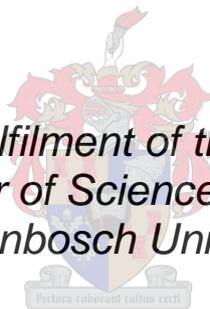


Investigating the Mechanism of Transcriptional Regulation of the Gonadotropin-Releasing Hormone Receptor (GnRHR) gene by Dexamethasone.

by

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Degree of Master of Science (Biochemistry) at
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 18 November 2008

This thesis is dedicated, in loving memory, to my Father Uwe.

ABSTRACT

Gonadotropin-releasing hormone (GnRH) acting through the cognate GnRH receptor (GnRH-R) plays an important role in the regulation of mammalian reproductive function by regulating the synthesis and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The sensitivity of pituitary gonadotropes to GnRH depends on the number of GnRH receptors present on the gonadotrope cell surface. GnRH-R is regulated at a transcriptional, post-transcriptional and post-translational level. Hormones such as GnRH and glucocorticoids (GCs) regulate GnRH-Rs in a time- and dose-dependent manner. Previous studies have shown that the GnRH-R promoter confers glucocorticoid-dependent activation via the activating protein 1 (AP-1) site in the non-gonadotrope GGH₃ cell line. The mechanism by which GCs regulate the GnRH-R promoter is not precisely known as the literature is contradictory. Therefore this study investigates the mechanism of transcriptional regulation of the mouse GnRH-R promoter in the mouse gonadotrope cell line LβT2, treated with the synthetic GC dexamethasone (dex). Assays used include promoter-reporter studies, Western blotting, endogenous mRNA expression studies, electrophoretic mobility shift assay (EMSA) as well as the *in vivo* chromatin immunoprecipitation (ChIP) assay. A transfected promoter-reporter plasmid containing 600 bp of the mouse GnRH-R promoter was used to investigate the effect of dex on transcriptional regulation. Previously it was determined in our laboratory that the GnRH-R promoter is activated via an AP-1 binding site in the LβT2 cell line, and is regulated in a time- and dose-dependent manner by dex. In the present study in the LβT2 cell line a small induction was indeed seen upon dex treatment. Cotransfecting a expression vector for rat GR succeeded in inducing a 2 fold positive dex response. Western blot analysis revealed that GR levels remain consistent even after 8 hours dex induction. The effect of dex on the endogenous GnRH-R gene was investigated by means of real-time RT-PCR. Dex did indeed upregulate the gene in a time-dependant manner. Maximal induction (7.4 fold) was obtained after at least 12 hours of dex treatment. Untreated LβT2 nuclear extracts were investigated using EMSA, for protein binding to the mouse GnRH-R promoter AP-1 binding site, and these proteins were identified as c-Fos and GR. This suggests that the GR interacts with the AP-1 transcription factor via a tethering mechanism to mediate the positive dex response. The results of an *in vivo* ChIP assay were consistent with this hypothesis, showing that the GR interacted with a genomic fragment containing

the AP-1 site, in response to dex. The transactivation of the GnRH-R promoter by means of the GR tethering to AP-1 has not been shown before in the L β T2 cell line.

OPSOMMING

Die voortplantingsfunksies van soogdiere word grotendeels deur die gonadotropien-vrystellingshormoon (GnVH) gereguleer. GnVH se werking word bemiddel deur die gonadotropien-vrystellingshormoon reseptoor (GnVH-R) wat die sintese en vrystelling van follikelstimuleringshormoon (FSH) en luteniseringshormoon (LH) reguleer. Die aantal GnVH reseptore op die pituitêre gonadotroopsellyn oppervlak bepaal die sensitiwiteit vir GnVH. GnVH-Re word gereguleer op n transkripsionele-, post-transkripsionele- en post-translasievlak. Hormone, insluitende GnVH en glukokortikoïede (GK), reguleer die GnVH-R vlakke op 'n tyd en dosis afhanklike wyse. Vorige studies in die nie-gonadotroopsellyn GGH₃ toon aan dat GK aktiveering van die GnVH-R deur die aktivering proteïen 1 (AP-1) bemiddel word. Die meganisme van hierdie regulasie is nie bekend en die literatuur is teenstrydig. Hierdie studie ondersoek dus die meganisme van transkripsie waarby die sintetiese GK, dexamethasone (dex) 'n muis GnVH-R promotor in die muis gonadotroopsellyn L β T2 reguleer. Tegnieke gebruik sluit in promotor-rapporteurder-studies, Western-klad, endogene mRNA uitdrukking-studies, EMSA om *in vitro* proteïen-DNS-bindingstudies te ondersoek en *in vivo* ChIP essays. Die getransfekteerde promotor-rapporteurderkonstruk wat die 600 bp muis GnVH-R promoter fragment insluit is gebruik om die dex regulasie van transkripsie te ondersoek. Daar is voorheen in ons laboratorium bepaal dat die dex aktivering van die muis GnVH-R promotor deur die AP-1 bemiddel word in die L β T2 sellyn; en die regulasie is tyd en dosis afhanklik. 'n Klein positiewe dex respons was inderdaad gevind in die huidige studie in die L β T2 sellyn. Deur rot GK reseptoor (GR) in te sluit by die transfekteerde promotor-rapporteurderkonstruk is 'n positiewe respons van 2 foud verkry. Western-klad het getoon dat GR proteïen vlakke onveranderd bly selfs na 8 ure dex behandeling. Daarna is die effek van dex op die endogene GnVH-R ondersoek met behulp van intydse RT-PCR. Dex het die geen in n tyd afhanklike manier opgereguleer. Die maksimale opregulasie (7.4 foud) was na ten minste 12 ure van dex stimulasie waargeneem. Onbehandelde L β T2 selkern ekstrakte was gebruik om proteïen binding aan AP-1 DNS fragmente te ondersoek met behulp van EMSA. Proteïenbinding het inderdaad plaasgevind aan die AP-1 binding setel van die muis GnVH-R promotor en die proteïene was as c-Fos en GR geïdentifiseer. Die positiewe dex respons word moontlik bemiddel deur 'n GR-AP-1 interaksie. Die resultate van die *in vivo* ChIP essai, waar die GR interaksie toon met n genoom fragment wat die AP-1 setel

bevat, met dex regulasie, ondersteun die hipotese. Opregulering van die GnVH-R geen deur middel van die direkte interaksie tussen die GR en AP-1 is nog nie vantevore in die L β T2 sellyn gewys nie.

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AF	N-terminal transactivation domain
ANOVA	analysis of variance
AP-1	Activator Protein-1
ATP	adenosine triphosphate
AVP	arginine vasopressin
BTF	basal transcriptional factors
bp	base-pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CG	chorionic gonadotropin
ChIP	chromatin immunoprecipitation
CP	crossing point
CRH	corticotropin-releasing hormone
CRE	cAMP response element
CREB	cAMP response element binding protein
CT-FCS	charcoal treated FCS
DAG	diacylglycerol
DARE	down-stream activin regulatory element
DBD	DNA-binding domain
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
E	efficiency
E ₂	estradiol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid

EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FCS	fetal calf serum
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GDP	guanosine diphosphate
GRAS	GnRHR-activating sequence
GRE	glucocorticoid response element
GSE	gonadotrope-specific element
GTM	general transcription machinery
GTP	guanosine triphosphate
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRIP	GR interacting protein
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
HAT	histone acetylase
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
hsp	heat shock proteins
IgG	immunoglobulin G
LBD	ligand binding domain
IP ³	inositol-(1,4,5)-triphosphate
JNK	c-Jun N-terminal kinase
kb	kilobasepair
LB	Luria-Bertani
LBD	ligand binding domain

LH	luteinizing hormone
LUC	luciferase
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
M-MLV	Moloney murine leukemia virus
MMTV	mouse mammary tumour virus
mRNA	messenger ribonucleic acid
NF-Y	nuclear factor-Y
nGRE	negative GRE
NRE	nuclear response element
NTC	no template control
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PIP ²	phosphatidylinositol 4,5-bisphosphate
PKA	protein Kinase A
PKC	protein Kinase C
PLC	phospholipase C
POMC	pro-opiomelanincortin
R	relative response
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SF-1	Steroidogenic Factor-1
SRC	steroid receptor coactivator
STAT	signal transduction-activated transcription factor
SURG	sequence underlying responsiveness to GnRH
TAE	tris-acetate EDTA
TBS	tris-buffered saline

TE	tris-EDTA
TM	transmembrane domain
T _m	melting temperature
TRAF	TNF receptor associated factor
UTR	untranslated region

CHAPTER 1

LITERATURE REVIEW

1. HPG-HPA integrated

Mammalian reproductive function is controlled by the hypothalamo-pituitary-gonadal (HPG) axis (Figure 1) (1). The pulsatile release of GnRH from the hypothalamus acts via the GnRH-R situated on the surface of gonadotrope cells, found in the anterior pituitary. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized and secreted from these cells and stimulate the gonads to produce sex steroids. These hormones are vital for sexual maturation and sexual function in mammals. The hypothalamo-pituitary-adrenal (HPA) axis (Figure 1) is important for maintaining stress responses in mammals. The hypothalamus is induced by a stressor to release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). This induces the synthesis of pro-opiomelanocortin (POMC), the precursor of adrenocorticotrophic hormone (ACTH) in anterior pituitary corticotrophs (2). ACTH acts on the adrenal cortex, stimulating the synthesis and release of glucocorticoids (GC).

Both the sex steroids and GCs feed back on the pathways by which they are induced. GCs have a negative feedback (Figure 1) on the production of CRF, AVP in the hypothalamus and POMC and ACTH production in the pituitary corticotrophs (Reviewed in 1). The hormones have been shown to have either a negative or positive feedback on reproductive function (see sections 6 and 11 on steroid regulation of GnRH and GnRH-R). Sex steroids exert either positive or negative feedback on the HPG axis at the hypothalamic or pituitary level (1). Crosstalk between the HPA and HPG axis occur when GCs act on the GnRH-R found on pituitary gonadotropes to modulate the receptor levels (1).

Although the regulatory effects of GCs on reproduction have ranged from induction, repression or no effect at all (3), GCs were found to down regulate the transcriptional activity of the GnRH-R promoter in the hypothalamic derived cell line, GT1 (4). Since these receptors are present in

hypothalamic and pituitary cells (1, 4, 5, 6) regulation of GnRH-R expression by GC's may occur at both the hypothalamic and pituitary level.

GC receptors (GR) require ligand binding for the activation of the receptor (7) The identification of GR expressing neurons and pituitary cells in rainbow trout strongly suggest a neuroendocrine role for the GR in reproduction (8). GCs are important in physiological development, cellular proliferation as well as differentiation. These hormones have a widespread pharmacological application in the treatment of conditions such as rheumatoid arthritis and asthma to name but a few (9).

1.1. The effects of stress on reproduction

The effect of stress hormones on reproductive function has been studied extensively. Stressors reduce gonadotropin secretion in combination with the activation of the HPA axis and is mediated by GCs (3,10). GCs have inhibitory and stimulatory effects on the hypothalamic and pituitary sites of action involved in reproduction (1,11). This includes reducing GnRH pulse generating activity (16) and synthesis in the hypothalamus (15).

The synthetic GC, dexamethasone was found to suppress gonadotropin secretion by reducing GnRH secretion as well as dissociating gonadotropin receptor responsiveness to GnRH (12) at both the pituitary and hypothalamic level (13). Hypothalamic neurones secreting GnRH were found to contain GR, which could account for the negative regulation of dex on the HPA axis (4). Furthermore, dex was found to repress the endogenous mouse GnRH gene by decreasing mRNA levels (12). Acute stress induced by either dex or cortisol was found to stimulate reproduction in animal models and cell cultures (Reviewed in 3). Repeated acute stress was found to suppress reproduction in various animal models (Reviewed in 3).

Chronic stress is believed to suppress reproduction, through actions mediated at the hypothalamus or pituitary, by suppressing gonadotropin secretion (3,14). Acute stress was found to enhance reproductive function (3). High GC levels of cortisol in ovariectomized ewes (10), wethers (15) and women (16) suppressed pituitary responsiveness to GnRH and decreased LH secretion.

Furthermore the GC, corticosterone augmented the inhibitory effect of testosterone and reduced estradiol induced LH secretion (17) In contrast, cortisol increased FSH β mRNA and LH and FSH concentrations in pituitary tissues (15) yet the GnRH responsiveness was diminished (16).

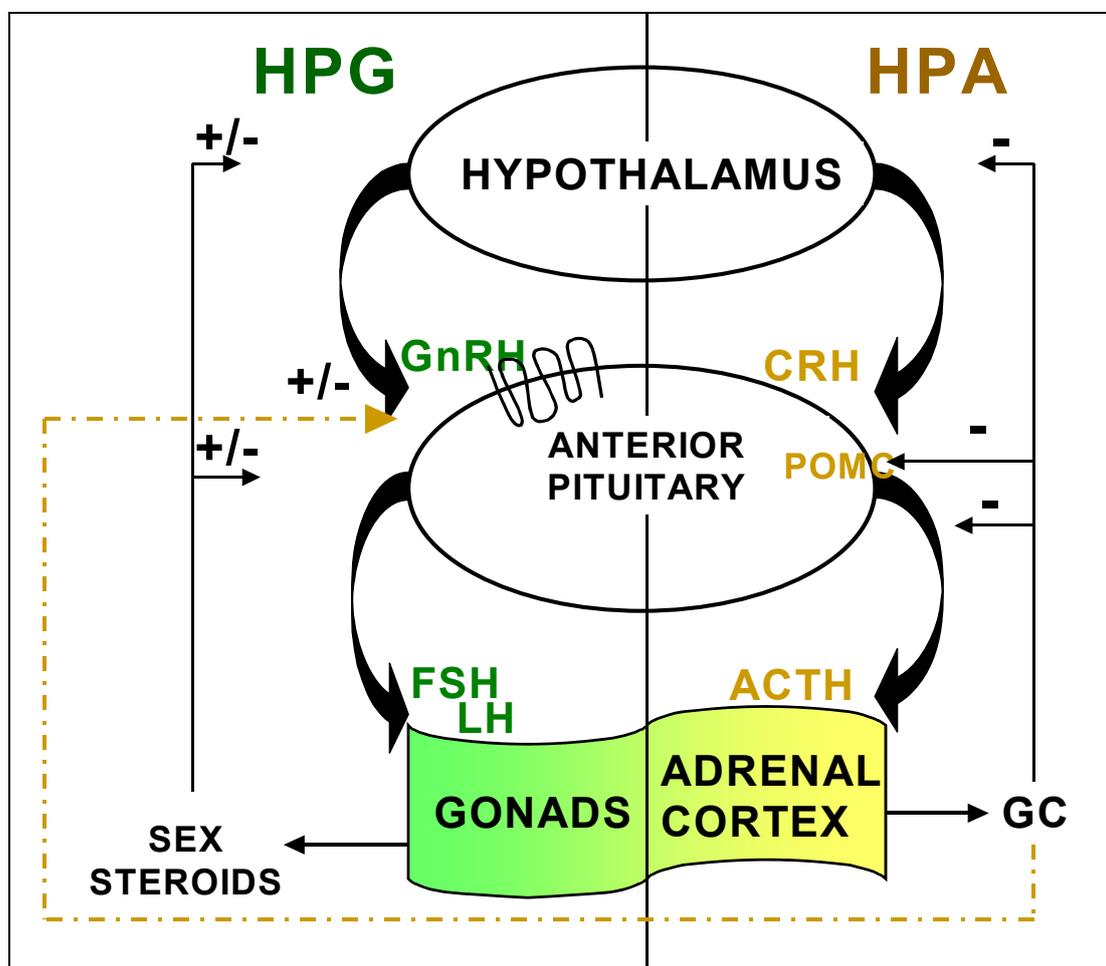


Figure 1. The HPG-HPA axes integrated. Left-hand side: The hypothalamo-pituitary gonadal (HPG) axis involves the pulsatile release of GnRH from the hypothalamus, acting via the GnRH-R to induce the synthesis and release of FSH and LH from anterior pituitary gonadotropes. This regulates the release of sex steroids from the gonads. Right hand side: the hypothalamo-pituitary-adrenal (HPA) axis involves a stressor activating the release of CRH from the hypothalamus, which induces the release of POMC from corticotrophs in the anterior pituitary, a precursor for ACTH. ACTH stimulates the synthesis and release of GCs from the adrenal cortex. The feedback mechanism of the sex steroids and GCs are shown by the arrows (+ indicates upregulation and - repression) Crosstalk between the HPA and HPG axis is shown by the dashed line. (1,18). Figure by S von Boetticher as adapted from Tilbrook, AJ *et al* (1) and Newton, R (18).

In summary chronic stress was found to consistently suppress reproduction usually by down regulating gonadotropin secretion, whereas acute stress resulted in either an suppression or up regulation of reproduction and is usually mediated by altered gonadotropin secretion (1, 3). These

responses as well as the mechanisms, by which they occur, vary according to the species, sex of the animal and sex steroids (1).

2. The glucocorticoid receptor (GR)

The numerous regulatory functions of GCs are mediated by the GR, a member of the nuclear receptor family (19). Transcription factors included in this family are the mineralocorticoid, progesterone, androgen and estrogen receptors amongst others (20). Nuclear receptors bind as homo- or heterodimers to cognate DNA binding elements to act as ligand dependent transcription factors (21). The GR has been shown to both induce and repress gene expression (22).

2.1. GR Structure

The modular GR contains three domains as shown in figure 2 (Reviewed in 23). The variable N-terminal transactivation domain contains the AF-1 transcriptional activation domain, needed for transcriptional enhancement (24) and for association with basal transcriptional factors (BTF). The central DNA-binding domain (DBD) that consists of two conserved zinc fingers (20) needed for dimerisation, target site binding, transcriptional activation and repression. The conserved C-terminal ligand-binding domain (LBD) is a binding site for hormones, chaperone proteins and coactivators and contains the ligand-dependent activation function domain (AF-2) (20). The C-terminal domain distinguishes between ligands and determines agonist and antagonist transactivation (25). The transcriptional activity of AF-2 is dependent on GC activation whereas AF-1 can act independent of GC activation (19). Post-translational modifications such as phosphorylation and ubiquitination modulate the receptor stability and function (26).

2.2. Transcriptional regulation by the GR

GCs are able to passively diffuse through the plasma membrane due to their lipophilic nature, and bind to the GR. The GR is found in an inactive conformation in the cytoplasm, bound to chaperone proteins such as heat shock proteins 90 (hsp90), hsp 70, hsp56, hsp40, phosphoprotein p23 and immunophilins (Figure 3) (20,23,26). The GR undergoes a conformational change upon ligand activation and dissociates from the multimeric complex proteins (23,26). Nuclear translocation

proteins shuttle the GR into the nucleus (26), where acting as an activated transcription factor the GR modulates transcriptional responses by binding to cis-acting DNA sequences, such as glucocorticoid-response element (GREs) to activate transcription (transactivation), and negative glucocorticoid-response element (nGREs) to repress transcription (transrepression); or by means of protein-protein interactions with transcription factors such as NF- κ B, AP-1 and Smad and signal transduction and activator of transcription (STAT) as illustrated in figure 3 (20,23,26). GR regulation depends on the specific cellular mechanisms of the promoter or cell type (19), which in turn alters the activity of the general transcription machinery (GTM) (23). In the present study dex transactivates through mechanism previously described for transrepression and for this reason section 2.2.2 will be discussed in more detail.

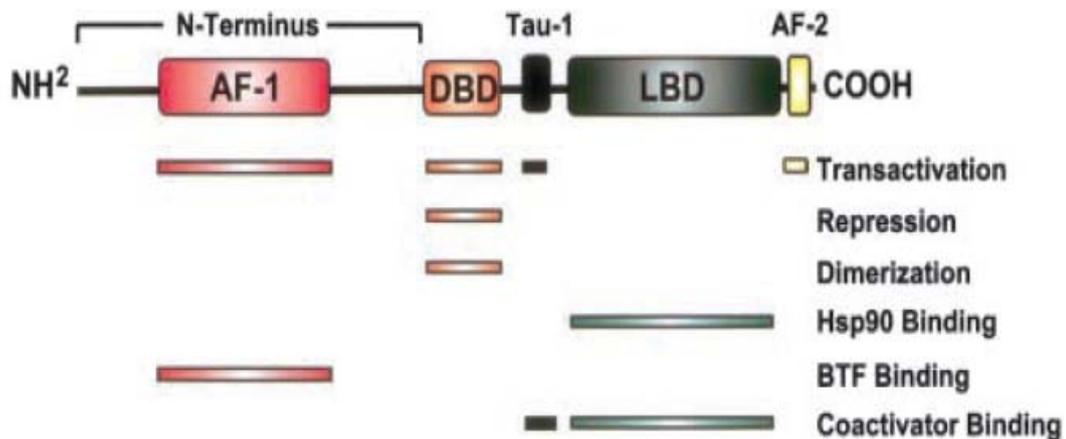


Figure 2. Three domains making up the glucocorticoid receptor. The N-terminal transactivation domain (AF-1), DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD) are shown above. Horizontal lines indicate the domain involved in the GR function such as the basal transcriptional factors (BTF). Figure taken from Necela, BM *et al* (23).

2.2.1. Transactivation

The GR modulates transactivation as shown in figure 4, A to C (Reviewed in 18). In figure 4 A the GR dimer binds to a classical GRE as a homodimer. The consensus palindromic GRE sequence is 5'-GGTACAnnnTGTCT-3', where n can be any nucleotide. The GR can bind a composite GRE to interact with a transcription factor as shown in figure 4 B. The interaction of GR with transcription factors such as STAT or Smad has been found to enhance GRE transcriptional activation (20). The GR undergoes a conformational change once bound to the GRE and coactivator recruitment is promoted. These include GR interacting protein 1 (GRIP-1), CBP/p300, P/CAF and steroid receptor

coactivator 1 (SRC-1) (27). The cofactors have histone acetylase (HAT) activity or mediate histone-modifying enzymes (28), important for chromatin remodelling allowing basal transcription machinery access to the promoter (16,20,24). Lastly, as shown in figure 4 C, the GR can tether to a transcription factor to upregulate transcription without direct DNA binding.

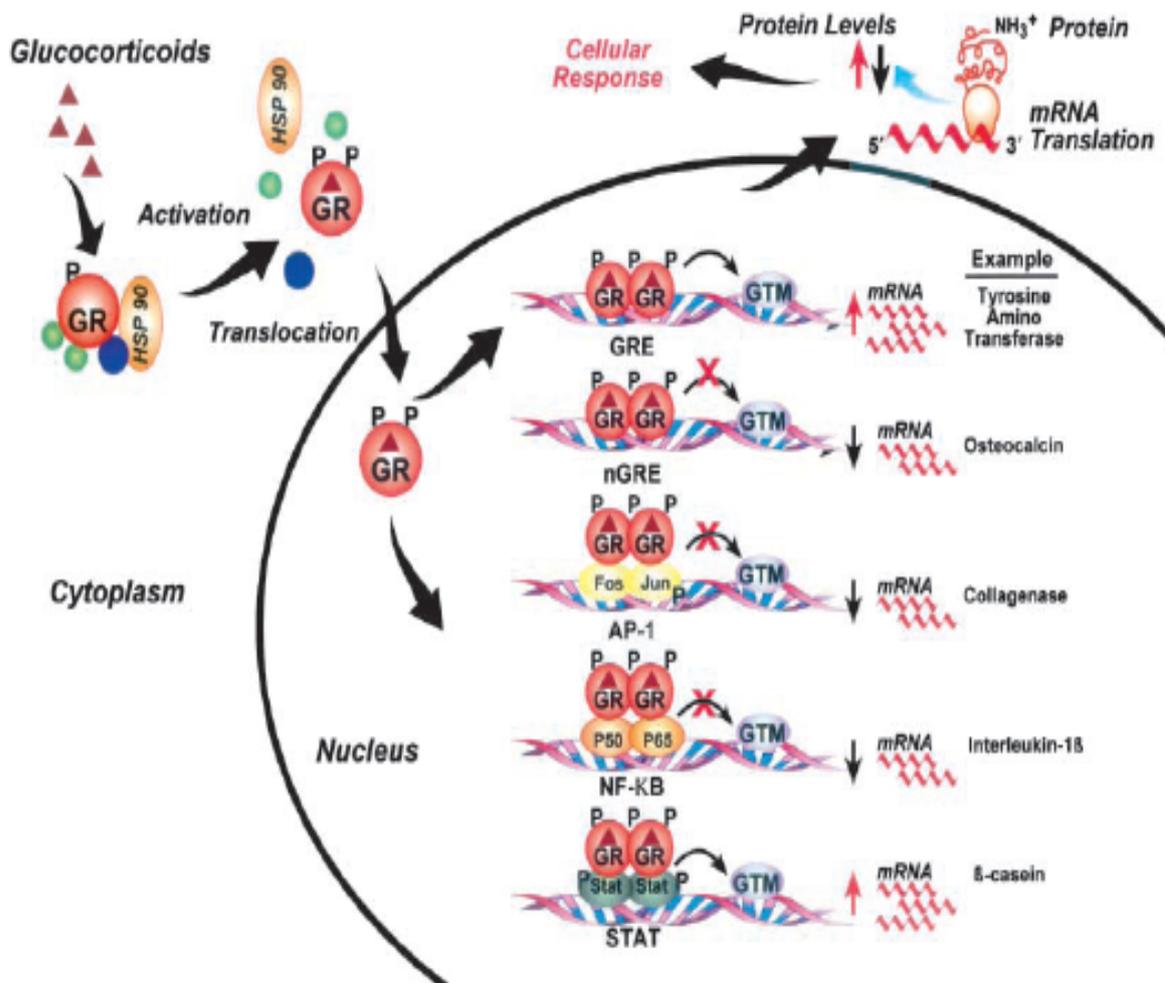


Figure 3. Mechanisms of transcriptional regulation by the GR. Cytosolic GR dissociate from chaperone proteins upon ligand binding (activation) and translocates into the nucleus. The dimerized GR modulates gene transcription by interacting directly with *cis*-elements or transcription factors. This interaction alters the gene transcription by altering the regulation of the general transcription machinery (GTM), which in turn regulates translation and the subsequent cellular response. Examples of genes regulated by the GR are shown (Reviewed in 23). Figure taken from Necela, BM *et al* (23).

