Transcriptional regulation of the mouse gonadotropin-releasing hormone receptor gene in pituitary gonadotrope cell lines

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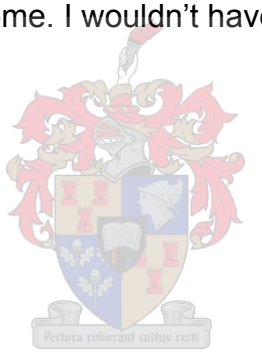
I, the undersigned, hereby declare that the work contained in this thesis is my own original work. The result presented in figure 3.1 was obtained by Gustav Styger (previously from the research group of the present author). The results presented in figures 3.2 and 3.3 were originally presented in my M.Sc. thesis. Regarding the remainder of the results, I hereby declare that I have not previously submitted any part of it at any university for a degree.

Signature…… Date…….

To everybody who has walked alongside me on this journey:

Thanks, it has been awesome. I wouldn't have wanted it any other way.



ABSTRACT

Gonadotropin-releasing hormone (GnRH), acting via its cognate receptor (GnRHR) is the primary regulator of mammalian reproductive function. Pituitary sensitivity to GnRH can be directly correlated with GnRHR levels on the surface of the pituitary gonadotrope cells, which can be regulated at transcriptional, post-transcriptional and post-translational levels. This study investigated mechanisms of transcriptional regulation of mouse GnRHR expression in two mouse gonadotrope cell lines, αT3-1 and LβT2, using a combination of endogenous mRNA expression studies, promoter-reporter studies, a two-hybrid protein-protein interaction assay, Western blotting, and *in vitro* protein-DNA binding studies. In the first part of the study, the role of two GnRHR promoter nuclear receptor binding sites (NRSs) and their cognate transcription factors in basal and Protein Kinase A (PKA)-stimulated regulation of GnRHR promoter activity was investigated in αT3-1 cells. The distal NRS was found to be crucial for basal promoter activity in these cells. While the NRSs were not required for the PKA response in these cells, results indicate a modulatory role for the transcription factors Steroidogenic Factor-1 (SF-1) and Nur77 via these promoter elements. The second part of the study focused on elucidating the mechanism of homologous regulation of GnRHR transcription in LβT2 cells, with a view to defining the respective roles of PKA and Protein Kinase C (PKC) in the transcriptional response to GnRH. In addition, the respective roles of the NRSs, the cyclic AMP response element (CRE) and the Activator Protein-1 (AP-1) promoter *cis* elements, together with their cognate transcription factors, in basal and GnRH-stimulated GnRHR promoter activity, were investigated. Homologous upregulation of transcription of the endogenous gene was confirmed, and was quantified by means of real-time RT-PCR. The GnRH response of the endogenous gene and of the transfected promoter-reporter construct required PKA and PKC activity, and the GnRH response of the promoter-reporter construct was found to be dependent on a functional AP-1 site. Furthermore, GnRH treatment resulted in increased binding of phosphorylated cAMP-response element binding protein (phospho-CREB) and decreased expression and binding of SF-1 to their cognate *cis* elements *in vitro*, and stimulated a direct interaction between SF-1 and CREB, suggesting that these events are also required for the full transcriptional response to GnRH. This study is the first providing detail regarding the mechanism of transcriptional regulation of GnRHR expression in LβT2 cells by GnRH. Based on results from this study, a model has been proposed which outlines for the first time the kinase pathways, the promoter

cis elements and the cognate transcription factors involved in homologous regulation of GnRHR transcription in the LβT2 cell line. As certain aspects of this model have been confirmed for the endogenous GnRHR gene, the model is likely to be physiologically relevant, and provides new ideas and hypotheses to be tested in future studies.

OPSOMMING

Voortplantingsfunksies in soogdiere word primêr beheer deur die gonadotropien-vrystellingshormoon (GnRH), wat sy funksie verrig deur middel van die GnRH-reseptor (GnRHR). Pituïtêre sensitiwiteit vir GnRH hou direk verband met die aantal GnRH reseptore op die oppervlak van die pituïtêre gonadotroop-selle, wat gereguleer kan word op die vlakke van transkripsie, post-transkripsie and post-translasie. Hierdie studie het die meganismes van transkripsionele regulering van muis GnRHR uitdrukking in twee muis-gonadotroopsellyne, αT3-1 and LβT2, ondersoek. 'n Kombinasie van tegnieke, naamlik endogene mRNA uitdrukking-studies, promotor-rapporteerder-studies, 'n twee-hibried proteïen-proteïen-interaksie-essai, Western-klad en *in vitro* proteïen-DNA-bindingstudies, is gebruik. Die muis GnRHR promotor bevat twee selkernreseptor-bindingsetels, en in die eerste deel van hierdie studie is ondersoek ingestel na die rol van hierdie bindingsetels, tesame met die transkripsie-faktore wat daaraan bind, in basale en Proteïen Kinase A (PKA)-gestimuleerde regulering van GnRHR promotoraktiwiteit in αT3-1 selle. Daar is bevind dat die distale bindingsetel noodsaaklik is vir basale promotoraktiwiteit in hierdie selle. Alhoewel die bindingsetels nie benodig word vir die PKA respons in hierdie selle nie, dui die resultate daarop dat die transkripsiefaktore Steroidogenic Factor-1 (SF-1) en Nur77 die respons via hierdie setels moduleer. Die fokus van die tweede deel van die studie was om die meganisme van homoloë regulering van GnRHR transkripsie in LβT2 selle toe te lig, met die doel om die onderskeie rolle van PKA en Proteïen Kinase C (PKC) in die transkripsionele GnRH respons te definieer. Verder is ondersoek ingestel na die onderskeie rolle van die selkernreseptor-bindingsetels, die sikliese AMP respons-element (CRE) en die Activator Protein-1 (AP-1) promotor *cis* elemente, tesame met die ooreenstemmende transkripsiefaktore wat aan hierdie elemente bind, in basale en GnRH-gestimuleerde GnRHR promotoraktiwiteit. Homoloë verhoging in transkripsie van die endogene GnRHR geen is bevestig, en is gemeet deur middel van intydse RT-PCR. PKA- en PKC-aktiwiteit was benodig vir die GnRH respons van beide die endogene geen en die getransfekteerde promotor-rapporteerderkonstruk, en daar is aangetoon dat die GnRH respons van die promotor-rapporteerderkonstruk afhanklik is van 'n funksionele AP-1 bindingsetel. Bykomende resultate het getoon dat toediening van GnRH verhoogde binding van gefosforileerde CREB (cAMP response element binding protein) en verminderde uitdrukking en binding van SF-1 aan hul ooreenstemmende bindingsetels *in vitro* tot gevolg gehad het, en dat GnRH 'n direkte interaksie

tussen SF-1 en CREB geïnduseer het. Hierdie resultate dui aan dat hierdie gebeure ook benodig word vir die volle transkripsionele GnRH respons. Hierdie studie verskaf die eerste detail aangaande die meganisme van transkripsionele regulering van GnRHR uitdrukking in LβT2 selle deur GnRH. Op grond van die resultate van hierdie studie word 'n model vir die homoloë regulering van GnRHR transkripsie in die LβT2 sellyn voorgestel, waarin die onderskeie rolle van die kinase-paaie, die promotor *cis* elemente en hul ooreenstemmende transkripsiefaktore vir die eerste keer uitgespel word. Aangesien sekere aspekte van die model reeds bevestig is vir die endogene GnRHR geen, is die model waarskynlik fisiologies relevant, en verskaf dit nuwe idees en hipoteses wat in toekomstige studies getoets kan word.

ACKNOWLEDGEMENTS

A PhD is by no means a solo journey, and there are many people who I have to thank for helping me reach this point in my academic career:

First, I have to thank Our Heavenly Father, who has guided me along this process, always blessing me with strength, enthusiasm and inspiration.

Then, to my supervisor, teacher and mentor, Prof Janet Hapgood: Thank you for your guidance, patience, inspiration, positive attitude and encouragement, and for being so incredibly good at what you do. Everything I know about science and about being a scientist, I learnt from you. It is a gift that can never be repaid.

To my parents, thank you for providing me with every opportunity in life, for your emotional and financial support, and for your unshakable faith in my ability to achieve. (When I had just started writing this thesis, my dad once told me: “There is only one way to eat an elephant: one bite at a time.” This line has gotten me through many moments when the task ahead loomed too large.)

I have to specially thank Emerentia Hutchinson, Donita Africander and Carmen Langeveldt, who have managed our laboratory and maintained our cell cultures through the years. Ladies, your hard work behind the scenes created an environment where we could focus on the science. The time and effort that you have put in, have made the road less bumpy and the load much lighter for the rest of us.

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My academic career and this project would not have been possible without the financial support of the National Research Foundation of South Africa, providing both personal scholarships and research funding, and for this I am grateful.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP-1	Activator Protein-1
BIM	bisindolylmaleimide
bp	base-pair
CAD	constitutive activation domain
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
ChIP	chromatin immunoprecipitation
CRE	cAMP response element
CREB	cAMP response element binding protein
CBP	CREB-binding protein
DAG	diacylglycerol
DAX-1	dosage-sensitive sex reversal - adrenal hypoplasia congenita critical region on the X chromosome - 1
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FSH	follicle-stimulating hormone
GDP	guanosine diphosphate
GRAS	GnRHR-activating sequence
GSE	gonadotrope-specific element
GTP	guanosine triphosphate

GnRH	gonadotropin-releasing hormone
GnRHR	GnRH receptor
GPCR	G-protein coupled receptor
hCG	human chorionic gonadotropin
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
IgG	immunoglobulin G
IP_3	inositol-(1,4,5)-triphosphate
JNK	Jun N-terminal kinase
kb	kilobasepair
KID	kinase-inducible domain
LB	Luria-Bertani
LH	luteinizing hormone
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MEK	MAPK kinase
M-MLV	Moloney Murine Leukemia Virus
MOPS	4-morpholine-propanesulfonic acid
mRNA	messenger ribonucleic acid
m/v	mass per volume
N-CoR	nuclear receptor co-repressor
NF-Y	nuclear factor-Y
nNOS	neuronal nitric oxide synthase
NRS	nuclear receptor binding site
PACAP	pituitary adenylate cyclase activating polypeptide
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase anti-peroxidase
PBS	phosphate-buffered saline

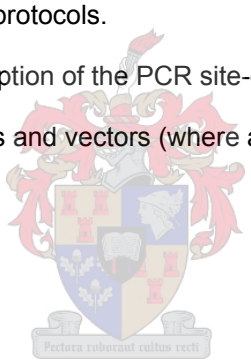
PKA	Protein Kinase A
PKC	Protein Kinase C
PVR	PACAP/VIP receptor
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SF-1	Steroidogenic Factor-1
SRC-1	steroid receptor co-activator-1
SSC	sodium chloride/sodium citrate buffer
SURG	Sequence underlying responsiveness to GnRH
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TE	Tris-EDTA
TSH	thyroid-stimulating hormone
UTR	untranslated region
UV	ultraviolet
VIP	vasoactive intestinal polypeptide
v/v	volume per volume

THESIS OUTLINE

This thesis contains the following sections:

1. An **Introduction**, which will briefly outline the pertinent issues in the field of GnRHR gene expression and regulation, and which will state the aims of the thesis;
2. Chapter 1: **Literature review**. This chapter gives a detailed overview of the relevant knowledge currently available in the literature, with a particular focus on the expression and transcriptional regulation of the mouse GnRHR in pituitary cells. Some sections of this chapter formed part of a review article published in Journal of Neuroendocrinology in 2005; a copy of the printed article is attached at the back of the thesis. The published review article also covered the expression and regulation of the GnRHR in other species, in the pituitary as well as in extra-pituitary tissues; these topics will not be covered in Chapter 1.
3. Chapter 2: **Materials and Methods.** This chapter gives details regarding the experimental protocols used to obtain the results presented in chapters 3 and 4.
4. Chapter 3: **Regulation of mouse GnRHR gene transcription by PKA in αT3-1 cells**. The first part of this project utilized the αT3-1 gonadotrope cell line as a model system for studying mouse GnRHR gene regulation. A brief introduction to the specific aims of these studies and the results of these studies are presented in this chapter. These results were published in Endocrinology in 2003; a copy of the printed article is attached at the back of the thesis. The result presented in figure 3.1 was obtained by Gustav Styger (previously from the research group of the present author). The results presented in figures 3.2 and 3.3 were from the M.Sc. thesis of the present author, and are included for the sake of completeness. The cell culture work was performed by Emerentia Hutchinson and Donita Africander.
5. Chapter 4: **Homologous regulation of mouse GnRHR gene transcription in LβT2 cells.** Certain issues that emerged from the results obtained in αT3-1 cells (chapter 3) were further investigated in a different gonadotrope cell line, LβT2. A brief introduction to the specific aims of these studies and the results of these studies are presented in this chapter. These results will shortly be submitted for publication. All the results presented in this chapter were obtained by the present author, and the cell culture work was performed by Carmen Langeveldt.

6. Chapter 5: **Discussion and Conclusions.** In this chapter, the results presented in chapters 3 and 4 are discussed. This chapter also contains a proposed model for homologous regulation of GnRHR gene transcription in LβT2 cells. In addition, some perspectives about future research efforts are included.
7. Addendum A: **LightCycler Real-time RT-PCR**. This chapter contains detailed theory and background on RT-PCR and on absolute and relative quantification of gene expression by means of LightCycler real-time RT-PCR. It also covers technical issues with regards to optimization of LightCycler PCR protocols, and gives a detailed account of the optimization of GnRHR transcript quantification.
8. **Addendum B:** This addendum contains sequences of all primers and oligonucleotides used in this study.
9. **Addendum C:** This addendum contains detailed information regarding buffers and other mixes used in the experimental protocols.
10. **Addendum D:** A detailed description of the PCR site-directed mutagenesis protocol
11. **Addendum E:** Maps of plasmids and vectors (where available) used in the present study



INTRODUCTION

Gonadotropin-releasing hormone (GnRH), in conjunction with the GnRH receptor (GnRHR) is the primary regulator of reproduction in vertebrates. It is well established that GnRH is released from the hypothalamus in a pulsatile fashion, from where it travels to the pituitary gland via the portal hypophyseal vasculature (Fink G, 1988). Upon GnRH binding to its G protein-coupled receptor (GPCR) on the plasma membranes of pituitary gonadotrope cells, a range of intracellular signalling pathways are activated that ultimately regulate the synthesis and secretion of the gonadotropin hormones, LH and FSH. Gonadotropins in turn stimulate sex hormone synthesis and gametogenesis in the gonads to ensure reproductive competence.

Besides the well established role for GnRH and GnRHR in gonadotropin regulation in the pituitary, the expression of both the hormone and the receptor in multiple mammalian non-pituitary tissues and cells suggests numerous and diverse autocrine, paracrine and endocrine extra-pituitary roles for GnRHs and GnRHRs. These include: neuronal migration during development (Romanelli RG *et al*., 2004), neuromodulation in the brain to affect sexual behavior (Millar RP, 2003), digestive tract function (Huang W *et al*., 2001), inhibition of gastric acid secretion (Chen L *et al*., 2005), regulation of T cell function (Chen A *et al*., 2005), hCG release in the placenta (Raga F *et al*., 1998), steroidogenesis in ovarian cells (Guerrero HE *et al*., 1993; Gaetje R, 1994), sperm function (Morales P, 1998) and growth inhibition in reproductive tumors (Grundker C *et al*., 2002; Franklin J *et al*., 2003; Harrison GS *et al*., 2004; Finch AR *et al*., 2004). However, the specific roles of GnRH and the GnRH receptor in these various tissues and cells remain to be elucidated.

GnRH and its analogs are extensively used in the treatment of hormone-dependent diseases, as well as for assisted reproductive techniques (Millar RP *et al*., 2004). More recently they have been proposed as novel contraceptives in men and women (Anderson RA and Baird DT, 2002; Millar RP *et al*., 2004). The finding that naturally occurring mutations of the GnRHR are linked to the disease hypogonadotropic hypogonadism, which results in delayed puberty, has also recently stimulated interest in GnRHR function (Iovane A *et al*., 2004). Thus, the emerging multiple roles of GnRH and the

GnRHR have presented new therapeutic targets and intensified the search for novel interventive GnRH analogs.

A central issue in the field is to understand the extracellular signals and the intracellular mechanisms that regulate expression of GnRHRs in these diverse tissues and cells. Responsiveness to GnRH depends on the number of GnRHRs on the cell surface. In turn, GnRH appears to be an important regulator of receptor levels on the gonadotrope cell surface (Yasin M *et al*., 1995). Several lines of evidence indicate that the number of GnRHRs is partially dependent upon the level of GnRHR mRNA, which appears to be regulated at least in part at the transcriptional level in gonadotropes, including by GnRH itself (Kaiser UB *et al*., 1993; Padmanabhan V *et al*., 1995; Yasin M *et al*., 1995; White BR *et al*., 1999; Norwitz ER *et al*., 1999a). In addition to being regulated by its homologous hormone, new insights have recently been obtained about regulation of GnRHR expression by other hormones, such as melatonin (Woo MMM *et al*., 2001), adrenal and sex steroids (Maya-Nunez G and Conn PM, 2003; Cheng CK *et al*., 2003; An BS *et al*., 2005), activin (Norwitz ER *et al*., 2002a) and hCG (Peng C *et al*., 1994; Li X *et al*., 1996). The intracellular signalling pathways (McArdle CA *et al*., 2002; Ruf F *et al*., 2003; Navratil AM *et al*., 2003; Caunt CJ *et al*., 2004) and transcription factors involved (Norwitz ER *et al*., 2002b; Ellsworth BS *et al*., 2003a; Ellsworth BS *et al*., 2003b; Kam KY *et al*., 2005) in regulating mammalian GnRHR transcription in diverse tissues from different species have also been the focus of intense investigation in recent years. Although further research is necessary to understand the mechanisms of regulation of GnRHR expression, the GnRHR is emerging as a potential target gene for facilitating cross-talk between neuroendocrine, immune and stress-response systems in multiple tissues via autocrine, paracrine and endocrine signalling. Knowledge of these mechanisms is important to fully understand both the physiological and the therapeutic actions of GnRH and GnRHRs, given the central role of GnRHRs in reproductive endocrinology and the widespread use of GnRH analogues in endocrine and anti-cancer therapy.

AIMS AND HYPOTHESES

The aim of this project was to study the mechanism of transcriptional regulation of the mouse GnRHR gene in response to physiological hormones in two immortalized mouse pituitary gonadotrope cell lines. In particular, the aim was to identify the intracellular signalling pathways mediating the hormonal responses, and also to identify the nuclear transcription factors and promoter *cis* elements involved in this regulation.

To this end, the following hypotheses were formulated and subsequently investigated:

1) that GnRHR transcription is regulated by GnRH and by pituitary adenylate cyclase activating polypeptide (PACAP);
2) that the Protein Kinase A (PKA) and Protein Kinase C (PKC) pathways play important roles in regulation of GnRHR gene transcription, via targeting several transcription factors;
3) that nuclear receptors are important in mediating the transcriptional effects of GnRH via these pathways by binding to two nuclear receptor binding sites in the GnRHR promoter;
4) that the CREB transcription factor is involved in mediating the transcriptional effects of GnRH via the PKA pathway, by binding to the cAMP-response element in the GnRHR gene promoter;
5) that AP-1 transcription factors are involved in mediating the transcriptional effects of GnRH via the PKC pathway, by binding to the AP-1 response element in the GnRHR gene promoter;
6) and that the two mouse model cell lines chosen, i.e. the αT3-1 precursor gonadotrope cell line and the more differentiated LβT2 gonadotrope cell line, will exhibit differences in their mechanisms of GnRHR gene regulation, given their different states of differentiation.

The first part of the research project focused on the regulation of GnRHR gene transcription by the PKA pathway in the αT3-1 precursor gonadotrope cell line. The second part of the project utilized the LβT2 cell line, representing more differentiated gonadotropes, as a physiologically more relevant system. This part of the research project investigated the transcriptional regulation of GnRHR expression by GnRH in LβT2 cells, with a particular focus on the respective role of the PKA and PKC pathways in this regulation.

The aim of the first part of the study was to investigate the mechanism of basal and PKA-mediated transcriptional regulation of the mouse GnRHR gene in αT3-1 cells. More specifically, the project focused on the respective roles of the nuclear receptor binding sites -15/-7NRS and -244/-236NRS, together with the transcription factors that could potentially recognize them, in particular SF-1 and Nur77, in basal and PKA-regulated promoter activity. This was achieved by overexpressing SF-1 and Nur77 proteins in αT3-1 cells, individually and in combination with each other, in order to define specific roles for each of them in the transcriptional activity of transfected mouse GnRHR promoter-reporter constructs. Mutations abolishing SF-1 binding were also introduced into the -15/-7NRS and -244/-236NRS promoter *cis* elements, to investigate the role of SF-1 protein in determining promoter activity via these elements.

The aim of the second part of the project was to investigate GnRH-mediated transcriptional regulation of the mouse GnRHR gene in LβT2 cells, and to determine whether PKA plays a role in mediating the downstream transcriptional effects of GnRH. In addition, the study focused on the role of four GnRHR promoter *cis* elements, namely the two NRSs mentioned above, the cAMP response element (CRE) and the AP-1 element, in mediating basal and GnRH-mediated transcriptional regulation. The binding of cognate transcription factors to these promoter elements, under basal and GnRH-stimulated conditions, was also investigated.

A major portion of the data regarding transcriptional regulation was obtained by transient transfections of GnRHR promoter-reporter constructs, and these results were correlated with data obtained for the regulation of endogenous GnRHR mRNA levels in the two cell lines. Additional information regarding the specific roles of promoter *cis* elements and their cognate transcription factors was obtained by means of *in vitro* protein-DNA binding experiments. The results obtained in LβT2 cells, along with selected findings from the literature, were incorporated into a proposed model for homologous regulation of GnRHR gene transcription in these cells.

CHAPTER 1*

LITERATURE REVIEW

1.1 THE GnRH RECEPTOR: A MEMBER OF THE GPCR FAMILY

1.1.1 G-protein coupled receptors

The G-protein coupled receptor (GPCR) family constitutes the largest family of plasma membrane receptors, and form a key component of the cell's capacity to respond appropriately to external stimuli, such as light, odorants, neurotransmitters, hormones and growth factors (reviewed in Bockaert J & Pin JP, 1999; Marinissen MJ & Gutkind JS, 2001). All GPCRs consist of 7 transmembrane helices spanning across the plasma membrane, connected by three intracellular and three extracellular loops. In addition, they contain an extracellular N-terminus and an intracellular C-terminal tail, of which the length varies between different GPCRs (Bockaert J & Pin JP, 1999). Because of this unique structure, these receptors are also sometimes referred to as seven-transmembrane, serpentine or heptahelical receptors (Marinissen MJ & Gutkind JS, 2001).

GPCRs couple to G-proteins in order to initiate an intracellular signalling cascade. G-proteins are heterotrimeric, consisting of an α, β and γ subunit, and in the inactive (basal) state, the α-subunit is associated with one molecule of GDP. Ligand binding to the GPCR induces a conformational change in the cytoplasmic domains of the receptor, enabling the receptor to couple to a specific G-protein. This interaction facilitates the exchange of the GDP on the α-subunit for GTP, which in turn leads to the dissociation of the G-protein from the GPCR, as well as the dissociation of the α and associated $\beta\gamma$ subunits. Both the α- and the $\beta\gamma$-subunits can interact with downstream effector molecules, which includes adenylyl and guanylyl cyclases, phospholipases and phosphodiesterases. This results in increased or decreased production of second messenger molecules, such as cyclic AMP (cAMP), cGMP, diacylglycerol (DAG) and inositol (1,4,5)-triphosphate. In addition, certain G-proteins facilitate increases in intracellular calcium levels, as well as the opening or closing of ion channels.

* Note that sections of this chapter were published in: J.P. Hapgood, H. Sadie, W. van Biljon and K. Ronacher 2005 Regulation of expression of mammalian GnRH receptor genes. Journal of Neuroendocrinology 17 p. 619-638 (see attached manuscript)

The combined effects of these events lead to the activation or inhibition of intracellular signalling pathways, which will ultimately impact on the expression levels of genes involved in cellular processes, such as cell growth and proliferation, differentiation and development (reviewed in Marinissen MJ & Gutkind JS, 2001).

G-proteins can contain any one of four different Gα-subunits, namely $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. These subunits preferentially regulate specific effector molecules, and therefore have different downstream effects on intracellular signalling. Adenylyl cyclase, the enzyme responsible for cAMP production, is stimulated by $G\alpha_s$ and inhibited by $G\alpha_i$, respectively. $G\alpha_q$ activates phospholipase C, which leads to the increased production of DAG and inositol triphosphate (reviewed in Ostrom RS *et al.*, 2000).

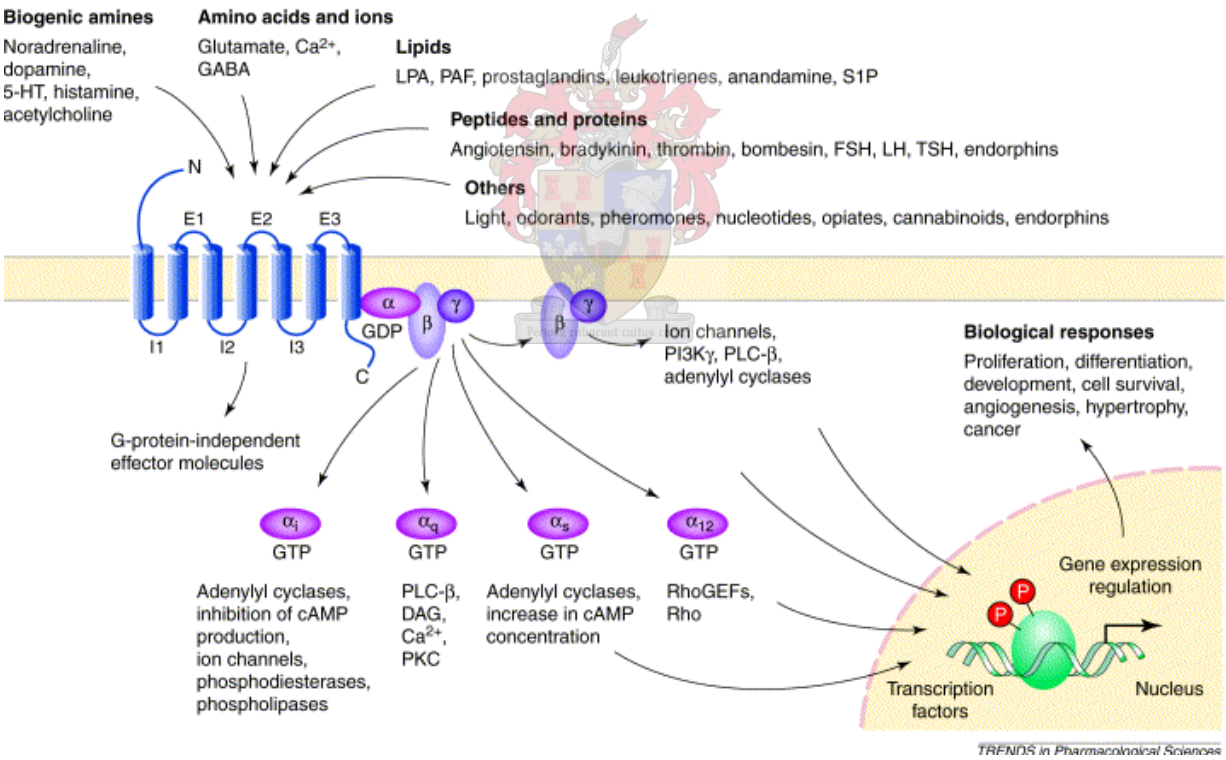
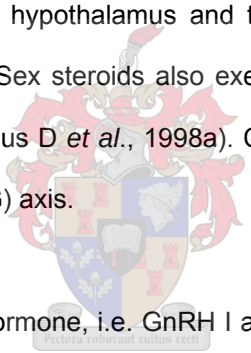


Figure 1.1 The diversity of G-protein coupled receptors (GPCRs). A wide variety of ligands use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways. Such signalling pathways regulate key biological functions, such as cell survival, proliferation and differentiation. (From Marinissen MJ & Gutkind JS, 2001)

1.1.2 Gonadotropin releasing hormone and its receptor

Gonadotropin-releasing hormone (GnRH), in conjunction with the GnRH receptor (GnRHR) is the primary regulator of reproduction in vertebrates. This peptide hormone consists of 10 amino acid residues and is synthesized in specialized neurons of the pre-optic area of the hypothalamus. It is released from the hypothalamus in a pulsatile fashion and travels through the hypophyseal portal circulation to the gonadotrope cells of the anterior pituitary, where it binds and activates its cognate G-protein-coupled receptor on the plasma membrane of the pituitary gonadotrope cells (reviewed in Conn PM & Crowley WF, 1994; Kaiser UB *et al*., 1997a). Upon GnRH binding to the GnRHR, a range of intracellular signalling pathways is activated that ultimately regulate the synthesis and secretion of the gonadotropins, LH and FSH. These hormones are released into the general circulation, and act specifically on the gonads, where they regulate gametogenesis and sex steroid production (reviewed in Kaiser UB *et al*., 1997a). The concentration of sex steroids in the bloodstream provides both positive and negative feedback on the hypothalamus and the pituitary, thereby modulating GnRH release and responsiveness to GnRH. Sex steroids also exert feedback control on the GnRH pulse generator in the hypothalamus (Stanislaus D *et al*., 1998a). Collectively, this system is referred to as the hypothalamic-pituitary-gonadal (HPG) axis.



At least two forms of the decapeptide hormone, i.e. GnRH I and GnRH II, as well as the receptor, i.e. GnRHR I and GnRHR II, have been found in most vertebrates, including mammals, increasing the potential for diverse physiological actions (Neill JD, 2002; Millar RP, 2003; Pawson AJ *et al*., 2003; Millar RP *et al*., 2004; Neill JD *et al*., 2004). GnRH I released from the hypothalamus is the hormone that appears to be sufficient for gonadotropin regulation in the mammalian pituitary (Millar RP, 2003). GnRH II was originally isolated from chicken brain and its precise roles in mammals remain to be elucidated (Morgan K *et al*., 2003). However, both GnRH peptides can bind to and activate both receptor forms, with GnRH I exhibiting a greater affinity and potency for GnRHR I and GnRH II exhibiting a greater affinity and potency for GnRHR II (Millar RP *et al*., 2001). While GnRHR II transcripts have been detected in pituitary as well as extra-pituitary tissues and cell lines from several mammalian species (Millar RP, 2003), expression of endogenous GnRHR II protein has not been conclusively shown in any mammalian species. Although recent indirect evidence suggests that a functional human GnRHR II protein may be expressed in cancer cells (Enomoto M *et al*., 2004), to

date no definitive functional role has been established for any mammalian GnRHR II (Neill JD *et al*., 2004).

The GnRHR I was first cloned from the mouse (Reinhart J *et al*., 1992; Tsutsumi M *et al*., 1992), and subsequently from the rat (Eidne KA *et al*., 1992; Kaiser UB *et al*., 1992), human (Kakar SS *et al*., 1992) and many other mammalian and non-mammalian species. The GnRHR I protein consists of 327 to 328 amino acids, and share >80% homology across species (reviewed in Stojilkovic SS *et al*., 1994). The mammalian GnRHR I lacks a cytoplasmic C-terminal tail, setting it apart from non-mammalian GnRHRs and all other GPCRs, and making it one of the smallest GPCRs known (reviewed in Kaiser UB *et al*., 1997a).

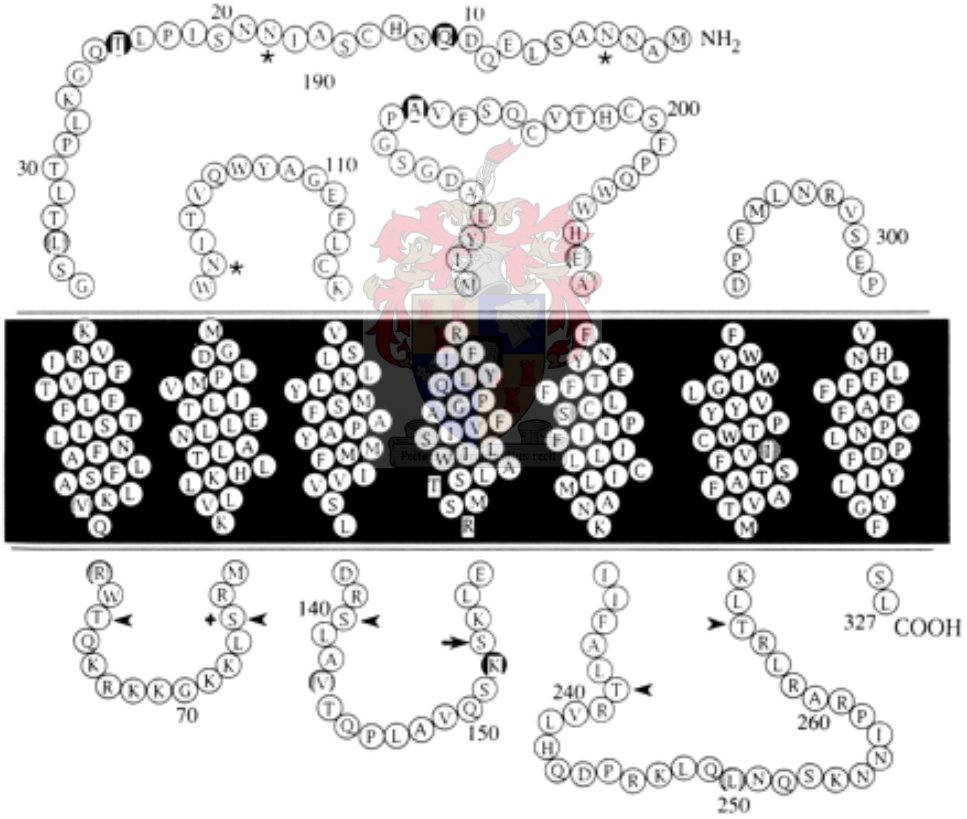


Figure 1.2 Model of the rat GnRHR. Amino acids residues in black represent non-conserved amino acids between the rat and mouse GnRHR; shaded amino acid residues are non-identical but conserved between the two species. Asterisks indicate potential glycosylation sites. Potential phosphorylation sites are indicated for PKC (arrowheads), casein kinase II (arrow) and PKA (cross). (From Kaiser UB *et al*., 1997a)

In the anterior pituitary, GnRHR I is primarily expressed in the gonadotrope cells (Hyde CL *et al*., 1982), but GnRHR I immunoreactivity has also been detected in human thyrotropes and somatotropes (La Rosa S *et al*., 2000). GnRHRs are also expressed in various brain areas and reproductive tissues, as well as in many other tissues and cell-types. (Please refer to Table 1 in the review manuscript attached to the thesis for a comprehensive list of sites of GnRHR expression.) *In vivo*, pituitary GnRHR numbers are regulated during fetal development, during sexual maturation (showing differential patterns between sexes), and during the reproductive cycle, pregnancy and lactation in the adult (Bauer-Dantoin AC *et al*., 1993; Zapatero-Caballero H *et al*, 2003; also reviewed in Stojilkovic SS & Catt KJ, 1995). Castration, which increases GnRH secretion from the hypothalamus, has also been found to increase GnRHR levels *in vivo* (Kaiser UB *et al*., 1993).

