16]. These individual domains execute separate functions in tandem to regulate the transcriptional and signal transduction activities of GR.

	Exon 1	Exon 2	Exon3	Insertion	Exon4	Exon5	Exon 6	Exon 7	Exon 8	Exon 9
Primary	+	+	+	—	+	+	+	+	+	9α + 9β
GRα	+	+	+	—	+	+	+	+	+	9α
GRβ	+	+	+	—	+	+	+	+	+	9β
GRγ	+	+	+	gua	+	+	+	+	+	9α or 9β
GRA	+	+	+	—	+	—	—	—	+	9α or 9β
GRP	+	+	+	—	+	+	+	+	—	—
GR-Low Sensitivity	+	—	+	—	+	+	+	+	+	9α or 9β

PROTEIN

hGRα-A (1 – 777)
hGRα-B (27 – 777)
hGRβ-A (1 – 742)
hGRβ-B (27 – 742)
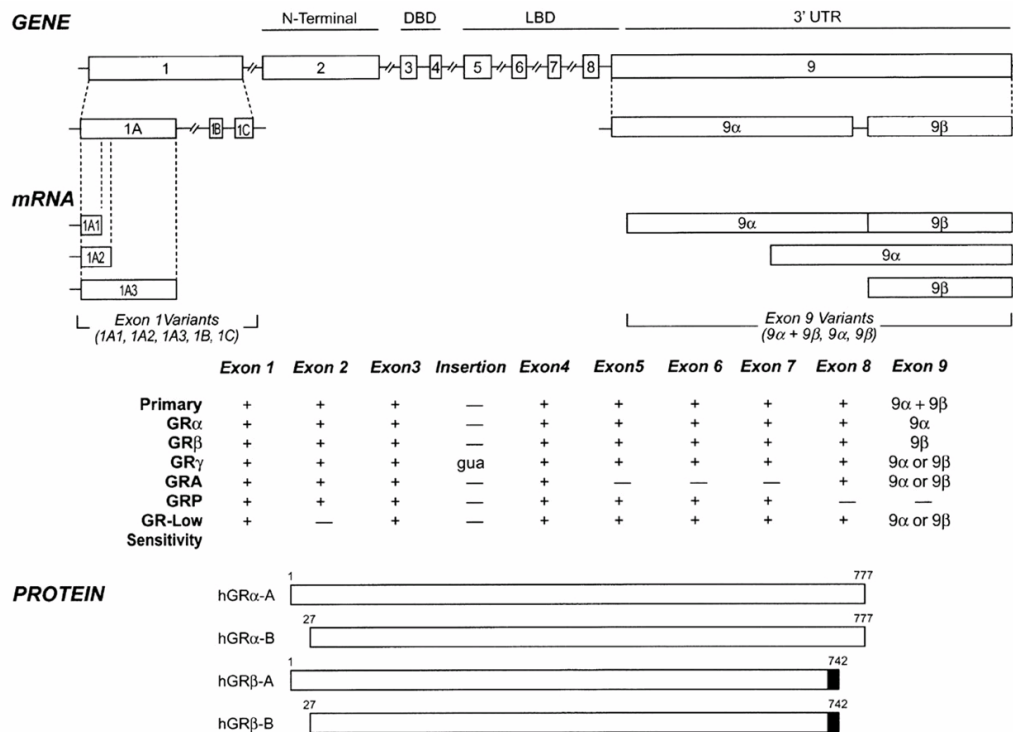
Figure 1.2. The primary human GR gene is comprised of 9 exons, coding various regions of the receptor, such as the DNA binding domain (DBD), ligand binding domain (LBD), and untranslated regions (UTR). The presence of 3 promoters in Exon 1gives rise to several GH isoforms differing in the 5'-UTR. Alternative splicing of Exon 9 generates mRNAs coding for hGRα and hGRβ. In addition, alternative splicing can cause the insertion of an additional codon (GRγ), exon skipping (GRA) or exon deletion (GRP). These receptor isoforms have blunted activity. The A and B forms of both hGRα and hGRβ are generated from alternative translation initiation start sites, corresponding to methionine 1 or 27. *From Lu and Cidlowski 2004 Ann NY Acad Sci 1024: 102-123*

N-Terminal Domain (NTD)

As it relates to both size and sequence homology the NTD region epitomizes the most capricious domain among the NRs, even between different species of the GR[17, 18]. The major function of the NTD, also called the A/B region, is in transcription activation. It includes a section that regulates a constitutive transcriptional activity and is also a target of several interaction proteins and kinases. In all members of the nuclear receptor superfamily there are at least two transcription activating domains (TADs), one of which is located in the A/B domain. This amino-terminal section is referred to as the activation function-1 (AF-1) or TAD1 region[19]. AF1 is cell and promoter specific, but its activity is independent of ligand binding. It can act constitutively in the absence of the LBD, and is fairly active in provoking transcription from simple promoters containing similar glucocorticoid response elements (GREs).[20] The amino terminus of GR also contains a PEST domain. This name arises from the one-letter designation of the four amino acids prevalent in this domain: proline (P), glutamic acid (E), serine (S), and threonine (T). These regions manage the stability of the GR[21]. Lastly, the NTD is very immunogenic and includes the major known sites of phosphorylation in the GR[22-24].

DNA-Binding Domain (DBD)

As you proceed toward the C-terminal end of the GR, the next protein module contains the DNA binding domain (DBD). This area, sometimes referred to as the C region, is the most conserved domain among the nuclear receptors and consists of two α-helical structures termed "zinc fingers".[25] Zinc fingers were so named because of the requirement for zinc as the coordinating group to stabilize these helical regions.[26] The

DBD contains amino acids that make contact with particular bases in the GRE sequence to impart site specificity for GR:DNA binding. These amino acids are positioned in the zinc finger closest to the amino-terminal, where the "P box" is responsible for GRE distinction.[27, 28] The second zinc finger stabilizes the DBD:GRE contacts, and has five amino acids that comprise the "D box" which participates in an important role in homodimerization at the GRE.[27, 28] Additionally, a well-defined dimmer interface is formed as two DBD subunits bind the DNA helix in adjacent major grooves[28].

Ligand-Binding Domain (LBD)

The C-terminal LBD or E region is responsible for identifying and binding to steroid hormone ligands, chaperones and other proteins.[17] Additionally, the LBD contains the 2nd transcription activation domain (AF-2). In contrast to AF-1, the activity of AF-2 is ligand-dependent, and folds as to complete binding surfaces for other proteins such as coactivators and corepressors via their LXXLL motifs[29 30].

Isoforms

Alternative splicing of the human (h) GR gene gives rise to various GR isoforms. When the GR was cloned back in 1985 two GR gene splicing products were detected, these were termed hGRα and hGRβ. Alternative splicing of the 9th exon generates these two highly homologous isoforms which are identical through amino acid 72, but vary at their carboxy-terminus. hGRα has an additional 50 amino acids, while hGRß possesses an additional nonhomologous 15 amino acids[31]. The α isoform of hGR is ubiquitously expressed in almost all human tissues and cells, and represents the classic GR that

operates as a ligand-dependent transcription factor. A diagram of the primary GR gene and the alternative splicing event that produces hGRβ and other variants is shown in Figure 1.2.

hGRβ does not bind hormone or activate gene transcription. As a result, it was originally labeled as a minor gene product and perhaps even a cloning artifact. It took the work of Bamberger and Oakley, 10 years later, to show that hGRβ was an endogenous hGR gene product and further characterized its role and expression[32, 33]. Unlike hGRα, hGRβ is located in the nucleus in the absence of ligand. In spite of this, it is the ability of hGRβ to exert a dominant negative on hGRα -mediated transactivation of gene expression that is most fascinating and likely physiologically important. The latest investigations examining the mechanism of the dominant negative activity of hGRβ propose that hGRβ exerts its effect on hGRα by forming hGRαβ heterodimers that are incapable of binding coactivators or by competing with hGRα for combining to glucocorticoid receptor-interacting protein 1(GRIP-1) and other p160 coactivators[34, 35].

FROM THE CYTOPLASM TO THE NUCLEUS

In cellular states where ligand is absent or deficient, the preponderance of the glucocorticoid receptor population dwells in the cytoplasm complexed to heat shock proteins (hsp) forming a large multiprotein complex [36-38]. This complex consists of the receptor, two molecules of HSP-90, and several other proteins. There is one model that proposes that binding of ligand to the receptor causes the liberation of the bound hsp. As a result the nuclear localization sites within the LBD of the GR become uncovered thus

allowing the activated GR to translocate into the nucleus from the cytoplasm. In spite of this proposed mode of action, the role of hsp is almost certainly more convoluted because hsp have also been associated with having an active role within the transcriptional complex[39] and aiding transport and cytoplasmic nuclear shuttling of GR[40]. One other corollary resulting from ligand association is the creation of both receptor hetero- or homodimers[41].

Ligand binding to the GR also initiates the phosphorylation of threonine and serine residues in the AF-1 domain[42]. Mitogen-activated protein kinase (MAPK), cyclin-dependent kinase (Cdk), c-Jun N-terminal kinase (JNK) are examples of some kinases that have been shown to regulate the action of ligand-bound GR[43-46]. The role of phosphorylation of GR is well documented, however very little facts are known bout the molecular relationship between phosphorylation and ligand binding.

THE ACTIVATION DOMAINS

After moving from the cytoplasm into the nucleus, the GR is able to attach to GREs, and as a result the gene is either up- or down-regulated (Figure 1.3). Although binding to DNA is generally a necessary prerequisite for GR to activate transcription, it is clearly not in itself sufficient for this to occur. Ensuing binding to GREs, GR must somehow regulate transcription by directly activating RNA polymerase itself or by assisting the binding of other proteins and the creation of a secure transcriptional complex. As mentioned before, the GR is a transcription factor that is modular in structure such that a particular region of the GR mediates DNA binding (DBD) while another may mediate

binding of a co-factor (AF-1/2). In the preponderance of cases, the AF-1/2 activation

domains are distinct from those of the DBD.
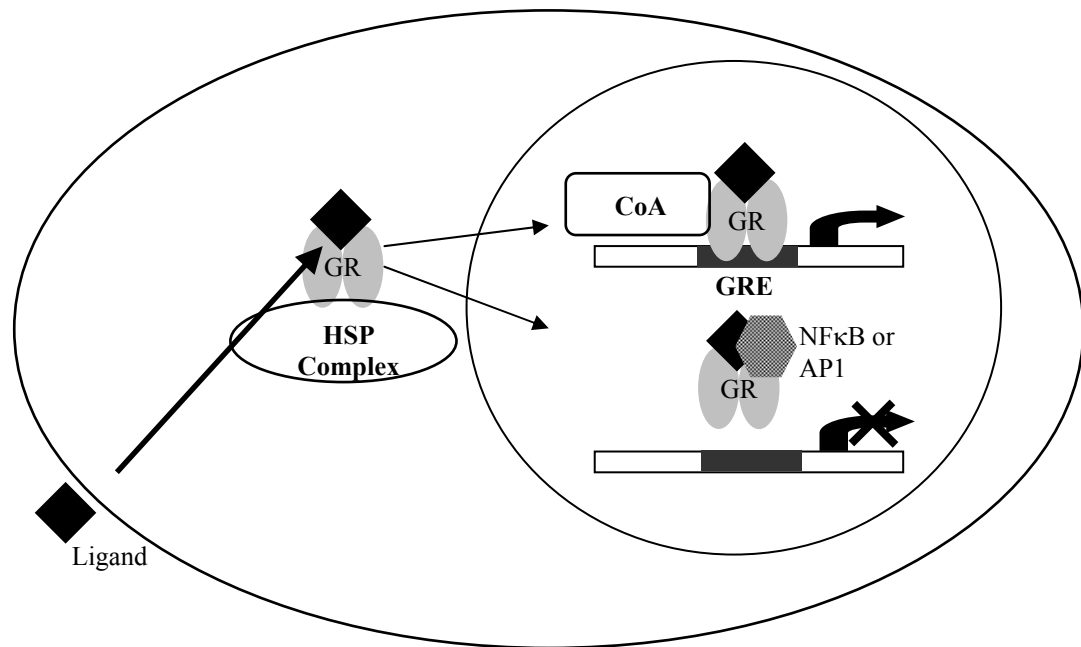
Figure 1.3. Simplified schematic of GR transactivation or transrepression activities. GR typically resides in the cytoplasm with heat shock proteins (HSP complex) until ligand association stimulates nuclear transport. Two of the most important activities of GR are (1) direct interaction with coactivators (CoA) and DNA response elements, such as GREs and negative GREs, to enhance or diminish transcription and (2) interaction with transcription factors (such as NFκB or AP-1) to diminish gene transcription.

The discovery of the activation domains of the GR ushered in a new era into gene transcription by not only GR, but other members of the NR family. In contrast to the DNA binding regions, the AF-1/2 domains of the GR that are responsible for gene activation cannot be ascertained on the basis of a simple assay of , for example, the capacity to bind DNA or another protein. More accurately, a functional assay that measures gene activation following DNA binding was needed. The activation regions of GR and other transcription factors have been detected on the results of so-called "domain-swap" experiments (Figure 1.4). In such experiments, the DNA binding region of one transcription factor is combined with an assortment of regions of another factor and the capacity to initiate transcription of a gene containing the binding site for the first factor is gauged. The two independent activation domains of the GR, each able to produce gene activation, were discovered in this way.

Once the GR is activated by ligand binding, the transcription activation domains of the GR are now able to recruit coactivator proteins that are needed for transcription to occur. Many of these accessory proteins, for example Steroid receptor coactivator-1 (SRC-1), possess chromatin-modifying activities such as histone acetylation[47]. The importance of histone acetylation in gene activation will be discussed later in this chapter. SRC-1, like many other coactivator proteins, possess a LXXLL motif or an NR box[48]. This unique pattern is a short, leucine-rich, amphipathic alpha-helix which allows the protein to dock to the ligand-bound GR. Joining of the ligand to the GR allows the GR to assume a conformation that produces a shallow hypdrophobic groove that can house the leucines of the LXXLL motif, thereby successfully linking the two proteins together.
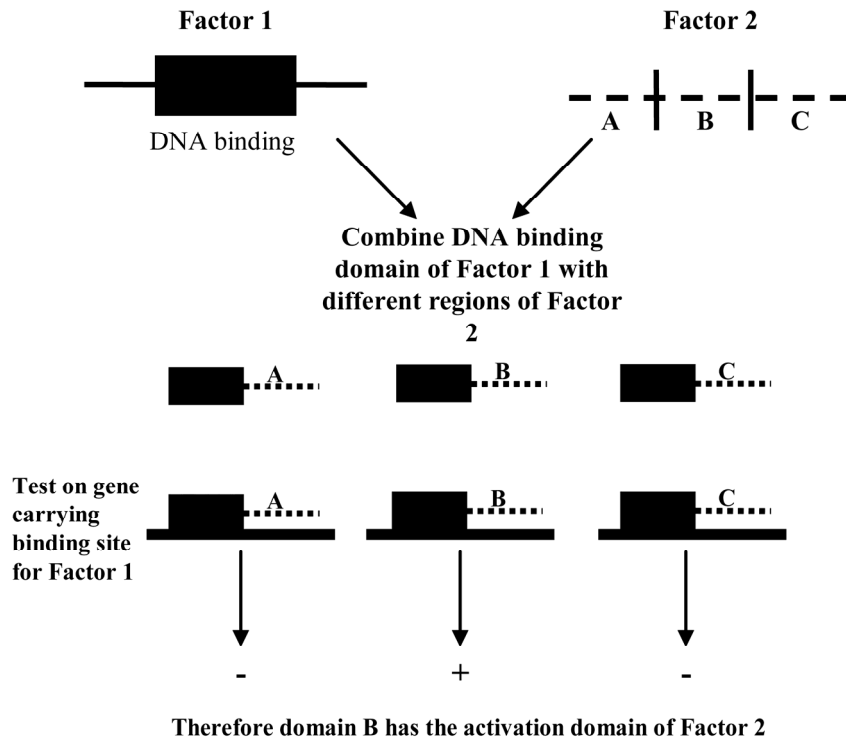
Figure 1.4. Domain swapping experiment, in which the activation domain of Factor 2 is mapped by combining different regions of Factor 2 with the DNA binding domain of Factor 1 and assaying the hybrid proteins for the ability to activate transcription of a gene carrying the DNA binding site for Factor 1.

THE REMODELING OF THE CHROMATIN STRUCTURE

The DNA inside the nucleus of the cell is strongly compressed around a protein core resulting in a structure called chromatin. This chromatin configuration is organized into nucleosomes, which are elements consisting of ~146 of bp DNA associated with an octamer of 2 molecules each of core histone proteins (H2A, H2B, H3, and H4). Gene expression and repression is linked with alterations in chromatin structure by enzymatic modification of core histones[49]. When genes are not being transcribed, the DNA is firmly compressed around these histone proteins. This configuration prevents the binding of RNA polymerase II, which is one of the enzymes that is needed for the formation of messenger RNA. This conformation of the chromatin structure is described as closed and is associated with suppression of gene expression[49] (Figure 1.5).

The stabilization of chromatin thread in the closed state is supplemented by contacts between the nucleosomes and linker histones such as H1. For some time now, histone H1 has been considered a repressor of transcription. However, recent evidence has indicated that it may have a positive role in transcriptional regulation[50]. In this more recent function of histone H1, it performs as a "gate" to nucleosomal DNA. When bound to DNA, histone H1 inhibits the binding of transcription factors to the DNA. In order for so called "opening factors" to bind, histone H1 removal is required. These opening factors, for example high mobility group (HMG)B proteins, bind to the same sites as histone H1 and generate a nucleosomal structure that is conducive to transcription factor binding in order to stimulate transcription. The phosphorylation of histone H1 may play a crucial function in the instigation of gene transcription[50].

Histone Deacetylation
(Gene repression)

Histone Acetylation
(transcriptional activation)

Histone Deacetylase
Class 1: HDACs 1-3, 8
Class 2: HDACs 4-7, 9, 10
Novel: HDAC11
HDACs 12-17 unclassified

Histone Acetyltransferase
p300/CBP, PCAF,
SRC-1, NCOA3, TIP60
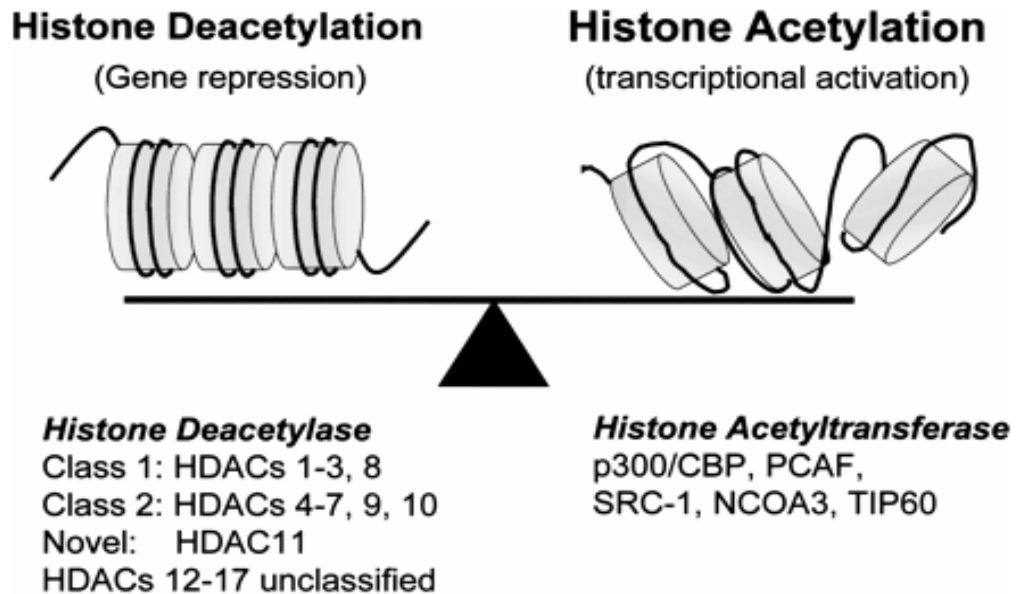
Figure 1.5. Gene repression and activation are regulated by acetylation of core histones. In the inactive state, DNA is tightly coiled around histones, forming a dense nucleosomal structure due to electrostatic attraction between negatively charged DNA and positively charged lysine residues. Acetylation of histones removes this charge, allowing loosening of the nucleosomal structure. Histone acetylation is mediated by transcriptional coactivators, which have intrinsic histone acetyltransferase (HAT) activity, whereas repression is induced by histone deacetylases (HDACs), which reverse this acetylation, allowing repackaging of the nucleosomes. *From Adcock et.al. 2004, The Proceedings of the American Thoracic Societ.*

DEACETYLATION/ACETYLATION OF HISTONES AND GENE ACTIVATION/REPRESSION

The amino-terminal tails of the core histone proteins contain particular amino acid residues (namely arginines, serines, and lysines) that can undergo post-translational modifications. These post-translational modifications include methylation, phosphorylation, ubiquintination, and acetylation. All of these alterations of histones have been linked to the regulation of gene expression[49]. Acetyl groups contain negative charges, consequently attachment of these molecules to histones reduces the charge on the histones (i.e., makes them more negative in charge) and this repels the negative charge of the DNA molecules. As a result, the tightly wound DNA assumes a more open configuration allowing the acetylation of the ε-group on lysines reduces the charge of the histone residue and subsequently releases the tightly wound DNA, allowing large protein complexes to bind to the DNA[49]. An important innovation in the role of histone acetylation was the experiment that demonstrated that transcriptional coactivators such as CREB-binding protein (CBP) and p300/CBP-associated factor contain intrinsic histone acetyltransferase (HAT) activity. So when transcription factors bind to DNA, this HAT activity is brought to the site of gene transcription. In addition, the connection of coactivator and transcription factor my further augment the HAT activity of the coactivator[50, 51].

The reversal of the acteylation process is associated with repression of gene transcription, or histone deacteylation. Histone deacetylation is regulated by HDACs[52]. HDACs are grouped into two classes according to their homology with yeast HDACs. Class 1 HDACs (HDAC1, 2, 3 and 8) are the most closely related to the yeast

transcriptional regulator RPD3. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) share

domains with similarity to HDA1, another deacetylase found in yeast[52]. Class I HDACs

are more ubiquitously expressed than class II HDACs. The removal of acetyl groups

from histones causes the DNA to become more tightly wound around the histone

proteins, this results in a more dense chromatin structure and diminishes the accessibility

of the DNA to binding by transcription factors.

In spite of this, the simple model described above does not reveal the full picture.

Under inactive conditions, less than half of the prospective lysine residues available for

acetylation are in fact acetylated, and these residues have a fast turnover[53]. This condition

implies that even subtle changes above or below the resting level are sufficient enough to

cause an activated chromatin state. Furthermore, this model predicts that changes in the

"histone code" must be transformed into downstream events very quickly[53, 54]. The

"histone code" applies to the varied range of histone tail post-translational modifications

such as methylation, phosphorylation, ubiquintination, and acetylation, which are set and

maintained by histone-modifying enzymes and contribute to coactivator recruitment and

subsequent increases in transcription.

GENE TRANSCRIPTION INDUCTION BY GLUCOCORTICOIDS

Glucocorticoids generate the cell-specific effects by activating the GR to either

directly or indirectly control the transcription of target genes[55]. It is estimated that the

number of genes directly regulated by glucocorticoids is between 10 and 100. However,

many genes can be indirectly regulated though GR interaction with other transcription

factors and coactivators.

Similar to other transcription factors, activated GR increases gene transcription through chromatin remodeling and the recruitment of RNA polymerase II to the site of local DNA unwinding. GR is able to bind with CBP and other coactivator proteins, including SRC-1, TIF-2, p300/CBP cointegrator protein, and GRIP-1, that increase local HAT activity[56, 57]. For example, it has been shown that Dexamethasone in A549 cells enhances the binding of activated GR to CBP and/or associated coactivators. The consequence of this action is the acetylation of H4 on lysines 5 and 16, the recruitment of the activated transcription complex RNA polymerase II, and finally increased gene transcription[56].

The phosphorylation of Histone H1 may also play a pivotal role in GR-mediated gene expression[58]. Histone H1 can only be removed from the mouse mammary tumor virus (MMTV) promoter by GR only when it is in the phosphorylated form. What's more, there is a so-called "refractory" state of the MMTV promoter resulting from prolonged exposure to glucocorticoids. This may be explained by the fact that long exposure to glucocorticoids leads to dephosphorylation of histone H1.

GENE TRANSCRIPTION REPRESSION BY GLUCOCORTICOIDS

In spite of the fact of the classical model of glucocorticoid action involves an increase in gene activation, glucocorticoids possess action that can lead to repression of gene transcription. It is this gene repression characteristic of glucocorticoids that is primarily responsible for the anti-inflammatory actions of glucocorticoids. Glucocorticoids exert their negative gene effects by either cis or trans-repression. Cis-mechanisms involve the activated GR binding directly to DNA, while trans-mechanisms do not.

The cis-repression by which glucocorticoids regulate gene transcription is by binding to negative GREs (nGREs). The DNA sequence of nGREs is distinct from the GREs which activate glucocorticoid-inducible genes, although the two are related (Table 1.1). This has led to the suggestion that the sequence difference causes the receptor/hormone complex to bind to the nGRE in a configuration in which its activation domain cannot interact with other transcription factors to activate transcription as occurs following binding to the positive GRE[59]. In agreement with this idea, the GR has been shown to bind to the nGRE in the POMC gene as a trimer rather than the dimer form which binds to the GRE and stimulates transcription[60]. The receptor bound in this configuration to the nGRE can also act by preventing binding of a positive acting factor to this or an adjacent site, thereby preventing gene induction. For example, the nGRE in the human glycoprotein hormone alpha subunit gene, which overlaps a cyclic AMP response element (CRE), is only able to inhibit gene expression when the CRE is left intact[61]. Hence, it is likely that receptor bound at the negative element prevents binding of a transcriptional activator to the CRE and thereby inhibits gene expression.

Binding site for positive regulation	RGRACANNNTGTYCY
Binding site for negative regulation	ATYACANNNTGATCW

Table 1.1. Relationship of the sites in DNA which mediate gene activation or repression by binding the glucocorticoid receptor. Note that the sites are related but distinct.

Trans-repression by glucocorticoids can inhibit transcription thru competition with other transcription factors for co-activator proteins. It has been known for some time that glucocorticoids are potent inhibitors of the induction of the collagenase gene by phorbol

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esters, owing to their anti-inflammatory effects. This inhibition is mediated by the GR

which inhibits the activity of the Jun and Fos proteins that normally activate the

collagenase gene via the AP-1 sites in its promoter. However, the collagenase promoter

does not contain any binding sites for GR adjacent to its AP-1 sites, nor does the GR bind

to the collagenase promoter. Interestingly, like the GR, the Fos/Jun complex requires the

CREB binding protein (CBP) as a co-activator to activate transcription of the collagenase

gene. Hence, the GR may compete with Fox/Jun for the limited quantities of the CBP co-

activator which are present in the cell[62]. However, De Bosscher and colleagues have cast

doubt on this hypothesis, showing that overexpression of CBP and other cofactors did not

affect the glucocorticoid-concentration response curve[63]. It was thought for a while that

trans-repression by glucocorticoids involved preventing nuclear translocation of other

transcription factors, it appears this is not the case. Recent data suggests that

glucocorticoids affect only steps downstream of transcription factor binding, such as

recruitment of HDACs to the transcription initiation complex, the inhibition of the HAT

activity of co-activators such as CBP, and preventing P-TEFb phosphorylation of RNA

polymerase II (Figure 1.6)[56, 64, 65].
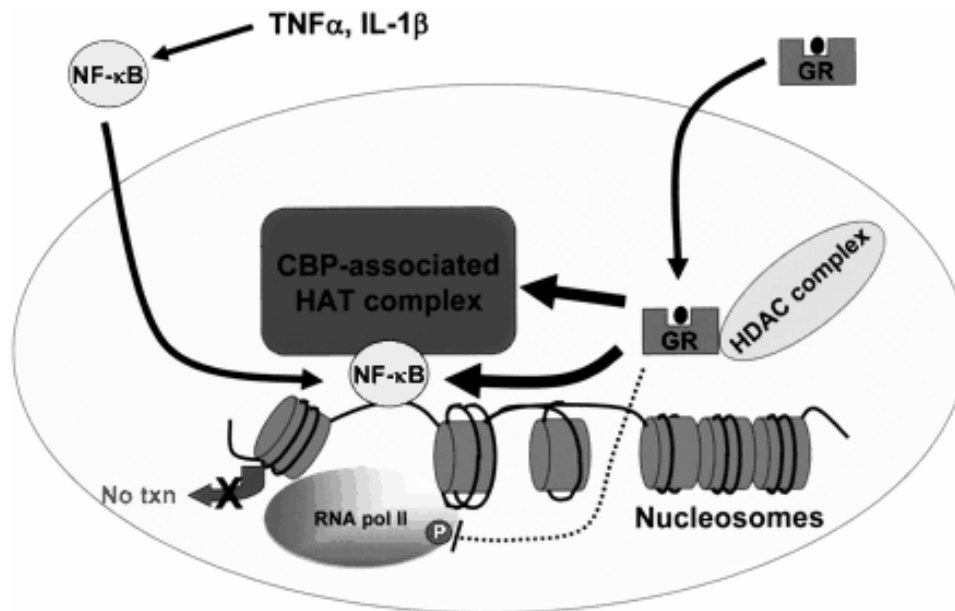
Figure 1.6. How glucocorticoids switch off inflammatory gene expression. Many inflammatory genes are activated by stimuli, such as interleukin (IL)-1ß or tumor necrosis factor (TNF)-α, and activate NF-κB, which translocates to the nucleus. NF-κB binds to specific κB recognition sites in the promoter regions of responsive genes and subsequently recruits transcriptional coactivators, such as CBP or p300/CBP-associated factor, that have intrinsic HAT activity. This results in acetylation of lysines in core histones, leading to recruitment of large protein complexes, including RNA polymerase II (RNA pol II), and in turn leading to increased transcription of inflammatory genes (txn). Glucocorticoid receptors translocate to the nucleus after activation by corticosteroids and act as monomers, reducing histone acetylation. The activated GR monomer interacts with and inhibits the HAT activity of coactivator complexes. In addition, the GR is able to recruit HDAC to the NF-κB complex, leading to suppression of inflammatory genes (no txn). Furthermore, the GR may also be able to reduce phosphorylation of serine 2 residues within the C-terminal repeat region of RNA polymerase II, reducing its capacity to cause mRNA elongation and reinitiation. *From Hayashi et.al. 2004 European Journal of Pharamacology.*

NONGENOMIC ACTION OF GLUCOCORTICOIDS

From the initial examinations on the actions of glucocorticoids, it became evident that steroid hormones can evoke cellular effects that occur in a very short amount of time, within minutes or even seconds of their treatment. These rapid actions do not conform to the classical "genomic" model of steroid action. Glucocorticoids effects on mRNA stability can be also included in the classical mode of action, because they affect gene expression and may be sensitive to protein synthesis inhibitors. To distinguish between the genomic and nongenomic effects of glucocorticoids, the definition recently given by Lösel and Wehling is very useful[66]. According to them, nongenomic effects can be "Any action that does not directly and initially influence gene expression, but rather drives more rapid effects such as the activation of signaling cascades". This definition illustrates the fact that the difference between the two modes of action is not black and white. However, the nongenomic effects of glucocorticoids do have some distinguishing characteristics. In addition to the short time frame in which the effects occur, these characteristics include: (1) a different pharmacologic profile, because the effects are insensitive to transcriptional and protein synthesis inhibitors; (2) action on nonnucleated cells, such as platelets, erythrocytes, and spermatozoa; and (3) the ability of steroid analogs (such as bovine serum albumin-conjugated steroid molecules) that cannot access the intracellular compartment to elicit a response[67].