2.2.2. Transrepression

The GR modulates transrepression as shown in figure 4, D to G (Reviewed in 18). The GR dimer can bind to a nGRE as represented by figure 4, D. The POMC gene transcription was repressed in such a manner when GR multimers bound to a nGRE. The sequences of nGREs are variable and

therefore the mechanism of repression can vary. One possibility is preventing basal transcriptional machinery from binding if the transcription factor binding sites overlap with that of the nGRE (20). The GR may compete for binding with transcription factors as shown by figure 4, E. An example of this is the promoter of the glycoprotein hormone α -subunit that contain overlapping binding sites for the cAMP response element binding protein (CREB) and the GR. GR binding is proposed to inhibit CREB binding thereby inhibiting transcriptional activation (18). Tethering of the GR to transcription factors can result in the repression of a gene without direct binding of the GR to DNA and is represented by figure 4, F. This regulatory mechanism has been characterized for nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (18,20). The interaction of DNA bound GR and transcription factors may result in transrepression of a gene as is shown by figure 4, G. The proliferin gene promoter contains a GRE and AP-1 binding site adjacent to one another. The GR does not influence AP-1 DNA binding and is therefore thought to mediate the repressive action of the AP-1 by affecting its ability to activate transcription (18,20).

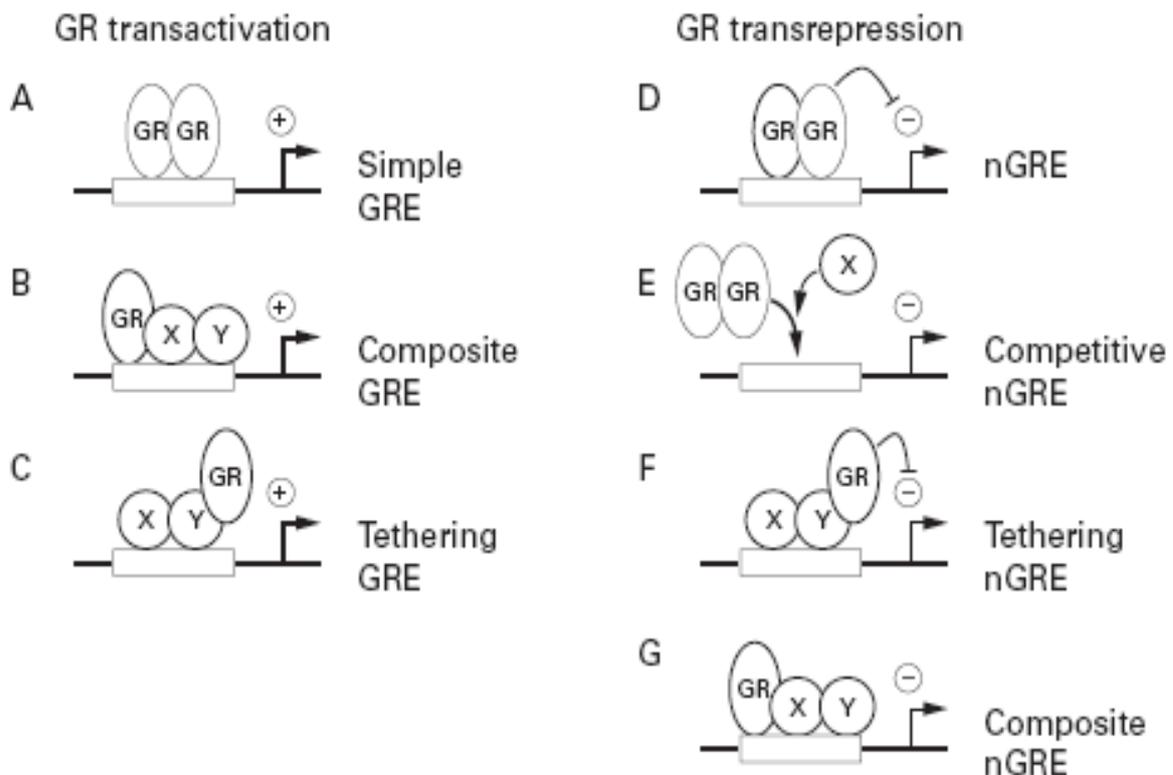


Figure 4. Models by which the GR modulates transcriptional regulation. Figures A to C represent transactivation by the GR whereas figures D to G show transrepression. For more detail refer to section 3.2.1 and 3.2.2. Figure taken from Newton, R (18).

3. AP-1 transcription factor

The AP-1 consensus binding site is 5'-TGAG/CTCA-3' (29,30). AP-1 is a member of the basic region-leucine zipper family of DNA binding proteins (29,31). Two families of proteins make up the AP-1 transcription factor (Figure 5). The components of these transcription factors are encoded by c-fos and c-jun proto-oncogenes (32). Fos proteins include: Fos, c-Fos, Fra-1, Fra-2 and Fos-B while the Jun proteins are: Jun, c-Jun, Jun-B and Jun-D (29,33). Most genes of the AP-1 components are rapidly induced by almost any possible external stimuli (Reviewed in 34).

AP-1 dimerisation is the result of a leucine zipper formation between the two subunits and this in turn mediates DNA binding (33,34). Fos homodimers are unstable (33) and must dimerize with a Jun protein in order to bind DNA (29,34). The subunit composition of AP-1 determines the degree of binding activity, where Jun-Fos heterodimers having higher DNA binding activity than the combination of the other subunits (20, 33, 34). Fos is the only subunit that contains a transactivation domain and only heterodimers containing Fos can therefore activate gene transcription (23,31,34) and it is this characteristic rather than DNA binding that determines AP-1 mediated regulation (Reviewed in 34).

Jun Proteins	Fos-Related Antigens
	(FRA)
c-Jun	c-Fos
Jun B	Fos B
Jun D	FRA-1 and FRA-2

Figure 5. Jun and Fos proteins making up the AP-1 family of regulatory proteins. The consensus binding sequence for AP-1 proteins is: 5'-TGA~~CT~~CA-3'. Figure reproduced from Pennypacker, KR *et al* (29).

3.1. AP-1 activation

AP-1 subunit activity is regulated by transcriptional and posttranslational modifications (Reviewed in 34, 35). Activation occurs by means of a signal transduction cascade: The mitogen-activated

protein kinase superfamily MAPK protein family member, c-Jun N-terminal kinase (JNK), is primarily stimulated by cytokines such as TNF- α and IL-1 β , but cross talk with other MAPK pathways can occur (20,31). c-Jun is phosphorylated by JNK, becomes activated, and is able to dimerize with c-Fos (20,23)

3.2. AP-1 and GCs

AP-1 oncogene transcription is under GC regulation (20,22,36) and this further determines the AP-1 composition as was shown in anterior pituitary corticotrophs, AtT-20, treated with dex (37). The GR and Jun reciprocally repress one another by protein-protein interactions involving the GR ligand binding domain (LBD) and c-Jun leucine zipper (22,38). Tuckermann *et al* (39) also found AP-1 activity was repressed *in vivo* by the GR. High doses of dex had no effect on the basal c-Fos mRNA expression. However, c-Jun was transiently down regulated (37).

The GR modulates AP-1 activated gene expression in the absence of a GRE by interacting with Jun homodimers (40). Furthermore c-Jun was found to disrupt GR-GRE complexes in *in vitro* studies (38). König *et al* (43) found that the GR was not able to interfere with the DNA binding of Jun homodimers, but Kerppola *et al* (42) found the GR DNA binding domain was able to inhibit the Fos/Jun heterodimer from binding to the AP-1 element.

The cell context plays an important role in determining the type of regulation. An example of this is the mouse proliferin gene that is either upregulated by GCs in the presence of only c-Jun compared to repression when c-Jun and high levels of c-Fos is present via a GRE element in the gene promoter (41,42). Dex mediated receptor activation of a proliferin gene promoter fragment (41) occurred only in the presence of Jun (homodimers). The promoter activation by the Fos/Jun heterodimer was suppressed upon dex treatment (41).

Fos and Jun proteins mediate the type of regulation the GR exerts at the AP-1 binding site (41,42). The GR preferentially interacts with Fos monomers before Fos/Jun heterodimers and Jun homodimers (42,43) Fos monomers cannot bind DNA, therefore Jun homodimers or Fos/Jun heterodimers are responsible for transcriptional regulation (29,34). The bifunctional role of Jun in

either activation or repression of transcription could therefore be selected for by c-Fos (44). It is clear from the studies that the GR, Fos and Jun interact on various levels to regulate the gene transcription, their activity as well as the type of regulation they exert at a promoter level.

4. G protein coupled receptors (GPCR)

The superfamily of rhodopsin GPCRs is characterized by an amino terminal followed by 7 α -helical transmembrane domains, with 3 extracellular and 3 intracellular loop domains and a carboxyl-terminal domain (Figure 6) (45,46). The GnRH-R falls under this grouping (47). The extracellular domains and external areas of the transmembrane domains are mostly involved in binding of peptide hormones whereas the transmembrane domains are involved in receptor configuration and activation. Conformational changes of the intracellular domains allow for G-protein interaction involved in signal transduction (45). GPCRs are phosphorylated by G-protein receptor kinases, second messenger-regulated kinases, such as protein kinase A (PKA) or PKC or casein kinases (47). Activation of the GPCR also leads to desensitisation and internalisation of the receptor. β -Arrestins mediate desensitisation, downregulation and internalisation of GPCRs (47). The action of β -arrestin requires the association with phosphorylated GPCR sites located within the C-terminal tail of the receptor. Mammalian receptors lack this C-terminal tail and therefore internalise in an β -arrestin independent manner (47).

4.1. Signalling via GPCRs

GPCR activation is mediated through heterotrimeric proteins (48), GTP-binding proteins (G proteins), consisting of an α -subunit ($G\alpha$) and a β - and γ -subunit ($G\beta\gamma$) (48). Upon receptor activation $G\alpha$ dissociates from the heterotrimeric complex and GDP is replaced with GTP. Membrane bound phosphatidylinositol 4,5-bisphosphate (PIP^2) is hydrolysed to inositol-(1,4,5)-trisphosphate (IP^3) and diacylglycerol (DAG) (45,49). Intracellular calcium is also increased (45). Although the $G\beta\gamma$ dimer is still attached to the plasma membrane, it is able to initiate downstream signalling, as does $G\alpha$ (50). GTPase hydrolysis of GTP to GDP allows the $G\alpha$ - $G\beta\gamma$ complex to re-

associate, inactivating the receptor (45). Figure 7 illustrates the GPCR: GnRH-R signal transduction cascade.

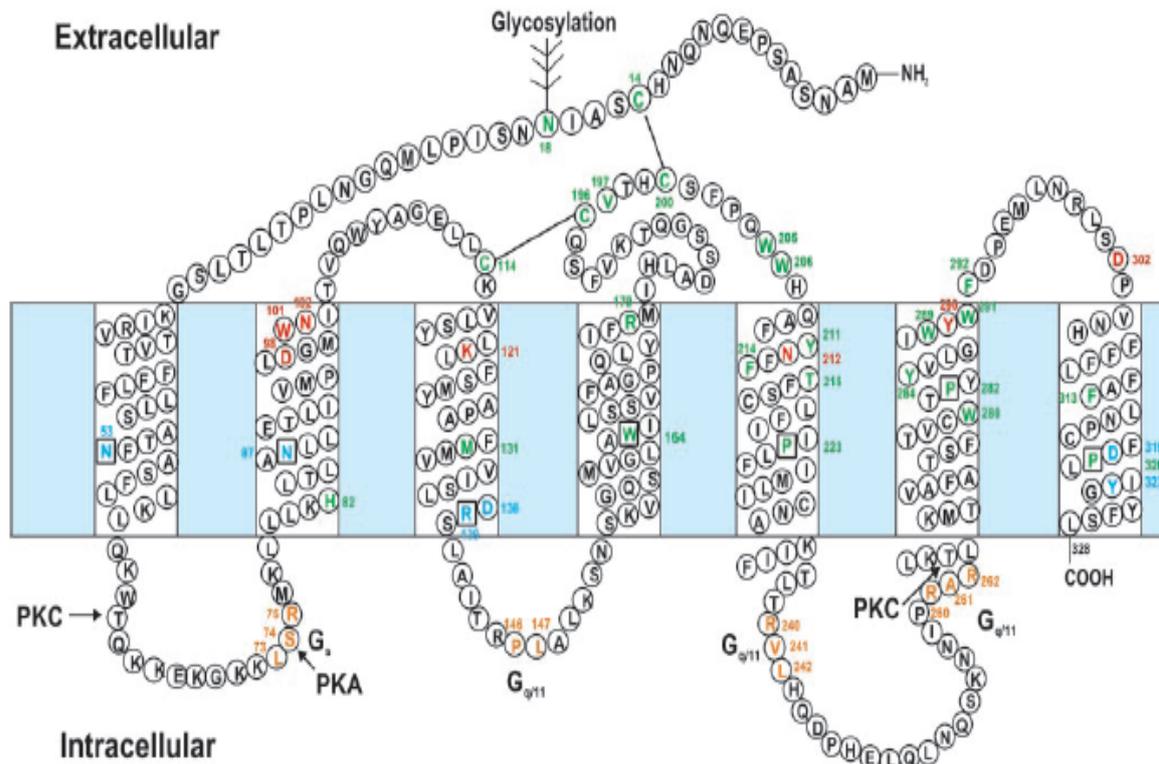


Figure 6. Two-dimensional structure of the human GnRH-R. Transmembrane domains are boxed and connected by extracellular and intracellular domains. Red denotes the ligand binding sites whereas green indicates residues thought to be important for the receptor structure or the formation of a binding pocket. Residues shown in blue are involved in receptor activation. Residues in squares are highly conserved amongst GPCRs. Orange residues are involved in coupling to G proteins. The phosphorylation sites for PKC and PKA are indicated by arrows. Figure taken from Millar, RP *et al* (45).

4.2. Gonadotropin releasing hormone receptor (GnRH-R)

Many GnRH and receptor isoforms have been isolated from different vertebrates, with most having at least three receptor isoforms (45). In humans GnRH I and its receptor, GnRH-R I are functionally expressed yet the GnRH-R II gene remains transcriptionally active and may play a neuromodulatory role and possibly regulate GnRH-R I expression (45,51,52). GnRH II and GnRH-R II have been identified in primates (rhesus monkey) and non-primates (pig) (47,53, Reviewed in 54) but are completely absent from the mouse genome (53). GnRH III is only functional in teleosts (45,52) whereas human and bullfrog GnRH-R III splice variants have shown an inhibitory effect on GnRH-R function (51). For simplicity only the type I GnRH and GnRH-R will be discussed.

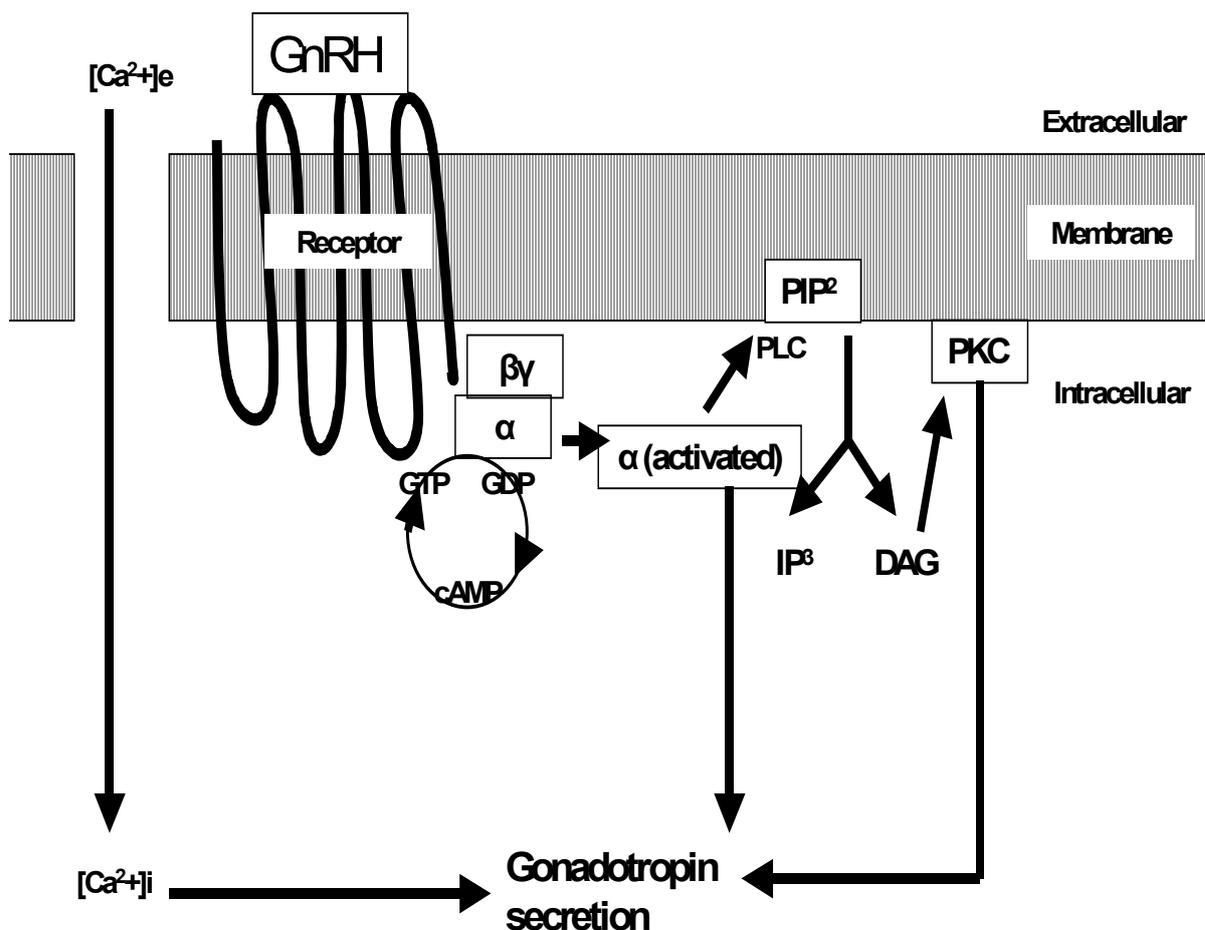


Figure 7. GnRH-induced signal transduction of the GnRH-R GPCR, in gonadotropes. The GnRH-R is activated upon GnRH binding. The receptor in turn stimulated the G-proteins ($\alpha\beta\gamma$), which stimulate PLC hydrolysis of PIP^2 to IP^3 and DAG. DAG activates PKC and this can stimulate gonadotropin secretion. Intracellular calcium levels are also increased by GnRH and also result in gonadotropin secretion. Figure adapted from Hawes, BE *et al* (49).

Various mammalian GnRH-R genes have been characterized including that of the mouse and human (Reviewed in 54). The mouse GnRH-R, cloned into the pituitary α T3-1 gonadotrope cell line was the first receptor of which the amino acid sequence was deduced (55). The 327-amino acid protein displayed the GPCR characteristic seven transmembrane domains, but lacks the C-terminal tail (47). All vertebrate GnRH receptors lack this C-terminal tail as can be seen from the human GnRH-R in figure 6 (45,47,55). This gene exists as a single copy containing three exons (46), separated by two introns (56) with high sequence and structural homology amongst species (Figure 8). Variability is seen in intron size and the 5' and 3' untranslated region (UTR) sequence and length. The GnRH-R exon 1 encodes the N-terminal tail and transmembrane domain (TM)

1,2,3 and part of TM 4. Exon 2 encodes the remainder of TM 4 and TM 5. TM 6 and 7 is encoded by exon 3 (47, 53).

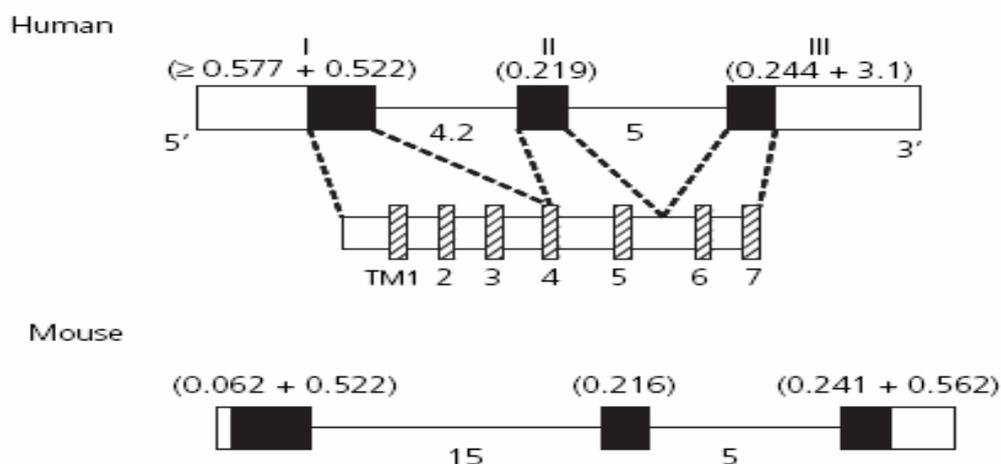


Figure 8. The human and mouse GnRH-R gene structure. Blocks indicate exons, with coding regions in black. Untranslated regions (UTR) are shown as white blocks. Exon size is shown as the sum of the noncoding and coding portion in kilobasepairs. Solid lines represent introns, with size shown below. Figures (not drawn to scale) were taken from Hapgood, JP *et al* (54).

5. Gonadotropin releasing hormone (GnRH)

The primary endocrine regulator of mammalian reproduction is the GnRH that is synthesized by the mediobasal hypothalamus neurons (16) and is released from the median eminence (45) in a pulsatile manner (57) from where it travels via the hypothalamo-hypophyseal portal to the anterior pituitary. The hormone binds to its cognate receptor, GnRH-R, on cells within the pituitary termed gonadotropes to stimulate the synthesis and release of FSH and LH (48,52,53,57). FSH and LH in turn regulate gonadal steroidogenesis and gametogenesis (46,52). Figure 10 shows the genomic structure of the human GnRH gene. The gene consists of 4 exons and 3 introns as described in the figure legend (46). The primary amino acid sequences for the GnRH decapeptide found in various species are shown in figure 9 (45,46,47). The amino- and carboxyl-termini are indicated in the grey boxes. Sequence conservation within the GnRH is important for receptor binding and activation of the GnRH-R. The residue variation at position 8 influences the ligand selectivity of the receptor (45). GnRH isoforms in other species have neuroendocrine, paracrine, autocrine and neuromodulatory roles in the central and peripheral nervous system compared to the mammalian

GnRH that plays a primary role in the regulation of LH and FSH (46,47). GnRH distribution is found primarily in brain tissue (46). The GnRH amino- and carboxyl-termini are important for receptor binding, but only the carboxyl-terminal is involved in receptor activation (45).

	1	2	3	4	5	6	7	8	9	10	
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH ₂
Guinea Pig	pGlu	Tyr	Tyr	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH ₂
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH ₂
Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH ₂
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH ₂
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH ₂
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH ₂
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH ₂
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH ₂
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH ₂
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH ₂
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH ₂
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH ₂
Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH ₂
Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH ₂
Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH ₂
Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH ₂
Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH ₂
Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH ₂
Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH ₂
Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH ₂
Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH ₂

Figure 9. Amino acid sequence of GnRH structural variants. Amino- and carboxyl-terminal amino acids, highlighted in the grey boxes display sequence conservation. The hormones are named according to the species in which it was first discovered, but may be present in more than one species. Figure taken from Millar, RP *et al* (45).

6. Steroidal regulation of GnRH

Steroids differentially regulate GnRH: Chorionic gonadotropin (CG) down-regulate GnRH-I in a dose dependent manner in humans (46). Estradiol was found to regulate gonadotropin production

at the translational level, the type of regulation being dependent on the concentration and duration of estradiol administered (58). Estrogen acts via a negative feedback loop at the hypothalamic level (58) to alter the secretion of GnRH I and LH (59) whereas the positive feedback regulation acts at pituitary levels (58). Furthermore 17β -estradiol (E_2) down-regulates the steady-state GnRH-I mRNA levels in various cell types and is believed to do so via the estrogen receptor (ER). E_2 was also shown to repress mRNA expression and promoter activity of GnRH in a mouse cell line (46). Androgens regulate the GnRH (46) and LH (17) transcription by acting at the hypothalamic level to slow the GnRH pulse frequency (58). Progesterone is capable of regulating the hypothalamic-pituitary functions thereby inhibiting the pulsatile secretion of GnRH and LH (58,59).

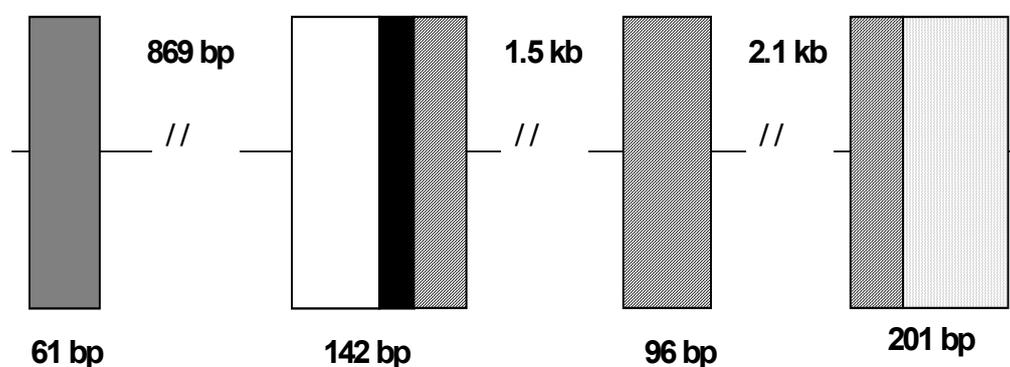


Figure 10. Genomic structure of the human GnRH gene. Exons are indicated by boxes and introns by the lines. Genes are organized in the following manner: the signal sequence (white box) and the GnRH decapeptide (Black box), GAP (striped box) and the untranslated regions (UTR) in the grey and dotted boxes. Figure adapted from Cheng, CK *et al* (46).