GnRH is released from the hypothalamic neurons in a pulsatile fashion. The pulse frequency and amplitude are regulated by a number of sex steroids and gonadal peptides, and therefore change over the course of the reproductive cycle (Shupnik MA, 1996). The GnRH pulse frequency typically varies between once every 30 to 120 minutes (Fink G, 1988). The frequency of the pulses has a differential effect on the numbers of GnRH receptors (Katt JA *et al*., 1985), as well as on the synthesis rate of LHβ and FSHβ in the gonadotropes (Shupnik MA, 1996). GnRH receptor synthesis and LHβ synthesis is stimulated at higher pulse frequencies, while a lower pulse frequency favours FSHβ synthesis (Kaiser UB *et al*., 1997b). The pulsatile nature of GnRH release is fundamental for maintaining reproductive function, since continuous GnRH administration desensitizes gonadotropes, ultimately resulting in a shut-down of gonadal function (reviewed in Millar RP *et al*., 2004), a reversible condition referred to as “biochemical castration” (Conn PM & Crowley WF, 1994).

1.1.3 Physiological roles of mammalian GnRHRs

1.1.3.1 Physiological actions in the pituitary

Multiple GnRHR I mRNA transcripts coding for full-length protein are detected in normal mammalian pituitary tissue and cell lines. In addition, several species, including humans, express splice variants, which may code for functionally relevant truncated GnRHR I proteins (reviewed in Norwitz ER at al., 1999b; Cheng CK and Leung PCK, 2005). It is well established that the primary role of GnRHR I protein in mammalian pituitary gonadotropes is to regulate LH and FSH synthesis and release (Fink G,

1988). The important role played by the GnRHR in gonadotropin regulation is illustrated by the findings that several naturally-occurring mutations in the human GnRHR I result in hypogonadotropic hypogonadism, with symptoms of delayed sexual development, low or apulsatile gonadotropin and sex steroid hormone levels, in the absence of abnormalities in the hypothalamic-pituitary axis (Seminara SB *et al*., 1998; Millar RP *et al*., 2004). The majority of these mutated receptors are mislocalized proteins, exhibiting altered membrane trafficking (Knollmann PE *et al*., 2005) and endoplasmic reticulum retention that can be restored to function by pharmacological chaperones (Brothers SP *et al*., 2004).

1.1.3.2 Physiological action in extra-pituitary tissues

1.1.3.2.1 Reproductive tissues

GnRHR I mRNA and/or protein has been detected in normal human breast tissue, several ovarian compartments, in endometrial tissue and in the placenta (see table 1 in Hapgood JP *et al*., 2005 (manuscript attached), also reviewed in Cheng CK and Leung PCK, 2005). In these tissues, paracrine/autocrine actions of GnRH via GnRHR I play a role in normal breast (Kottler ML *et al*., 1997) and ovarian (Kogo H *et al*., 1999) development, regulation of the menstrual cycle (Raga F *et al*., 1998) and establishment and maintenance of pregnancy (reviewed in Rama S *et al*., 2001). Additional functions for GnRHRs in the human ovary include inhibition of gonadotropin-regulated steroidogenesis and suppression of hCG-stimulated progesterone production in granulosa-luteal cells (Guerrero HE *et al*., 1993; Gaetje R, 1994).

The human testis and prostate express GnRH or GnRH-like peptides (Morales P and Llanos M, 1996) and GnRHR I (Kakar SS *et al*., 1992), and these all form part of complex autocrine and paracrine regulatory circuits in male reproductive tissues. Paracrine/autocrine actions of GnRH I via GnRHR I play a role in testicular development (Cheung TC and Hearn JP, 2003) as well as in sperm motility and sperm-oocyte interactions (Morales P, 1998).

1.1.3.2.2 Non-reproductive tissues

Hypothalamic GnRH neurons have been found to express GnRHR I, which is proposed to function in an autocrine fashion to regulate GnRH release (Martinez-Fuentes AJ *et al*., 2004). The detection of

both GnRHR I (see table 1 in review manuscript) and GnRHR II (Millar RP, 2003) transcripts in many mammalian brain tissues has also supported a role for GnRH I and/or GnRH II as a neurotransmitter or neuromodulator. This hypothesis is supported by functional evidence *in vivo* for an integral role for GnRH in sexual behavior in mammals via several different brain tissues (Millar RP, 2003). In addition, the detection of GnRH and GnRHR I transcripts and/or protein in T-cells (Chen A *et al*., 2002), spleen (Jacobson JD *et al*., 1998) and gastric parietal cells (Chen L *et al*., 2005), combined with functional evidence, suggests other autocrine/paracrine roles for GnRHRs in immunomodulation (Jacobson JD *et al*., 1998; Chen HF *et al*., 1999), such as adhesion chemotaxis and homing in T cells (Chen A *et al*., 2002), and inhibition of gastric acid secretion (Chen L *et al*., 2005).

1.1.3.3 Cancer cells

It is widely accepted that continuous administration of GnRH analogs inhibits growth of several reproductive tissue-derived tumors and that this effect may be mediated via GnRHRs expressed on these cells (Grundker C *et al*., 2002; Franklin J *et al*., 2003; Limonta P *et al*., 2003; Harrison GS *et al*., 2004; Finch AR *et al*., 2004). However, the anti-proliferative effects of GnRH analogs on human melanoma cells (Limonta P *et al*., 2003) suggest that such GnRHR-mediated growth effects are not unique to reproductive tissue-derived cancer cells. The GnRHR-mediated intracellular pathways involved may include nuclear GnRH binding sites (Szende B *et al*., 1991) and/or interaction and interference with epidermal growth factor receptor (EGFR) mitogenic signalling (Roelle S *et al*., 2003; Kraus S *et al*., 2003).

1.2 GONADOTROPES AND GONADOTROPE CELL LINES

1.2.1 Cell-types of the anterior pituitary

The anterior pituitary consists of a heterogeneous population of well-differentiated endocrine cell-types, each responsible for the production of different hormones and the control of different endocrine systems. The corticotropes differentiate first during pituitary development (Horn F *et al*., 1992), and produce a precursor peptide, pro-opiomelancortin (POMC), which is cleaved into several products, including adrenocorticotrophic hormone (ACTH), endorphins and enkephalin. Other cell-types that arise later in development are the thyrotropes, which secrete thyroid-stimulating hormone (TSH), somatotropes, responsible for growth hormone (GH) production, lactotropes, which produce prolactin

(PRL), and the gonadotropes, which produce the gonadotropins LH and FSH (Horn F *et al*., 1992). Anterior pituitary cells have also been shown to secrete more than one hormone, for instance LH together with FSH, or GH together with PRL (reviewed in Kaiser UB *et al*., 1997a).

1.2.2 Gonadotrope cell lines

Gonadotropes only make up 6% to 15% of the total anterior pituitary cell population (Childs GV *et al*., 1987; Ibrahim SN *et al*., 1986). Historically, experiments on the pituitary control of reproduction were performed *in vivo,* or in primary pituitary cultures. However, the heterogeneity of the anterior pituitary cell population limited the interpretation of results derived from these systems. Furthermore, primary pituitary cells cannot be maintained in continuous culture, creating practical constraints on experimental design. It was therefore necessary to engineer immortalized pituitary cell lines, specifically of the gonadotrope cell-type, to use as a model system for the investigation of the regulation of reproductive function by GnRH and the GnRHR (reviewed in Kaiser UB *et al*., 1997a).

In the past, studies on the regulation of GnRHR gene expression have been predominantly carried out in the αT3-1 gonadotrope cell line (Duval DL *et al*., 1997b; White BR *et al*., 1999; Norwitz ER *et al*., 2002a). Recently, several studies dealing with the expression of other gonadotrope genes, such as the gonadotropin genes (Pernasetti F *et al*., 2001; Curtin D *et al*., 2001; Fowkes RC *et al*., 2002) and the neuronal nitric oxide synthase (nNOS) gene (Bachir LK *et al*., 2003), have utilized the LβT2 gonadotrope cell line. However, very little data have been published on the regulation of the GnRHR gene in this cell line.

1.2.2.1 The αT3-1 cell line

The αT3-1 clonal cell line was created by means of targeted tumourigenesis in transgenic mice, using the promoter of the human glycoprotein hormone α-subunit (α-GSU) gene (Windle JJ *et al*., 1990). These cells represent precursor gonadotrope cells, retaining some differential gonadotrope functions, such as the expression of functional GnRH receptors and GnRHR-mediated responsiveness to GnRH administration. In addition, they express several activin receptor subunits, as well as the β-subunit, but not the α-subunit, of inhibin (Fernandez-Vazquez G *et al*., 1996). These cells also express, synthesize

and secrete the glycoprotein hormone α-subunit, but they do not express either of the glycoprotein hormone-specific β-subunits, LHβ and FSHβ (Windle JJ *et al*., 1990).

1.2.2.2 The LβT2 cell line

Similar to αT3-1 cells, the LβT2 cell line was generated by targeted tumourigenesis. However, in this instance, the rat LHβ promoter was used, generating a clonal line representing more mature and differentiated gonadotropes (Turgeon JL *et al*., 1996). These cells express functional GnRH receptors, as well as the glycoprotein hormone α-subunit, LHβ and FSHβ (Turgeon JL *et al*., 1996; Pernasetti F *et al*., 2001). In addition, they express activin and activin receptors, as well as inhibin and follistatin, thus displaying all the hallmarks of fully differentiated gonadotropes (Pernasetti F *et al*., 2001).

1.2.3 GnRH signaling in gonadotropes and gonadotrope cell lines

GnRH can regulate a wide variety of responses in gonadotropes. The mechanism by which a single ligand can facilitate such a range of responses through the activation of a single class of receptors is an intriguing question.

As already mentioned, different GnRH pulse frequencies and amplitudes have different effects on gonadotropin expression, with LHβ synthesis being stimulated at higher pulse frequencies, while a lower pulse frequency favours FSHβ synthesis (Kaiser UB *et al*., 1997b). This could at least in part be attributed to the fact that modulation of pulse frequency regulates GnRH receptor numbers, since differences in receptor numbers have been demonstrated to have differential effects on LHβ and FSHβ promoter activity. High receptor numbers were found to stimulate LHβ promoter activity, while low receptor numbers preferentially stimulated FSHβ promoter activity (Kaiser UB *et al*., 1995). Modulation of GnRH pulse frequency therefore definitely contributes to the ability of GnRH to regulate multiple physiological events.

It has also been proposed that GnRHR numbers could influence coupling to G proteins (Stanislaus D *et al*., 1998a). This was demonstrated for G_s-coupled receptors, which were shown to activate PLC in addition to adenylate cyclase, but only at high receptor densities (Zhu X *et al*., 1994). In addition, the levels of different G proteins in the pituitary vary significantly during the different phases of the rat

estrous cycle, providing another possible mechanism whereby GnRHR I could couple to different G proteins under different conditions (Bouvier C *et al*., 1991). Furthermore, modulation of G-protein activity, through mechanisms such as phosphorylation and palmitoylation, could add another dimension in ultimately determining the downstream signaling events initiated by GnRH (Stanislaus D *et al*., 1998a).

In rat pituitary cell cultures, GnRHR I can couple to several different G proteins, including G_i/G_o, G_q/G_{11} and G_s (Hawes BE *et al*., 1992; Stanislaus D *et al*., 1998b). GnRH has also been shown to increase intracellular cAMP (Borgeat P *et al*., 1972) and activate PKC (McArdle CA and Conn PM, 1986), ERK (Haisenleder DJ *et al*., 1998) and JNK (Mulvaney JM and Roberson MS, 2000) in primary rat pituitary cultures.

GnRH stimulation increases IP3 turnover and activates PKC in αT3-1 cells, but it does not elevate cAMP levels in these cells (Horn F *et al*., 1991). Although initial results suggested that GnRH also signalled via G_i, (Sim PJ *et al*., 1995), it has since been established that the GnRHR couples exclusively to G_q/G_{11} in αT3-1 cells (Grosse R *et al*., 2000). GnRH can activate several members of the MAP kinase family via PKC, including ERK (Sim PJ *et al*., 1995; Reiss N *et al*., 1997), p38 MAPK (Roberson MS *et al*., 1999) and JNK (Levi NL *et al*., 1998). PKC also regulates the affinity of GnRHR for GnRH, suggesting a novel form of “inside-out” signalling (Caunt CJ *et al*., 2004). GnRH also induces influx of extracellular calcium and mobilization of intracellular calcium stores (reviewed in Kraus S *et al*., 2001). In αT3-1 cells, the GnRHR is not desensitized by chronic stimulation with GnRH, but the GnRH-mediated calcium responses are downregulated, due to a loss of type I IP3 receptor induced by chronic GnRH stimulation (Willars GB *et al*., 2001). The appropriate organization of GnRH receptors into low density membrane microdomains on the cell surface appear to be critical in mediating GnRH-induced intracellular signalling in αT3-1 cells (Navratil AM *et al*., 2003).

In LβT2 cells, GnRHR can signal via both G_s and G_q G-proteins (Liu F *et al*., 2002b). While some investigators have found no role for pertussis toxin-sensitive G proteins (such as G_i) (Liu F *et al*., 2002b), others have found that G_i plays a role in GnRH-mediated ERK activation (Yokoi T *et al*., 2000; Bonfil D *et al*., 2004). In LβT2 cells, GnRH activates ERK, JNK and p38 MAPK (Yokoi T *et al*., 2000;

Liu F *et al*., 2002a; Liu F *et al*., 2003). ERK activation is dependent on PKC, whereas JNK activation is independent of PKC and calcium (Yokoi T *et al*., 2000, Liu F *et al*., 2002a). GnRH can also activate ERK in a PKA-dependent manner, through G_s-mediated increases in cAMP levels (Liu F *et al*., JBC 2002b). Long-term chronic GnRH stimulation of LβT2 cells decreases GnRHR expression and G_q/G_{11} expression, but not G_s expression, and it downregulates PKC, cAMP and calcium signaling (Liu F *et al*., 2003).

1.3 REGULATION OF MOUSE GnRHR GENE EXPRESSION

1.3.1 Mammalian GnRHR genes

The structures of the mouse (Zhou W and Sealfon SC, 1994), rat (Reinhart J *et al*., 1997), human (Fan NC *et al*., 1995), pig (Jiang Z *et al*., 2001) and sheep (Campion CE *et al*., 1996) GnRHR I genes have been characterized. In these species, the genes exist as a single copy, and have a high degree of sequence homology in the coding regions. They are structurally similar and consist of 3 exons separated by 2 introns. The exon-intron boundaries are conserved between the species, but the genes differ with regard to the size of the introns, as well as the sequence and length of the 5' and 3' untranslated regions (UTR) (see figure 1). Exon 1 encodes the N-terminal tail as well as transmembrane helices (TM) 1, 2, 3 and part of TM 4 of the GnRHR I protein. Exon 2 encodes the rest of TM 4 and the whole of TM 5 while exon 3 encodes TM 6 and 7 (Fan NC *et al*., 1994).

Candidate genes for GnRHR II can be found on chromosomes 1 and 14 in the human genome (Neill JD, 2002; Millar RP, 2003), and GnRHR II genes have also been detected in other mammalian genomes. The GnRHR II gene has the same exon/intron structure as GnRHR I, except that exon 3 includes a cytoplasmic C-terminal tail, which is absent in GnRHR I (Faurholm B *et al*., 2001). Premature stop codons occur in the human and chimpanzee GnRHR II genes (Neill JD, 2002; Morgan K *et al*., 2003), whereas a fully functional GnRHR II gene is present in other primates, like marmoset monkey (Millar RP *et al*., 2001), rhesus monkey and African green monkey (Neill JD *et al*., 2001). The GnRHR II gene is completely deleted from the mouse genome (Millar RP 2003), but a gene remnant is present in the rat genome (Pawson AJ *et al*., 2003).

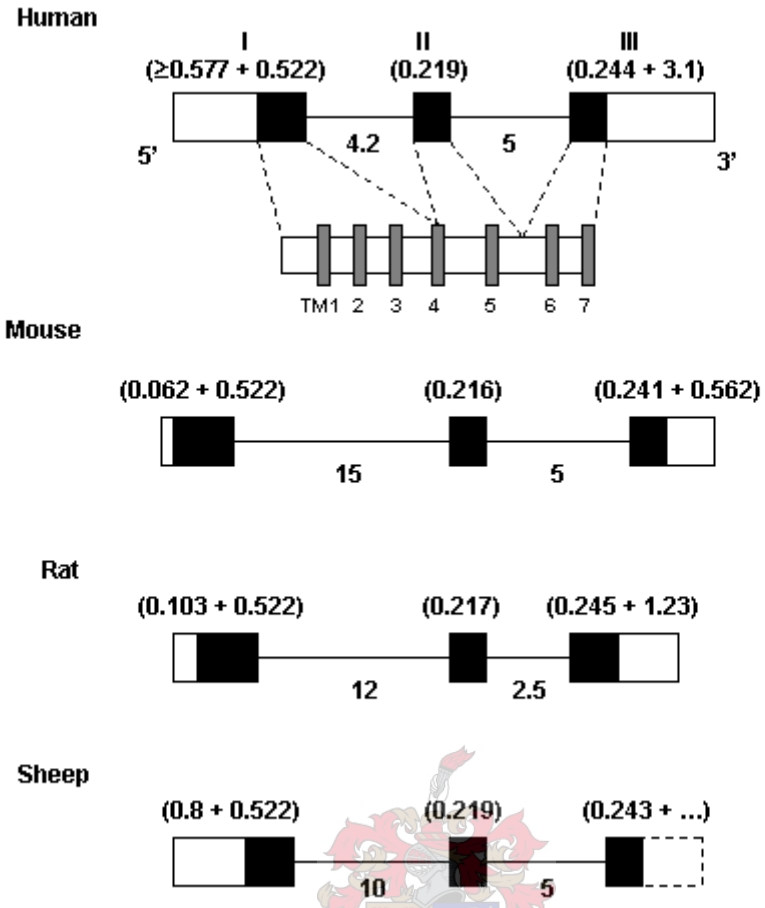


Figure 1.3: Structural organization of the GnRHR I gene in human, mouse, rat and sheep.
Note that this figure is not drawn to scale. Exons are represented by blocks, with portions of exons containing coding sequences shown as dark areas, and untranslated regions (UTR) shown as light areas. Sizes of coding portions are indicated above, and sizes of 5' and 3' UTR below each block, where established. Introns are represented by solid lines, with sizes as indicated. All sizes are indicated in kilobasepairs. For the human gene, the size of the 5' UTR is given relative to most 3' transcription start site as identified by Kakar SS, 1997 for human pituitary tissue, and the size of the 3' UTR is as established by Fan NC *et al*., 1995. For the mouse gene, the 5' UTR is relative to the major transcription start site as identified by Albarracin CT *et al*., 1994 for αT3-1 cells. For the rat gene, the size of the 5' UTR is given relative to the major transcription start site as identified by Reinhart J *et al*., 1997 for rat pituitary tissue. This figure was compiled from Reinhart J *et al*., 1997; Pincas H *et al*., 1998; Fan NC *et al*., 1994; Fan NC *et al*., 1995; Zhou W and Sealfon SC, 1994; Kakar SS, 1997; Campion CE *et al*., 1996; Pincas H *et al*., 1998; Eidne KA, 1994; Albarracin CT *et al*., 1994. (From Hapgood JP *et al*., 2005; see manuscript attached)

To date, the 5' flanking regions of the mouse (Albarracin CT *et al*., 1994), rat (Reinhart J *et al*., 1997), human (Fan NC *et al*., 1995; Kakar SS, 1997) and sheep (Campion CE *et al*., 1996) GnRHR I genes have been characterized. While the mouse and rat promoters share > 80% homology over 1.9 kb, the rat promoter shares 55% homology with the human promoter over 2.2 kb, and 63% homology with the sheep promoter over 0.9 kb (Pincas H *et al*., 1998). There are several highly homologous regions within the proximal 500 basepairs of the mouse, rat, human and sheep promoters (Pincas H *et al*., 1998). A number of *cis*-elements have been conserved, in sequence as well as position, supporting their role as important functional elements (see figure 1.4). No functional characterization of mammalian GnRHR II promoters has as yet been published. Figure 1.4 represents a detailed schematic of the human, mouse and rat proximal GnRHR I promoters, indicating the presence and position of the *cis*-elements that have been characterized in the literature.

1.3.2 Mouse GnRHR promoter characterization and gonadotrope-specific expression

The mouse GnRHR proximal promoter was the first to be isolated and characterized (Albarracin CT *et al*., 1994). The major transcription start site in primary pituitary tissue (Sadie H *et al*., 2003) and αT3-1 cells (Albarracin CT *et al*., 1994) is located at -62 relative to the start codon[†], and is not associated with a consensus TATA box. In addition to this site, Clay *et al*. identified other pituitary transcription start sites at -90 and -200 bp (Clay CM *et al*., 1995) in αT3-1 cells. The presence of multiple transcription start sites and the absence of consensus TATA box elements have also been described for genes encoding other G-protein-coupled receptors, such as the receptors for LH, FSH and TSH (reviewed in Albarracin CT *et al*., 1994).

[†] Note that all numbering of promoter elements is relative to the translation start site.

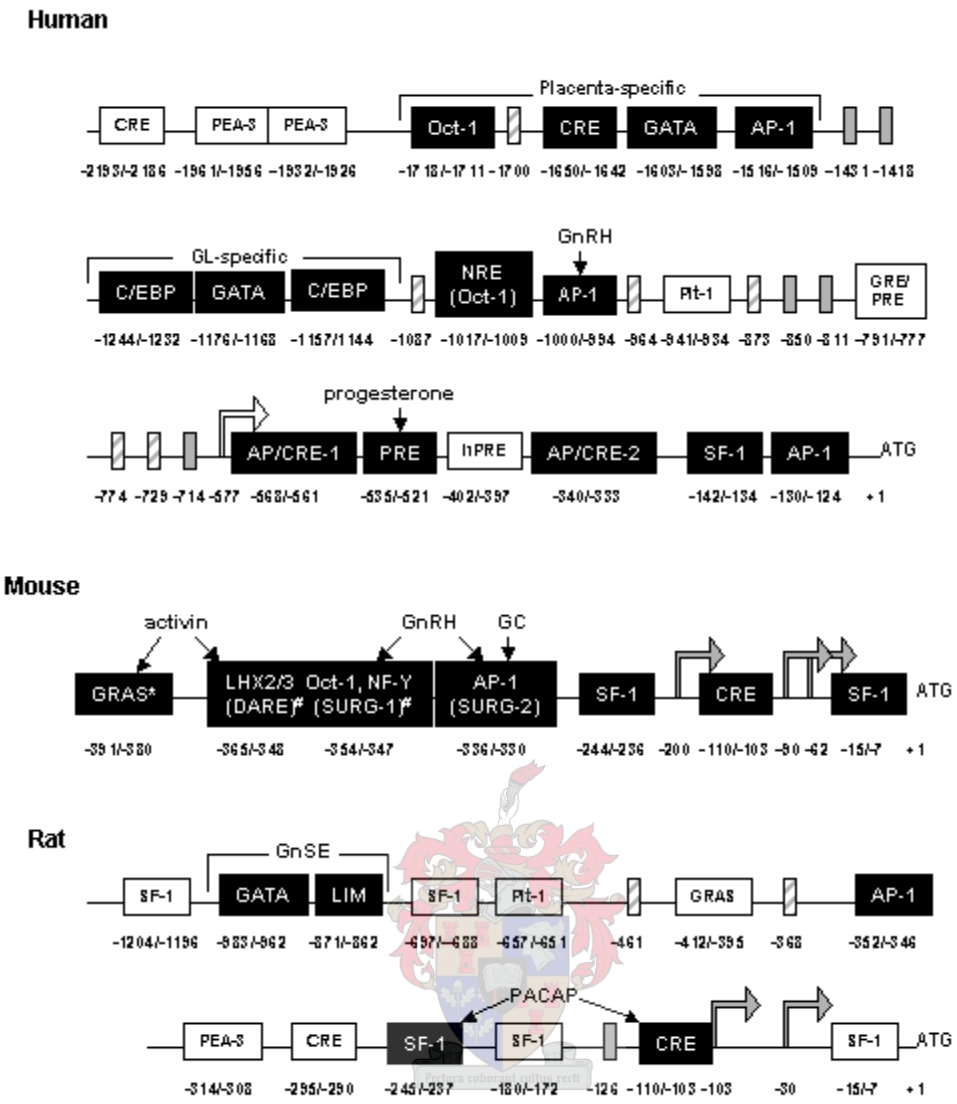


Figure 1.4: Functional elements in the GnRHR I promoter regions of human, mouse and rat. Hormone responses and their corresponding *cis*-elements established in functional studies in pituitary cell lines are indicated. Black boxes represent elements that have been functionally characterized. White boxes represent putative elements that have been identified through promoter sequence analysis. Shaded boxes and striped boxes represent TATA and CCAAT elements, respectively. Transcription start sites are indicated with arrows, and the translation start site with "ATG". For the human gene, the most 3' transcription start site, as identified by Kakar SS, 1997 for human pituitary tissue, is indicated. Other transcription start sites, as identified for human brain (Fan NC *et al*., 1995), pituitary (Kakar SS, 1997) and placental tissues (Cheng KW *et al*., 2001), are not indicated. Hormone responses and their corresponding *cis*-elements established in functional studies in pituitary cell lines are indicated. The mouse promoter has not been functionally characterized upstream of the GRAS element. This figure was compiled from Cheng CK and Leung PCK, 2005 and Norwitz ER *et al*., 1999b, and other references quoted in the text. *GRAS contains binding sites for SMAD, AP-1 and FoxL2 proteins (Norwitz ER *et al*., 2002b; Ellsworth BS *et al*., 2003a). # Several functional elements overlap in this region. The positions for DARE and SURG-1 are indicated. LHX2 was specifically

shown to bind DARE (Cherrington BD *et al*., 2005), but LHX3 was shown to bind an overlapping site (McGillivray SM *et al*., 2005). Abbreviations: SF-1 = Steroidogenic Factor-1 binding site; PRE = progesterone response element; hPRE = PRE half-site; CRE = cAMP response element; AP-1 = Activator Protein 1 binding site; C/EBP = CCAAT/enhancer binding protein motif; GRE/PRE = glucocorticoid response element/progesterone response element; PEA-3 = phorbol ester response element; Pit-1 = Pit-1 transcription factor binding site; Oct-1 = octamer transcription factor-1 binding site; GATA = GATA transcription factor binding site; LIM = LIM-homeodomain factor binding site; GRAS = GnRH receptor activating sequence; DARE = downstream activin response element; SURG = sequence underlying responsiveness to GnRH; GnSE = GnRHR-specific enhancer; NF-Y = nuclear factor-Y binding site; NRE = negative regulatory element; GL-specific = granulosa-luteal cell-specific; GC = glucocorticoid. Note that this figure is not drawn to scale. (From Hapgood JP *et al*., 2005; see manuscript attached)

Albarracin *et al.* demonstrated that a 1.2 kb mouse GnRHR genomic fragment, containing 1.1 kb of 5' flanking region and the full-length (62 bp) 5' untranslated region (UTR), was sufficient to confer gonadotrope-specific expression in αT3-1 cells (Albarracin CT *et al*., 1994). These investigators also identified a gonadotrope-specific element (GSE)-like sequence at position -15/-7, similar to the GSEs found in the promoters of the glycoprotein hormone α-subunit (Horn F *et al*., 1992) and the luteinizing hormone β-subunit (Halvorson LM *et al*., 1996). This element is conserved between the mouse and rat promoters in both sequence and position, and *in vitro* protein-DNA binding experiments have shown that the orphan nuclear receptors Steroidogenic Factor-1 (SF-1) can recognize this element in the mouse promoter (Duval DL *et al*., 1997a). However, Clay *et al*. demonstrated that this GSE-like element was not necessary for high basal and cell-specific expression of the mouse GnRHR (Clay CM *et al*., 1995). Instead, gonadotrope-specific activity of the mouse GnRHR promoter in αT3-1 cells was found to be conferred by a tripartite basal enhancer, which includes another SF-1 binding site at -244/-236 (Duval DL *et al*., 1997a), a consensus activator protein-1 (AP-1) site at -336/-330 and an element originally termed GnRHR-activating sequence (GRAS) at -391/-380 (Duval DL *et al*., 1997b) (see figure 1.4). The pan-pituitary homeobox transcription factor Pitx-1 has been shown by chromatin immunoprecipitation (ChIP) assay to interact with AP-1 in intact LβT2 cells, and functional evidence in other cell types indicate that this interaction might be important for GnRHR gonadotrope-specific, basal promoter activity (Jeong KH *et al*., 2004). In addition, the promoter region around -360, shown to bind LHX3 homeodomain protein *in vitro* and in intact cells, was demonstrated to be important for mouse GnRHR basal promoter activity in αT3-1 cells (McGillivray SM *et al*., 2005).

1.3.3 Regulation of mouse GnRHR gene expression in gonadotropes by physiological compounds

Regulation of GnRHR numbers on the cell surface has been shown to occur at the transcriptional, translational and post-translational level. A well-known mechanism for ligand-mediated posttranslational downregulation of GPCR numbers on the cell surface involves desensitization, internalization and degradation. Mammalian type I GnRH receptors have been shown to internalize slowly due to the absence of a C-terminal tail (McArdle CA *et al*., 2002). Homologous regulation of translation efficiency from GnRHR mRNA has also been shown to occur in αT3-1 cells (Tsutsumi M *et al*., 1995). However, very little research has been reported on post-transcriptional regulation of GnRHR I gene expression, and this section will therefore focus on the transcriptional regulation of GnRHR I gene expression.

1.3.3.1 Homologous regulation of GnRHR gene expression

Homologous regulation of the GnRHR is a physiologically relevant mechanism for increasing pituitary sensitivity to GnRH during ovulation (White BR *et al*., 1999). Understanding the mechanism of regulation of GnRHR gene transcription by GnRH and the intracellular pathways that mediate this response has been a focus of several groups in the field, since this hormone appears to have a significant effect on GnRHR levels in *vivo* (Kaiser UB *et al*., 1993; Yasin M *et al*., 1995). It is widely accepted that pulsatile GnRH stimulation (as delivered by the hypothalamus) is essential in facilitating and maintaining GnRHR upregulation while avoiding receptor down-regulation due to continuous hormonal stimulation (Weiss J *et al*., 1995; Liu F *et al*., 2003). GnRH pulse frequency and amplitude vary with physiological state, the estrous cycle in mammals and the menstrual cycle in humans, puberty and menopause (Norwitz ER *et al*., 1999a), and is regarded as one of the most important factors in regulating GnRHR numbers and activity.

1.3.3.1.1 Homologous regulation *in vivo*

Studies in rats found that continuous stimulation with GnRH was ineffective in increasing pituitary GnRHR mRNA levels, but that pulsatile administration of GnRH upregulated GnRHR expression (Yasin M *et al*., 1995). While the maximal increases in response to pulsatile GnRH stimulation were found to be the same in both sexes, responses to differences in pulse amplitude and frequency clearly

differed between the sexes. For both sexes, increases in GnRHR mRNA levels were already detectable after 4 hours, and continued to rise, even after 24 hours of pulsatile stimulation (Yasin M *et al*., 1995). In contrast, several days of continuous GnRH administration in sheep (Wu JC *et al*., 1994) and cows (Vizcarra JA *et al*., 1997) greatly reduced GnRHR mRNA and protein levels, clearly demonstrating the downregulatory effect of continuous GnRH treatment *in vivo*.

In transgenic mice expressing a luciferase transgene driven by 1900 bp of the proximal mouse GnRHR promoter, immunomeutralization of GnRH led to decreased luciferase expression in the pituitary (McCue JM *et al*., 1997). It can therefore be concluded that this fragment of the proximal promoter contains the *cis*-elements required to confer GnRH regulation in the pituitary *in vivo*. Pituitary-specific expression of this promoter fragment in transgenic mice was not dependent on an intact AP-1 site. This is consistent with results in αT3-1 cells (Duval DL *et al*., 1997b), showing that mutation of the AP-1 site decreased promoter activity, but did not completely abolish it. However, the AP-1 element was found to be required for GnRH responsiveness of the promoter in transgenic mice (Ellsworth BS *et al*., 2003b).

1.3.3.1.2 Homologous regulation in primary pituitary cultures

Studies by different groups investigating homologous regulation of GnRHR expression in primary pituitary cultures have not always yielded consistent results. Some early reports indicate that continuous administration of low (nanomolar and subnanomolar) doses of GnRH increased GnRHR numbers in cultured rat pituitary cells over several hours (Loumaye E and Catt KJ, 1982; Loumaye E and Catt KJ, 1983; Young LS *et al*., 1984). However, Kaiser *et al*. found that continuous GnRH administration had no effect on GnRHR mRNA levels in rat pituitary primary cultures, but that pulsatile stimulation by GnRH increased GnRHR mRNA already after the first pulse, reaching a maximum after 4 hours (Kaiser UB *et al*., 1993). These apparently conflicting results emphasize the fact that GnRHR numbers are regulated by both transcriptional and post-transcriptional mechanisms.