A categorization of the rapid steroid effects has recently been proposed[68]. The mechanisms of action, with different specificity according to the cell type and the steroid studied, are frequently mediated by the production of a variety of second-messenger

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systems, by the activation of different kinase pathways, and by alterations in ion fluxes[67]. In several of the studies, the rapid effects were shown to be regulated by the classical GR. In these examinations, the effects were highly sensitive to the action of nuclear receptor antagonists. However, some of the nongenomic effects appear to be nuclear antagonist–independent. This finding implies that a different, membrane-bound GR mediates the nongenomic action[69, 70]. These receptors were originally described in amphibians, but have subsequently been depicted in mammalian cells. These receptors have very unique hormone binding properties compared the classical cytoplasmic receptor. They are also associated with a variety of intracellular signaling pathways that act thru G-protein coupled receptors and kinase pathways[69-72].

A certain number of the nongenomic effects of steroids appear to be controlled by alterations in the physicochemical properties of the cell membrane, without the involvement of the GR. Due to their highly lipophilic nature, steroids can easily diffuse though the plasma membrane of cells, where they are believed to interfere with the function of membrane-bound molecules, such as ion channels or receptors. These effects have been observed in vitro in several cell types[73]. However, in the preponderance of the studies concentrations that were required to achieve these effects in vitro were above those of physiologic and therapeutic ranges ($> 10 \ \mu M$)[74]. Therefore, their relevance in vivo is questionable.

Some of the nongenomic effects of glucocorticoids are characterized by their action through the classical GR. For example, the effect of glucocorticoids on the activation of endothelial nitric oxide synthase (eNOS). Glucocorticoids have been shown to have

some acute cardioprotective effects on cardiac ischemia[75], and nitric oxide is a key

mediator of cardiovascular protection[76]. Therapeutic concentrations of dexamethasone

activated eNOS production in human endothelial cells within 10 minutes and up to 24

hours after stimulation[77]. When the cells were pretreated with the GR-antagonist RU486,

Glucocorticoid-induced early eNOS activity and nitric oxide production were eliminated.

However, the activity was not affected by treatment with actinomycin D. These results

suggest a GR-dependent and transcription-independent action. In addition, this response

was significantly suppressed by specific inhibitors of phosphatidyl inositol 3-kinase

(PI3K), as well as by a specific inhibitor of eNOS. In contrast, it took at least 4 hours for

the same concentration of dexamethasone to activate the transcription of a reporter

construct driven by multiple glucocorticoid response elements, an effect that was

sensitive to RU486 and actinomycin D but insensitive to PI3K inhibitors. The authors

concluded that eNOS activation by dexamethasone involves rapid, nontranscriptional

mechanisms.

Two candidates for a membrane-associated glucocorticoid (GC) receptor have been so

far studied. The first is a 63-kD acidic glycoprotein, which has been identified in

neuronal plasma membranes of the amphibian Taricha granulosa. It has been functionally

characterized as a putative membrane receptor for GC, with pharmacologic characteristics

completely distinct from the known GR[78]. The second candidate has been classified in

mammalian cells as an altered form of the classical GR, which has been hypothesized to

function as a membrane-bound glucocorticoid receptor (mGR)[79]. Several studies have

characterized differences between the GR and mGR (cell localization, molecular size, and

specificity to glucocorticoid) while these same studies have also highlighted several

characteristics common to both receptors, for example, shared epitope recognition for different antibodies directed against the GR, similar ability to bind to heat shock proteins or DNA, and similar phosphorylation patterns[76]. The existence of mGR has been functionally linked to glucocorticoid-induced lysis of lymphoma cells, and it has been suggest to play a vital role in thymic involution and apoptosis[79, 80].

CONCLUSION

The method in which glucocorticoids regulate gene transcription is in no way a simple model that once was believed. For example, how can the GR interact with its GRE when the DNA is in the condensed conformation. It is possible that the GR may attach to a GRE located within a linker DNA molecule between nucleosomes. Or it may bind to GRE wrapped around histones, just as long as the core residues are facing outward[81]. Binding to the GRE can then possibly alter the local chromatin structure, allowing a configuration that will permit GR right of entry. Another uncertainty that needs to be attended to is whether there is a particular sequence of recruitment of co-factors to the activated GR complex. There has been recent work with thyroid and androgen receptors that showed that the nuclear receptors do not in themselves recruit all of the co-activators required for gene activation at target promoters[82]. Lastly, it has been demonstrated that the stable association of GR with GRE is not required in some instances. In some fascinating work by Hager and colleagues, it was shown that GR has a "hit and run" mechanism of action[83]. They used fluorescence recovery after photobleaching and fluorescence loss in photobleaching to study green fluorescent protein/GR association with a multimer of 200 copies of stably integrated MMTV-LTR. Their work showed that GR existed on DNA for less than 10 seconds before being expelled and replaced by

another GR. Despite these lingering questions, progress in the basic mechanism of glucocorticoid-regulated gene transcription, particularly in recruitment of histone-modying cofactors and some of the non-genomic effects, have provided researchers with an improved comprehension of the molecular pathways in which glucocorticoids suppress/induce gene regulation.

Chapter 2: Growth Hormone Gene Regulation: A Review

INTRODUCTION

   Differentiation of growth hormone (GH)-secreting cells (somatotrophs) in the anterior

lobe of the pituitary occurs during rat fetal and chick embryonic development. The

theory that somatotroph differentiation is regulated by glucocorticoids and thyroid

hormones has been supported in numerous studies. In cultures of chick embryonic and

rat fetal pituitary cells, the differentiation of somatotrophs can be induced prematurely

with adrenal glucocorticoids (cortisol for rat, corticosterone for chicken). In addition, the

GH-inducing effect of glucocorticoids can be augmented by co-treatment with thyroid

hormones. The plasma levels of glucocorticoids, adrenocorticotropic hormone (ACTH)

and thyroid hormones (T_3 and T_4) increase during development, and during this period

when these hormones are elevated there is a simultaneous increase in the number of

somatotrophs. Premature induction of somatotrophs with treatment of either

glucocorticoids or thyroid hormones occurs in vivo as well. Suppression of endogenous

thyroid hormone production through the use of methimazole inhibits somatotroph

differentiation in vivo, suggesting that endogenous thyroid hormone synthesis contributes

to normal somatotroph differentiation. Our working model for the regulation of

somatotroph differentiation during normal development includes modulation by elements

of the hypothalamo–pituitary–adrenal and hypothalamo–pituitary–thyroid axes. Further

studies are presented in this review that characterizes the mechanism of action for these

peripheral hormones in induction of pituitary GH gene expression during development.

GROWTH HORMONE GENE STRUCTURE

Growth hormone (GH), a polypeptide hormone synthesized in and secreted by the anterior lobe of the pituitary gland, influence a vast assortment of physiological parameters, such as growth performance, carcass composition, and milk production[84-86]. The chicken growth hormone (cGH) gene is thought to be one of the most essential candidate genes that can impact chicken performance traits because of its vital function in growth and metabolism[87, 88]. First isolated and sequenced in 1988[89], the cGH locus spans approximately 4.1 kb and encodes a 191 amino acid mature growth hormone protein and a 25–amino acid signal peptide. The cGH differs from its mammalian equivalent in that it consists of five exons and four introns[90, 91] and to date only one cGH has been identified on chromosome 1[92] while in human there are 5 highly conserved GH genes[93]. The positions of one Pit-1 binding site at –113/–104, and a suppressive thyroid hormone response element (TRE) at –137/–74 have been identified in the cGH promoter[94]. In addition to Pit-1 and TRE, other regulatory elements, such as glucocorticoid response elements (GRE) and cAMP response element (CRE), also play an important role in the regulation of GH gene expression. In the rat GH gene there have been two GREs identified, and a mutation in the proximal element is sufficient to abolish the ability of mediating glucocorticoid (GC) induction of gene expression[95]. It has also been demonstrated that glucocorticoids can act synergistically with cAMP on the rainbow trout GH gene[96]. To date, no proposed or functional GRE has been identified in the cGH gene.

SOMATOTROPH DIFFERENTIATION

Even though GH-containing cells are present in the caudal lobe of the anterior pituitary of early chick embryos[97], these cells are unlikely to secrete GH. Mainly because GH cannot be detected in the serum until embryonic 16 (e16)[98]. In 1995, Porter et al. showed that GH-secreting somatotrophs are occasionally present on e12 and comprise a statistically significant proportion of the pituitary cell population on e16[99]. On e16 the majority of somatotrophs are able to respond to GHRH, somatostatin and insulin-like growth factor-I (IGF-1)[99-101], but the ability to respond to other GH regulating hormones, such as thyrotropin-releasing hormone (TRH), is hindered until later in embryonic development[101, 102].

The capability of somatotrophs to differentiate, which is a post-mitotic event, does not happen autonomously. Indeed, it appears to be reliant on an external signal[103]. The serum from older embryos has the capacity to induce premature differentiation of somatotrophs from e12 embryos, implying that this external signal is blood borne. As a matter of fact, serum for e16 embryos is more successful at inducing differentiation than serum from e12 embryos, and the pituitary cells from older embryos are more reactive to this blood borne signal[103]. When serum from e16 embryos was heated or digested with trypsin, the ability to induce GH cells in vitro remained intact. The potential involvement of a binding protein that was suppressing the somatotroph-inducing activity of e12 serum was revealed when parallel treatments (i.e., heating and trypsin digestion) on e12 serum uncovered it[104]. In addition, the serum bioactivity was preferentially soluble in ether, suggesting it was steroidal in nature. Lastly, preabsorption of e16 serum with an antiserum against the adrenal glucocorticoid corticosterone (CORT) suppressed the

capacity of the embryonic serum to induce GH cell differentiation[104]. The concentration of CORT in the circulation of chick embryos increases dramatically between e12 and e16[105], concurrent with somatotroph differentiation. Taken together, these results indicate that an increase in adrenal glucocorticoid secretion of CORT during development may induce pituitary GH cell differentiation.

Glucocorticoids can stimulate the differentiation of GH cells in e12 or e14 pituitary cell cultures, even in the absence of GHRH[104, 106]. The somatotroph inducing ability of e16 serum is blocked by RU486, which specifically inhibits glucocorticoid receptors in chickens[107]. Trypsin augments the effectiveness of serum; as a result, the ontogenic changes in corticoid-binding proteins may also be crucial, as suggested by levels of serum activity following trypsin digestion between e12 and e16, which were similar[104]. CORT has been shown to be a GH-differentiation factor in vivo, in that injection of e11 eggs with serum from e12 or e16 or with CORT itself increases the proportion of GH-secreting cells on e14[108]. The serum from e16 embryos also expands the number of GH-containing cells[108]. However, the synergistic effect of GHRH on CORT or serum-induced somatotroph differentiation in vitro[106] is not observed in vivo[108].

Even though CORT can induce somatotroph differentiation during the 2nd trimester of incubation, it is ineffective during the last trimester. CORT cannot increase the number of GH-cells from e17 pituitary cultures[106]. GHRH by itself cannot induce somatotrophs, but can act synergistically with CORT to increase differentiation in e12 but not e14 pituitary cells[106]. There is actually a decrease in the number of GHRH-responsive somatotrophs in vitro from e17 embryos in response to pre-treatment with GHRH[106].

The fraction of somatotrophs responding to GHRH is about 50-70% on e16[99]. In addition, at e16 and e18 there are more somatotrophs that respond to GHRH than to TRH. Conversely, by e20, the proportions of GHRH and TRH-responsive somatotrophs are nearly equal (40%). These findings suggests that there is a heterogeneity in GH cell populations[100]. These outcomes may demonstrate a delayed acquirement of TRH responsiveness in a single somatotroph population or it may show that there are two distinct somatotroph populations; one that is responsive to only one secretagogue and another that is responsive to both secretagogues. As a matter of fact, early embryonic somatotrophs are responsive to GHRH[100], while responsiveness to TRH does not happen until just prior to hatching[102].

Piper and Porter in 1997 revealed that somatotroph populations may also vary in their responsiveness to inhibitory factors such as IGF-1 and somatostatin (SRIF)[101]. Both SRIF and IGF-1 decreased both basal and GHRH-stimulated GH release from e16 and e18 embryos. Although, only the basal number of GH cells was lowered in e20 pituitary cells and to a lesser extent than in earlier embryos. The elevation in GH secretion that is observed at hatching may reflect a reduction in sensitivity to inhibitory factors. In addition, Piper and Porter also noticed that IGF-1 activated a basally dormant somatotroph population due to the fact that treatment of e20 pituitary cells increased the ratio of GH secretors.

Lastly, there may be other factors that are required for somatotroph differentiation and/or maintenance. For example, the ontogenic appearance of somatotrophs that is seen on e12 in chickens is complemented with the entry of macrophages and other

hematopoietic cells into the anterior pituitary[109], thus incriminating the immune system in GH cell development.

EFFECTS OF OTHER HORMONES ON GLUCOCORTICOID ACTION

The ability of glucocorticoids to act together with other hormones in order to induce their cellular actions is well known, this fact is also evident in their ability to induce somatotroph differentiation. GHRH is the prime hypothalamic inciter of GH secretion from the pituitary. Despite the fact that GHRH alone had no direct effect on somatotroph number, GHRH in combination with CORT increased the number of cells that expressed GH mRNA and secreted GH above the number of somatotrophs induced with CORT alone in cultured e12 pituitary cells[106, 110]. Through binding and activating the type 2 somatostatin receptor, SRIF is able to suppress GH secretion in chickens[111]. SRIF also lowers the secretion of GH from somatotrophs during late embryonic development[101]. The addition of SRIF to e12 pituitary cell cultures failed to affect the CORT induction of GH-containing cells (unpublished finding). In conclusion, GHRH can augment the CORT induction on somatotroph number, however SRIF appears to have no effect.

It appears that in addition to hypothalamic and adrenocortical hormones, thyroid hormones can act to regulate somatotroph abundance during embryonic development. In cultures of fetal rat pituitary glands, simultaneous treatment with cortisol and T_3 was able to increase both the number of GH cells and GH secretion above that with cortisol alone. However, T_3 treatment by itself was unsuccessful[112]. Similar observations were seen in chickens. Treatment of chick embryonic pituitary cells with T_3 or T_4 alone had negligible effects on somatotroph abundance in vitro[113]. On the other hand, the combination of the thyroid hormones and CORT was able to augmented GH cell numbers above that found

with CORT alone. In one peculiar observation, higher levels of T_3 (10^{-8} M) partially

suppressed the induction of GH cells by CORT. This inhibitory effect of high T_3 levels

on CORT induction of GH cells was more pronounced after 6 days of treatment. Similar

inhibitory effects of high T_3 levels on CORT induced GH secretion from existing

somatotrophs occur later in development as well[114]. When cultured pituitary cells were

pretreated with the monodeiodinase inhibitor iopanoic acid, the stimulatory effect of T_4

on CORT induction of somatotrophs was ablated. This finding implies that conversion of

T_4 to T_3 within the pituitary is required for the augmented synergistic effect of T_4 and

CORT. Taken all together, these results show that glucocorticoids and thyroid hormones

could act together to control somatotroph number.

As well as the exogenous effect of the thyroid hormones to augment GH cell

differentiation, there appears to be a function for the endogenous production of thyroid

hormone in the timing of somatotroph differentiation during normal development.

Pregnant rats that were treated with dexamethasone in their drinking were injected with

T_3, this treatment resulted in an increase in the number of somatotrophs on e17 of fetal

development above that found with dexamethasone alone[115]. In contrast, suppression of

endogenous thyroid hormone production by injections of the thyroid hormone synthesis

inhibitor methimazole reduced the number of somatotrophs on fetal day 19 and

suppressed the dexamethasone effect on day 18. Injection of T_3 or T_4 into the egg

albumen of chick embryos on e9 was able to increase somatotroph cell numbers on

e13[116]. As seen in the rat, suppression of endogenous thyroid synthesis through

administration of methimazole on e9 decreased somatotroph abundance on e14. This

effect could be reversed by concurrent treatment with T_3. In conclusion, thyroid hormone secretion is required for normal somatotroph ontogeny in both rats and chickens.

As a whole, the results that were obtained from both *in vivo* and *in vitro* examinations in rat and chicken embryos point to the observation that glucocorticoid and thyroid hormones moderate the timing of somatotroph differentiation during embryonic development. The development of GH cells may also be regulated by the actions of GHRH. The model depicted in Figure 2.1 shows the effects of endocrine signals on somatotroph differentiation in the chick embryo. In this representation, somatotroph differentiation occurs in reaction to elevated levels of adrenocortical production of CORT, which is the result of increased secretion of ACTH from the anterior pituitary. Levels of both CORT and ACTH have been shown to increase significantly prior to the increase in GH cell number during development[99, 105, 116]. Injection of ACTH or CORT before the endogenous elevation in their levels results in an early increase in somatotroph abundance[108, 117]. These GH cells which are induced by CORT or ACTH are most likely already destined to differentiate into somatotrophs, with CORT acting as the final signal to induce the expression of the GH gene. Despite CORT acting the as the principal signal in somatotroph differentiation, its effects are co-regulated by thyroid hormones. It has been shown that the synthesis of thyroid hormone is needed for normal somatotroph ontogeny[116]. Lastly, the normal ontogeny of GH cells during development may be affected by hypothalamic GHRH release. While this effect has not been proved *in vivo*, simultaneous treatment with GHRH and CORT in vitro results in a further increase in GH cell numbers above CORT alone[106, 110]. Also, somatotroph abundance is reduced in the little mouse, which lacks a functional GHRH receptor[118, 119].
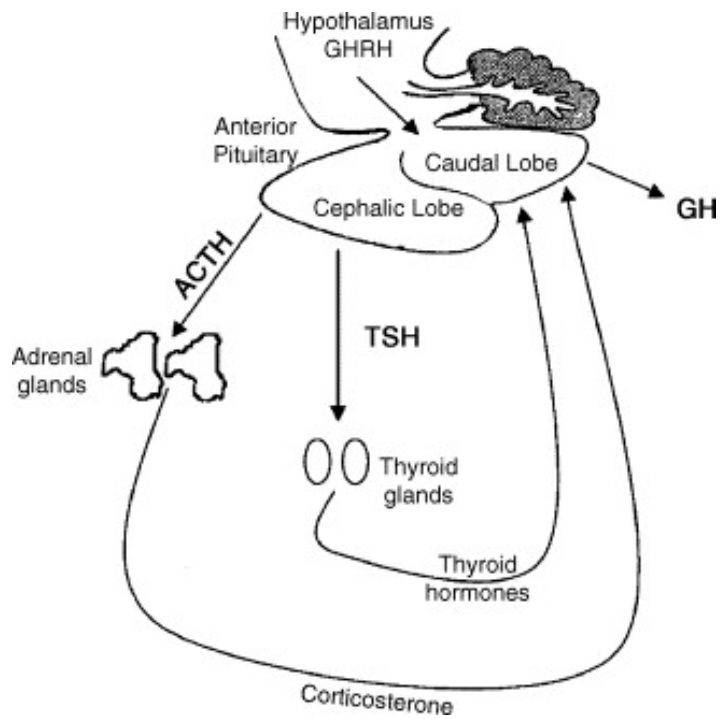
Figure 2.1. Working model of the regulation of pituitary GH cell differentiation during chick embryonic development by adrenal corticosterone and thyroid hormones. *From Porter, TE 2005 Domest Anim Endocrinol 29(1):52-62.*

CORT INDUCTION OF GH GENE EXPRESSION

The ability of glucocorticoids to increase somatotrophs prematurely during development in rats and chickens is well known, although the mechanism underlying glucocorticoid induction of GH gene expression is not as apparent. The GH inducing effect of CORT appears to be indirect. When cultures of either fetal (rat) or embryonic (chicken) pituitary cells have been pretreated with protein synthesis inhibitors (puromycin in rats, cycloheximide in chickens), the CORT stimulated increase in GH mRNA is ablated[115, 120]. This finding indicated that synthesis of some intermediate protein(s) induces GH gene expression in response to glucocorticoids. It was originally thought that this intermediate protein was Pit-1 because Pit-1 has been shown to be upregulated by glucocorticoids and Pit-1 could directly increase Prolactin mRNA in fish[121]. However this is not likely the case because Pit-1 levels were not increased by CORT treatment[122].

If CORT increases GH gene expression during development through the induction of an go-between protein, and this protein is not Pit-1 which is known to be vital for the expression of the GH gene[123-125], then what is the identity of this protein? At present, the answer to this question is the focus of several open research projects. The GHRH receptor was one apparent candidate due to the fact that glucocorticoids significantly increase GHRH receptor expression in rat pituitary cell lines and rat fetal pituitaries in culture[126, 127]. On the contrary, research using cultured chick embryonic pituitary cells does not support the hypothesis that the GHRH receptor is involved in CORT induction of GH mRNA. As stated earlier, co-treatment with CORT and GHRH increases GH mRNA levels synergistically above those induced by CORT treatment of e12 pituitary

cells alone[120]. As well, treatment with activators of protein kinase A (PKA) and cAMP

production increased GH mRNA levels above that with CORT alone. However, the

treatment of these activators in the absence of CORT failed to elevate GH mRNA levels.

In addition, inclusion of the PKA pathway inhibitor H-89 or the PKC pathway inhibitor

calphostin C had no affect on the ability of CORT to induce GH mRNA, although H-89

did impede the synergistic effect of GHRH and CORT on GH mRNA[120]. In summary,

these outcomes imply that CORT stimulated GH gene expression does not happen

through induction of GHRH receptors. Nevertheless, these findings do not rule out the

possibility of GHRH receptors having a role in the synergistic actions of CORT and

GHRH on GH mRNA levels. In contrast to the data on the PKA and PKC pathways,

recent studies have shown that Ras or a Ras-related enzyme may be involved. For

example, the Ras inhibitor manumycin suppressed CORT stimulated increases in GH

mRNA levels[120]. CORT may induce GH gene expression through activation of the Ras

signal transduction cascade. Alternatively, CORT might induce the rapid expression of

another factor and then this unidentified factor acts in cohort with Ras to stimulate GH

gene expression. Additional research in this area is required.

There has been one piece of the puzzle of CORT induction of somatotroph

differentiation that has been solved. Usually, it has been thought that CORT binds to and

functions through primarily to the glucocorticoid receptor (GR). However, it is well

known that glucocorticoids show a high affinity for both GR (type II) and the mineral

corticoid (type I) receptor.   There has been recent analysis showing that CORT

stimulation of GH mRNA occurs through the MR as well as the GR. Pretreatment of

cultures of chicken pituitary cells with the GR specific antagonist ZK98299 or the MR

antagonist spironolactone failed to block CORT induction of somatotroph differentiation or increases in GH mRNA[128]. However, combined treatment with both ZK98299 and spironolactone completely eliminated the CORT induction of GH mRNA and GH-containing cells. The presence of MR within the embryonic somatotrophs induced by CORT was verified by dual immunofluorescence for MR and GH. Consequently, these findings indicate that glucocorticoids can induce somatotroph differentiation during development by acting through either GR or MR. Interestingly, the embryonic development period between e10 and e12 is when pituitary expression of MR occurs[128] and when somatotroph differentiation is responsive to CORT treatment *in vivo*[117].

CONCLUSION

Glucocorticoid induction of somatotrophs has been well characterized during both rat fetal and chick embryonic development. Glucocorticoids possess the aptitude to provoke somatotroph differentiation *in vitro* and advance GH cell ontogeny *in vivo*. Endogenous production of CORT through treatment with ACTH can also increase the number of GH cells. One question that remains is whether suppression of endogenous glucocorticoid production or ablation/antagonism of glucocorticoid receptors impedes or hinders somatotroph differentiation. Quite the opposite occurs when thyroid hormone synthesis is suppressed, as there is a decrease in the number of GH cells that initially appear, implying that thyroid hormones are required in normal somatotroph ontogeny. The identity of the unknown intermediatiory protein that functions in CORT induction of GH mRNA during development remains to be solved. As of right now, all that is proven is that this it is not Pit-1, nor the GHRH receptor, and it does not function through the PKA

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or PKC pathways. Identification of the intermediary protein(s) and pathways involved in glucorticoid induction of cGH gene expression during development is a major research focus in our laboratory. As a final point, interaction between glucocorticoids and the MR in glucocorticoid regulation of GH production during early development and in adults deserves additional research.

Chapter 3: Cloning and characterization of a chicken dexamethasone-induced ras-
related 1 (Dexras1) cDNA

INTRODUCTION

The members of the Ras protein family are small guanine nucleotide-binding factors that play central roles in various cellular signal transduction pathways that regulate processes such as apoptosis, proliferation, and cell differentiation[129, 130]. Ras proteins serve as binary switches in signal transduction by rotating between inactive GDP-bound forms and active GTP-bound forms. They can be found in almost all cell types, where they have critical functions in various cellular pathways, including cytoskeletal organization, intracellular transport, and vesicle formation[131]. The mRNA levels of ras genes remain relatively constant in cells, however some ras proteins can be upregulated in response to serum growth factors[132] or in cells that have become tumorigenic[131]. In 1998, Kemppainen and Behrend identified a novel mouse gene that was rapidly induced in a murine corticotroph cell line (AtT-20) in response to glucocorticoid treatment (dexamethasone, Dex)[133]. This protein was named Dexamethasone-induced Ras related protein 1, or Dexras1. More recently, the human homologue of Dexras1 was identified as a ligand-independent activator of heterotrimeric G proteins in a pheromone assay in yeast[134] and subsequently named Activator of G protein signaling (AGS1).

The predicted Dexras1/AGS1 protein contains patterns that are typical of the small G-protein Ras-superfamily, although there is the presence of an extended C-terminus compared to that of H-ras. In addition to functioning as a ligand-independent activator of G-proteins, Dexras1/AGS1 may act to suppress G-protein signaling downstream of

ligand binding[135, 136]. Dexras1/AGS1 was also shown to be a protein-binding partner for CAPON, which is a protein known to associate with neuronal nitric oxide synthase[137]. Lastly, Dexras1 might have a potential function as a mediator of glucocorticoid negative feedback regulation of ACTH secretion from pituitary corticotrophs. Dexras1/AGS1 is abundantly expressed in the pituitary and is quickly induced (within 15 min) in response to glucocorticoids[133, 138]. In AtT-20 cells, this induction is glucocorticoid-specific, occurs in a dose-dependent manner, and appears mediated at the level of gene transcription[133, 139]. What's more, the expression of a constitutively active form of Dexras1 in AtT-20 cells suppressed cAMP-stimulated secretion of transiently expressed growth hormone, corroborating the perception that Dexras1/AGS1 might work to impede regulated endocrine secretion in corticotrophs[138].

To date, Dexras1 has been identified in mice, rats, and humans, but not in other vertebrates. Moreover, pituitary expression of Dexras1 has not been characterized during normal embryonic/fetal development in any species.

We report here the cloning and characterization of a chicken Dexras1 cDNA. This is the first time that Dexras1 has been characterized in chickens or in any other avian species. We show that Dexras1 expression in the chicken is pituitary-specific, regulated by glucocorticoids and increases at the end of embryonic development concomitant with the known increase in endogenous CORT.

MATERIAL AND METHODS

Animals

All animals used were Avian x Avian chicken embryos. All procedures with chicken embryos were approved by the Institutional Animal Care and Use Committee on this campus. All hormones and other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated. Embryonic day 0 (e0) was defined as the day when the eggs were placed in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 C. The typical length of embryonic development for chickens is 21 days.

Chicken Dexras1 cDNA cloning and sequencing

A cDNA library was produced from chicken pituitary, hypothalamic, and pineal gland RNA, and clones from this library were sequenced at random as described previously[140]. One clone from this library (pgp1n.pk012.a14) with limited predicted amino acid homology to human, mouse, and rat Dexras1 was selected for the current study. Oligonucleotide primers for chicken Dexras1 were designed and purchased from Sigma-Genosys (The Woodlands, TX). Plasmid and PCR products were sequenced in both directions by the University of Maryland DNA Sequencing Facility using AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP (Perkin-Elmer/Applied Biosystems; Foster City, CA) and an Applied Biosystems DNA sequencer (model 3730). DNA sequence assembly and alignments were conducted using Vector NTI Advance 9.0 software (Invitrogen; Carlsbad, CA) and the overlapping sequences were assembled into a contig. The sequence of chicken Dexras1 (Accession# DQ516976) was compared to

that of human (Accession# BCO18041), mouse (Accession# BCO34166), and rat (Accession# AF239157) Dexras1 using the BLASTN and BLASTX programs of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequence of chicken Dexras1 was predicted (SIXFRAME) and the sequence alignment (CLUSTALW) and comparison (TEXSHADE;DRAWTREE) with those of human, mouse, and rat were done using the tools within Biology Workbench maintained by the San Diego Super Computer Center (http://workbench.sdsc.edu/).