7. Expression and regulation of the GnRH receptor

The GnRH-R is highly expressed in gonadotropin cells found in the anterior-pituitary (60) but transcripts of the receptor and hormone isoforms have been found in various mammalian tissues (Reviewed in 54).

Receptor expression in extrapituitary tissue is low with binding sites having lower affinity for GnRH (61). Extrapituitary tissue where the receptor is expressed includes reproductive, non-reproductive as well as cancerous tissues: GnRH-R has been detected in human breast tissue, the ovary, endometrial tissue, placental trophoblasts, cytotrophoblasts and syncytiotrophoblasts (Reviewed in 54).

GnRH and GnRH-R is also expressed in the testes and prostate (62) and plays a role in testis and sperm development and function (63,64). GnRH receptors are thought to have autocrine and paracrine roles in the non-reproductive tissues in which they occur, such as hypothalamic neurons (6). Furthermore it has been suggested that neuronal differentiation and migration may be in part regulated by GnRH receptors (5) and the presence in mammalian brain tissue and T-cells, indicate a possible neuromodulatory and immunomodulatory (65) role respectively (Reviewed in 54).

GnRH and its receptor have been found in breast, endometrium, prostate and ovary cancers that are gonadal steroid dependent (47,53,62,66). The GnRH-R found in cancer cells has a lowered affinity for agonists compared to those found in the pituitary (47), suggesting higher GnRH levels are required to exert an response and in some tumour cells GnRH is indeed overexpressed (46). GnRH analogues show antiproliferative effects when administered to cancer cells (47, 54).

8. Model systems to investigate GnRH-R promoter transcriptional regulation

Specific cell types can be immortalized from transgenic mice by targeted expression of oncogenesis. Target cells can be immortalized at a particular developmental stage to represent the chronological differentiation stages of a cell line (67). Mouse pituitary cell lines have been used to study the mechanism of gene regulation of the mouse, rat and human GnRH receptor (54).

8.1. α T3-1 cells

The protein coding sequence of the SV40 T-antigen oncogene linked to the 1.8 kb of the 5'-flanking sequences of human glycoprotein α -subunit gene was used to induce oncogenesis in the pituitary of transgenic mice (67,68). This gonadotrope precursor cell line synthesizes and secretes the α -subunit but not the β -subunit needed to synthesize LH and FSH, as well as expressing a functional GnRH-R (67,68). These cells show dose responsiveness to GnRH with maximal induction at 100 nM GnRH (69). GnRH responsiveness of the GnRH-R promoter is mediated via PKC activation of MAPK and is mediated through an AP-1 site in α T3-1 cells (69,70).

8.2. L β T2 cells

In order to obtain a mature differentiated gonadotrope cell line, T antigen expression, as for α -T3-1 cells, was targeted using the rat LH β -subunit regulatory region (67,71,72). This cell line is more differentiated than α T3-1 cells in terms of expression of a functional GnRH-R, expression of the α -subunit and LH β subunit and secretion of LH (11,67,72,73). L β T2 cells respond to pulsatile GnRH administration, by increasing GnRH-R mRNA (11). The cell line differs from normal pituitary gonadotropes in that it undergoes mitosis (11). The fact that the cell line resembles a mature gonadotrope supports its use in gonadotrope research (71). However this cell line is notoriously difficult to work with and steroid induced responses are small with large experimental errors (personal communication, Professor JP Hapgood, 74).

9. Signalling in gonadotropes

The exact mechanism whereby signalling occurs in L β T2 cells is not known as research has been done mainly in the gonadotrope cell line α T3-1. Figure 11 illustrates the pathways whereby signalling takes place in α T3-1 cells. A general model for signalling is also shown in figure 7 and is described in more detail below.

Binding of GnRH to its receptor on pituitary gonadotropes results in G-protein activation, which in turn leads to the activation of phospholipase C (PLC) (49,50,57,75). Activated PLC, in turn generates the second messengers, inositol 1,4,5-trisphosphate (IP³) and DAG, required for Ca²⁺ mobilization and PKC activation. Both Ca²⁺ and PKC regulate gonadotropin secretion (49) (See Figure 7). Following this, the major members of the MAPK superfamily including: ERK, JNK and p38MAPK, are activated (52) (See figure 11). The PKC activation of the MAPK cascades confers GnRH responsiveness to GnRH-R (50,69) to mediate GnRH-induced gonadotropin synthesis and release (52). In the α T3-1 and L β T2 cell lines, GnRH stimulates ERK, JNK, p38 and ERK5 (Reviewed in 52).

In α T3-1 cells the murine human GnRH-R was found to signal exclusively through the G_{q/11} G-protein (76), whereas in L β T2 cells the receptor signals through G_{q/11} and G_s (75). G_{q/11} activates

PLC and this increases the IP^3 production as well as the intracellular Ca^{2+} concentration (72). PKC is activated (figure 11) and either directly activates gene expression, or the MAPK cascade. G_s in turn lead to adenylyl cyclase activation and cAMP production, that in turn increases expression of the α -subunit gene in $\alpha T3-1$ cells (72).

In the somatolactotrope cell line, GGH₃ (77), MAPK is activated in a PKA dependent manner but can do so via PKC depending on the G protein subunit through which the GnRH-R signals (77). The further activation may include either ERK- or JNK-mediated signalling cascades being activated, or both which could then converge at AP-1 (45,69). ERK activation induces c-Fos and LH β production in these cells (75). These divergent signalling pathways provide an insight into the mechanism whereby a single hormone; GnRH can regulate reproductive function via a single receptor.

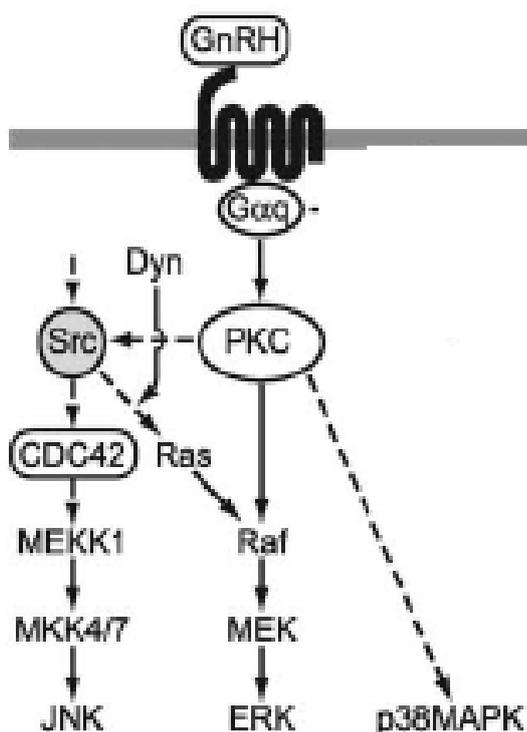


Figure 11. Signal transduction cascades in the $\alpha T3-1$ pituitary cell line resulting in gonadotropin synthesis and secretion (52). Upon GnRH binding to the receptor $G_{\alpha q}$ protein and activates PKC with the resulting cascades: c-Src, CDC42/Ras1 and MEKK1, MKK4/7 and finally the activation of JNK; alternatively Raf activates MEK, which activates ERK; or p38MAPK is activated via a pathway that is still unclear. These pathways mediate the differential gonadotropin synthesis (50,52). Figure taken from Dobkin-Bekman, M *et al* (52).

10. GnRH-R promoter characterisation and regulation

The 5' region of the human (56,78) and mouse (60) GnRH-R promoters have been characterized. The human 5' promoter region is more complex than that of the mouse, as can be seen in figure 12 (54). The mouse GnRH-R promoter contains three transcriptional start sites (Figure 12) (54), whereas the human promoter contains five consensus TATA regions in the 5' region of the gene associated with multiple transcription initiation sites (56). The *cis*-acting regulatory sequences involved in the mouse GnRH-R expression, have been studied extensively in pituitary cell lines, whereas those for the human GnRH-R have been identified in non-pituitary tissues as well (54).

10.1. Human

The human GnRH-R gene utilizes promoter elements and transcription factors differentially depending on the tissue, specifically in reproductive tissues, to regulate the gene expression (Reviewed in 54). The human GnRH-R gene requires two promoter regions, located at $-774/-557$ and $-1351/-1022$ for expression (79). The proximal 173 bp of the human promoter is critical for basal promoter activity in α T3-1 cells whereas the Steroidogenic Factor-1 (SF-1) site, at $-142/-134$, mediates cell-specific expression (80). Furthermore, basal activity of the human promoter in α T3-1 cells, requires Oct-1 binding at $-1718 / -1711$ (81). When Oct-1 binds to the negative regulatory element (NRE) at $-1017/-1009$, the human GnRH-R promoter activity is repressed (82). The cAMP response element (CRE) and Oct-1 regulate basal GnRH-R gene expression in a cell or even species-specific manner (54).

Activin, a growth factor secreted by the gonads initiate intracellular signalling (83) to stimulate FSH secretion (84) by recruiting and phosphorylating the GnRH-R. Inhibin, structurally related to activin (84), upregulates the GnRH-R (85,86), and in the absence of inhibin, GnRH binding was decreased (86).

10.2. Mouse

The mouse GnRH-R proximal promoter was isolated and characterized first (60). Pituitary tissues have a transcriptional start site 62 bp upstream from the translational start site as opposed to the

human transcriptional start site at the consensus TATA box (60,87). The 1.2-kb 5'-flanking mouse GnRH-R gene was found to have regulatory elements important for tissue-specific expression and GnRH regulation (60).

10.2.1. Basal regulation of the mouse GnRH-R

The mouse GnRH-R promoter basal activity is mediated by a tripartite enhancer in α T3-1 cells (88). This enhancer contains binding sites for the orphan nuclear receptor steroidogenic factor-1 (SF-1) at -244/-236 (89), activator protein-1 (AP-1) at -336/-330 and the GnRH-R-activating sequence (GRAS) at -391/-380 (Figure 12) (54,88). Mutational studies showed a loss of basal promoter activity when all three sites were mutated (88).

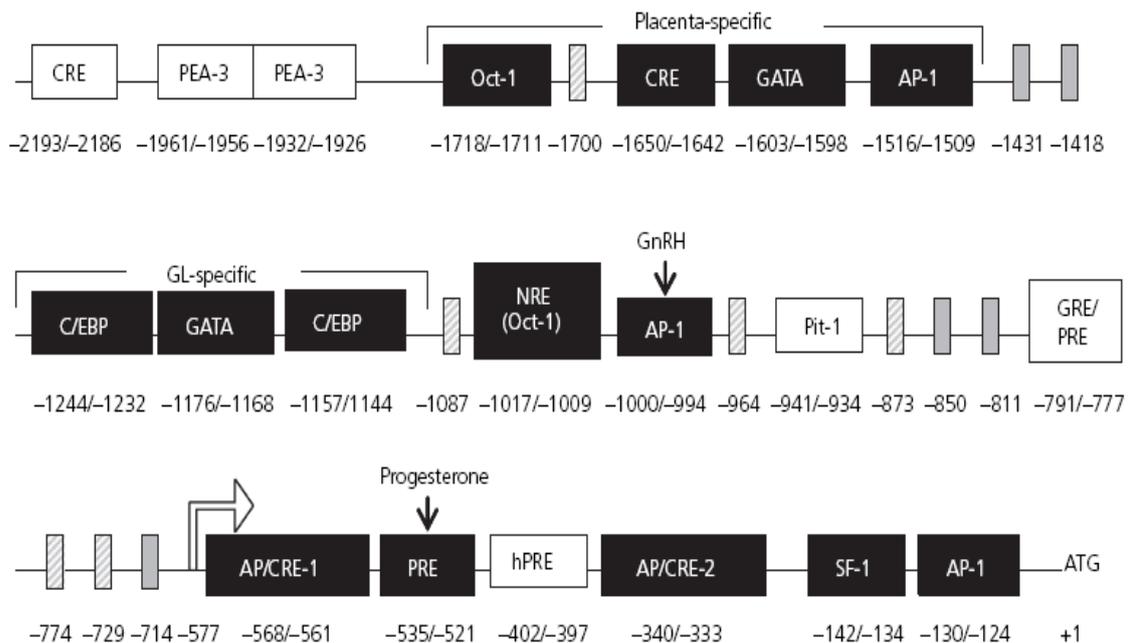
The nuclear factor Y (NF-Y) and Oct-1 binding to the Sequence Underlying Responsiveness to GnRH (SURG)-1 element (-354/-347) direct basal mouse GnRH-R gene expression in α T3-1 cells and do so possibly by interacting with the AP-1 element at (-274/-268) (90). Binding of LHX3 to one of four ATTA elements situated at -298 of the GnRH-R promoter in α T3-1 cells is sufficient to regulate the basal activity (91).

10.2.2. Homologous regulation of the mouse GnRH-R

Microarray analyses show that GnRH induction of the GnRH-R gene results in the transient upregulation of transcription factors (such as c-Fos), cytoskeletal proteins (such as β -actin) and modulators of signal transduction all of which regulate the activity of downstream secondary genes (92). Pituitary sensitivity to variations in GnRH concentration and frequency of administration is dependent on the numbers of GnRH-R present (16,69,93). Continuous exposure of α T3-1 cells to 1mM GnRH for 24 hours reduced GnRH-R binding sites by decreasing the receptor mRNA translational efficiency (47,94). GnRH-R numbers are downregulated upon continuous GnRH exposure and this desensitisation results in a reduced gonadotropin secretion (47). However frequent pulsatile GnRH administration increased GnRH-R mRNA expression (13,93).

GnRH responsiveness of the mouse GnRH-R in α T3-1 cells was located to two sequences, SURG-1 and SURG-2, and is mediated via PKC (95). SURG-1 is located at -354/-347 and SURG-2 at -336/-330 (Figure 12) (54,95). The DNA-protein complex formed at SURG-2 contains Fos/Jun

human



mouse

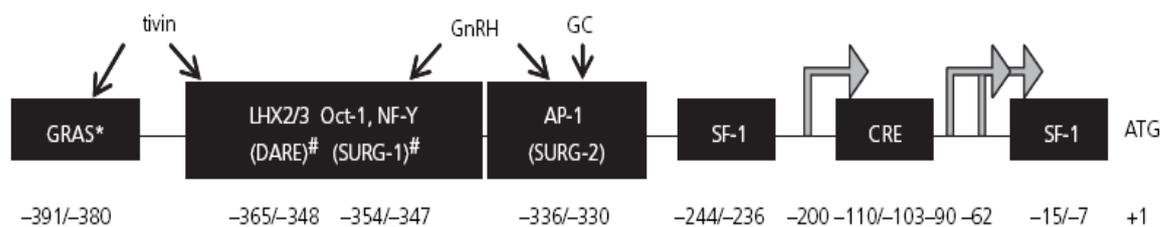


Figure 12. Regulatory elements of the GnRH-R promoter region for the human and mouse gene. Shaded boxes indicate TATA elements, whilst striped boxes indicate CCAAT elements. Functionally characterized elements are shown in the black boxes. Putative elements shown in white boxes have been identified by promoter sequence analysis. Transcriptional start sites are indicated by arrows and ATG the translational start site. The *cis*-elements required for hormone responses as established by functional studies in pituitary cell lines and the hormone is indicated. Figure (not drawn to scale) taken from Hapgood JP *et al* (54).

heterodimers, indicating that AP-1 confers GnRH responsiveness to the receptor gene (69,95). The down-stream activin regulatory element (DARE, -365/-348) overlaps with SURG-1, and contributes to GnRH responsiveness of the GnRH-R promoter and also interacts with NF-Y and Oct-1 (83,90). LHX2 was shown to bind DARE (83), whereas LHX3 binds an overlapping site (91). Both GRAS (96) and DARE are necessary for activin responsiveness of the mouse GnRH-R promoter (83). A cAMP response element (CRE) is found at -110/-103 (54) and mediates the cAMP and GnRH response (87). A repressor element exists in the mouse receptor promoter at -343/-335. When deleted a small increase in receptor transcription is seen with Buserelin stimulation via PKC and cAMP signal transduction (97).

11. Steroidal regulation of GnRH-R

GnRH-R sensitivity to GnRH is increased by steroids as LH secretion mediated by GnRH was increased 14-fold in the presence of dex (72) and this may in part be due to increased GnRH-R levels. Indeed, GnRH and gonadal steroids regulate receptor numbers by regulating the GnRH-R gene expression (93). GnRH was found to upregulate the levels of GnRH-R mRNA (97) at a transcriptional level (59). The estrous cycle influences the number of receptors as well as receptor RNA per cell (16). Not surprising, the GnRH-R proximal promoter has been shown to contain an estradiol responsive sequence in the ovine GnRH-R (16). Estradiol and GnRH synergistically upregulate GnRH-R mRNA production in female rats (93). The administration of dex to male rats resulted in a dose dependent suppression of the basal gonadotropin secretion (4). This is in contrast to what was shown by Rosen *et al* (13) where dex augmented the GnRH induced GnRH-R increase. GnRH-R induction in anterior pituitaries is GnRH dose dependent, but dex was found to augment this induction. In GGH₃ cells, containing the mouse GnRH-R promoter, a putative response element was identified that is able to confer glucocorticoid stimulation. The region between -331/-255 (relative to the transcriptional start site) was found to regulate basal and dex stimulation as determined by transient transfection studies using 5'-deletion mutants (16). The GnRH gene is suppressed by GCs in the hypothalamic neuronal derived cell line, GT1-7 (4).

GC actions range between acute or chronic and the questions remain to be asked if these actions occur via a genomic mechanism of action and what effect the cell/tissue or even animal has on this regulation. Most research has been done on the mouse GnRH-R gene in α T3-1 cells but not much is known about the regulation in L β T2 cells. The experimental work for this thesis was therefore carried out in L β T2 cells to determine the effect of dexamethasone on the GnRH-R promoter transcriptional regulation.

AIM

The aim of this project was to investigate the mechanism of transcriptional regulation by a synthetic GC, dexamethasone, of the mouse GnRH-R gene in L β T2 cells. The following stepwise experimental approach was followed: First GnRH-R promoter regulation was investigated in a transient system, and thereafter the endogenous GnRH-R promoter was studied.

A transiently transfected promoter-reporter plasmid pLG, containing 600 bp of the mouse GnRH-R promoter (Figure 13) located upstream of a luciferase gene (LUC) was used to investigate the transcriptional effect of dex on the promoter regulation in L β T2 cells. Thereafter exogenous rat GR was cotransfected along with the pLG construct into L β T2 cells to investigate the regulatory role of GR in L β T2 cells. GR expression is subject to homologous down-regulation when treated with dex (Reviewed in 98) and this was therefore investigated by means of western blot analysis.

Thereafter experimental work was done on the endogenous GnRH-R in L β T2 cells. The aim was to determine the effect dex has on GnRH-R transcription. Real time RT-PCR of mRNA harvested from dex or vehicle induced L β T2 cells were compared. L β T2 nuclear proteins such as GR and AP-1, were investigated for their ability to bind a region of the GnRH-R promoter that include the AP-1 and SF-1 binding sites (AP-1/SF-1; -345/-290). These transcription factors are thought to mediate the regulatory effect of dex on the GnRH-R promoter. The *in vivo* interaction of the GR with the mouse GnRH-R promoter region spanning a region containing SURG-2/AP-1 and SF-1 (Figure 12; -337/-170) was investigated in dex or vehicle treated L β T2 cells to further determine interaction of the GR with the GnRH-R promoter.

CHAPTER 2

Materials and Methods

Various assays were carried out at two laboratories and therefore two protocols may be described as the assays were carried out differently. Assays not carried out in Stellenbosch are written in *italics*.

2.1. Chemicals and Antibodies

Antibodies used for the CHIP assay included: GR H-300 (sc-8992), rabbit polyclonal IgG (sc-2027), c-jun (N) (sc-45 X), c-fos (K-25) (sc-253X), β -actin and normal rabbit IgG (sc-2027) all from Santa Cruz Biotechnology.

Trypsin-EDTA, F12 (Ham) nutrient mixture containing L-Glutamine as well as Dulbecco's modified eagle's medium (DMEM) containing 4500mg/L glucose and L-Glutamine, 200 mM was from GIBCO, Invitrogen life technologies.

Fetal Bovine Serum (FBS) (South American Origin) was from BIO WEST,

Hygromycin B in PBS (50mg/ml) was from Invitrogen (Carlsbad, CA 92008 USA)

Penicillin (10 000 Units / ml) – Streptomycin (10 000 μ g / ml) was from GIBCO, Invitrogen Corporation.

Dexamethasone and PMSF were purchased from SIGMA-ALDRICH.

Tissue culture plates (24 well, 6 cm and 10 cm) and flasks were either from TPP Switzerland or Falcon.

2.2. Plasmids

Plasmid maps are shown in Addendum B. pGL2-basic promoterless luciferase expression vector (5.6 kb) and pSV- β -galactosidase expression vector (6.8 kb) were from Promega. Construct R10, containing the mouse GnRHR cDNA (1.2 kb) (55) in the pcDNA1 expression vector (total construct size 6 kb), as well as clone 111, containing 1.1 kb of the mouse GnRH receptor gene were obtained from Dr. SC Sealfon (Mt Sinai Medical School, New York, N.Y.) (55). Constructing the pLG construct is described by Sadie *et al* (87). The pSTCGRN 795 wt construct containing the wild

type rat GR cDNA insert was a gift from Dr S Rusconi, Institute of Biochemistry, University of Fribourg, Perolles, 1700 Fribourg, Switzerland.

2.3. Plasmid preps:

Small-scale plasmid purification from bacterial constructs was performed according to the manufacturers protocol at room temperature unless stated otherwise. Bacterial cells were grown overnight at 37°C in 10 ml LB medium for mini preps or 50 ml LB medium for midi preps, containing 50µg / ml ampicillin. The bacterial culture was then centrifuged at 15 000 X g and used as indicated:

2.3.1. GenElute™ Gel Extraction Kit (SIGMA) for R10 fragments

Plasmids obtained from the R10 construct (74,99) were separated on a 1% low melting agarose gel. The band containing the plasmids was cut from the gel and weighed. Gel Solubilization Buffer was added to the gel in the ratio 3 parts (volume) buffer to 1 part (weight) gel. The mixture was kept at 60°C for 5 minutes until the gel had completely melted. Sodium acetate (pH 5.2) should be added to the mixture if the colour does not remain yellow. One gel volume isopropanol was then added to the mixture. The column is prepared by the addition of 500µl Column Preparation Solution and centrifugation at 12 000 rpm for 30 seconds and the flow through discarded. The gel mixture is loaded onto the column and centrifuged at 12 000 rpm for 1 minute and the flow through discarded. Wash Solution (500µl) was added to the column and centrifuged at 12 000 rpm for 30 seconds and the flow through discarded. Elution Buffer (50µl) is added to the column and the DNA eluted by centrifuging at 12 000 rpm for one minute. DNA was stored at -20°C.

2.3.2. Wizard Plus SV Miniprep Purification Systems (Promega)

The bacterial cell pellet was resuspended in 250µl Resuspension Solution and 250µl Cell Lysis Solution was added. The mixture was inverted four times, 10µl Alkaline Protease Solution was added and the mixture again inverted four times and incubated for five minutes. Neutralization Solution (350µl) was added to the mixture and inverted a further four times. The mixture was centrifuged at 15 000 X g for 10 minutes and the clear lysate transferred to a spin column. The spin column was centrifuged at top speed for one minute. The spin column was washed twice with

750µl Wash Solution by centrifugation at 15 000 X g for one and two minutes respectively. Nuclease-free water (100µl) was added to the spin column and allowed to stand for 15 minutes in order to increase the yield before centrifugation at 15 00 X g for one minute. DNA was stored at – 20°C.

2.3.3. Pure Yield Plasmid Midiprep kit (Promega)

The bacterial cell pellet was resuspended in 3 ml Cell Resuspension Solution and 3 ml Cell Lysis Solution was added. The mixture was inverted five times and incubated for three minutes at room temperature. Thereafter 5 ml Neutralization Solution is added and the mixture again gently inverted four times. The lysate is allowed to incubate in an upright position to allow for a white precipitate to form. A blue PureYield Clearing Column is placed into a clean 50 ml Falcon tube and the lysate poured into the column. The lysate is allowed to incubate for two minutes in the column before centrifugation at 1500 X g for 5 minutes. A white PureYield Binding Column is placed into a clean 50ml Falcon tube and the filtered lysate poured into the column. The column is centrifuged at 1500 X g for three minutes. Endotoxin Removal Wash solution (5ml) is added to the column and centrifuged again at 1500 X g for 3 minutes and the flowthrough discarded. Column Wash solution (20ml) is added to the column and centrifuged at 1500 X g for 5 minutes and the flowthrough discarded. The column is centrifuged at 1500 X g for a further 10 minutes to ensure ethanol removal. The column was then placed into a clean 50ml Falcon tube and 600 µl Nuclease-Free Water added to the column membrane. The column was centrifuged at 1500 X g for 5 minutes and the filtrate collected from the tube. DNA was stored at – 20°C.