1.3.3.1.3 Homologous regulation in gonadotrope cell lines

Homologous regulation of the GnRHR at the transcriptional level in αT3-1 cells is the subject of some controversy. Similar to what has been reported for primary pituitary cultures, some investigators report

changes in GnRHR mRNA levels in αT3-1 cells in response to GnRH, whereas others observe changes in receptor numbers without concomitant changes in mRNA levels. Tsutsumi *et al.* found that short-term stimulation with low concentrations of GnRH increased GnRHR numbers, but not GnRHR mRNA levels (Tsutsumi M *et al*., 1993), while continuous stimulation with high concentrations of GnRH over 24 hours greatly decreased GnRHR numbers without any effect on GnRHR mRNA levels (Tsutsumi M *et al*., 1995). Another group found that continuous long-term stimulation with high concentrations of GnRH caused a steady reduction in GnRHR numbers and GnRHR mRNA levels in αT3-1 cells, even after only 2 hours of hormone treatment. However, the maximal reduction of mRNA levels was consistently found to be less than the reduction in receptor numbers, indicating that the lowered receptor numbers were also due to mechanisms other than transcriptional regulation, such as endocytosis and degradation (Mason DR *et al*., 1994). In contrast to these findings, GnRHR mRNA levels in αT3-1 cells were found to increase after stimulation with 100 nM GnRH for 4 hours, only decreasing after longer incubations (Norwitz ER *et al*., 1999a).

Consistent with the results published by Norwitz et al. for GnRHR mRNA levels in αT3-1 cells, mouse GnRHR promoter-reporter constructs transfected into αT3-1 cells significantly responded to continuous treatment with 100 nM GnRH after 4h (Norwitz ER *et al.,* 1999a; White BR *et al.,* 1999). The GnRH responsiveness of the mouse promoter was mapped to two regions, designated SURG-1 and SURG-2 (Sequence Underlying Responsiveness to GnRH) (Norwitz ER *et al.,* 1999a). SURG-1 contains binding sites for nuclear factor Y (NF-Y) and Oct-1 (Kam KY *et al.,* 2005), while SURG-2 contains the AP-1 site previously described as part of the tripartite basal enhancer (Duval DL *et al.,* 1997b). GnRH I responsiveness via SURG-2 appears to be predominantly mediated by PKC-induced activation of JNK. This results in increased expression, activity and binding of AP-1 proteins to SURG-2 (Ellsworth BS *et al.,* 2003b). GnRH also leads to increased binding of NF-Y and Oct-1 to SURG-1, as was shown by chromatin immunoprecipitation assays in LβT2 cells (Kam KY *et al.,* 2005). SURG-1 and SURG-2 can respond to GnRH independently, but the AP-1 element is critical for conferring GnRH responsiveness in αT3-1 cells (Norwitz ER *et al.,* 1999a), in agreement with results obtained in transgenic mice (Ellsworth BS *et al.,* 2003b). In addition, responsiveness of the mouse GnRHR promoter to GnRH also involves binding of Smad and AP-1 proteins to the GRAS element described earlier (Norwitz ER *et al.,* 2002b).

Very few studies have been published on homologous regulation of GnRHR gene expression in LβT2 cells. Endogenous GnRHR mRNA and protein levels are upregulated upon pulsatile GnRH stimulation over several days (Turgeon JL *et al*., 1996). Consistent with these findings, Bedecarrats and Kaiser found that GnRHR protein levels in LβT2 cells were modestly upregulated over several hours of pulsatile GnRH stimulation. Pulsatile GnRH stimulation also upregulated the activity of a mouse GnRHR promoter-reporter construct transfected into these cells, while continuous GnRH administration had little effect on reporter gene expression (Bedecarrats GY and Kaiser UB, 2003).

1.3.3.2 GnRHR gene regulation by pituitary adenylate cyclase activating polypeptide (PACAP)

PACAP was originally isolated from sheep hypothalamic tissue as a potent activator of cyclic AMP production in the anterior pituitary. Since then, PACAP has been shown to exert an effect in several other tissues, including the brain, adrenals, gut and lung. PACAP occurs as two isoforms, consisting of either 38 amino acids (PACAP-38), or of the N-terminal 27 amino acids (PACAP-27), and share high sequence homology with vasoactive intestinal polypeptide (VIP) (reviewed in Rawlings SR and Hezareh M, 1996).

Three types of PACAP/VIP receptors (PVRs) have been cloned. In gonadotropes, PACAP appears to function primarily via PVR1 receptors, which have a much higher affinity for PACAP than for VIP, and can potentially activate both adenylate cyclase and phospholipase C (reviewed in Rawlings SR and Hezareh M, 1996). In αT3-1 cells, it has been shown that PACAP is a more potent stimulator of cAMP production than of IP turnover (Schomerus E *et al*., 1994). However, the LβT2 cell line has been found to express extremely low levels of PVR1 receptor, and PACAP fails to stimulate cAMP production in these cells. The down-stream effects of PACAP stimulation on gene expression and other physiological events can therefore not be studied in these cells (Fowkes RC *et al*., 2003).

The human (Cheng KW and Leung PCK, 2001) and rat (Pincas H *et al*., 2001b) GnRHR I promoters have been shown to be regulated by PACAP in αT3-1 cells, via a mechanism that involves PKA. Two promoter elements, designated PARE (PACAP response element) I and PARE II, are required for the PACAP response of the rat GnRHR I promoter. PARE I includes a SF-1 binding site at position -245/-237, along with binding sites for additional factors, while PARE II contains an imperfect cAMP

response element (CRE) at position -110/-103 that can bind CREB (see Figure 1.4) (Pincas H *et al*., 2001b). Both the SF-1 site and the imperfect CRE are conserved in relative position in the mouse GnRHR promoter, raising the possibility that the activity of the mouse promoter could be regulated by PACAP via a similar mechanism.

1.3.3.3 GnRHR gene regulation by activin

Activin is a member of the transforming growth factor (TGF)-beta family of proteins, is produced by primary gonadotrophs (Roberts VJ *et al.,* 1992), αT3-1 (Fernandez-Vazquez G *et al.,* 1996) and LβT2 cells (Pernasetti F *et al.,* 2001), and exert autocrine/paracrine effects on pituitary cells. Activin-A stimulates the rate of synthesis of new GnRHRs in rat pituitary cell cultures (Braden TD and Conn PM, 1992). In αT3-1 and LβT2 cells, long-term stimulation with activin-A upregulates endogenous GnRHR mRNA synthesis and mouse GnRHR promoter-reporter activity (Fernandez-Vazquez G *et al.,* 1996, Pernasetti F *et al.,* 2001), and pretreatment of αT3-1 cells with activin enhances the response of the mouse GnRHR promoter to GnRH (Norwitz ER *et al.,* 2002b). Follistatin blocks the activin-mediated stimulation at both mRNA and promoter level. In addition, follistatin decreases the basal activity of the mouse GnRHR promoter in αT3-1 and LβT2 cells, indicating that endogenous activin maintains basal GnRHR expression levels in these cells (Norwitz ER *et al.,* 2002b; Fernandez-Vazquez G *et al.,* 1996; Pernasetti F *et al.,* 2001).

Activin responsiveness of the mouse GnRHR promoter was mapped to the GRAS element (Duval DL *et al.,* 1999) described earlier, together with a region immediately downstream from GRAS, termed DARE (down-stream activin regulatory element) (Cherrington BD *et al.,* 2005) (see figure 1.4). The mouse GRAS element is a composite regulatory element for which the functional activity in αT3-1 cells depends on the proper organization and assembly of a multi-protein complex, which includes Smad, AP-1 and FoxL2 proteins (Ellsworth BS *et al.,* 2003a). Basal GnRHR promoter activity, as well as responsiveness to GnRH and to activin require binding of Smad factors to the Smad binding element (SBE), as well as binding of AP-1 to a novel AP-1 element contained within GRAS (see figure 1.4) (Norwitz ER *et al.,* 2002a and 2002b). The LIM-homeodomain protein LHX2 was shown to bind the DARE sequence in vitro (Cherrington BD *et al.,* 2005). It has been postulated that activin responsiveness requires a specific configuration of multiple transcription factors on these distinct

elements, to form a complex activin-responsive "enhanceosome" (Cherrington BD *et al.,* 2005). Interestingly, the sequence of the corresponding GRAS element in the rat GnRHR I promoter differs from the mouse GRAS by only one base-pair, but does not confer activin responsiveness to the rat promoter (Pincas H *et al.,* 2001a; Cherrington BD *et al.,* 2005), suggesting that the rat DARE sequence is non-functional for activin responsiveness.

1.3.4 Transcription factors involved in GnRHR gene regulation in gonadotrope cell lines

1.3.4.1 Steroidogenic Factor-1

1.3.4.1.1 Introduction

SF-1 is a member of the nuclear receptor superfamily of transcription factors (Mangelsdorf DJ *et al*., 1995). Classically, SF-1 has been regarded to form part of the orphan receptor class of nuclear receptors, since no specific ligand has conclusively been identified for SF-1 (Mangelsdorf DJ and Evans RM, 1995; Mangelsdorf DJ *et al*., 1995). However, recent evidence suggests that phosphatidyl inositols could serve as ligands for SF-1 and other orphan receptors (Krylova IN *et al.,* 2005). Unlike most other nuclear receptors, SF-1 binds to its consensus DNA recognition sequence (5' TCA AGG TCA 3' (Morohashi K *et al*., 1992)) as a monomer. SF-1 is expressed in all steroidogenic tissues, as well as in the ventromedial hypothalamus and the pituitary gonadotrope cells (Luo X *et al*., 1994; Barnhart KM and Mellon PL, 1994; Parker KL, 1998), and regulates the expression of many genes involved in reproduction, steroidogenesis and endocrine function, including the gonadotropin subunit genes, the cytochrome P450 hydroxylases and the receptors for prolactin and GnRH I (reviewed in Parker KL, 1998). It is also responsible for coordinating the proper development and organogenesis of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes (Luo X *et al*., 1994; Wong M *et al*., 1996), and is crtical for male sexual development (Luo X *et al*., 1994). Since SF-1 was found to be expressed at all levels of the HPA and HPG axes, and shown to regulate expression of GnRHR, gonadotropins and steroidogenic enzymes, it was postulated that SF-1 functions as a global coordinator of reproductive function (Barnhart KM and Mellon PL, 1994).

1.3.4.1.2 The role of SF-1 in regulation of pituitary GnRHR expression

As already mentioned in section 1.3.2, two SF-1 consensus sites have been identified and characterized in the mouse GnRHR promoter. The proximal site is located at -15/-7 (within the 5'

untranslated region) (Albarracin CT *et al*., 1994), and does not appear to be involved in gonadotrope-specific expression of the mouse GnRHR gene (Clay CM *et al*., 1995). This is in contrast to results obtained for the human GnRHR I promoter, where an SF-1 binding site located in the 5' UTR is largely responsible for mediating gonadotrope-specific expression (Ngan ESW *et al*., 1999). The distal SF-1 consensus site in the mouse GnRHR promoter is located at -244/-236, and forms part of the tripartite basal enhancer that confers gonadotrope-specific activity on the mouse GnRHR promoter (Duval DL *et al*., 1997b). However, this site does not appear to be critical for mouse GnRHR promoter activity in αT3-1 cells, since mutation of this element only reduced promoter activity, but did not abolish it entirely (Duval DL *et al*., 1997b). This element was also found not to be involved in the response of a mouse GnRHR promoter-reporter construct to GnRH stimulation in αT3-1 cells (White BR *et al*., 1999). The two SF-1 elements in the mouse GnRHR I promoter are conserved in sequence and position in the rat promoter (Reinhart J *et al*., 1998). While no function has been demonstrated for the proximal site in the rat promoter, the distal SF-1 site has been shown to be involved in gonadotrope-specific expression (Pincas H *et al*., 2001a), as was shown for the mouse promoter. The cAMP/PACAP response of the rat promoter in αT3-1 cells is also mediated via this SF-1 site, together with a cAMP-response element at -110/-103 (Pincas H *et al*., 2001b). In addition, overexpression of SF-1 increases the transcriptional activity of the rat GnRHR I promoter in both αT3-1 and LβT2 cells (Pincas H *et al*., 2001a), and similar findings have been reported for the human promoter in αT3-1 cells (Ngan ESW *et al*., 1999).

1.3.4.2 Nur77

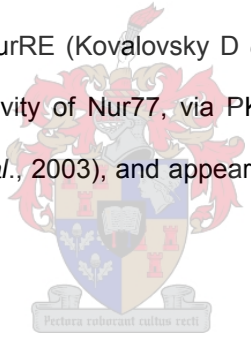
1.3.4.2.1 Introduction

Nur77 (also known as NGFI-B) is another orphan member of the nuclear receptor superfamily (reviewed in Maruyama K, 1998). It is widely expressed, in particular in the hypothalamus (Parkes D *et al*., 1993), adrenal cortex (Wilson TE *et al*., 1993b), gonads (Song KH *et al*., 2001) and pituitary (reviewed in Maruyama K 1998). In the pituitary, Nur77 expression has been specifically shown in the corticotropes (Okabe T *et al*., 1998). Nur77 protein is an immediate-early gene product of which the expression can be rapidly induced by a variety of stimuli, such as growth factors (Hazel TG *et al*., 1988), cAMP (Murphy EP and Conneely OM, 1997) and peptide hormones (Murphy EP and Conneely OM, 1997; reviewed in Maruyama K, 1998).

Nur77 protein can bind to promoter elements as monomers (Wilson TE *et al*., 1991) or homodimers (Philips A *et al*., 1997), or as heterodimers with other transcription factors (Perlmann T and Jansson L., 1995; Wu Q *et al*., 1997). The monomeric Nur77 binding site is referred to as an NBRE (NGFI-B response element), with the consensus sequence defined as 5' AAAAGGTCA 3' (Wilson TE *et al*., 1991), but Nur77 has also been shown to recognize SF-1 binding sites (Wilson TE *et al*., 1993b; Crawford PA *et al*., 1995).

1.3.4.2.2 The role of Nur77 in PKA-mediated gene regulation in the pituitary

Nur77 expression in pituitary corticotropes is rapidly induced by CRF and by forskolin (Murphy EP and Conneely OM, 1997), via a mechanism involving PKA and calcium (Kovalovsky D *et al*., 2002). In primary corticotropes and in the AtT20 corticotrope cell line, the proopiomelanocortin (POMC) gene is regulated by Nur77 via two promoter elements: a monomeric NBRE and a NurRE that can bind Nur77 homo- or heterodimers (Philips A *et al*., 1997; Maira M *et al*., 1999). CRF/cAMP rapidly but transiently induces Nur77 binding to the POMC NurRE (Kovalovsky D *et al*., 2002). Acute treatment with CRF also potentiates the transactivation activity of Nur77, via PKA- and MAPK-dependent mechanisms (Kovalovsky D *et al*., 2002; Maira M *et al*., 2003), and appears to enhance co-activator recruitment by Nur77 (Maira M *et al*., 2003).



1.3.4.3 Cyclic AMP response element binding protein (CREB)

1.3.4.3.1 General mechanism of transcriptional activation by CREB

The classical mechanism of cyclic AMP-mediated gene regulation is mediated via a promoter *cis* element called the cAMP-response element (CRE). A consensus CRE has an 8 base-pair palindromic sequence TGACGTCA (reviewed in Montminy M, 1997), but the CRE can also occur as a "half-site" CGTCA (reviewed in Mayr B and Montminy M, 2001). The transcription factor CREB (cAMP-response element binding protein) (Montminy MR and Bilezikjian LM, 1987) binds to the CRE primarily as a homodimer (Dwarki VJ *et al*., 1990). CREB contains a carboxy-terminal basic region/leucine zipper (bZIP) motif that is involved in homodimerization and DNA binding (Dwarki VJ *et al*., 1990), and is also conserved in other eukaryotic transcription factors, such as Jun and Fos family members (listed in Meyer TE *et al.,* 1993).

Phosphorylation of CREB on serine 133 (Ser-133) is crucial for transcriptional activation via PKA and other pathways (Gonzalez GA and Montminy MR, 1989). A rise in intracellular cAMP levels leads to rapid dissociation of the PKA regulatory and catalytic subunits (reviewed in Montminy M, 1997). The catalytic subunit translocates to the nucleus and phosphorylates CREB inside the nucleus (where it is constitutively targeted to (Waeber G and Habener JF, 1991)), reaching a maximum after approximately 30 minutes (Hagiwara M *et al*., 1993). In addition to phosphorylation via PKA-dependent mechanisms, many other signals have been identified that can induce CREB Ser-133 phosphorylation in different cellular systems (reviewed in Mayr B and Montminy M, 2001; Johannessen M *et al*., 2004). These signals utilize different intracellular messengers and kinases for the phosphorylation step, and include growth factors, peptide hormones and even environmental stress factors.

The transactivation domain of CREB consists of two distinct functional regions, a glutamine-rich constitutive activation domain (CAD) and a kinase-inducible domain (KID). CAD facilitates the assembly of a transcriptional complex containing RNA polymerase II and the transcription factor TFIID (TFIIB together with the TATA-box binding protein (TBP)). This occurs in the absence of hormonal stimuli, independent of the phosphorylation status of CREB, and is sufficient for the initiation of basal transcription by CREB. The KID contains the Ser-133 phosphoacceptor site and can modify the activity of the basal transcriptional complex, as recruited by CAD. KID mediates signal-induced activation of CREB-mediated gene regulation in a strictly phosphorylation-dependent manner, by facilitating the isomerization of the basal transcription complex, promoter clearance, transcriptional elongation and re-initiation of multiple rounds of transcription (Kim J *et al*., 2000; Felinski EA *et al*., 2001).

Phospho-CREB interacts with another nuclear factor, CREB binding protein (CBP), specifically through P-Ser-133 (Chrivia JC *et al*., 1993). CBP possesses intrinsic histone acetyltransferase (HAT) activity that can facilitate chromatin remodelling, making it more accessible for the transcriptional machinery. In addition, CBP is believed to exist as a component of RNA polymerase II complexes, further promoting recruitment of these complexes to cAMP-responsive regions (Kee BL *et al*., 1996).

1.3.4.3.2 Role of CREB in regulation of GnRHR gene expression in pituitary cell-lines

CREB has been shown to regulate human (Cheng KW and Leung PCK, 2001) and rat (Pincas H *et al*., 2001b) GnRHR I promoter activity in αT3-1 cells. The human promoter contains two atypical CREB binding sites that both contribute to PKA-mediated stimulation of promoter activity (Cheng KW and Leung PCK, 2001). Activators of the PKA pathway, including PACAP, also stimulate rat GnRHR I promoter activity in αT3-1 cells via a bipartite element containing a CREB binding site and an SF-1 binding site.

In GGH_3 cells (rat GH_3 pituitary somatolactotrope cells stably transfected with rat GnRHR I cDNA (Stanislaus D *et al*., 1994)), basal and GnRH-mediated regulation of the activity of a transfected mouse GnRHR promoter-reporter construct was shown to involve the CRE at -107/-100 (Maya-Nunez G and Conn PM, 1999). However, no results on the role of the CRE in regulating mouse GnRHR promoter activity in gonadotrope cell lines have been published.

1.3.4.4 Activator Protein-1 (AP-1)

1.3.4.4.1 Introduction

The transcription factor AP-1 is a dimer that consists of two members of the Fos (Zerial M *et al*., 1989; Cohen DR *et al*., 1989) and/or Jun (Nakabeppu Y *et al*., 1988; Hirai SI *et al*., 1989) protein subfamilies. These proteins are the products of primary response (immediate-early) genes (Cesnjaj M *et al*., 1994), and the increases in their mRNA expression levels are often quite dramatic, but also very transient (reviewed in Wisdom R, 1999). The transcriptional activity of AP-1 proteins can also be upregulated via post-translational mechanisms, such as the phosphorylation of c-Jun by JNK (Ellsworth BS *et al*., 2003b; Yokoi T *et al*., 2000).

Jun and Fos proteins contain leucine zipper domains, required for their homo- and heterodimerization (Turner R and Tjian R, 1989), which is essential for their DNA binding (Gentz R *et al*., 1989). AP-1 can bind to its cognate DNA element (5' TGACTCA 3') (Nakabeppu Y *et al*., 1988) either as Jun-Jun homodimers or as Jun-Fos heterodimers; the Fos leucine zipper is incapable of forming homodimers (Turner R and Tjian R, 1989). Fos proteins have been demonstrated to increase the DNA binding activity of Jun proteins (Zerial M *et al*., 1989; Nakabeppu Y *et al*., 1988), and Jun/Fos heterodimers

appear to occupy the DNA binding site longer than Jun/Jun homodimers (Ryseck RP and Bravo R, 1991).

1.3.4.4.2 Regulation of AP-1 expression and activity in gonadotrope cells

As discussed in sections 1.3.3.1.1 and 1.3.3.1.3, the AP-1 element in the mouse GnRHR promoter is essential for homologous regulation of promoter-reporter expression, both in transgenic mice (Ellsworth BS *et al*., 2003b) and in transfected αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a). GnRH induces the expression and/or activity of several Fos and Jun family members in gonadotrope cell lines, providing a mechanism whereby the transcriptional effects of GnRH can be mediated via AP-1. In αT3-1 cells, serum causes high constitutive expression levels of Fos and Jun proteins. Serum starving decreases mRNA levels substantially, thereby increasing responsiveness to other stimuli. In serum-starved αT3-1 cells, GnRH was shown to cause a rapid induction of junB, c-jun and c-fos mRNA levels, reaching a maximum after 30 minutes, and similar effects were observed in primary pituitary cells from female rats (Cesnjaj M *et al*., 1994). One hour of GnRH treatment also increased c-Fos protein levels in αT3-1 cells, and this was sustained even after 6 hours of GnRH stimulation. In contrast, 6 hours of GnRH treatment did not affect FosB and JunD protein levels, but induced the transcriptional activity of FosB and the phosphorylation of JunD protein (Ellsworth BS *et al*., 2003b). Regulation of Jun and Fos expression by GnRH in LβT2 cells has been investigated by microarray analysis (Wurmbach E *et al*., 2001; Kakar SS *et al*., 2003). JunB and c-Jun mRNA levels were increased after 1 hour of GnRH treatment, but JunD mRNA levels remained unchanged, even after 6 hours of GnRH treatment. Expression levels of FosB and c-Fos mRNA also increased rapidly in response to GnRH (Wurmbach E *et al*., 2001; Kakar SS *et al*., 2003). Correlating with results obtained in these microarray studies, GnRH was found to stimulate c-Fos, FosB, c-Jun and JunB protein expression in LβT2 cells, but not JunD protein expression. The GnRH-mediated upregulation of JunB protein expression was found to be MEK-dependent (Coss D *et al*., 2004). Similarly, the GnRH-mediated upregulation of c-Fos protein expression was demonstrated to be MEK- and calcium-dependent, but independent of PKC (Liu F *et al*., 2002a).

1.3.4.4.3 Cross-talk between AP-1 and CREB

The consensus sequences of AP-1 binding sites (5' TGA(C/G)TCA 3') and CREB binding sites (5' TGACGTCA 3') are quite similar (Nakabeppu Y *et al*., 1988). For this reason, in addition to binding to AP-1 sites, Jun homodimers (Nakabeppu Y *et al*., 1988) and Jun/Fos heterodimers (Sassone-Corsi P *et al*., 1990) can also bind to consensus CREs, although with lower affinity (Nakabeppu Y *et al*., 1988). Furthermore, CREB, Jun and Fos proteins all contain leucine zipper dimerization domains (Benbrook DM and Jones NC, 1990), and are capable of forming selective cross-family heterodimers (Benbrook DM and Jones NC, 1990; Macgregor PF *et al*., 1990; Hai T and Curran T, 1991). These heterodimers can bind to either AP-1 or CREB consensus sequences, depending on the dimer composition, but appear to generally prefer CREB sites (Hai T and Curran T, 1991).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Antiserum against SF-1 protein was a kind gift from Dr Ken-ichirou Morohashi, Department of Molecular Biology, Kyushu University, Japan. CCRF-CEM nuclear extracts (5 µg/µl) and purified anti-Nur77 antibodies (cat. no. 1600045) were from Geneka Biotechnology, Montreal, Quebec, Canada, unless stated otherwise in figure legend. Antibodies against CREB (cat. no. 9192) and phosphorylated CREB (Ser-133) (cat. no. 9191) were purchased from Cell Signalling Technology, Inc., Beverly, Maryland, USA. Antibodies against c-Jun (cat. no. sc-44X) and c-Fos (K-25) (cat. no. sc-253X) were purchased from Santa Cruz Biotechnology. Rabbit pre-immune serum was from Dr Bellstedt, Department of Biochemistry, University of Stellenbosch, South Africa. Horseradish peroxidase-linked anti-rabbit IgG (cat. no. NA934V) was purchased from Amersham.

Test compounds: Forskolin (cat. no. F6886), 8-Br-cAMP (cat. no. B7880), GnRH (cat. no. L7134), PACAP-27 (cat. no. A9808) and the PKC pathway inhibitor bisindolylmaleimide (BIM) (cat. no. B3931) were purchased from Sigma. The PKA pathway inhibitor H-89 (cat. no. 371962) was purchased from Calbiochem.

Oligonucleotides and primers were purchased from Integrated DNA Technologies, Inc., USA. Primers used to amplify β-actin transcripts with LightCycler real-time PCR were purchased from Roche. See addendum B for sequences of all primers and oligonucleotides used.

2.2 Plasmids

pGL2-basic promoterless luciferase expression vector (5.6 kb), pGL2-basic vector-specific primers GL1 (sense) and GL2 (antisense) and pSV-β-galactosidase expression vector (6.8 kb) were from Promega. The pCMV-SF-1 expression construct (6.8 kb) containing the full-length (2.1 kb) human SF-1 cDNA (Ikeda Y *et al.,* 1993) was from Dr K Parker, University of Texas, Texas, USA. For *in vitro* transcription-translation, the full-length cDNA (2 kb) was excised with *EcoR*I and cloned into the *EcoR*I

site of pSPT19 (3.1 kb) (Roche). The mouse pCMX-Nur77 (6.7 kb) and pRSV-PKI expression constructs were gifts from Dr. Jacques Drouin, IRCM, Montreal, Quebec, Canada. The pFC-PKA catalytic sub-unit expression vector (7.2 kb), originally from Stratagene, was a gift from Dr Vanden Berghe, Department of Molecular Biology, Royal University of Gent, Belgium. The full-length (2.1 kb) human fibroblast cytoplasmic β-actin cDNA in the Okayama-Berg expression vector (total construct size 5 kb) (Gunning P *et al.,* 1983) was from Dr MI Parker, Department of Medical Biochemistry, University of Cape Town, South Africa. The pG5-luciferase (5 kb), pACT (5.6 kb) and pBIND (6.4 kb) mammalian two-hybrid vectors, originally from Promega Corporation, were a gift from Dr D Stocco, Texas Tech University Health Sciences Center, Texas, USA. The pACT-CREB and pACT-CREBm constructs, containing the coding regions for wild-type or mutated (S133A) CREB (1.1 kb), respectively, cloned into pACT, as well as the pBIND-SF-1 construct, containing the SF-1 coding region (2 kb) cloned into pBIND (Manna PR *et al.,* 2003), were also from Dr Stocco. Construct R10, containing the full-length (1.2 kb) mouse GnRHR cDNA (Tsutsumi M *et al.,* 1992) 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 in pBluescript®SK, were obtained from Dr. SC Sealfon, Mt Sinai Medical School, New York, USA. The ATG start codon of clone 111 in pBluescript®SK was mutated to a *BglII* site using oligonucleotide 1S, to yield plasmid pGB. Plasmid pGBM, where the two central Cs of the -15/-7NRS region were mutated to two Ts, as well as the ATG start codon mutated to a *BglII* site, was also created from clone 111 in pBluescript®SK by a similar strategy, using oligonucleotide 2S. Thereafter, 591 bp *BglII/BamHI* fragments were excised from pGB and pGBM and cloned into the *BglII* site of pGL2-basic to yield pLG and pLGM1 (6.3 kb), respectively. The sequence in the region of the -15/-7NRS for pLG is as follows: GCCTGTCCTTGGAGAAAAT**Aga**tctaag, where capital letters denote the wild type GnRHR sequences, bold letters denote the original position of the ATG codon, and the underlined region denotes the wild type -15/-7NRS region.

(Note that the preparation of constructs pLG and pLGM1 were originally described by Carmen Pheiffer, M.Sc. thesis, University of Cape Town, 1998).

2.3 PCR mutagenesis

(See addendum D for detailed mutagenesis protocol)

The -244/-236NRS was mutated to a *PstI* site by PCR, using overlapping primers -244/-236mps and -244/-236mpa, containing the modified -244/-236NRS sequence. Using pLG as template, two separate PCR reactions were performed, with either GL1 and -244/-236mpa, or GL2 and -244/-236mps as primers, generating products of approximately 400 bp and 300 bp, respectively. Subsequently, these products were used in a PCR fusion reaction with primers GL1 and GL2, generating a product of approximately 700 bp, containing the -579/+1 promoter sequence with the modified -244/-236NRS. The product was purified by preparative low melting agarose gel electrophoresis, using the Macherey-Nagel NucleoSpin Extract 2 in 1 kit, and subsequently cloned back into S*acI* and *HindIII* sites of pGL2-basic, to yield pLGM2. Construct pLGM1+2, containing mutations in both the -15/-7NRS and -244/-236NRS, was constructed as described for pLGM2, using pLGM1 as a template. For pLGcrem, primer sets GL1/PCRmcrea and GL2/PCRmcres were used to generate a mutation (Pincas H *et al.,* 2001b) in the CRE. For pLG-mAP1, primer sets GL1/PCRmAP1a and GL2/PCRmAP1s were used to generate a mutation (Duval DL *et al.,* 1997b) in the AP-1 site. All reporter constructs were sequenced for confirmation.

2.4 Electrotransformation of bacterial cells and plasmid preparation

Plasmids were transformed into *Escherichia coli* JM109 cells by electroporation with a Savant GTF100 gene transformer/electroporator. The protocols for the preparation and transformation of electrocompetent cells, as described in the Savant Instruction Manual, were strictly followed. Immediately after transformation, cells were mixed with 1 ml ice-cold SOC medium (Sambrook J *et al.,* 1989), and incubated for 1 hour at 37 °C while shaking at 200 rpm. Cells were subsequently plated out on LB agar plates (Sambrook J *et al.,* 1989) containing 50 µg/ml ampicillin (Sigma) and allowed to grow overnight at 37°C. Positive clones (single colonies), as selected for by ampicillin, were picked and resuspendend in 20 µl sterile water. LB medium (400 µl, containing 50 µg/ml ampicillin) was added and cells were grown overnight at 37 °C while shaking at 200 rpm. Afterwards, 100 µl 80% (v/v) glycerol was added, the suspension was vortexed, and stored at -70 °C. For mid-scale plasmid preparations, LB medium containing ampicillin was inoculated, grown overnight at 37 °C while shaking

at 200 rpm, and plasmids were purified with the Promega Wizard Midiprep or Promega Pureyield plasmid purification kits, according to the protocols provided.

2.5 Cell culture

Dr. PL Mellon, University of California, San Diego, California kindly supplied αT3-1 and LβT2 mouse pituitary gonadotrope cells. Both cell lines were maintained in high glucose (4.5 g/ml) DMEM (GibcoBRL). Medium was supplemented with 10% fetal calf serum (FCS) (Delta Bioproducts, Johannesburg, South Africa), 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL), unless stated otherwise. Cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-one) at 37 °C, in an atmosphere of 90% humidity and 5% CO_2. αT3-1 cells were cultured and used for experiments up to a maximum of 10 passages. LβT2 cells were used for experiments from passage 3 onwards, with no upper limit to passage number established. Both cell lines were passaged with 0.25% trypsin / 0.1% versene EDTA in calcium- and magnesium-free PBS (Highveld Biologicals, Lyndhusrt, South Africa) (2 ml in a 75 cm^2 flask). Cells were very sensitive to overtrypsinizing, and were therefore not incubated in the trypsin solution for longer than 2 minutes. Both cell lines were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney RI, 1987), and only mycoplasma-negative cells were used in experiments.

2.6 Incubation with test compounds

PACAP and GnRH were purchased as lyophilized salts and dissolved in H_2O, to final concentrations of 20 µM and 100 µM, respectively. Forskolin and 8-Br-cAMP were purchased as lyophilized products and dissolved in DMSO, to final concentrations of 10 mM and 1 M, respectively. H89 and BIM were purchased as concentrated solutions in DMSO, and were diluted in DMSO to final concentrations of 1 mM and 100 µM, respectively. All test compounds were administered to cells as 1/1000 dilutions in culture medium, as indicated in figure legends. The concentration of DMSO in culture medium was 0.1%. Vehicle controls were either culture medium only, or culture medium containing 0.1% DMSO, depending on the test compounds added.

2.7 Isolation of total RNA

Note that these steps were performed under RNAse-free conditions. Gloves were worn at all times and changed regularly. RNAse-free microcentrifuge tubes and filter tips were used throughout. Glassware and metal weighing spoons were wrapped in foil and baked overnight at 100 °C. Water was treated with diethylpyrocarbonate (DEPC) (1/1000) by incubating at 37 °C for 2 hours, and was subsequently autoclaved twice. All solutions were made up in DEPC-treated water. The pH meter electrode was incubated in 0.5 M NaOH for 30 minutes, and rinsed with DEPC-treated water before use. Gel equipment was rinsed with 3% H_2O_2. All experiments were performed in a laminar flow cabinet.