Tissue Distribution by Northern Blot and RT-PCR

Northern Blot Analysis

Total RNA was isolated from spleen, pituitary, lung, brain, adrenal, kidney, and heart of juvenile chickens using the Trizol procedure according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Isolated RNA was quantified using a UV spectrophotometer (Genequant II; Pharmacia Biotech, Piscataway, NJ). For the Northern Blot analysis, 10 μg of total RNA from the aforementioned tissues were separated by formaldehyde 1% agarose gel electrophoresis and transferred to a nylon membrane (Zeta-Probe® Blotting Membranes, Bio-Rad, Hercules, CA) by vacuum blotting using a Bio-Rad Model 785 Vacuum Blotter System (Hercules, CA). The membrane was crosslinked using a UV stratalinker (Stratagene, La Jolla, CA). The membrane was then probed with a ^{32}P-labeled full-length chicken Dexras1 cDNA probe generated by PCR using the pgp1n.pk012.a14 plasmid and vector-specific primers. The 20 μl PCR reaction consisted of 2 μl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton-X-100, and dH$_2$O), 1 μl of 25mM MgCl$_2$, 2 μl of 1mM dNTPs (A, T, G), 2 μl of 70 μM dCTP, 1 μl

each of plasmid-specific primer at 0.5 μg/μl, 2 μl containing 1 pg of purified PCR product, 2 μl of 3.3 μM [α^{32}P]dCTP, 1 μl of 2 U/μl Taq Polymerase, and 6 μl of nuclease-free water. Hot start PCR was performed using a commercial thermal cycler (Techne Progene; Princeton, NJ). PCR thermal cycling parameters were as follows: one cycle at 94°C for 3 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 3 min, with a final extension at 72 °C for 7 min. The PCR product was purified by passage through a spin column-30 (Sigma, St. Louis, MO) according to the manufacturer's protocol. Incorporation of radioactivity was determined by β-counter. Hybridization was performed overnight at 65 °C using PerfectHyb™ Plus 1X Hybridization Buffer (Sigma, St. Louis, MO). The membrane was washed twice under low stringency conditions (2X SSC, 0.1% SDS, room temperature) and 3 times under high stringency conditions (0.2X SSC, 0.1% SDS, 65 °C) and bands were visualized by phosphorimager (Molecular Dynamics PhosphorImager™, Amersham Biosciences, Piscataway, NJ). The membrane was stripped (0.02X SSC, 0.1% SDS at 90°C) and re-hybridized with a ^{32}P-labeled chicken β-actin probe.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

For the RT-PCR analysis, 1 μg of total RNA from the same tissues used in the Northern analysis was subjected to RT-PCR using an anchored oligo-dT primer. 2 μl of the RT reaction were then used in a 25 μl PCR reaction using 1 set of Dexras1-specific primers (Sense: 5'-AAGCAGCAGATCCTGGAGAC-3', Antisense: 5'-CAGAGTCCGTGCACAGCTTC-3'). Genomic liver DNA (DNA) was used a negative control.

Developmental profile of Dexras1 expression

Embryos were removed from the incubator on e11, e14, e17, and e20. The pituitaries

were isolated using a dissecting microscope, immediately frozen in liquid nitrogen, then

stored at -80 °C until RNA extraction. 5 pituitaries were pooled together for each

replicate at each age (n=3).

CORT induction of Dexras1

Eggs were removed from the incubator on e12. Fertile eggs were then injected into

the small apical end with either 1500 ng (in 100 μl of 2.9% ethanol solution) of CORT or

100 μl of 2.9% ethanol in deionized water and then immediately placed back into the

incubator for 2 hours.   After the 2 hours, the pituitaries were isolated using a dissecting

microscope, immediately frozen in liquid nitrogen, and then stored at -80 °C until RNA

extraction. 5 pituitaries were pooled together for each replicate of each treatment group

(n=3).

RNA extraction, Reverse transcription, and Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from the pituitaries using the RNeasy Mini Kit (Qiagen,

Valencia, CA) according to the manufacturer's protocol, and then quantified by

measuring the absorbance at 260 and 280 nm using a UV spectrophotometer (Genequant

II; Pharmacia Biotech, Piscataway, NJ). 1 μg of the total RNA was then reverse-

transcribed (RT) with SuperScript II (Invitrogen, Carlsbad, CA) using an oligo-dT primer

in a 20 μl reaction. Following reverse transcription, 2 μl of the RT reaction was used per

25 μl of real-time PCR reaction employing SYBR green detection. Chicken Dexras1

specific primers (Sense: 5' – GGTCTACCAGCTCGACATCC-3', Antisense: 5'-TGAACACGAGGATGAAAACG-3') were designed using Beacon Designer 2.1 software (Bio-Rad Laboratories). Primers were also designed for chicken Beta-actin, which was used as a normalizing gene (Sense: 5'CGCATAAAACAAGACGAGA-3', Antisense:5'-GTTTTTAAGGCGGAAGATACA-3'). PCR standards were prepared for both Dexras1 and Beta-actin using the cDNA from one of the control groups using the same primers. The PCR standards were purified using a spin column, their concentrations determined, and then diluted to contain 1×10^2 to 1×10^8 transcripts/µl. Real-time PCR was performed in a Bio-Rad iCycler using iQ SYBR Green Supermix (Bio-Rad Laboratories) and analyzed by iCycler iQ Real-time PCR Detection System Software Version 3.0 A. The quantity of cDNA in unknown samples was calculated from the appropriate external standard curve run simultaneously with the samples. Samples and standards were assayed in duplicate. Relative expression levels were calculated with the following formula: number of Dexras1 transcripts/number of Beta-actin transcripts.

Statistical Analysis

Statistical analyses were performed using Statistical Analysis System (SAS) version 8.02 (SAS Institute, Cary, NC). Levels of mRNA reported are the means and standard errors of the relative expression levels described above. Prior to statistical analysis, qRT-PCR data were transformed to correct for heterogeneity of variance among treatment groups by taking the $\log_{10}$ of the relative expression levels. Results were then analyzed by analysis of variance using the PROC MIXED procedure of the Statistical Analysis

System (SAS; SAS Institute; Cary, NC). Differences between treatments were compared using the PDIFF procedure (SAS). Differences were considered significant at P≤0.05.

RESULTS

Sequence comparison to other species

We have recently isolated a chicken Dexras1 clone from a chicken neuroendocrine library constructed in our lab. The cDNA encodes an open reading frame of 837 bp that encodes a putative chicken protein with a predicted molecular mass of approximately 31.5 kDa. The nucleotide and predicted amino acid sequences are available in Genbank (Accession# DQ516976). Sequence analysis has shown that chicken Dexras1 is highly homologous at both the nucleic acid and protein level to human, mouse, and rat Dexras1 (Table 3.1). All motifs that are known to be vital for Ras functions, including the P-loop GXXXXGK(S/T) (residues 31-38), the guanine base binding loops NKXD (residues 145-148) and EXSAK (residues 175-179) and the C-terminal farnesylation site CAXX (residues 278-281) are well conserved in the chicken Dexras1 protein (Figure. 3.1).

Species	% Identity Nucleic Acids	% Identity Amino Acids
Human	87	87
Mouse	82	87
Rat	82	86

Table 3.1. Chicken Dexras1 cDNA and predicted amino acid sequences were compared with those of Human, Mouse, and Rat Dexras1 sequences. The comparisons were made across the protein coding open reading frames.

A

```
Mouse_Dexras1   MKLAAMIKKMCPSDSELSIPAKNCYRMVILGSSKVGKTAIVSRFLTGRFE
Rat_Dexras1     --------------------------MVILGSSKVGKTAIVSRFLTGRFE
Human_Dexras1   MKLAAMIKKMCPSDSELSIPAKNCYRMVILGSSKVGKTAIVSRFLTGRFE
Chicken_Dexras1 MKLAAMIKKMCPSEAELSIPAKNCYRMVILGSSKVGKTAIVSRFLTGRFE

Mouse_Dexras1   DAYTPTIEDFHRKFYSIRGEVYQLDILDTSGNHPFPAMRRLSILTGDVFI
Rat_Dexras1     DAYTPTIEDFHRKFYSIRGEVYQLDILDTSGNHPFPAMRRLSILTGDVFI
Human_Dexras1   DAYTPTIEDFHRKFYSIRGEVYQLDILDTSGNHPFPAMRRLSILTGDVFI
Chicken_Dexras1 EQYTPTIEDFHRKFYSIRGEVYQLDILDTSGNHPFPAMRRLSILTGDVFI

Mouse_Dexras1   LVFSLDNRDSFEEVQRLKQQILDTKSCLKNKTKENVDVPLVICGNKGDRD
Rat_Dexras1     LVFSLDNRDSFEEVQRLKQQILDTKSCLKNKTKENVDVPLVICGNKGDRD
Human_Dexras1   LVFSLDNRDSFEEVQRLRQQILDTKSCLKNKTKENVDVPLVICGNKGDRD
Chicken_Dexras1 LVFSLDNRDSFEEVQRLKQQILETKSCLKNKTKENIEVPLVICGNKGDRD

Mouse_Dexras1   FYREVEQREIEQLVGDDPQRCAYFEISAKKNSSLDQMFRALFAMAKLPSE
Rat_Dexras1     FYREVEQREIEQLVGDDPQRCAYFEISAKKNSSLDQMFRALFAMAKLPSE
Human_Dexras1   FYREVDQREIEQLVGDDPQRCAYFEISAKKNSSLDQMFRALFAMAKLPSE
Chicken_Dexras1 FYREVQPREIEQLVGADPKKCAYFEISAKKNSSLDQMFQALFAMAKLPSE

Mouse_Dexras1   MSPDLHRKVSVQYCDVLHKKALRNKKLLRAGSGGGG-DHGDAFGILAPFA
Rat_Dexras1     MSPDLHRKVSVQYCDVLHKKALRNKKLLRAGSGGGG-DHGDAFGILAPFA
Human_Dexras1   MSPDLHRKVSVQYCDVLHKKALRNKKLLRAGSGGGGGDPGDAFGIVAPFA
Chicken_Dexras1 MSPDLHRKVSVQYCDILHKKALKGKKLLKEGGRGG---TEEAYGVVAPFA

Mouse_Dexras1   RRPSVHSDLMYIREKTSVGSQAKDKERCVIS
Rat_Dexras1     RRPSVHSDLMYIREKTSVSSQAKDKERCVIS
Human_Dexras1   RRPSVHSDLMYIREKASAGSQAKDKERCVIS
Chicken_Dexras1 RRPSVHSDLMYIREKAIGGGHGKEKDRCVIS
```

B
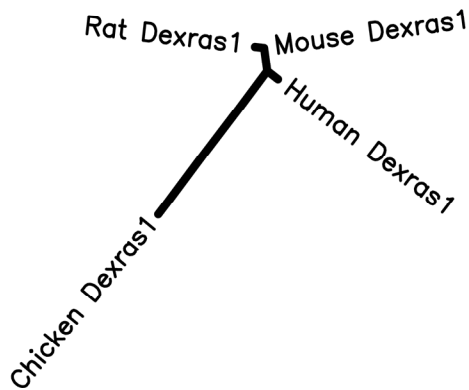
Figure 3.1. (A) Predicted amino acid sequences for mouse, rat, human, and chicken Dexras1, were compared using the CLUSTALW command of Biology Workbench. Boxed groups of amino acids show motifs that are known to be essential for Ras functions. (B) Phylogenetic tree representing Dexras1 amino acid sequence comparisons among species is also shown.

Tissue distribution of Dexras1

Previous studies have shown that Dexras1 is expressed in a variety of tissues in human and mice, including the brain, ovary, heart, kidney, skeletal muscle, pancreas, and liver[133, 141]. Logically we wanted to determine which tissues expressed Dexras1 in the chicken. Total RNA was isolated from spleen, pituitary, lung, brain, ovary, adrenal, kidney, and heart of juvenile chickens, and tissue distribution was determined by both Northern blotting and RT-PCR analysis. In contrast to mouse and human tissues, both types of analysis showed that Dexras1 could only be detected in the pituitary in the chicken (Figure 3.2). This finding, however, does not rule out the possibility that Dexras1 can become expressed at a later age in the chicken (i.e., sexual maturity), as the research from the human and mouse studies were done with adult tissues.
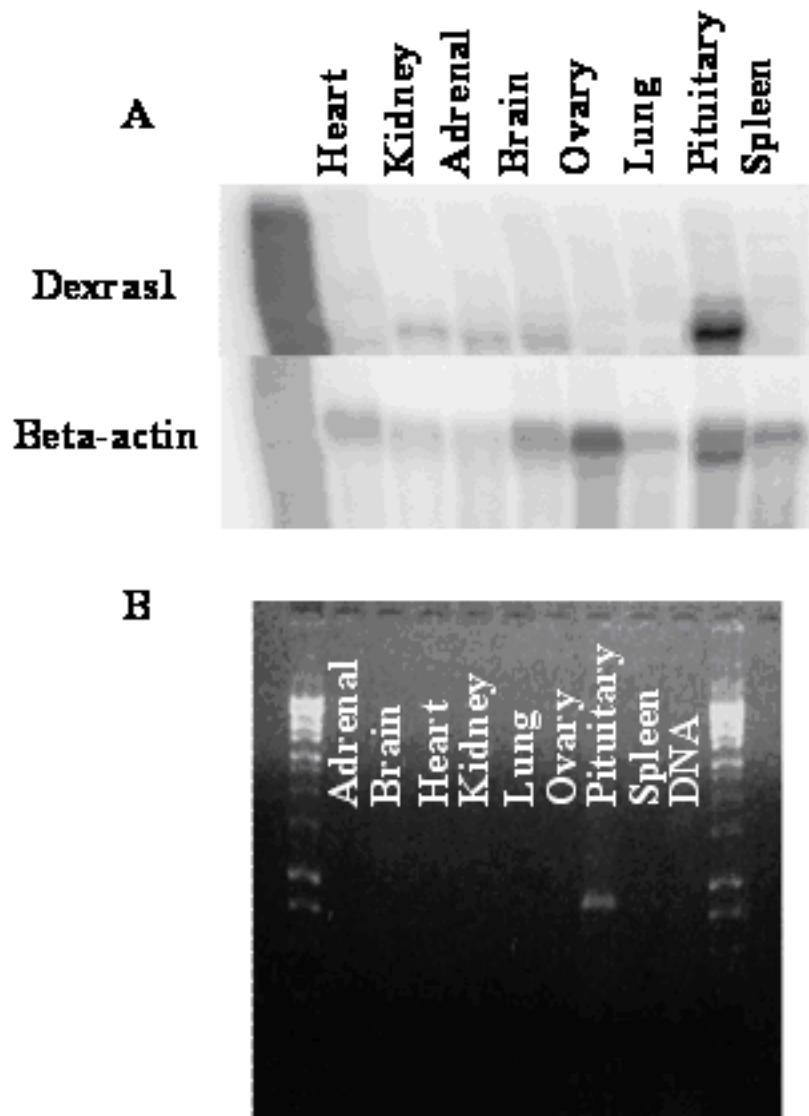
Figure 3.2. Tissue Distribution of Dexras1. (A) 10 µg of RNA from adrenal, brain, heart, kidney, lung, ovary, pituitary, and spleen were subjected to Northern blotting for Dexras1. Dexras1 mRNA is expressed in high levels in the pituitary. B) 1 µg of RNA from these tissues were subjected to RT-PCR using an anchored oligo-dT primer for the RT reaction and 1 set of Dexras1-specific internal primers for the PCR reaction. Genomic liver DNA (DNA) was used a negative control. Dexras1 cDNA was detected only in the pituitary. The product was the predicted size of 890 bp.

Developmental profile of Dexras1 expression during embryonic development

During the course of chicken embryonic development, plasma concentrations of glucocorticoids show two distinct patterns of increase. The first occurs between e14 and e16 of embryonic development[142], and the second occurs immediately before hatching (e20 through e21)[143-145]. Knowing that the expression of Dexras1 is increased in response to glucocorticoids in mammals, we wanted to ascertain if the mRNA expression levels of Dexras1 fluctuated in conjunction with the plasma concentrations of glucocorticoids. We isolated pituitaries from embryos on e11, e14, e17, and e20. Next, we extracted the RNA and determined the levels of Dexras1 using qRT-PCR analysis. We found that Dexras1 showed two significant increases in its expression, the first occurring between e14 and e17, and the next occurring between e17 and 20 (Figure 3.3). Thus the Dexras1 mRNA expression profile shown here is consistent with the reported plasma glucocorticoids levels seen in chickens during their embryonic development.
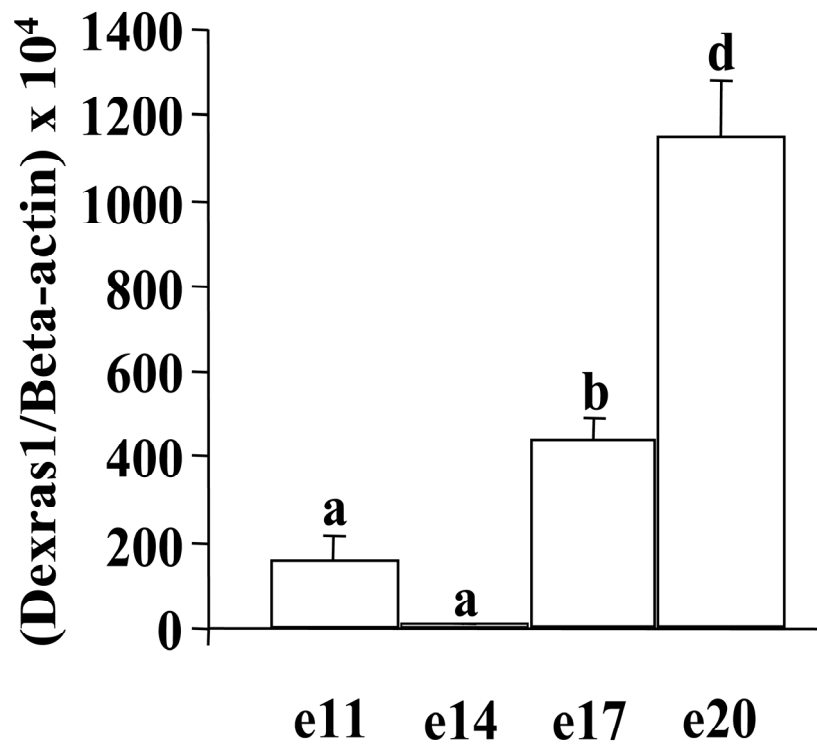
Figure 3.3. Developmental expression profile of Dexras1. On e11, e14, e17, and e20, pituitary glands were isolated and total RNA extracted mRNA was reverse transcribed Using Dexras1 specific primers, qRT-PCR was performed to determine the amount of Dexras1 cDNA present. Means without a common superscript differ (P<0.05). *CORT induction of Dexras1 in vivo*

To investigate the effect of CORT on the expression of Dexras1 in vivo, we injected 1500 ng of CORT into the egg albumen of e12 embryos. Two hours later, we isolated the pituitaries and extracted mRNA from CORT injected embryos as well as embryos injected with a control solution of 2.9% ethanol in deionized water. The expression levels of chicken Dexras1 mRNA in the CORT treated and control treated e12 embryos were analyzed by quantitative real-time PCR (qRT-PCR) with Dexras1 specific primers. The results show that CORT was able to increase the amount of Dexras1 mRNA transcripts more than two-fold compared to the control injected group (Figure 3.4). The findings show that CORT can increase pituitary Dexras1 mRNA expression quickly and significantly *in vivo* in embryonic chicken.
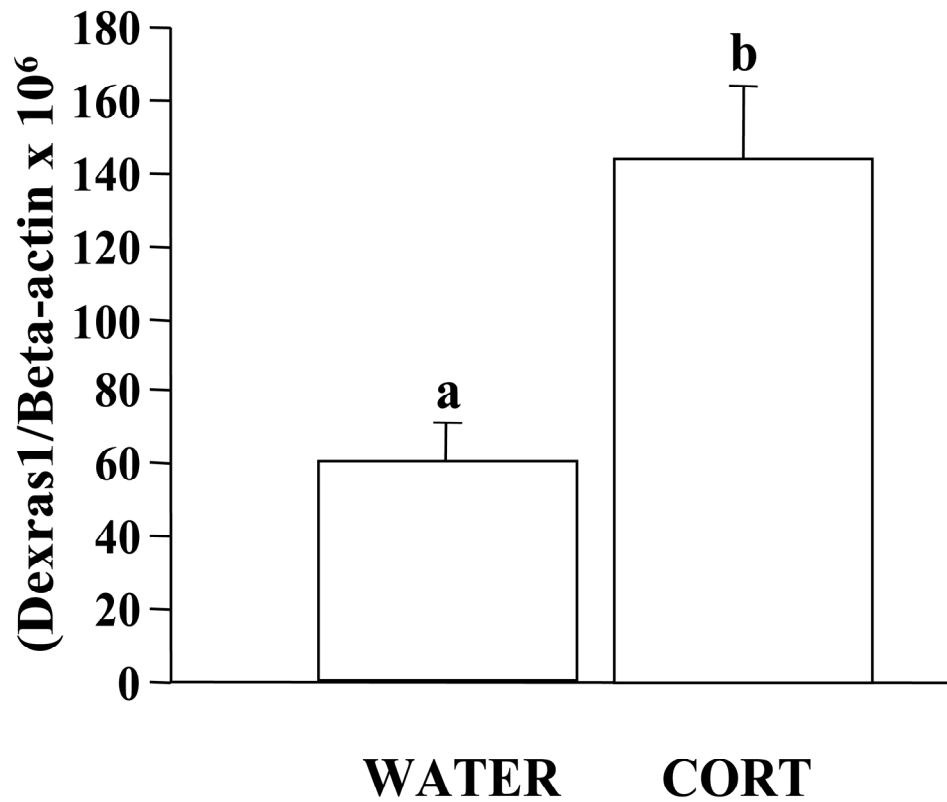
Figure 3.4. Dexras1 is upregulated by CORT treatment in e12 chicken pituitaries. Fertile e12 eggs were injected either with 1500 ng of corticosterone (black bar, n=3) or 2.9% ethanol (white bar, n=3) which served as a negative control. 2 hours later the pituitaries were isolated and RNA extracted. Total RNA was reverse transcribed into cDNA using oligo-dT primers. Using Dexras1 specific primers, qRT-PCR was performed to determine the amount of Dexras1 cDNA present. Means without a common superscript differ ($P<0.05$).

DISCUSSION

In the present study we cloned and characterized the Dexras1 cDNA found in chicken. Our findings show that chicken Dexras1 is highly homologous at both the nucleic acid and protein levels to those of the Dexras1 forms found in human, mouse, and rat. In contrast to other studies on the tissue distribution of Dexras1, our findings indicate that Dexras1 expression is limited to the pituitary gland (at least in 3-week old chickens). The expression profile of Dexras1 in the anterior pituitary gland during embryonic development appears to be positively correlated to circulating levels of CORT in the plasma of chickens. Through *in ovo* injection of CORT we showed that Dexras1 is quickly and significantly induced *in vivo*.

Ras proteins have a very ubiquitous cell type distribution and are involved in a variety of cellular activities. It is not surprising that Dexras1 has been proposed to have multiple diverse cell-specific functions. Previous studies in our lab have shown that CORT can induce growth hormone gene expression in the chicken embryo both *in vitro*[104] and *in vivo*[108]. However, this effect of CORT requires the synthesis of one or more proteins[120]. We have also shown that the Ras inhibitor manumycin suppressed CORT stimulated increases in GH mRNA levels[120]. These findings combined with our current results make it tempting to speculate that Dexras1 might be involved in glucocorticoid induction of GH gene expression. However research needs to be done to define the exact role of Dexras1 in the chicken embryonic pituitary gland and to identify the anterior pituitary cell type(s) in the chicken pituitary that express Dexras1.

The present study, as well as previous examinations, has shown that Dexras1 is quickly and strongly up-regulated in response to glucocorticoids. This induction has been shown to occur in as little as 15 minutes[133, 138], suggesting a direct action of glucocorticoids on Dexras1. A functional glucocorticoid response element (GRE) has been found in the human Dexras1 gene in the 3' flanking region (2.3 kb downstream of poly(A) signal)[146]. This GRE conferred rapid glucocorticoid responsiveness when inserted into a homologous promoter-driven luciferase reporter, and a point mutation within the 15-bp GRE abolished this glucocorticoid responsiveness. However, analysis of the 5' and 3' flanking region (5 kb of each region) and the 2 exons and 1 intron of the chicken Dexras1 gene using the vertebrate TRANSFAC transcription factor matrix within TESS (http://www.cbil.upenn.edu/cig-bin/tess/tess33/) yielded only multiple potential GRE half-sites. In spite of this observation, it has been shown that glucocorticoids can regulate the expression of various genes through trans-activation mechanisms[147, 148].

In summary, chicken Dexras1 is highly similar in nucleic acid and amino acid sequence to those of the human, mouse, and rat forms. As seen with *in vitro* and *in vivo* models from other species, chicken Dexras1 is positively regulated by exposure to glucocorticoids. Its tissue distribution restriction to the pituitary gland and lack of any potential GRE sequences within 1 kb of the gene (both 5' and 3') might imply a distinctive mechanism of glucocorticoid action on chicken Dexras1 gene expression. Its ontogeny of expression during embryonic development of the chick anterior pituitary gland suggests that Dexras1 might function in glucocorticoid regulation of pituitary differentiation or gene expression, including negative feedback on POMC expression or

induction of GH expression. Future research is needed targeting the functional role of Dexras1 in the chicken embryonic pituitary gland.

CHAPTER 4: Characterization of Glucocorticoid-Induced Changes in Gene Expression in the Embryonic Pituitary Gland

INTRODUCTION

The embryo of the domestic chicken (*Gallus domesticus*) provides a fitting vertebrate model to study pituitary cell differentiation in that 1) it can easily be manipulated without confounding maternal interferences and 2) the pattern of anterior pituitary cell differentiation is similar to that of mammals[149]. The pituitary gland is a highly vascularized structure that is composed of two parts from two different embryonic germ layers. The posterior portion of the gland is derived from neural origin, while the anterior portion, called the adenohypophysis, originates from oral ectoderm. The fully differentiated anterior lobe of the gland is composed of the following five different cell phenotypes: 1) Lactotrophs, which secrete prolactin (PRL); 2) Thyrotrophs, which secrete thyroid stimulating hormone (TSH); 3) Gonadotrophs, which secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH); 4) Corticotrophs, which secrete adrenocorticotropic hormone (ACTH); and 5) Somatotrophs, which secrete growth hormone (GH).

We have previously established that between embryonic day (e) 13 and e16 of chicken embryonic development, a considerable rise in the number of somatotrophs takes place, even though an occasional somatotroph can be detected earlier[97, 99]. Somatotrophs lack the ability to differentiate spontaneously and require a blood-borne extrapituitary signal for their induction in culture[103]. We demonstrated that serum from e16 embryos but not that from e12 embryos was able to stimulate somatotroph differentiation in cultures of e12 pituitary cells, which is four days in advance to the time that somatotroph differentiation would typically occur in the chick embryo[103]. This factor contained in the

serum of e16 embryos was ether-soluble, bound by a trypsin-sensitive protein, and was heat-stable[104]. These characteristics suggested that this blood-borne factor was a steroid. Consequently, we showed that the blood-borne signal capable of prematurely inducing somatotroph differentiation was the adrenocortical glucocorticoid CORT[104].

The ability of glucocorticoids to stimulate somatotroph differentiation during embryonic development is not restricted to chickens. Oral administration of a synthetic glucocorticoid, dexamethasone (Dex), to pregnant rats induces early GH expression in the corresponding day 17 or day 18 fetuses[150, 151]. It has also been reported that glucocorticoids can effectively stimulate GH cell differentiation in pituitary cultures derived from rats[112, 150, 152].

When e11 chicken embryos were treated *in vivo* with CORT, the number of cells that secreted GH was augmented on e13, and this reaction involved elevated expression of GH mRNA in the caudal portion of the anterior pituitary gland[108, 117]. In cultures of e12 pituitary cells, CORT was able to increase GH mRNA due to an increase in the amount of cells expressing GH mRNA[110, 120]. In addition, treatment with the protein synthesis inhibitor cycloheximide (CHX) completely blocked CORT induction of GH mRNA, indicating that the response requires the ongoing synthesis of one or more proteins. Furthermore, treatment with CORT failed to induce somatotrophs prior to e11, and premature somatotroph induction by CORT does not does not alter the quantity of GH cells later in development[117]. Taken together, these results indicate that corticosteroids are involved in the terminal steps of somatotroph differentiation in the chicken embryo, that the corticosteroid-induced increase in GH gene expression and GH secretion is limited to a group of cells that are already committed to become somatotrophs, and that

glucocorticoid induction of GH gene expression is indirect, involving synthesis of another protein(s).

Our hypothesis is that this unknown "intermediate" protein(s) synthesized in response to CORT ultimately induces GH gene expression and somatotroph differentiation. The purpose of this study was to identify this unknown protein(s) that is involved in CORT induction of GH mRNA during chicken embryonic development. To help in identifying potential candidate genes for this intermediate protein(s), we used a high-density chicken cDNA microarray. The DEL-MAR 14K Integrated Systems Microarray contains 14,053 cDNAs assembled from the neuroendocrine system, the reproductive system, the liver, adipose tissue, and skeletal muscle. Based on this microarray analysis, candidate genes were identified, and the effects of overexpression of these genes in transient transfection assays were determined.

MATERIALS AND METHODS

Animals and pituitary dispersions

All animals were Ross broiler strain chicken embryos purchased from Allen's Hatchery (Seaford, DE). All procedures were approved by the Institutional Animal Care and Use Committee. All hormones and other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Embryonic day 0 (e0) was defined as the day when the eggs were placed in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 °C. The typical incubation length for chickens is 21 days. On e11, the embryos were removed and their pituitary glands isolated using a dissecting microscope. The pituitaries

were dispersed into individual cells by trypsin digestion and mechanical agitation as described previously[103].