2.3.4. Plasmid Maxi Kit (QIAGEN)

Large-scale plasmid purification was performed according to the manufacturers protocol. Bacterial cells from glycerol stocks were grown in a 5ml LB medium starter culture containing 50µg/ml ampicillin for about 6 hours at 37°C. The starter culture was transferred to 500ml LB medium containing 50µg / ml ampicillin and allowed to grow overnight at 37°C. The bacterial culture was centrifuged at 6 000 X g for 15 minutes at 4°C in a Beckman JA-10 rotor. The pellet was resuspended in Buffer P1 containing Rnase A. Buffer P2 (10 ml) was added to the mixture and inverted gently four times and incubated for 5 minutes at room temperature. Cold Buffer P3 (10 ml)

was added to the mixture and was gently inverted four times and incubated on ice for 20 minutes. The mixture was centrifuged twice at 20 000 X g for 30 minutes at 4°C using a Beckman JA-20 rotor. A QIAGEN-tip 500 column was equilibrated by the application of 10ml Buffer QBT and allowing it to empty by gravity flow. Supernatant was then applied to the column and allowed to enter the resin by means of gravity flow. Thereafter the column was washed twice with 30ml Buffer QC. DNA was then eluted with 15ml Buffer QF and precipitated by the addition of 10.5ml isopropanol at room temperature. The mixture was centrifuged at 15 000 X g for 30 minutes at 4°C in a Beckman JA-20 rotor after which the supernatant was discarded. The DNA pellet was carefully washed with 5 ml 70% (v/v) ethanol and centrifuged at 15 000 X g for 10 minutes in a Beckman JA-20 rotor. The supernatant was discarded and the pellet air dried for 10 minutes after which the DNA was resuspended in 500µl TE buffer, pH 8.0.

2.4. Cell line and culture conditions

LβT2 cells were a gift from Dr Pamela Mellon from the University of California, San Diego, California. Cells were maintained in 75-cm² culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (SIGMA-ALDRICH) supplemented with 10% FCS or FBS, 50 IU / ml penicillin and 50 µg / ml streptomycin unless stated otherwise. Cells were grown in a humidified (90%) air atmosphere at 37°C in 5% CO₂.

2.5. Charcoal-Dextran treatment of Fetal Bovine Serum (100)

Fetal Bovine Serum was treated with charcoal dextran in order to remove trace steroids, which could possibly influence the LβT2 response seen with the compound of interest. The charcoal-dextran solution used consisted of 10% wt/vol activated charcoal (Sigma Chemical Co, St Louis, Missouri) and 0.05% wt/vol dextran-T70 (Pharmacia Biotech AB, Uppsala, Sweden) in water and was kept at 4°C. HCl was used to lower the pH of 500 ml Fetal Bovine Serum (FBS) (Biowest) to pH 4.2. The FBS was kept at 0°C for 30 min after which 25 ml dextran – charcoal solution was added. The pH was again set to 4.2 and the solution stirred overnight at 4°C. Charcoal was removed by centrifugation at 13 000 rpm for 20 min at 4°C. NaOH was used to increase the pH of the serum to 7.2. The serum was then sterile filtered through a 75 mm NALGENE Filter Unit (Nalge Nunc International corp. USA) and aliquots frozen for later use.

2.6. Transient Transfections

L β T2 cells were plated in 24-well culture plates (Nunc, Naperville, IL) with 1×10^5 cells per well in 500 μ l DMEM supplemented with 10% fetal calf serum (Delta Bioproducts, Johannesburg, South Africa) and 50 IU / ml penicillin and 50 μ g / ml streptomycin (Invitrogen Corporation, GIBCO). At least 24 hours after plating, medium was replaced with antibiotic free DMEM containing 10 % charcoal treated FCS (CT-FCS). Transfections were performed according to the Fugene 6 Transfection Reagent protocol (Roche). DNA was transfected along with Fugene 6 reagent at a ratio of 1 μ g DNA/ 2 μ l Fugene 6 in 50 μ l serum free DMEM per well. At least 24 hours after transfecting the medium was again replaced with antibiotic- and serum-free medium. Incubations were performed with test compounds for various time points as indicated in the figure legends. Cells were harvested in 50 μ l reporter lysis buffer (Promega) per well. The luciferase assays (Promega Corp. Luciferase Assay System) and β -galactosidase assays (Galacto-Star, Tropix, Bedford, MA) were carried out in 10 μ L cell extract in white 96-well plates in a Veritas Microplate Luminometer (Turner Biosystems). Luciferase values were normalized to corresponding β -galactosidase values and expressed relative to control values, which were set as 1 unless stated otherwise.

Cells were harvested in 200 μ l Lysis Solution (Tropix, Bedford, MA) per well. The luciferase assay (Luciferase Assay Kit A, Bio Thema AB, Sweden) was carried out in white 96-well plates (White Cliniplate, Thermo Labsystems Oy, Finland) where the reaction mix consisted of 100 μ l lysate and 100 μ l Luciferin substrate. ATP substrate was injected by the luciferase machine at a volume of 100 μ l per well before measuring the luciferase readings. Readings were expressed relative to vehicle treated controls, which were set to one.

2.7. RNA isolation

L β T2 cells were plated out at 9×10^5 per well in 6-well plates in 2 ml medium. At least 24 hours after cells were plated ligand or vehicle treatment was done as indicated in the figure legends. There after cells were washed with PBS and 185 μ l Tri Reagent added per well. Wells were scraped and incubated for 5 minutes before transferring the well content to microcentrifuge tubes. Subsequent steps were performed at 4°C. To each sample 40 μ l chloroform was added and tube

vortexed for at least 15 seconds. Samples were incubated for 15 minutes before centrifugation at 13 000 rpm for 15 minutes. The supernatant was transferred to a new tube and an equal volume isopropanol added. Samples were frozen at -20°C and thawed on ice. The RNA was pelleted by centrifugation at 14 000 rpm for 20 minutes. The supernatant was aspirated and 500 µl Ethanol (70% v/v) added per sample and again centrifuged. Supernatant was aspirated, ethanol added and samples vortexed for 1 minute before centrifugation. Supernatant was removed and the RNA pellet allowed to air dry. The pellet was dissolved in 100 µl DEPC-H₂O. Samples were stored at -20°C. RNA integrity was analysed on a 1% agarose gel.

LβT2 cells were seeded into 6 well plates at 9×10^5 cells per well in 2 ml medium. At least 24 hours after seeding out, cells were induced in 2 ml DMEM containing 10 % CT-FBS containing either 10^6 Dex or a vehicle control for each time point as indicated in the results. The medium was then aspirated and the wells washed with cold PBS. mRNA was isolated by making use of the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Cat No. 74104). The protocol for animal cells was modified as follows: Fresh RLT buffer was made up by the addition of mercaptoethanol as indicated in the protocol. To each well 600 µl RLT Buffer was applied. Wells were scraped and the buffer-cells mixture transferred to sterile microcentrifuge tubes and vortexed to dissolve clumps. All subsequent steps were performed at 4 ° C. Cells were homogenized by sonicating each sample for 10 seconds at the highest intensity. One volume of 70 % ethanol was added to each sample and mixed by pipetting. Samples were applied 700 µl at a time to corresponding RNeasy mini columns in 2 ml tubes. These were centrifuged at 10 000 rpm for 15 seconds and the flow-through discarded. To each column 700 µl RW1 Buffer was added and samples again centrifuged as before. The columns were transferred to new 2 ml tubes and 500 µl RPE Buffer applied to each and centrifuged as before. Flow-through was discarded and 500 µl RPE Buffer once again applied to each column. Samples were centrifuged at 10 000 rpm for 2 minutes. Columns were transferred to new 2 ml tubes and centrifuged for 1 minute at 13 000 rpm. Columns were transferred to 1.5 ml tubes and the RNA eluted in 50 µl RNase free water by centrifugation at 10 000 rpm for 1 minute. Samples were stored at -70 ° C. The optical density of the samples was measured to determine the concentration of RNA per sample.

2.8. cDNA Synthesis

SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, life technologies Cat No. 11904-018) was used to reverse transcribe cDNA from L β T2 mRNA. The maximum volume RNA that could be used was 10 μ l and samples were therefore normalised to the concentration of the lowest sample. The first reaction mixture per nuclease-free microcentrifuge tube was as follows: 10 μ l mRNA, 1 μ l Random Hexamers (50 ng / μ l), 1 μ l dNTP mix (10 mM of each dATP, dCTP, dGTP, dTTP at neutral pH) and if necessary DEPC treated water to a final volume of 10 μ l. The reaction mixture was heated at 65°C for five minutes and then incubated on ice for one minute. The microcentrifuge tubes were briefly centrifuged and 9 μ l of the second reaction mixture was added to each. The Second reaction mixture consisted of: 2 μ l RT Buffer (10 X), 4 μ l Mg Cl₂ (25 mM), 2 μ l DTT (0.1 M) and 1 μ l RNase OUT™ Recombinant RNase Inhibitor (40U / μ l). Samples were then incubated at 25°C for 2 minutes before 1 μ l SuperScript™ III RT (200U / μ l) was added and further incubated for 10 minutes. Samples were then kept at 42°C for 50 minutes and at 70°C for 15 minutes after which they were placed on ice. Samples were briefly centrifuged before the addition of 1 μ l E.coli RNase H (2 units) after which samples were kept at 30°C for 20 minutes. Optical density for each sample was determined and the cDNA concentration normalised to 1 μ g / μ l. Samples were stored at -20°C.

Alternatively the M-MLV Reverse Transcriptase Rnase H minus kit (Promega) was used to transcribe RNA to cDNA according to the protocol: 1 μ g RNA and 1 μ g oligo-dT primer (1 μ g/ μ l) was mixed to a final volume of 14 μ l using PCR water. The mixture was incubated at 70°C for 5 minutes after which the mixture was chilled on ice and briefly centrifuged. The following was then added: 5 μ l 5x buffer, 5 μ l dNTP (10mM) and 1 μ l enzyme to a final volume of 25 μ l. The mixture was then incubated at 42°C for 1 hour. The cDNA concentration was determined and samples stored at -20°C.

2.9. Polymerase Chain Reaction (PCR)

Each PCR reaction was made up to a final volume of 50 μ l using PCR-grade water:

4 μ l template (only 2 μ l input DNA was used)

2 μ l sense primer (100 μ M)

2 μ l antisense primer (100 μ M)

1 μ l dNTP mix (10 mM; Sigma)

5 μ l 10X Taq polymerase buffer (Promega)

2 μ l MgCl₂ (Promega, stock solution 25 mM)

0.25 μ l Taq polymerase (Promega, 5U/ μ l)

Table 1. Conventional PCR conditions.

Temperature (°C)	Minutes	Number of cycles
95	2.5	1
95	1	35
Tm of amplicon*	1	
72	1	
72	10	1

***Melting temperature (T_m) of the amplicon:**

SURG-2 primer product: 51°C

SF-1 primer product: 54°C

The PCR product was visualised on a 1 % agarose gel.

2.10. Real Time-PCR (RT-PCR)

LightCycler® real-time quantitative RT-PCR was set up by H. Sadie (74) according to the guidelines set out by the ROCHE LightCycler FastStart DNA Master PLUS SYBR Green I kit.

Isolated mRNA from ligand or vehicle treated L β T2 cells were reverse transcribed to cDNA and amplified by means of PCR using primers designed to the target gene. The target gene was that of the mouse GnRHR promoter (74) with sequence 5'-CCACAGTGGTGGCATCAGGCCTTC-3'

(sense strand) and 5'-TAGCGTTCTCAGCCGAGCTCTTGG-3' (anti-sense strand). The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene with sequence 5'-ATTGTCAGCAATGCATCCTG-3' (sense strand) and 5'-ATGGACTGTGGTCATGAGCC-3' (anti-sense strand), is not under dex regulation and was therefore used as reference gene (101).

A cDNA dilution series was made of a time zero ethanol induced sample and was used to construct a standard curve for both primer sets. PCR amplification was performed in 96-well Optical Reaction Plates with Barcode (code 128, Applied Biosystems). The reaction mixture in each well were as follows: 1 μ l sense primer and 1 μ l antisense primer (both 200 nM final concentration), 8.5 μ l PCR water, 2 μ l cDNA (1 μ g/ μ l) and 12.5 μ l Power SYBR®Green PCR Master Mix (Applied Biosystems, Warrington, UK). The final volume for each reaction was 25 μ l, and each reaction was performed in duplicate (triplicate for samples used to construct the standard curve). Wells were sealed with Optical Caps (Applied Biosystems, USA) before centrifugation at 1000 rpm for two minutes. The plate was subjected to RT-PCR.

A vial of SYBR Green I was allowed to thaw and 13 μ l FastStart Taq polymerase added to prepare the SYBR Green I Master Mix. This master mix is light and temperature sensitive and was therefore protected from light and stored at 4°C for no longer than 2 weeks. Reaction mixtures were prepared in glass capillary tubes kept in centrifuge adaptors. The capillaries were kept on an aluminium-cooling block while the reaction mixtures were added to maintain the mixture at 4°C.

Table 2. Reaction mixture for the LightCycler® real-time quantitative RT-PCR

Total volume of 20 μl.	
cDNA template	2 μ l
sense primer (5 μ M)	2 μ l
antisense primer (5 μ M)	2 μ l
LightCycler SYBR Green I Master Mix	4 μ l
PCR H ₂ O	10 μ l

The PCR reaction mixture excluding the cDNA was prepared for all reactions and 18µl pipetted into each capillary tube. Thereafter the corresponding 2µl cDNA was pipetted into each capillary. Capillaries were sealed with caps and centrifuged at 3000 rpm for 5 seconds to ensure the mixture collected at the bottom of the capillary.

The LightCycler protocol is as follows: The PCR samples are incubated at 95°C for 10 minutes to activate the Roche FastStart Taq polymerase. The subsequent PCR amplification steps are indicated in table 3. The annealing temperature is determined by the % GC content of the primers. Fluorescence is measured at the end of each extension step. Typically there would be 35 repeats of the three PCR steps. During amplification the temperature transition rate is set to 20°C per second. In order to generate the melting curve the temperature transition rate is set to 0.2°C per second and the fluorescence is measured throughout this melting step.

Table 3. RT-PCR conditions.

PCR Steps	Denaturation		Annealing		Extension	
	Time (s)	°C	Time (s)	°C	Time (s)	°C
mouse GnRH-R (S4/AS4) primers	8	95	10	58	8	72
GAPDH primers	8	95	10	50	5	72

2.11. Western blotting

LβT2 cells were plated out at 9×10^6 per well in a 6 well plate in 2ml medium. At least 24 hours after plating out cells were induced, as indicated in figures, in medium containing only 10% CT-FCS. Cells were washed twice in cold PBS and all subsequent steps were performed on ice. Per well 400µl CREB-lysis buffer (refer to Addendum C3: SDS-PAGE buffers) was added and placed on a shaker for 15 min. The CREB-lysis buffer proved most effective to lyse LβT2 cells and for this reason was used throughout the protocol. Cells were scraped into microcentrifuge tubes and vortexed. Samples were allowed to freeze at -80°C after which they were thawed on ice and again vortexed. Samples were centrifuged for 10 minutes at 10 000 rpm at 4°C and the supernatant stored at -80°C. Samples were subjected to a Bradford protein determination and equal concentrations of respective samples prepared to a final volume of 10µl containing 2µl of a 5X

loading buffer and boiled for 10 minutes prior to loading. Proteins were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 70 V for 45 min and then at 200 V for 60 min in 1X Tris-glycine and 10% sodium dodecyl sulfate. Proteins were transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences) by means of electroblotting for 45 min at 100V in 1X Tris-glycine containing 20% (vol/vol) methanol. The membrane was blocked overnight at 4°C in 2% ECL advanced blocking agent (Amersham Biosciences) in TBS containing 0.1% (vol/vol) Tween. Antibody dilutions and washes were made in TBS containing 0.1% (vol/vol) Tween. There after the membrane was incubated with primary antibody (dilution indicated in figure legends) for 60 min and washed 3X for 5 min each. Subsequently the membrane was incubated with secondary antibody, anti-rabbit HRP (horse radish peroxidase) (1:10000) in 2% ECL advanced blocking agent for 60 min and washed as before. Proteins were visualized on Hyperfilm with the use of Lumigen TMA 6 Solutions A and B (Amersham Biosciences). Membranes to be reprobred with different antibody were stripped in 50 ml stripping solution at 50°C for 10 minutes. The membrane was then washed twice in 20ml TBS for ten minutes at room temperature. The membrane was again blocked overnight in 2% ECL Advanced Blocking Solution and the steps followed as described above.

2.12. Nuclear Extract preparation

Nuclear extracts were prepared according to the protocol of Schreiber et al (1989) as modified in (74). L β T2 cells were plated out at 3×10^6 per 10cm dish and grown until confluent. Ligand treatment is indicated in the figure legends. Cells were washed with cold PBS and scraped into microcentrifuge tubes and thereafter centrifuged at 1 000 rpm for 5 minutes at 4°C. The PBS was aspirated and the cells frozen at – 70°C. Cells were thawed on ice and washed by centrifugation in 2 ml TBS at 1 000 rpm for 5 min at 4°C. TBS was aspirated and cells were resuspended in 1 ml TBS and pelleted for 15 seconds. TBS was aspirated and cells were gently resuspended in 400 μ l cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). Samples were incubated on ice for 15 minutes after which 25 μ l Nonident NP-40 (10 % v/v) was added and the tubes vortexed for 10 seconds. Samples were centrifuged at 12 000 rpm for 5 min at 4°C. The supernatant was aspirated and the pellet resuspended in 50 μ l cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). Samples were

placed on a shaker for 15 minutes at 4°C. Samples were centrifuged at 13 000 rpm for 5 minutes at 4°C and the supernatant stored at – 70°C.

2.13. Electrophoretic Mobility Shift Assay (EMSA)

Equimolar amounts of the sense and antisense single stranded oligonucleotides comprising the proximal (–345 /-290) mouse GnRHR gene promoter were annealed in a PCR reaction. Successful annealing was confirmed on a 2% agarose gel and staining with ethidium bromide. Double stranded oligonucleotides were 5'-end labeled using 100 µCi [γ -³²P] ATP (NEN Life Science) and 10U Escherichia coli polynucleotide kinase (Roche). The unincorporated nucleotides were removed by passing the labeling mixture through a 1 ml G50 Sephadex (Pharmacia) spin column in TAE buffer. DNA probe was incubated with nuclear extracts (2 µg / µl) prepared from LβT2 cells as described by Sadie *et al* (87). Protein-DNA incubations were performed in a final volume of 5 µl in a reaction buffer specific for either oligonucleotide AP-1/SF-1 (Figure 12; –345/-290) that contains binding sites for AP-1 and SF-1, or oligonucleotide AP-1 (as described in addendum C2) together with 0.6 µg BSA (Roche, molecular biology grade). Unless indicated otherwise, 1 µg pdIdC was used. A 100-fold excess unlabeled double stranded oligonucleotide was added to the protein-DNA complexes for the competition studies. Antibody shifts were performed as described above with minor alterations as described: 1 µl of the specific antibody or rabbit pre-immune serum was added to the cocktail and incubated at room temperature for 20 min before electrophoresis. The complexes were resolved on polyacrylamide gels (29:1 acrylamide / bisacrylamide) in 1X TAE buffer at 100 V for 225 min. Gels were dried under vacuum for 30 min at 74°C and exposed to Hyperfilm (Amersham Biosciences). Conditions subjected to variation are indicated in figure legends. Unless indicated, buffer and protocols are according to Sambrook J. 1989 (102).

2.14. Genomic DNA isolation

Genomic DNA was prepared according to the GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA-Aldrich, Saint Louis, Missouri). The protocol was as follows: Respective cell cultures were plated out at 3 X 10⁶ and grown until confluent. Cells were collected into microcentrifuge tubes and pelleted at 2 000 rpm for 5 minutes. Resuspension Solution (200 µl) was used to resuspend the cell pellet. Lysis was achieved by the addition of 20µl Proteinase K solution followed by 200µl Lysis

Solution C. The sample was vortexed and incubated at 70°C for 10 minutes. The GenElute Miniprep Binding Column assembled in a 2 ml collection tube, was prepared by the addition of 500 µl Column Preparation Solution and centrifugation at 13 000 rpm for 1 minute. To the lysate 200µl absolute ethanol was added and again vortexed after which the mixture was loaded onto the binding column and centrifuged at 10 000 rpm for 1 minute. The binding column was transferred to a new 2 ml collection tube and washed by the addition of 500µl Wash Solution and centrifugation at 10 000 rpm for 1 minute. The wash step was repeated but the column centrifuged for 3 minutes at maximum speed and the flow through discarded. The binding column was centrifuged for an additional minute at maximum speed to ensure ethanol removal. Elution Solution (200µl) was applied to the centre of the binding column, incubated for 5 minutes at room temperature and then centrifuged at 10 000 rpm for 1 minute. DNA was stored at – 20°C.

2.15. Chromatin immunoprecipitation (ChIP) assay

LβT2 cells were seeded out in 150 mm dishes at 3.5×10^6 and the medium replaced with Phenol red free medium, 10% CT-FCS, 50 IU / ml penicillin and 50 µg / ml streptomycin, 2mM L-Glutamine medium on day three. Cells were then serum starved on day four and induced with 100 nM Dex or ethanol vehicle at 37°C for one hour on day five. Crosslinking was performed by the addition of 1% Formaldehyde (final concentration) to the plate and incubation at 37°C for 10 minutes. The reaction is quenched by the addition of 0.125M glycine (final concentration) for 5 minutes at room temperature with mild shaking. Thereafter cells are washed twice with ice cold PBS and scraped in 5 ml PBS containing Complete, Mini Protease Inhibitor Cocktail Tablet (Roche). Subsequent steps are performed at 4°C. Cells are pelleted at 1200g for 10 minutes and resuspended in 500µl nuclear lysis buffer containing Complete, Mini Protease Inhibitor Cocktail Tablet (Roche). Lysed cells are sonicated for 20 seconds with 40 seconds incubation repeated for 10 cycles. Sonicated lysate is centrifuged at 15,000 g for 10 minutes at 4°C. A concentration of 16 µg of the sonicated chromatin is removed as the input, and 90µl IP elution buffer (1% SDS, 100nM NaHCO₃, 300nM NaCl) is added and crosslinks reversed overnight at 65°C. The mixture is pre-incubated for 1 hour at 45°C with the addition of 10µl EDTA (0.5M), 40µl Tris-HCl (1M, pH 6.5) and 2µl proteinase K (stock

10µg/µl). Thereafter the input DNA is purified using the QIAquick® PCR Purification Kit (QIAGEN) according to the Microcentrifuge protocol provided:

To the chromatin mixture 5 volumes buffer PBI is added and transferred to a QIAquick column in a 2ml collection tube. The column is then centrifuged at 13 000m rpm for 1 minute. The flow through is discarded and the column washed by adding 750µl buffer PE (included with kit) to the column and centrifuging as before. The flow through is again discarded and the column centrifuged for an additional 1 minute. The column is then transferred to a clean 1.5ml microcentrifuge tube and 30µl EB (included with kit) is added to the centre of the column and incubated for 1 minute. The column is then centrifuged as before to elute the DNA

Protein G beads are pre-blocked as follows: 1ml beads + 50µg sheared salmon sperm DNA (11mg / ml, Invitrogen) + 1mg/ ml BSA (Fraction V, ROCHE) + 1ml IP dilution buffer on a rotating wheel for 4°C for 1 hour. The beads are centrifuged and resuspended as 50% slurry in IP dilution buffer. A concentration of 100µg of the sonicated chromatin was taken from the remainder of the supernatant. Pre-cleared protein G beads (20µl) was added to this and incubated at 4°C for 1 hour with nutating^b. The mixture is centrifuged at 15 000 g for 10 minutes and the supernatant transferred to a new tube. GR antibody (5µg) is added to the supernatant and the sample volume made up to a final volume of 1ml using IP dilution buffer. The chromatin-antibody mixture is incubated at 4°C overnight with nutating. The following day pre-blocked protein G beads (40µl) is added to the mixture and incubated for 6 hours at 4 °C with nutating. The mixture is then centrifuged at 5500g for 1 minute and the pellet washed sequentially with 1ml of each wash buffer I, II, III, and three times with 1ml TE (pH 8.0). Chromatin is eluted twice with 150µl elution buffer by nutating for 15 minutes at room temperature. To the 300µl elution buffer 300nM NaCl (final concentration) is added and incubated at 65°C overnight to reverse the crosslinks. The mixture is pre-incubated for 1 hour at 45°C with the addition of 5 µl EDTA (0.5M), 20µl Tris-HCl (1M, pH 6.5) and 1µl proteinase K (stock 10µg/µl). The chromatin is purified from the mixture using the QIAquick® PCR Purification Kit (QIAGEN) according to the Microcentrifuge protocol provided.

^b Nutating is the term used to describe the process during which the chromatin containing tube is rotated top over bottom in order to ensure maximal homogeneity is achieved.

2.16. Statistical analysis

GraphPad PRISM™ (version 4.1) (GraphPad Software, Inc., San Diego, CA) was used to analyse data. The statistical analysis used was one-way ANOVA. Unless stated otherwise in the figure legends Bonferroni's post-test (compare all values to each other) was used. Statistical significance was assumed when $P < 0.05$.

CHAPTER 3

Results and Discussion

3.1. The synthetic glucocorticoid, dex, upregulates expression of a GnRH-R promoter-reporter construct in a gonadotropin cell line

Several reports in the literature on anterior pituitary cell cultures and non-gonadotrope cell lines suggest that the transcription of the GnRH-R gene is regulated by glucocorticoids (12,16,72).

Whether the GnRH-R promoter is regulated by glucocorticoids was therefore investigated in this study by means of transiently transfecting a GnRH-R promoter-reporter construct into L β T2 cells. Identifying *cis* elements important for the regulation of the GnRH-R promoter is possible as the effect of mutating the potential binding sites can be compared to the response of the wild type construct. The promoter-reporter plasmid pLG contains 600 bp of the mouse GnRH-R promoter (Figure 13) located upstream of a luciferase gene (LUC). The GnRH-R promoter region contains the following known transcription factor binding sites: AP-1 (-336/-330), SF-1 (-244/-236) (Figure 13, see also Figure 12) (54,87). For simplicity no other binding sites are indicated in figure 13 (compared to figure 12), as they were not the focus of the present study.