For Northern blotting, αT3-1 cells were plated in 100 mm petri dishes at a density of 2 X 10^6 cells per dish and grown overnight. Incubations with test compounds were performed in fresh medium supplemented with 10% serum, as described in figure legends. Subsequently, the medium was removed and the cells were harvested in 1 ml TRI reagent (Sigma) per dish. Total RNA was isolated according to the TRI reagent protocol (Chomczynski P, 1993). Briefly: cell lysates were thereafter transferred into microcentrifuge tubes, 200 µl chloroform was added to each sample and vortexed for 30 seconds. Samples were incubated at room temperature for 15 minutes and centrifuged for 15 minutes at 12 200g (4 °C). The top aqueous layer was transferred into clean microcentrifuge tubes, and an equal volume of ice-cold isopropanol was added. Samples were stored overnight at -20 °C. RNA preciptates were collected by centrifugation for 30 minutes at 20 000g (4 °C). RNA pellets were washed twice by adding ice-cold 70% ethanol and centrifuging for 15 minutes each at 20 000g (4 °C). Subsequently, pellets were air-dried for 5 minutes, dissolved in 50 µl Formazol (Molecular Research Center) and stored at -20 °C. The concentration of the RNA was determined by measuring the optical density at 260 nm, and the purity was determined by calculating the ratio of the optical densities at 260 nm and 280 nm. The integrity of the RNA was confirmed by means of denaturing formaldehyde agarose gel electrophoresis (Sambrook J *et al.,* 1989). A 1% agarose gel was prepared as described in addendum C, section C3. For sample preparation, 15 µl sample loading buffer (section C3) was added to each RNA sample (3 to 5 µg, in a maximum volume of 7 µl). Samples were incubated at 65 °C for 10 minutes to denature secondary RNA structures and cooled on ice, after which 2.5 µl sample loading dye (section C3) was added to each sample. Samples also contained 0.5 µl ethidium bromide

(10 µg/µl in DEPC-treated H_2O) for visualization under UV illumination. Gel electrophoresis was performed in 1X MOPS buffer (diluted in DEPC-treated H_2O) at 65V for approximately 1.5 hours (Sambrook J *et al.,* 1989).

For RT-PCR, LβT2 cells were plated in 6-well culture plates (Greiner Bio-one Cellstar) at a density of 9 X 10^5 cells per well. Forty-eight hours after plating, medium was replaced with fresh serum-free medium. Incubations with test compounds were performed as described in figure legends, after which the medium was removed and the cells harvested with a sterile cell scraper, in 185 µl TRI reagent per well. Cell lysates were transferred into microcentrifuge tubes and 40 µl chloroform was added to each sample. Total RNA was isolated as described above. The final RNA pellet was dissolved in 15 µl DEPC-treated water and stored at -20 °C.

2.8 Northern blotting

A mouse GnRHR cDNA fragment (1.2 kb) was excised from construct R10 by means of *Eco*RI/*Xho*I restriction enzyme digestion. Human β-actin cDNA (1.9 kb) was excised from the Okayama-Berg expression vector by means of *Bam*HI digestion. Fragments were separated and purified by preparative low melting agarose gel electrophoresis, as described in section 2.3. Thereafter 25 ng of each fragment was labelled with 50 µCi [α-^{32}P]-dCTP (AEC Amersham) using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). The reaction mix was made up to 100 µl with TE buffer (Sambrook J *et al.,* 1989), and unincorporated radioactivity was removed by passing the reaction mix through a 1 ml G50 Sephadex (Pharmacia) spin column (equilibrated with TE buffer). Total RNA (20 µg per well) was separated on a 1% denaturing formaldehyde agarose gel in 1X MOPS buffer (see section 2.7), transferred overnight onto Hybond N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting in 20X SSC buffer (Sambrook J *et al.,* 1989) and cross-linked to the membranes by UV cross-linking. The membranes were pre-hybridized in DIG EasyHyb solution (Roche) at 50 °C for 30 min. Labelled DNA probes (10^8 to 10^9 dpm/µg DNA) were denatured by incubating at 95 °C for 10 minutes, and hybridization (25 ng DNA/ 100 cm^2 membrane) was performed overnight at 50 °C. After hybridization, membranes were washed twice for 5 min at room temperature in a 2X SSC buffer containing 0.1% SDS, followed by two washes at 50 °C in a 0.1X SSC buffer containing 0.1% SDS. The washed membranes were wrapped in cling-film and exposed to Hyperfilm

at -80 °C. Membranes were stripped by immersing them in boiling 0.5% SDS and allowing the solution to cool for 3 hours, and were subsequently probed for β-actin transcripts. Quantification of signals was performed by densitometric analysis of the autoradiograms.

2.9 LightCycler® real-time quantitative RT-PCR

2.9.1 Reverse transcription (first strand cDNA synthesis)

Total RNA was reverse-transcribed with M-MLV Reverse Transcriptase (RNase H minus) (Promega), using oligo(dT) to prime the RT reaction. Of each sample, 1 µg total RNA, 1 µl oligo(dT) (1 µg/µl) and RNAse-free H_2O was mixed in a total volume of 14 µl and incubated for 5 minutes at 70°C. Samples were cooled on ice, and 5 µl dNTP mix (10 mM; Sigma), 5 µl M-MLV RT 5X reaction buffer (Promega) and 1 µl (200 U) M-MLV RT was added to each sample. Samples were incubated at 42 °C for 60 minutes.

2.9.2 Real-time PCR

Note that the LightCycler real-time PCR methodology for relative quantification of gene expression was set up and optimized by the present author. Details regarding optimization of Lightcycler PCR protocols and quantification of transcript levels are supplied in Addendum A.

Real-time PCR was performed on a Roche LightCycler, using the Roche LightCycler FastStart DNA Master^{PLUS} SYBR Green I PCR reaction mix and Roche LightCycler borosilicate glass capillaries, according to equipment and product specifications. GnRHR S4 and AS4 primers (standard desalted, purity confirmed by MALDI-TOF) were from IDT, and β-actin sense and anti-sense primers (HPLC-purified) were from Roche. The following reagent mix was prepared per reaction: 10 µl water (PCR-grade, supplied with FastStart kit); 2 µl sense primer and 2 µl anti-sense primer (primer stock concentration 5 µM, diluted in PCR-grade water); 4 µl SYBR Green Master Mix (supplied with FastStart kit). Of the reagent mix, 18 µl was pipetted into each capillary, along with 2 µl (0.4 µg) cDNA. The PCR protocol was as follows: a single incubation at 95 °C for 10 minutes (no fluorescence measured); 40 amplification cycles of denaturation, annealing and extension (single fluorescence measurement after each cycle) (see table 2.1 for different conditions for GnRHR and β-actin transcript amplification), and a melting curve analysis between 50 and 95 °C, at a heating rate of 0.2 °C per second (continuous fluorescence measurement). Afterwards, samples were cooled down to 30 °C for

40 seconds. PCR products were analysed on a 1% agarose gel to confirm the size of the products. Relative GnRHR transcript levels were calculated with the "Fit Points" method described by Pfaffl MW, 2001, and were normalized to relative β-actin transcript levels.

	Denaturation		Annealing		Extension		Product size (bp)
Primer set	**Time (s)**	**°C**	**Time (s)**	**°C**	**Time (s)**	**°C**	
GnRHR S4/AS4	8	95	10	58	8	72	192
β-actin s/a	10	95	10	58	17	72	348

Table 2.1: Summary of conditions for amplification of mouse GnRHR and β-actin transcripts by LightCycler real-time PCR.

2.10 Transient transfections

2.10.1 Different types of transfection experiments

(Refer to sections 2.2 and 2.3 for detailed descriptions of constructs transfected).

Several different types of transfection experiments were performed in the present study. In order to investigate the respective roles of individual GnRHR promoter elements and transcription factor binding sites in the regulation of GnRHR transcription, GnRHR promoter-reporter constructs were prepared by cloning the proximal promoter upstream of the reporter gene luciferase The constructs were individually transiently transfected into the cells, after which the transcriptional activity of the promoter fragment could be measured by means of luciferase reporter assays. In some experiments, certain factors were overexpressed by transiently transfecting expression constructs (containing the relevant coding region cloned downstream of a constitutively active promoter) into the cells. For mammalian two-hybrid assays, the "bait" was the SF-1 coding region cloned downstream of the GAL4 DNA-binding domain, the "prey" was the CREB coding region cloned downstream of the VP16 activation domain, and the reporter construct contained 5 copies of the GAL4 binding site upstream of the luciferase reporter gene.

2.10.2 Transfection protocols

All transfections were performed according to the Fugene™ 6 (Roche) product protocol, at a ratio of 1µg DNA: 2 µl Fugene™ 6 in serum-free high-glucose DMEM.

αT3-1 cells: Cells were plated in 12-well culture plates (Nunc) at 2 X 10^5 cells/well, in a volume of 1 ml DMEM containing 10% FCS and antibiotics as described above. Twenty-four hours after plating, medium was replaced with fresh antibiotics-free medium and cells were transfected with promoter-reporter constructs and expression constructs, as indicated below the relevant figure legends. In order to correct for transfection efficiency, a β-galactosidade expression construct (pSV-β-galactosidase) was co-transfected. Thirty hours after transfection, medium was replaced with fresh antibiotics-free medium and cells were incubated with various test compounds, as indicated in the figure legends. Cells were harvested in 100 µl Lysis Buffer (Tropix Galacto-*Star*™ assay system) per well. The β-galactosidase assays (Galacto-*Star*™ assay system) and luciferase assays (Promega Luciferase Assay System) were performed with 20 µl cell extract in black 96-well plates, in a Labsytems Luminoskan RS luminometer. For each sample, the luciferase assay values were divided by the β-galactosidase assay values to correct for differences in transfection efficiency, and these values were then expressed relative to the pLG control value, which was taken as 1.

LβT2 cells: Cells were plated in 24-well culture plates (Nunc) at 1 X 10^5 cells/well, in a volume of 500 µl DMEM containing 10% FCS and antibiotics as described above. Twenty-four hours after plating, medium was replaced with fresh antibiotics-free medium containing 10% FCS, and transfections were performed as described above. Twenty-one to twenty-eight hours after transfections, medium was replaced with fresh antibiotics-free, serum-free medium and cells were incubated with various test compounds, as indicated in the figure legends. Cells were harvested in 50 µl 1X Reporter Lysis Buffer (Promega) per well. Luciferase assays (Promega Luciferase Assay System) were performed with 10 µl cell lysate in white 96-well plates, in a Veritas Microplate Luminometer (Turner Biosystems). Initial studies performed with pSV-β-galactosidase indicated that intra- and inter-assay variations in transfection efficiency in these cells were minimal, and therefore β-galactosidade co-transfection was not performed in subsequent experiments.

2.11 Preparation of nuclear extracts

2.11.1 Large-scale nuclear extracts preparation

(Note: All αT3-1 nuclear extracts were prepared with this method.)

Twenty 75cm^2 flasks of αT3-1 cells were grown to confluency in medium containing 10% FCS and antibiotics as described in section 2.5, and washed twice with PBS (Sambrook J et al., 1989). All subsequent steps were performed at 4 °C. The cells were harvested in PBS, pelleted by centrifugation at 1 000g for 10 minutes, and washed twice in 5 pellet volumes PBS. The cell pellet was resuspended in 5 pellet volumes Buffer A (10mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl, 0.5 mM DTT, 0.05 mM PMSF), and cells were incubated on ice for 20 minutes. Cells were pelleted by centrifugation at 1 000g, resuspended in 2 pellet volumes Buffer A and lysed by ten strokes in a Dounce homogenizer. A crude nuclear pellet was obtained by centrifugation at 2 000g for 10 minutes and resuspended in 4 pellet volumes Buffer A. Nuclei were collected by centrifugation at 25 000g for 20 min, and resuspended in 500 µl Buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 1 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT and 0.05 mM PMSF). Nuclei were lysed by adding 250 µl KCl (1 M) to the nuclear suspension (to a final KCl concentration of 0.33 M) and were gently rocked for 30 minutes. Subsequently, the insoluble debris was pelleted at 25000g for 30 min. The supernatant was dialysed for 3X 2 hours in 200 volumes Buffer D (10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, 3 mM DTT and 0.3 mM PMSF) and stored in aliquots at -80 ºC (Dignam JD et al., 1983). Proteins concentrations were determined by means of the Bradford protein assay (Bradford MM, 1976), and concentrations measured varied between 1 – 2 mg/ml.

2.11.2 Small-scale nuclear extract preparation

(Note: All LβT2 nuclear extracts were prepared with this method. All buffers contained phosphatase inhibitors as described, and all steps were performed at 4 °C.)

LβT2 cells were plated out in 100mm dishes in medium containing 10% FCS and antibiotics as described in section 2.5, at a density of 3 x 10^6 per dish. After 48 hours, medium was replaced with serum-free, antibiotics-free medium and cells were stimulated as indicated in figure legends. Cells were washed and harvested in 2 ml cold PBS containing the following phosphatase inhibitors: 100 µM sodium fluoride (Merck), 10 µM sodium orthovanadate (Sigma) and 10 µM β-glycerophosphate (Sigma). Sodium fluoride and β-glycerophosphate were dissolved in H_2O to a final concentration of 10

mM and 1 mM, respectively. Sodium orthovanadate was prepared according to Gordon JA, 1991, to a final concentration of 1 mM. All phosphatase inhibitors were added in a 1/100 dilution. Harvested cells were transferred into 2 ml microcentrifuge tubes, pelleted at 800g for 5 minutes (4 °C) and the cell pellets were stored at -80 °C. To prepare nuclear extracts, cell pellets were thawed on ice and washed with 2 ml cold TBS (Sambrook J et al., 1989) by centrifuging at 800g for 5 minutes. The cell pellets were resuspended in 1 ml TBS, and cells were pelleted by centrifuging at 20 000g for 15 seconds. The cells were resuspended in 400 µl ice-cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and incubated on ice for 15 minutes. Cells were lysed by adding 25 µl 10 % (v/v) NP-40 detergent, and were vortexed for 10 seconds. The nuclear pellet was collected by centrifuging at 10 000g for 5 minutes, and resuspended in 50 µl ice-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 vigorously rocked for 15 minutes, after which nuclear debris was pelleted by centrifuging at 12 000 g for 5 minutes. Supernatants containing nuclear proteins were transferred into clean microcentrifuge tubes, and stored at -80 °C (Schreiber E et al., 1989). Protein concentrations were determined by means of the Bradford protein assay (Bradford MM 1976), and varied between 0.5 – 1 mg/ml.

2.12 Electrophoretic mobility shift assays (EMSAs)

Probes (10 pmol of double-stranded oligonucleotides) were end-labeled by incubating together with 100 µCi γ-^{32}P-ATP (Amersham), 10 U E. coli polynucleotide kinase (PNK, Roche) and 2 µl 10X PNK phosphorylation buffer (Roche) in a total volume of 20 µl, at 37 °C for 30 minutes. The reaction mix was made up to 100 µl with TE buffer (Sambrook J et al., 1989), and passed through a G50 Sephadex spin column, as described in section 2.8. All protein-DNA incubations were performed at room temperature, in a final volume of 5 µl. For EMSAs with nuclear extracts, each lane for a particular experiment was loaded with the same amount of nuclear extract total protein, unless otherwise stated in the legends. This varied between experiments in the range of 1 to 2 µg protein. All incubations contained 0.5 µg poly(dIdC) (Roche) and 6 µg BSA (Roche, molecular biology grade), plus radiolabeled probe (0.1 pmol) (150 000 to 250 000 cpm). Incubations with the nuclear receptor binding site probes were performed for 10 min, in assay buffer containing 100 mM NaCl, 7.5 mM HEPES (pH 7.9), 10 mM Tris-HCl, 8% (v/v) glycerol, 1.25 mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.25 mM EGTA (pH

7.0), 0.67 mM PMSF, and 3.5 mM DTT. Incubations with the CRE probe were performed for 20 minutes, in assay buffer containing 20 mM NaCl, 20 mM KCl, 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 1 mM DTT, 1mM EDTA and 10% (v/v) glycerol (adapted from Cheng KW and Leung PCK, 2001). Incubations with the AP-1 probe were performed for 30 minutes, in assay buffer containing 120 mM NaCl, 20 mM HEPES (pH 7.9), 0.3 mM EDTA (pH 8.0), 0.3 mM EGTA (pH 7.0), 0.5 mM DTT, 0.3 mM PMSF and 5% (v/v) glycerol (adapted from White BR et al., 1999). In some experiments, 1.5 µl of in vitro transcribed-translated protein (diluted 1:7 in 1X assay buffer), or CCRF-CEM nuclear extracts (Geneka) (diluted 1:9 in 1X assay buffer, unless indicated otherwise in figure legends), were used. In vitro transcription-translation of SF-1 cDNA from the pSPT19-SF-1 expression construct (2 µg) was performed according to the Promega TnT® Quick Coupled Transcription/Translation protocol. For assays containing anti-SF-1, anti-Nur77 (Dr T Perlmann, Karolinska Institute, Stockholm, Sweden) or preimmune antisera (diluted 1:29 in 1X assay buffer), reactions were performed as described above, followed by further incubation for 10 minutes at room temperature after addition of 1 µl specific antibody or rabbit pre-immune serum. For AP-1 antibody incubations, specific anti-Jun or anti-Fos antibodies were preincubated with protein for 10 minutes at 4 °C, before labelled probe was added. For competition assays, double stranded oligonucleotide competitor DNA, labelled probe and proteins were added sequentially to the assay mix and incubated for 10 min at room temperature. EMSAs were performed using 4% (chapter 3) or 5% (chapter 4) poly-acrylamide gels (29:1 acrylamide: bisacrylamide) at 100 V in 1X TAE buffer (Sambrook J et al., 1989) for 3.5 to 4 hours at room temperature, using a BioRad Protean® II xi electrophoresis cell.

2.13 SDS-PAGE and Western blots

These protocols were adapted from Sambrook J *et al.,* 1989.

Chapter 3: Protein samples containing either 5 µl undiluted *in vitro* transcribed-translated protein or varying amounts of nuclear extracts were boiled for 10 minutes in 1X SDS sample loading buffer (1% SDS, 3% glycerol, 0.02 M Tris (pH 6.8), 0.1% (w/v) bromophenol blue, 1% (v/v) β-mercapto-ethanol (Sambrook J *et al.,* 1989) and separated on an 8% SDS-PAGE gel for 5 h at 120 V in 25 mM Tris-HCl, 250 mM glycine (Merck, reagent grade), 0.1% SDS, pH 8.3 (Sambrook J *et al.,* 1989), using a BioRad Protean® II xi electrophoresis cell. Proteins were transferred onto a nitrocellulose membrane (Schleicher-Schuell, BA 85) by electroblotting for 16 h at 120 mA in electroblotting buffer (see

addendum C, section C4), using a BioRad Mini Trans-Blot® cell. Membranes were blocked with casein buffer (154 mM NaCl, 0.5% casein (BDH), 10 mM Tris base, 0.02% thiomersal (BDH)) at room temperature for 1 hour and incubated overnight with anti-SF-1 antiserum (1:3000) at 4 °C, or blocked overnight in casein buffer at 4 °C and incubated with anti-Nur77 antibody (1:2000) at 37 ºC for 2 h. Incubations with the secondary goat anti-rabbit and rabbit PAP antibodies (Sigma) were each performed at 37 °C for 90 min, at 1:1000 and 1:5000 dilutions, respectively. All antibody dilutions were made in casein buffer. After each antibody incubation, the membrane was washed for 15 minutes in PBS and 5 minutes in PBS containing 0.1% (v/v) Tween (Merck), at room temperature. Protein-antibody complexes were visualized by ECL Detection (Amersham Pharmacia Biotech, #RPN2106).

Chapter 4: Protein samples were boiled as described above, and separated by SDS-PAGE (percentage acrylamide given in relevant figure legends) at 200 V in 25 mM Tris, 200 mM glycine and 0.1% SDS, using a BioRad Mini Protean® II electrophoresis cell. Proteins were electroblotted onto Hybond™-ECL™ (Amersham) nitrocellulose membrane for 1 h at 200 mA in 25 mM Tris, 200 mM glycine and 10% (v/v) methanol, using a BioRad Mini Trans-Blot® cell. Membranes were blocked overnight at 4 °C in 5% (m/v) milk powder in TBS, and subsequently incubated with primary antibody and secondary antibody (donkey anti-rabbit (Amersham), 1:10 000 diluted) for 1 hour each at room temperature, unless stated otherwise in figure legends. Primary antibody concentrations are indicated in relevant figure legends. All antibody incubations were performed in blocking solution. After each antibody incubation, membranes were washed with 3 x 5 minutes in 20 ml TBS-Tween (0.1% v/v), and 2 x 5 minutes in 20 ml TBS. Protein-antibody complexes were visualized with the ECL Advance™ Western Blotting Detection System (Amersham Biosciences RPN2135). For consecutive detection steps, membranes were stripped in 62.5 mM Tris (pH 8.6), 100 mM β-mercapto-ethanol and 2% SDS (Ismaili N *et al.,* 2005) for 2X 10 minutes at room temperature and blocked overnight before repeating antibody incubations.

2.14 Autoradiography and quantification

Autoradiography and ECL visualization were performed with Amersham Hyperfilm™ MP high performance autoradiography film. Autoradiograms presented in chapter 4 were photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290, and bands were quantified with Kodak 1D Image Analysis Software.

2.15 Statistical analysis

Results were analysed with GraphPad PRISM™ (version 4) software from GraphPad Software Inc, using One-way ANOVA, with either Dunnett's post-test (when comparing all values to a single control) or Bonferroni's post-test (when comparing all values to each other). Where statistical significance of difference was determined relative to a single control, the level of significance of the difference was indicated with asterisks, as explained in relevant figure legends. Where all values were compared to each other, statistical significance of difference was indicated by different lower-case letters, such that all the conditions with the same letter were found statistically not to be significantly different from each other (ie $P > 0.05$), while those having different letters were found statistically to be significantly different from each other (ie $P < 0.05$).

CHAPTER 3

INVESTIGATION INTO THE REGULATION OF MOUSE GnRHR GENE TRANSCRIPTION BY PROTEIN KINASE A IN THE αT3-1 GONADOTROPE CELL LINE

The results presented in this chapter were published in:

Sadie H, Styger G and Hapgood JP 2003 Expression of the mouse gonadotropin-releasing hormone receptor gene in αT3-1 gonadotrope cells is stimulated by cyclic 3',5'-adenosine monophosphate and Protein Kinase A, and is modulated by Steroidogenic Factor-1 and Nur77. Endocrinology 144 p.1958-1971.

3.1 BACKGROUND

The expression of the nuclear receptor transcription factor SF-1 in primary pituitary gonadotropes and in the αT3-1 mouse gonadotrope cell line is well documented (Ingraham HA *et al*., 1994; also reviewed in Parker KL 1998). Two monomeric SF-1 consensus sites have been identified in the proximal mouse GnRHR promoter, at -15/-7 and -244/-236 (see figure 1.4), and binding of SF-1 to the distal site has been demonstrated *in vitro* (Duval DL *et al*., 1997a). This element has also been shown to form part of a tripartite basal enhancer mediating gonadotrope-specific activity of mouse GnRHR promoter-reporter constructs in αT3-1 cells (Duval DL *et al*., 1997b). No results regarding the binding of transcription factors to the proximal site have been published, but this element does not appear to be necessary for high basal and cell-specific expression (Clay CM *et al*., 1995), nor for responsiveness to GnRH stimulation (Norwitz ER *et al*., 1999a) in αT3-1 cells. Nur77, another nuclear receptor transcription factor, has been shown to recognize SF-1 binding sites (Wilson TE *et al*., 1993a; Crawford PA *et al*., 1995). In the pituitary, functional Nur77 protein is known to be expressed in the corticotropes (Okabe T *et al*., 1998). The expression of Nur77 transcripts and their upregulation by GnRH have been reported in the LβT2 pituitary gonadotrope cell line (Wurmbach E *et al*., 2001), but the presence of functional Nur77 protein has not been demonstrated in αT3-1 cells or in LβT2 cells.

Both SF-1 and Nur77 have been shown to be involved in mediating transcriptional responses to PKA in several different cellular systems (Crawford PA *et al*., 1995; Carlone DL and Richards JS 1997; Michael MD *et al*., 1995). Transcriptional responses to PKA have been shown for rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK 2001) GnRHR I promoter constructs transfected into αT3-1 cells, and for mouse GnRHR promoter constructs transfected into GGH_3 pituitary somatolactotrope cells (Lin X and Conn PM, 1998).

3.2 AIMS

The aims of this study were the following:

- To determine whether endogenous mouse GnRHR gene transcription in αT3-1 cells is regulated by activators of the PKA pathway;
- To determine whether the transcriptional activity of a transfected mouse GnRHR promoter-reporter construct is regulated by activators of the PKA pathway, and by PKA itself;
- To demonstrate the protein expression of the nuclear receptor transcription factor Nur77 in αT3-1 cells;
- To investigate the *in vitro* binding of αT3-1 nuclear proteins, in particular the transcription factors SF-1 and Nur77, to two consensus nuclear receptor binding sequences (NRSs) in the proximal mouse GnRHR promoter;
- To investigate the respective roles of SF-1 and Nur77 via the NRSs in modulating the basal and PKA-mediated transcriptional response of the transfected GnRHR promoter-reporter construct in αT3-1 cells

3.3 RESULTS

3.3.1 Activators of the PKA pathway result in an increase in endogenous GnRHR mRNA

To determine whether the endogenous GnRHR gene in αT3-1 cells is responsive to elevated levels of cAMP and forskolin, the cells were incubated for 16 h in the absence and presence of either forskolin or 8-Br-cAMP. Total RNA was isolated and probed by Northern blotting with radiolabelled mouse GnRHR cDNA (figure 3.1A).

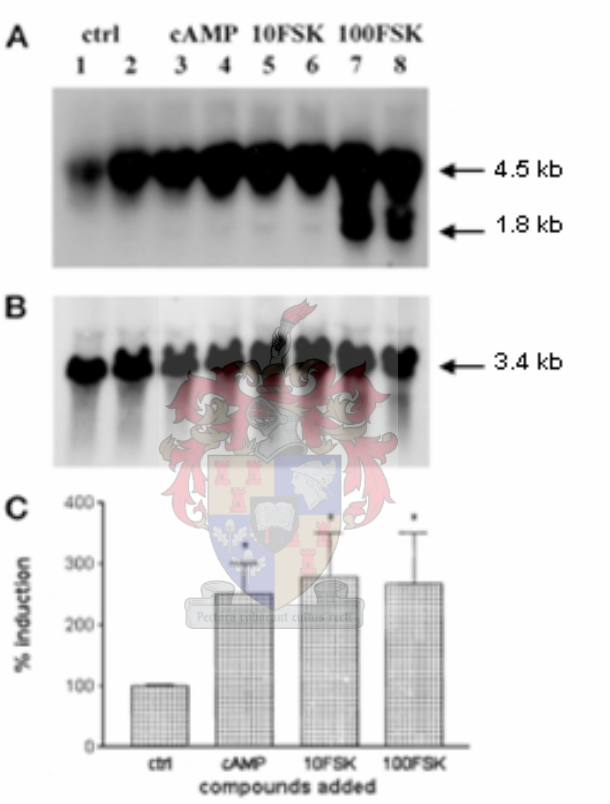


Figure 3.1: Levels of endogenous GnRHR mRNA in αT3-1 cells are increased by forskolin and 8-Br-cAMP

(A): αT3-1 cells were incubated for 16 h in the absence (ctrl, lanes 1 and 2) or presence of either 1 mM 8-Br-cAMP (cAMP, lanes 3 and 4), 10 μM forskolin (10FSK, lanes 5 and 6) or 100 μM forskolin (100FSK, lanes 7 and 8). Total RNA was probed for GnRHR mRNA by Northern blotting. (B): The membrane was stripped and reprobed for β-actin mRNA. Lane numbers are as in A. (C): The combined results of five independent experiments, similar to and including the experiment shown in panels A and B, where each incubation was performed in duplicate, were quantified by densitometric scanning. Note that the analysis was done on the signal corresponding to the larger (4.5 kb) GnRHR mRNA isoform. Labels are as described for A. The intensity of the GnRHR 4.5 kb signal, divided by that of the β-actin signal, both in the absence of forskolin, was taken as 100%. * = $P < 0.05$.

The results presented in figure 3.1 showed that both 8-Br-cAMP and forskolin resulted in a significant (approximately 2.5-fold) increase in GnRHR mRNA (4.5 kb) levels, relative to levels in unstimulated cells. Furthermore, forskolin induced the expression of a second, smaller mRNA isoform (1.8 kb) in a concentration-dependent fashion, with 100 μM forskolin resulting in markedly increased expression of this isoform, compared to expression at 10 μM. This may be due to the use of an alternative transcription start site or alternative RNA processing regulated by forskolin.

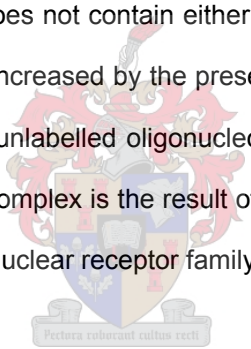
3.3.2 SF-1 and Nur77 proteins are present in αT3-1 nuclear extracts and recognize the -15/-7NRS *in vitro*

(Note that all oligonucleotide probes used consisted of 20 base-pairs containing the relevant transcription factor binding sites together with flanking sequences. Details of oligonucleotide sequences can be found in Addendum B).

Several DNA-protein complexes were detected (figures 3.2A and B) when αT3-1 nuclear extracts were incubated with a radiolabelled double-stranded probe containing the 20 base-pairs of promoter sequence encompassing the -15/-7NRS (figure 3.2A, lane 4; figure 3.2B, lane 5). Complex I was supershifted in the presence of anti-SF-1 antiserum, indicating that SF-1 protein in αT3-1 nuclear extracts binds to -15/-7NRS (figure 3.2A, lanes 5 and 6). However, no change in the intensity of complex II was observed in the presence of anti-SF-1 antibody.

Nur77 (also known as NGFI-B) is another nuclear receptor transcription factor that has previously been shown to recognize and bind to nuclear receptor half-sites as a monomer (Wilson TE *et al*., 1991; Wilson TE *et al*., 1993a; Wilson TE *et al*., 1993b; Zhang P and Mellon SH 1997; Okabe T *et al*., 1998). For this reason, the binding of Nur77 protein to the -15/-7NRS was also investigated. A consistent, reproducible decrease of complex II, with the concomitant appearance of a faint supershifted band, was observed in the presence of anti-Nur77 antiserum (figure 3.2B, lanes 6 and 7). The anti-Nur77 antiserum itself did not form any complexes with the -15/-7NRS probe (figure 3.2B, lane 4). Interestingly, a consistent increase in intensity of complex I was also observed in these lanes, most likely due to stabilization of the complex by serum proteins. *In vitro* transcribed-translated SF-1 protein (figure 3.2A, lanes 1 to 3) and Nur77-containing CCRF-CEM (a human T-cell leukemia cell line) nuclear extracts (figure 3.2B, lanes 1 to 3) were used as positive controls. The control protein-

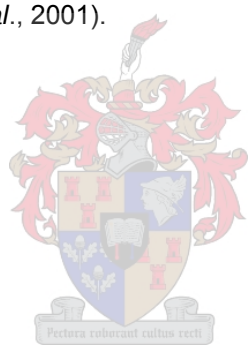
DNA complexes exhibited similar mobility on EMSAs as the respective complexes obtained with αT3-1 nuclear extract proteins (figures 3.2A and B). The SF-1 and Nur77 antisera did not cross-react with Nur77 and SF-1 control proteins, respectively (not shown). A similar pattern of complex formation as seen with αT3-1 nuclear extract proteins, was observed when SF-1 and Nur77 control proteins were mixed and incubated with the -15/-7NRS probe (figure 3.2C), providing further evidence that complexes I and II formed with αT3-1 nuclear extract proteins, contain SF-1 and Nur77, respectively. (A decrease in the intensity of the Nur77 complex caused by the presence of rabbit reticulocyte lysate proteins, was consistently observed). In addition to complexes I and II, other complexes were also observed in EMSAs with αT3-1 nuclear extract proteins. A slower migrating complex (above complex II) was sometimes observed, but the intensity of this complex was not reproducibly affected by the presence of SF-1 or Nur77 antisera (figures 3.2A and B). Similarly, the intensity of the faster migrating complex (below complex I) was not decreased by the presence of SF-1 or Nur77 antisera (figures 3.2 A and B), indicating that this complex does not contain either of these proteins. It is not clear why the intensity of this complex appears to be increased by the presence of anti-SF-1 antiserum (figure 3.2a, lanes 5 and 6). The variable effects of unlabelled oligonucleotide competitors on the intensity of this complex (figure 3.3) suggests that this complex is the result of non-specific protein binding, although it is also possible that it contains another nuclear receptor family member.



To serve as further confirmation that the protein in complex II was indeed Nur77, the binding of αT3-1 nuclear extract proteins to an established Nur77 binding site was investigated. As with the -15/-7NRS probe, several DNA-protein complexes were detected (figure 3.2D) when αT3-1 nuclear extracts were incubated with a radiolabelled double-stranded probe containing the Nur77 binding site from the human POMC promoter (NBRE) (Okabe T *et al*., 1998) (figure 3.2D, lane 4). One of these complexes, designated complex III, had similar mobility to that of one of the complexes formed between the NBRE probe and CCRF-CEM nuclear extracts (figure 3.2D, lane 1). The intensities of both these complexes decreased in the presence of anti-Nur77 antiserum (figure 3.2D, lanes 2 and 5). Although a decrease in intensity of the CCRF-CEM nuclear protein complex was consistently observed in the presence of pre-immune serum, this decrease was less pronounced than that seen for anti-Nur77 antiserum. The intensity of complex III was not influenced by anti-SF-1 antiserum (figure 3.2D, lane 6). In fact, it appears that complex IV, with higher mobility than complex III, contains SF-1 protein, since its intensity

was diminished by addition of anti-SF-1 antiserum. These results strongly indicate that complex III contains Nur77 protein from αT3-1 nuclear extracts. Furthermore, binding of the protein in complex III was competed for by the addition of 25-fold molar excess of non-radiolabelled -15/-7NRS oligonucleotide (figure 3.2D, lane 8), providing additional evidence that Nur77 protein from αT3-1 nuclear extracts can recognize the -15/-7NRS.