Cell culture

Dispersed pituitary cells were plated (~1 X 10^6 cells/well) in poly-L-lysine coated 12-well culture plates in serum-free medium (D-MEM/F-12 nutrient mixture; Invitrogen, Carlsbad, CA) supplemented with 0.1% bovine serum albumen (BSA), 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were allowed to attach for 1 h in a 37.5 °C, 5% CO_2 atmosphere (VWR Model 2250; VWR International, West Chester, PA). Cells were then either 1) pretreated for 1.5 h with 10 μg/ml CHX and then subsequently cultured in the presence of CORT (10^{-9}M) for 0, 1.5, 3, 6, 12, or 24 hrs, or (2) cultured with corticosterone (10^{-9}M) with no CHX pretreatment for 0, 1.5, 3, 6, 12, or 24 hrs. The concentration of CHX (10 μg/ml) was chosen because it was previously shown to block GH mRNA up regulation[120]. CORT stock solution (10^{-3}M) was prepared in absolute ethanol and further diluted in cell culture medium. Wells were randomly selected for each treatment/time point. Untreated cells served as a control. All cells were maintained in culture for 24 h regardless of treatment. At the end of the 24 h culture period, cells were retrypsinized and immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation.

RNA Isolation, Amplification, and In Vitro Transcription

Total RNA was isolated from the cultured pituitary cells using a RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol, and quantified using the RiboGreen RNA Quantitation Kit (Invitrogen). To remove any contaminating DNA, each sample was treated with DNAse I. The pituitary gland of a chicken embryo does not provide ample RNA for microarray analysis. For that reason we utilized an earlier detailed[153] variation of the Eberwine procedure[154] to amplify mRNA. Briefly, 0.5 μg of total RNA was reverse-transcribed with SuperScript II (Invitrogen, Carlsbad, CA) and an oligo(dT) primer containing a T7 promoter site (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGT$_{24}$-3', Affymetrix, Santa Clara, CA). After second strand synthesis, phenol-chloroform was used to extract the double-stranded cDNA; afterwards the cDNA was filtered with a Microcon-30 column (Millipore, Billerica, MA). Lastly, the double-stranded cDNA was used as a template for *in vitro* transcription with the T7 MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's protocol. The amplified RNA (aRNA) that resulted was phenol-chloroform extracted, purified with a Spin Column-30 (Sigma, St. Louis, MO), and then the quantity was measured using the RiboGreen RNA Quantitation Kit (Invitrogen). This amplification procedure was validated in a previous microarray study in our laboratory[155].

Production of the Del-Mar 14K Integrated Systems Microarray

The cDNA library used to make the microarrays was generated from RNA isolated from the neuroendocrine system (hypothalamus, anterior pituitary gland, and pineal gland), reproductive system (ovary, testes, and oviduct), and metabolic/somatic systems (liver, skeletal muscle, epiphyseal growth plate, abdominal fat) of chickens ranging in age from e12 to d63 and has been described previously[140, 153]. The construction and normalization of our chicken cDNA library was performed as a custom service by a commercial company (Life Technologies Inc., Rockville, MD; now Invitrogen) and has been described in detail elsewhere[156]. The DNA sequencing of the unigene clones was carried out at Dupont's high-throughput sequencing facility (Agricultural Products Division E.I. du Pont de Nemours & Co, Inc., Delaware Technology Park, Newark, DE) according to their protocols. The clone annotation process is described in detail by Carre and collegues[156].

The unigene clones were cultivated overnight in Terrific Broth (Sigma) in 96-well PCR plates. They were then lysed, and the inserts were PCR amplified from the pCMV·Sport6.1 vector (Invitrogen) using SP6 (5'-GGCCTATTTAGGTGACACTATAG-3') and T7 (5'-GCTTATAATACGACTCACTATAGGG-3') vector-specific primers (Sigma Genosys, The Woodlands, TX). The PCR products were detected by staining with ethidium bromide in 1% agarose gels. An annotated registry of the clones and their spot on the DEL-MAR 14K Integrated Systems microarrays can be found at the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) data repository (Platform Accession # GLP1731; National Center for Biotechnology Information (NCBI), Rockville, MD).

The GEO file also provides links to the cDNA sequence, contig sequence, BLASTN and BLASTX alignments, and chromosomal location.

Microarray hybridization and data analysis

Samples were hybridized to the microarrays using a reference design[157]. An internal reference sample was generated by labeling an aliquot of an RNA pool made from all the RNA samples with Cy5. The RNA from the experimental samples were tagged with Cy3 and hybridized to an array with an aliquot of the Cy5 labeled pool from each study. This design resulted in the use of 48 microarrays (12 treatment groups per replicate, n=4) for the 24 hr global analysis study.

The labeling of the cDNA with both Cy3 and Cy5, microarray hybridization, and image scanning were carried out at the University of Maryland Biotechnology Institute's Microarray Core Facility (http://www.umbi.umd.edu/~cbr/macore/macorestart.htm, Rockville, MD). Generation of the Cy3- or Cy5-labeled target cDNA was done in a two-step process. First, 1 μg aRNA was reversed transcribed using random primers with the Amino Allyl cDNA Labeling Kit (Ambion). Next, both Cy3 and Cy5 mono-reactive esters were attached to the cDNA (Amersham Biosciences, Piscataway, NJ). The labeled cDNA targets were then filtered from the unincorporated fluorescent dyes with the CyScribe GFX Purification Kit (Amersham). Microarray hybridization was carried out overnight at 42 °C with the Cy3-labeled experimental samples and an aliquot of the Cy5-labeled reference pool using microarray hybridization buffer (Amersham). After the overnight hybridization, the slides were washed with increasing salt sodium citrate

stringency and then scanned with a 418 confocal laser scanner (Affymetrix) at 550 nm for Cy3 and 649 nm for Cy5, generating two TIFF images for each slide.

The data obtained from the microarray analysis was processed and normalized using software that is part of the TM4 suite of microarray data analysis applications[158] offered by The Institute for Genomic Research (TIGR, Rockville, MD). Two TIFF images for each slide were processed using Spotfinder (version 2.2.4). Afterwards, the data that were determined with Spotfinder were exported to the Microarray Data Analysis System (MIDAS; version 2.18) to be normalized. Lowess normalization was carried out on the data from the Cy3 channel without background correction. Next, the data underwent standard deviation regularization first by block then by slide, with Cy5 (the pooled RNA sample) as the reference. The fluorescent intensity of each spot was calculated by dividing the total fluorescene (pixels) by the spot area. The background fluorescence was determined for each slide by taking the mean Cy3 and Cy5 fluorescence values of the 8 control spots (salmon testes DNA) on each slide. Any spot whose Cy3 or Cy5 fluorescence intensity levels were below background was deleted from further analysis. Spots whose fluorescence intensity levels (both Cy3 and Cy5) was above background were corrected by subtracting the average Cy3 and Cy5 background fluorescence of each slide from the corresponding Cy3 and Cy5 fluorescence intensity values for each spot. Data were then analyzed as $\log_2$(corrected Cy3/corrected Cy5), or $\log_2$-ratio, for each spot.

Plasmid constructs and transfection experiments

Plasmids containing the full length cDNAs for Dexras1, Prostaglandin-D Synthase, and RASDVA were obtained from the clones produced in our cDNA library (see above). Briefly, these clones were grown overnight in Terrific Broth (Sigma), and the plasmids were prepared for the transfection studies using a Nucleobond Maxiprep Plasmid Isolation Kit (Clontech, Palo Alto, CA). Plasmids were sequenced in both directions by the University of Maryland DNA Sequencing Facility using AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP (Perkin-Elmer/Applied Biosystems; Foster City, CA) and an Applied Biosystems DNA sequencer (model 3730) and the integrity of the plasmids was confirmed by gel electrophoresis. Dispersed e11 pituitary cells (~3 X 10^6 cells/treatment group) were cultured in serum-free Opti-MEM® I Reduced Serum Media (Invitrogen) and then were transfected with either an empty pCMV-Sport6.1 vector, Dexras1 (clone: pgp1n.pk012.a14, Accession# BX930456) Prostaglandin-D Synthase (clone: pgl1n.pk007.j7, Accession# CAA07005), or RASDVA (clone: pgp2n.pk003.j19, Accession# AY729886) plasmid along with a Golgi-targeted Green fluorescent protein (GFP)-expression vector[159], which served as a transfection control. The cells were transfected using the Lipofectamine[TM] 2000 Transfection Reagent (Invitrogen) according the manufacturer's instructions for a 6-well culture plate. The cells were transfected in suspension for 2 hrs at 37 °C, then plated into duplicate wells of a 6-well culture plate and placed back into the incubator for another 4 hrs. At the end of the 4 hr incubation, the Opti-MEM® I Reduced Serum Media was removed and the cells were re-cultured in serum free DMEM-F12 for 24 hrs at 37 °C. After 24

hrs, one well from each transfection group was treated with CORT (10^{-9} M) for 18 hrs while the other well of the same transfected group was left untreated.

After the 18 hr incubation period, the cells were retrypsinized as described above and sorted into GFP$^+$ and GFP$^-$ cell groups by fluorescence-activated cell sorting (FACS) at the laboratory of Dr. H. Dave Guthrie of the United States Department of Agriculture (Biotechnology and Germplasm Division, Beltsville, MD) according their protocols. Samples were analyzed on a MoFlo High Speed Flow Cytometer (Dako-Cytomation Inc., Fort Collins, CO) equipped with a 200 mW air-cooled 488 nm Coherent Innova 300 laser. Green GFP fluorescence was collected after a 530/30-nm bandpass filter. Cells were sorted under a flow rate of 500-800 cells/sec in a sheath of PBS under 40 psi. On each sample, a minimum of 30,000 GFP+ cells were collected. The MoFlo cytometer utilized Summit software from Dako-Cytomation to collect and analyze data. GFP$^+$ and GFP$^-$ cells were immediately frozen in liquid nitrogen and stored at -80 C until RNA extraction. RNA was extracted and quantified as described above, and 20 ng of total RNA was used for the reverse transcription reaction that was carried out with the oligo(dT) primer containing a T7 promoter. This experiment was repeated 4 times.

Primer Design

Primers (Sigma Genosys) used for PCR were designed using the PRIMER3 design tool within Biology Workbench maintained by the San Diego Super Computer Center (http://workbench.sdsc.edu/), and were designed from the contig or singlet sequence for that cDNA. All cDNA sequences are available from an online searchable database

(http://cogburn.dbi.udel.edu/). The sequences off all the primers that were used are listed

in Table 4.1.

Gene	Forward (5' - 3')	Reverse (5' - 3')
CCO	GCATGGAGTGGAAATACGGT	GCACATCCGAGAGACACAAG
SMARCD1	ACTCAGACTCGCCCAGTGAT	TCCGCTGGGATTCAAATATC
LSM7	GATGGCGGATAAGGAGAAGA	ACCACTTGCTTCTCTCCCAC
PLEKHB1	CTCTGGAGGCAGAGTTCCAT	GCACGAACCAATTCCTCTTC
CEPU	AGAAGGGCATCCTGATGTGT	CAGCCCTTTCTGTCCTTCAG
FBP	CAGGGACAGGTCATTTCACA	CAGCTGGATCGTACCTAGCC
PMPP22a	TCTCCTTCCTGGCCTTTGTA	TCATTCATTGCCCTCCTCTT
SUMO2	CCACAACCTTGGGAGAAGAG	GCCAGTGGAACCCTATCAAA
SEMA7A	CAAATTCCCTCGTCATCGTT	AGAGCTCTCCATCCCCTTTC
NOTCH	GAGCCAGTTGGAGAAGATGC	CTGCCATGTTACCCTCTGGT
Dexras1	GGTCTACCAGCTCGACATCC	TGAACACGAGGATGAAAACG
PDS2	ACGCTCCTGTCCTACAAAGC	CTCTGTCCCTGAGAGCCAAC
Ras-dva	AGGAAGCTCTCCATCCAGAA	GGAGGGAATTTGTCCTCCTT
Beta-actin	TTCTTTTGGCGCTTGACTCA	GCGTTCGCTCCAACATGTT
POMC	AGGGACCTCAGGGATCATCAA	TGTTCAAGGGCAGGTTGGA
PRL	ACCTGTGGGCTGCATTACTCA	AAGTTACTGATGATCCTGGTGCTGTA
GH	TTCAAGAAGGATCTGCACAAGGT	CTCAGATGGTGCAGTTGCTCTCT
IAP3	GAGCACAAGAGGCATTTTCC	ATTCTGCCATGGATGGATTC
Selenoprotein I	TTCCTGCTGCTTGTCTTCAA	GAGCTTGTTTGCCATCAACA
FKBP5	AGCACTGCATCCTCTACCTG	CTTTGGTGTCCATCTCCCAT

Table 4.1. Primers used in this study.

qRT-PCR

Two-step qRT-PCR was used to validate gene expression patterns from the microarray analysis and levels of gene expression from the overexpression studies. Reverse transcription reactions were carried out using SuperScript III (Invitrogen) with random primers (Invitrogen) for aRNA or the oligo-dT primer containing a T7 promoter site primer for T-RNA used in the transfection studies. Reverse transcription reactions (20 μl) were done using 500 ng aRNA for the microarray verification analysis or 20 ng T-RNA for the overexpression analysis. As a negative control for genomic DNA contamination, a pool of all the RNA from a given experiment was made, and the reaction conducted as the others except reverse transcriptase was not added. All reactions were diluted to 40 μl (2-fold) prior to PCR analysis. The diluted RT samples (2 μl) were then analyzed using the SYBR Green PCR Master Mix (Applied Biosystems) and a Bio-Rad iCycler.

Each PCR reaction (12 μl) contained 1 μl of diluted cDNA, 5 μM of each primer, and 6 μl of 2X PCR buffer (described below). Cycling parameters were: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 sec and 55 °C for 45 sec. Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reaction. The data were transformed using the equation 2^{-Ct}, where Ct is the threshold cycle, the fractional 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. For the microarray verification experiments, the data were divided by the mean of the highest level of gene expression for each technique for statistical analysis and comparison with the microarray

results. For the transfection experiments, the data were divided by the mean of the 2^{-Ct} value of the control group in a given experiment.

Statistical Analysis

Statistical analyses were performed using Statistical Analysis System (SAS) version 8.02 (SAS Institute, Cary, NC). Microarray data (Log_2 ratios) were subjected to one-way analysis of variance (ANOVA) to identify spots that were differentially expressed on at least one of the time points. Differences were considered significant at $P \leq 0.05$. Levels of mRNA reported are the means and standard errors of the relative expression levels described above. Prior to statistical analysis, qRT-PCR data were transformed to correct for heterogeneity of variance among treatment groups by taking the log_{10} of the relative expression levels. Results were then analyzed by analysis of variance using the PROC MIXED procedure of the Statistical Analysis System (SAS; SAS Institute; Cary, NC). Differences between treatments were compared using the PDIFF procedure (SAS). Differences were considered significant at $P \leq 0.05$.

RESULTS

Time course of CORT induction of GH mRNA

In order to ascertain which genes could be involved in CORT induction of GH mRNA during chicken embryonic development, the time course of CORT induction of GH mRNA was studied. To date the earliest time point at which CORT has been shown to stimulate GH mRNA in chicken embryos is 4 hrs[160]. Thus our first goal was to assess the expression of GH mRNA as a marker to identify genes regulated earlier by CORT. As shown in Figure 4.1, GH levels continued to significantly increase until 3 hrs after CORT treatment, where they no longer increased significantly, but GH mRNA levels remained elevated compared to the 0 hr time point throughout the 24 hr culture period. Interestingly, GH mRNA levels were induced more than 2 fold within 90 minutes of CORT treatment. In addition, CORT induction of GH mRNA was blocked by pretreatment with the protein synthesis inhibitor cycloheximide (CHX), as previously reported[120]. As a result of the data shown in Figure 4.1, we defined early CORT regulated genes as those whose expression was affected (induced or repressed) at least 2-fold within 3 hr after CORT treatment. Those genes whose expression levels were regulated at least 2-fold after 3 hrs (6, 12, and 24 hr) of CORT treatment were considered late CORT regulated genes.

Growth Hormone
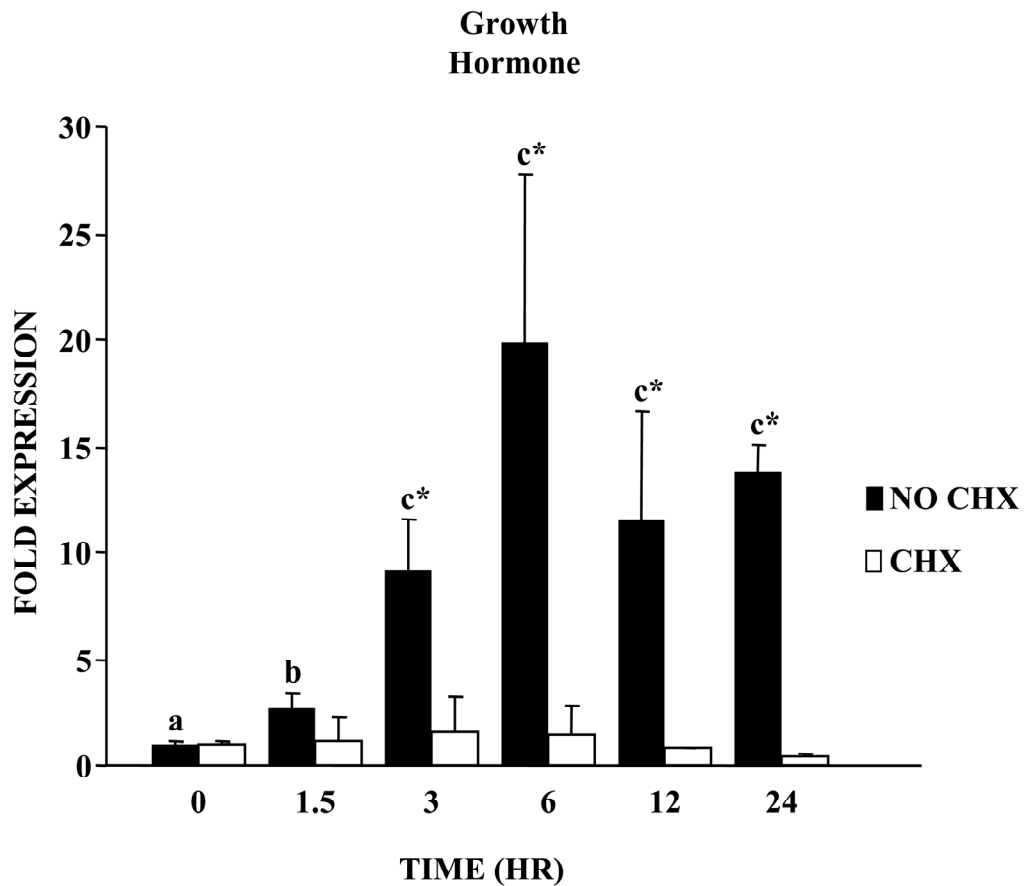
Figure 4.1. Time course of GH mRNA after CORT administration. Dispersed e11 pituitary cells were either pretreated for 1.5 h with 10 µg/ml Cycloheximide (CHX) and then subsequently cultured in the presence of CORT (10^{-9}M) for 0, 1.5, 3, 6, 12, or 24 hrs (open bars) or cultured with CORT (10^{-9}M) with no CHX pretreatment (black bars). Cells were then harvested, and total cellular RNA extracted. Levels of ACTB and GH mRNA were determined by qRT-PCR. Levels of GH mRNA were normalized to levels of ACTB mRNA, and results are expressed relative to the 0 hr time point. Results presented are the means and SE of the relative expression levels for 4 replicate experiments. Means without a common superscript differ (P<0.05) within the no CHX pretreatment group. Values denoted with an asterisk (*) were significantly different from CHX pretreated group at the same time point (P<0.05).

Microarray analysis after 24 h of CORT treatment

Because the goal of the microarray study was to identify candidate primary response genes that may mediate CORT induction of GH mRNA, we chose the 3 hr time point as the early time point marker and the time points 6, 12, and 24 hr as late markers. The Cy3-labeled aRNA that was prepared from RNA isolated from dispersed e11 pituitary cells either pretreated with CHX then CORT or CORT alone was competitively hybridized to our DEL-MAR 14K Integrated Systems microarray chips with Cy5-labeled aRNA from a pool of all experimental samples. Table 4.2 displays the output from the microarray analysis with the background corrected log2 Cy3/Cy5 ratios and p values for those spots on the array that showed a significant difference between any two time points, a total of 388 genes ($p < 0.05$).

From the 388 significant genes from table 4.2, we chose to display the distribution of those genes at each time point that were either initially upregulated or down regulated compared to the control time point 0 hr. As seen in figure 4.2, there were 49 transcripts that were at least 2-fold upregulated at 1.5 hr, that was nearly twice as much as those that were downregulated at the same time point (28 genes). At the 3 hr time point, there appeared to be an inverse of the proportion of transcripts that were up/down-regulated compared to the 90 min time point, with only 3 transcripts showing a significant increase in expression levels and 24 showing a reduction. At the later time points (6, 12, and 24 hr), the number of transcripts that were significantly induced or repressed gradually decrease, culminating at the 24 hr time point were only 4 genes showed an increase in expression levels and only 1 transcript was significantly repressed. We then decided to categorize our microarray data by grouping genes into one of 5 possible categories based

on the initial effect of CORT treatment on their levels of expression. This categorization

process excludes any biphasic expression pattern a gene may display in response to

CORT. The 5 categories that we chose were: (1) Genes that were induced early (i.e., at

least 2-fold within 3 hr), (2) Genes that were repressed early, (3) Genes that were induced

late (i.e., at least 2-fold between 6 – 24 hr), (4) Genes that were repressed late, and (5)

Genes that displayed no significant effect at any time point compared to basal (time point

0 hr). The microarray analysis detected a total of 49 transcripts that were significantly

increased early (i.e., within 3 hr), 48 that were significantly decreased early, 20 and 13

that were significantly increased and decreased late, respectively, after CORT treatment

(Table 4.3). The distribution of transcripts being either induced or repressed during the

24 hr culture period from figure 4.2 and table 4.3 implies a trend that CORT regulates the

expression of genes (either directly or indirectly) during shorter time intervals than at

later time points. This finding is not all that surprising given the fact that the lipophilic

nature of glucocorticoids (and other steroids) allows them to freely diffuse in and out of

the cell, which would allow their effects on gene expression to more immediate. It has

been shown previously that glucocorticoids can affect gene expression in a matter of

seconds/minutes[161, 162].

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
1	1.1.1	-4.04	-0.29	-1.24	-2.27	-2.38	-3.33	0.00	-2.26	0.20	0.47	2.37	0.74	0.95	0.01
9	1.1.9	-0.04	0.15	-0.09	-0.16	-0.01	-0.16	0.04	0.01	0.06	0.07	0.02	0.22	0.02	0.67
105	2.2.5	-0.50	0.33	-0.13	-1.11	0.08	-0.90	0.04	-0.52	0.77	-0.27	0.88	0.71	-0.07	0.28
302	4.4.2	-0.25	-0.04	-0.13	-0.28	0.13	-0.23	0.05	-0.14	0.00	-0.10	-0.11	0.23	-0.15	0.20
309	4.4.9	-0.44	0.45	-0.64	2.01	2.53		0.00	0.27	2.77		2.39	0.51		0.33
368	3.5.8	-0.26	-0.81	0.05	-0.12	-0.16	-0.16	0.03	-0.22	0.07	0.29	-0.21	-0.50	0.14	0.33
461	4.6.1	-0.10	0.11	-0.09	-0.20	-0.11	-0.39	0.04	0.06	0.12	0.05	0.13	0.34	0.03	0.54
481	1.7.1	-0.46	-0.25	-0.16	0.04	0.29	-0.04	0.02	-0.48	-0.31	-0.40	-0.21	0.15	-0.28	0.08
533	3.7.13	-0.26	0.08	-0.07	-0.13	0.09	-0.11	0.02	0.01	0.01	-0.03	-0.01	0.14	0.05	0.66
541	4.7.1	-0.29	0.32	-0.42	-1.35	1.03	-0.08	0.02	0.09	0.30	-0.09	0.68	0.69	-0.29	0.28
570	1.8.10	0.02	-0.71	0.12	0.05	0.25	-0.15	0.00	-0.18	-0.14	0.17	-0.48	-0.66	0.13	0.03
598	2.8.18	-0.09	-0.77	0.05	-0.07	-0.19	-0.03	0.01	-0.08	-0.12	0.25	-0.25	-0.66	0.30	0.00
614	3.8.14	0.00	-0.46	0.07	0.02	-0.22	-0.04	0.02	-0.08	-0.28	-0.06	-0.47	-0.57	-0.08	0.01
652	1.9.12	0.07	-0.47	0.22	0.15	0.02	0.19	0.04	0.10	0.09	0.42	-0.16	-0.66	0.34	0.03
758	2.10.18	0.02	-0.51	0.01	0.07	-0.09	0.06	0.02	-0.16	-0.26	0.06	-0.19	-0.48	-0.03	0.18
790	4.10.10	-0.06	-0.26	-0.03	-0.01	0.23	0.15	0.04	-0.02	0.07	0.04	0.00	0.12	-0.04	0.92
848	3.11.8	0.02	-0.37	0.11	0.05	-0.07	-0.13	0.04	-0.06	-0.03	0.11	-0.40	-0.35	0.11	0.05
1043	1.14.3	0.68	0.87	-0.21	0.20	-0.11	-0.07	0.02	0.51	0.51	-0.57	0.91	-0.15	-2.08	0.00
1078	2.14.18	-0.38	-0.03	-0.29	-0.34	0.73	0.01	0.00	-0.12	1.31	-0.02	0.72	0.70	0.03	0.45
1138	1.15.18	-0.12	-0.35	-0.05	0.05	-0.02	0.23	0.01	-0.17	-0.13	0.24	0.38	-0.11	0.10	0.00
1215	1.16.15	0.06	-0.37	0.23	0.18	-0.01	0.16	0.02	0.10	0.11	0.35	0.04	-0.20	0.32	0.20
1232	2.16.12	-0.02	-0.52	0.28	0.09	-0.03	0.13	0.03	0.10	-0.08	0.38	-0.04	-0.34	0.27	0.19
1255	3.16.15	-0.18	-0.77	0.18	0.09	-0.13	0.00	0.04	-0.04	0.00	0.31	-0.35	-0.40	0.12	0.22
1260	3.16.20	0.01	-0.35	0.06	0.06	-0.11	0.07	0.02	-0.06	-0.03	0.18	0.05	-0.31	-0.03	0.11
1405	3.18.5	-0.10	-0.23	0.08	0.05	-0.33	-0.11	0.02	-0.01	-0.12	0.22	-0.01	0.14	0.31	0.15
1637	6.1.17	-0.31	0.05	-0.23	-0.49	-0.15	-0.28	0.02	-0.07	0.14	0.10	0.36	0.28	0.19	0.83
1765	5.3.5	0.03	0.54	-0.55	0.65	0.77	3.31	0.03	0.01	-0.44	-1.62	-0.31	-1.04	-2.06	0.15
1808	7.3.8	0.22	0.15	0.21	-0.06	-0.45	-0.26	0.00	0.15	0.03	0.08	0.19	-0.26	-0.06	0.14
1818	7.3.18	0.49	0.97	-0.42	0.42	1.10	1.62	0.03	0.58	-0.03	-1.74	-0.97	-0.93	-0.38	0.31
1876	6.4.16	0.76	0.73	-0.11	-0.93		3.50	0.05	-0.09	0.41	0.15	2.17	-0.33	3.35	0.26
1951	6.5.11	0.06	0.44	0.03	-0.17		-0.08	0.00	0.14	0.10	0.00	0.21	0.36	0.00	0.09
1985	8.5.5	-0.02	0.09	-0.15	-0.12	0.35	-0.29	0.01	0.10	0.17	0.04	-0.07	0.33	-0.34	0.17
2081	5.7.1	-0.51	-0.35	-0.44	-0.60	-0.21	-0.29	0.04	-0.40	-0.11	-0.26	-0.24	0.04	-0.09	0.01
2172	5.8.12	0.05	-0.26	0.08	0.04	-0.02	-0.04	0.01	0.07	0.10	0.15	0.07	-0.21	0.18	0.01
2174	5.8.14	-0.88	-0.42	-0.25	0.61	-0.14	0.07	0.01	-1.52	-0.79	-0.15	0.66	0.60	0.65	0.00
2248	5.9.8	-0.06	0.03	0.14	0.09	-0.12	-0.14	0.02	-0.02	0.04	0.07	0.06	-0.05	0.09	0.52
2276	6.9.16	0.26	-0.38	0.37	0.36	-0.08	0.25	0.03	0.26	0.18	0.30	0.10	-0.48	0.30	0.03
2338	5.10.18	0.14	-0.46	0.09	0.06	-0.05	0.06	0.02	-0.14	-0.14	0.07	-0.15	-0.46	0.05	0.04
2446	7.11.6	-0.46	0.03	-0.29	-1.25	-0.09	-0.50	0.02	-0.23	-0.49	-0.17	-0.72	0.17	-0.42	0.29
2644	5.14.4	-3.05	0.35	-0.10	0.42	0.30	0.48	0.00	0.08	0.39	0.13	0.56	0.48	0.00	0.68
2647	5.14.7	-0.04	-0.52	-0.09	-0.09	-0.10	-0.10	0.05	-0.27	-0.15	0.18	-0.11	-0.45	0.08	0.00
2650	5.14.10	-0.15	-0.55	-0.04	-0.07	-0.19	-0.05	0.03	-0.13	-0.07	0.16	-0.06	-0.44	0.19	0.01
2671	6.14.11	-0.08	-0.71	-0.06	0.01	-0.14	0.03	0.05	-0.04	-0.18	0.19	-0.27	-0.52	0.06	0.06
2694	7.14.14	-0.14	0.03	0.11	-0.03	0.28	0.10	0.01	-0.02	0.08	0.06	-0.06	0.34	0.04	0.33
2756	6.15.16	-0.13	-0.83	-0.12	-0.18	-0.22	-0.07	0.05	-0.17	-0.25	0.10	-0.29	-0.53	0.08	0.13
2758	6.15.18	-0.19	-0.74	-0.33	-0.17	-0.31	-0.19	0.05	-0.18	-0.17	0.07	-0.13	-0.36	0.11	0.30
2811	5.16.11	-0.06	0.16	0.03	-0.29	0.21	-0.11	0.01	-0.01	-0.04	-0.02	0.33	0.36	-0.07	0.18
2815	5.16.15	-0.01	0.18	0.07	-0.32	0.37	1.76	0.02	0.40	0.76	0.44	0.77	0.85	0.10	0.92
2822	6.16.2	-0.93	-0.03	0.06	0.16	-0.32	-0.37	0.01	-1.08	-0.26	0.00	0.92	0.49	0.33	0.00