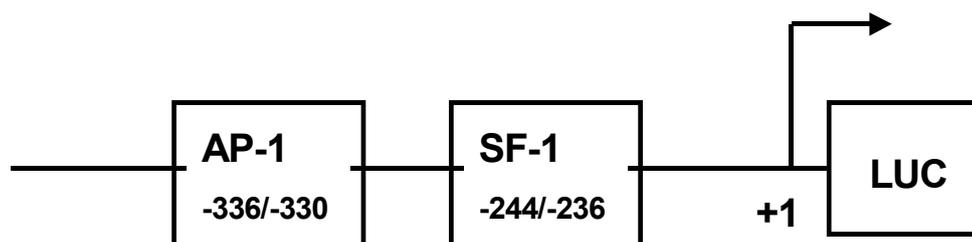


Figure 13. Diagrammatical representation of the transcription factor binding sites found in the pLG construct. The 600 bp promoter fragment of the mouse GnRH-R gene contains the following transcription factor binding sites, numbered relative to the transcriptional start site: AP-1 (-336/-330) and SF-1 (-244/-236). The promoter region is linked to a luciferase gene (LUC) (87,99). Figure not drawn to scale.

Construct pLGM1 was generated by others in our laboratory by means of PCR mutagenesis (74,87) and contains a mutation in the AP-1 binding site (-336/-330). Previous work in our laboratory

showed that GnRH positively induced the pLG construct, transfected into L β T2 cells. GnRH-R expression was induced by 100 nM GnRH 1.5 fold after 4 hours (74) and 2.5 fold after 8 hours induction (99) and 1 μ M Dex resulted in a 3- and 4.5-fold induction after 8 and 16 hours respectively (99). In addition it was shown that the basal as well as dex response obtained with the pLG construct, was lost when the mutant (pLGM1) construct was transfected into L β T2 cells (99).

3.1.1. Dex induction results in an increased transcription of the mouse GnRH-R promoter in L β T2 cells.

Assay conditions for transfecting L β T2 cells with pLG according to the Fugene 6 method, were previously established in our laboratory (99). In the present study, cells were induced with either ethanol vehicle or 1 μ M dex for 8 hours. Dex treatment induced a 1.4 fold response (compared to ethanol treated cells) in L β T2 cells transiently transfected with the GnRH-R promoter-reporter construct (pLG) as shown in figure 14. The fold increase is within the range of experimental error. Therefore significance was unable to be established, even with 8 experiments, each performed in duplicate or triplicate. Repeating the assay in a different laboratory resulted in a similar induction pattern (results not shown). Several parameters were varied to determine if a larger dex response could be obtained. These included serum conditions, the amount of construct transfected, ligand concentration and induction time. However, these changes did not increase the dex response (Results not shown).

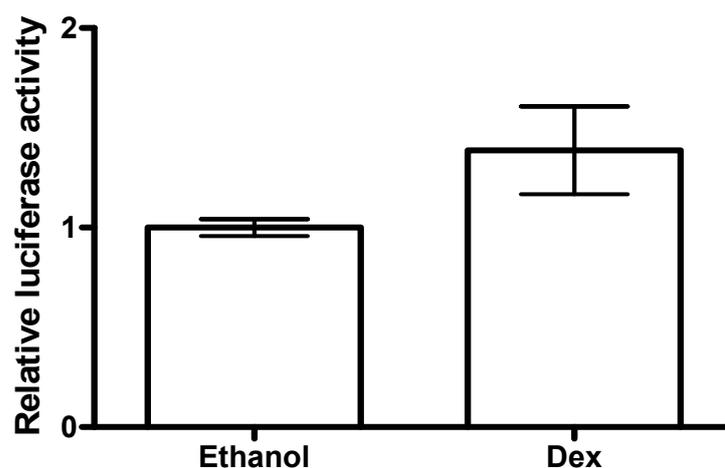


Figure 14. Transfected GnRH-R promoter-reporter construct, pLG induced with dexamethasone. L β T2 cells were transiently transfected with 1 μ g pLG construct. Transfected cells were then treated with 1 μ M dex or ethanol vehicle for 8 hours. Luciferase response was normalized to β -gal to control for transfection efficiency. Results are of 8 experiments performed either in duplicate or triplicate. Data was analysed by means of an unpaired t-test. $P > 0.05$.

3.1.2. Overexpression of exogenous rat GR increased the dex response of the GnRH-R promoter-reporter construct

The dex induction of the pLG promoter-reporter construct in L β T2 cells is small and the range of the experimental error was large as seen by others (74) as well as in the present study. Transiently transfected cells may not give a true indication of the endogenous response as the cellular context becomes altered with the addition of multiple copies of exogenous promoter constructs. The dex response may therefore be dependent on the levels of glucocorticoid receptor. Therefore a rat GR expression construct (pSTCGRN 795 wt) was cotransfected along with the pLG construct in an attempt to increase the response to dex.

Two amounts of rat GR expression construct (0.5 μ g and 1 μ g) were cotransfected in order to determine if this had an effect on the dex response. Cells were induced with either ethanol vehicle or 1 μ M dex for 8 hours, as before. Cotransfecting with 0.1 μ g pLG together with 0.5 μ g rat GR resulted in an increase in the dex response from 1.4 fold to 2 fold and cotransfecting with 1 μ g rat GR increased the dex response to 1.6 fold (compare Figure 14 to Figure 15). The fold dex response was altered when the amount of rat GR was varied, but cotransfecting 1 μ g rat GR increased the basal as well as dex response (Results not shown) compared to 0.5 μ g GR that resulted in the highest fold induction (Figure 15). The empty plasmid, PGL2 Basic, was used as an internal control to normalise the effect of transfecting increased plasmid DNA on the dex response. The ethanol and dex responses, for rat GR and pLG-cotransfected cells were normalised to the ethanol and dex responses of PGL2 Basic and pLG-cotransfected cells respectively. The dex response was then expressed relative to ethanol. The overexpression of rat GR resulted in a marginally better dex response and this result is consistent with a role for GR in mediating this response. The use of rat GR in a mouse cell line illustrates that the positive dex response is not necessarily species-specific.

The increased dex response was not significantly different from ethanol treated cells. Furthermore transiently transfected cells provide an artificial system to investigate cellular responses and this may further influence the discrepancies observed in this cell line. Limiting numbers of cellular factors may result in cells responding differently depending on the amount of plasmid transfected

into a cell. Passage number is also believed to play a role, as older passages tend to respond less to a given ligand (personal communication, Professor JP Hapgood).

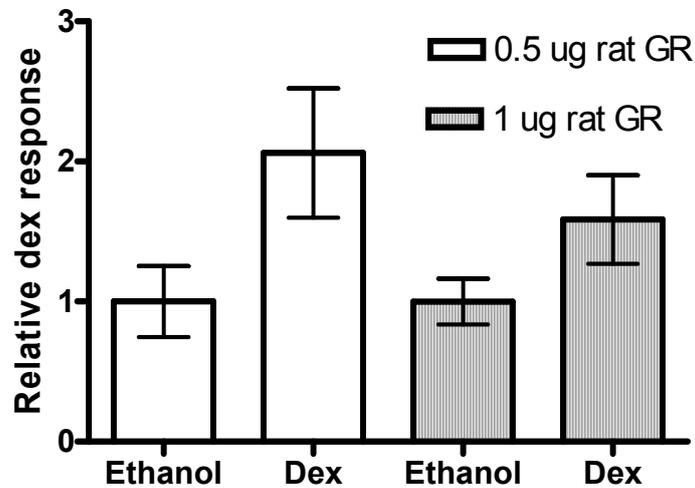


Figure 15. Dex induction of the GnRH-R promoter-reporter construct, pLG, cotransfected with different amounts of a rat GR expression vector. L β T2 cells were transiently transfected with 0.1 μ g pLG as well as 0.5 μ g or 1 μ g rat GR (pSTCGRN 795 wt). Cells were induced for 8 hours with either ethanol or dex. Pooled results of 2 experiments each performed in triplicate where ethanol was set as 1. Transfection efficiency was not determined. One-way ANOVA analysis and Bonferroni's post test was used to analyse the data. $P > 0.05$.

3.1.3. Continuous dex induction does not down-regulate GR protein levels in L β T2 cells

The homologous down-regulation of the GR by dex (Reviewed in 98) results in a decrease in GR levels. The small dex response (1.4 fold, Figure 14) seen with the pLG promoter-reporter construct over an 8 hour induction could be due to this down regulation of GR levels. Furthermore the cotransfected rat GR was constitutively expressed and an increased response was seen when the pLG promoter-reporter construct was dex induced. Therefore Western blot analysis of GR protein levels from L β T2 cell extracts was performed (Figure 16). Dex induced cell extracts did not have altered GR levels (lane 2, Figure 16) compared to ethanol treated cells (lane 1, Figure 16). β -actin protein levels were unaffected by ethanol or dex treatment (lane1 and 2, Figure 16, indicated by the arrow) and served as the loading control.

With so many different factors influencing the results of the transient transfections in the L β T2 cell line it was decided to proceed by investigating the transcriptional regulation of the endogenous GnRH-R gene via endogenous GR, by means of real time RT-PCR.

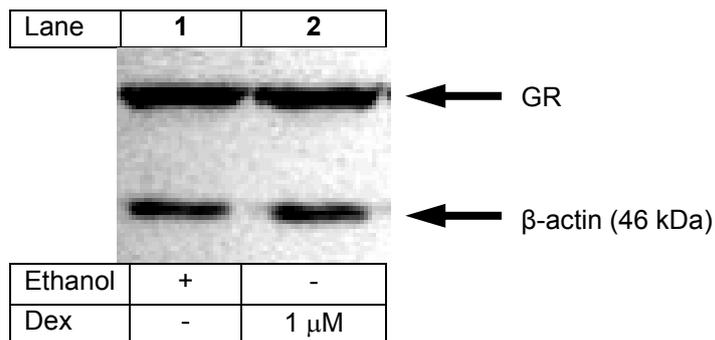


Figure 16. Western blot analysis of GR and β -actin levels. L β T2 cells were induced for 8 hours with ethanol or 1 μ M dex. Cell lysates were probed for with antibody specific for GR (1:2000) and β -actin (1:1000) proteins.

3.2. Dex upregulates transcription of the endogenous mouse GnRH-R in a dose-dependent manner

Although the promoter-reporter studies described above consistently showed a small increase in GnRH-R promoter expression in the presence of dex, this response was not statistically significant. This can be assessed when one compares expression of equal amounts of the β -gal expression vector transfected into COS cells versus L β T2 cells. COS cell lysates typically result in relative light units in the range of millions whereas those for L β T2 cells are in the range of thousands using the same transfection protocol and performing readings on the same luminometer (74, personal communication, Professor JP Hapgood). Thus the large error in the reporter assays may be due to poor and variable transfection efficiency. However, previous studies, carried out in L β T2 cells showed that the endogenous GnRH-R mRNA is significantly upregulation with dex treatment (11). Thus further studies were carried out on the endogenous GnRH-R promoter.

Initially Northern blotting was used in attempts to detect the GnRH-R mRNA. Although a 32 P radiolabelled GnRH-R cDNA probe with high specific activity was used GnRH-R transcripts could not be detected (results not shown). A possible reason for the inability to detect the mRNA could be because of a low abundance of these transcripts in L β T2 cells. The assay was instead replaced by the more sensitive RT-PCR assay.

3.2.1 L β T2 treatment and RNA isolation

L β T2 cells were induced with 1 μ M dex or vehicle as was done for the promoter-reporter assay, at various time-points, over a 24-hour time period including 30 minutes, 4-, 8-, 12-, 16- and 24-hours. The RNA was harvested as described in the Materials and Methods, Chapter 2, and the integrity thereof visualised on an agarose gel. Two clear RNA bands were detected, the 28S subunit and the 18S subunit (Figure 17) as expected. Eukaryotic ribosomes consist of two subunits: one large, containing the 28S rRNA, and a smaller subunit containing the 18S rRNA (30). The larger 28S subunit is present in a 2:1 ratio with the 18S subunit, and this was indeed seen in figure 17 for most samples. From figure 17 the absence of bands above the 28S band indicate the absence of contaminating genomic DNA or significant RNA degradation or indeed such low levels thereof that it is not visible on the agarose gel. Equal amounts RNA from each sample was then reverse transcribed to cDNA, as described in the Materials and Methods, Chapter 2. The cDNA was then further analysed by means of RT-PCR.

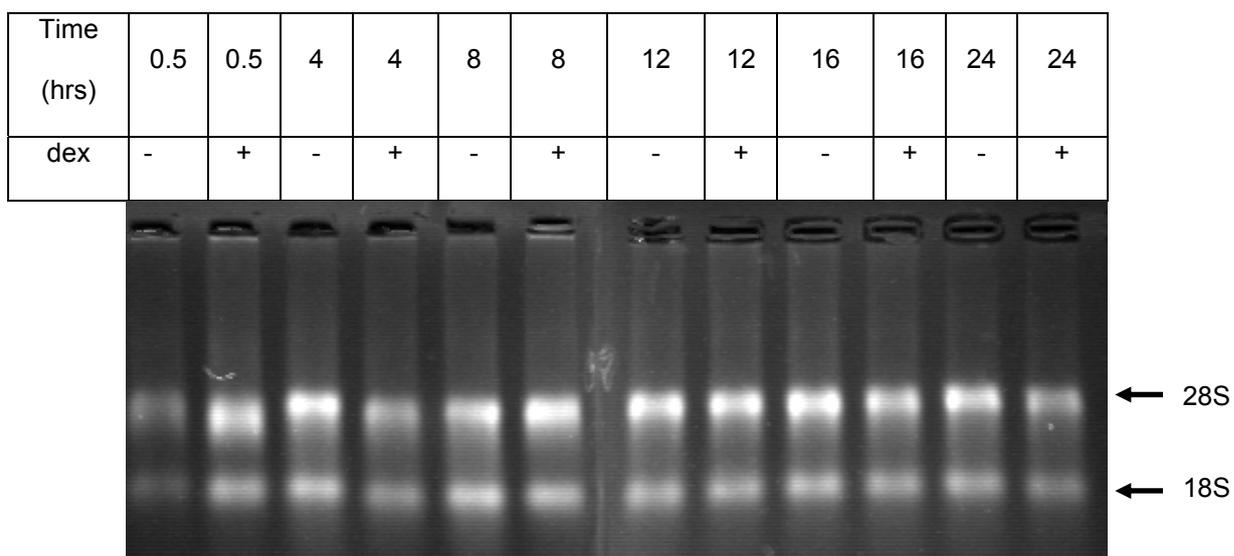


Figure 17. Visualising the integrity of RNA harvested from L β T2 cells. L β T2 cells were induced with ethanol (-) or dex (+) as indicated. RNA was harvested as set out in the Materials and Methods. A total of 2 μ g of RNA from each sample was electrophoresed on a 1 % agarose gel and RNA was visualised by ethidium bromide staining.

3.2.2. LightCycler® real-time quantitative RT-PCR

The efficiency for a PCR is normally assumed to be 2 where product is doubled with every PCR cycle. However, this depends on the efficiency of the primers and the PCR reaction and may therefore vary. The LightCycler® real-time quantitative RT-PCR allows for the differences of primer

efficiency to be taken into account and thereby a more accurate analysis is obtained. The PCR product can be visualised in real time as SYBR Green is incorporated into the PCR product and this fluorescence is then measured after each PCR cycle. A standard curve of samples of known concentration can be used to determine the efficiency of the primer sets.

3.2.3 Determining the efficiency of primer pairs

The cDNA was amplified using primers specific for the GnRH-R cDNA as designed by H Sadie (74) as well as for the mouse GAPDH gene (101). GAPDH has previously been used as a housekeeping gene in studies on L β T2 cells treated with GnRH (73) and is not regulated by dex (101). The GnRH-R sense primer S4 and antisense primer AS4 spans exon 2 and exon 3 to yield a 192 bp product (See Addendum A). Primers designed to span a large intron minimize amplification of contaminating genomic DNA resulting in a more accurate quantification. Primers for the GAPDH housekeeping gene yield a 113 bp product.

Vehicle treated RNA isolated from L β T2 cells, was used to generate standard curves for the respective primer sets by making a serial dilution series of the cDNA, and each was analysed in triplicate. Subsequent experiments were analysed in duplicate. The PCR protocol as part of the LightCycler® kit was adapted previously in our lab (99) and is described in the Materials and Methods, Chapter 2. The cDNA standard triplicates (as indicated by the coloured lines in Figure 18), have a similar PCR profile that confirms accurate pipetting, which plays an important role in reducing experimental error. The no template control (NTC) (Indicated by the black line in Figure 18) showed no product amplification, confirming that no primer self-amplification or possible contamination occurred.

The average cycle number, as determined from figure 18 by the LightCycler® software, was plotted against the log of the sample concentration to construct a cDNA standard curve for GAPDH (Figure 19 A) with a slope of -3.162 , and for the mouse GnRH-R (S4/AS4) primers (Figure 19 B) with a slope of -4.160 . The efficiency (E) was then calculated for the primer set by using the following equation (103):

$$(E) = 10^{[-1/\text{slope}]} \quad [1]$$

A new standard curve was created for each experimental repeat; as a result more than one E value was generated for the primer sets. This allows for experimental variation to be eliminated between subsequent repeats. The E for the GnRH-R primers was therefore between 2 and 2.07 while GAPDH was between 1.8 and 2.

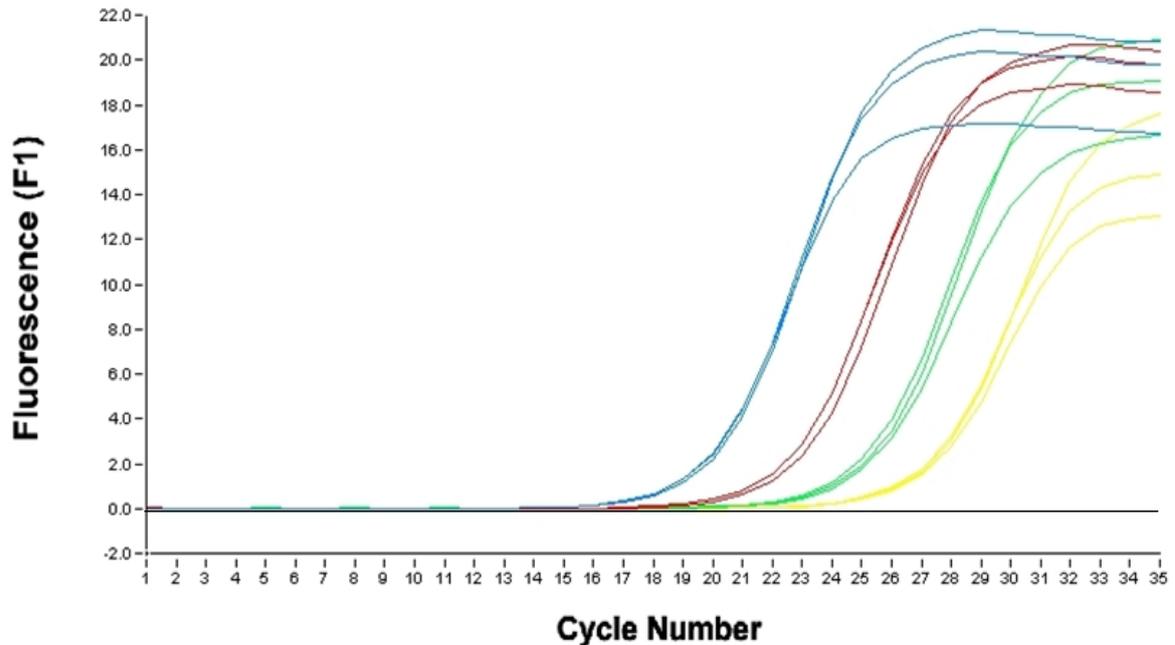
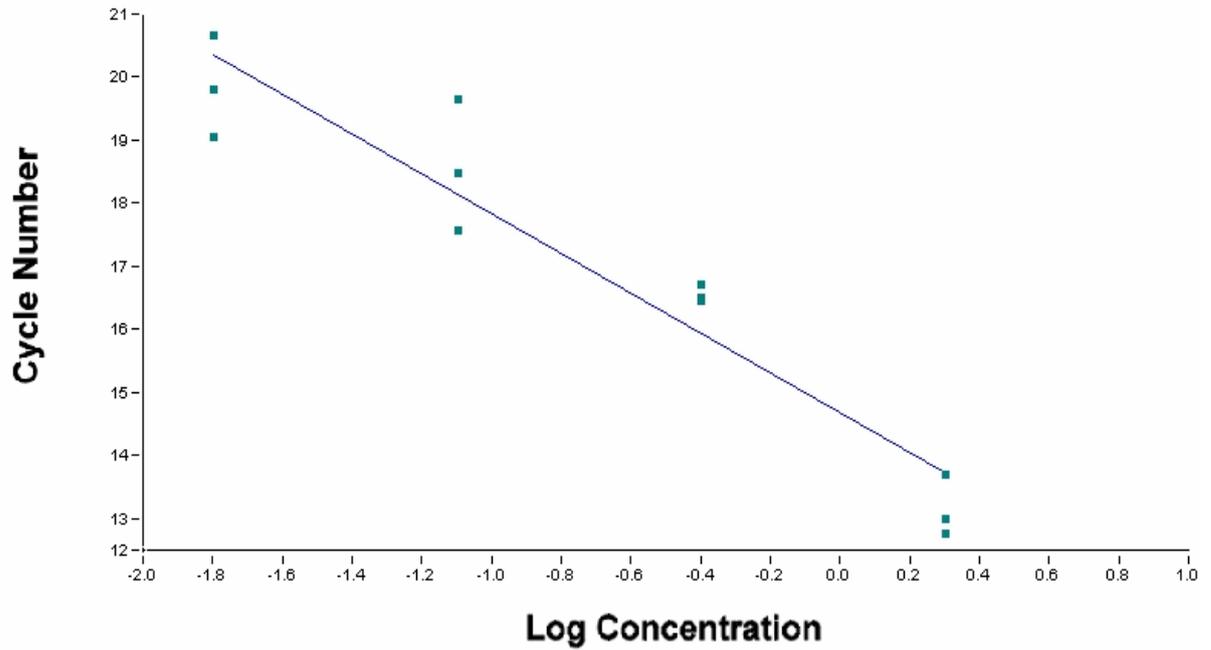


Figure 18. RT-PCR of a standard dilution series, amplified using the GAPDH primers. Samples were analysed in triplicate. The average cycle number (CP) was used to determine the relative expression. The blue lines represent 2 μg , red 0.4 μg , green 0.08 μg and yellow 0.016 μg . The no template control (NTC) is indicated by the black line. A similar dilution profile was created for the GnRH-R primers and is therefore not shown.

3.2.4 Relative expression

Once the primer efficiency was determined the dex and vehicle treated samples could be analysed. The amount of starting template determines how much product is formed, and this can be measured in terms of fluorescence. A typical graph, as seen in figure 20, has a linear range where the amount of input template is proportional to the PCR product (indicated by the dashed line). The crossing point (CP) of the sample fluorescence at this linear phase is measured as a cycle number and used to quantify the PCR product. When the plateau phase is reached the PCR product formation stops as components needed for the PCR step becomes depleted and is characterised by a flattening off of the fluorescence with subsequent cycles. Once the PCR reaches the linear range, the cycle number at that particular fluorescence can be used in equation 2 (103) to determine the relative expression (R):

A



B

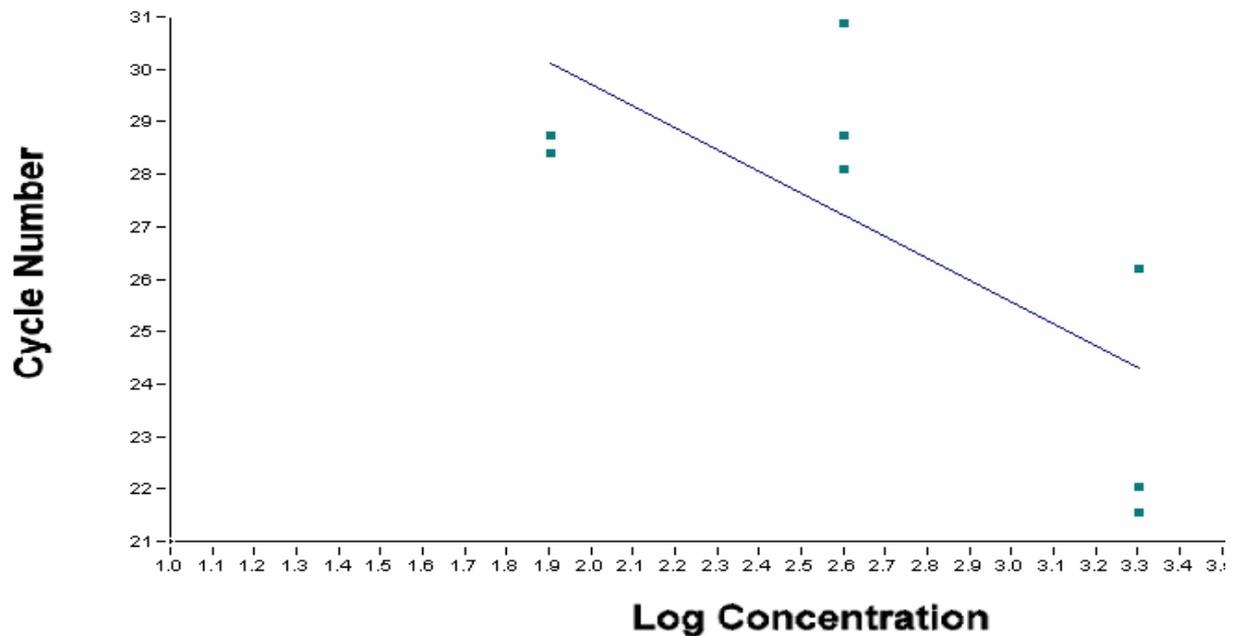
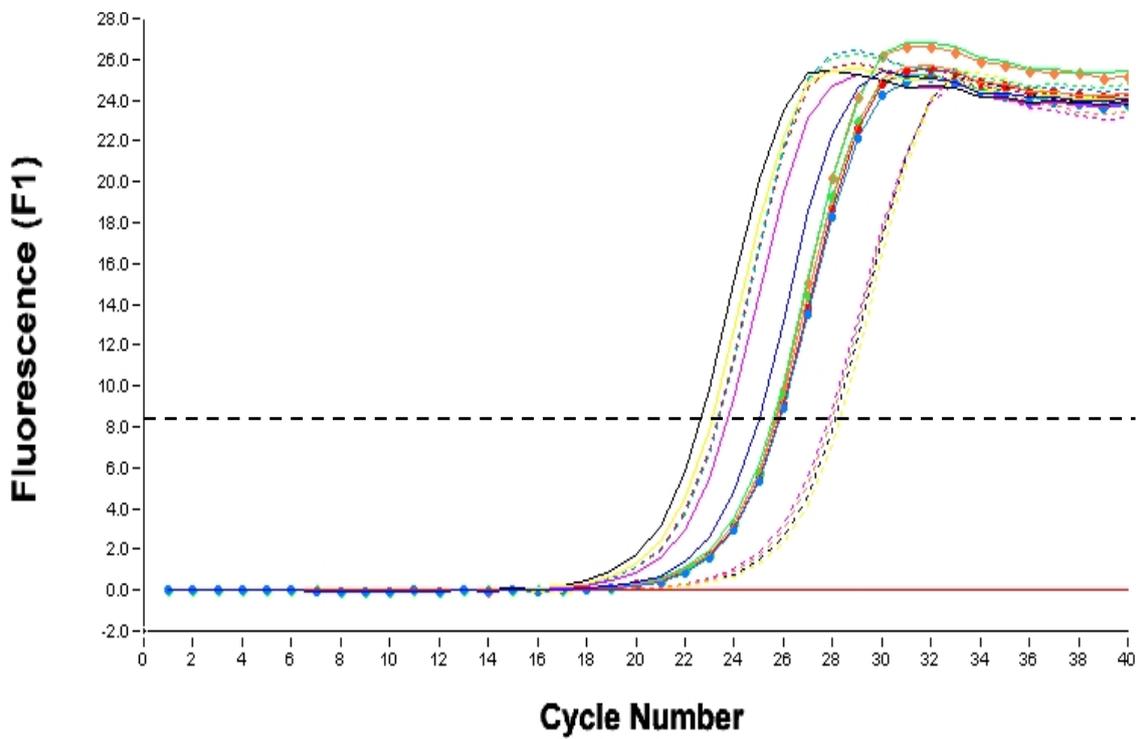


Figure 19. Standard curve showing cycle number versus log concentration of the cDNA amplified with the GAPDH primers (R^2 95) (A) and the mouse GnRH-R (S4/AS4) primers (R^2 72) (B). Template concentration was varied as follows: 2 μ g, 0.4 μ g, 0.08 μ g and 0.016 μ g and the cycle number obtained from figure 18. The slope obtained from the graph, -3.162, was used in equation [1]: $(E) = 10^{[-1/\text{slope}]}$ (103) to calculate the efficiency of the PCR. The slope of the mouse GnRH-R (S4/AS4) primers standard curve is -4.160.