The presence of both SF-1 and Nur77 proteins in αT3-1 nuclear extracts was confirmed by Western blotting (figure 3.2E). Once again, no cross-reactivity was observed for the SF-1 antiserum with the Nur77 control protein or the Nur77 antiserum with the SF-1 control protein (not shown). The detection of Nur77 protein and mRNA has not previously been reported in αT3-1 cells, although it is well known that Nur77 protein is expressed in pituitary corticotropes (Okabe T *et al*., 1998; Maruyama K *et al*., 1998). However, the presence of Nur77 transcripts, and their upregulation by GnRH, has been reported in LβT2 cells (Wurmbach E *et al*., 2001).



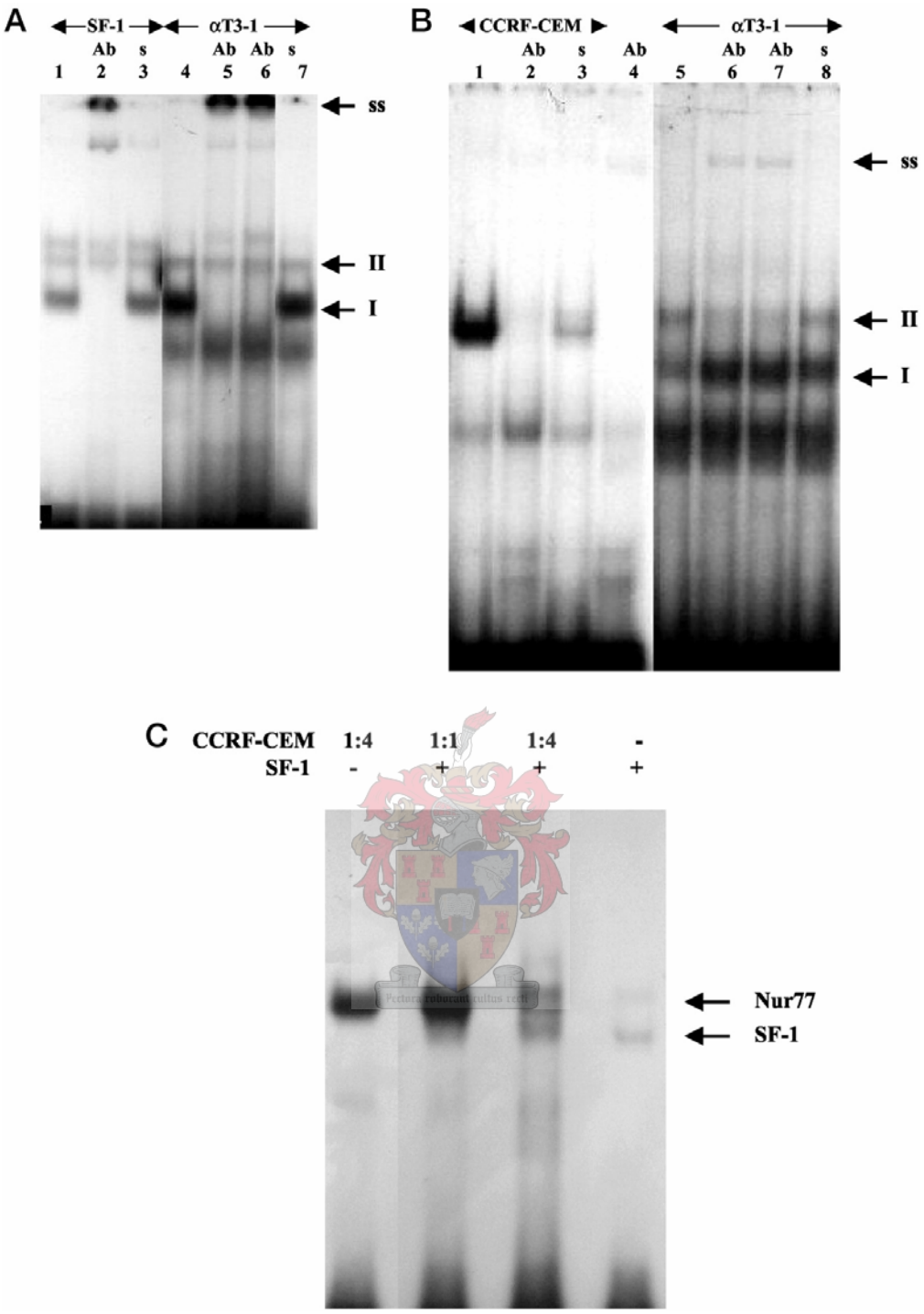


Figure 3.2 SF-1 and Nur77 proteins from αT3-1 nuclear extracts bind to the -15/-7NRS *in vitro*

(figure legend to follow on next page)

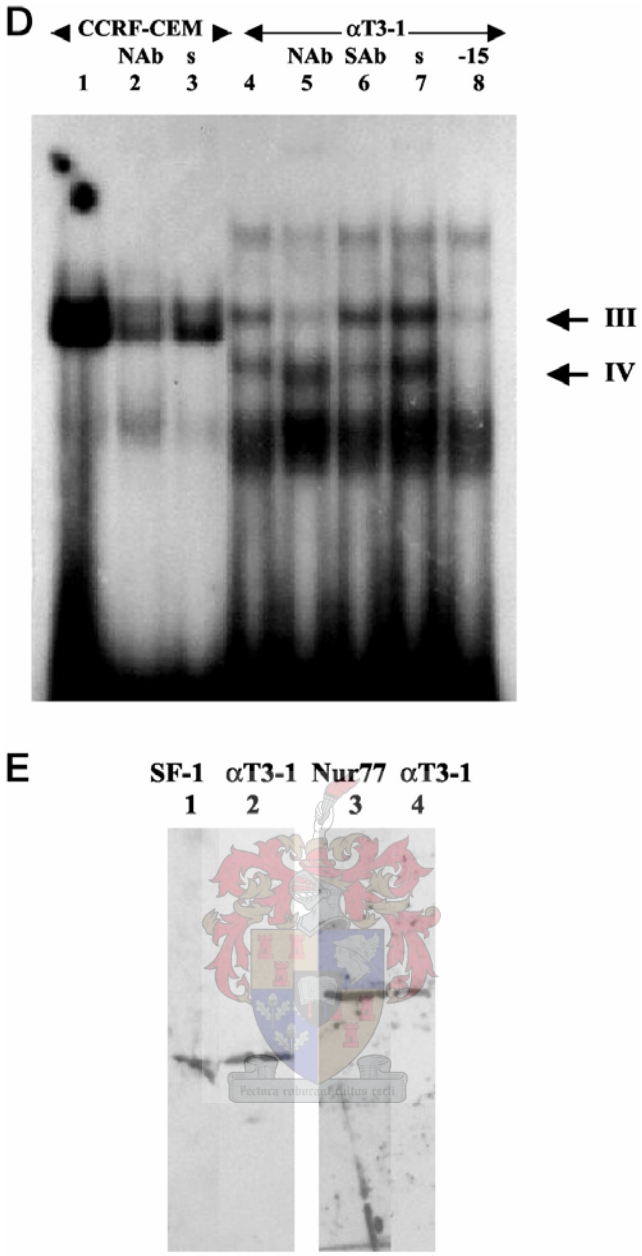


Figure 3.2: SF-1 and Nur77 proteins from αT3-1 nuclear extracts bind to the -15/-7NRS *in vitro*

Autoradiographs of EMSAs showing DNA-protein complex formation between αT3-1 nuclear extract proteins and the radiolabelled -15/-7NRS (A, B and C) and NBRE (D) probes. (A) Lanes 1 to 3 contained *in vitro* translated SF-1, with anti-SF-1 antiserum (Ab) added in lane 2 and pre-immune serum (s) added in lane 3. Lanes 4 to 7 contained αT3-1 nuclear extract proteins, with anti-SF-1 antiserum (Ab) added in lanes 5 and 6 (a duplicate incubation), and pre-immune serum (s) added in lane 7. Supershifted complex I is indicated by an arrow (ss). The positions of complex I (SF-1 with the -15/-7NRS probe) and complex II (Nur77 with the -15/-7NRS probe) are indicated with arrows. (B) Lanes 1 to 3 contained CCRF-CEM nuclear extracts (Geneka; positive control for Nur77), with anti-Nur77 antiserum (Ab) (from T. Perlmann; diluted 1:1.5 in 1X assay buffer) added in lane 2 and pre-immune serum (s) added in lane 3. Lanes 5 to 8 contained αT3-1 nuclear extract proteins, with anti-

Nur77 antiserum (Ab) added in lanes 6 and 7 (a duplicate incubation), and pre-immune serum (s) added in lane 8. Lane 4 contained anti-Nur77 antiserum in the absence of nuclear proteins. Complexes I and II are as for (A). Supershifted complex II is indicated by an arrow (ss). (C) Lanes 1 and 3 contained CCRF-CEM nuclear extracts (diluted 1:4 in 1X assay buffer), and lane 2 contained CCRF-CEM nuclear extracts (diluted 1:1 in 1X assay buffer). *In vitro* translated SF-1 was added in lanes 2 to 4. Complexes containing SF-1 and Nur77 protein, respectively, are indicated by arrows labelled correspondingly. (D) Lanes 1 to 3 contained CCRF-CEM nuclear extracts, with anti-Nur77 antiserum (NAb) added in lane 2 and pre-immune serum (s) added in lane 3. Lanes 4 to 8 contained αT3-1 nuclear extract proteins, with anti-Nur77 antiserum (NAb) added in lane 5, anti-SF-1 antiserum (SAb) added in lane 6 and pre-immune serum (s) added in lane 7. Lane 8 contained 25-fold molar excess of non-radiolabelled -15/-7NRS oligonucleotide (-15). The positions of complex III (SF-1 with the NBRE probe) and complex IV (Nur77 with the NBRE probe) are indicated with arrows. (E): The presence of SF-1 (53 kDa) and Nur77 (77 kDa) proteins in αT3-1 nuclear extracts was confirmed by Western blot analysis. The positive controls for SF-1 (lane 1) and Nur77 (lane 3) were 5 µl *in vitro* transcribed-translated SF-1 and 20 µg CCRF-CEM nuclear extracts, respectively. 30 µg αT3-1 nuclear extract protein was loaded in lanes 2 and 4. Lanes 1 and 2 were probed with anti-SF-1 antiserum, while lanes 3 and 4 were probed with anti-Nur77 antibody.

3.3.3 SF-1 and Nur77 proteins in αT3-1 nuclear extracts differ in their sequence requirements for binding to the -15/-7NRS and -244/-236NRS

The sequence requirements for binding of SF-1 (complex I) and Nur77 (complex II) from αT3-1 nuclear extracts to the -15/-7NRS were tested in an EMSA with radiolabelled -15/-7NRS oligonucleotide as probe (figure 3.3A), in the absence and presence of varying concentrations of non-radiolabelled competitor DNA containing either the wild-type -15/-7NRS sequence, or a mutated version thereof. Since the two adjacent cytosines within a nuclear receptor half-site had previously been shown to be essential for binding of SF-1 protein (Horn F *et al*., 1992), the corresponding bases in the -15/-7NRS were changed to two thymidines, to yield the oligonucleotide -15/-7NRSm. Although a 5-fold excess of non-radiolabelled -15/-7NRS oligonucleotide can effectively compete for SF-1 binding to radiolabelled -15/-7NRS (figure 3.3A, lanes 2 and 3), the -15/-7NRSm oligonucleotide was unable to compete for SF-1 binding, even at a 125-fold molar excess (lanes 4 to 6). In contrast, the -15/-7NRSm oligonucleotide was able to compete for Nur77 protein binding. However, Nur77 protein seemed to have a lower affinity for -15/-7NRSm than for wild-type -15/-7NRS, since complete competition for Nur77 binding by -15/-7NRSm was observed at 25-fold molar excess (figure 3.3A, lane 5), compared to 5-fold molar excess observed for wild-type -15/-7NRS (figure 3.3A, lane 2).

The results presented in figure 3.3B show that a 25-fold molar excess of the -244/-236NRS oligonucleotide competed for binding of both SF-1 and Nur77 proteins to -15/-7NRS (figure 3.3B, lane 2). When the -244/-236NRS sequence was modified to yield a *PstI* restriction digestion site, the resulting oligonucleotide (-244/-236NRSm) failed to compete for SF-1 binding to the -15/-7NRS probe (figure 3.3B, lanes 4 to 6), even at 125-fold molar excess (figure 3.3B, lane 6). Once again, the -244/-236NRSm oligonucleotide competed very effectively for Nur77 binding, with complete competition observed at 5-fold molar excess. Therefore, these results show that SF-1 and Nur77 proteins bind to both the -15/-7NRS and the -244/-236NRS, and that Nur77 protein recognizes mutated versions of these sites that are not recognized by SF-1.

These experiments were subsequently repeated using the -244/-236NRS oligonucleotide as radiolabelled probe (figures 3.3C and 3.3D). Complexes I and II, as formed by the -15/-7NRS probe, were also observed for the -244/-236NRS probe, and the proteins in the complexes were confirmed by specific antibodies to be SF-1 and Nur77, respectively (data not shown). Once again, 5-fold molar excess of both -15/-7NRS (figure 3.3C, lane 2) and -244/-236NRS (figure 3.3D, lane 2) were able to completely compete for binding of both SF-1 and Nur77. As seen before, 125-fold molar excess of either mutated competitor was unable to compete for binding of SF-1 (figure 3.3C, lane 6, and figure 3.3D, lane 6), whereas 5-fold molar excess was sufficient to compete for Nur77 binding (figure 3.3C, lane 4, and figure 3.3D, lane 4). Finally, the competition results for Nur77 binding were confirmed by using the NBRE oligonucleotide as probe (figure 3.3E), in the absence and presence of each of the four competitor oligonucleotides. Both the wild-type and mutated oligonucleotides competed for Nur77 binding (figure 3.3E, lanes 2-5).

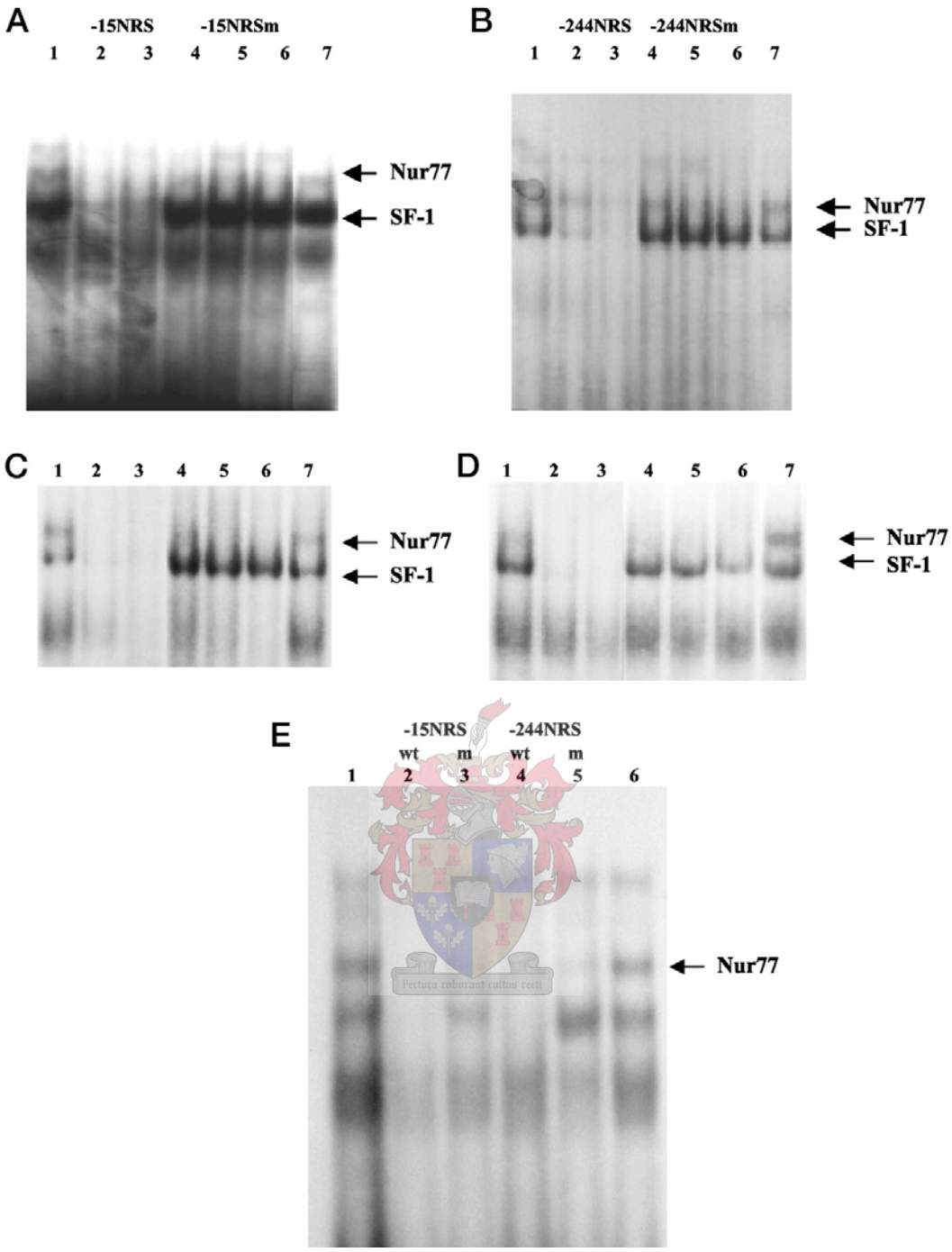


Figure 3.3: SF-1 and Nur77 proteins from αT3-1 nuclear extracts bind to both the -15/-7NRS and the -244/-236NRS, but have different sequence requirements for DNA binding

(A+B): Autoradiographs of competition EMSAs, using radiolabelled -15/-7NRS as probe. (A) Lanes 1 and 7 contained no competitor DNA. Lanes 2 and 3 contained 5- and 25-fold molar excess of -15/-7NRS, respectively; lanes 4 to 6 contained 5-, 25- and 125-fold molar excess of -15/-7NRSm, respectively. (B) Lanes 1 and 7 contained no competitor DNA. Lanes 2 and 3 contained 5- and 25-fold molar excess of -244/-236NRS, respectively; lanes 4 to 6 contained 5-, 25- and 125-fold molar excess of -244/-236NRSm, respectively. (C+D): Autoradiographs of competition EMSAs, using radiolabelled

-244/-236NRS as probe. Lane numbers for C are as for A, and numbers for D are as for B. (E): Autoradiograph of competition EMSA, using radiolabelled NBRE as probe. Lanes 1 and 6 contained no competitor DNA. Lanes 2 and 3 contained 25-fold molar excess of -15/-7NRS (wt) and -15/-7NRSm (m), respectively, and lanes 4 and 5 contained 25-fold molar excess of -244/-236NRS (wt) and -244/-236NRSm (m), respectively.

3.3.4 The -579/+1 GnRHR promoter is responsive to stimulation by forskolin, PACAP and GnRH

To investigate the response of the proximal mouse GnRHR promoter to activators of the PKA pathway, a promoter-reporter construct, designated pLG, was prepared by inserting 580 base-pairs of the mouse GnRHR gene, from position -579 to +1 relative to the translation start site, into the promoterless pGL2-basic luciferase reporter vector. αT3-1 cells were transfected with pLG, followed by incubation for varying times with 10 μM forskolin (figure 3.4A). No significant effect was observed on promoter activity until 16 hours of forskolin stimulation, at which time an approximately 2-fold increase in activity was obtained. In order to determine whether PACAP has a similar stimulatory effect on the mouse GnRHR proximal promoter as has been shown for the rat promoter (Pincas H *et al*, 2001b), a similar experiment was performed with PACAP on the mouse pLG construct (figure 3.4B). A significant increase in promoter activity of pLG was observed after only 30 min of stimulation with PACAP, with maximum activity (about 5-fold) observed after 8 hours (figure 3.4B). After 16 hours, the activity was still significantly higher (approximately 2-fold) than that of uninduced pLG (figure 3.4B). To compare the response of the mouse promoter to forskolin and PACAP with the response to GnRH, a similar experiment was performed on pLG with GnRH (figure 3.4C). While the promoter responded to GnRH with a maximal increase of about 1.5-fold after 4 hours, no significant effect was observed after 8 or 16 hours.

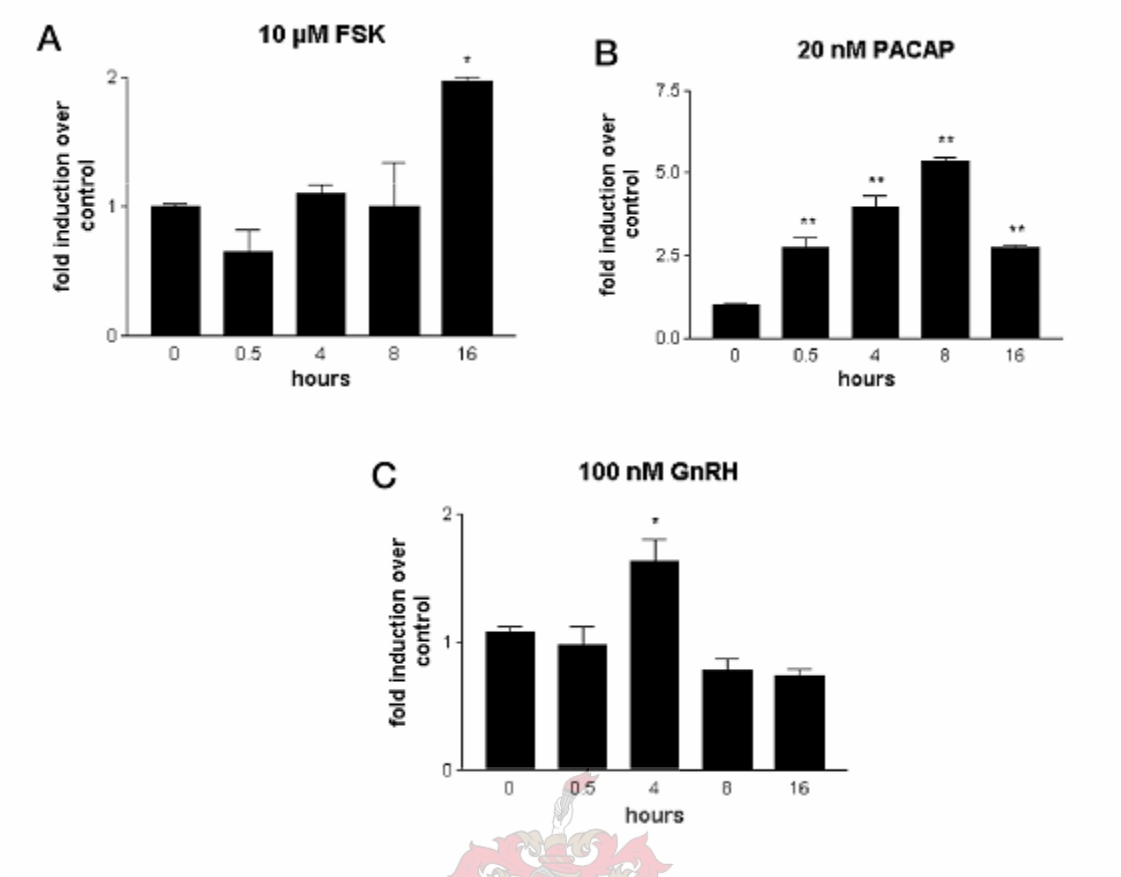


Figure 3.4: The -579/+1 GnRHR promoter responds to stimulation by forskolin, PACAP and GnRH.

αT3-1 cells were transfected with 0.5 µg per well of pSV-β-gal and 1 µg per well of pLG and stimulated for varying times with either (A) 10µM forskolin (B) 20 nM PACAP or (C) 100 nM GnRH, in medium containing 10% FCS. Control incubations for each time point were performed with mock additions of test compounds in solvent only i.e. DMSO (final concentration of 0.1% (v/v)) (A) or water (B and C). Cell extracts were harvested and assayed for luciferase and β-galactosidase activity. Luciferase assay values were normalized by β-galactosidase assay values to correct for differences in transfection efficiency between wells. Time points (in hours) are indicated below each bar. Values are expressed as normalized induced value relative to normalized control value for each time point, which was taken as equal to 1 for the zero time point. The results of a single typical experiment are shown, where each time point was performed in duplicate. Each experiment was performed at least twice. * = $P < 0.05$, ** = $P < 0.01$.

3.3.5 The -579/+1 GnRHR promoter responds to activation of the PKA pathway via a mechanism that is modulated by SF-1 binding to the -15/-7NRS and -244/-236NRS

To investigate the role of the -15/-7NRS and -244/-236NRS in basal and forskolin-induced expression of the mouse GnRHR gene, mutated versions of pLG were prepared, where either the -15/-7NRS, or the -244/-236NRS, were mutated. These constructs were designated pLGM1 (with the -15/-7NRS mutated) and pLGM2 (with the -244/-236NRS mutated), and are schematically represented in figure 3.5A. The mutations introduced were shown in figure 3.3 to result in loss of SF-1 binding *in vitro*. The constructs were individually transfected into αT3-1 cells, followed by overnight incubation (16 hours) with either 10 μM forskolin in 0.1% DMSO (v/v), or 0.1% DMSO (v/v) alone (figure 3.5B). Mutating the -15/-7NRS did not have any significant effect on the basal transcriptional activity of the -579/+1 promoter fragment. However, when the -244/-236NRS was mutated, the -579/+1 promoter fragment did not retain any basal activity whatsoever. This is consistent with results obtained by others (Duval DL *et al*., 1997a; Duval DL *et al*., 1997b), demonstrating that the -244/-236NRS is necessary for basal GnRHR promoter activity.

The wild type promoter was stimulated 2- to 2.5-fold by forskolin, relative to uninduced pLG. Interestingly, when the -15/-7NRS was mutated in such a way that SF-1 protein could no longer bind there, the response of the -579/+1 promoter fragment to forskolin stimulation was increased to about 3.5-fold above basal expression levels. This result indicates that binding of SF-1 to the -15/-7NRS is not necessary for the forskolin response, but that prevention of binding of SF-1 to this site removes an inhibitory element. No such increase in the forskolin response was observed for the pLGM2 construct containing the mutated -244/-236NRS. However, pLGM2 responded to forskolin treatment with an increase in activity to a level similar to that of unstimulated pLG, indicating that binding of SF-1 to the -244/-236NRS is also not essential for the forskolin response. The promoterless pGL2-basic vector did not respond to forskolin (not shown).

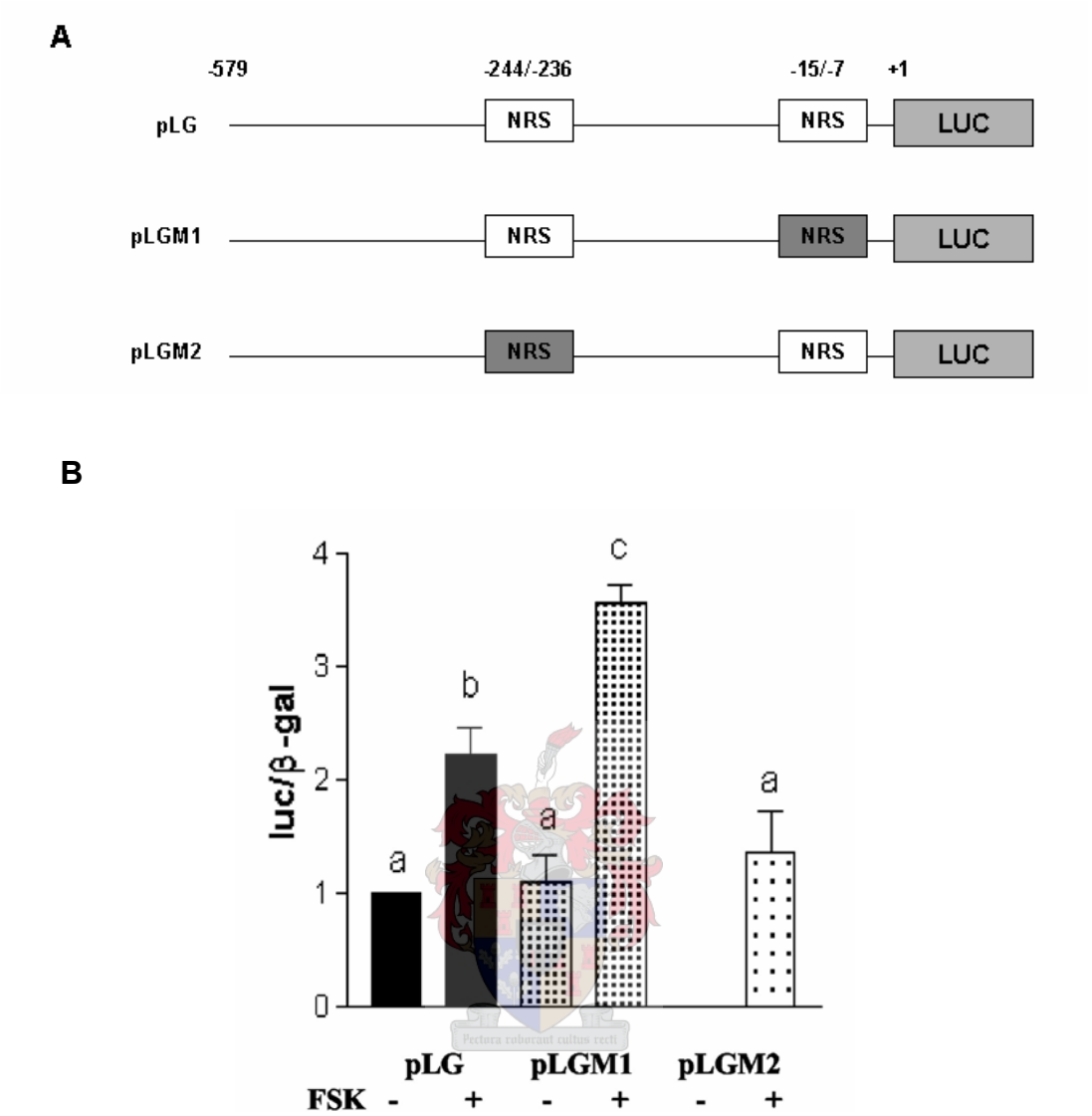


Figure 3.5: The response of the -579/+1 GnRHR promoter to forskolin stimulation is modulated by SF-1 binding to the -15/-7NRS and -244/-236NRS

(A) Diagrammatic representation of the luciferase expression constructs used in the transfection experiments. LUC = luciferase coding sequence; NRS = nuclear receptor binding site. (B) αT3-1 cells were transfected with pSV-β-gal (0.5 µg per well) plus GnRHR promoter reporter constructs (1 µg per well), as indicated below the graph bars. Cells were stimulated overnight with 10 µM forskolin (FSK) in 0.1% DMSO (v/v) (+), or with 0.1% DMSO (v/v) alone (-), as indicated, in medium containing 10% FCS. Cell extracts were harvested and analyzed as described for figure 3.4. The graph shows the combined results of 4 independent experiments, all performed in duplicate. Statistically significant differences ($P < 0.05$) are indicated by different lower case letters above the graph bars.

To confirm that the forskolin-induced increase in transcriptional activity of the -579/+1 promoter fragment is the result of activation of the Protein Kinase A (PKA) pathway by elevated cAMP levels, the same constructs described above were successively transfected into αT3-1 cells, together with a construct expressing the PKA catalytic subunit. To investigate the role of SF-1 protein in the response of the -579/+1 promoter fragment to PKA, a construct expressing SF-1 cDNA was also co-transfected in some experiments. The wild-type -579/+1 GnRHR promoter fragment (pLG) responded strongly to PKA (figure 3.6A). While overexpression of SF-1 had no effect on basal -579/+1 promoter activity, it substantially (about 2-fold) enhanced the PKA response of pLG. As already shown in figure 3.5B, mutating -15/-7NRS such that SF-1 could no longer bind there (pLGM1) had no effect on basal levels of transcriptional activity of the -579/+1 promoter fragment. Furthermore, overexpression of SF-1 had no effect on basal pLGM1 activity. Similar to the result obtained for forskolin, the PKA response of pLGM1 was substantially increased (figure 3.6B), compared to that of pLG, i.e. about 32-fold as compared to about 13-fold. This is consistent with the result presented in figure 3.5B, indicating that binding of SF-1 at the -15/-7NRS negatively modulates the PKA response. Overexpression of SF-1 increased the PKA response of pLGM1 to about 100-fold above basal pLG activity, or about 3-fold above that seen in the absence of overexpressed SF-1, as found for pLG.

Once again, as was shown before in figure 3.5B, mutation of the -244/-236NRS so as to prevent SF-1 protein from binding, resulted in the loss of basal -579/+1 promoter activity (figure 3.6C). pLGM2 retained its ability to respond to PKA, as it did with stimulation by forskolin (figure 3.5B). However, unlike pLG and pLGM1, overexpression of SF-1 did not result in any increase in the response of pLGM2 to PKA. This would be consistent with a mechanism whereby occupation of the -244/-236NRS by SF-1 in the wild type promoter results in enhancement of the PKA response. The results with pLGM1 would indicate that concomitant binding of SF-1 at site 1 is not required for this effect. As was found for forskolin, pGL2-basic was not responsive to overexpression of PKA or SF-1 (not shown).