Table 4.2. The output from the microarray analysis. The table shows the spot number of the clone (Spot), the position on the array that each clone occupied (ID), and the background corrected log2 Cy3/Cy5 ratios for each spot for the 0, 1.5, 3, 6, 12, and 24 hr time points under basal (B, with CORT alone) and cycloheximide pretreated (CHX) conditions. In addition, the p values (p) for both treatment conditions are also given.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
2862	8.16.2	-0.84	-0.14	-0.04	-0.05	-0.33	-0.11	0.01	-1.02	-0.21	0.02	1.05	0.46	0.01	0.01
2898	5.17.18	1.31	0.66	1.36	2.71	-0.24	7.26	0.00	1.14	0.47	0.94	1.66	10.11	-0.02	0.09
2953	8.17.13	0.08	0.04	0.49	-0.60	2.61	1.82	0.00	0.05	0.22	-4.10	0.59	-1.04		0.07
3205	9.1.5	-0.01	-0.42	-0.06	-0.12	-0.14	-0.13	0.03	-0.28	-0.09	0.02	-0.22	-0.33	0.01	0.03
3225	10.1.5	-0.30	0.03	0.14	-1.11	1.10	3.82	0.01	0.39	0.85	1.25	0.24	0.70	0.62	0.99
3296	9.2.16	0.04	0.34	-0.01	-0.13	-0.11	-0.14	0.03	0.19	0.15	0.21	0.04	0.09	0.22	0.90
3320	10.2.20	0.17	0.15	0.07	0.04	-0.42	-0.35	0.01	-0.08	0.13	0.16	0.12	-0.15	-0.06	0.24
3338	11.2.18	0.24	0.18	-0.38	0.06	-0.60	2.07	0.01	0.14	0.96	-0.22	-0.21	-1.04		0.74
3404	11.3.4	-0.31	0.19	-0.23	-0.32	1.23	0.22	0.01	-0.36	-0.12	-0.10	0.51	1.38	0.09	0.10
3408	11.3.8	-1.31	2.57	1.62	1.16	1.18	2.49	0.01	-0.37	1.54	-0.69	-1.62	-3.07	-2.63	0.12
3492	11.4.12	0.20	-0.18	0.07	0.13	-0.03	0.29	0.04	0.02	-0.07	0.06	-0.38	-0.78	-0.15	0.04
3528	9.5.8	0.01	0.12	0.04	0.02	-0.09	-0.18	0.01	0.07	0.03	0.05	0.13	0.09	0.00	0.76
3572	11.5.12	-0.27	0.63	-0.15	-0.23	0.22	-0.21	0.00	0.08	0.04	-0.04	0.16	0.45	-0.13	0.31
3581	12.5.1	0.24	0.63	0.22	0.33	2.97	7.31	0.01	0.74	0.10	-0.26	0.08	1.54	1.10	0.46
3623	10.6.3	0.18	0.70	0.07	-0.20	0.02	-0.16	0.04	0.18	0.28	0.02	0.37	0.53	-0.26	0.06
3643	11.6.3	-0.27	0.53	-0.04	-0.10	-0.07	0.03	0.03	0.25	0.01	-0.09	0.03	-0.03	-0.02	0.74
3644	11.6.4	-0.48	0.60	-2.17	0.28	1.03	1.36	0.03	0.52	0.60	0.06	1.82	3.14	0.13	0.14
3839	12.8.19	-0.06	-0.27	0.19	0.07	-0.10	-0.02	0.04	-0.17	-0.11	0.15	-0.07	-0.64	-0.12	0.03
3858	9.9.18	0.05	-0.29	0.09	0.05	-0.02	0.18	0.02	0.21	-0.04	0.21	0.10	-0.22	0.29	0.41
3966	11.10.6	-0.14	0.24	0.07	-0.10	1.06	-0.43	0.03	0.67	0.25	-0.11	0.40	0.74	0.02	0.86
3998	12.10.18	-0.25	-0.38	0.09	0.11	-0.20	0.07	0.04	-0.32	-0.17	0.17	0.65	-0.16	-0.14	0.22
4081	9.12.1	-0.92	-0.24	-0.34	-0.05	0.80	0.15	0.02	0.05	-0.01	0.23	-0.65	0.24	-0.50	0.70
4236	12.13.16	1.09	1.18	-0.13	-0.16	-0.29	0.25	0.04	0.47	0.25	-0.58	0.72	-1.79	-3.72	0.00
4270	10.14.10	-0.23	-1.08	-0.13	-0.33	-0.32	-0.08	0.04	-0.16	-0.25	0.25	-0.38	-1.08	0.09	0.03
4432	10.16.12	0.03	-0.90	0.17	0.04	-0.21	0.02	0.00	-0.15	-0.16	0.36	0.30	-0.62	0.27	0.03
4494	9.17.14	0.07	0.26	0.16	-0.12	0.47	-0.04	0.05	-0.23	0.13	-0.13	-0.09	0.57	0.20	0.01
4518	10.17.18	0.59	0.89	0.27	-0.31	0.63	1.21	0.03	0.33	0.62	-0.23	-0.70	-0.78	-1.05	0.14
4617	11.18.17	-0.07	-0.74	0.00	-0.01	-0.27	0.07	0.01	-0.07	-0.05	0.26	-0.33	-0.88	0.13	0.00
4654	9.19.14	0.03	0.09	-0.19	4.58	-0.53	0.83	0.02	0.40	0.93	0.06	1.12	5.87	0.66	0.49
4861	16.1.1	-0.34	0.10	0.08	0.09	-0.24	-0.33	0.05	-0.19	0.42	0.36	1.00	0.32	0.13	0.02
4875	16.1.15	-0.07	-0.16	0.14	0.25	0.06	0.08	0.04	-0.25	-0.01	0.19	0.18	-0.02	-0.06	0.12
4891	13.2.11	0.00	0.30	-0.09	-0.15	0.12	-0.19	0.04	0.00	0.01	-0.23	0.25	0.30	-0.18	0.10
5047	13.4.7	-1.37	0.22	0.97	1.46	0.43	0.82	0.00	-1.24	-0.79	-0.17	0.32	-0.68	-0.78	0.04
5048	13.4.8	-0.93	-0.08	-0.06	0.12	-0.32	-0.38	0.01	-0.40	-0.28	0.40	1.43	0.55	0.23	0.09
5050	13.4.10	-4.36	-1.59	-0.07	0.33	-1.01	-1.11	0.03	-3.37	-1.34	-0.37	1.52	1.39	0.86	0.00
5088	15.4.8	-2.15	-0.84	-0.06	0.07	-0.72	-1.00	0.00	-2.92	-0.71	0.11	1.68	0.28	0.69	0.00
5120	16.4.20	-0.08	-0.09	0.02	0.53	0.23	0.18	0.00	-0.19	-0.12	0.16	-0.28	-0.07	-0.26	0.26
5123	13.5.3	-0.37	-0.55	-0.15	0.05	-0.31	-0.12	0.02	-0.30	-0.32	0.11	0.13	-0.18	0.19	0.02
5228	14.6.8	-0.21	0.21	-0.41	-1.11	0.22	-0.35	0.04	-0.40	-0.05	-0.33	-0.25	0.61	-0.22	0.03
5264	16.6.4	-0.12	0.26	0.05	-0.03	0.08	-0.23	0.04	0.06	-0.05	-0.04	-0.05	0.25	0.06	0.51
5289	13.7.9	0.65	1.28	-0.25	0.19	-0.15	0.11	0.00	0.43	0.78	-0.58	1.14	0.34	-2.04	0.00
5290	13.7.10	0.17	0.12	0.09	0.08	-0.03	-0.14	0.04	0.01	0.03	0.02	0.00	-0.03	0.02	0.99
5296	13.7.16	0.75	1.15	-1.80	1.13	0.60	1.33	0.04	0.43	0.70	-0.70	1.16	-0.14	-1.85	0.10
5310	14.7.10	-0.01	-0.90	-0.13	-0.13	-0.30	-0.05	0.01	-0.20	-0.24	0.08	-0.36	-0.86	0.00	0.03
5345	16.7.5	-0.17	0.02	0.00	-0.33	0.42	-0.33	0.05	0.31	0.03	0.07	0.39	0.56	0.20	0.88
5408	15.8.8	0.08	0.32	0.06	-0.10	-0.13	-0.22	0.01	0.24	0.22	0.08	0.31	0.11	-0.22	0.19
5466	14.9.6	0.31	-0.11	0.32	0.30	0.00	0.27	0.05	0.24	0.16	0.24	0.15	-0.38	0.25	0.11
5519	16.9.19	0.45	0.53	-0.02	0.04	-0.04	-0.02	0.01	0.33	0.18	0.21	0.26	-0.26	-1.03	0.00
5531	13.10.11	-0.27	0.09	-0.13	-1.43	0.40	0.01	0.01	0.20	2.73	0.34	0.00	0.27	-0.11	0.03
5534	13.10.14	0.09	-2.26	-0.58	-0.73	-0.20	0.31	0.05	-0.97	-0.25	0.07	-1.64	0.06	0.69	0.28

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
5564	15.10.4	0.24	-0.30	0.28	0.19	-0.09	0.25	0.04	0.15	0.19	0.35	0.10	-0.60	0.33	0.05
5583	16.10.3	-0.02	-0.42	0.10	0.02	-0.03	0.11	0.05	0.10	0.00	0.17	-0.19	-0.50	0.12	0.04
5600	16.10.20	0.53	1.07	-0.20	0.07	0.04	0.65	0.02	0.55	0.02	-1.04	0.20	-0.91	-0.48	0.03
5606	13.11.6	-0.24	-1.01	-0.31	-0.27	-0.29	-0.41	0.04	-0.20	-0.33	0.06	-0.48	-0.67	0.16	0.00
5780	13.13.20	-0.22	0.36	-2.20	-1.53	-0.71	0.08	0.03	0.27	0.65	-0.32	0.88	0.13	-0.56	0.53
5888	15.14.8	0.05	-0.73	-0.01	-0.06	-0.28	-0.03	0.01	-0.05	-0.01	0.25	-0.18	-0.78	0.19	0.03
5899	15.14.19	-0.44	-0.07	0.14	-2.21	0.11	-0.21	0.01	-0.09	-0.12	-0.02	-0.73	0.45	-0.30	0.30
5936	13.15.16	0.00	-0.07	0.25	0.02	-0.12	-0.05	0.01	0.06	0.04	0.14	0.06	-0.01	0.16	0.61
5952	14.15.12	-0.17	-0.81	-0.06	-0.17	-0.34	-0.20	0.03	-0.24	-0.19	0.12	-0.30	-0.72	0.07	0.05
6001	13.16.1	-0.04	0.06	-0.16	-0.39	0.07	-0.17	0.02	-0.11	0.11	-0.08	0.03	0.22	-0.04	0.06
6007	13.16.7	-0.45	-0.66	-0.50	-2.60	-0.27	-1.41	0.01	-0.24	1.48	0.10	0.94	0.72	1.21	0.59
6096	13.17.16	0.29	-0.12	0.37	0.21	0.10	0.32	0.02	0.10	0.19	0.42	0.05	-0.38	0.28	0.04
6130	15.17.10	-0.14	-0.61	0.15	0.06	-0.02	0.09	0.01	-0.15	-0.03	0.25	-0.35	-0.67	-0.01	0.01
6173	13.18.13	0.85	1.67	0.77	0.39	0.44	4.08	0.02	0.55	0.27	-0.35	0.76	-0.45	-0.55	0.12
6222	16.18.2	0.05	-0.64	0.08	0.03	-0.11	0.00	0.03	0.03	-0.02	0.34	-0.14	-0.65	0.14	0.02
6552	20.2.12	-0.13	0.04	-0.18	-0.28	-0.30	-0.02	0.03	-0.08	0.07	0.17	0.34	0.29	0.33	0.29
6585	18.3.5	-0.10	0.36	-0.34	-0.25	-0.13	-0.18	0.03	-0.02	0.11	0.02	0.43	0.48	0.08	0.06
6677	18.4.17	-0.15	0.38	-0.15	-0.10	0.19	-0.23	0.00	0.02	-0.03	-0.15	-0.29	0.49	-0.10	0.12
6695	19.4.15	-0.12	0.67	0.06	-0.36	-0.02	-0.09	0.04	0.06	-0.06	-0.01	-0.18	0.12	-0.13	0.79
6848	19.6.8	-0.13	0.17	-0.07	-0.09	0.19	0.11	0.04	-0.05	0.04	-0.06	0.45	0.42	-0.03	0.17
6854	19.6.14	0.13	-0.30	-0.85	-0.45	2.55	-0.52	0.01	-0.50	-0.38	-0.24	-0.12	0.86	-0.46	0.04
6867	20.6.7	-0.06	-0.49	0.05	0.01	-0.11	0.02	0.03	-0.03	-0.03	0.27	-0.27	-0.46	0.04	0.04
6917	18.7.17	0.06	-0.10	0.03	0.17	0.12	0.49	0.02	0.19	-0.03	0.15	0.14	-0.26	-0.03	0.16
7050	17.9.10	-0.25	0.42	-0.18	-0.42	-0.20	-0.28	0.04	-0.51	0.00	0.12	0.30	0.35	0.11	0.20
7068	18.9.8	0.15	0.47	0.17	0.01	-0.21	-0.14	0.02	0.33	0.31	0.14	0.48	0.52	0.54	0.46
7097	19.9.17	0.01	-0.65	0.06	-0.09	-0.22	0.05	0.03	-0.10	-0.12	0.30	-0.24	-0.73	0.18	0.01
7128	17.10.8	0.87	0.54	0.33	0.67	5.54	1.25	0.00	0.55	1.34	-0.98	0.95	-0.29	-1.10	0.24
7154	18.10.14	-0.35	-1.31	-0.33	-0.34	-0.56	0.33	0.04	-0.41	-0.95	0.05	-0.63	-0.73	-0.15	0.30
7284	17.12.4	-0.12	0.15	-0.05	0.00	0.18	-0.13	0.02	-0.06	0.15	-0.02	0.09	-0.07	0.20	0.20
7346	20.12.6	-0.04	0.21	-0.01	-0.05	-0.08	-0.17	0.02	0.16	-0.04	-0.06	-0.09	0.18	0.05	0.44
7450	17.14.10	-0.02	-0.68	0.04	-0.04	-0.32	0.02	0.04	-0.14	-0.15	0.25	-0.26	-0.51	0.17	0.02
7468	18.14.8	0.10	-0.69	0.06	0.02	-0.29	-0.11	0.01	-0.13	-0.21	0.21	-0.11	-0.21	0.12	0.26
7490	19.14.10	-0.03	-0.65	0.03	0.00	-0.22	-0.03	0.02	-0.15	-0.11	0.28	-0.21	-0.73	0.22	0.02
7619	17.16.19	1.21	0.46	2.20	-0.10	0.66	1.77	0.05	2.67	0.77	-0.36	-0.16	0.85		0.02
7659	19.16.19	0.05	-0.74	0.09	0.05	-0.17	0.02	0.03	-0.12	-0.10	0.28	-0.28	-0.64	0.25	0.04
7664	20.16.4	0.02	0.18	-0.02	0.07	0.17	-0.08	0.01	0.06	0.09	0.03	0.09	0.28	0.06	0.29
7709	18.17.9	-0.08	0.09	-1.17	-0.31	1.64	-0.13	0.02	-0.31	0.26	0.05	-0.07	0.32	0.78	0.67
7739	19.17.19	1.07	0.91	0.43	-0.90	0.45	-0.38	0.04	0.61	0.22	-0.71	1.76	0.70		0.09
7782	18.18.2	0.18	0.17	0.29	0.30	-0.02	0.04	0.04	0.19	0.14	0.10	0.19	-0.07	0.30	0.33
8159	24.2.19	0.21	0.74	-0.76	-1.34	-0.26	1.65	0.02	0.53	0.47	0.22	1.37	1.09	1.11	0.68
8212	23.3.12	0.27	0.56	1.00	0.46	0.74	3.75	0.02	2.97	0.78	0.24	0.94	0.29	-0.38	0.92
8215	23.3.15	-0.02	-0.47	-0.01	-0.04	-0.14	-0.10	0.05	-0.12	-0.16	0.14	-0.25	-0.57	-0.01	0.01
8302	24.4.2	0.52	0.76	0.20	0.13	0.85	3.53	0.02	1.13	0.73	-1.00	0.27	-1.16	-0.28	0.40
8331	21.5.11	0.24	0.18	-0.13	-0.09	-0.15	-0.09	0.02	0.25	0.30	0.04	0.18	-0.04	0.01	0.20
8409	21.6.9	-0.17	-0.31	0.02	0.25	0.04	0.19	0.02	-0.33	-0.18	0.20	0.13	-0.16	-0.08	0.19
8427	22.6.7	0.11	-0.51	0.13	0.10	-0.15	-0.01	0.01	-0.06	-0.06	0.20	-0.10	-0.48	0.25	0.03
8436	22.6.16	-0.20	0.01	-1.10	-0.22	0.39	0.31	0.00	0.13	0.53	-0.28	1.94	0.58	-0.13	0.38
8450	23.6.10	0.06	0.01	-0.89	-0.46	1.57	0.53	0.01	-0.17	0.38	0.13	-0.32	2.52		0.20
8535	23.7.15	0.79	2.40	0.54	0.60	0.45	1.21	0.01	2.60	1.55	0.83	1.02	1.61	2.27	0.81
8555	24.7.15	0.36	-0.58	0.05	0.34	0.16	0.78	0.02	0.20	0.04	0.44	0.68	-0.17	0.62	0.30
8613	23.8.13	0.15	0.30	0.04	0.05	0.03	-0.07	0.00	0.11	-0.06	-0.04	-0.05	0.06	-0.23	0.10

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
8624	24.8.4	-0.17	-0.11	-0.18	-0.39	0.26	-0.92	0.04	-0.50	-0.46	0.19	-0.39	0.33	0.40	0.54
8644	21.9.4	0.40	0.73	0.04	0.12	-0.06	-0.15	0.04	0.08	-0.20	-0.60	0.63	-0.23	0.38	0.12
8664	22.9.4	0.33	-0.30	0.29	0.29	0.04	0.29	0.04	0.10	0.07	0.29	0.20	-0.44	0.22	0.03
8666	22.9.6	0.12	-0.48	0.23	0.15	-0.09	0.02	0.03	0.04	0.05	0.33	0.01	-0.42	0.29	0.03
8797	24.10.17	-0.09	0.50	1.60	0.53	-1.34	-0.37	0.03	0.50	2.92	1.71	-0.14	0.54		0.54
8859	23.11.19	0.00	0.07	-0.41	-0.15	0.73	0.24	0.03	-0.59	0.47	0.39	-0.22	0.58	0.36	0.09
8869	24.11.9	3.90	1.77	0.22	0.83	2.33		0.04	1.12	2.02	-0.18	-0.61		-1.57	0.10
8883	21.12.3	-0.07	-0.41	-0.11	0.03	-0.04	0.00	0.02	-0.17	-0.08	0.12	-0.18	-0.28	0.30	0.00
8903	22.12.3	0.27	-0.40	0.25	0.56	1.46	1.01	0.03	0.52	-0.29	0.46	1.21	-0.38	1.14	0.10
8964	21.13.4	1.51	-1.13	-3.28	-1.18	0.45	0.75	0.05	-0.36	0.63	-2.29	-0.11	-0.54	-1.61	0.24
9067	22.14.7	0.01	-0.42	0.02	0.04	-0.23	0.03	0.04	-0.12	-0.16	0.17	-0.13	-0.39	0.09	0.02
9202	21.16.2	0.01	0.18	-0.21	-0.20	0.08	-0.32	0.02	0.01	-0.04	-0.17	0.09	0.35	0.04	0.02
9208	21.16.8	0.05	-0.15	0.04	0.09	-0.12	-0.02	0.04	-0.01	-0.02	0.13	0.20	-0.12	0.23	0.06
9246	23.16.6	-0.28	1.04	-1.16	0.06	-0.82		0.03	-0.01	1.73	0.67	0.02	-0.04	0.21	0.88
9252	23.16.12	0.19	-0.07	0.26	0.21	0.00	0.10	0.04	0.12	0.16	0.29	0.06	-0.22	0.27	0.03
9279	24.16.19	-0.38	-1.70	-0.71	0.47	-0.20	0.14	0.01	-0.63	-0.73	-0.13	-0.05	-1.65	-0.14	0.00
9314	22.17.14	-0.37	-0.24	-0.34	-0.20	0.01	0.00	0.00	-0.33	-0.26	-0.14	-0.17	-0.07	-0.19	0.46
9382	22.18.2	0.13	-0.17	0.19	0.04	-0.11	0.04	0.01	0.06	0.12	0.21	0.06	-0.16	0.15	0.31
9396	22.18.16	0.06	-0.50	0.13	0.07	-0.16	0.02	0.01	-0.05	-0.02	0.24	-0.13	-0.50	0.14	0.02
9779	25.3.19	0.03	-0.54	0.11	0.11	-0.06	0.07	0.01	-0.03	0.00	0.24	-0.05	-0.38	0.14	0.04
9792	26.3.12	-0.01	-0.34	0.08	-0.03	-0.16	0.20	0.04	-0.09	-0.06	0.21	-0.10	-0.27	0.06	0.09
9837	28.3.17	0.08	-0.23	0.17	0.07	-0.06	0.06	0.03	0.14	0.15	0.26	0.30	-0.11	0.30	0.48
9873	26.4.13	0.02	-0.32	0.05	0.09	-0.22	0.20	0.02	0.08	-0.02	0.18	0.02	-0.24	0.13	0.38
9928	25.5.8	-0.15	-0.43	-0.04	0.11	-0.11	0.10	0.05	-0.12	-0.04	0.16	-0.08	-0.30	0.07	0.08
9955	26.5.15	-0.01	-0.30	0.05	0.00	-0.26	0.10	0.01	0.01	-0.05	0.17	-0.07	-0.16	0.10	0.40
9956	26.5.16	-0.04	-0.22	0.00	-0.01	-0.26	-0.10	0.04	0.15	-0.01	0.12	0.06	-0.13	0.16	0.66
9976	27.5.16	-0.05	-0.67	-0.02	-0.04	-0.14	-0.02	0.00	-0.13	-0.12	0.14	-0.32	-0.61	0.26	0.04
9987	28.5.7	-0.05	-0.43	-0.02	-0.01	-0.17	-0.05	0.05	-0.05	0.06	0.13	0.02	-0.22	0.09	0.07
10038	26.6.18	-0.41	0.15	-1.30	-0.31	0.74	0.08	0.02	-0.25	-2.29	-0.19	0.01	0.28	-0.27	0.65
10040	26.6.20	-0.29	-0.05	-0.33	0.30	0.49	0.16	0.04	-0.07	-0.02	-0.29	-0.13	0.27	-0.70	0.02
10065	28.6.5	-0.14	-0.60	-0.03	-0.11	-0.22	-0.09	0.05	-0.13	-0.08	0.24	-0.08	-0.45	0.13	0.04
10086	25.7.6	0.23	0.27	-0.81	-0.47	0.90	0.56	0.05	0.01	0.90	0.28	1.58	1.20	0.16	0.09
10110	26.7.10	0.00	-0.51	0.04	0.02	-0.27	0.01	0.01	-0.19	-0.12	0.17	-0.18	-0.36	0.08	0.03
10138	27.7.18	-0.30	-0.06	-0.22	-0.31	-0.06	0.10	0.04	-0.13	-0.08	-0.17	0.06	0.25	0.35	0.03
10251	25.9.11	-0.01	-1.00	0.28	0.21	-0.14	-0.19	0.02	-0.20	-0.21	0.30	-0.16	-0.63	0.27	0.12
10261	26.9.1	0.12	-0.55	0.07	0.16	-0.13	0.04	0.01	0.06	0.02	0.22	-0.06	-0.33	0.16	0.13
10270	26.9.10	-0.01	1.06	-0.47	-0.12	0.01	1.74	0.02	-0.27	-0.14	0.28	1.18	0.39	-0.34	0.41
10273	26.9.13	0.17	-0.26	0.16	0.23	-0.16	0.35	0.01	0.02	0.20	0.25	0.10	-0.25	0.20	0.24
10275	26.9.15	0.07	-0.50	0.17	0.15	-0.17	0.10	0.02	0.07	0.05	0.26	-0.03	-0.35	0.20	0.16
10333	25.10.13	0.89	0.90	-0.71	0.33	1.32	2.84	0.03	1.75	0.59	-1.59	-0.17			0.51
10336	25.10.16	-0.05	-0.61	0.00	0.02	-0.11	-0.09	0.03	-0.05	-0.10	0.21	-0.11	-0.41	0.19	0.04
10351	26.10.11	-0.34	-0.33	-0.73	-0.79	-0.01	-0.28	0.01	-1.12	-0.31	0.01	-0.21	-0.10	-0.20	0.11
10426	26.11.6	0.04	-0.27	0.10	0.05	-0.18	0.02	0.02	-0.16	-0.16	0.06	-0.13	-0.09	0.03	0.27
10430	26.11.10	-0.09	-1.03	-0.08	-0.21	-0.35	-0.38	0.05	0.16	-0.02	0.26	0.03	-0.13	0.13	0.75
10474	28.11.14	-0.10	2.27	-0.48	0.65	8.04	0.33	0.00	-0.14	0.47	-0.18		0.76	0.62	0.11
10500	25.12.20	-0.28	1.26	0.00	0.59	1.17	8.05	0.01	0.21	0.69	-0.02	1.02	1.72	-1.59	0.45
10535	27.12.15	-0.32	0.70	0.02	-0.19	-0.24	-0.11	0.02	0.11	-0.07	-0.06	0.60	0.34	0.60	0.20
10652	25.14.12	-0.19	-0.88	-0.08	-0.14	-0.30	-0.27	0.02	-0.25	-0.30	0.11	-0.41	-0.54	0.08	0.04
10656	25.14.16	-0.17	-0.53	-0.01	-0.07	-0.13	-0.12	0.02	-0.24	-0.24	0.12	-0.19	-0.10	0.04	0.44
10737	25.15.17	0.06	-0.21	-0.02	0.04	-0.05	0.03	0.02	-0.02	0.07	0.21	0.04	-0.04	0.16	0.62
10810	25.16.10	-0.10	0.61	0.00	-0.03	0.02	-0.06	0.03	0.06	-0.08	-0.01	0.00	0.21	-0.02	0.75