A



B

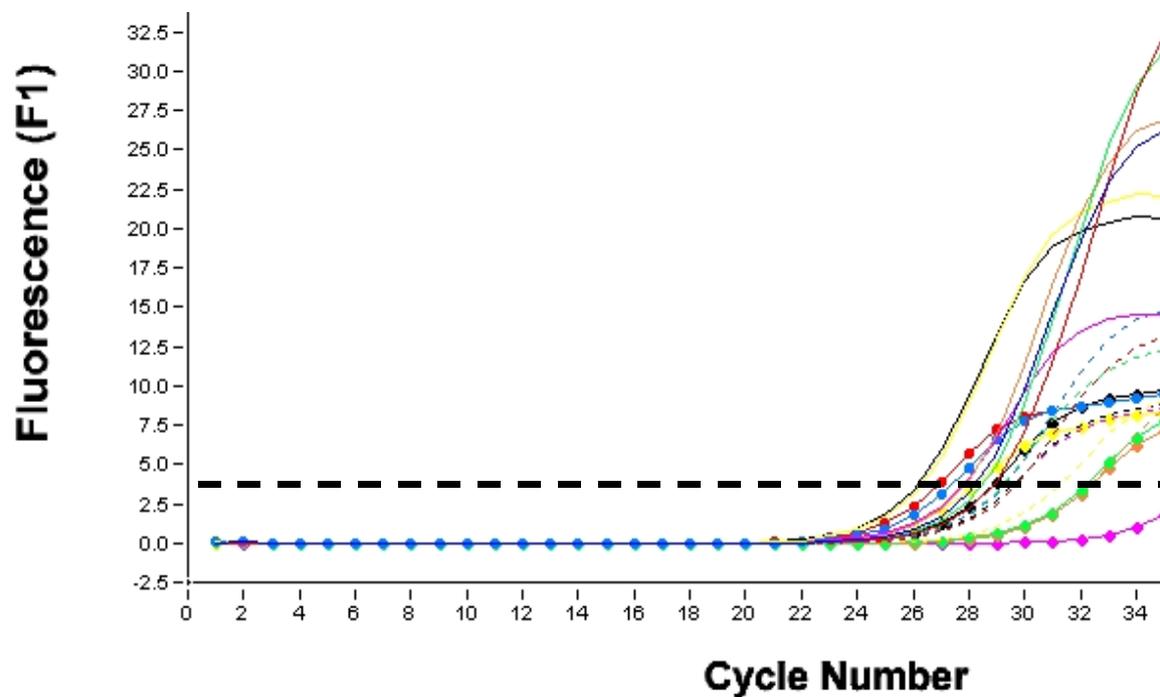


Figure 20. RT-PCR of cDNA samples amplified using the mouse GAPDH primers (A) and the mouse GnRH-R (S4/AS4) primers (B). The increase in PCR product is indicated as an increased fluorescence. The crossing point is chosen as the cycle number of the sample at a particular fluorescence where all the samples show linear amplification, as indicated by the dashed line. For simplification none of the samples have been labelled.

$$R = \frac{(E_{\text{target gene}})^{\Delta\text{CP}(\text{control} - \text{sample})}}{(E_{\text{reference gene}})^{\Delta\text{CP}(\text{control} - \text{sample})}} \quad [2]$$

The relative response (R) is therefore expressed as a ratio of dex (sample in equation 2) normalised to ethanol (control in equation 2) for the target gene (mouse GnRH-R promoter) after it has been normalized to the reference gene (GAPDH). ΔCP is therefore calculated by subtracting the average dex CP from the average ethanol CP. E is determined using equation 1, and this was done for every experimental repeat for which a standard curve dilution series was generated. A ratio of 1 means there is no difference between dex or ethanol treated cells for the GnRH-R primers normalised to the GAPDH housekeeping gene.

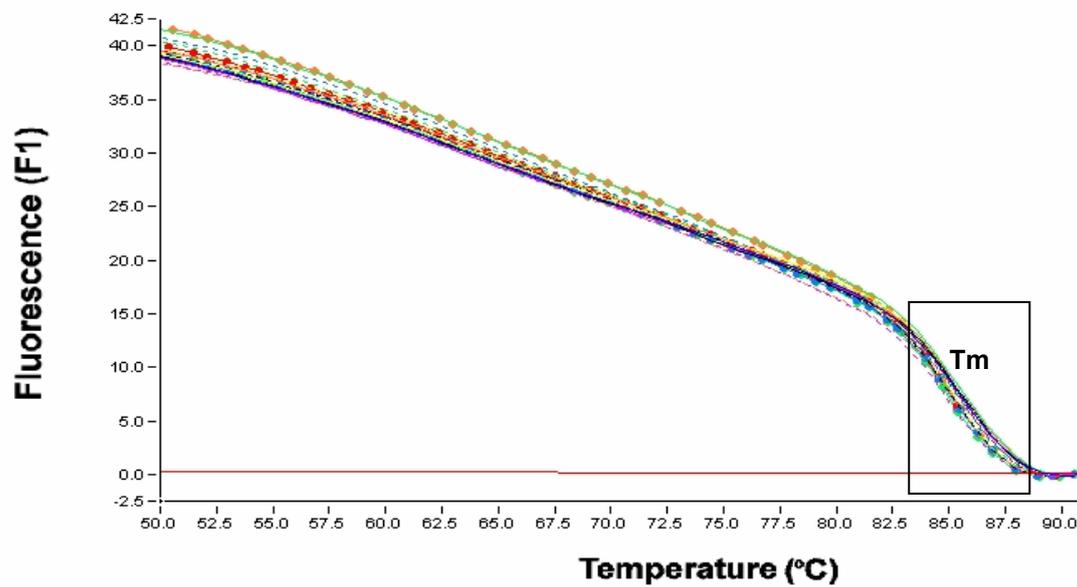
3.2.5 Product analysis

The PCR product was analysed for purity by means of a melting curve and melting peak generated by the LightCycler® as described in the Materials and Methods, Chapter 2. Fluorescent molecules incorporated into the GnRH-R primer PCR product are released as the double stranded DNA separates with increased temperature. The melting curve therefore indicates the decrease in fluorescence as the temperature is increased and is an indication of the purity of the PCR product as the PCR products should all have a similar melting curve profile, indicating a single product was formed.

The melting curve of the GAPDH and mouse GnRH-R (S4/AS4) primer products (Figure 21) demonstrates this decrease in fluorescence (y-axis) as temperature increases (x-axis). The point where the double stranded DNA separates (T_m) can be seen as a steep decrease in fluorescence (indicated by the square labelled T_m). The GAPDH no template control (NTC) does not display this melting profile (Figure 21 A) as no product was amplified (Figure 20 A). The mouse GnRH-R primer did amplify product in the no template control (NTC) as can be seen from figure 20 B. The amplification only occurs after 35 PCR cycles and primer self-amplification could possibly have occurred. The different amount of product formed can also be seen from figure 21 B, as the starting fluorescence is different for the samples. In contrast to this the GAPDH primer product (Figure 21

A) that is not regulated by dex displays not only a similar melting profile, but all samples have a similar fluorescence starting point.

A



B

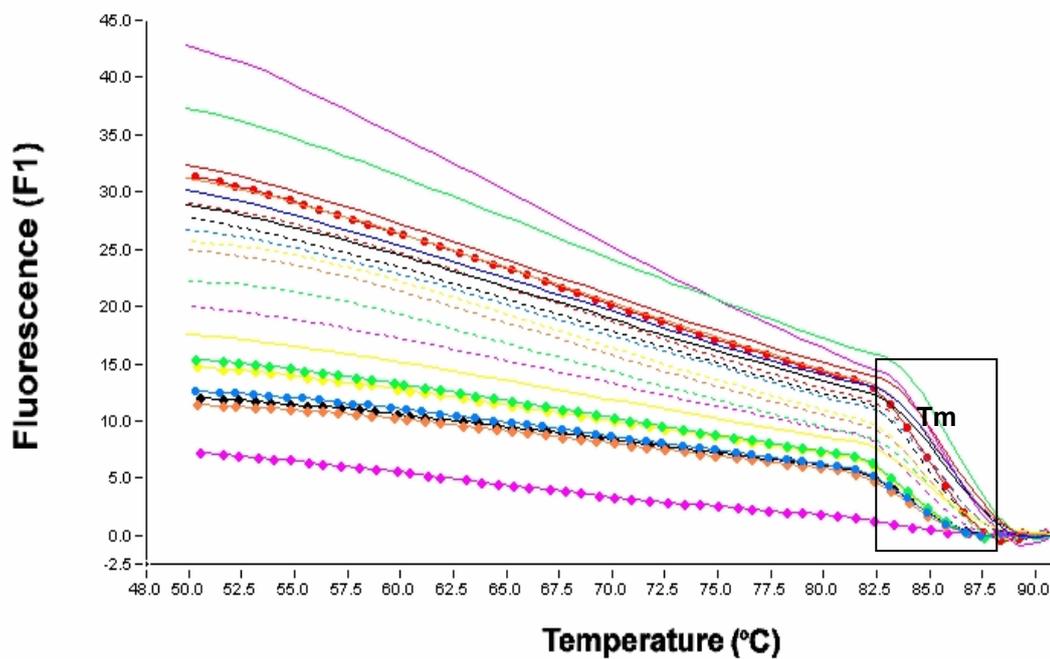


Figure 21. The RT-PCR melting curve for the GAPDH primer product (A) and the mouse GnRH-R (S4/AS4) primer product (B): A decrease in fluorescence is observed as the temperature is increased. The box labelled T_m indicates the melting temperature range for the samples. The no template control (NTC) is indicated by the red line (Figure 21 A) and the pink dotted line (Figure 21 B). For simplification none of the unknown samples have been labelled.

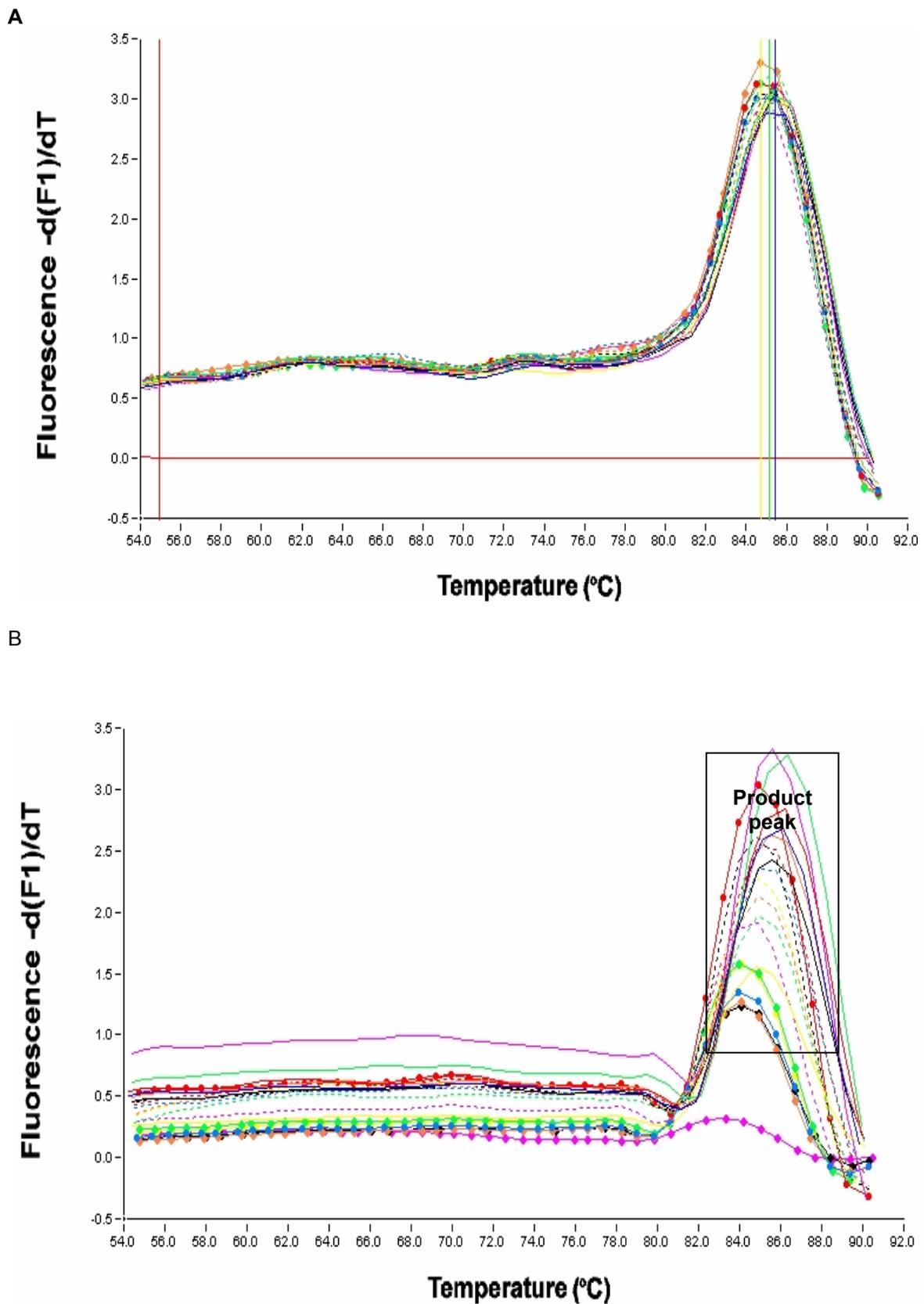


Figure 22. Melting peak of the mouse GAPDH PCR products (A) and the mouse GnRH-R (S4/AS4) primer PCR products (B) plotted as $-d(F1)/dT$. The vertical coloured lines indicate the melting temperature range for the GAPDH PCR products that ranged from 84.7 (yellow line) to 85.4 °C (blue line). The no template control (NTC) is indicated by the red line (A) and the pink dotted line (B). For simplification none of the unknown samples have been labelled.

Replotting the fluorescence, as $-d(F1)/dT$ (Figure 22 A and B) by the LightCycler® software, gives a melting peak. The presence of only one peak per sample confirms that only one product was amplified in the PCR reaction and no primer dimers were formed. The no template control (NTC) as indicated by the red line in figure 22 A and the pink dotted line in figure 22 B was not expected to peak at all, yet a small peak can be observed in figure 22 B due to the minimal product amplification observed in figure 20 B. Melting temperatures for the GAPDH product ranged between 84.7 – 85.4 °C and 84 - 86 °C for the GnRH-R product.

3.2.6. Normalising and processing of raw data

The gene transcript levels of dex treated cells were quantified and normalised to that of ethanol treated cells. This was further normalised to the housekeeping gene, GAPDH, as it is not under transcriptional regulation by dex (11,101).

Table 4. The cycle number determined for the crossing point (CP) for each sample when the mouse GnRH-R primers PCR products were present in equal amounts. Samples were analysed in duplicate and the average CP value used in equation 2 (page 45) (103) to determine the relative response. CP values shown are of one experiment performed in duplicate.

Time	Ligand	CP	Average CP
30 minutes	Ethanol	24.08	24.05
		24.01	
	Dex	23.18	23.26
		23.34	
4 hours	Ethanol	23.37	23.36
		23.34	
	Dex	21.29	21.70
		22.11	
8 hours	Ethanol	24.44	24.73
		25.01	
	Dex	22.83	22.75
		22.67	
12 hours	Ethanol	22.67	23.21
		23.10	
	Dex	21.40	21.42
		21.43	
16 hours	Ethanol	22.76	22.96
		23.17	
	Dex	21.07	21.23
		21.44	
24 hours	Ethanol	24.99	25.20
		25.42	
	Dex	22.66	23.38
		24.09	

The CP is chosen at a particular fluorescence where all the samples show linear amplification. An example of the cycle number obtained from the mouse GnRH-R primer set is indicated in Table 4 and that of GAPDH is shown in Table 5. The sample duplicates have CP values that are very similar, for example ethanol treatment for 30 minutes (Table 4) yielded CP values of 24.08 and 24.01, respectively. The differences in the duplicates depend on accurate pipetting of the sample. The same samples amplified by the GnRH-R primers were subjected to amplification with GAPDH primers (Table 5). GAPDH is ubiquitously expressed and the CP value was expected to remain unchanged, yet there was some variation, from 18.53 to 23.89 (Table 5), but this could be due to experimental error. The average CP values for GAPDH are within the same range, for the different incubation conditions.

Table 5. The CP determined for each sample of the GAPDH primers PCR products. Samples were analysed in duplicate and the average CP value was used in equation 2 (page 46) (103) to determine the relative response. CP values shown are of one experiment performed in duplicate.

Time	Ligand	CP	Average CP
30 minutes	Ethanol	19.28	19.34
		19.39	
	Dex	22.78	20.55
		18.31	
4 hours	Ethanol	18.16	18.85
		19.54	
	Dex	17.72	18.53
		19.33	
8 hours	Ethanol	20.92	22.74
		24.55	
	Dex	23.97	23.12
		22.27	
12 hours	Ethanol	20.88	20.75
		20.62	
	Dex	21.65	21.61
		21.57	
16 hours	Ethanol	20.36	20.32
		20.27	
	Dex	20.47	20.45
		20.42	
24 hours	Ethanol	20.17	20.13
		20.08	
	Dex	23.99	23.89
		23.78	

The relative response is then calculated for each time point using the average CP values and equation 2 (page 45) as shown below:

$$\begin{aligned}
 \text{Relative response (103) at 30 minutes} &= \frac{(2)^{(24.05 - 23.26)}}{} \\
 &= (1.8)^{(19.34 - 20.55)} \\
 &= 1.98
 \end{aligned}$$

The calculation was performed for all time points to obtain the relative dex response (R) over the 24 hour time period (Table 6). Three experimental repeats were performed (Table 6 and Figure 23). The L β T2 endogenous GnRH-R mRNA showed a clear time response to dex induction (Figure 24). At 30 minutes an average response of 1.2 fold was seen (Table 6). The average relative response at four hours increased to 1.9 fold and at 8 hours to 5 fold (Table 6). The time course was performed in triplicate yet individual results for the time points differ as internal (SYBR green age) and external (pipetting) factors play a role in the results (74). The response at 12 hours varied between experiments, as it decreased in experiment R2 (Table 6 and Figure 23), yet increased in R3 (Table 6 and Figure 23). Yet, at 12 hours, 1 μ M dex induced an average induction of about 7.4-fold (average of R1, R2 and R3 in Table 6 and Figure 24). Previously, expression of the transiently transfected mouse GnRH-R promoter in GGH₃ cells were maximally induced by 1 μ M dex after 12 hours (16). In the present study a 5.9 fold response was seen at 16 hours (average of R1, R2 and R3 in Table 6 and Figure 24) and it decreased at 24 hours to 4.9 fold (average of R1, R2 and R3 in Table 6 and Figure 24). It is unclear if the maximal induction occurred at 8 hours and the response reached a plateau, or if the maximal induction occurred at 12 hours after which the response decreased. Previously it was seen that the maximal response seen at 12 hours decreased to that of untreated cells over a 72-hour time period (16), which might not be evident at the 24 hours investigated here. Furthermore, previous studies demonstrated that dex induced the GnRH-R mRNA levels 5-fold in L β T2 cells (11), which is similar to the results in figure 24. This study shows that the expression of the endogenous GnRH-R gene is upregulated by dex. This regulation may be via a direct transcriptional effect, where the activated GR upregulates GnRH-R gene transcription directly. Alternatively, activated GR may upregulate another gene and this gene product could effectively increase the GnRH-R gene transcription.

Table 6: The relative dex response of L β T2 cells over various time points. The experiment was repeated three times, where R1 represents the results for experiment 1.

Sample	R1	R2	R3	Average
30 min	0.016	1.987	1.558	1.187
4 hours	0.163	2.784	2.753	1.900
8 hours	4.065	4.462	6.744	5.090
12 hours	4.127	3.299	14.85	7.425
16 hours	7.704	5.468	4.435	5.869
24 hours	4.340	4.211	6.214	4.922

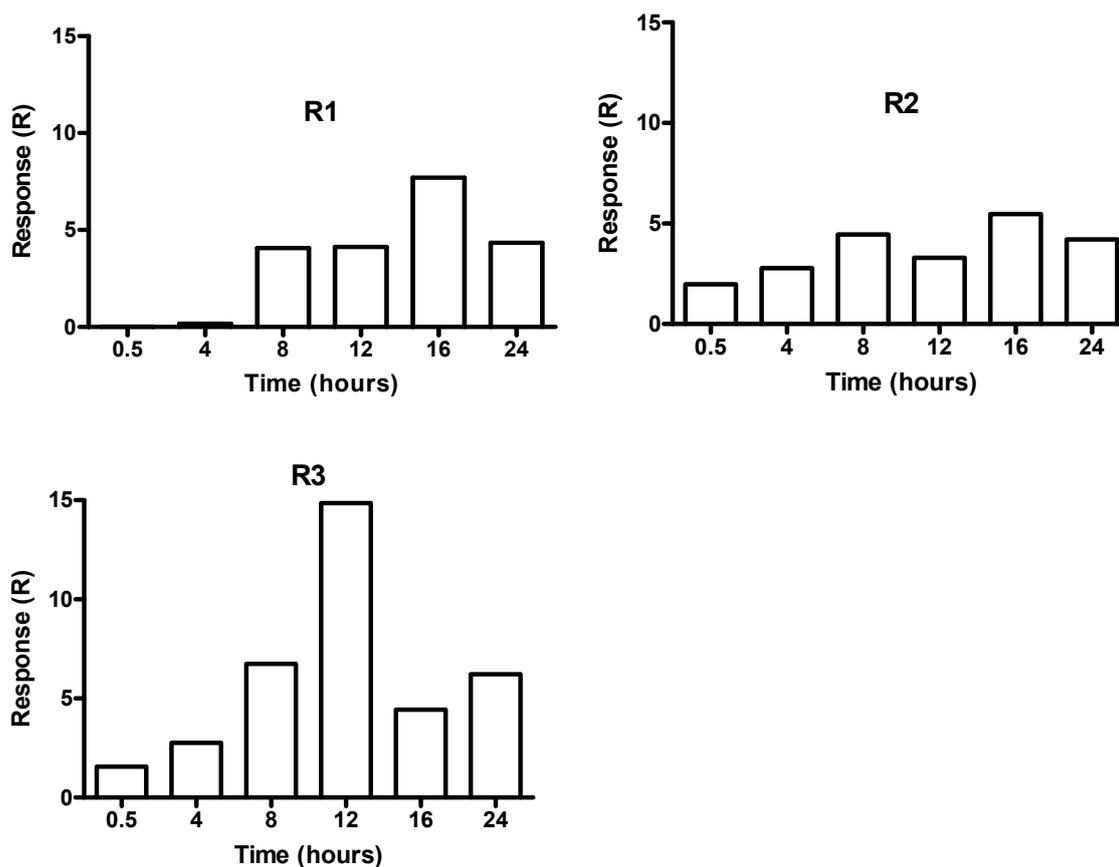


Figure 23. Response of dex treated L β T2 cells normalised to ethanol and GAPDH. The CP values for each sample were used in equation 2 to calculate the relative response (R). The experiment was repeated three times and the time response to 1 μ M dex is represented in separate graphs, R1, R2 and R3.