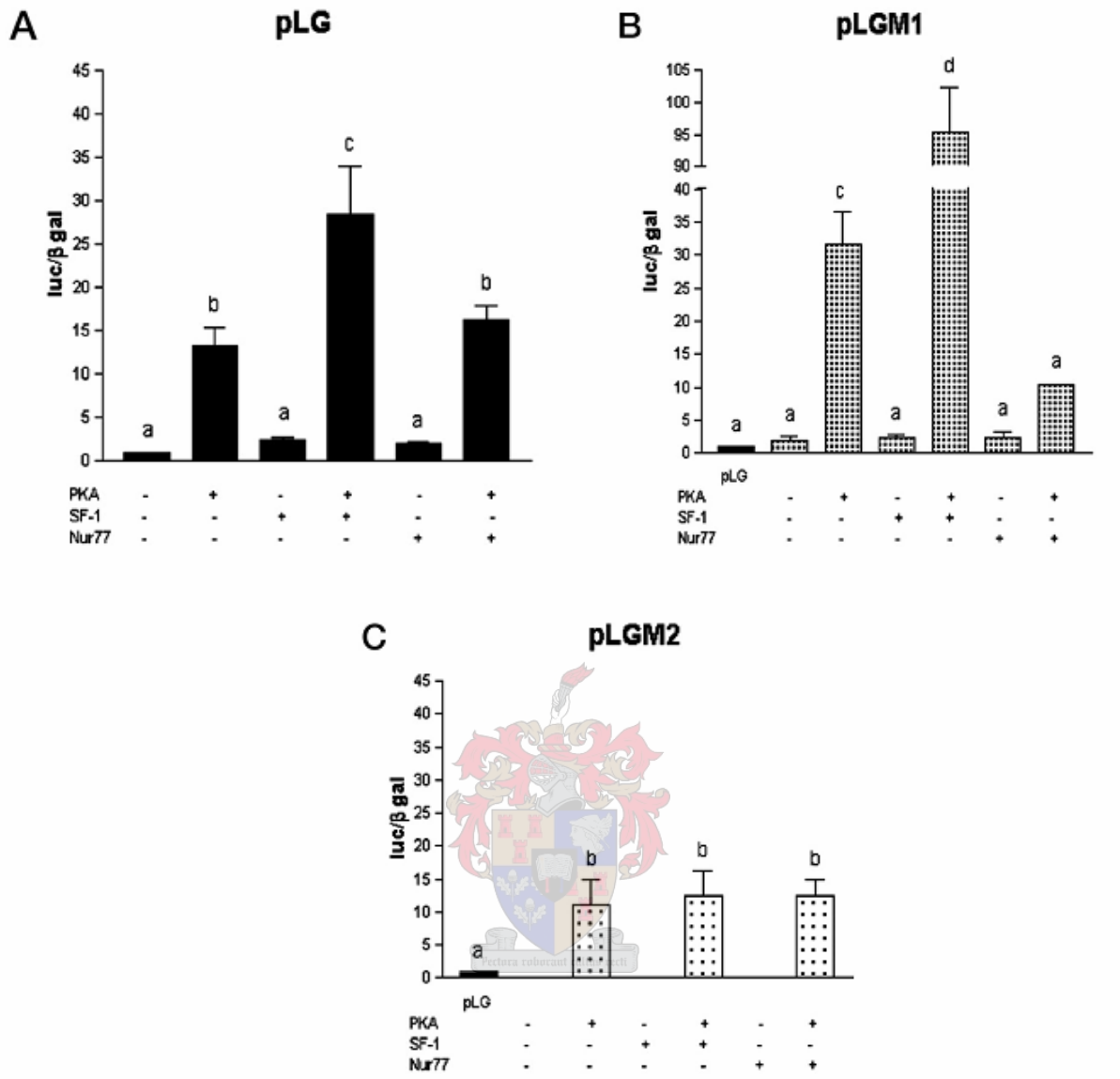


Figure 3.6: The -579/+1 GnRHR promoter responds to activation of the Protein Kinase A pathway and is modulated by SF-1 and Nur77

αT3-1 cells were transfected with 0.5 µg pSV-β-gal per well plus 1 µg per well of pLG (A), pLGM1 (B), or pLGM2 (C), together with 0.25 µg of pFC-PKA (PKA) and 0.5 µg of either pCMV-SF-1 (SF-1) or pCMX-Nur77 (Nur77), as indicated below the graph bars. Cell extracts were harvested and analyzed as described for figure 3.4. The results of a single representative experiment are shown, where each point was performed in duplicate. Each experiment was repeated at least three times. For each of the mutant constructs, a reference point (uninduced pLG) was taken and set as 1. Although the magnitudes of the responses differed between experiments, the general trend was well established. Statistically significant differences ($P < 0.05$) are indicated by different lower case letters above the graph bars.

3.3.6 The PKA response of the -579/+1 GnRHR promoter can be repressed by Nur77

To investigate the role of Nur77 protein in basal activity and in the response of the -579/+1 promoter fragment to PKA, similar experiments to those described above were performed, with a construct expressing the Nur77 cDNA (figure 3.6). Overexpression of Nur77 had no effect on basal activity of all three of the promoter constructs. Unlike SF-1, Nur77 had no influence on the PKA response of pLG. In experiments performed with pLGM2, where responsiveness to SF-1 was lost, Nur77 overexpression also had no effect on the PKA response (figure 3.6C). However, an unexpected and very interesting result was obtained for the pLGM1 construct, where it was found that overexpression of Nur77 resulted in a substantial (about 3-fold) attenuation of the PKA response (figure 3.6B). This result indicates that, in the absence of SF-1 binding at the -15/-7NRS, Nur77 is able to repress the PKA response.

The effect of overexpression of Nur77 in the presence of both overexpressed PKA plus SF-1 was also investigated for pLG and pLGM1 (figure 3.7). Since overexpressed SF-1 could not enhance the PKA response of pLGM2, this construct was not investigated. As seen previously in figure 3.6, the PKA response of both pLG and pLGM1 was enhanced by overexpressing SF-1 (figure 3.7). Overexpressed Nur77 completely abrogated the enhancing effect of SF-1 on the PKA response of pLG. A small reduction in the SF-1-enhanced PKA response of pLGM1 was also consistently observed in the presence of overexpressed Nur77, although this effect was not statistically significant.

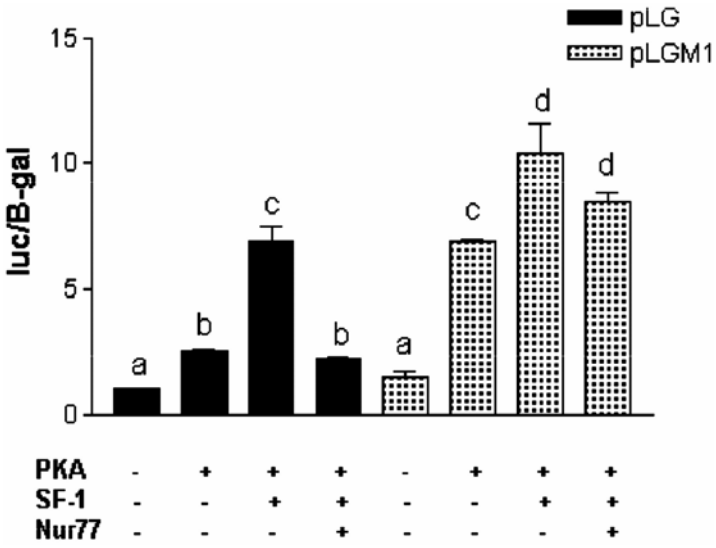


Figure 3.7: Nur77 antagonizes the SF-1-enhanced PKA response of the -579/+1 GnRHR promoter

αT3-1 cells were transfected with 0.5 µg pSV-β-gal per well plus 1 µg per well of either pLG or pLGM1, together with 0.25 µg of pFC-PKA (PKA) and 0.5 µg of pCMV-SF-1 (SF-1) in the absence or presence of 0.5 µg pCMX-Nur77 (Nur77), as indicated below the graph bars. Cell extracts were harvested and analyzed as described for figure 3.4. The results of a single representative experiment are shown, where each point was performed in duplicate. Each experiment was repeated at least three times. Although the magnitude of the responses differed between experiments, the general trend was well established. Statistically significant differences ($P < 0.05$) are indicated by different lower case letters above the graph bars.

3.3.7 SF-1 mRNA levels increase in response to forskolin

To gain insight into the mechanism whereby SF-1 modulates the response of the GnRHR promoter to stimulation of the PKA pathway, we investigated the effect of forskolin on endogenous SF-1 mRNA levels in αT3-1 cells. Total αT3-1 RNA was isolated after overnight incubation in the absence or presence of increasing concentrations of forskolin and was probed for SF-1 mRNA by Northern blotting. The result in figure 3.8 clearly shows that SF-1 mRNA (approximately 3 kb) levels increase in the presence of forskolin in a concentration-dependent manner (figure 3.8A), while β-actin mRNA levels were unchanged (figure 3.8B).

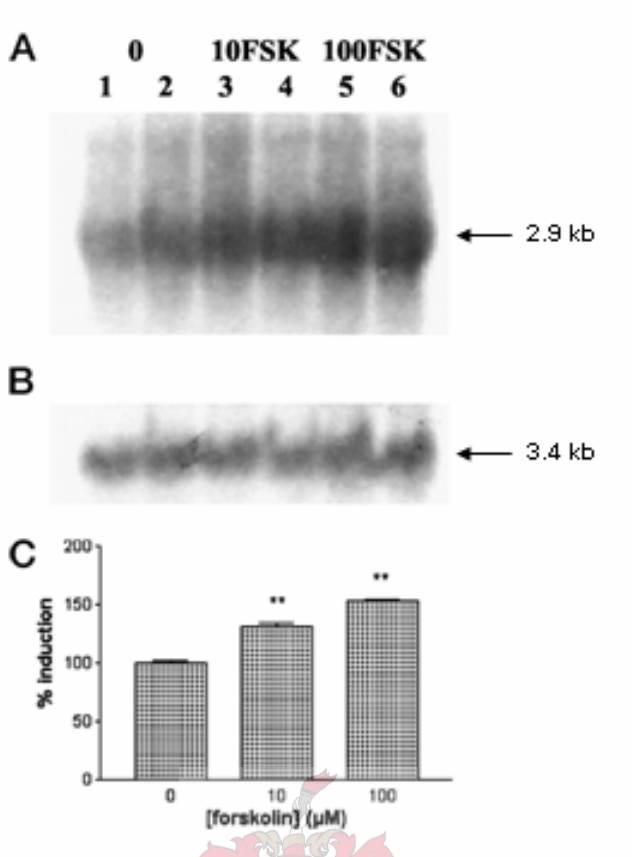


Figure 3.8: Levels of endogenous SF-1 mRNA in αT3-1 cells are increased by forskolin

(A): αT3-1 cells were incubated for 16 h in the absence (ctrl, lanes 1 and 2) or presence of either 10 µM forskolin (10FSK, lanes 3 and 4) or 100 µM forskolin (100FSK, lanes 5 and 6), in medium containing 10% FCS. Total RNA was analyzed and probed for SF-1 mRNA by Northern blotting. (B): The membrane was stripped and reprobed for β-actin mRNA levels, to serve as internal control. Lane numbers are as in A. (C): Quantification by densitometric scanning of the results shown in panels A and B. Labels of bar graphs are as described for A. The intensity of the SF-1 mRNA signal, divided by that of the β-actin signal, both in the absence of forskolin, was taken as 100%. ** = $P < 0.01$. The experiment was performed twice, the result of a single representative experiment is shown.

CHAPTER 4

AN INVESTIGATION INTO HOMOLOGOUS REGULATION OF THE MOUSE GnRHR GENE IN THE LβT2 GONADOTROPE CELL LINE

4.1 BACKGROUND

While homologous regulation of the mouse GnRHR gene has been well studied in αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a; Ellsworth *et al*., Endo 2003b), very little information is available regarding the effects of GnRH on GnRHR transcription in LβT2 cells. Pulsatile GnRH treatment of LβT2 cells over several days resulted in an increase in GnRHR mRNA levels (Turgeon JL *et al*., 1996). Pulsatile administration of GnRH over a 10 hour period also upregulated the activity of a GnRHR promoter-reporter construct transfected into LβT2 cells, and increased GnRHR levels on the cell surface (Bedecarrats GY and Kaiser UB, 2003). In the same study, continuous GnRH stimulation was found to have very little effect on the activity of the transfected GnRHR promoter-reporter construct, and actually down-regulated GnRHR numbers. However, to date, the kinase pathways involved in homologous regulation of GnRHR expression, and detailed mechanisms of transcriptional regulation of the GnRHR gene in LβT2 cells have not been published. Results from chromatin immunoprecipitation assays in intact LβT2 cells indicate that c-Jun protein occupy the GnRHR promoter *in vivo* in the absence of hormonal stimuli (Jeong KH *et al*., 2004), but the impact on transcriptional regulation is not clear.

The GnRHR can couple to both G_s and G_q G-proteins in LβT2 cells (Liu F *et al*., 2002b), and GnRH can activate several MAP kinase cascades in LβT2 cells, via both PKC-dependent and -independent mechanisms (reviewed in Ruf F *et al*., 2003). It has been shown that GnRH-mediated upregulation of endogenous LHβ expression and LHβ promoter activity in LβT2 cells involves MAPK and c-Jun (Yokoi T *et al*., 2000; Liu F *et al*., 2002a). GnRH also stimulates the expression of several AP-1 family members in these cells, and this has been shown to form part of the mechanism of GnRH-mediated upregulation of endogenous FSHβ expression and FSHβ promoter activity in these cells (Coss D *et*

al., 2004). In addition, the GnRH-mediated upregulation of nNOS promoter activity in LβT2 cells was shown to be PKA-dependent, and a CRE overlapping the transcription start site in this promoter was found to be crucial for basal and GnRH-stimulated promoter activity (Bachir LK *et al*., 2003). However, it is not known whether any of these kinase pathways or transcription factors is involved in transcriptional regulation of the GnRHR in LβT2 cells.

In chapter 3, results were presented that the GnRHR gene in the αT3-1 precursor gonadotrope cell line was upregulated by activators of the PKA pathway (forskolin, PACAP), as well as by overexpressed PKA itself. In addition, it was found that SF-1 binding to two nuclear receptor binding sites was not required for the PKA response, although a modulatory role for SF-1 in this response was demonstrated. However, the *cis* elements in the GnRHR promoter required for the PKA response were not identified. Also, the endogenous ligand(s) that activate the PKA pathway in these cells was not conclusively identified.

In αT3-1 cells, the GnRH response of the GnRHR I gene (Norwitz ER *et al*., 1999a) was found to be mediated via PKC, with PKA having no involvement. This can be explained by the fact that the endogenous GnRHR in αT3-1 cells has been found to couple exclusively to $G_{q/11}$, and not to G_s G protein (Grosse R *et al*., 2000). GnRH-mediated activation of the GnRHR in these cells could therefore not result in increased intracellular cAMP production and activation of the PKA pathway via G_s. However, the mouse GnRHR promoter (see chapter 3) and the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I promoters have been demonstrated to respond to activation of the PKA pathway in αT3-1 cells. Given that the GnRHR in LβT2 cells has been shown to signal via both $G_{q/11}$ and G_s (Liu F *et al*., 2002b), and that the upregulation of nNOS promoter activity by GnRH in these cells was shown to be PKA-dependent (Bachir LK *et al*., 2003), the possibility existed that regulation of GnRHR promoter activity by GnRH in LβT2 cells could be mediated via the PKA pathway.

4.2 AIMS

The aim of the present part of the project was to investigate transcriptional regulation of the mouse GnRHR gene by GnRH in LβT2 cells, and to determine whether PKA plays a role in mediating the downstream transcriptional effects of GnRH. It was decided to utilize the LβT2 cell line as a model system, since these cells display more characteristics of mature gonadotropes than the αT3-1 cell line, and the results obtained in LβT2 cells may therefore be physiologically more relevant.

In addition, the project aimed at determining the GnRHR promoter elements and their cognate transcription factors mediating transcriptional regulation of the GnRHR by GnRH and/or PKA. In this regard, four promoter *cis* elements were identified to be of particular interest. The two nuclear receptor binding sites, at -15/-7 and -244/-236 (relative to the ATG initiation codon), respectively, were included because of the modulatory role identified for them in the PKA response of the mouse GnRHR promoter in αT3-1 cells (chapter 3). The cAMP response element (CRE) at -107/-100 was investigated, since PKA classically signals via this promoter element (reviewed in Montminy M, 1997), and since CREs have been shown to be involved in the PKA-mediated transcriptional regulation of the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I genes. Because GnRH-mediated upregulation of mouse GnRHR promoter activity in αT3-1 cells was found to require the AP-1 element at -336/-330 (Norwitz ER *et al*., 1999a), and because c-Jun protein has been shown to occupy a region of the GnRHR promoter encompassing this AP-1 element in intact cells (Jeong KH *et al*., 2004), the role of this *cis* element in GnRHR transcriptional regulation was also investigated in LβT2 cells.

The detailed aims of the second part of the study were the following:

- To optimize the GnRH response of a transfected GnRHR promoter-reporter construct and of the endogenous GnRHR gene in LβT2 cells;
- To investigate the respective roles of PKA and PKC in the transcriptional response to GnRH;
- To identify the specific transcription factors in LβT2 nuclear extracts binding to the abovementioned promoter *cis* elements *in vitro*;
- To investigate the respective roles of the promoter *cis* elements in basal and GnRH-regulated transcriptional activity of the transfected GnRHR promoter-reporter construct in LβT2 cells;

- To investigate the protein expression levels and binding of LβT2 nuclear proteins to these promoter elements *in vitro*, under basal and GnRH-stimulated conditions

In each experiment, the effects of GnRH were investigated in the absence or presence of PKA pathway inhibitors. Since homologous regulation of the GnRHR I gene in αT3-1 cells was shown to be mediated via the PKC pathway, parallel experiments were performed in the presence of a PKC inhibitor.

4.3 RESULTS

4.3.1 Homologous regulation of mouse GnRHR gene expression

4.3.1.1 Optimization of conditions for maximal response of a GnRHR promoter-reporter construct to GnRH, PACAP and forskolin

In αT3-1 cells, GnRH-mediated regulation of the transcriptional activity of mouse GnRHR promoter-reporter constructs has been observed by several groups (White BR *et al*., 1999; Norwitz ER *et al*., 1999a; also see figure 3.4). It appears that only one study investigating homologous regulation of GnRHR promoter-reporter activity has been performed in LβT2 cells (Bedecarrats GY and Kaiser UB 2003). These authors found that a 1.2 kb GnRHR promoter-luciferase construct responded very weakly (increased activity by 1.5-fold) to continuous stimulation by 10 nM GnRH over 10 and 20 hours (Bedecarrats GY and Kaiser UB, 2003). In order to optimize the transcriptional response of the GnRHR promoter-reporter construct to GnRH in the present system, cells were transfected with the wild-type -579/+1 mouse GnRHR promoter-luciferase construct pLG (described in chapter 3), and stimulated with GnRH under different culture conditions for varying times. In the presence of 10% FCS, administration of 100 nM GnRH only resulted in a weak, non-reproducible response, even after 10 hours (not shown). For this reason, cells were serum-starved to different extents, and for different periods of time, in order to determine the optimal conditions for studying the response of the GnRHR I promoter to GnRH. As can be seen in figure 4.1, the serum concentration present during transfection did not have a significant influence on the magnitude of the GnRH response. However, the serum concentration present during GnRH administration was clearly a key determinant of responsiveness to GnRH. When the cells were treated with GnRH in the absence of serum, a 2- to 3-fold increase in GnRHR promoter activity was observed after 8 hours. For all subsequent experiments, transfections

were performed in medium containing 10% FCS, and incubations with compounds were performed in serum-free medium.

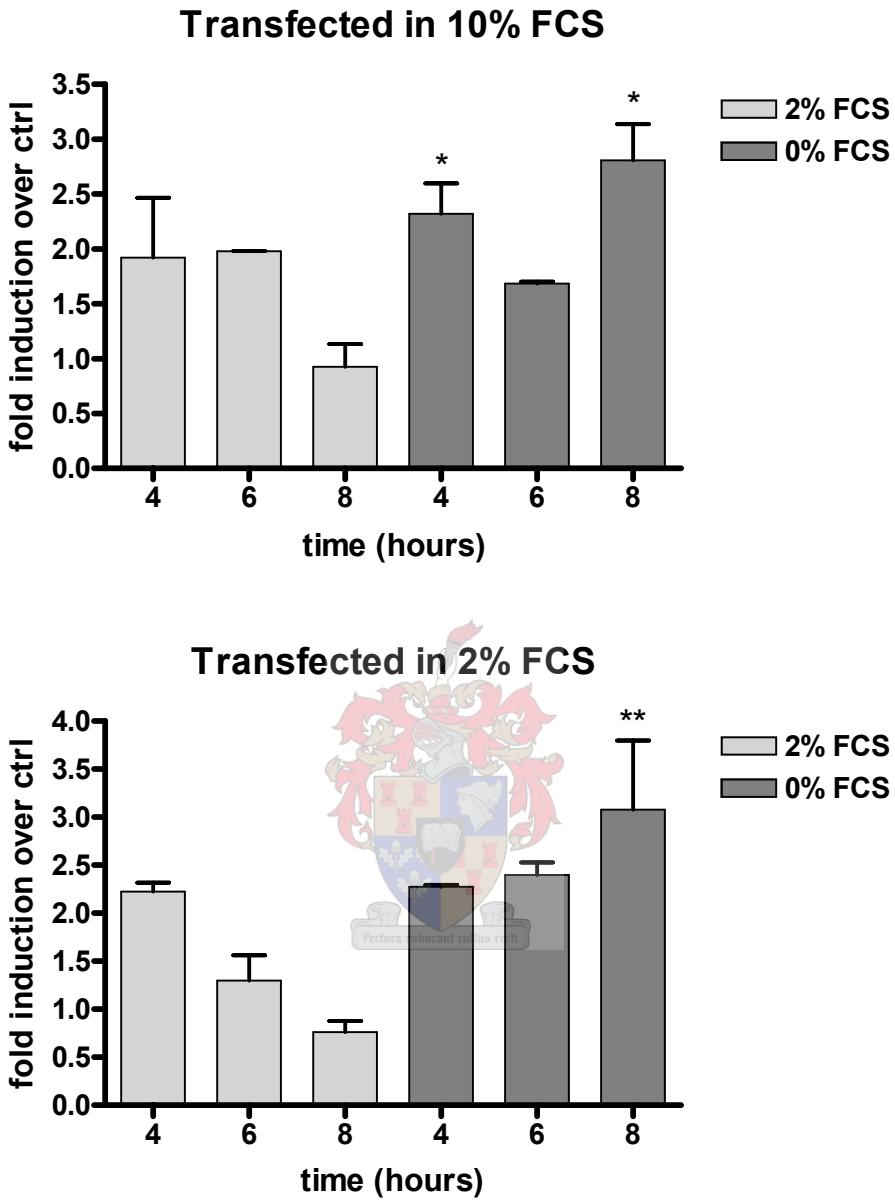


Figure 4.1: The serum concentration in culture medium influences the GnRH response of the GnRHR promoter. LβT2 cells were transfected with 100 ng pLG, in the presence of either 10% FCS or 2% FCS, as indicated above the respective graphs. After approximately 24 hours, medium was replaced, and cells were treated with 100 nM GnRH, for different periods of time, in the absence or presence of 2% FCS, as indicated in the figure legend. For each condition and each time point, the luciferase activity of vehicle-treated pLG was set as 1, and the fold induction in response to GnRH treatment was calculated relative to no hormone at that condition. The figure shows the result of one experiment, performed in duplicate. Asterisks above bars indicate statistical significance of responses: * = $P < 0.05$; ** = $P < 0.01$.

In order to confirm that the conditions chosen above were sufficient to yield an optimal response to GnRH, cells transfected with pLG were incubated in serum-free medium containing 100 nM GnRH, for varying times. In parallel, the regulation of pLG activity by PACAP and by forskolin (an adenylyl cyclase activator) was investigated under the same conditions.

As can be seen in figure 4.2, four hours of continuous GnRH administration resulted in a small increase in promoter activity, with a maximal response observed after 8 hours. Promoter activity had returned to near basal levels after 12 hours. A smaller response was observed with forskolin, with a maximum (~1.8 fold) also reached after 8 hours. No effect on GnRHR promoter activity was observed in the presence of PACAP. This is in agreement with earlier results published by Fowkes RC *et al*., 2003, showing that LβT2 cells express extremely low levels of PACAP receptors, and that PACAP is therefore unable to elicit a measurable intracellular response in these cells. This is in contrast to the situation in αT3-1 cells, where PACAP-mediated activation of both adenylyl cyclase and phospholipase C has been detected (Schomerus E *et al*., 1994), and the upregulation of mouse (figure 3.4), rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR promoter activity by PACAP has been demonstrated. Based on these results, no further experiments were performed with PACAP, and the 8 hour time point was chosen for all subsequent incubations with GnRH and forskolin.

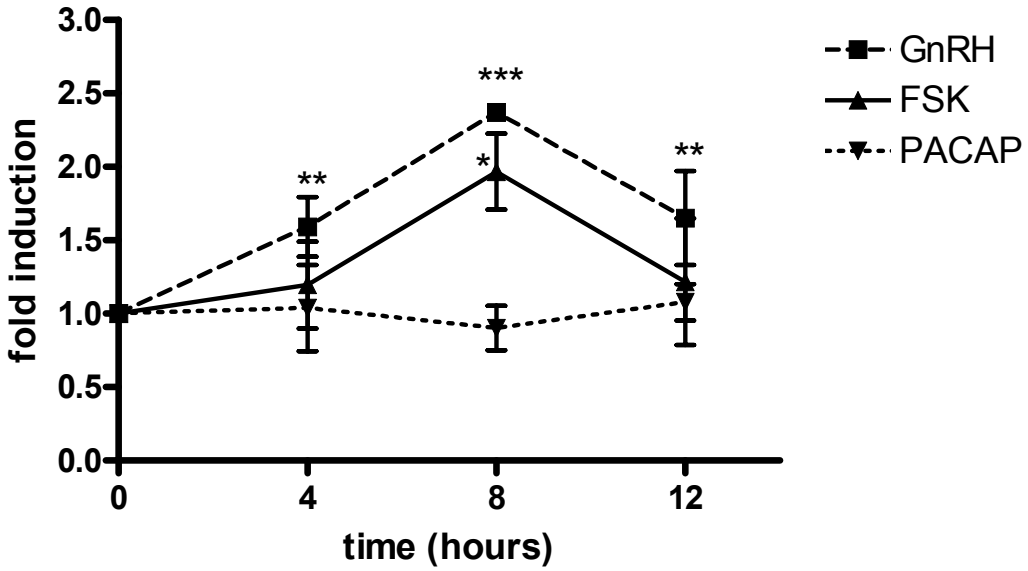


Figure 4.2: The GnRHR promoter responds to GnRH and forskolin, but not to PACAP in LβT2 **cells.** LβT2 cells were transfected with 100 ng pLG, in the presence of 10% FCS. After approximately 24 hours, medium was replaced, and cells were incubated for different periods of time in serum-free medium containing vehicle, 100 nM GnRH, 10 μM FSK or 100 nM PACAP. For each time-point, the luciferase activity of vehicle-treated pLG was set as 1, and the hormone-stimulated increase in pLG activity was calculated relative to the vehicle-treated control for each time-point. Figure shows combined results of three experiments, all performed in triplicate. * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

4.3.1.2 The transcriptional response to GnRH involves both the PKA and PKC pathways

The previous experiments established that the pLG GnRHR promoter construct responded maximally to GnRH after 8 hours of continuous administration. In order to investigate whether the PKA pathway was involved in mediating the response to GnRH, cells transfected with pLG were stimulated with GnRH in the absence or presence of H89, a specific PKA inhibitor. In addition, since the GnRH response of the GnRHR promoter in αT3-1 cells was shown by others to be mediated by the PKC pathway (White BR *et al*., 1999; Norwitz ER *et al*., 1999a), parallel experiments were performed in the presence of the PKC inhibitor bisindolylmaleimide (BIM). As can be seen in figure 4.3, both BIM and H89 inhibited the transcriptional response to GnRH, such that in the presence of these inhibitors, GnRH failed to stimulate promoter activity above basal levels. However, since H89 can also inhibit other pathways, albeit at higher concentrations (Sigma product information), a different approach was used in parallel to confirm involvement of the PKA pathway. Experiments were also performed where

the specific PKA inhibitory peptide, PKI, was overexpressed. The GnRH response was completely abrogated in the presence of overexpressed PKI, providing strong evidence that the PKA pathway is specifically required for the GnRH response in LβT2 cells. Results from control experiments where cells were treated with kinase inhibitors, in the absence of GnRH stimulation, showed that these inhibitors had no effect on the basal transcriptional activity of pLG (figure 4.3, dark bars), indicating that activity of PKA and PKC are not required for maintaining basal GnRHR expression. Taken together, these results clearly demonstrated a critical role for both the PKA and PKC pathways in mediating the transcriptional response of the GnRHR promoter to GnRH in LβT2 cells.

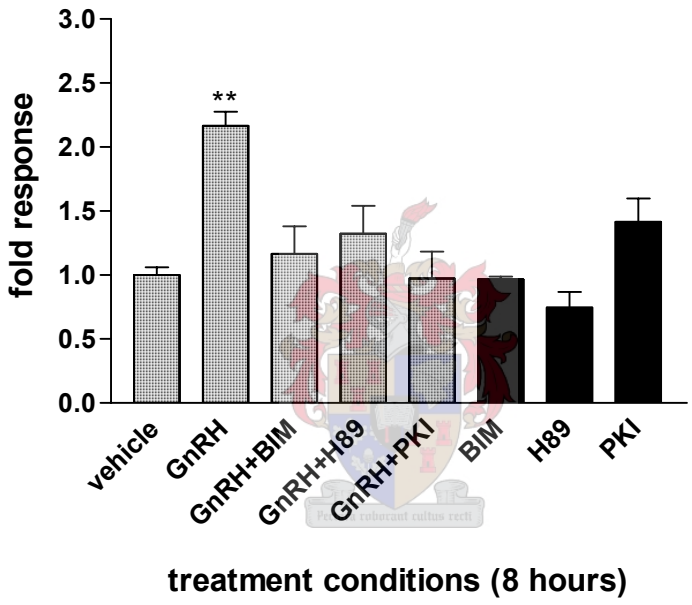


Figure 4.3 Inhibitors of both the PKA and the PKC pathways inhibit the transcriptional response to GnRH. LβT2 cells were transfected with pLG (100 ng) or with pLG and pRSV-PKI (2.5 ng), as indicated below the relevant bars, in the presence of 10% FCS. After approximately 24 hours, medium was replaced and cells were incubated for 8 hours in serum-free medium containing either vehicle, 100 nM GnRH, GnRH plus 1 μM H89, or GnRH plus 100 nM BIM, as indicated. Control incubations with kinase inhibitors, in the absence of GnRH stimulation, were also performed and are indicated by the dark bars. Luciferase activity of vehicle-treated pLG was set as 1. Figure shows combined results of 3 experiments, all done in triplicate. ** = $P < 0.01$.

4.3.1.3 Regulation of endogenous GnRHR mRNA levels by GnRH

Studies performed by Tsutsumi *et al.* in αT3-1 cells showed that endogenous GnRHR mRNA levels in these cells remained unchanged after short-term (Tsutsumi M *et al*., 1993) or long-term (Tsutsumi M

et al., 1995) continuous GnRH treatment. Results obtained by a different group showed a modest elevation (~ 1.8-fold) in GnRHR mRNA levels after 4 hours of continuous GnRH treatment (Norwitz ER *et al*., 1999a). The direct effect of continuous GnRH administration on GnRHR mRNA levels in LβT2 cells has not been reported. As was shown in figure 4.2, the activity of the pLG GnRHR promoter-luciferase construct was upregulated 2-fold by GnRH in LβT2 cells after 8 hours. To determine whether GnRH upregulates expression of the endogenous GnRHR gene in LβT2 cells in the same way as the transiently transfected GnRHR promoter-reporter construct, GnRHR mRNA levels were measured by means of LightCycler real-time RT-PCR. Total RNA was isolated from LβT2 cells that had been treated with GnRH for 8 hours, under the conditions established for the promoter assays. An upregulation of GnRHR mRNA levels by GnRH was observed in several repeat experiments, although the magnitude of the response varied between individual experiments (figure 4.4).

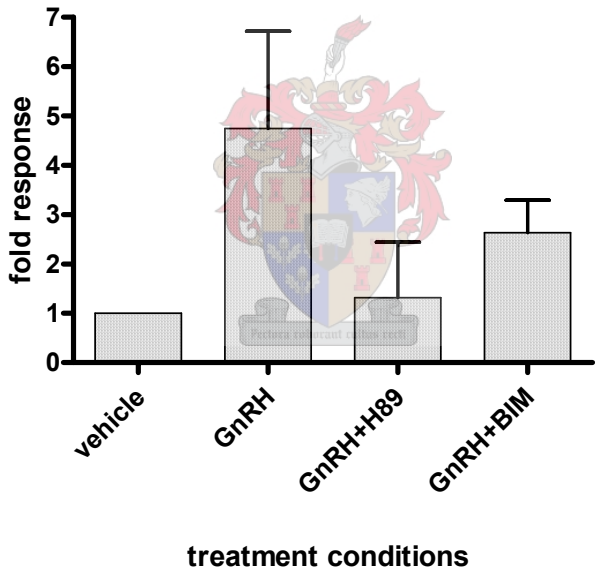


Figure 4.4: Regulation of endogenous GnRHR mRNA levels in LβT2 cells by GnRH. Cells were grown for 48 hours in medium containing 10% FCS, after which medium was replaced. Total RNA was isolated from cells treated with test compounds in serum-free medium for 8 hours (as described for figure 4.3). RNA was reverse-transcribed, and relative levels of GnRHR transcripts were determined by LightCycler real-time RT-PCR. Relative levels of β-actin transcripts were determined to serve as internal control. For each sample, 400 ng of unpurified cDNA was used as PCR template. GnRHR mRNA levels present in vehicle-treated samples were taken as 1, and fold changes in mRNA levels were calculated relative to vehicle-treated samples, and were normalized against the relative levels of β-actin transcripts measured in each sample (refer to Addendum A for full details on calculating

relative mRNA expression from LightCycler PCR data). Graph shows combined results of 2 experiments.