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
10812	25.16.12	-0.02	-0.62	0.18	0.07	-0.12	0.09	0.00	-0.06	-0.15	0.28	-0.24	-0.46	0.09	0.05
10823	26.16.3	0.12	-0.22	-0.03	0.00	-0.13	0.23	0.02	-0.08	0.04	0.09	0.05	-0.05	0.05	0.56
10837	26.16.17	-0.15	0.41	-0.10	-0.10	-0.14	0.32	0.03	0.01	-0.03	-0.01	-0.02	-0.07	0.08	0.87
10852	27.16.12	0.14	-0.29	0.12	0.09	-0.10	0.05	0.00	-0.03	0.05	0.24	-0.11	-0.34	0.10	0.01
10861	28.16.1	-0.19	-0.56	-0.13	-0.06	-0.22	-0.23	0.04	-0.31	-0.22	0.16	-0.20	-0.49	0.05	0.02
10869	28.16.9	0.72	0.39	-0.62	-0.26	-0.94	3.15	0.03	0.33	-0.85	0.02	1.22	1.44	2.38	0.15
11027	28.18.7	-0.14	-0.60	-0.02	-0.11	-0.16	0.04	0.05	0.10	-0.01	0.20	-0.18	-0.54	0.19	0.02
11076	26.19.16	0.03	-0.79	0.02	-0.10	-0.23	0.38	0.03	-0.07	-0.09	0.17	-0.31	-0.69	0.16	0.04
11262	32.1.2	-0.16	-0.48	0.00	0.14	-0.11	-0.08	0.01	-0.32	-0.22	0.09	0.02	-0.38	0.21	0.00
11297	29.2.17	0.10	0.42	0.12	0.07	0.39	0.14	0.02	0.07	0.10	-0.13	-0.38	0.36	-0.02	0.47
11404	31.3.4	-0.04	0.32	-0.31	-0.08	0.12	-0.09	0.01	0.09	0.15	-0.06	0.53	0.39	0.17	0.01
11477	30.4.17	-0.04	-0.37	0.02	0.03	-0.25	0.02	0.01	-0.09	-0.08	0.14	-0.09	-0.37	0.08	0.12
11481	31.4.1	0.01	-0.56	0.06	0.11	-0.18	-0.02	0.01	-0.06	-0.03	0.25	0.03	-0.54	0.17	0.04
11505	32.4.5	1.00	0.99	0.01	0.32	0.29	0.05	0.05	1.37	0.26	-0.16	0.24	-0.27	-1.84	0.00
11549	30.5.9	-0.10	-0.39	-0.15	-0.02	-0.26	0.02	0.03	0.08	-0.04	0.13	-0.06	-0.33	0.10	0.02
11576	31.5.16	0.03	-0.46	0.02	0.01	-0.11	-0.04	0.03	-0.10	-0.04	0.21	-0.16	-0.53	0.13	0.01
11581	32.5.1	-0.10	0.40	0.08	-0.02	0.12	-0.13	0.01	0.07	-0.07	-0.05	0.04	0.09	-0.11	0.53
11599	32.5.19	-0.05	0.22	-0.04	-0.06	0.04	-0.24	0.02	-0.06	-0.03	-0.07	-0.08	0.19	0.02	0.31
11644	31.6.4	0.19	0.39	0.17	0.23	0.04	-0.10	0.00	0.17	0.37	0.22	0.57	0.26	0.43	0.19
11741	32.7.1	-0.30	0.41	0.05	0.13	0.08	-0.06	0.01	0.28	0.06	-0.11	-0.32	0.16	-0.51	0.20
11803	31.8.3	0.15	-0.38	0.22	0.11	-0.05	0.22	0.03	0.11	0.13	0.28	0.00	-0.41	0.22	0.14
11904	32.9.4	0.26	1.79	0.38	0.45	0.23	0.58	0.04	1.07	0.08	-0.28	0.84	1.19	0.11	0.41
11905	32.9.5	0.91	8.88	1.68	1.40	2.54	2.82	0.00	1.95	1.07	2.87	1.50	0.09		0.24
11909	32.9.9	-0.09	-0.53	0.05	0.04	-0.14	0.04	0.03	-0.13	-0.10	0.19	-0.13	-0.36	0.01	0.20
11924	29.10.4	-0.07	-0.77	0.02	-0.01	-0.34	-0.10	0.03	-0.15	-0.16	0.19	-0.33	-0.66	0.20	0.03
11943	30.10.3	0.23	-0.55	0.14	0.24	-0.02	0.17	0.04	0.06	0.07	0.29	-0.12	-0.43	0.26	0.22
12009	29.11.9	-0.12	0.16	0.09	-0.17	0.31	-0.33	0.01	-0.05	0.11	-0.02	0.12	0.34	0.16	0.05
12270	30.14.10	3.54	2.81	3.51	1.19	1.71	0.67	0.03	1.86	0.95	-0.69	1.96	0.48	1.45	0.20
12272	30.14.12	0.06	-0.54	-0.02	-0.08	-0.17	-0.06	0.04	-0.20	-0.04	0.14	-0.22	-0.52	0.08	0.05
12404	29.16.4	-0.06	-0.66	-0.09	-0.11	-0.24	-0.09	0.01	-0.23	-0.03	0.25	-0.21	-0.36	0.22	0.02
12405	29.16.5	0.01	-0.39	0.13	0.04	-0.11	0.15	0.01	-0.15	-0.16	0.15	-0.28	-0.41	0.11	0.03
12419	29.16.19	0.23	0.61	-0.10	0.27	1.41	0.05	0.02	0.98	0.23	0.35	0.36	1.00	-0.18	0.53
12474	32.16.14	0.13	-0.51	0.00	0.04	-0.04	0.04	0.02	-0.12	-0.10	0.13	-0.13	-0.64	-0.18	0.02
12638	32.18.18	0.12	-0.12	0.05	-0.29	-0.62	-0.17	0.04	-0.18	-0.24	0.02	0.23	0.01	0.12	0.23
12806	33.1.6	0.55	2.31	-0.69	0.04	-0.40	0.55	0.01	0.51	0.33	-0.58	1.13	-0.34	0.62	0.37
12816	33.1.16	-0.02	-0.15	-0.09	-0.03	-0.01	0.19	0.00	0.10	-0.05	-0.07	0.04	-0.02	0.17	0.09
12827	34.1.7	-0.06	0.25	-0.04	-0.07	0.11	-0.10	0.01	-0.06	0.01	-0.04	-0.01	0.23	-0.10	0.20
12844	35.1.4	-0.01	0.57	-0.21	-0.31	0.10	-0.18	0.00	-0.05	0.03	-0.05	0.19	0.41	-0.19	0.27
12871	36.1.11	0.12	-0.13	0.20	0.32	0.08	0.15	0.04	-0.09	0.02	0.16	0.26	-0.22	0.04	0.00
12878	36.1.18	-0.33	1.17	1.86	0.17	0.10	0.34	0.04	1.05	0.81	0.50	1.26	-0.44	-2.13	0.07
12909	34.2.9	0.60	3.14	0.76	0.08	0.88	0.93	0.04	0.71	1.03	-0.52	1.36	-0.13	-0.41	0.39
13016	35.3.16	0.00	-0.56	-0.02	0.00	-0.22	-0.12	0.04	-0.12	0.00	0.13	-0.20	-0.64	0.15	0.01
13031	36.3.11	0.01	-0.55	-0.11	-0.05	-0.18	-0.05	0.01	-0.12	-0.11	0.11	-0.05	-0.53	-0.01	0.03
13033	36.3.13	-0.05	1.50	-0.76	-0.68	0.17	0.44	0.03	-0.24	-0.46	-0.65	-0.02	-0.02	-1.31	0.73
13034	36.3.14	0.15	-0.19	0.04	0.02	-0.07	0.02	0.05	-0.02	-0.01	0.16	0.00	-0.20	0.05	0.13
13101	36.4.1	0.59	3.74	0.50	0.09	0.25	1.43	0.01	0.75	0.36	0.68	0.59	-0.38	-1.24	0.30
13150	34.5.10	0.38	1.34	-0.98	-0.18	0.36	0.51	0.02	0.44	0.76	0.68	0.59	0.10	0.06	0.90
13155	34.5.15	-0.03	-0.42	-0.04	0.05	-0.20	0.00	0.03	-0.15	-0.08	0.20	-0.20	-0.35	0.07	0.09
13166	35.5.6	1.00	2.93	-0.14	1.04	0.26	0.73	0.04	2.40	0.85	0.40	3.01	0.33	1.24	0.57
13221	34.6.1	-0.21	0.36	-0.18	-0.22	0.00	-0.33	0.03	-0.09	0.18	0.03	0.28	0.67	0.10	0.10
13300	33.7.20	-0.56	0.12	-2.49	-0.62	1.82	-0.86	0.02	-0.01	0.30	-0.62	0.79	0.67	0.73	0.53

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
13330	35.7.10	-0.07	-0.39	-0.08	0.01	-0.23	0.03	0.04	-0.08	-0.16	0.11	-0.19	-0.35	-0.10	0.12
13362	33.8.2	-0.56	-0.34	-1.65	-1.90	-0.18	-0.49	0.04	-0.35	-0.14	-0.26	-0.18	0.22	0.79	0.88
13374	33.8.14	-0.07	-0.46	-0.04	-0.17	-0.07	-0.04	0.04	-0.07	0.14	0.08	-0.18	-0.31	0.10	0.07
13441	33.9.1	-0.03	0.22	-0.16	-0.10	0.01	-0.25	0.02	-0.05	-0.01	-0.15	0.06	0.28	0.12	0.19
13446	33.9.6	-0.03	-0.10	0.07	0.28	0.33	0.15	0.02	-0.27	-0.24	0.01	-0.21	-0.31	-0.25	0.13
13525	33.10.5	-0.20	-0.73	-0.29	-0.06	-0.21	0.12	0.02	-0.14	-0.17	0.00	-0.35	-0.46	-0.18	0.27
13554	34.10.14	0.00	1.76	0.77	0.91	0.05	1.03	0.03	1.18	1.58	-4.10	0.08	0.86	0.29	0.52
13583	36.10.3	0.12	-0.21	0.14	0.23	-0.07	-0.05	0.03	0.14	-0.07	0.19	0.06	-0.56	0.04	0.00
13654	35.11.14	-0.19	-1.71	-0.09	-0.08	-0.32	-0.52	0.01	-0.65	-0.64	0.17	-0.72	-0.70	-0.22	0.21
13683	33.12.3	0.29	1.95	-1.20	0.06	2.44	1.59	0.04	0.62	0.10	-0.19	1.63	0.80	-1.06	0.59
13892	35.14.12	-0.02	-0.68	-0.05	-0.10	-0.29	-0.09	0.05	-0.07	-0.02	0.20	-0.14	-0.69	0.02	0.04
13914	36.14.14	-0.27	-0.80	-0.14	-0.14	-0.29	-0.14	0.03	-0.25	-0.35	0.12	-0.36	-0.88	0.08	0.01
13937	33.15.17	-0.03	-0.57	0.00	-0.01	-0.22	-0.11	0.03	-0.12	-0.06	0.18	-0.13	-0.28	0.10	0.05
13939	33.15.19	-0.27	-0.23	0.04	0.06	-0.01	-0.11	0.04	-0.19	0.13	0.02	0.13	-0.11	0.08	0.14
13987	36.15.7	0.13	0.33	0.04	0.12	0.13	-0.01	0.03	0.23	0.24	0.03	0.14	0.23	-0.11	0.08
14010	33.16.10	-0.05	0.01	-0.03	-0.33	1.34	-0.10	0.00	0.04	0.43	-0.14	0.57	0.29	-0.03	0.53
14014	33.16.14	-0.13	-0.41	-0.03	-0.03	-0.18	-0.11	0.03	-0.15	-0.04	0.10	-0.13	-0.18	0.06	0.14
14020	33.16.20	0.00	-0.38	0.10	0.07	-0.10	-0.03	0.02	-0.05	-0.09	0.18	-0.13	-0.24	0.17	0.03
14034	34.16.14	0.00	-0.90	-0.08	-0.08	-0.39	-0.13	0.02	-0.21	-0.12	0.10	-0.39	-0.85	0.14	0.01
14069	36.16.9	-0.04	0.07	-0.19	-0.21	0.08	-0.23	0.01	0.06	-0.02	0.02	-0.03	-0.08	-0.04	0.83
14182	34.18.2	0.87	1.34	0.46	0.12	2.56	0.07	0.03	1.83	1.55	0.87	2.39	1.47	1.10	0.63
14183	34.18.3	-0.08	0.47	0.06	-0.24	0.13	-0.31	0.04	-0.06	0.27	0.12	0.70	0.57	0.45	0.78
14186	34.18.6	-0.02	-0.87	-0.07	-0.04	-0.29	0.04	0.03	-0.05	-0.06	0.29	-0.30	-0.86	0.11	0.03
14217	35.18.17	0.18	-0.28	0.20	0.04	-0.05	0.19	0.01	0.05	0.13	0.26	0.03	-0.24	0.21	0.18
14454	39.1.14	-0.11	0.14	-0.11	-0.09	0.14	-0.08	0.02	0.00	-0.04	-0.11	-0.03	0.38	-0.03	0.07
14463	40.1.3	0.06	-0.23	0.07	0.11	0.01	0.09	0.02	-0.04	-0.01	0.02	-0.18	-0.19	-0.09	0.52
14587	38.3.7	0.01	-0.18	-0.50	0.95	1.54	-0.37	0.03	-0.12	0.46	0.16	-2.33	0.13	-0.23	0.38
14616	39.3.16	-0.11	-0.28	0.06	0.17	-0.09	0.00	0.04	-0.17	-0.04	0.11	0.02	-0.25	0.04	0.20
14636	40.3.16	-0.03	-0.44	-0.07	-0.16	-0.14	-0.11	0.04	-0.20	-0.09	0.07	-0.02	-0.27	0.03	0.02
14639	40.3.19	0.03	-0.28	0.04	0.01	0.01	0.15	0.03	-0.04	-0.03	0.17	-0.13	-0.26	0.08	0.02
14641	37.4.1	-0.39	-0.50	-0.01	0.06	0.12	0.32	0.00	-0.38	-0.01	0.22	0.36	-0.22	-0.01	0.04
14704	40.4.4	-0.07	-0.63	-0.03	-0.08	-0.06	-0.15	0.01	-0.05	-0.14	0.01	-0.24	-0.72	0.08	0.01
14723	37.5.3	-0.16	-0.71	-0.09	-0.17	-0.21	-0.10	0.03	-0.21	-0.21	0.11	-0.27	-0.43	0.07	0.07
14730	37.5.10	0.36	-0.06	0.01	-0.08	-0.17	0.13	0.05	0.30	0.07	0.15	-0.09	-0.34	-0.17	0.11
14755	38.5.15	-0.15	-0.62	-0.08	-0.12	-0.25	-0.17	0.05	-0.24	-0.15	0.22	-0.27	-0.44	0.08	0.02
14756	38.5.16	-0.08	-0.54	-0.09	-0.09	-0.15	-0.07	0.05	-0.12	-0.08	0.23	-0.24	-0.47	0.08	0.01
14759	38.5.19	-0.04	0.09	-0.02	-0.03	-0.14	-0.24	0.05	-0.04	0.05	0.04	0.13	0.14	0.17	0.38
14822	38.6.2	0.13	0.28	0.02	0.26	0.07	0.03	0.04	0.16	0.08	0.13	0.16	0.34	0.09	0.51
14869	40.6.9	0.02	-0.36	0.03	-0.04	-0.09	-0.01	0.02	-0.04	0.01	0.15	0.07	-0.15	0.16	0.25
14889	37.7.9	0.01	-1.68	-0.75	-0.49	0.27	-0.39	0.02	-0.17	-0.11	0.19	0.30	-0.48	0.74	0.45
14912	38.7.12	0.10	-0.50	-0.10	0.02	-0.02	-0.09	0.01	-0.23	-0.19	0.06	-0.33	-0.49	0.02	0.03
14930	39.7.10	-0.07	-0.68	-0.15	-0.20	-0.24	-0.19	0.01	-0.17	-0.16	0.11	-0.12	-0.72	0.15	0.01
14973	37.8.13	0.11	-0.33	0.08	0.13	-0.07	0.15	0.03	0.02	0.05	0.15	0.27	-0.12	0.16	0.28
14992	38.8.12	-0.09	-0.68	0.04	-0.03	-0.08	-0.08	0.02	-0.15	-0.11	0.22	-0.22	-0.62	0.11	0.05
15057	37.9.17	-0.28	-0.43	0.01	0.29	0.10	0.05	0.05	-0.25	-0.10	0.22	-0.01	-0.34	0.12	0.16
15058	37.9.18	0.13	-0.55	0.11	0.03	-0.07	0.07	0.01	0.02	0.01	0.18	-0.14	-0.56	0.08	0.00
15089	39.9.9	-0.07	0.32	0.18	0.13	-0.01	-0.16	0.05	0.00	0.02	-0.04	0.04	0.21	-0.02	0.38
15092	39.9.12	-0.09	0.17	-0.19	-0.14	-0.08	-0.13	0.04	0.03	0.04	0.04	0.34	0.35	0.31	0.01
15109	40.9.9	0.08	-0.24	0.14	0.12	0.00	-0.02	0.03	0.00	0.00	0.19	-0.01	-0.29	0.07	0.25
15145	38.10.5	-0.28	-0.73	-0.15	-0.16	-0.25	-0.24	0.04	-0.22	-0.27	0.11	-0.43	-0.60	-0.06	0.08
15188	40.10.8	0.66	0.25	0.11	0.20	-0.13	-0.48	0.01	0.24	0.22	0.15	1.15	-0.15	-0.47	0.00

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
15222	38.11.2	0.40	1.24	0.12	0.52	0.37	0.79	0.05	0.62	-0.04	-0.53	-0.17	-0.14	-0.54	0.21
15263	40.11.3	-0.26	-1.84	-0.63	-0.18	-0.76	-0.95	0.01	-0.60	-0.39	-1.63	-0.08	-0.67	-0.11	0.61
15266	40.11.6	-0.13	-0.24	0.28	0.25	-0.01	-0.01	0.01	-0.15	-0.02	0.11	0.22	-0.24	-0.02	0.11
15281	37.12.1	-0.11	1.28	-1.20	0.27	0.42	-0.35	0.03	0.20	0.23	-0.23	1.49	1.29	-0.17	0.19
15282	37.12.2	-0.49	0.34	-0.30	-0.21	-0.22	-0.50	0.00	-0.78	0.51	0.31	0.89	0.74	0.14	0.00
15330	39.12.10	-0.53	0.01	-1.34	-0.51	0.42	-0.54	0.04	0.16	0.02	0.54	0.21	0.74	-0.37	0.82
15430	40.13.10	-0.11	-0.51	-0.09	-0.11	-0.17	-0.08	0.02	-0.10	-0.12	0.05	-0.26	-0.55	0.12	0.00
15447	37.14.7	-0.51	0.00	-0.14	-0.34	0.02	-0.25	0.02	0.02	0.35	0.18	-0.08	0.22	-0.12	0.04
15569	39.15.9	-0.23	-0.55	-0.09	0.00	-0.20	-0.09	0.03	-0.26	-0.19	0.08	-0.19	-0.43	0.08	0.07
15608	37.16.8	0.16	0.14	-0.24	-0.26	-0.41	-0.05	0.04	0.17	0.38	0.00	0.50	0.36	0.27	0.37
15663	40.16.3	-0.75	6.52	0.05	-0.94	1.32	-0.99	0.04	0.70	-0.12	0.01	-0.14	-0.99		0.93
15751	40.17.11	0.05	-0.38	-0.11	-0.02	-0.03	-0.06	0.00	-0.09	-0.16	-0.01	-0.23	-0.25	0.00	0.62
15774	37.18.14	0.68	3.69	-0.30	-0.66	-0.01	0.66	0.02	0.32	0.26	-0.06	0.60	0.01	-0.37	0.82
16043	43.1.3	-0.08	-0.55	0.02	0.04	-0.10	0.01	0.00	-0.03	0.07	0.11	-0.20	-0.47	0.05	0.07
16045	43.1.5	-0.07	-0.32	0.01	0.00	-0.12	-0.01	0.03	-0.04	-0.03	0.05	-0.14	-0.29	-0.06	0.05
16053	43.1.13	-0.12	-0.09	-2.02	-1.11	-1.40	-0.25	0.02	-0.53	0.12	0.38	1.57	1.12	0.08	0.12
16061	44.1.1	-0.14	0.45	-0.56	-0.74	-0.35	-0.18	0.03	-0.41	-0.47	-0.14	-1.02	0.49	0.02	0.09
16124	43.2.4	-0.33	0.11	0.08	-0.10	-0.43	-0.17	0.00	-0.14	0.48	0.16	0.11	-0.34	-0.34	0.00
16177	41.3.17	0.28	2.55	-0.08	0.51	3.61	1.59	0.03	0.33	1.02	0.75	5.58	5.12	0.57	0.15
16211	43.3.11	-0.30	0.31	-0.58	-0.74	-0.05	0.20	0.04	-0.16	-0.24	-0.09	-0.91	0.20	-0.65	0.12
16256	41.4.16	-0.07	1.02	-0.11	-0.35	0.05	-0.04	0.02	0.18	-0.17	-0.02	-0.32	0.31	0.05	0.69
16259	41.4.19	-0.22	0.46	-0.02	-0.37	0.01	0.02	0.01	0.10	-0.11	-0.02	-0.43	0.07	-0.13	0.20
16271	42.4.11	0.04	-0.54	-0.02	-0.07	-0.10	-0.02	0.01	-0.09	-0.10	0.15	-0.17	-0.54	0.21	0.01
16273	42.4.13	0.04	-0.45	0.08	0.07	-0.08	0.13	0.00	-0.01	-0.07	0.15	-0.09	-0.43	0.04	0.08
16277	42.4.17	-0.04	0.47	0.10	0.01	0.05	0.12	0.03	0.10	0.01	-0.06	0.26	0.29	0.00	0.25
16278	42.4.18	-0.31	0.67	-0.13	-0.79	-0.24	0.33	0.04	-0.20	-0.27	-0.05	-0.67	-0.08	-0.01	0.40
16302	44.4.2	0.01	-0.48	-0.02	-0.03	0.02	0.08	0.00	-0.19	-0.02	0.18	-0.28	-0.59	-0.11	0.05
16340	41.5.20	-0.15	-0.78	-0.04	-0.26	-0.23	-0.07	0.01	-0.07	0.13	0.25	0.03	-0.41	0.14	0.05
16539	43.7.19	0.10	-0.28	-0.16	-0.10	0.11	-0.38	0.01	-0.19	-0.24	-0.32	-0.36	-0.01	-0.51	0.51
16577	41.8.17	0.17	2.61	0.02	-0.66	0.35	2.03	0.05	0.51	1.19	-0.07	0.91	1.11	-0.30	0.58
16595	42.8.15	0.14	-0.04	0.04	0.04	-0.07	0.01	0.03	0.07	0.03	0.09	0.05	-0.06	0.10	0.56
16599	42.8.19	-0.05	-0.37	0.02	0.04	-0.11	-0.11	0.03	0.00	-0.02	0.11	0.09	-0.27	0.08	0.02
16620	43.8.20	-0.20	-0.72	-0.19	-0.24	-0.14	0.02	0.05	-0.24	-0.20	0.10	-0.13	-0.55	0.31	0.02
16626	44.8.6	-0.45	-3.03	-0.50	-0.79	-1.54	0.21	0.00	-0.85	-0.28	-0.61	-0.77	-0.57	-1.25	0.75
16635	44.8.15	-0.13	-0.63	-0.03	-0.07	-0.10	-0.17	0.02	-0.16	-0.16	0.11	-0.24	-0.60	0.09	0.00
16694	43.9.14	-0.30	0.16	-0.51	-0.37	-0.23	-0.47	0.01	-0.08	0.05	-0.12	0.32	0.63	0.21	0.01
16719	44.9.19	0.59		0.82	0.05	-0.25	0.94	0.03	0.60	0.18	-0.02	0.51	-1.36	-1.98	0.43
16754	42.10.14	-0.12	-0.59	-0.07	-0.05	-0.09	-0.12	0.03	-0.08	-0.15	0.19	-0.20	-0.35	0.18	0.03
16776	43.10.16	0.18	-0.43	-0.03	0.13	-0.01	0.33	0.01	0.03	-0.19	0.11	-0.38	-0.88	-0.03	0.04
16802	41.11.2	0.64	0.89	-0.26	-0.94		0.22	0.01	0.60	0.16	-0.35	1.45	1.26	2.54	0.32
16837	42.11.17	-0.14	0.20	0.02	-0.02	-0.16	-0.33	0.02	0.01	0.06	0.16	0.23	0.17	0.15	0.75
16992	42.13.12	-0.13	-0.58	-0.07	-0.06	-0.19	-0.03	0.05	-0.10	-0.10	0.17	-0.21	-0.45	0.13	0.02
17169	43.15.9	-0.24	-0.60	-0.06	-0.08	-0.24	-0.11	0.04	-0.25	-0.15	0.21	-0.08	-0.42	0.04	0.03
17213	41.16.13	-0.08	-0.43	0.07	0.11	-0.02	0.02	0.04	0.10	0.16	0.20	-0.01	-0.42	0.15	0.02
17383	42.18.3	-0.10	-0.54	0.11	0.06	-0.13	0.05	0.04	-0.14	-0.19	0.21	-0.20	-0.42	0.10	0.23
17626	46.1.6	0.62	1.78	0.34	-0.09	0.53	0.56	0.01	0.82	0.58	-0.37	-0.28	-2.20	-2.37	0.01
17639	46.1.19	0.42	1.48	0.00	0.05	0.12	0.31	0.03	0.29	-0.35	-1.01	0.27	-0.61	-1.39	0.33
17645	47.1.5	0.68	1.28	0.23	0.04	0.14	0.07	0.02	0.07	-0.16	-0.67	0.47	-0.34	-0.46	0.19
17664	48.1.4	-0.08	0.28	-0.57	-0.41	-0.08	-0.14	0.04	-0.09	-0.45	-0.16	-0.32	0.10	-0.37	0.70
17816	47.3.16	-0.18	-0.54	-0.12	-0.18	-0.17	-0.11	0.04	-0.10	-0.07	0.19	-0.13	-0.44	0.14	0.00
17822	48.3.2	0.23	0.66	-0.23	-0.04	-0.11	-0.18	0.01	0.31	0.11	0.05	0.29	0.43	0.50	0.02

Table 4.2 continued.

Spot	ID	0_B	1.5_B	3_B	6_B	12_B	24_B	p_B	0_CHX	1.5_CHX	3_CHX	6_CHX	12_CHX	24_CHX	p_CHX
17842	45.4.2	0.56	3.06	-0.21	0.35	0.17	0.87	0.02	0.01	0.24	-0.17	1.18	-0.27	0.85	0.07
17853	45.4.13	0.62	0.45	0.32	-0.04	0.28	-0.94	0.01	0.96	1.00	0.40	-0.18	-1.08	0.11	0.05
17877	46.4.17	0.07	0.26	-0.06	0.01	0.01	-0.02	0.02	0.07	0.00	-0.06	0.09	0.15	-0.06	0.27
17891	47.4.11	0.17	0.65	0.08	0.00	0.16	-0.10	0.05	-0.10	0.15	-0.05	-0.57	0.23	-0.15	0.23
17907	48.4.7	-0.15	3.68	-1.77	-0.60	-0.73	0.89	0.02	0.20	0.89	-0.59	0.93	-0.86	1.13	0.57
17968	47.5.8	-0.22	0.32	0.04	-0.67	-0.04	-0.22	0.03	-0.11	-0.34	-0.05	-0.44	0.17	-0.10	0.41
18126	47.7.6	0.38	4.40	-0.18	0.51	0.47	0.13	0.01	0.81	1.20	0.58	1.13	1.10	0.09	0.93
18157	48.7.17	0.43	2.02	-2.37	-2.55	6.62	-0.42	0.00	0.90	1.03	-0.02	0.24	1.74		0.47
18259	45.9.19	-0.02	-0.47	0.06	-0.01	-0.09	0.16	0.04	0.01	0.04	0.24	-0.03	-0.38	0.17	0.04
18279	46.9.19	-0.07	-0.50	0.11	-0.02	-0.17	-0.03	0.04	-0.06	-0.07	0.22	-0.11	-0.33	0.19	0.07
18295	47.9.15	-0.17	-0.63	-0.01	-0.13	-0.19	-0.05	0.04	-0.18	-0.17	0.22	-0.35	-0.63	0.10	0.03
18333	45.10.13	-0.03	-0.39	0.04	0.06	0.25	0.05	0.01	0.02	0.07	0.15	0.10	0.12	-0.04	0.61
18339	45.10.19	-0.03	-0.48	0.04	-0.04	-0.11	0.08	0.02	-0.08	-0.12	0.21	-0.09	-0.28	0.24	0.08
18341	46.10.1	-0.05	-0.76	0.10	-0.06	-0.10	-0.48	0.02	0.15	0.02	0.15	0.15	-0.33	0.65	0.01
18379	47.10.19	-0.32	-0.71	-0.08	0.03	-0.17	-0.18	0.02	-0.26	-0.25	0.14	-0.33	-0.47	0.03	0.21
18380	47.10.20	-0.21	-0.62	-0.12	-0.19	-0.03	-0.38	0.04	-0.25	-0.15	0.15	-0.20	-0.44	0.18	0.04
18411	45.11.11	-0.06	-1.19	0.13	-0.17	-0.25	-0.33	0.00	-0.34	-0.38	0.07	-0.58	-0.80	0.09	0.06
18430	46.11.10	-0.30	-0.85	-0.16	-0.18	-0.34	-0.19	0.05	-0.26	-0.31	0.16	-0.39	-0.55	0.04	0.11
18441	47.11.1	-0.06	-0.75	0.02	0.02	-0.03	0.10	0.04	0.06	0.09	0.47	0.20	-0.31	0.18	0.38
18451	47.11.11	-0.27	-1.24	-0.61	-0.58	-0.47	-0.54	0.03	-0.13	-0.28	-0.09	-0.59	-0.75	-0.24	0.04
18473	48.11.13	-0.25	-1.50	-0.48	-0.51	-0.38	-0.53	0.01	-0.90	-0.52	-0.15	-0.33	-0.70	0.02	0.04
18512	46.12.12	-0.63	-0.18	-2.24	-0.76	1.33	-0.58	0.03	-1.15	-0.25	-0.35	-0.38	1.04	1.18	0.61
18599	46.13.19	1.22		0.75	0.79	1.66	2.90	0.01	4.07	1.32		0.35	-0.04	-1.56	0.18
18614	47.13.14	-0.09	-0.53	-0.02	-0.05	-0.16	-0.08	0.05	-0.12	-0.13	0.15	-0.18	-0.39	0.02	0.08
18648	45.14.8	-0.20	-2.06	-0.06	-0.18	-0.22	0.43	0.01	0.19	-0.04	0.33	-0.14	-2.54	-0.34	0.20
18668	46.14.8	0.21	0.72	-0.36	-0.23	-0.11	-0.10	0.02	0.55	0.40	-0.05	0.80	-0.54	-1.05	0.00
18758	46.15.18	-0.05	0.30	0.04	0.25	-0.01	0.00	0.03	-0.07	-0.04	0.02	0.15	0.26	-0.05	0.01
18780	47.15.20	-0.51	-0.05	0.02	-0.03	-0.07	-0.13	0.00	-0.40	-0.11	-0.01	0.40	0.30	0.20	0.00
18813	45.16.13	-0.02	-0.74	0.12	0.08	-0.08	0.05	0.03	0.03	-0.10	0.35	0.09	-0.44	0.20	0.19
18820	45.16.20	-0.18	-0.47	0.05	-0.04	-0.13	-0.05	0.04	-0.10	-0.13	0.28	-0.22	-0.38	0.02	0.09
18825	46.16.5	-0.07	-0.48	0.11	0.01	-0.06	0.05	0.05	-0.10	0.04	0.26	-0.08	-0.31	0.19	0.36
18917	46.17.17	-0.01	1.21	0.43	0.79	0.56	1.61	0.03	0.02	0.08	-0.02	1.01	-1.00	-1.41	0.15
19025	48.18.5	-0.34	-0.39	-1.27	0.48	-0.57	0.08	0.02	-0.37	0.06	0.46	-0.43	-0.45	-0.41	0.71
19050	45.19.10	-0.06	-0.52	0.11	0.05	-0.09	0.06	0.02	-0.09	-0.05	0.22	-0.09	-0.38	0.19	0.10

Table 4.2 continued.