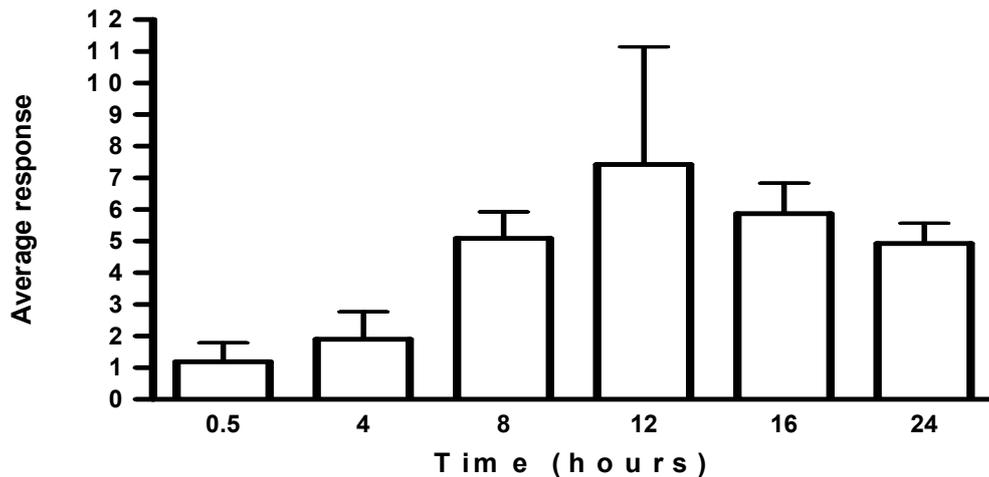


Figure 24. The average time response of endogenous GnRH-R promoter transcription. L β T2 cells were induced with 1 μ M dex as indicated. The average relative response for three repeats, R1, R2 and R3 (Table 6) is shown here. One-way ANOVA analysis and Bonferroni's post test was used to analyse the data. $P > 0.05$.

Therefore interaction of the GR with the GnRH-R promoter or associated factors, such as AP-1, could be responsible for the increase in endogenous GnRH-R mRNA. The presence of AP-1 proteins and GR in was then investigated in L β T2 nuclear extracts as the dex upregulation of the GnRH-R promoter is thought due to interaction of these transcription factors.

3.3. L β T2 nuclear proteins bind *in vitro* to the GnRH-R promoter region spanning -345/-290

Both an AP-1 and SF-1 site were investigated for their ability to bind L β T2 gonadotrope nuclear extracts as the dex response was shown to require the AP-1 binding site (99) of the mouse GnRH-R promoter. The potential SF-1 site was included as previous studies (99) implicated SF-1 in the basal regulation of the GnRH-R promoter and the full dex response could require this element as well. A double stranded oligonucleotide (oligo) of 7 bp containing the AP-1 binding site at -336/-330 (AP-1), or a larger oligo of 56 bp, AP-1/SF-1, containing binding sites for AP-1 and a potential SF-1 binding site, spanning -345/-290 relative to the mouse GnRH-R transcriptional start site, were used in the EMSA. These oligo's (sequences shown in Addendum A) were [γ ³²P]-radioactively labelled and used to identify binding proteins in unstimulated L β T2 nuclear extracts.

Binding conditions for AP-1 proteins differ from SF-1 binding conditions, therefore the assay conditions for the AP-1/SF-1 oligo were optimised. A series of parameters were varied in order to improve complex formation and visualization by clearing up background (Results not shown). This included varying the sample incubation time, incubation temperature and the cocktail composition (Addendum C2). Reducing the amount of probe added to each lane reduced the non-specific interactions and complex formation was improved (Results not shown). L β T2 nuclear protein interaction with AP-1/SF-1 was visualised in figure 25. Lane 1 (Figure 25) contains only the labelled AP-1/SF-1 probe to determine non-specific complex formation. Co-incubating nuclear extract and probe, without unlabeled competitor, resulted in the formation of at least 5 complexes (lane 2, Figure 25). The addition of excess unlabeled AP-1/SF-1 oligo resulted in the loss of complexes I, II and III (lane 3, Figure 25). Excess unlabeled AP-1 oligonucleotide on the other hand, partially competed away complex I and II (lane 4, Figure 25). Complex I and II returned when the mutated AP-1 oligonucleotide was used (lane 5, Figure 25). L β T2 gonadotrope cell nuclear extracts therefore contain proteins that specifically bind the regions spanning AP-1 (-336/-330) and AP-1/SF-1 (-345/-290) as was seen when complexes I, II and III were competed away by excess unlabelled oligo's. Complexes IV and V are most likely non-specific as lane 1 (Figure 25) containing only free probe have these complexes, and these complexes were not competed away by excess unlabeled probe.

Previous studies by others carried out in the GGH₃ cell line showed that nuclear extracts, stimulated with dex, were able to bind oligo's that contain the AP-1 site. Mutating this AP-1 site resulted in a loss of binding (16). The proteins within the oligo - nuclear extract complexes were identified using antibodies.

3.3.1. Proteins bound to the AP-1 oligonucleotide contain c-Fos and GR

The purpose of this experiment was therefore to identify AP-1 proteins, as well as other possible transcription factors from L β T2 cell extracts that interact with the AP-1 and AP-1/SF-1 mouse GnRH-R promoter sites. The addition of GR antibody decreased the intensity of the apparent non-specific complex IV (Figure 26) resulting in the formation of a supershift (lane 2, Figure 26).

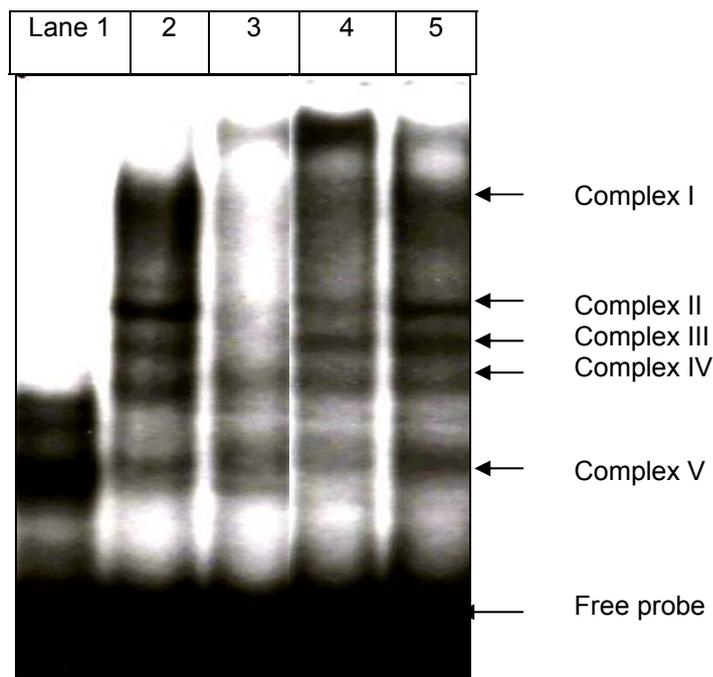


Figure 25. Electrophoretic mobility shift assay, showing unstimulated L β T2 nuclear extract complex formation. All lanes contain 0.5 μ l labelled AP-1/SF-1 probe and lane 2 to 5 included the addition of 1.5 μ l nuclear extracts. A 100 fold molar excess of unlabeled competitor: AP-1/SF-1 (lane 3), AP-1 (lane 4) and mutated AP-1 (lane 5) was added.

Complex IV was not competed away by either AP-1/SF-1 or AP-1 oligo (lanes 3 and 4, Figure 25) possibly indicating that the GR does not interact directly with these binding sites. The addition of c-Fos antibody created a supershift of complex I (Figure 26, lane 3) indicating that complex I, competed away in the competition assay (lane 4, Figure 25), contains c-Fos proteins. The presence of c-Fos in L β T2 cells was thought to be dependent on GnRH induction (104). In the present experiment c-Fos was indeed present in unstimulated L β T2 cell extracts (lane 3, Figure 26). Previously the presence of Jun (74) and Fos (16, 74) proteins was shown by western blot analysis of GnRH induced L β T2 cell extracts and Fos and Jun did bind the AP-1 element in vitro as deduced from EMSAs (74).

From this experiment it is clear that unstimulated L β T2 nuclear extracts contain c-Fos and GR proteins and the positive dex response may therefore be mediated via these transcription factors, as glucocorticoid regulation has been associated with the protein-protein interaction of the GR and AP-1 proteins (18,20), whereas the type of regulation depends on the cellular contexts (44).

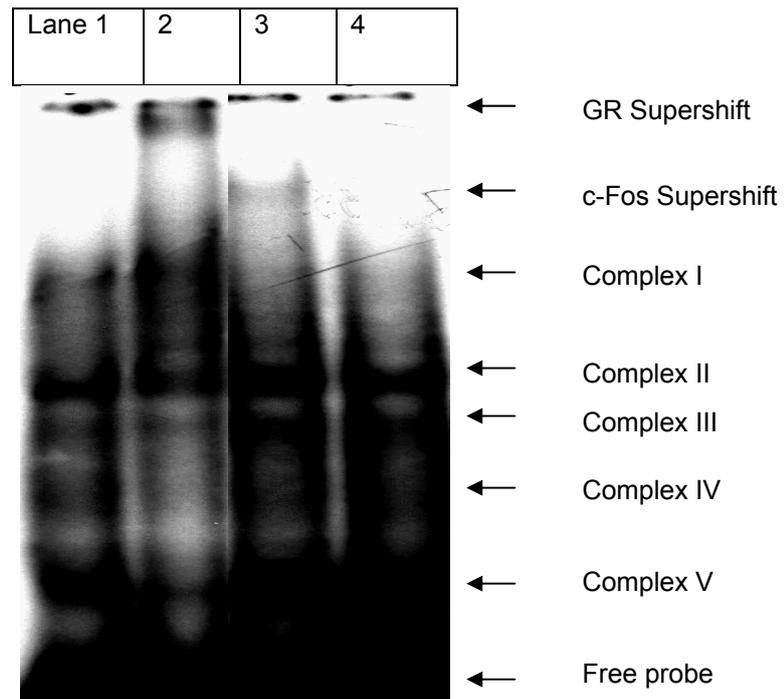


Figure 26. Electrophoretic mobility shift assay identifying proteins within unstimulated L β T2 nuclear extract complexes. Each lane contains 0.5 μ l labelled AP-1/SF-1 probe. Lane 1 contains unstimulated L β T2 nuclear extracts without antibody. Lane 2 contains GR antibody. Lane 3 the c-Fos (AP-1) antibody. Lane 4 contains pre-immune serum. A volume of 1 μ l of the respective antibody was added to each sample and incubated for 10 min before electrophoresing.

3.4. GR binds to the AP-1 site in response to dex treatment in whole cells

GR tethering to a region of the mouse GnRH-R promoter region where AP-1 binding occurs, was investigated *in vivo* in the L β T2 cell line as the GR could possibly interact with the Jun or Fos AP-1 components to regulate transcription. The GR interaction with the c-Jun DNA binding domain has previously been shown to interfere with the DNA binding activity (22). Interestingly c-Jun has been implicated in GnRH-R gene regulation by interacting with the GR (16) but from figure 26 it is clear that L β T2 cells contain the c-Fos AP-1 subunit.

3.4.1. GR binds to the AP-1 region in the GnRH-R promoter in whole cells in response to dex treatment

The chromatin immunoprecipitation (ChIP) assay is a novel way of investigating the spatial and sequential *in vivo* interaction of protein complexes with a promoter area of interest (105). The assay is described in the Materials and Methods, Chapter 2, but a brief description follows: Cells

were induced with 100 nM dex or ethanol for 1 hour and *in vivo* cross-linking was performed by the addition of formaldehyde. The formaldehyde cross-linking is very effective as it easily penetrates living cell membranes to bind molecules closely associated, and crosslinking is easily reversed (105). Formaldehyde crosslinking occurs due to the dipolar characteristic of this compound that forms a Schiff's base with the amino group of lysine, as well as adenine, guanine and cytosine side chains^a. Cells were collected and lysed after which the chromatin-protein complexes are sheared into fragments shorter than 500 bps by means of sonication.

These DNA fragments serve as template for the subsequent PCR step and it was therefore important to ensure optimal sonication was achieved. Determining the optimal sonication conditions was a lengthy process as each sonicator yields different results. Therefore cell lysates were subjected to a range of differing combinations of sonication conditions and the fragmentation thereof compared to determine the actual sonicator settings required. Altering the pulse frequency at which energy is released into the lysate or the power output of the sonicator probe allows for the sonication conditions to be varied. It was essential to ensure the lysate did not heat up, as protein-DNA crosslinks would be reversed. Optimally sonicated chromatin is visualised in figure 27 (and the sonication conditions described in the figure legend); the majority of the chromatin fragments are smaller than 500 bps but not smaller than 100 bps.

Once the chromatin shearing was optimised the functionality of the primers were confirmed using L β T2 genomic DNA in the PCR reaction. The PCR conditions were optimised to ensure the best possible product formation, for this reason the MgCl₂ concentration was varied (results not shown) and it was found that 3 μ M MgCl₂ results in the highest PCR product yield. Subsequent PCR reactions were carried out using 3 μ M MgCl₂ per sample cocktail.

An aliquot of the sonicated lysate, or input, is removed before immunoprecipitation. This input was used as a positive control in the PCR assay. The remaining sonicated lysate was immunoprecipitated overnight with GR antibody. The antibody-chromatin complexes were pulled down with agarose beads and unbound chromatin and proteins removed by buffered wash steps.

^a Jason Matthews, Department of Biosciences, Karolinska Institutet. CHIP assays to study Transcriptional activation. (Lecture) 10 November 2005.

The chromatin crosslinks were reversed by maintaining the samples at 65°C overnight. The fragments were purified from the mixture using the QIAquick® PCR Purification Kit (QIAGEN).

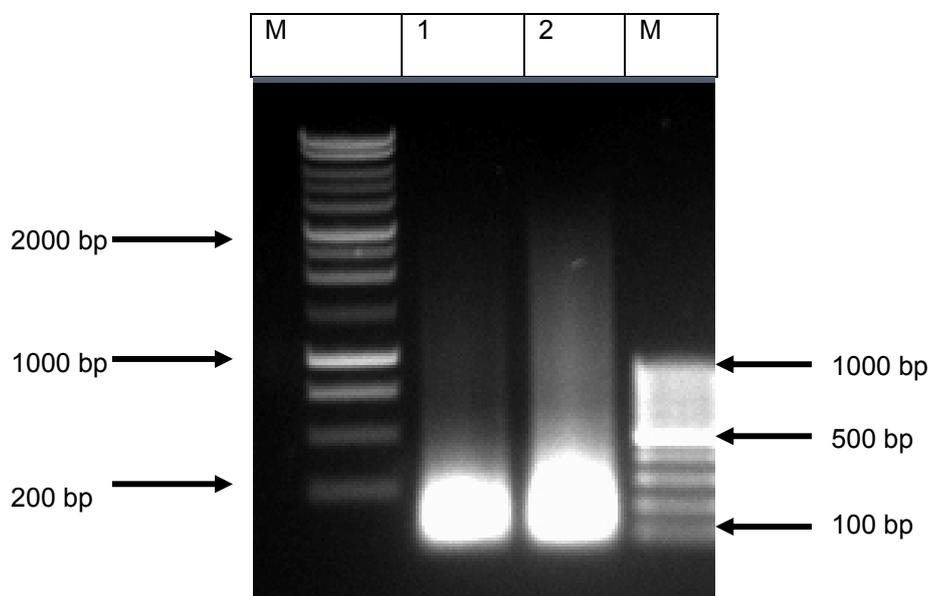


Figure 27. Confirming optimal L β T2 chromatin shearing. Cells were induced for 1 hour with ethanol (lane 1) or 100 nM dex (lane 2). Lysed cells were sonicated at power output 30, for 20 seconds and incubated for 40 seconds and repeated for 10 cycles. Chromatin fragments were electrophoresed on a 1% agarose gel and visualised by ethidium bromide staining. The fragment size was determined from the markers (M).

The purified ChIP DNA fragments immunoprecipitated with GR antibody, were amplified by conventional PCR. The SURG-2 primers span the GnRHR promoter region –337/–170 relative to the transcriptional start site. The SURG-2 primers include the SURG 2 binding site (Refer to Figure 12), and the AP-1 binding site. The product size (167 bp fragment size) as well as the quantity of PCR product amplified was visualised on an agarose gel (Figure 28 and 29). Equal volumes of each PCR product were electrophoresed and the differences in band intensity compared. Input DNA had a high PCR product yield and there is no difference between ethanol (lane 1, Figure 28) and dex (lane 2, Figure 28) induced L β T2 cells. The dex treated L β T2 cells, after immunoprecipitation gave a higher PCR product (lane 4, Figure 28) compared to ethanol treated cells (lane 3, Figure 28). This indicates GR enrichment of the GnRH-R promoter area spanning –337/–170 upon dex treatment but not ethanol treatment. The no template control (lane 5, Figure 28) confirms that the product formation was due to template amplification. Only one band is seen per lane (Figure 28) indicating single product amplification and no primer dimer formation.

The samples were then PCR amplified using primers to SF-1 (-151/-7) as a negative control (Figure 29). Loading equal amounts of the respective PCR products allowed for a comparison of GR immunoprecipitated fragments induced with either ethanol or dex. Lanes 1 and 2 (Figure 29) contain the input DNA induced with ethanol and dex respectively. The inputs are not immunoprecipitated and therefore the SF-1 product (143 bp) is clearly visible in lanes 1 and 2 (Figure 29). Unfortunately primer dimer formation occurred, and is evident in all lanes as a band less than 100bps in size, compared to the SF-1 product of 143 bps (indicated by the arrow in Figure 29). Furthermore no product was seen in lanes 3 or 4 (Figure 29) irrespective of ethanol or dex treatment indicating no GR is associated with the SF-1 region. Lane 5 (Figure 29) represents the no template control, and except for the primer dimer, no product is seen.

To further confirm this result the samples were subjected to real time RT-PCR. This is a far more sensitive method to quantify the relative levels of immunoprecipitated fragments that does not rely on gel electrophoresis and accurate loading thereof to differentiate between samples. The SURG-2 primers (-337/-170) were once again used to amplify input and immunoprecipitated fragments. Table 7 gives the relative crossing point (CP) values determined for the RT-PCR products. Once immunoprecipitation takes place only fragments associated with the GR remains and can be amplified. The CP value is therefore a comparison of the amount of product formed relative to all samples. The lower the CP the more product was formed (refer to the discussion of RT-PCR, section 3.2.4 Relative expression). The values for the inputs are similar, ethanol: 22,82 and dex: 22,42; and quite low indicating a high RT-PCR product yield. The IP dex product has a lower CP value (29.60) than the IP ethanol product (33.63) confirming the result shown in figure 29 (lane 3 and 4).

The raw data was analysed as follows:

$$\frac{(\text{ethanol CP})}{(\text{dex CP})}$$

$$\frac{(\text{input ethanol CP})}{(\text{input dex CP})}$$

The dex response is measured relative to the ethanol treated samples as the IP of samples with GR antibody prevents the use of a housekeeping gene as a control for the RT-PCR assay. The fold

increase of fragment association with the GR is 12.4 fold higher when cells were induced with dex than when induced with ethanol.

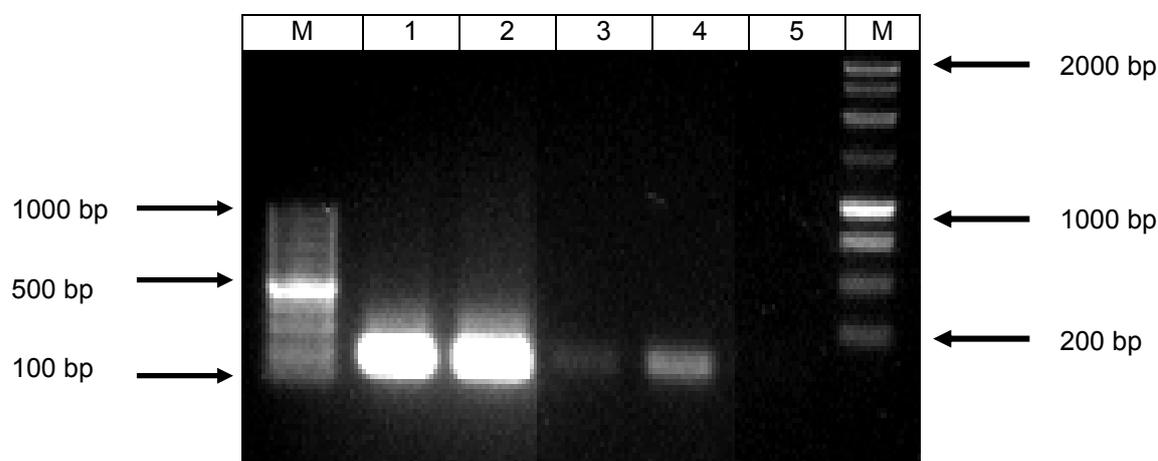


Figure 28. PCR amplification of GR immunoprecipitated chromatin fragments using SURG-2 primers. L β T2 cells were induced for 1 hour with ethanol (lane 1 and 3) or 100 nM dex (lane 2 and 4). A volume of 25 μ l of each PCR product was electrophoresed on a 1% agarose gel. Input chromatin was used as template for the PCR products in lane 1 and 2, whereas lanes 3 and 4 immunoprecipitated chromatin was used as PCR template. No product was amplified in lane 5, the no template control. The fragment size was determined from the markers (M).

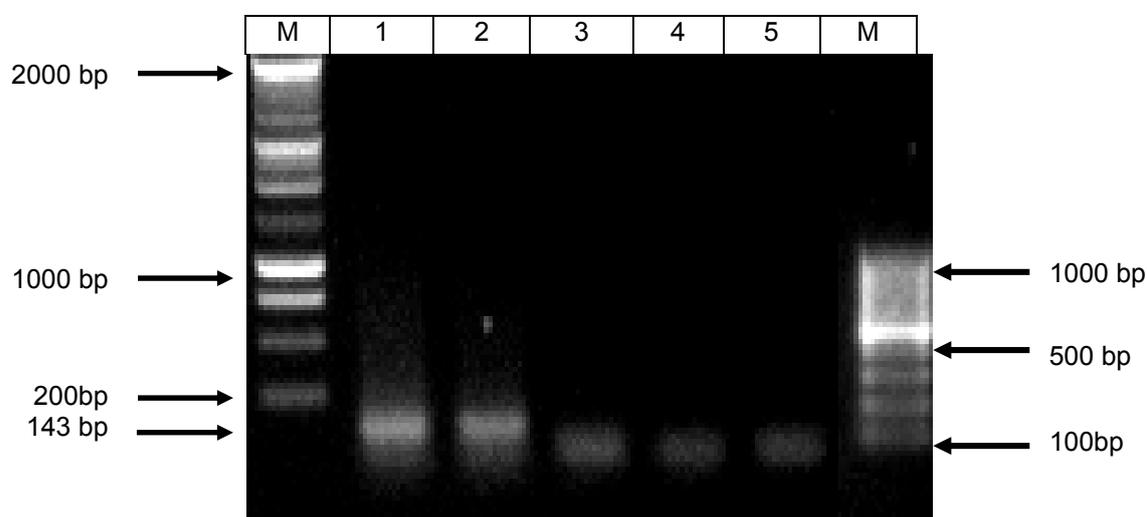


Figure 29. PCR amplification of GR immunoprecipitated chromatin fragments using SF-1 primers. L β T2 cells were induced for 1 hour with ethanol (lane 1 and 3) or 100 nM dex (lane 2 and 4). A volume of 25 μ l of each PCR product was electrophoresed on a 1% agarose gel. Input chromatin was used as template for the PCR products in lane 1 and 2, whereas for lanes 3 and 4 immunoprecipitated chromatin was used as PCR template. Lane 5 is the no template control. The PCR product size (143 bps) was confirmed from the markers (M). The samples were prepared and electrophoresed by Andrea Kotitschke.

The ChIP assay needed to be set up in the laboratory and various steps needed to be optimised. Once the conditions were determined the response was lost and many months were spent altering parameters in order to regain this. It was discovered that the antibody used for the immunoprecipitation step was responsible for the variable results. This was corrected but unfortunately I was unable to repeat more ChIPs. Due to the time constraints only one successful experiment performed by the candidate is shown and discussed (Figure 28, 29 and Table 7). Andrea Kotitschke, (a PhD student of the same laboratory) was, however, able to successfully repeat the ChIPs and a reproducible GR enrichment was obtained at the L β T2 promoter region spanning –337/–170 when induced with dex.

Table 7. RT-PCR amplification of GR immunoprecipitated chromatin fragments. The SURG-2 primers were used to amplify the chromatin fragments as described for RT-PCR in the materials and methods.