These results indicated that the upregulation of GnRHR promoter activity, as observed by promoter-reporter assays, correlated with transcriptional regulation of the endogenous gene, and was therefore physiologically relevant. Interestingly, the response of the endogenous gene was often greater than that of the -579/+1 promoter, possibly suggesting that promoter regions further upstream from -579 are involved in facilitating maximal transcriptional activation of the GnRHR gene by GnRH.

The stimulatory effect of GnRH on GnRHR mRNA was at least partially inhibited by both the PKA and PKC inhibitors, although these inhibitory effects varied between experiments. This inhibition of the transcriptional response to GnRH of endogenous mRNA levels is similar to what was found for the transfected pLG GnRHR promoter-reporter construct (figure 4.3), and suggests that the results obtained for the transfected promoter are not an artefact of the transient transfection assay. Although the effects of the kinase inhibitors alone on GnRHR transcript levels were not investigated, the results obtained for the transfected pLG construct (figure 4.3) suggest that these inhibitors would not have an effect on basal levels of GnRHR transcription. However, this would still need to be confirmed experimentally for the endogenous gene. Due to variability in the extent of the response between experiments, when the results from different independent experiments were combined, the changes in GnRHR mRNA levels were not statistically significant. However, these results follow a trend similar to that observed for the transfected pLG construct (figure 4.3), and are most likely physiologically relevant. In three experiments performed in LβT2 cells, GnRH consistently upregulated endogenous GnRHR mRNA. In one experiment, the addition of H89 together with GnRH increased GnRHR mRNA levels even more than GnRH alone, but in two subsequent experiments, both H89 and BIM inhibited the stimulatory effects of GnRH. It is clear that more experiments would be needed to confirm the inhibitory effects of H89 and BIM on GnRH stimulation of GnRHR mRNA levels, and to establish statistical significance of the response to GnRH.

4.3.2 *Cis*-elements and transcription factors involved in basal promoter activity and homologous regulation

4.3.2.1 Binding of LβT2 nuclear proteins to different *cis* elements in vitro

(Please note that, as mentioned in Chapter 3, all oligonucleotide probes used in electrophoretic mobility shift assays consisted of 20 base-pairs containing the specific transcription factor site together with flanking sequences. Detailed sequences of oligonucleotides can be found in Addendum B.)

In Chapter 3, different lines of evidence were provided to show that Nur77 protein was expressed in αT3-1 cells, and that it recognized both the -15/-7NRS and the -244/-236NRS. The presence of SF-1 protein in these cells, and its binding to the nuclear receptor binding sites were also demonstrated. SF-1 protein has also been demonstrated to be present in LβT2 cells (Fowkes RC *et al*., 2002), although its role in the transcriptional regulation of the GnRHR gene has not been investigated. While Nur77 mRNA expression in LβT2 cells has been detected by microarray analysis (Wurmbach E *et al*., 2001), the presence and function of Nur77 protein in these cells has apparently not been established.

As observed by electrophoretic mobility shift assay (EMSA) for αT3-1 cells (chapter 3, figure 3.2), nuclear proteins from LβT2 cells also formed two major complexes (figure 4.5A) with the -15/-7NRS probe (lanes 1 to 3), as well as with the -244/-236NRS probe (lanes 4 to 6). The lower complex (complex I) was of comparable intensity for both probes, and was supershifted with anti-SF-1 antiserum (figure 4.5A, lanes 2 and 5), confirming the identity of the protein in complex I as SF-1. The upper complex (complex II) formed with the -244/-236NRS probe was of markedly lower intensity than that formed with the -15/-7NRS probe (compare lanes 1 and 4). The formation of complex II, but not complex I, was completely inhibited by the presence of anti-Nur77 antibody (figure 4.5B, lane 3).

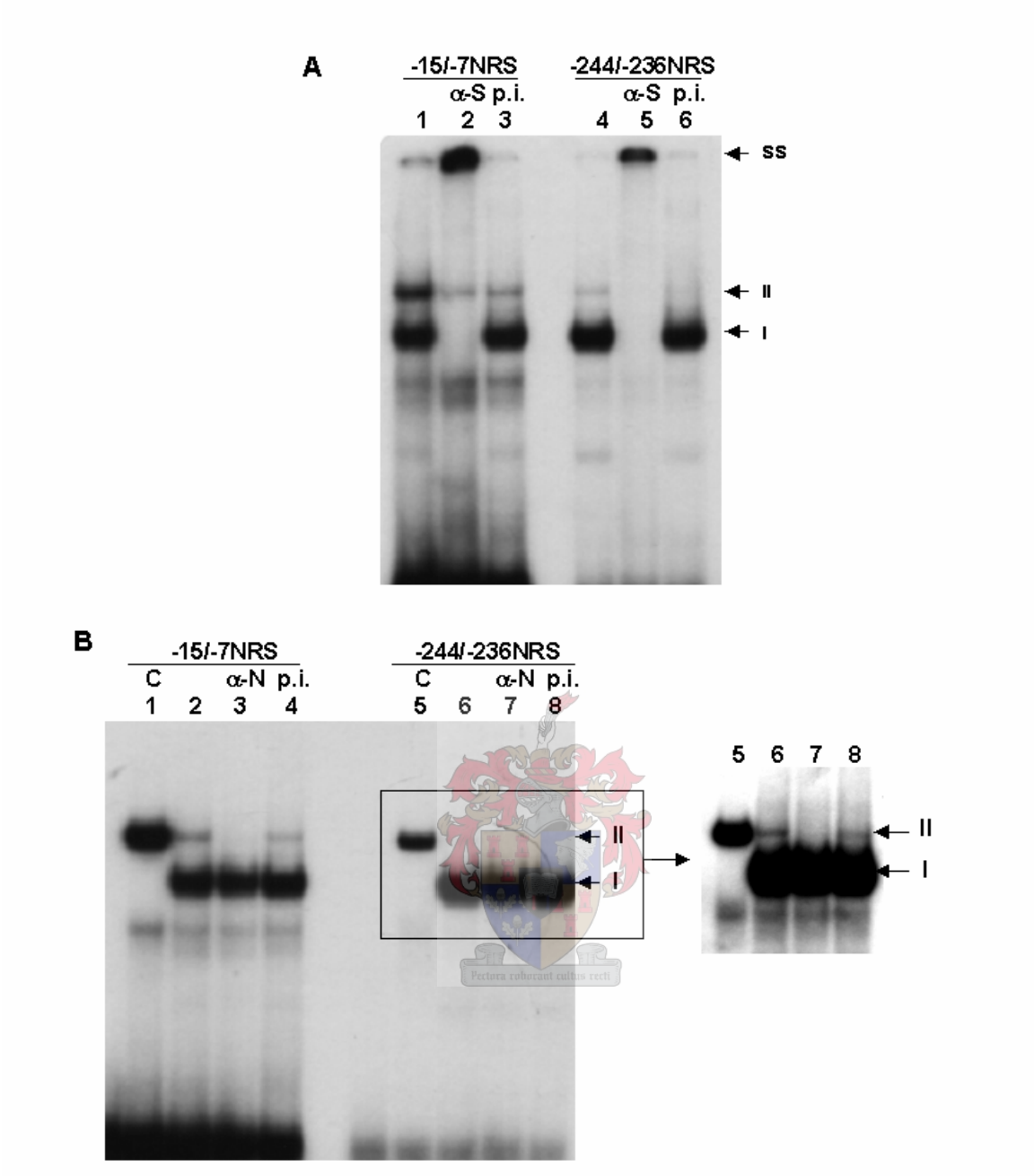


Figure 4.5: Binding of LβT2 nuclear proteins to the -15/-7NRS and -244/-236NRS. Autoradiographs of EMSAs showing binding of LβT2 nuclear proteins to radiolabeled -15/-7NRS and -244/-236NRS probes, as indicated above each image. All lanes contained LβT2 nuclear extracts, unless otherwise indicated. (A) In lanes 2 and 5, 1 µl anti-SF-1 antiserum was added (lanes labelled "α-S"), and in lanes 3 and 6, 1 µl pre-immune serum was added ("p.i."). Supershifted complexes are indicated by "ss". (B) Lanes 1 and 5 contained 0.75 µg CCRF-CEM nuclear extracts (lanes labelled "C"). In lane 3, 1 µl anti-Nur77 was added (lane labelled "α-N"), and in lane 4, 1 µl pre-immune serum was added. (Note that anti-nur77 also abrogated complex II formed with the -244/-236NRS probe; however, due to the low intensity of this complex, this could only be observed after much longer exposure of the autoradiograph, and is shown in the inset.)

In addition, CCRF-CEM nuclear extracts, containing high levels of Nur77 protein, formed a single complex of the same mobility as complex II with both NRS probes (figure 4.5B, lanes 1 and 5), indicating that complex II contained Nur77 protein. As seen with LβT2 nuclear extracts, the intensity of the complex formed between CCRF-CEM nuclear extracts and the -244/-236NRS probe was also lower than that formed with the -15/-7NRS probe (figure 4.5B, compare lanes 1 and 5). Interestingly, the intensity of complex II was also reduced by anti-SF-1 anti-serum (figure 4.5A, lanes 2 and 5), but since a similar effect was observed with the pre-immune serum (lanes 3 and 6), this effect is most likely non-specific.

Previously, it was established that Nur77 protein from αT3-1 cells recognized mutated versions of the two nuclear receptor binding sites that SF-1 protein could not recognize (figure 3.3), and thus it was of interest to see whether this was also true for Nur77 protein from LβT2 cells. In figure 4.5C, it can be seen that excess unlabelled competitor with the mutated sequences (see Addendum B) of -15/-7NRS (lane 3) and -244/-236NRS (lane 5) reduced the intensity of complex II, but not complex I. Taken together, the results in figure 4.5 show that both SF-1 and Nur77 proteins are expressed in LβT2 cells, and that both proteins can recognize the -15/-7NRS and the -244/-236NRS in the mouse GnRHR promoter *in vitro,* thereby mirroring results obtained in αT3-1 cells (figure 3.2). In addition, these results confirm that Nur77 protein in LβT2 cells can bind to the mutated versions of the nuclear receptor binding sites *in vitro*, while SF-1 protein is unable to do so. However, the excess unlabelled wild-type -244/-236NRS competitor does not completely inhibit the formation of complex II, suggesting that Nur77 might be binding non-specifically to the oligonucleotide, possibly recognizing a different part of the oligonucleotide sequence.

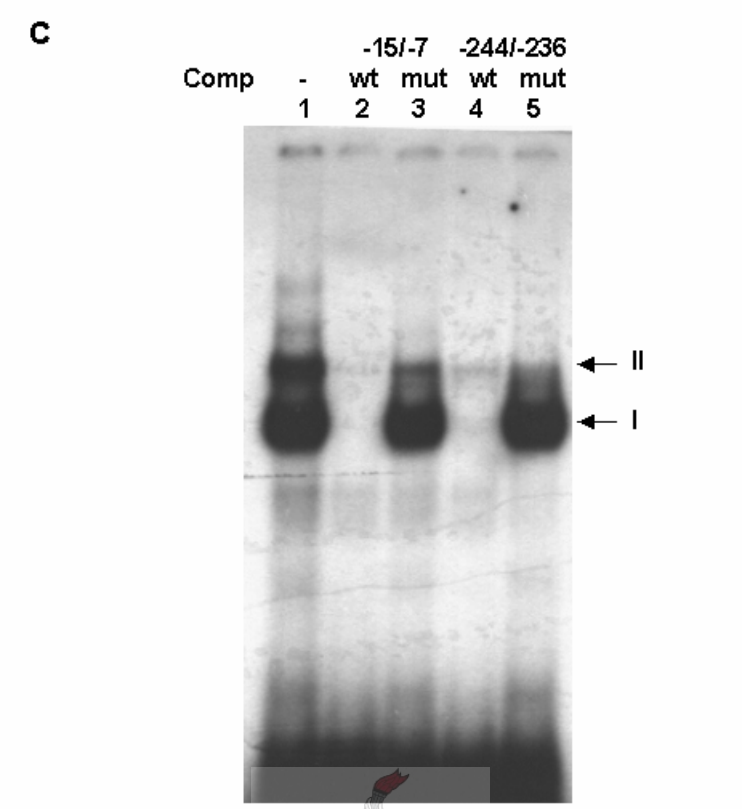


Figure 4.5 (C) All lanes contained LβT2 nuclear extracts, complexed with the -15/-7NRS probe. All unlabelled competitor (Comp) double-stranded oligonucleotides were added at 50-fold molar excess. Lanes 2 and 3 contained wild-type (wt) and mutated (mut) -15/-7NRS competitors, respectively, and lanes 4 and 5 contained wild-type and mutated -244/-236NRS competitors, respectively.

Specific *in vitro* binding of AP-1 proteins to the AP-1 element at -336/-330 has been shown for αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a). *In vivo* binding of c-Jun protein to a broad region of the mouse GnRHR promoter, encompassing the AP-1 element, has also been demonstrated by chromatin immunoprecipitation (ChIP) in LβT2 cells (Jeong KH *et al*., 2004). However, the specific binding of AP-1 proteins to the AP-1 element has not been investigated in LβT2 cells. Interestingly, we observed that nuclear extracts prepared from unstimulated LβT2 cells had very low AP-1 binding activity, and therefore nuclear extracts from GnRH-treated cells were used to investigate AP-1 binding. Two major complexes were formed with a radiolabelled oligonucleotide probe containing the AP-1 site (figure 4.6A), of which the intensity of the lower complex (complex II) appeared to vary between experiments. Both anti-Jun and anti-Fos antibodies interfered with the formation of complex I, with anti-Fos causing a supershift of complex I (figure 4.6A, lane 3), and anti-Jun resulting in the

disappearance of complex I (lane 2). The presence and intensity of complex II were not appreciably influenced by the addition of the anti-Jun or anti-Fos antibodies. Since the consensus sequences of CRE and AP-1 elements are quite similar (Nakabeppu Y *et al*., 1988), and since CREB and AP-1 proteins have been reported to recognize each other's binding sequences, anti-CREB antibodies were included in lane 4 to detect possible binding of CREB to the AP-1 probe. The intensity of neither complex I nor complex II appeared to be influenced by the presence of anti-CREB antibody, indicating that CREB protein did not recognize the AP-1 sequence under the assay conditions. This is also in agreement with results obtained in αT3-1 cells (White BR *et al*., 1999). Taken together, these results show that AP-1 proteins (both Jun and Fos family member proteins) from LβT2 cells can recognize the -336/-330 AP-1 element *in vitro*. These results therefore support earlier *in vivo* findings in LβT2 cells (Jeong KH *et al*., 2004), and also correlate with *in vitro* data obtained for αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a).

Previously, it had been established that the two nuclear receptor binding sites played a modulatory role in the response of the mouse GnRHR promoter to PKA in αT3-1 cells (see chapter 3). However, the primary promoter element involved in mediating this response was not identified in these cells, but it was hypothesized that the cAMP response element (CRE) would be a likely candidate. The role of the CRE in transcriptional regulation of the mouse GnRHR has not been investigated in pituitary gonadotrope cells, but this element has been demonstrated to play a role in both basal and GnRH-regulated transcriptional activity of the mouse GnRHR promoter in GGH_3 pituitary somatolactotrope cells (Maya-Nunez G and Conn PM 1999). When the binding of LβT2 nuclear proteins to a radiolabelled oligonucleotide probe containing the mouse GnRHR CRE was investigated *in vitro,* one major complex (complex I) and two minor complexes (II and III) were observed (figure 4.6B, lane 1). As already mentioned above, CREB and AP-1 proteins have been reported to recognize each other's binding sites, and therefore antibodies against CREB and AP-1 proteins were used to establish the identity of the proteins in these complexes. Inclusion of anti-CREB (figure 4.6B, lane 2) caused a clear supershift of complex I, while anti-Jun (lane 3) and anti-Fos (lane 4) had no effect on this complex. The intensity of complex III was diminished by the specific antibodies (lanes 2 to 4) as well as by non-specific pre-immune serum (lane 5), indicating that this effect is non-specific. The intensity of complex II was not affected by any of the antisera included in the protein-DNA incubations. These results show

that *in vitro,* CREB is the major transcription factor recognizing the mouse GnRHR promoter CRE, and that AP-1 proteins apparently do not recognize this element. This is in agreement with *in vitro* results obtained for the CREs from the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I promoters. In addition, the combined results presented in figure 4.6 show that CREB and AP-1 proteins from LβT2 cells specifically bind to their respective cognate promoter *cis* elements *in vitro*, and do not display cross-reactivity for each other's binding sequences.

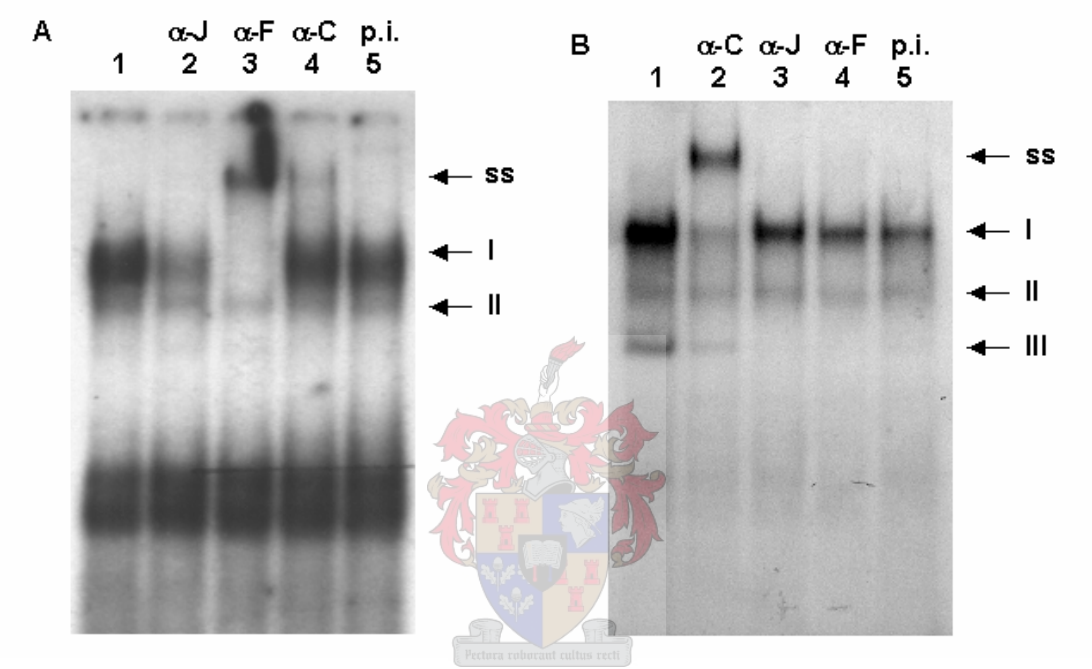


Figure 4.6 Binding of LβT2 nuclear proteins to the AP-1 element and the CRE *in vitro*. Autoradiographs of EMSAs showing binding of LβT2 nuclear proteins to radiolabeled AP-1 (A) and CRE (B) oligonucleotide probes. In lanes labelled "αJ" and "αF", 2 µg anti-Jun or anti-Fos antibodies were added, respectively. In lanes labelled "αC", 1 µl anti-CREB was added, and in lanes labelled "p.i.", 1 µl pre-immune serum was added. For (A), "ss" refers to the supershift with anti-Fos antibody in lane 3. For (B), "ss" refers to the supershift with anti-CREB antibody in lane 2.

4.3.2.2 The contribution of various *cis* elements to basal GnRHR promoter activity

In order to investigate the role of the abovementioned promoter elements in basal GnRHR promoter activity as well in the transcriptional response to GnRH, mutations (see Addendum B) were generated in these elements by PCR mutagenesis, within the context of the -579/+1 GnRHR promoter construct (see figure 4.7). Since it was found that the GnRH response of the GnRHR promoter involved PKA (figure 4.3), the role of the CRE was also investigated. The mutations introduced into the CRE and

AP-1 elements were previously shown by others (Pincas H *et al*., 2001b; White BR *et al*., 1999), and confirmed by us (not shown) by means of EMSAs to disrupt binding of the cognate transcription factors, CREB and AP-1. Cells were transfected with relevant wild-type or mutant promoter constructs (figure 4.7), and approximately 22 hours after transfection, cells were incubated in the absence or presence of 100 nM GnRH in serum-free medium for 8 hours before being harvested. The basal transcriptional activity of the mutant constructs, relative to that of the wild-type construct pLG, is graphically represented in figure 4.8. Mutation of the -15/-7NRS (construct pLGM1) had no significant effect on basal promoter activity in LβT2 cells (figure 4.8), in agreement with results obtained in αT3-1 cells (chapter 3, figures 3.5 and 3.6). Mutation of the -244/-236NRS (construct pLGM2) caused a modest decrease in promoter activity in LβT2 cells (figure 4.8); however, this decrease was found to not be statistically significant, even after several experiments. Previously, it was demonstrated that this site was essential for basal promoter activity in αT3-1 cells (chapter 3, figures 3.5 and 3.6) Other investigators have also indicated a role for the -244/-236NRS in basal GnRHR promoter activity in αT3-1 cells, with mutation of this site leading to a 60% decrease in promoter activity (Duval DL *et al*., 1997a, Duval DL *et al*., 1997b). The results presented in figure 4.8 suggest that the -244/-236NRS is involved in basal GnRHR promoter activity in LβT2 cells, but that the decrease in promoter activity observed with the pLGM2 promoter-luciferase construct is too small, relative to the experimental error and/or variability, to establish statistical significance. Simultaneous mutation of the two NRS elements (construct pLGM1+2) did not have any additive or synergistic effects on basal promoter activity.

Promoter activity of the pLGmAP1 construct in LβT2 cells was not significantly different to that of the wild-type pLG (figure 4.8), as was also found by Norwitz *et al.* in αT3-1 cells (Norwitz ER *et al*., 1999a). However, this is in contrast with earlier results obtained by others, showing that AP-1 forms part of a tripartite basal transcriptional enhancer, and that mutation of the AP-1 site leads to a marked decrease in basal promoter activity in αT3-1 cells (Duval DL *et al*., 1997b; White BR *et al*., 1999), suggesting that basal GnRHR promoter activity possibly involves different mechanisms in αT3-1 cells as compared to LβT2 cells. In addition, the corresponding AP-1 element in the rat GnRHR I promoter (-352/-346) was found to be involved in basal promoter activity in both αT3-1 and LβT2 cells (Pincas H *et al*., 2001a), pointing to species-specific differences in maintaining basal GnRHR expression. Mutation of the CRE resulted in a dramatic reduction of basal GnRHR promoter activity in LβT2 cells

(figure 4.8), with resulting luciferase expression levels similar to those of the promoterless pGL2-basic vector. The finding that the CRE is essential for basal GnRHR promoter was unexpected, since the results presented in figure 4.3 clearly demonstrated that PKA activity is not required for basal promoter activity. Previously, it was found that mutation of the CRE caused a decrease, but not a complete loss, of mouse GnRHR promoter activity in GGH_3 somatolactotrope cells (Maya-Nunez G and Conn PM 1999). Nevertheless, the results presented in figure 4.8 clearly show that the CRE is indispensable for basal GnRHR promoter activity in LβT2 cells, with possible minor contributions from the -244/-236NRS, as well as possibly other *cis* elements not investigated here.

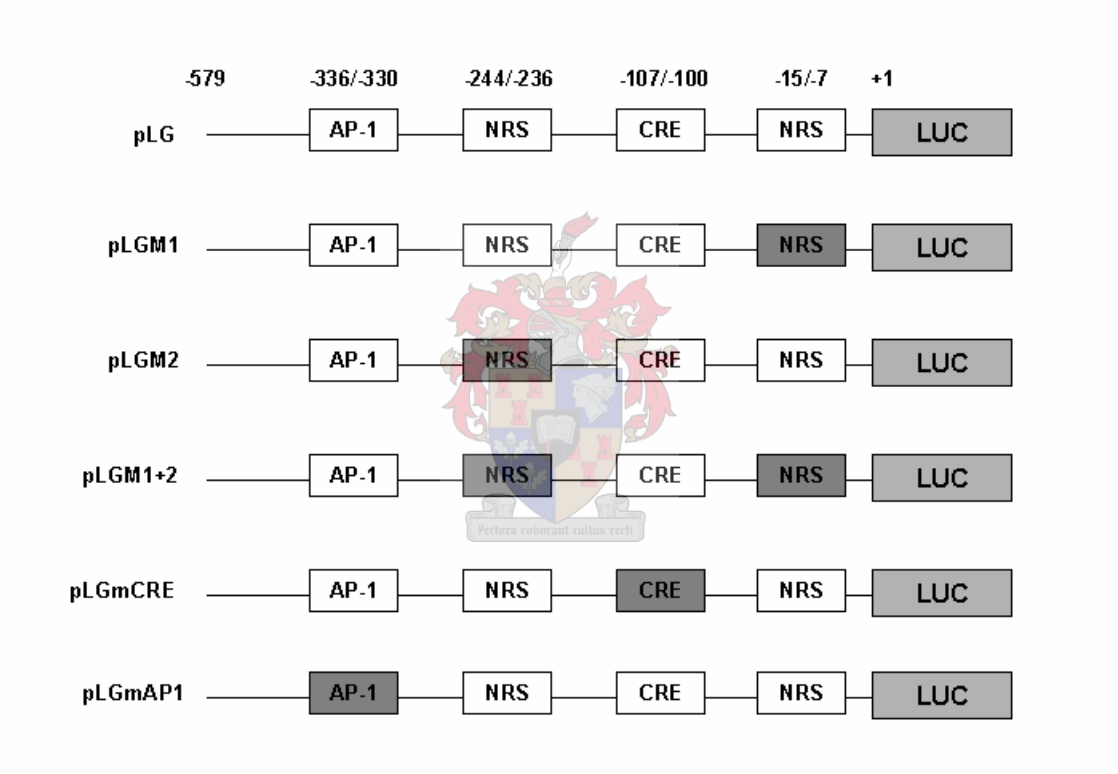


Figure 4.7: Schematic of mouse GnRHR promoter-luciferase constructs. Transcription factor binding sites are indicated as boxes, with positions relative to translation start site indicated at the top. Dark boxes represent sites that have been mutated (see Addendum B for mutated sequences). Note that this figure is not drawn to scale.

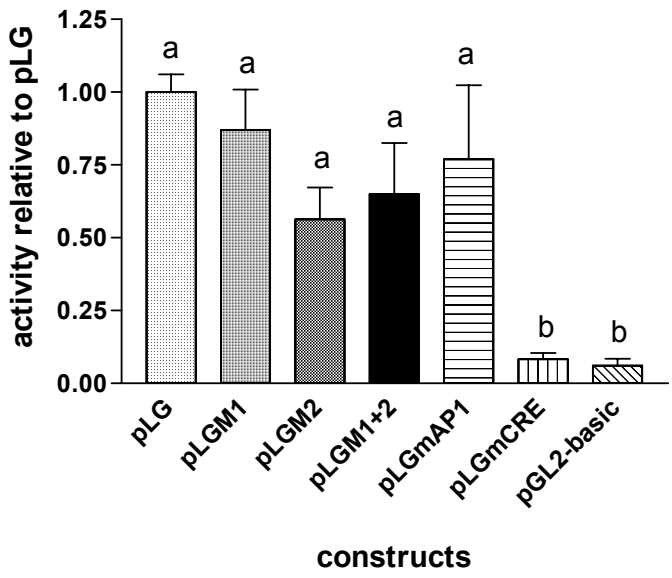


Figure 4.8 The contribution of different *cis* elements to basal GnRHR promoter activity. LβT2 cells were transfected with 100 ng of wild-type or mutant GnRHR promoter constructs, as indicated below the relevant bars, in the presence of 10% FCS. After approximately 24 hours, medium was replaced, and cells were incubated for 8 hours in serum-free medium, Luciferase activity of untreated pLG was set as 1. Graph shown combined results of at least 4 experiments, all performed in triplicate. ** = P < 0.01.

Construct	Activity relative to pLG	Std deviation	n	P value (vs pLG)
pLG	1.000	0.150	6	
pLGM1	0.870	0.341	6	> 0.05
pLGM2	0.563	0.269	6	> 0.05
pLGM1+2	0.650	0.352	4	> 0.05
pLGmAP-1	0.769	0.510	5	> 0.05
pLGmCRE	**0.083**	**0.049**	**5**	**< 0.01**
Inhibitor	**Effect on basal pLG activity**			
H89	0.747	0.213	3	> 0.05
PKI	1.417	0.257	2	> 0.05
BIM	0.966	0.032	2	> 0.05

Table 4.1 Summary of results obtained for basal activity of mutant promoter constructs relative to wild-type promoter activity, and wild-type promoter activity in the presence of kinase inhibitors.

4.3.2.3 The involvement of various *cis* elements in responsiveness to GnRH and forskolin

When the transcriptional response of these mutant constructs to GnRH was investigated, it was observed that binding of SF-1 to the two nuclear receptor binding sites was not required for this response, either individually or in combination with each other (figure 4.9A). Mutation of the CRE markedly decreased the transcriptional response to GnRH (figure 4.9A). Similarly, mutation of the AP-1 site caused a significant decrease in the GnRH response of the GnRHR promoter-luciferase construct in LβT2 cells (figure 4.9A). In αT3-1 cells, mutation of the AP-1 element was found to result in a complete loss of GnRH responsiveness of GnRHR promoter-luciferase constructs (Norwitz ER *et al*., 1999a; White BR *et al*., 1999), correlating with observations made in transgenic mice harbouring a mouse GnRHR promoter-luciferase construct (Ellsworth BS *et al*., 2003b), indicating that in these systems a functional AP-1 site is the sole requirement for the transcriptional response to GnRH. However, from the results presented in figure 4.9A and table 4.2, it appears that both the pLGmCRE and pLGmAP1 promoter-reporter constructs retained some responsiveness to GnRH in LβT2 cells (approximately 60% and 35%, respectively) although these responses were not statistically significant. These results indicate that both these elements contribute to the full transcriptional response to GnRH in LβT2 cells.

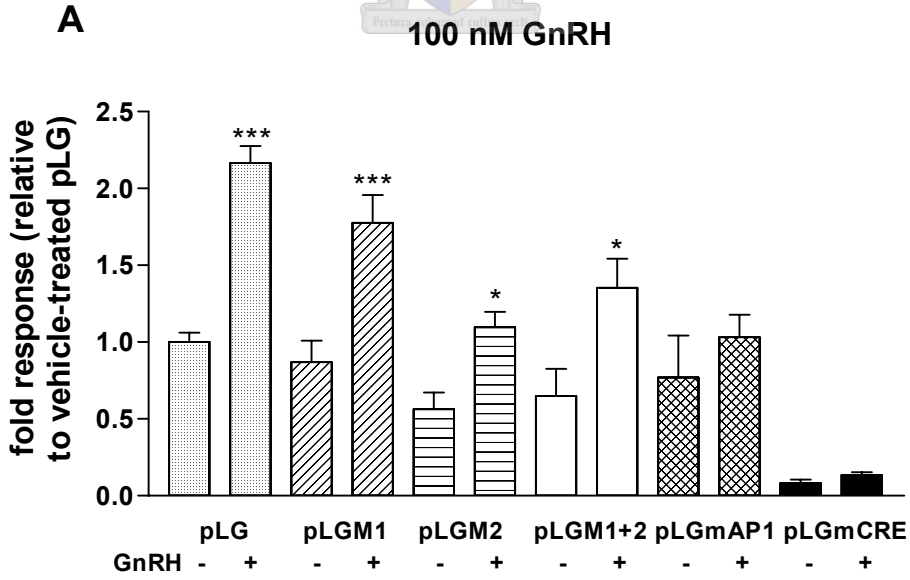


Figure 4.9 (continued on next page)

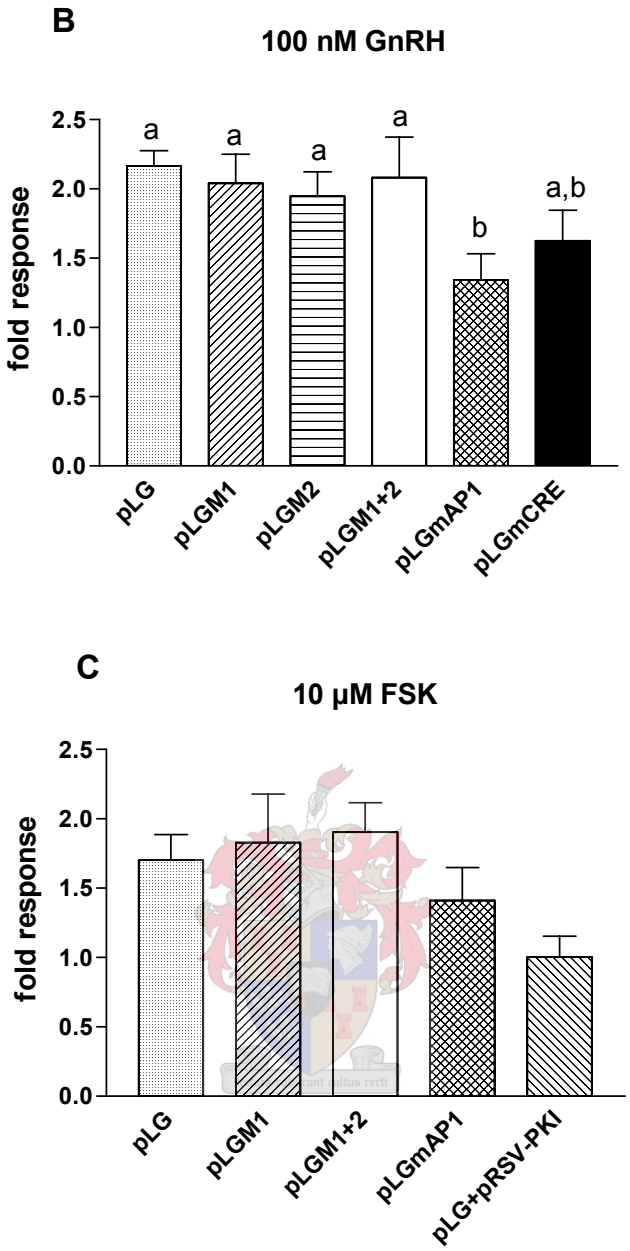


Figure 4.9 The role of different *cis* elements in GnRHR promoter responsiveness to GnRH and forskolin. LβT2 cells were transfected with 100ng of wild-type or mutant GnRHR promoter constructs, as indicated below the relevant bars, in the presence of 10% FCS. After approximately 24 hours, medium was replaced, and cells were incubated for 8 hours in serum-free medium containing 100 nM GnRH (A and B) or 10 µM forskolin (FSK) (C). (A) Graph showing transcriptional responses to GnRH of individual promoter constructs, relative to vehicle-treated pLG. For each construct (vehicle-treated and GnRH-treated), the luciferase activity was calculated relative to vehicle-treated pLG, which was set as 1. For each construct, statistically significant differences were determined for the GRH-induced increase in activity relative to the vehicle-treated control (ie pLGM1 plus GnRH vs pLGM1 minus GnRH). (B) Graph showing actual GnRH responses of individual promoter constructs. For each construct, the luciferase activity of the vehicle-treated control was set as 1. Statistically significant differences were determined relative to the GnRH response of pLG. (C) Graph showing actual FSK

responses of individual promoter constructs. For the bar labelled “pLG+pRSV-PKI”, 5 ng of the PKI expression construct was co-transfected together with pLG. For each construct, the luciferase activity of the vehicle-treated control was set as 1. Statistically significant differences were determined relative to the GnRH response of pLG. Graphs show combined results of at least 3 experiments, all done in triplicate. * = P < 0.05; *** = P < 0.001; a vs b = P < 0.05.