Category	Number
Time points with CORT	6
CHX treated or Control groups	2
Replicates	4
Total spots on array	19200
Unique cDNA clones on array	14053
Total arrays	48
Total Dyes (Sample and Reference pool)	2
Total data points	1,843,200
Spots with significant difference between any two time points ($p<0.05$) in the absence of CHX	457
Spots with at least half of Observations in absence of CHX ($n \geq 12$)	388
Spots with less than a 2-fold Effect	251
Genes Induced Early (In presence of CHX)	51 (13)
Genes Induced Late (In presence of CHX)	20 (9)
Genes Repressed Early (In presence of CHX)	52 (12)
Genes Repressed Late	14

Table 4.3. Number of transcripts that were either induced early, induced late, repressed early, repressed late, or showed no significant effect across at either time point during the 24 hr culture period in the presence of CORT (10^{-9} M).
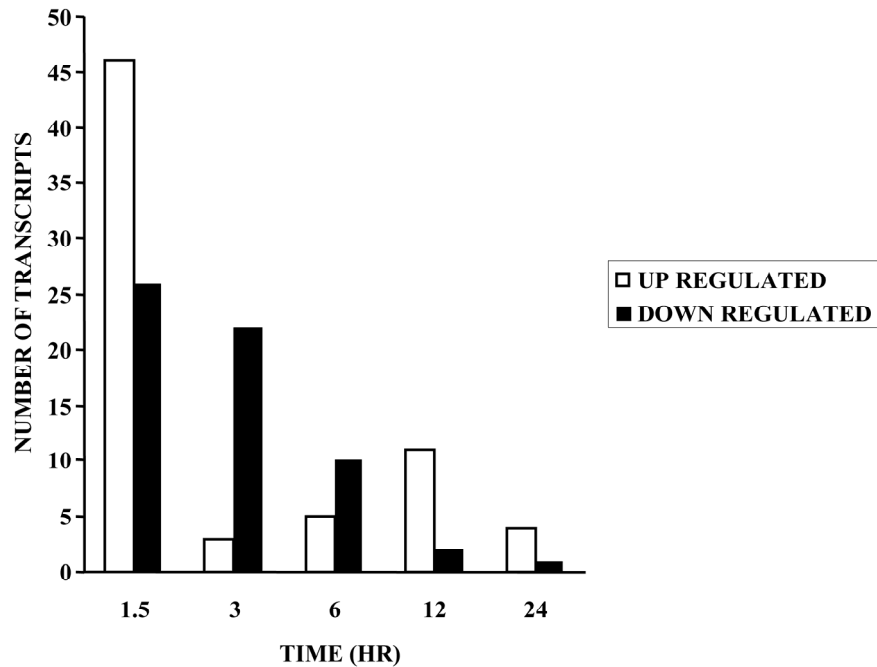
Figure 4.2. Distribution of transcripts in e11 chicken pituitary cells during a 24 hr culture in the presence of CORT (10^{-9} M). Data represents the number of transcripts that were first affected by CORT at the particular time points. Open bars represent transcripts that were ≥2-fold increased in the presence of CORT. Black bars represent transcripts that were ≥2-fold down regulated in the presence of 10^{-9} M CORT.

To verify the data obtained from the microarray, qRT-PCR analysis was performed on genes from each gene expression category and the results shown in figure 4.3. The genes that we verified for the no significant effect category are Cytochrome c oxidase subunit VIc (CCO) and SMARCD1 protein (SMARCD1). Cytochrome c oxidase is the terminal enzyme of the mitochondrial electron transfer chain and is composed of 10 to 13 subunits in eukaryotes[163]. SMARCD1 is a nuclear protein that is a member of the SWI/SNF-related, matrix-associated, actin-dependent regulators of chromatin protein family[164]. LSM7 and PLEKHB1 are two of the transcripts that we verified for genes belonging to our repressed early category. LSM7 is a member of the eukaryotic Sm and Sm-like (LSm) U6 small nuclear RNA associated protein group. These proteins associate with RNA to form the core domain of the ribonucleoprotein particles involved in a variety of RNA processing events including mRNA degradation, replication of telomeres, and pre-mRNA splicing[165]. Pleckstrin homology domain containing, family B (evectins) member 1 (PLEKHB1) belongs to a group of proteins that have so called pleckstrin homology (PH) domains. PH domains are only found in eukaryotes and possess a variety of functions that allow proteins with this domain to be involved in processes such as regulating the actin of the cytoskeleton, AP1 regulation, apoptosis, and muscle differentiation[166-168]. Genes that were repressed late were next verified by qRT-PCR. Two genes belonging to that category were assayed. The first transcript is CEPU-Se alpha 2 isoform (CEPU) which belongs to the IgLON family, which is a subfamily of the Ig superfamily[169]. These proteins are cell adhesion molecules that are involved in neuronal guidance and have been studied in chick embryos[170, 171]. The 17 kD fetal brain protein (FBP) is a product of the chromosome 9 open reading frame 19 gene. FBP

contains a SCP-like extracellular protein domain that is found in proteins such as human glioma pathogenesis-related protein (GliPR), the human testis specific glycoprotein (TPX-1), and several other extracellular proteins from rodents (SCP), insects venom allergens (Ag5, Ag3), and plants pathogenesis proteins (PR-1)[172]. The induced late category was validated with Peripheral myelin protein 22 a (PMP22a) and SUMO1/sentrin/SMT3 specific peptidase 2 (SUMO2). PMP22a is a tetraspan membrane glycoprotein whose mutated form is associated with hereditary demyelinating neuropathies[173, 174], while SUMO2 is a small ubiquitin-like protein that covalently conjugates to other proteins and is involved in protein/protein interactions, intracellular localization, and prevents some of its binding-targets from ubiquitin-dependent degradation[175]. Interestingly, SUMO2 has been shown to interact with the MR and GR[176]. The last category that we validated entailed those genes who induction occurred early (within 3 hrs). qRT-PCR validated two genes in this category, Semaphorin 7A precursor (SEMA7A) and Notch homolog 2 (NOTCH). SEMA7A is a member of a large protein family involved in axon guidance signaling[177], while NOTCH is a product of a gene that encodes a conserved family of receptors that influence developmental fate of various cell lineages in many species, including chickens[178]. As figure 4.3 shows, gene expression levels between the two different techniques were highly comparable.

CCO
SMARCD1 NO EFFECT
LSM7
PLEKHB1 REPRESSED EARLY
CEPU
FBP REPRESSED LATE
PMP22a
SUMO2 INDUCED LATE
SEMA7A
NOTCH INDUCED EARLY
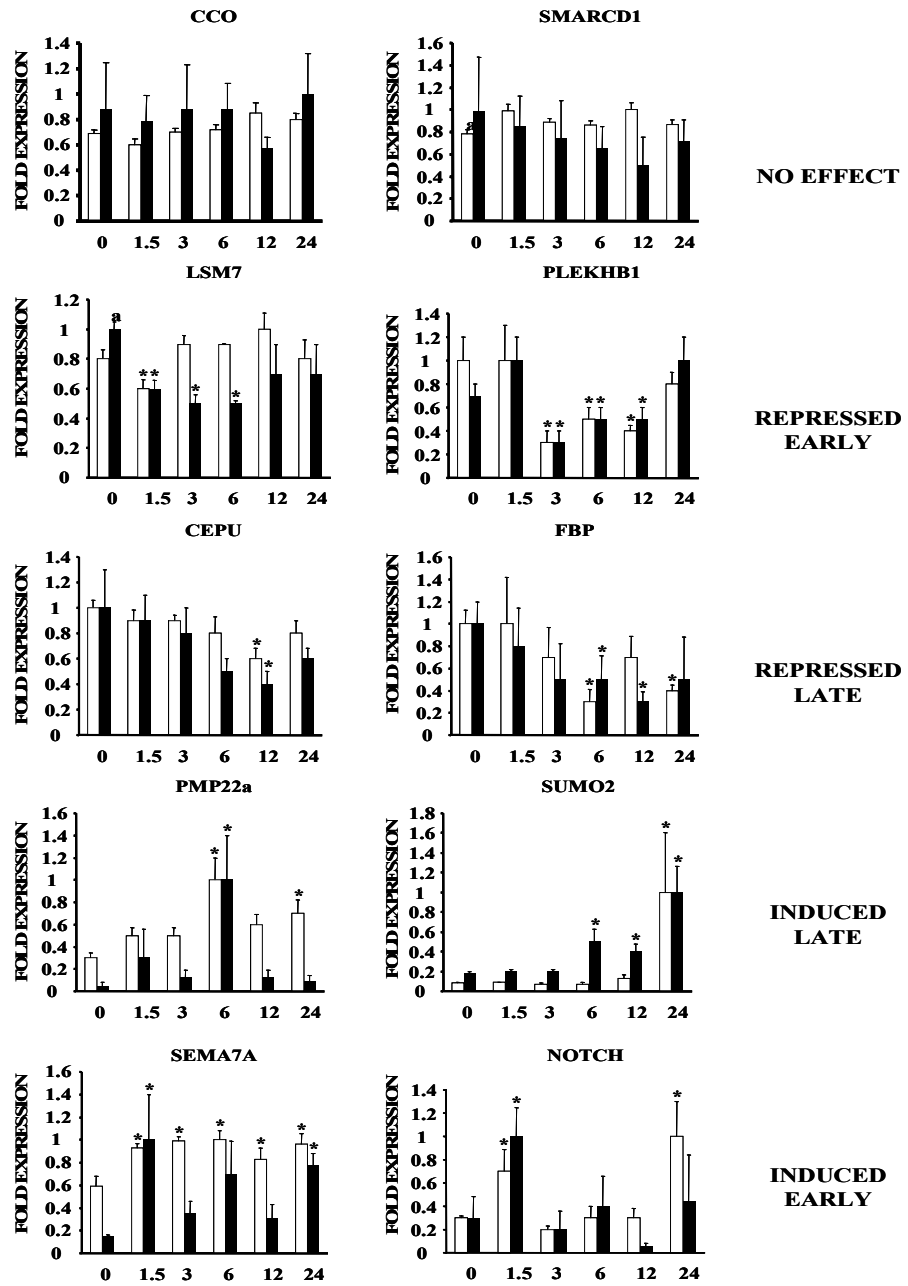
(legend on following page)

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Figure 4.3. Comparison of the microarray analysis (open bars) of anterior pituitary expression levels for 4 different categories of gene expression with results from quantitative real-time reverse-transcription PCR (qRT-PCR) analysis (black bars) on the same genes. Genes from the microarray analysis were put into 1 of 4 categories: No effect, Repressed Early, Repressed Late, Induced Late, and Induced Early. Levels of the analyzed genes were normalized to levels of beta-actin mRNA, and results are expressed relative to the highest expression level for each technique. Results are presented for Cytochrome c Oxidase (CCO), SMARCD1 protein (SMARCD1), LSM7 homolog (LSM7), Pleckstrin homology domain containing family B member 1 (PLEKHB1), CEPU gene (CEPU), 17kD fetal brain protein (FBP), Peripheral myelin protein 22 a (PMP22a), SUMO1/sentrin/SMT3 specific peptidase 2 (SUMO2) , Semaphorin 7A precursor (SEMA7A), and Notch homolog 2 (NOTCH) . The qRT-PCR analysis for all genes was conducted on amplified RNA. Values within a given technique with an asterisk are significantly different from control (0 hr) ($p \leq 0.05$, n=4).

Narrowing down the candidate gene list

As stated above, our hypothesis is that CORT induces the synthesis of one or more

proteins and then these protein(s) initiate GH mRNA production. The results from figure

4.1 show that GH mRNA is induced significantly within 3 h of CORT treatment. In

addition, we noticed a slight but significant increase in GH mRNA within 1.5 hr. Given

these 2 observations we next theorized that our candidate gene(s) must be up-regulated in

response to CORT within 1.5 hr. In addition, our candidate gene(s) must also be a direct

target of CORT, meaning that it must be induced in the presence of cyclocheximide

(CHX). Table 4.3 shows that 51 transcripts fall into the category of being induced early

in response to CORT. Of these 51, only 13 showed at least a 2-fold induction in the

presence of CHX. These direct early inducted transcripts are listed in table 4.4. The

identities of 2 of these transcripts are presently unknown. Interestingly the unknown

transcript corresponding to spot 1 on our microarray chip has the 2nd highest fold

induction of the directly induced CORT genes. However, we could not validate this

induction by qRT-PCR (data not shown). The transcripts that showed the highest fold

induction on our microarray was the scavenger receptor cysteine-rich type 1 protein.

This receptor belongs to the scavenger receptor cysteine-rich superfamily (SRCR-SF),

whose members are a highly conserved group of membrane and/or secreted proteins

related to the innate and adaptive immune system[179]. Unfortunately, induction of this

transcript could not be verified with qRT-PCR as well (data not shown).

Spot	Gene Name	Fold induction
1	Unknown	13
105	Cellular FLICE-like inhibitory protein long form	2
309	P450 (cytochrome) oxidoreductase	2
2822	FK506 binding protein 5	2
2862	Semaphorin 7A precursor	2
3408	RAS, dexamethasone-induced 1	15
5047	Ras-dva small GTPase	5
5048	Unknown	2
5050	Scavenger-receptor protein	20
5088	FK506-binding protein 5	4
8797	Unknown	3
9246	Mitogen-activated protein kinase-activated protein kinase 3	2
15282	Pyruvate dehydrogenase kinase-like protein	2

Table 4.4. Transcripts that are directly up-regulated by CORT treatment within 3 h.

To aid us identifying potential candidate genes, we undertook a clustering method to group genes based on their expression profiles during their 24 hr period. Because we only interested in expression levels compared to the basal time point and not differences that may be observed between the other time points (i.e., 3 hr vs. 6 hr, 6 hr vs. 12 hr, 12 vs. 24 hr) we did not refer to common clustering procedures such as SOMS analysis and hierarchical clustering. To cluster our transcripts, we compared the background corrected Cy3/Cy5 ratios of our control time point (0 hr) to every other time point (i.e., 0 hr vs. 1.5 hr, 0 hr vs. 3 hr, 0 hr vs. 6 hr, 0 hr vs. 12 hr, and 0 hr vs. 24 hr) and determined the levels of induction/repression with the following formula (Cy3/Cy5 ratio of 0 hr – Cy3/Cy5 ratio of sample time point). If the difference was $\leq$ -.99, that correlated to a 2-fold induction (Cy3/Cy5 ratios are on a log2 scale) and that particular time interval was categorized as induced (UP). If the difference was $\geq$ .99, that correlated to a 2-fold decrease or repression, and that particular time interval was categorized as repressed (DOWN). If the difference was between -.99 and .99, then that time interval was perceived as having no significant effect (NA). With 5 possible time intervals, 3 possible outcomes in each time interval, the number of possible different combinations totaled 243. Out of those 243 possible combinations, only 58 had at least one 1 gene in its cluster. Because we theorized that our candidate gene must be significantly affected within 1.5 hr, those clusters who had no significant effect between 0 and 1.5 hr could be eliminated. That left us with 27 possible clusters, whose expression profiles and the number of genes in the cluster is shown in table 4.5.

CLUSTER	0-1.5	0-3	0-6	0-12	0-24	# OF GENES
82	UP	NA	NA	NA	NA	16
83	UP	NA	NA	NA	UP	3
85	UP	NA	NA	UP	NA	1
86	UP	NA	NA	UP	UP	1
92	UP	NA	UP	NA	UP	1
95	UP	NA	UP	UP	UP	1
101	UP	NA	DOWN	NA	UP	1
109	UP	UP	NA	NA	NA	1
112	UP	UP	NA	UP	NA	1
113	UP	UP	NA	UP	UP	1
118	UP	UP	UP	NA	NA	2
119	UP	UP	UP	NA	UP	1
121	UP	UP	UP	UP	NA	1
122	UP	UP	UP	UP	UP	5
136	UP	DOWN	NA	NA	NA	4
137	UP	DOWN	NA	NA	UP	1
140	UP	DOWN	NA	UP	UP	1
142	UP	DOWN	NA	DOWN	NA	1
149	UP	DOWN	UP	UP	UP	1
154	UP	DOWN	DOWN	NA	NA	1
159	UP	DOWN	DOWN	UP	DOWN	1
163	DOWN	NA	NA	NA	NA	21
169	DOWN	NA	NA	DOWN	NA	1
172	DOWN	NA	UP	NA	NA	1
181	DOWN	NA	DOWN	NA	NA	1
217	DOWN	DOWN	NA	NA	NA	1
241	DOWN	DOWN	DOWN	DOWN	NA	1

Table 4.5. Clustering of genes into expression profiles across the 24 hr culture period.
Genes were clustered into one of out 243 possible expression profiles according to their
expression levels relative to control (0 hr) from the microarray analysis. During each
time point comparison (0 – 1.5 hr, 0 – 3hr, 0 – 6 hr, 0 – 12 hr, and 0 -24 hr), the
expression levels of genes could either show no 2 fold difference from the control (Not
affected, NA), exhibit at least a 2-fold increase (UP), or exhibit at least a 2-fold decrease
(DOWN) in relative expression levels. The table shows 27 categories in which there was
at least a 2-fold increase or decrease in expression levels during the first 90 minutes of
culture in the presence of CORT (10^{-9} M).

As seen in figure 4.1, expression levels of GH remain elevated throughout the 24 hr culture period. Noticeably, one of our clusters (Cluster 122) contains 5 transcripts whose expression levels remain elevated throughout the 24 hr culture period. Two of these transcripts represent members of the Ras superfamily of signal transduction proteins. RAS, dexamethasone-induced 1 (also known as Dexras1) was originally identified in 1998 as a gene in a murine corticotroph cell line (AtT-20) that was rapidly induced in response to glucocorticoid treatment[133]. Dexras1 has been shown to have a variety of possible functions such as a suppressor of G-protein signaling downstream of ligand binding[135, 136] and as a protein-binding partner for CAPON, which is a protein known to associate with neuronal nitric oxide synthase[137]. The other ras protein is Ras-dva small GTPase, which has been shown to be an essential component of the signaling network that patterns the early anterior neural plate and the adjacent ectoderm in Xenopus laevis embryos[180]. In addition to these proteins being involved in signal transduction pathways, these two genes are ideal candidates because previous research in our laboratory has shown that that the Ras inhibitor manumycin suppressed CORT stimulated increases in GH mRNA levels[120]. The third transcript in this cluster is FK506-binding protein 5 (FKBP5). FKBP5 is an immunophilin that binds FK506, and is an ideal candidate because it has been shown to be involved in nuclear translocation of the glucocorticoid receptor (GR)[181], and FK506 has been shown to stimulate GH release from rat somatotrophs[182]. The cDNA clone of FKBP5 from our cDNA library is not of full length and we are currently in the process of cloning the full length cDNA into an expression vector. The fourth transcript is corresponds to spot 2644 on our microarray and is an unknown transcript. However, its induction was blocked by CHX and thus was

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eliminated from further analysis. The last transcript in this category is Scavenger

receptor protein (SR), which is a membrane receptor that binds lipoproteins and are

involved in their uptake into cells[183], and SR has been characterized in chickens[184].

Unfortunately, we could no verify the expression levels of SR with qRT-PCR.

We also decided to choose a transcript that was directly repressed by CORT within 3 h

to support the hypothesis that CORT treatment can repress the expression of an inhibitor

that suppresses GH mRNA production. One interesting candidate that fit this profile was

Prostaglandin-D synthase (PGDS). PGDS synthase catalyzes the isomerization of

Prostaglandin H2, a common precursor of various prostanoids, to produce Prostaglandin-

D 2 (PGD2) in the presence of sulfhydryl compounds. PGDS is considered to be a dual-

function protein; it acts as a PGD2-producing enzyme and also as a lipophilic ligand-

binding protein, because the enzyme binds retinoids, thyroids, and bile pigments with

high affinities[185]. The fact that PGDS can bind thyroids is of particular interest to us

because previous research in our laboratory has shown that treatment of chick embryonic

pituitary cells with T_3 or T_4 alone had negligible effects on somatotroph abundance *in

vitro*[113]. On the other hand, the combination of the thyroid hormones and CORT were

able to augment GH cell numbers above that found with CORT alone.

Before we could proceed with further analysis of our candidate genes (Dexras1,

PGDS, Ras-dva), we had to verify the microarray analysis of these genes by qRT-PCR.

As shown in figure 4.4, expression levels of both Dexras1 and Ras-dva are significantly

increased within 1.5 hr in both techniques. Microarray analysis of PGDS shows that its

expression levels doesn't become significantly decreased until 3 hr after CORT

treatment. However, qRT-PCR shows that PGDS expression levels are significantly

repressed within 90 min. Despite this discrepancy, both techniques show that PGDS is

repressed within 3 hr.
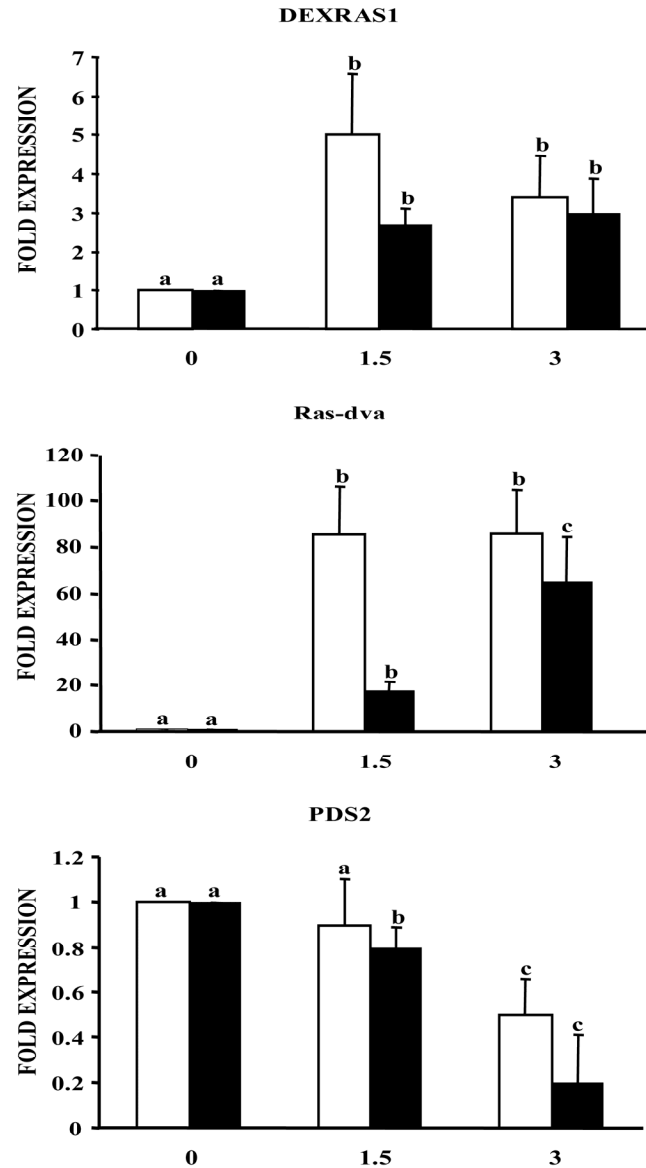
Figure 4.4. Comparison of the microarray analysis results (open bars) with results from quantitative real-time reverse-transcription PCR (qRT-PCR) analysis (black bars) Dexras1, Ras-dva, and Prostaglandin-D synthase (PDS2). Levels of the analyzed genes were normalized to levels of beta-actin mRNA, and results are expressed relative to 0 hr time point. The qRT-PCR analysis for all genes was conducted on amplified RNA. Values within a given technique with different letters are significantly different from one another ($p \leq 0.05$, n=4).

Effect of overexpression of candidate genes on GH mRNA

If the candidate gene directly induces GH mRNA, then overexpression of that gene should lead to an increase in GH mRNA even in the absence of CORT. Furthermore, if overexpression of a candidate gene enhances the effect of CORT, then that gene may play a role in CORT induction of GH mRNA. All of the cDNAs in our library are in a eukaryotic expression vector, pCMV-Sport6.1. The clones for the candidate genes that we have chosen (Dexras1, PGDS, and Ras-dva) contain the full length cDNA for their respective genes, and as a result no further cloning was necessary to express the gene product. e11 pituitary cells were transfected with either an empty pCMV-Sport6.1 vector, or this vector containing Dexras1, PGDS, or Ras-dva. A Golgi-targeted GFP-expressing plasmid was co-transfected to facilitate isolation of transfected cells. After 24 hrs, one well from each transfection group was treated with CORT for 18 h, while the other well of the same transfected group was left untreated. After the 18 hr incubation period, the cells were sorted into GFP$^+$ and GFP$^-$ cell groups. RNA was then extracted from only those cells that were GFP$^+$, and qRT-PCR analysis was performed to determine relative mRNA levels. A result from one FACS experiment is shown in figure 4.5. Cells that are positive for GFP are contained within the large rectangle on the right side of each graph, while GFP- negative cells are located outside of the rectangle. The top graph represents cells that were non-transfected, while the bottom graph represents cells transfected with GFP and Dexras1. As figure 4.5 illustrates, the percentage of fluorescent cells increases (~18%) dramatically in the culture transfected with the GFP expression vector, relative to non-transfected cells (< 4% of all cells).

Non-transfected
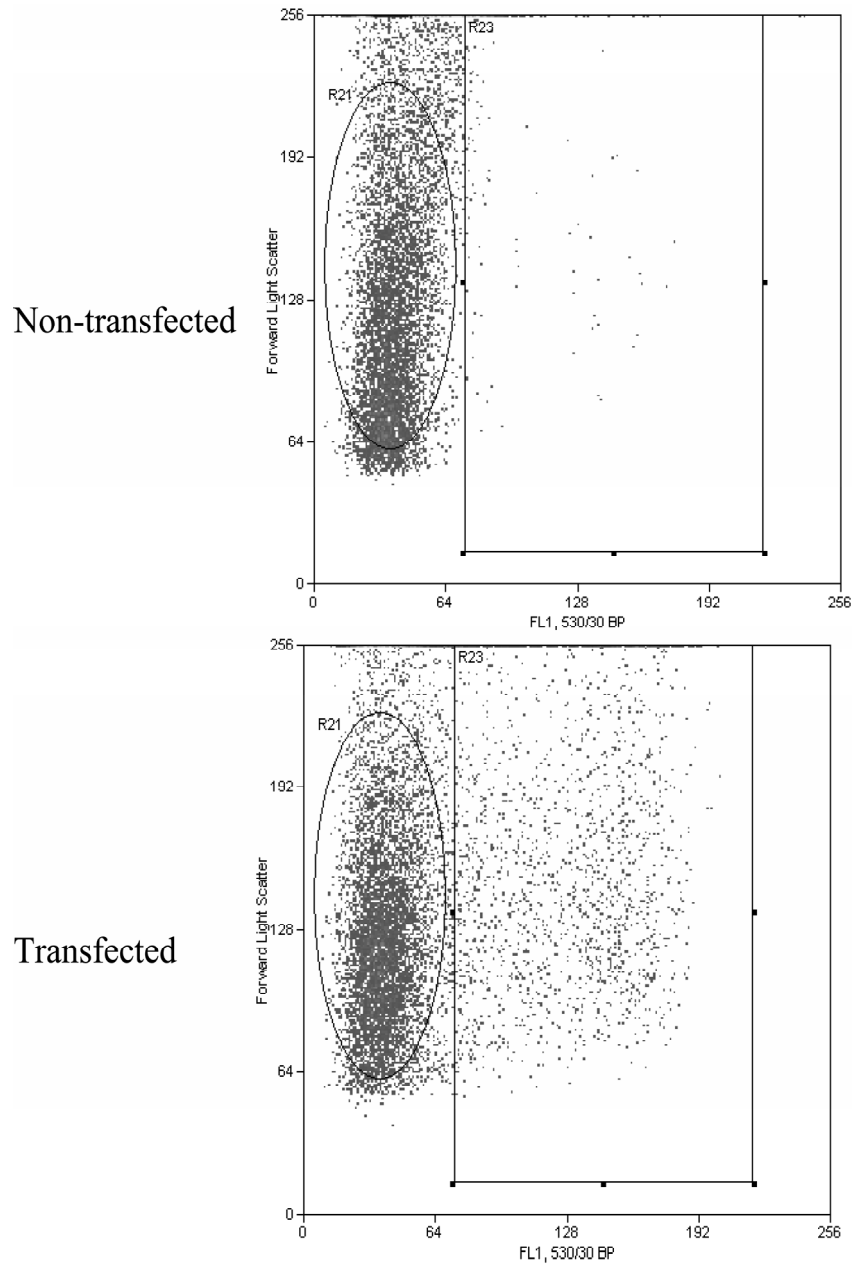
Transfected

Figure 4.5. FACS analysis of cells transfected with Dexras1 and a Golgi-targeted GFP-expressing plasmid. The top panel represents cells that were non-transfected and the bottom represents cells transfected with Dexras1 and GFP. Fluorescent cells are contained in a rectangle on the right side of each graph, and non-fluorescent cells are located outside of the rectangle.