Sample	CP value
Input ethanol	22.82
Input dex	22.42
IP ethanol	33.63
IP dex	29.60

CONCLUSION

Previously a transfected promoter-reporter construct of the rat GnRH-R gene in the pituitary somatolactotrope cell line, GGH₃, was shown to be under transcriptional regulation by GCs (16). The mechanism of this upregulation appeared to be mediated via the AP-1 binding site, as the dex response was lost when the AP-1 site (-336/-330) was mutated (99). The results presented in this thesis indicate that dex upregulates transcription of the transfected GnRH-R promoter fragment (Figure 14) in a promoter-reporter assay and shows conclusively that this occurs at the endogenous mouse GnRH-R gene (Figure 24) in a pituitary gonadotrope cell line, L β T2. Dex induction of a transiently transfected GnRH-R promoter fragment in L β T2 cells resulted in a 1.4 fold dex response (Figure 14). The response in this cell line is small and falls within the range of error and statistical significance could not be determined. Differences in the amount of exogenous rat GR transfected influenced the dex response (compare 0.5 μ g rat GR to 1 μ g rat GR, Figure 15). Overexpressing rat GR together with this GnRH-R promoter-reporter fragment resulted in a 2 fold (0.5 μ g rat GR) and 1.6 fold (1 μ g rat GR) increase in dex induction (Figure 15). The small dex response seen in the pLG promoter reporter construct was thought to be due to the homologous down-regulation of GR by dex (Reviewed in 106). The rat GR promoter-reporter construct (pSTCGRN 795 wt) is not subject to this down regulation by dex as it was constitutively expressed, and an increased response was seen when cotransfected with the pLG promoter-reporter construct and induced with dex. Western blot analysis revealed that GR levels are not altered by 8 hours dex induction, compared to ethanol treatment (Figure 16) and is therefore not the reason for the small dex response seen in L β T2 cells. A possible explanation for the big endogenous response compared to the weak response seen in the reporter-promoter assay might be due to the involvement of *cis*-elements, which are not present in the promoter-reporter fragment. Furthermore the chromatin could influence the magnitude of the endogenous response as the upstream organization and structure of the chromatin could promote recruitment of remodeling proteins.

The effect of dex on the regulation of the endogenous GnRH-R promoter was investigated by means of real time RT-PCR. This assay is very sensitive and as it investigates the endogenous gene it is more reliable. A time response curve of the GnRH-R transcription to 1 μ M dex was for the

first time to our knowledge, established in L β T2 cells with maximal induction seen between 8 and 16 hours (5- and 5.9-fold respectively, Figure 24). This is in agreement with the literature where dex induced the GnRH-R mRNA levels 5-fold in L β T2 cells (16,11). Furthermore in the somatolactotrope cell line GGH₃ GnRHR expression was maximally induced by 1 μ M dex after 12 hours (16).

Unstimulated L β T2 nuclear extracts were previously shown to bind the AP-1 region (-336/-330) of the mouse GnRH-R promoter (74,99). This is in agreement with the result seen in figure 25. The proteins that bind to the AP-1 fragment were identified as c-Fos and GR (Figure 26) and these results are in agreement with the hypothesis that the dex response is mediated via the GR directly interacting with the AP-1 transcription factor. Fos contains a transactivation domain (23,31,34) and GR activation may very well result in transcriptional upregulation of the gene of interest although some studies have found that high levels of Fos mediate negative GC regulation (41). Previously, Jun and Fos proteins were identified in L β T2 cell extracts, and these proteins were shown to interact with the AP-1 binding region in GnRH induced L β T2 cells, *in vitro* (74). In the anterior pituitary corticotroph cells AtT-20, the AP-1 composition depends on hormonal treatment such as dex (107) and the presence of c-Fos in L β T2 cells was thought to be determined by GnRH induction (104). In the present study Fos protein was detected in uninduced L β T2 cell extracts. Since no GRE is present in the GnRH-R promoter it is likely the GR interacts with Jun (40) and the presence of c-Fos (74, present study) implies the presence of the Fos/Jun heterodimer in L β T2 cells, although previously dex was shown to suppress the heterodimer activation in a mouse proliferin gene promoter fragment (41). The outcome may be promoter-specific or cell-specific and depends on the presence of the *cis*-elements and transcription factors.

The direct interaction of the dex activated GR with AP-1 was investigated *in vivo* in L β T2 whole cells. GR interaction with the AP-1 binding region of the mouse GnRH-R promoter in L β T2 cells was indeed enriched upon dex induction (lane 4, Figure 28). Real time RT-PCR allowed for a relative quantification of these ChIP fragments, whereby dex induction resulted in a 12.4 fold enrichment of GR associated with AP-1 fragments, as compared to ethanol treated cells. This is the first time an upregulation by dex of the endogenous mouse GnRH-R gene was demonstrated

by tethering of the GR to the AP-1 binding region in L β T2 cells. This interaction could prove to be yet another means of hormonal regulation of the mouse GnRH-R gene in the L β T2 pituitary gonadotrope cell line.

The ChIP result was not reproducible in the candidate's hands. Using the particular antibody did however produce reproducible results (Andrea Kotitschke, a PhD student of the same laboratory). The receptor-AP-1 interaction is possibly not very stable and may require additional cofactors. This is illustrated in figure 30. The bound AP-1 transcription factor possibly interacts with transcription machinery and an unknown associated factor (factor Y, Figure 30) may promote tethering to GR. Furthermore co-factors that interact with both the GR and AP-1 (represented as factor X, Figure 30) could promote transactivation, these include SWI.SNF components and CBP/p300: A component of the SWI.SNF chromatin-remodelling complex, BAF60a, has high binding affinity to c-Fos/c-Jun and is required for GR transactivation in yeast (33). The Brm subunit of SWI.SNF is required for c-Fos/c-Jun transactivation and interacts with GR as well (19,33).

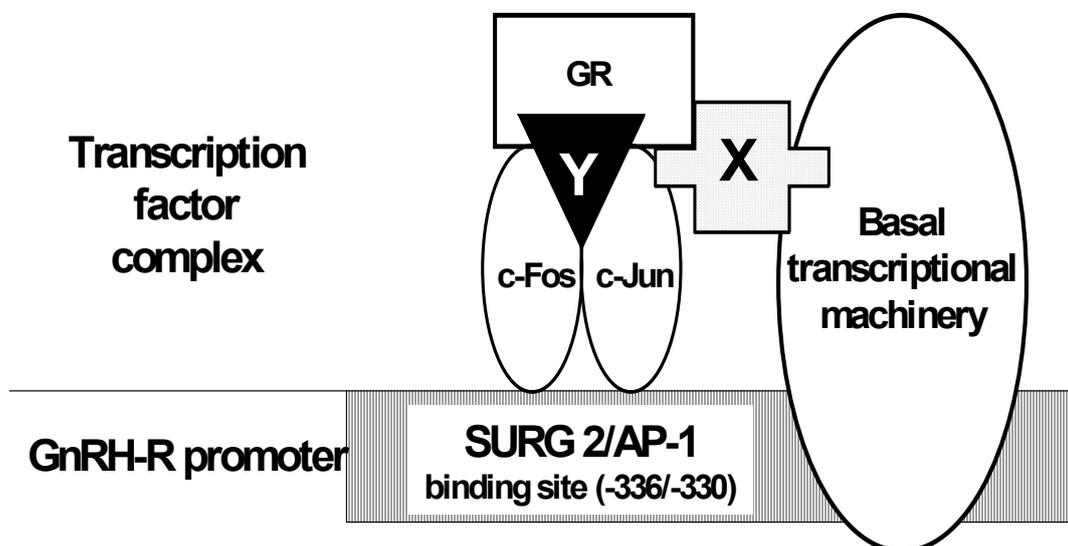


Figure 30. Proposed model for factors interacting at the AP-1 site of the mouse GnRH-R promoter to result in upregulation by dex. The AP-1 heterodimer, c-Fos/c-Jun is bound at SURG 2, which contains the AP-1 binding site (-336/-330). The GR tethered to the AP-1 is possibly stabilised by a cofactor (Y). Depending on the cofactor associated (X) with the transcription initiation complex, such as Brm (19,33), CBP (19,21) or BAF60a (33), the GR-AP-1-Y-X complex possibly upregulates transcriptional activation in L β T2 cells.

The levels of cofactors such as SWI.SNF may therefore influence cell specific regulation seen by dex. In other systems CBP is believed to connect the phosphorylated activation domains of c-Jun with the basal transcriptional machinery (34). In these systems GR and AP-1 functionality requires SRC-1 and CBP/p300, and overexpression of CBP and SRC-1 freed AP-1 from GR repression, but the combination of the AP-1 dimer further determines CBP recruitment (19,21). Therefore a particular dimer composition could recruit CBP and in the process abolish GR inhibition. Recently GC gene activation of the human MKP-1 gene was shown to involve the tethering of a promoter bound C/EBP to the GR (108). A similar mechanism may be involved in the dex upregulation of the GnRH-R gene, but involving AP-1 rather than C/EBP.

The β -casein promoter is activated by means of the GR tethering to STAT5 transcription factors (23) (Figure 3) yet this interaction results in a repression of the MMTV promoter activity (23). Similarly GR tethered to AP-1 typically results in transrepression in the presence of Jun and high levels of Fos (41). However in L β T2 cells the GnRH-R promoter was transactivated by dex (99 and present study). It is possible that an as yet unidentified factor (factor Y, Figure 30) stabilizes the binding of the GR to the AP-1 transcription factor dimer. Yet another cofactor (factor X, Figure 30), or indeed the same one, may interact with the basal transcriptional machinery and this transcription factor complex composition may determine whether the outcome by dex is transactivation or transrepression. Taking this into consideration the weak response seen with promoter-reporter assay may indeed be due to the lack of cis elements found in the endogenous gene or the possible effect of the chromatin on the organisation and recruitment of co-factors.

FUTURE RESEARCH

The mechanism of transcriptional regulation of the GnRH-R gene needs to be further investigated in order to determine the composition of the transcription factor complex responsible for the dex induced upregulation seen in L β T2 pituitary gonadotrope cells.

The ChIP assay allows for investigation of the *in vivo* interaction at the promoter level to be determined. The GR was shown to interact with the area spanning -337 to -170 of the GnRH-R promoter. Chromatin co-immunoprecipitation did confirm the GR tether to the AP-1 complex (Andrea Kotitschke, a PhD student of the same laboratory). The composition of the AP-1 dimer could also be determined as specific monomers, such as c-Jun and c-Fos can be immunoprecipitated. Whether other factors such as C/EBP, CBP and SRC-1 (19,21, 108) are involved in the regulatory complex could also be investigated for.

The role of Fos/Jun as well as other co-factors in the upregulation of the GnRH-R promoter by dex needs to be further determined. Yang-Yen *et al* (22) found that the GR and AP-1 was mutually inhibitory but this inhibition was lowered when exogenous c-Fos and c-Jun was cotransfected. They postulate that as c-Fos occurs mostly as a monomer, it could possibly interact with the GR more readily (22) than c-Jun to lift the inhibition seen in GR repressed promoters. Since the GR interacts with the GnRH-R promoter area containing the AP-1 binding site in the present study, Fos/Jun probably does not sequester the GR off the promoter in this cell system, but could rather promote GR-co-factor interaction.

Small interfering RNAs (siRNA) specific to the GR, c-Jun and c-Fos or any factor of interest could also be used to determine the involvement in the transcriptional regulation of the GnRH-R gene in L β T2 cells. siRNA results in the degradation of mRNAs (109), the corresponding RNA-product level is decreased and the cellular response can be studied. If a single factor such as c-Fos or GR is indeed required for the transcriptional upregulation of the GnRH-R gene, decreasing the levels of this factor using siRNA specific to cFos RNA, will abolish the transcriptional upregulation.

ADDENDUM A

OLIGONUCLEOTIDE AND PRIMER SEQUENCES

Sequences denoted by **s** indicates the sense strand while **a** denotes the antisense strand. All sequences are in a 5' to 3' direction. Sequences are identified by the name used in the assay or alternatively the position relative to the transcriptional start site.

A1: Oligonucleotides used for the EMSA studies

Transcription factor binding sites are indicated in bold and mutated bases are underlined. Oligonucleotides were obtained from Integrated DNA technologies, Inc. (Whitehead Scientific, South Africa) and primers from Roche.

AP-1/SF-1 (-345/-290)

s- GATATTAT**AGAGTCA**CTTTTCGACATCAGAATTAGACTCCAAGT**GTCC**TT**CCTCACC**

a- CTATAATACTCAGTGAAAGGTGTAGTCTTAATCTGAGGTT**CACAGGAAGGAGTGG**

AP-1

s- GATATTAT**AGAGTCA**CTTTTCGA

a- CTATAATACTCAGTGAAAGGT

mAP-1

s- GATATTAG**GAATTC**TTTCGA

a- CTATAACCTTAAGGAAAGCT

A2: Primers used for ChIP assay

SURG-2 (-337 /-170) (90)

s- GTATCTGTCTAGTCACAACAG

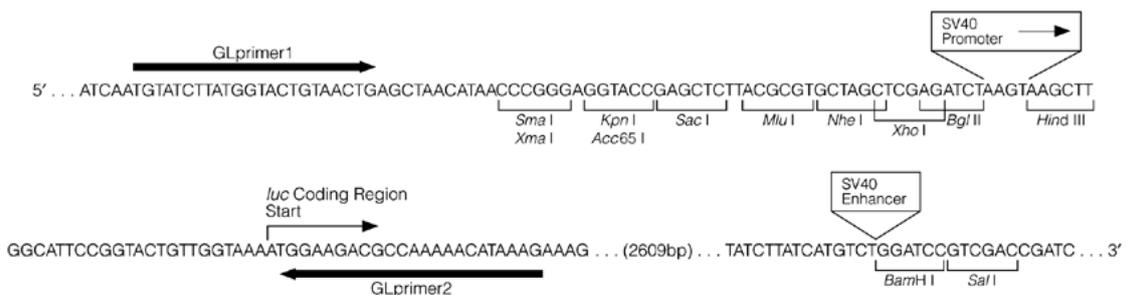
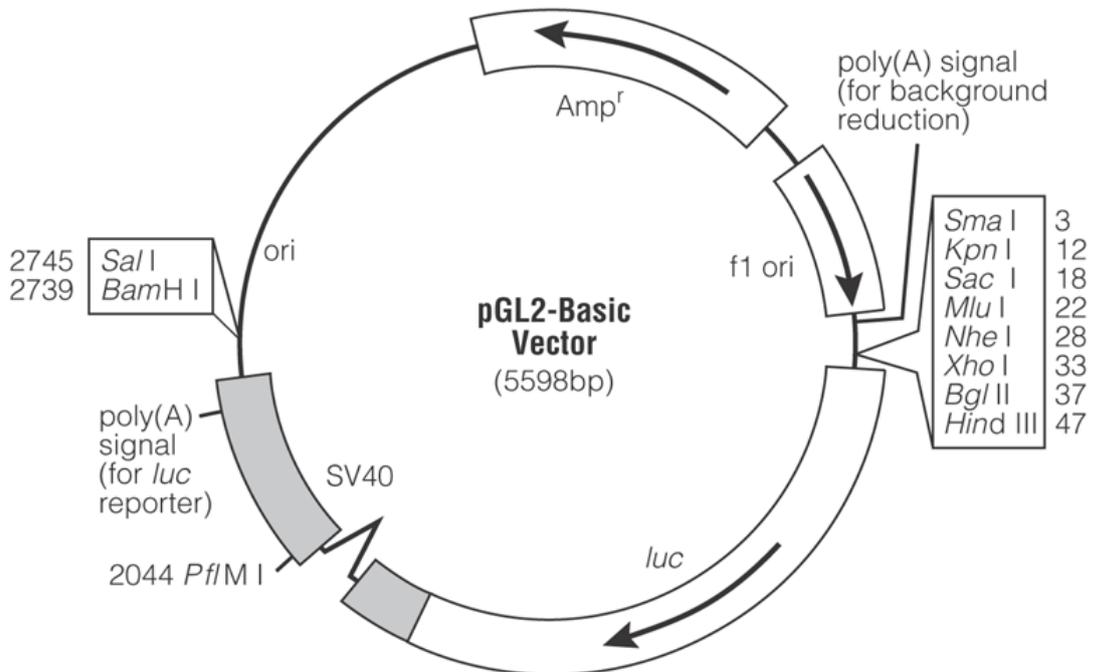
a- TCCTGAAGGCCAAGTGTAAACC

SF-1 (-151/-7)**s-** CGCGTTCAGTTATGATAAAACATCA**a-** TGTATCAAGGGGATGCTGTTGTT**A3: Primers used for RT-PCR and conventional PCR****GAPDH** (101)**s-**ATTGTCAGCAATGCATCCTG**a-**ATGGACTIONGTGGTCATGAGCC**mouse GnRH-R (S4/AS4)** (74)**s-**CCACAGTGGTGGCATCAGGCCTTC**a-**TAGCGTTCTCAGCCGAGCTCTTGG **β -actin****s-**TGGAATCCTGTGGCATCCAGAAAC**a-**TAAAACGCAGCTCAGTAACAGTCCG

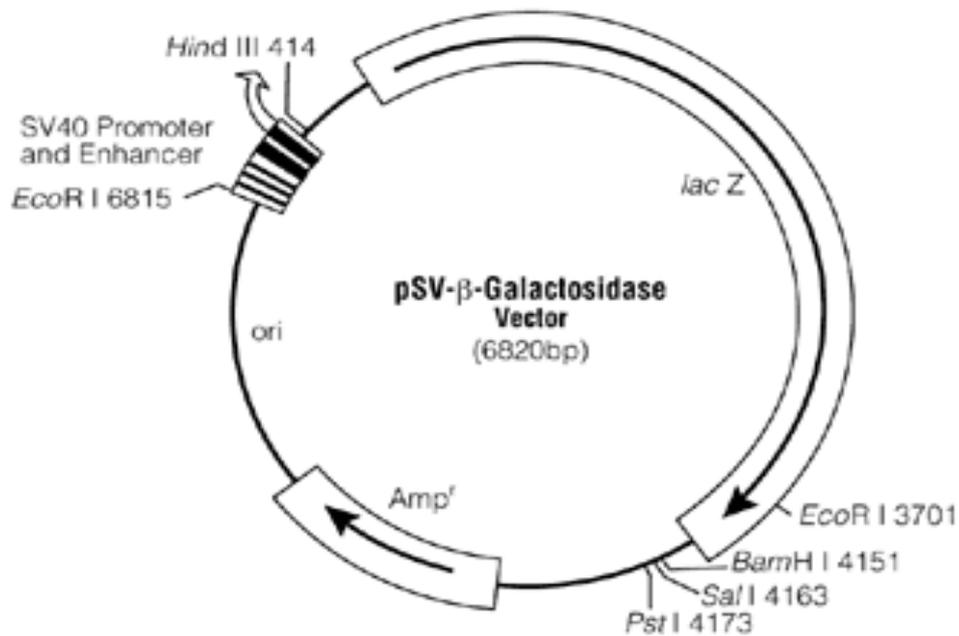
ADDENDUM B

PLASMIDS

B1: pGL2 Basic expression vector and multiple cloning sequences

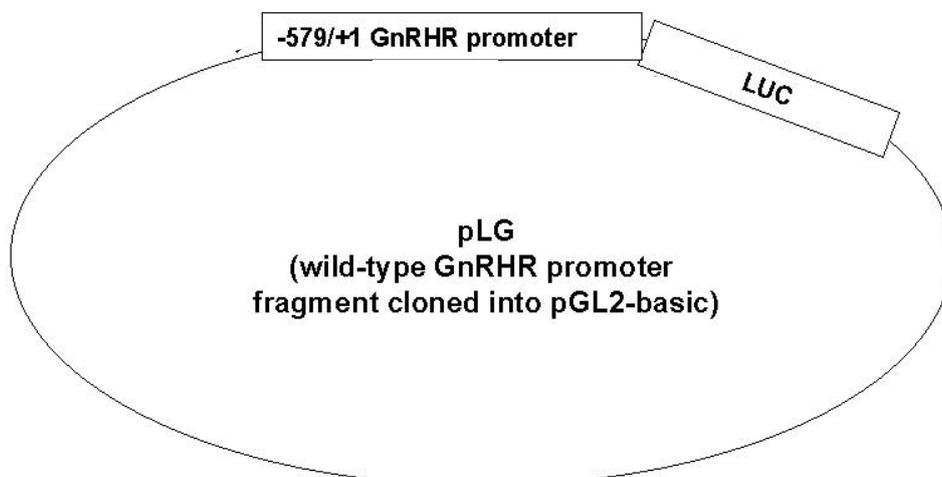


B2: pSV- β -galactosidase expression vector used to control for transfection efficiency



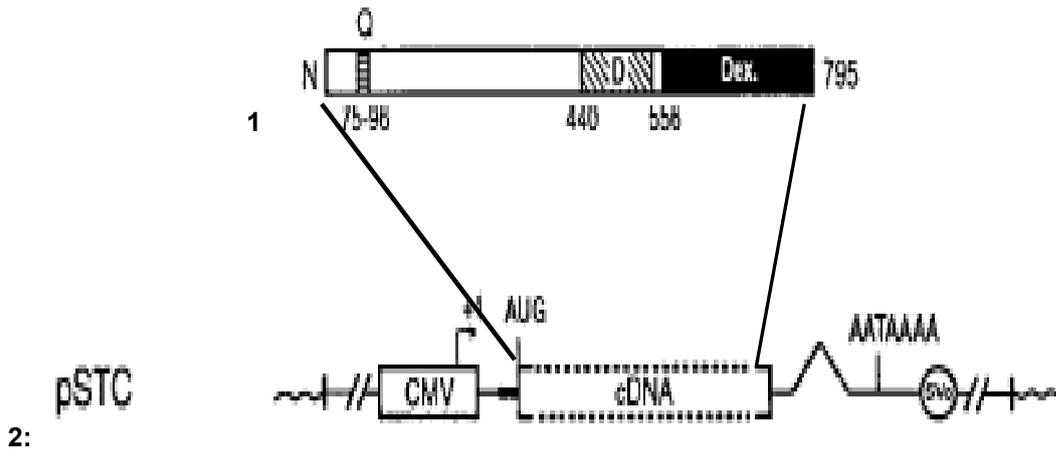
B3: pLG (87, 99, 110)

The wild type mouse GnRH-R promoter fragment (-579/ +1) was cloned into *Bgl*II within the multiple cloning site of the pGL2-basic vector (110).



B4: pSTCGRN 795 wt (25)

1: Structure of the full-length rat GR cDNA, Q represents a Gln repeat between residues 75 and 96.



2: The expression vector pSTC with a CMV promoter/enhancer region at -522 to +72.

Transcription initiation at +1 and AUG indicates the translation start site.

Broken line indicates rat GR cDNA insert.

AATAAAA indicates the genomic sequence of rabbit 6-globin (905-2080) with signals for splicing and polyadenylation.

SVo: 188 base pairs of SV40 sequence spanning origin of replication.

Wavy line: bacterial plasmid (pSP65) conferring ampicillin resistance.

ADDENDUM C

Buffers and solutions

C1: Working buffers and growth medium

50x TAE

242 g Tris Base

57.1 ml Glacial acetic acid

100 ml 0.5M EDTA (pH 8)

Final volume to 1 L using deionised water.

TBS (pH 7.4)

25 mM Tris

137 mM NaCl

2.7 mM KCl

Final volume to 1 L using deionised water.

TE buffer (pH 8)

1 mM Tris.HCl (pH7.5)

0.5 mM EDTA (pH8)

The pH was set using NaOH.

LB Medium

10g Tryptone

5g yeast extract

10g NaCl

Final volume 1L. Autoclave and use aseptically

C2: EMSA buffer conditions and gel mixture

A final volume of 5 μ l of each sample was electrophoresed. This consists of: 1.5 μ l nuclear extract, 1.5 μ l cocktail, 1 μ l labelled probe and 1 μ l of the unlabelled competitor oligonucleotide/antibody/water.

Cocktail

1 μ g poly dIdC

0.3 μ l BSA (20 μ g/ μ l)

0.7 μ l Reaction buffer

Oligonucleotide AP-1/SF-1 (-345/-290) Reaction buffer (adapted from 69,74 and 111)

10 mM Hepes (pH 7.9)

1 mM EDTA (pH 8.0)

100 mM NaCl

10% (v/v) glycerol

10 mM Tris-HCl (pH 8.0)

1.25 mM MgCl₂

0.25 mM EGTA (pH 7.0)

0.675 mM PMSF

3.5 mM DTT

PMSF and DTT are added immediately before the reaction buffer is used.

5% Poly-acrylamide gel (40 mL)

6.7 ml Acrylamide: bisacrylamide (29:1)

32.5 Deionised water

400 μ l 50X TAE

70 μ l TEMED

400 μ l 10% (w/v) AMP

Electrophoresis buffer

1X TAE

C3: SDS-PAGE buffers

5x Loading buffer

100 mM Tris (pH 6.8)

5% SDS

0.1% Bromophenol blue

20% glycerol

0.5 ml Mercaptoethanol

Final volume: 10 ml.

10x Tris-Glycine

30.2g Tris

188g Glycine

Final volume: 1L.

Electrophoresis buffer

200 ml 10x Tris-Glycine

20 ml 10% SDS

Final volume: 2L.

Transfer buffer

200 ml 10x Tris-Glycine

200 ml methanol

Final volume: 2L.

Membrane stripping solution

62.5 mM Tris (pH 8.6)

100 mM β -mercaptoethanol

2% SDS

CREB-lysis buffer

1 M Tris (pH 7.5)

5 M NaCl

0.5 M EDTA

0.1 M EGTA

1% (v/v) Triton X100

0.1115 g Sodumpyrophosphate

2.1 mg β -Glycerolphosphate

C4: ChIP assay buffers:**Nuclear lysis buffer**

1% SDS

50 mM Tris-HCL, pH 8.0,

10 mM EDTA

one Complete, Mini Protease Inhibitor Cocktail Tablet (Roche)

IP dilution buffer

0.01% SDS

20 mM Tris-HCL, pH 8.0

1.1% Triton X-100

167 mM NaCl

1.2 mM EDTA

one Complete, Mini Protease Inhibitor Cocktail Tablet (Roche).

Wash buffer I:

0.1% SDS

1% Triton X-100

2 mM EDTA

20 mM Tris-HCL, pH 8.0

150 mM NaCl

Wash buffer II

0.1% SDS

1% Triton X-100

2 mM EDTA

20 mM Tris-HCL, pH 8.0

500 mM NaCl

Wash buffer III

250 mM LiCl

1% NP-40

1% sodium deoxycholate

1 mM EDTA

10 mM Tris-HCL, pH 8.0

TE buffer

10 mM Tris-HCL, pH 8.0

1 mM EDTA

IP elution buffer

1% SDS

100 mM NaHCO₃

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