Table 4.5 shows that the genes transfected into the e11 pituitary cells are indeed being over-expressed. Results presented in figure 4.6 show that overexpression of either Dexras1, PGDS, or Ras-dva did not cause an elevated amount of GH mRNA in e11 pituitary cells in the absence of CORT, meaning that none of these genes can directly stimulate GH mRNA production. However, there was a significant increase in GH mRNA in cells that were transfected with the Dexras1 plasmid in the presence of CORT, relative to cells transfected with the empty vector and treated with CORT. This observation shows that Dexras1 can act synergistically with CORT to increase GH mRNA levels in e11 pituitary cells. Perhaps there is an additional unknown gene that CORT induces along with Dexras1 that allows the increase in GH mRNA in response to Dexras1. We also tested what effect overexpression of our candidate genes would have on two other pituitary hormones, prolactin and pro-opiomelanocortin (POMC). However, transfecting with our candidate genes had no significant effect on the levels of mRNA for transcription of these genes. In addition, we chose two other genes that were indirectly induced late in response to CORT to see if overexpression of our candidate genes affected their expression levels. The two additional genes that we chose to analyze were Selenoprotein I and Inhibitor of apoptosis 3 (IAP3). The function of Selenoprotein I is currently unknown but has been isolated in a chicken bursal lymphocyte cDNA library[186]. IAP3 is, as its name suggests, an anti-apoptotic factor whose expression was found elevated during the differentiation of the developing chick lens[187]. As the graph in figure 4.7 shows, overexpression of our candidate genes had no effect on Selenoprotein or IAP3 mRNA levels either in the absence or presence of CORT.

mRNA quantified	Gene transfected	CORT Treated	Fold Expression ± SEM
Prostaglandin-D Synthase	Dexras1	Yes	1.5 ± 1.0
Prostaglandin-D Synthase	Dexras1	No	.7 ± .2
Prostaglandin-D Synthase	PGDS	Yes	261.7 ± 115.5
Prostaglandin-D Synthase	PGDS	No	380.1 ± 126.3
Prostaglandin-D Synthase	Ras-dva	Yes	4 ± 2.3
Prostaglandin-D Synthase	Ras-dva	No	.6 ± .2
Prostaglandin-D Synthase	Sport6.1	Yes	.9 ± .5
Prostaglandin-D Synthase	Sport6.1	No	1 ± 0.0
Dexras1	Dexras1	Yes	131 ± 30.0
Dexras1	Dexras1	No	119.9 ± 25.5
Dexras1	PGDS	Yes	.6 ± .3
Dexras1	PGDS	No	.5 ± .1
Dexras1	Ras-dva	Yes	.5 ± .1
Dexras1	Ras-dva	No	.7 ± .1
Dexras1	Sport6.1	Yes	4.8 ± 1.5
Dexras1	Sport6.1	No	1 ± 0.0
Ras-dva	Dexras1	Yes	3.2 ± 1.9
Ras-dva	Dexras1	No	1.8 ± 0.4
Ras-dva	PGDS	Yes	3.3 ± 2.3
Ras-dva	PGDS	No	1.5 ± 0.2
Ras-dva	Ras-dva	Yes	600.3 ± 339.9
Ras-dva	Ras-dva	No	248.6 ± 106.2
Ras-dva	Sport6.1	Yes	1.4 ± 0.5
Ras-dva	Sport6.1	No	1 ± 0.0

Table 4.6. Confirmation of overexpression of transfected genes. Dispersed e11 pituitary cells were cultured in serum-free media and then transfected with either an empty pCMV-Sport6.1 vector, or the same vector containing cDNAs for chicken Dexras1, PGDS, or Ras-dva, along with a Golgi-targeted GFP-expressing plasmid. After 24 hrs, one well from each transfection group was treated with CORT (10^{-9} M) for 18 h while an identical well was left untreated. After the 18 hr incubation period, the cells were retrypsinized and sorted into GFP[+] and GFP[-] cell groups. Finally, RNA was extracted from only those cells that were GFP[+] and qRT-PCR analysis was performed to determine relative mRNA levels. Levels of the analyzed genes were normalized to levels of beta-actin mRNA, and results are expressed relative to cells transfected with empty pCMV-Sport6.1 vector with no CORT treatment.
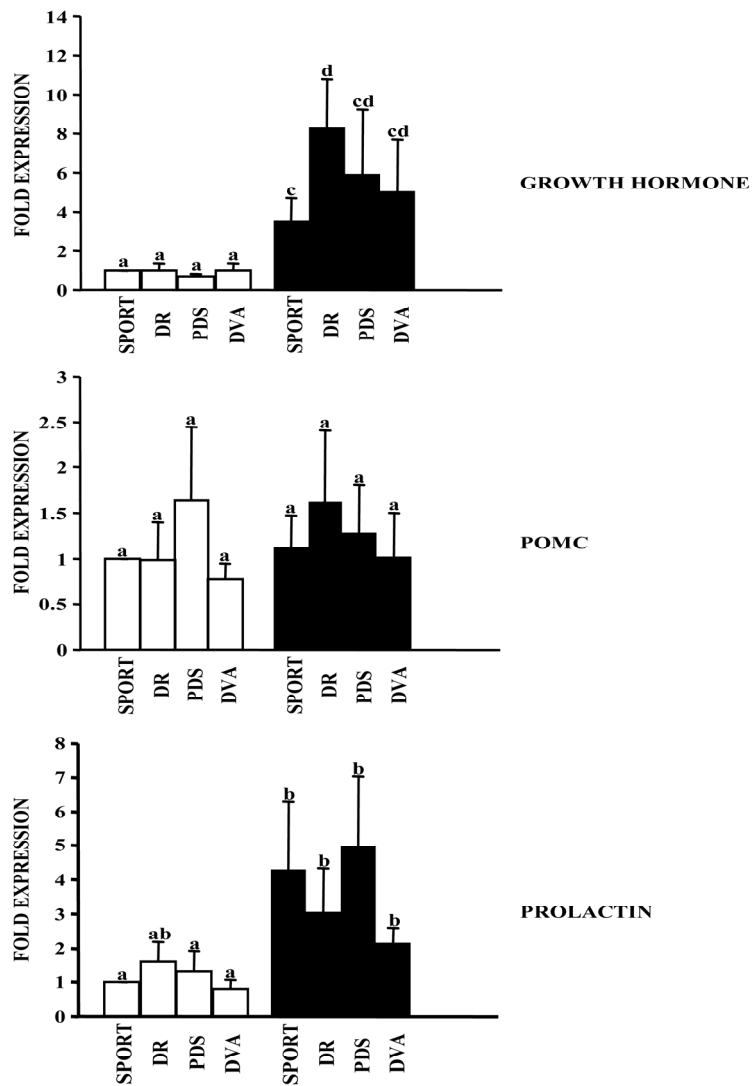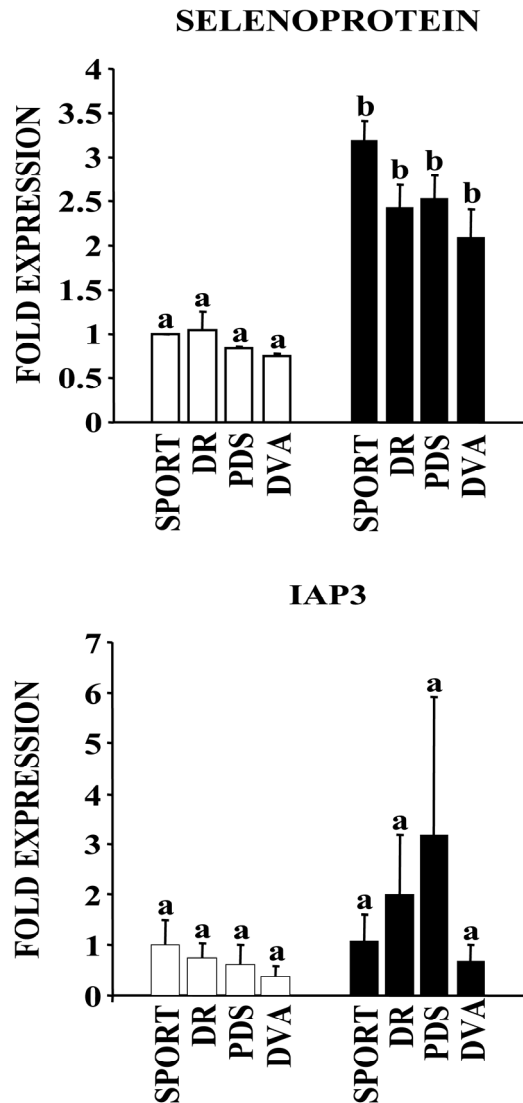
Figure 4.6. Effect of overexpression of candidate genes on pituitary hormone mRNA levels. Dispersed e11 pituitary cells were cultured in serum-free media and then transfected with either an empty pCMV-Sport6.1 vector (SPORT), or Dexras1 (DR), PGDS (PDS), or Ras-dva (DVA) plasmid along with a Golgi-targeted GFP-expressing plasmid. After 24 hrs, one well from each transfection group was treated with CORT $(10^{-9}$ M) (black bars) for 18 h while the other was left untreated (open bars). After the 18 hr incubation period, the cells were retrypsinized and sorted into GFP^+ and GFP^- cell groups. Finally, RNA was extracted from only those cells that were GFP^+ and qRT-PCR analysis was performed to determine relative mRNA levels of Growth Hormone, POMC, and Prolactin. Levels of the analyzed genes were normalized to levels of beta-actin mRNA, and results are expressed relative to cells transfected with empty pCMV-Sport6.1 vector with no CORT treatment. Values with different letters are significantly different from one another ($p \leq 0.05$, n=4).

SELENOPROTEIN

FOLD EXPRESSION

4
3.5
3
2.5
2
1.5
1
0.5
0

b
b b
b

a a a a

SPORT DR PDS DVA SPORT DR PDS DVA

IAP3

FOLD EXPRESSION

7
6
5
4
3
2
1
0

a
a
a
a
a a a a

SPORT DR PDS DVA SPORT DR PDS DVA

Figure 4.7. Effect of overexpression of candidate genes on CORT induced genes. Dispersed e11 pituitary cells were cultured in serum-free media and then transfected with either an empty pCMV-Sport6.1 vector (SPORT), or Dexras1 (DR), PGDS (PDS), or Ras-dva (DVA) plasmid along with a Golgi-targeted GFP-expressing plasmid. After 24 hrs, one well from each transfection group was treated with CORT (10^{-9} M) (black bars) for 18 h while the other was left untreated (open bars). After the 18 hr incubation period, the cells were retrypsinized and sorted into GFP$^+$ and GFP$^-$ cell groups. Finally, RNA was extracted from only those cells that were GFP$^+$ and qRT-PCR analysis was performed to determine relative mRNA levels of Selenoprotein, and Inhibitor of Apoptosis 3 (IAP3). Levels of the analyzed genes were normalized to levels of beta-actin mRNA, and results are expressed relative to cells transfected with empty pCMV-Sport6.1 vector with no CORT treatment. Values with different letters are significantly different from one another ($p \leq 0.05$, n=4).

DISCUSSION

We have used a chicken DEL-MAR 14K Integrated Systems cDNA microarray to assess global gene expression in chicken embryonic anterior pituitary cells treated with CORT. Our custom microarray contains 14,053 cDNAs assembled from the neuroendocrine system, the reproductive system, the liver, adipose tissue, and skeletal muscle. It is estimated that the chicken genome contains between 20,000 to 23,000 genes[188], so our microarray covers more than half of the chicken genome.

Glucocorticoids are capable of inducing somatotroph differentiation in both fetal rats and chicken embryos[189]. Inhibition of protein synthesis blocks glucocorticoid induction of GH mRNA in both chickens[120] and rats[115], entailing that an intermediary protein is required for GH expression. The aim of the present study was to identify genes that are involved in CORT induction of GH mRNA expression.

Since the aim of our study was to identify possible genes that may regulate CORT induction of GH, we had to establish the time course of CORT induction of GH mRNA, because any gene involved would have to be induced before or coincident GH. We cultured e11 pituitary cells with CHX pretreatment then CORT or just with CORT alone. As the results from figure 4.1 show, GH is significantly induced within 90 minutes and continues to rise significantly until 3 hr, where the levels remain statistically similar between 3 and 24 hr but stay well above the levels at the 0 hr time point. As we stated earlier, CHX treatment blocks CORT induction of GH mRNA, and the results from figure 4.1 confirm that. Glucocorticoids have to been shown to increase gene expression even earlier than 90 minutes. For example Dexamethasone can increase the dual-specificity MAPK phosphatases MKP-1 10-fold within 30 minutes[190]. However, rapid

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mRNA induction does not necessarily translate into increased gene transcription because glucocorticoids do have some nongenomic effects such as regulating mRNA stability[191, 192].

The microarray analysis that we performed was done on 19,200 genes, of which 14,053 represent unique chicken cDNAs. Of those 14, 053 cDNAs only 388 showed a significant difference in expression between any two time points ($p<0.05$) and were observed in at least half of the potential observations ($n \leq 12$; maximum = 24). The use of a rich time-series design for the examination of hormone effects using high-throughput methods such as microarrays provides the advantages that multiple independent measurements taken over relatively short time intervals abrogate the need for independent confirmation of gene regulation necessary with single-point studies, and provides a definitive analysis of the pattern of regulation that is not available or may even be obscured by single time point studies. However, the extremely large datasets generated by such a design provides challenges in analysis. As a result, we did not perform common clustering analysis methods, such as SOMS and hierarchical clustering, but instead group genes based on the magnitude of expression and time of expression. We first categorized the gene expression data into 5 groups, (1) Genes that were induced at least 2-fold within 3 hr were considered Induced early; (2) Genes that were repressed at least 2-fold within 3 hr were considered repressed early; (3) Genes that were induced late (after 3 hrs); (4) Genes that were repressed late; and (5) Genes that showed no 2-fold induction/repression. As seen in table 4.3, the majority of the 388 significant genes belong to the category 5, 251 genes. As can been seen from table 4.3 and figure 4.2, the majority of the genes that were regulated by CORT (either directly or indirectly) were

regulated relatively early (within 3 hr). This finding is consistent with the lipophilic

nature of steroid hormones such as glucocorticoids, which are able to diffuse easily into

and out of the cell. One would expect their effect on gene transcription to be more

immediate and brief compared to protein hormones. On the other hand, protein

hormones have into bind to a membrane receptor and initiate a signaling transduction

cascade to affect gene transcription, and require an energy-requiring process to be able to

enter the cell. Of the 388 genes that we categorized, only 51 were induced early (i.e.,

within 3 hr) and of those 51 only 13 were induced in the presence of CHX. We also had

to account for genes who even though were directly induced early in response to CORT,

might exhibit an biphasic expression profile that would be inconsistent with the

expression profile seen with GH during the 24 hr culture period. To cover for this

possibility, we performed a cluster analysis were genes were clustered according to their

expression levels at various time intervals (i.e., 0 hr to 1.5 hr, 0 hr to 3 hr, 0 hr to 6 hr, 0

hr to 12 hr, 0 to 24 hr). Due to the fact we had 5 time intervals and 3 possible outcomes

in each interval (up-regulated, down-regulated, or no 2-fold difference) this gave us 243

different possible combinations. Because our candidate gene(s) had to be significantly

affected within 90 min, this allowed us to eliminate the majority of the 243 possible

expression patterns until we were left with 27 clusters that had at least one gene in them

(Table 4.5). Of these 27 clusters, cluster 122 was the most interesting. This was so

because its expression profile was consistent with the expression profile of GH, meaning

gene expression levels remained above basal (0 hr) throughout the 24 hr culture period.

Before we could proceed with further analysis, the results obtained from the microarray

had to be verified by another technique, in this case qRT-PCRT. As the results showed

from figure 4.3, the two methods are decidedly similar in measuring gene expression.

As mentioned previously, our microarray analysis identified 13 transcripts that were

directly induced by CORT (shown in table 4.4), and of those 13, 4 occupied cluster 122.

Two of the genes in cluster 122 are Ras proteins, Dexras1 and Ras-dva. These two genes

made ideal candidates because we have previously shown that the ras inhibitor

manumycin blocks CORT induction of GH mRNA[120]. Another transcript in this cluster

represents FK506 binding protein 5 (FKBP5), which is an immunophilin that binds

FK506. In addition to FKBP5 being directly and rapidly induced by CORT, it is of

particular interest because it is involved in nuclear translocation of the glucocorticoid

receptor (GR)[181] and FK506 can incite GH release from rat somatotrophs[182]. However,

the cDNA clone from our library does not contain the full length sequence. We choose a

3rd gene, prostaglandin-D synthase (PGDS), because it was directly repressed in response

to CORT as wells as the fact that this protein binds thyroid hormones. This was of

interest because we have previously shown that treatment of chick embryonic pituitary

cells with T_3 or T_4 alone had minor effects on somatotroph abundance *in vitro*[113].

Conversely, the combination of CORT and the thyroid hormones were able to increase

the number of somatotrophs above that found with just CORT alone.

All of the cDNAs in our library are in a eukaryotic expression vector, pCMV-

Sport6.1, and the clones for our 3 candidate genes (Dexras1, PGDS, and Ras-dva) contain

the full length cDNA. We transfected into e11 pituitary cells the plasmids for Dexras1,

PGDS, and Ras-dva, and then we treated the cells either for 18 hrs in the presence of

CORT or with no treatment. Confirmation that the plasmids were being transcribed is

shown in table 4.6. None of the genes that we tested had any direct effect on GH mRNA (Figure 4.6), however there appears to be a synergistic effect with Dexras1 and CORT on GH mRNA. It is possible that CORT induces another gene in addition to Dexras1 and that these two induce GH mRNA. We also tested to see if our genes had any direct effect on the genes of two other pituitary hormones, prolactin and POMC, and two CORT regulated genes, Selenoprotein I and IAP3. However, overexpression of our candidate genes had no significant effect on these genes.

Currently, we are in the process of cloning additional candidate genes into expression to ascertain if any of these genes are involved in CORT induction GH. Genes such as FKBP5 will be of particular interest because of the previous findings of its involvement in GR translocation and GH release from rat somatotrophs. FKBP5, Ras-dva, and Dexras1 were also found to be up-regulated in a developmental study in our lab using another of our custom microarrays[155]. In that study, changes in gene expression were assayed with a 5K array from e10 to e17 of chicken embryonic development. During time period of e12 to e14, there was a significant increase in expression levels of those 3 genes. This finding is consistent with the rise in serum glucocorticoids that occurs in the chick embryo on e14[105]. Future work will also attempt to determine the exact role of Dexras1. For example investigators could use either siRNA or antisense RNA to knock-down Dexras1 transcript levels and see what effect this would have on GH production. Because Dexras1 is a member of the Ras superfamily, it is most likely involved in a number of different signal transduction pathways and may have numerous functions in the cell. Indeed, it has been shown to have a very diverse range of tasks. Dexras1 has been shown to function as a ligand-independent activator of G-proteins and it may act to

suppress G-protein signaling downstream of ligand binding[135, 136]. Last but not least, Dexras1/AGS1 might have a potential function as a mediator of glucocorticoid negative feedback regulation of ACTH secretion from pituitary corticotrophs. Dexras1/AGS1 is abundantly expressed in pituitary and is quickly induced (within 15 min) in response to glucocorticoids[133, 138]. In AtT-20 cells, this induction is glucocorticoid-specific, occurs in a dose-dependent manner, and appears mediated at the level of gene transcription[133, 139]. What's more, the expression of a constitutively active form of Dexras1 in AtT-20 cells suppressed cAMP-stimulated secretion of transiently expressed growth hormone, corroborating the perception that Dexras1/AGS1 might work to impede regulated endocrine secretion in corticotrophs[138].

   Based on the available data, we have proposed 2 possible mechanisms by which Dexras1 could act in conjunction with the unknown CORT induced production to stimulate GH mRNA production (Figure 4.8). The first example we called the "Genomic model", and it involves the activated GR binding to the promoter elements of CORT responsive genes and influencing transcription. In the "Genomic model", CORT diffuses into the cell where it binds to and activates the GR. The activated GR then initiates transcription of both Dexras1 and the unknown gene. Afterwards both Dexras1 and the unknown protein act synergistically to stimulate GH mRNA production. In the "Nongenomic model", CORT regulates Dexras1 protein levels in one of two ways. CORT can 1) increase the stability of Dexras1 mRNA leading to an increase in translation of Dexras1 mRNA. Glucocorticoids have been shown to affect the mRNA stability of various genes[193-195]. Or 2) CORT/GR can directly activate the Dexras1 protein through protein/protein interaction. In various experimental systems GR has been
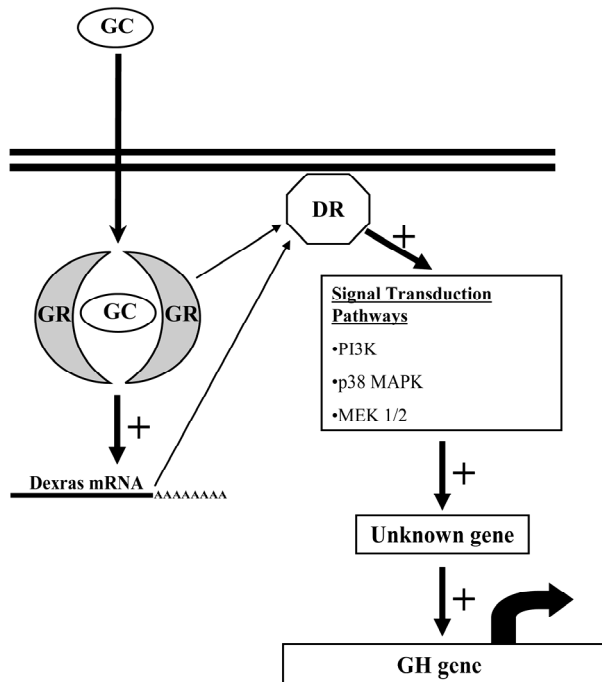
reported to associate with proteins involved in other signaling pathways such as NF-κB[196, 197], STAT3[198], and STAT5[199]. In the end result of either "nongenomic" mechanism, Dexras1 can then initiate various signal transduction cascades which leads to the transcription and translation of an unknown protein(s) and this unknown protein(s) stimulates the transcription of the GH gene. The unknown protein(s) is most likely regulated by the PI3K, p38 MAPK, MEK 1/2, and Ras signal transduction pathways because previous research in our lab have shown that inhibitors of these pathways blocks CORT induction of GH[120, 200].

Genomic Model

GC

GR GC GR

UG DR

Unknown gene

Dexras gene

GH gene

+

Nongenomic Model

GC

GR GC GR

DR

+

Signal Transduction Pathways
•PI3K
•p38 MAPK
•MEK 1/2

Dexras mRNA AAAAAAAA

+

Unknown gene

+

GH gene

+
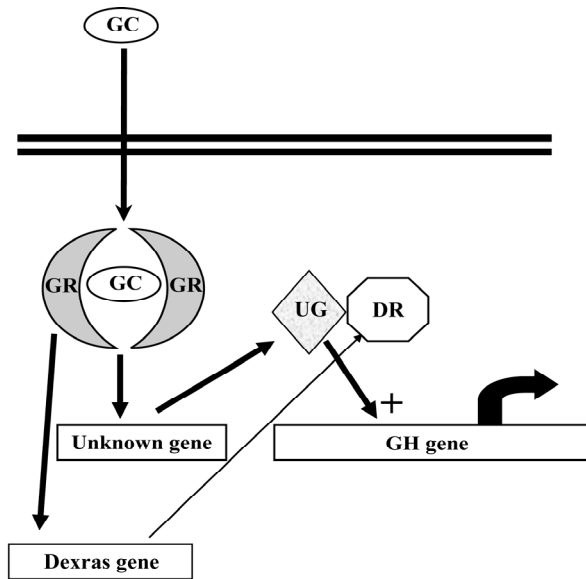
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Figure 4.8. Possible mode of action of Dexras1 on CORT induced GH mRNA expression. Genomic Model (top): Glucocorticoids (GCs) freely diffuse through the cell membrane to bind and activate the cytoplasmic glucocorticoid receptor (GR). The activated GR can then induces the transcription of an unknown gene leading to production of its protein (UG), and the activated GR can then either induce the expression of Dexras1 by binding to cis-elements located in its promoter or activate its expression by some unknown trans-mechanisms. The unknown protein and Dexras1 can then act synergistically to induce GH mRNA. Nongenomic Model (bottom): GCs again freely diffuse through the cell membrane to bind and activate the GR. The activated GR can increase Dexras1 protein levels either through increasing mRNA stability of Dexras1 or activating the Dexras1 protein directly through protein/protein interaction. The activated Dexras1 protein can then initiate signal transduction cascades such as the PI3K, p38 MAPK, and MEK 1/2 pathway which then stimulates the transcription of an unknown gene. The protein product of the unknown gene can subsequently induce GH gene transcription.

It is still unknown at the present time whether it is necessary for the activated GR to bind to the promoter region of Dexras1 in order to stimulate transcription of the gene. To date, only one functional glucocorticoid response element (GRE) has been discovered in a Dexras1 gene. In a study by Kemppainen et al, a GRE was discovered in the 3' flanking region (2.3 kb downstream of poly(A) signal) of the human Dexras1 gene[146]. Based on the present evidence, we can hypothesize that glucocorticoids can stimulate Dexras1 mRNA expression by binding to GREs in cis-elements in the Dexras1 gene or activate it by some unknown trans-mechanism. We have proven that glucocorticoids must be able to interact with the GR in order to induce GH mRNA[128], but one must also consider the possibility that the mechanism by which the GR induces GH mRNA might be independent of its binding to DNA. GREs have been discovered in the rat and human growth hormone gene[95, 201], but to date no functional GRE has been found in the chicken. Present work in our laboratory is currently underway to identify a GRE in the chicken growth hormone gene.

In conclusion, we used a custom chicken cDNA microarray, the Del-Mar 14K Integrated Systems Microarray, to analyze global gene expression in the chicken embryonic pituitary cells and to identify possible candidate genes that are involved in the CORT induction of GH mRNA. We identified 25 direct targets of CORT that were rapidly induced or repressed (within 3 hr) in the presence of CHX. From these possible candidates, we measured the effect of overexpression on GH mRNA of three of these genes (Dexras1, Ras-dva, and Prostaglandin-D Synthase). We found that overexpression of these genes had no direct effect on GH mRNA, but Dexaras1 was able to act synergistically with CORT to induce GH. We are currently cloning the genes of other potential candidates and will determine their effect on GH and other genes as well.

GENERAL DISCUSSION

The aims of this project were simple in design, although the actual execution of it was going to be a major undertaking. We started the construction of our DEL-MAR 14K Integrated Systems microarray back in 2002 as a consortium project, and at that time there were no microarray chips available for chicken. Our hope was to initiate a new era in the functional genomics of poultry by supplying genomic resources [expressed sequence tags (EST) and DNA microarrays] and by investigating global gene expression in various target tissues of chickens. The use of DNA microarrays has been a prolific approach for the discovery of functional genes in several model organisms (i.e., human, mice, frog, zebrafish, fruit fly, etc.), and our current study has again shown the value microarrays.

In the current study we decided to pursue possible candidate genes that might be involved in CORT induction of GH mRNA during chicken embryonic development. In addition, we also tried to analyze global gene expression in response to CORT treatment with the hope of possibly identifying new CORT regulated genes. Without the use of our microarray, this project would have taken a lot longer because we would have had to pursue genes one at a time and given the fact that there is estimated to be ~20,000 – 23,000 genes in the chicken genome, needless to say this project would have taken some time. Also, it would have been very difficult to identify genes that might be involved in various pathways without being able to analyze global gene expression. Some of the directly induced early genes that are presented in table 2 have not been shown to be regulated by glucocorticoids previously These include genes such as Mitogen-activated

protein kinase-activated protein kinase 3, Ras-dva small GTPase, and Semaphorin 7A

The fact we showed have shown these genes to be directly regulated by glucocorticoids can provide new insights and information into their function, how they are regulated, and what cellular pathways/processes they could be involved in in chickens, as well as other species.

We chose to analyze gene expression over a 24 hr culture period. This design would have been all right, but we were also pre-treating cells with a protein synthesis inhibitor (CHX) and the deleterious effects of this inhibitor over a 24 hr span make interpreting the results difficult. Furthermore, we were looking for possible candidate genes involved in GH induction and these genes would have to be significantly up/down-regulated in a short amount of time. In hind site, the culture period should have been limited to 12 hr. In addition, we might have also tried to analyze gene expression in time points earlier than 1.5 hr. For example, we could have substituted the 24 hr time point with a 45 min time point. This earlier time point could have provided some more information on genes that are possibly induced by glucocorticoids through a non-genomic pathway. For example, Dexras1 has been shown to be induced within 15 min in AtT-20 cells, and this time period is probably too short to be the result of the GR binding to DNA and inducing transcription.

Also in hind site, it would have been better to clone those candidates that weren't full length into the Sport 6.1 vector in the beginning. This would have resulted in all of the candidate genes in the same expression vector and would have maintained uniformity

across all of the candidates in the transfection experiments. In the end, this would have saved valuable time.

The clustering method that we decided to use was intriguing, especially the fact that the most promising candidate genes (Dexras1, Ras-dva, and FKBP5) were all in the same cluster. Those who are going to follow my work, or anyone who is going to perform a time-series microarray experiment will benefit highly from this clustering method. Especially if they are using fewer time or data points than we decided to use. When we were using SOMS and hierarchical clustering, the candidate genes that we chose never ended up in the same cluster. SOMS analysis and hiearchical clustering are most likely not the most ideal method to use in a time-series design, although there have been previous time-series microarray studies that have used SOMS and hiearchical clustering. The only limitation with these techniques in a time-series format is that genes that differ in expression levels but behave similarly across the 24 hr culture peroid, are usually put into different clusters. In the format that we used in our study, the expression pattern of the genes outweigh their expression levels. The clustering method that we used is an example of when a very simple method may be more effective than a complicated one. In addition to studying genes involved in somatotroph differentiation, our microarray results and clustering methods will be of use in the examination of genes involved in the differentiation of the other pituitary cell types.

Lastly, the use of a pituitary cell-line would be an invalubable tool in the identification of glucocorticoid-regulated genes invovled in somatotroph differentiation. We have tried

in the past to create one, and obviously we did not succeed. However, the time and effort

that is required to create a cell line would be greatly outweighed by its beneifts. The use

of a cell line would not only save time and money in the long run, but would also remove

the necessity of having to amplify the RNA for microarray analysis or dilute RT samples

in order to be able peform more qRT-PCR analysis. Even though the use of amplfiied

RNA in microarray analysis and diluted RT samples has become a common practice, if

these steps could be eliminated the interpretation of the results would be more direct and

clearer than if these procedures were retained.

In summary, we used a custom chicken cDNA microarray, the Del-Mar 14K

Integrated Systems Microarray, to analyze global gene expression in the chicken

embryonic pituitary cells and to identify possible candidate genes that are involved in the

CORT induction of GH mRNA. We detected 25 direct targets of CORT that were

rapidly induced or repressed (within 3 hr) in the presence of CHX. From these possible

25 candidate genes, we measured the effect of overexpression on GH mRNA of three of

these genes (Dexras1, Ras-dva, and Prostaglandin-D Synthase). We found that

overexpression of these genes had no direct effect on GH mRNA, but Dexaras1 was able

to act synergistically with CORT to induce GH. We are currently cloning the genes of

other potential candidates and will determine their effect on GH and other genes as well.

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