Construct	GnRH response	Standard deviation; n value	FSK response	Standard deviation; n value
pLG	2.166	0.270; n = 6	1.704	0.446; n = 6
pLGM1	2.041	0.513; n = 6	1.828	0.699; n = 4
pLGM2	1.946	0.436; n = 6	ND	ND
pLGM1+2	2.083	0.582; n = 4	1.904	0.473; n = 5
pLGmAP1	1.342	0.426; n = 5	1.411	0.585; n = 6
pLGmCRE	1.625	0.494; n = 5	ND	ND
pLG + pRSV-PKI	0.974	0.365; n = 3	1.004	0.259; n = 3

Table 4.2: Summary of results obtained for responses of wild-type and mutant GnRHR promoter constructs to GnRH or forskolin treatment (responses calculated as fold induction over unstimulated construct). (ND = not determined).

The transcriptional response of the pLG GnRHR promoter-luciferase construct to forskolin in LβT2 cells (figure 4.9B) was weak (1.6- to 2-fold), and not reproducible between different experiments. In αT3-1 cells, it was observed that pLGM1 had a stronger response to forskolin than pLG (figure 3.5B), but this effect was not observed in LβT2 cells (figure 4.9B). Mutation of both the -15/-7NRS and the -244/-236NRS did not have any effect on the magnitude of the forskolin response in LβT2 cells (figure 4.9B), similar to what was observed in αT3-1 cells (figure 3.5). Mutation of the AP-1 element resulted in a modest reduction of the FSK response in LβT2 cells (figure 4.9B), and overexpression of the PKA inhibitory peptide PKI resulted in a similar reduction. However, since the magnitude of the FSK responses varied considerably between individual experiments, the reduced responses observed with pLGmAP-1, or with overexpressed PKI, were found not to be statistically significant, even after several repeat experiments, and it would therefore be inappropriate to draw any conclusions from these data.

4.3.3 Mechanism(s) of transcriptional activation by GnRH

4.3.3.1 GnRH stimulates binding of phosphorylated CREB to the GnRHR promoter CRE *in vitro,* and induces a direct interaction between CREB and SF-1

The results presented in figures 4.8 and 4.9A demonstrate that the CRE is essential for basal GnRHR promoter-reporter activity, and indicate that the CRE contributes to the transcriptional response to GnRH. However, while mutation of the CRE appeared to result in partial loss of the GnRH response, due to the extremely low basal activity of the pLGmCRE mutant, the extent of the loss could not be firmly established, even with multiple experiments. Thus another approach was employed to investigate a role for CREB and the CRE in the GnRH response. It is well established that phosphorylation of CREB on serine-133 induces its transactivation potential, and that phospho-CREB is considered to be the transcriptionally active form of CREB (Montminy M, 1997). Originally, PKA was identified as the intracellular effector inducing this activation, but several other kinases have since been demonstrated to also phosphorylate CREB on serine-133 (Mayr B and Montminy M, 2001; Johannessen M *et al*., 2004). The ability of GnRH to induce phosphorylation of this residue was measured by investigating the binding of phospho-CREB to the CRE probe, in response to GnRH stimulation. Previously, studies by Hagiwara M *et al.* indicated that CREB phosphorylation by PKA reaches a maximum after 30 minutes (Hagiwara M *et al.,* 1993), and for this reason the 30 minute time-point was chosen for the present experiments. LβT2 cells were treated with GnRH for 30 minutes, after which nuclear extracts were prepared. These extracts were used in an EMSA (figure 4.10A), in the absence or presence of antibodies against CREB and phospho-CREB (phosphorylated at Ser-133). For each sample, the intensities of the respective supershifted bands (figure 4.10A, complexes labelled “ss”) were quantified and were expressed as the ratio between phospho-CREB and total CREB (figure 4.10B). GnRH treatment clearly caused an increase in the binding of phospho-CREB to the CRE (figure 4.10A, lanes 5 and 6, compared to lanes 2 and 3). Both the PKA inhibitor H89, as well as the PKC inhibitor BIM partially inhibited the GnRH-induced phosphorylation of CREB (figure 4.10A, lanes 8 and 9, and lanes 11 and 12, also see figure 4.10B). Although the differences in CREB phosphorylation were not statistically significant, the effects of the different compounds on CREB phosphorylation were reproducible (figure 4.10B). This result is consistent with the hypothesis that the transcriptional effects of GnRH on GnRHR promoter activity involve increased phosphorylation of CREB and increased binding of phospho-CREB to the CRE in the GnRHR promoter, and supports

the role of the CRE in the GnRH response, as indicated in figure 4.9A. Furthermore, the finding that both PKA and PKC are involved in increased binding of phospho-CREB to the CRE *in vitro* (figure 4.10) is consistent with the results presented in figures 4.3 and 4.4, showing that both PKA and PKC are involved in the GnRH-mediated upregulation of GnRHR transcription.

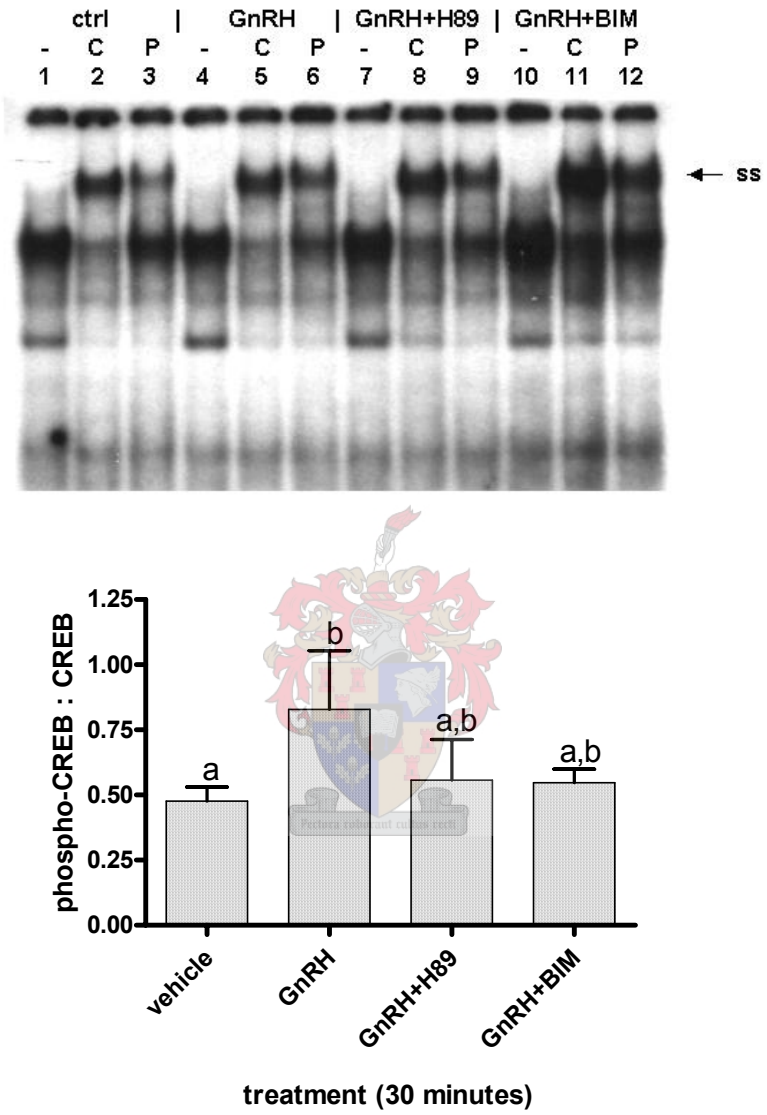


Figure 4.10: GnRH stimulates binding of phospho-CREB to the mouse GnRHR promoter CRE in vitro. Autoradiograph of an EMSA showing the binding of CREB and phospho-CREB to a radiolabelled oligonucleotide probe representing the CRE in the mouse GnRHR promoter. Nuclear extracts were prepared from LβT2 cells treated for 30 minutes with vehicle (lanes 1-3), 100 nM GnRH (lanes 4-6), 100 nM GnRH plus 1 μM H89 (lanes 7-9) or 100 nM GnRH plus 100 nM BIM (lanes 10-12), all in serum-free medium. For each sample, the first lane contained no antibodies (-), the second lane contained 1 μl anti-CREB (C) and the third lane contained 1 μl anti-phospho-CREB (Ser-133) (P). Supershifted complexes are indicated by "ss". The autoradiograph shows a representative experiment. For each condition, 1.5 μl of nuclear extract was used per incubation, corresponding to total protein of

0.67 µg for lanes 1-3, 0.69 µg for lanes 4-6, 0.69 µg for lanes 7-9 and 0.71 µg for lanes 10-12. Supershifted complexes were quantified, and results were expressed as a ratio between the intensity of the phospho-CREB and CREB complexes for each condition. The graph shows the combined results of 2 experiments. a vs b = $P < 0.05$.

Since GnRH treatment resulted in an increase of phospho-CREB binding to the mouse GnRHR promoter CRE (figure 4.10), it was also of interest to determine whether GnRH stimulated interactions between phospho-CREB and other transcription factors. As described in chapter 3, the distal SF-1 binding site at -244/-236 in the mouse GnRHR promoter was found to enhance the PKA response of this promoter in αT3-1 cells (figure 3.6). Although the primary promoter element mediating the PKA response was not identified, the CRE was regarded as a likely candidate. Similarly, the maximal response of the rat GnRHR I promoter to PACAP and PKA in αT3-1 cells depends on SF-1 and CREB binding to sites corresponding to the -244/-236NRS and the CRE in the mouse promoter described above (Pincas H *et al*., 2001b). In addition, several components of the steroidogenesis pathway, such as the steroidogenic acute regulatory protein (StAR) (Manna P *et al*., 2003) and aromatase enzyme (Carlone DL and Richards JS, 1997) genes are regulated by SF-1 and CREB in a cooperative fashion. For these reasons, whether GnRH stimulated a functional interaction between SF-1 and CREB in LβT2 cells was investigated with mammalian two-hybrid assays, using an artificial luciferase reporter containing five GAL4 binding sites. LβT2 cells were transfected with this reporter, along with the expression vectors pBIND-SF-1 (SF-1 fused to the GAL4 DNA binding domain) and pACT-CREB (CREB fused to the VP16 activation domain). In figure 4.11 it can be seen that GnRH treatment caused an interaction between SF-1 and CREB that could be at least partially inhibited by the PKC inhibitor BIM, while the PKA inhibitor H89 had no effect on this interaction. Interestingly, mutation of the crucial serine-133 residue in CREB did not inhibit this interaction, indicating that phosphorylation of CREB on this residue was not required for the interaction with SF-1. The specificity of the SF-1-CREB interaction was confirmed by the fact that GnRH did not induce an interaction between the empty pBIND and pACT vectors (see last two bars of figure 4.11). However, the effect of GnRH on either pBIND-SF-1 or pACT-CREB alone was not tested in these experiments.

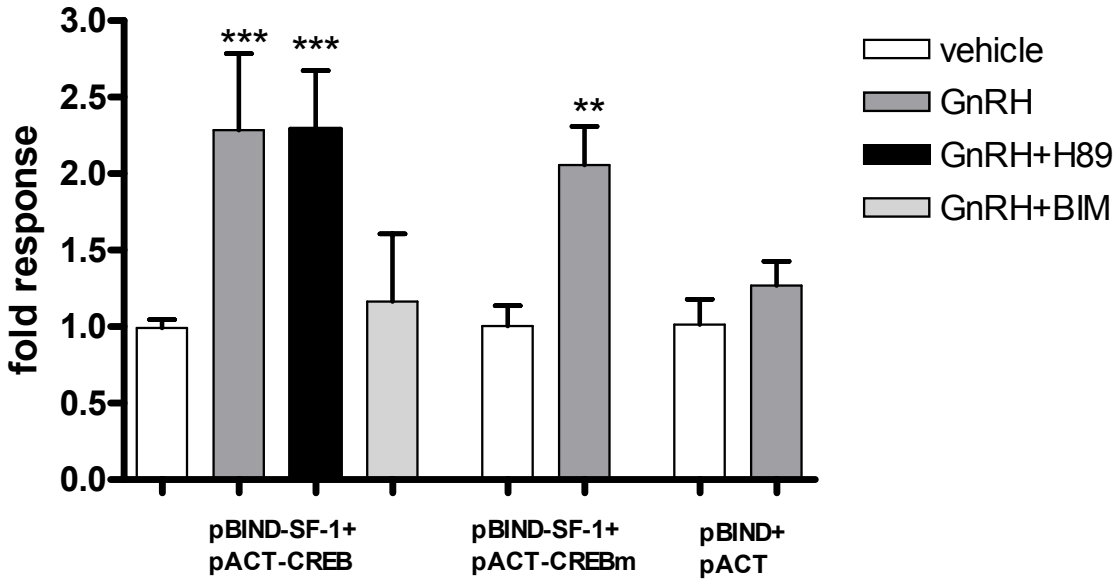


Figure 4.11: GnRH induces a direct interaction between SF-1 and CREB. LβT2 cells were transfected with 400 ng of pG5-LUC reporter, together with 5 ng pBIND-SF-1 and either pACT-CREB or pACT-CREBm, as indicated below the relevant bars, in the presence of 10% FCS. Approximately 22 hours after transfection, medium was replaced and cells were incubated in serum-free medium containing GnRH for 8 hours, in the absence or presence of kinase inhibitors, as indicated in the legend. (Concentrations of compounds were as described for other figures: 100 nM GnRH, 1 μM H89 and 100 nM BIM.) For each condition, as indicated below the X-axis, the luciferase value of the vehicle-treated samples were set as 1, and interactions were quantified relative to the control for each condition. Graph shows combined results of 3 experiments, performed in triplicate. *** = $P < 0.001$, relative to vehicle-treated pBIND-SF-1 + pACT-CREB; ** = $P < 0.01$, relative to vehicle-treated pBIND-SF-1 + pACT-CREBm.

4.3.3.2 GnRH decreases SF-1 protein expression and *in vitro* binding to both nuclear receptor binding sites in the mouse GnRHR promoter

The combined results of figures 4.8 and 4.9 indicate that SF-1 binding to the nuclear receptor binding sites at -15/-7 and -244/-236 is not required for basal or GnRH-stimulated GnRHR promoter activity in LβT2 cells. However, since Nur77 protein can recognize the mutated nuclear receptor binding sequences *in vitro*, it is possible that *in vivo*, transcriptional regulation by GnRH involves the action of Nur77, via one or both these sites. In addition, the results obtained from the two-hybrid experiments (figure 4.11) clearly demonstrated that GnRH induced a functional interaction between CREB and SF-1, pointing to a possible role for SF-1 in the transcriptional response to GnRH. For these reasons,

nuclear extracts from GnRH-treated LβT2 cells were analysed by EMSA for their binding activity to the -15/-7NRS and -244/-236NRS probes.

From figure 4.12A it can be seen that GnRH treatment (8 hours) caused a decrease in SF-1 (lower complex) binding to the -15/-7NRS probe (figure 4.12A, compare lanes 1 and 2), and this decrease was reversed by the PKC inhibitor BIM (compare lanes 2 and 4). The effect of the PKA inhibitor H89 on the GnRH-mediated reduction in SF-1 binding to this probe varied between experiments, displaying either a slight inhibition of the GnRH effect (typical result shown in figure 4.12A, compare lanes 2 and 3), or no effect at all (result not shown). GnRH had no clear reproducible effect on the binding of Nur77 (upper complex) to this site *in vitro.*

Similarly, GnRH treatment caused a decrease of SF-1 binding to the -244/236NRS (figure 4.12, panel B1, compare lanes 1 and 2). Once again, this effect could be reversed by the PKC inhibitor (compare lanes 2 and 4), while the PKA inhibitor did not have a substantial and reproducible effect (compare lanes 2 and 3). Since the complex formed between Nur77 and this oligonucleotide probe was of such a low intensity (also see figure 4.5), the autoradiographs of these experiments had to be overexposed in order to properly visualize this complex (figure 4.12, panel B2). Similar to what was observed for the -15/-7NRS, GnRH treatment did not appear to have any reproducible effect on Nur77 binding (upper complex) to the -244/-236NRS *in vitro*. The effects of the kinase inhibitors alone on the binding of SF-1 and Nur77 to the nuclear receptor binding site probes were not investigated in these experiments.

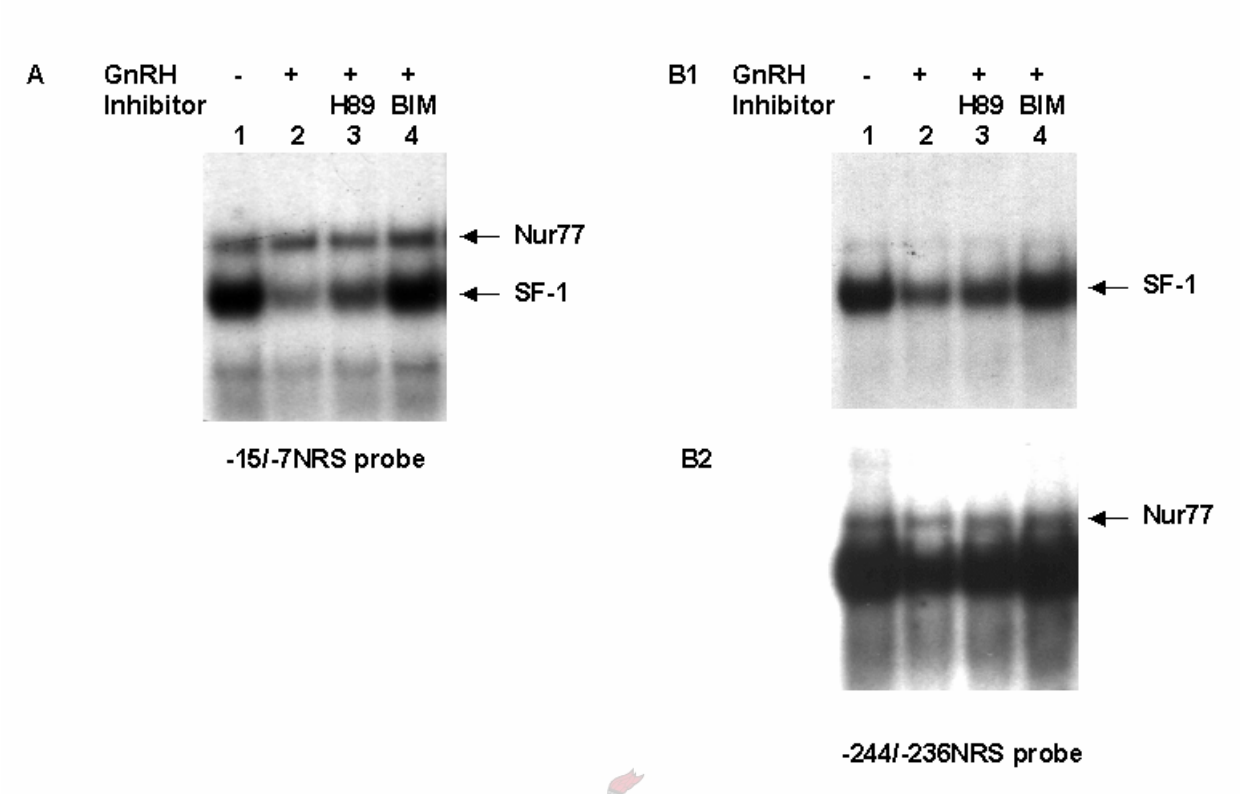


Figure 4.12 The effect of GnRH treatment on *in vitro* binding of LβT2 nuclear proteins to -15/-7NRS (A) and -244/-236NRS (B) probes. LβT2 cells were grown for 48 in medium containing 10% FCS, after which medium was replaced with serum-free medium and cells were treated for 8 hours as described. For each experiment, lane 1 contained nuclear extracts from vehicle-treated cells (-), while lanes 2 to 4 contained extracts from cells stimulated with 100 nM GnRH in the absence (lane 2) or presence of 1 μM H89 (lane 3) or 100 nM BIM (lane 4), as indicated above the autoradiographs. Panels B1 and B2 respectively show shorter and longer exposures of the same experiment. Experiments were repeated four times; one representative experiment is shown for each probe.

Taken together, the results presented in figure 4.12 showed that 8 hours of GnRH treatment resulted in decreased binding of SF-1 protein to both the -15/-7NRS and the -244/-236NRS *in vitro*. It was therefore of interest to determine whether this reduced binding activity was due to decreased levels of SF-1 protein expressed in LβT2 cells, or due to post-translational modification of SF-1 in response to GnRH. Western blot analysis on nuclear extracts from these cells confirmed that SF-1 protein expression was decreased after 8 hours of GnRH treatment (figure 4.13). In agreement with results obtained for *in vitro* binding to the two nuclear receptor sites, this downregulation of SF-1 protein expression could be at least partially reversed by the PKC inhibitor, but not by the PKA inhibitor. Earlier findings demonstrated that GnRH pulses increased pituitary GnRHR mRNA levels in female rats (Haisenleder DJ *et al.,* 1996), and the present results are apparently at odds with these findings.

However, since GnRH pulses *in vivo* affect the general hormonal milieu, it is possible that the effects on pituitary GnRHR mRNA levels *in vivo* could be due to indirect effects, and that might explain the apparent inconsistency between results obtained by Haisenleder DJ *et al.* and results obtained in the current system.

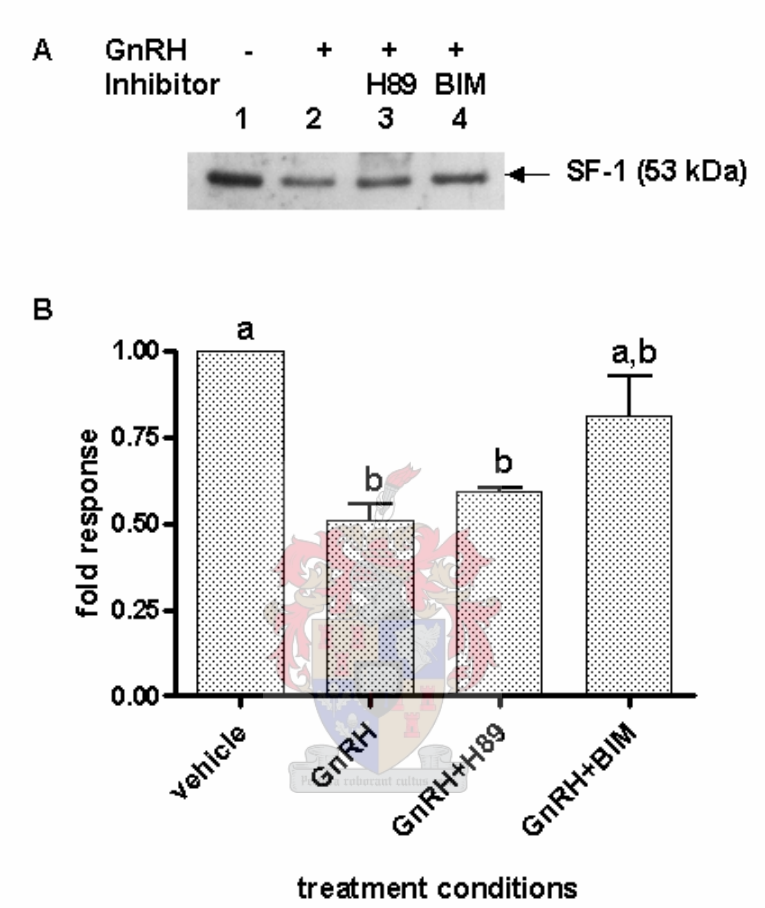


Figure 4.13 The effect of GnRH treatment on SF-1 protein levels in LβT2 cells. (A) Nuclear proteins from LβT2 cells, as prepared for figure 4.12, were separated on a 10% SDS-PAGE gel, blotted onto a nitrocellulose membrane and probed with anti-SF-1 antiserum (1:2500). Lane 1 contained extracts from vehicle-treated cells (-). In lanes 2 to 4, cells had been stimulated with 100 nM GnRH for 8 hours, in the absence (lane 2) or presence of 1 μM H89 (lane 3) or 100 nM BIM (lane 4), as indicated above the autoradiograph. (B) Quantification of SF-1 protein levels in LβT2 nuclear extracts, in the absence and presence of GnRH treatment. Experimental conditions were as for (A). Graph shows combined results of two experiments, performed as single incubations.

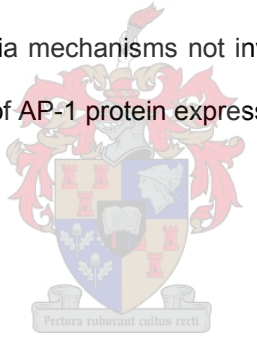
4.3.3.3 GnRH increases AP-1 protein expression levels and binding to the GnRHR promoter AP-1 site *in vitro*

As already mentioned in section 4.3.2.1, when the binding of AP-1 proteins to the AP-1 site of the mouse GnRHR promoter was investigated *in vitro*, nuclear extracts from unstimulated LβT2 cells displayed almost no AP-1 binding activity, and GnRH treatment was required to induce binding of AP-1 proteins *in vitro.* This is in contrast to what was found in LβT2 cells *in vivo*, where it was demonstrated by means of chromatin immunoprecipitation (ChIP) assays that c-Jun occupied a region of the mouse GnRHR promoter encompassing the AP-1 binding site, even in unstimulated cells (Jeong KH *et al*., 2004). In light of the fact that the AP-1 element was clearly required for the transcriptional response of the mouse GnRHR promoter to GnRH in LβT2 cells (figure 4.9), the effects of the PKA and PKC pathway inhibitors on the GnRH-induced AP-1 binding were determined. In figure 4.14, the induction of AP-1 protein binding to the AP-1 element *in vitro* after 8 hours of GnRH treatment can be clearly observed (compare lanes 1 and 2). Interestingly, the PKA and PKC pathway inhibitors did not inhibit this GnRH-induced increase in protein binding to the AP-1 element (figure 4.14, compare lanes 3 and 4 to lane 2). These results clearly demonstrate that GnRH strongly induces binding of AP-1 proteins from LβT2 cells to the GnRHR promoter AP-1 element, but that neither PKA nor PKC is required for this effect. An increase in the intensity of the AP-1 complex was sometimes observed in the presence of H89 (figure 4.14A, lane 3), but this increase was not always reproducible between experiments. Nevertheless, in two repeat experiments, the induction of AP-1 protein expression and binding to the AP-1 probe was not inhibited by H89 or by BIM.

In order to investigate whether the GnRH-mediated increase in AP-1 binding was the result of increased AP-1 protein levels, Western blot analyses were performed on the same nuclear extract samples used in the EMSA in figure 4.14A. In figure 4.14B, an anti-Jun antibody, specific for all Jun family members, was used. Based on their primary amino acid sequence, all three Jun proteins (c-Jun, JunB and JunD) have a size of approximately 40 kDa. In figure 4.14B, it can be seen that GnRH induced the expression of a ~40 kDa protein that was undetectable in extracts from unstimulated cells (figure 4.14B, compare lanes 1 and 2). This result is consistent with the observation that nuclear extracts from unstimulated LβT2 cells had virtually undetectable AP-1 binding activity (figure 4.14A), and indicates that the GnRH-induced increase in AP-1 protein binding to the AP-1 site is most likely

due to the induction of AP-1 protein expression by GnRH. The presence of the PKA or PKC inhibitors did not inhibit the induction of AP-1 protein expression by GnRH (figure 4.14B, compare lanes 3 and 4 with lane 2), indicating that this induction occurs via a mechanism that does not involve these kinases. This result is also consistent with the *in vitro* binding results in figure 4.14A, demonstrating that these inhibitors did not inhibit the GnRH-induced increase in AP-1 protein binding to the AP-1 site.

Similarly, in figure 4.14C it can be seen that GnRH treatment strongly induced the expression of Fos proteins that were undetectable in unstimulated cells (figure 4.14C, compare lanes 1 and 2). Note that, in figure 4.14C, two protein bands can be seen, which were in the approximate range of 55 – 80 kDa. Since the anti-Fos antibody used in this experiment does not discriminate between different Fos family members, it is likely that these bands represent different Fos proteins. Once again, the PKA and PKC inhibitors did not inhibit the GnRH-mediated induction of Fos protein expression (figure 4.14C, compare lanes 3 and 4 with lane 2), providing additional evidence that GnRH induces the expression of Jun and Fos proteins in LβT2 cells via mechanisms not involving PKA or PKC. The effects of the kinase inhibitors alone on the induction of AP-1 protein expression and binding to the AP-1 probe were not investigated in these experiments.



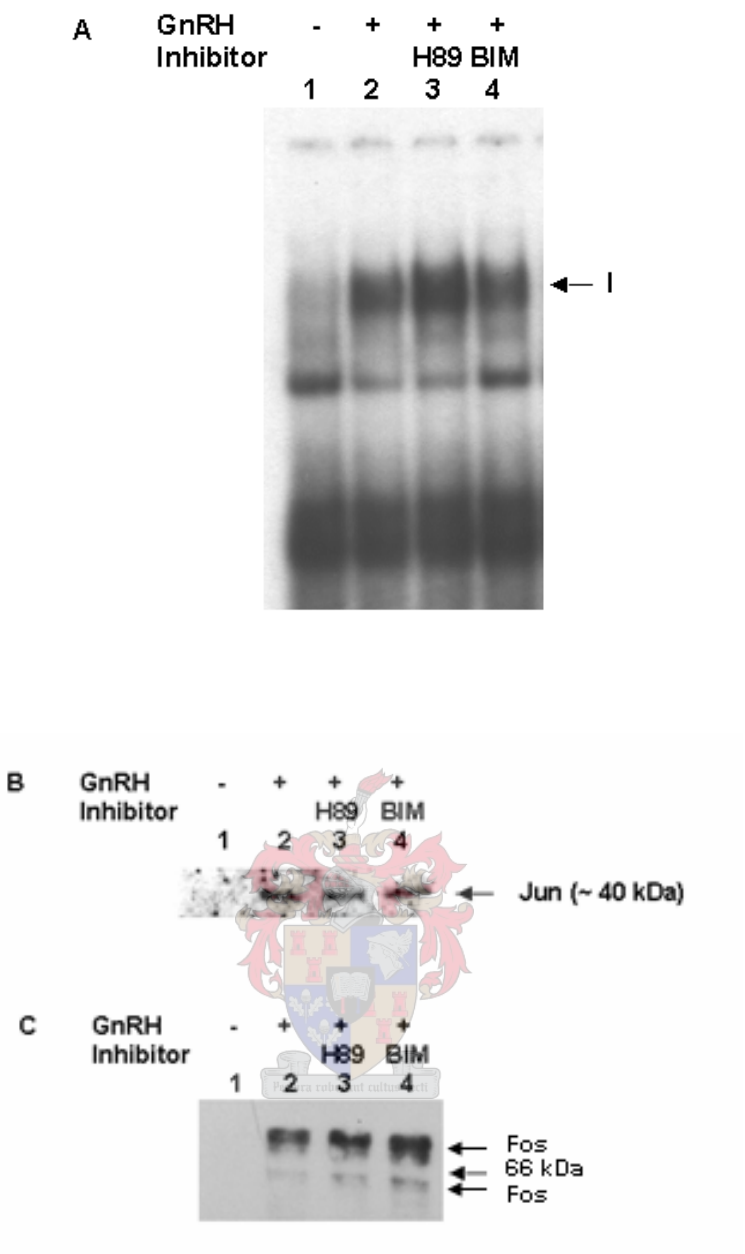


Figure 4.14 Effect of GnRH treatment on AP-1 protein expression and binding activity in LβT2 cells. LβT2 cells were grown up and treated as described in figure 4.12, before nuclear extracts were prepared. (A) Autoradiograph of EMSA performed with LβT2 nuclear extracts and the radiolabelled AP-1 oligoncleotide probe. Lane 1 contained extracts from vehicle-treated (-) cells. In lanes 2 to 4, cells were treated with 100 nM GnRH for 8 hours, in the absence or presence of kinase inhibitors, as indicated above the autoradiograph. Concentrations of inhibitors added were as described for other figures. Complex I, as denoted in figure 4.6, is indicated by an arrow. (B+C) Western blot analyses of LβT2 nuclear extracts. Proteins, as prepared for (A), were separated on a 10% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane and probed with anti-Jun (1:3000) (B) or anti-Fos (1:3000) (C) for 1 hour at room temperature. All other steps were performed as described under Materials and Methods. For (C), the 66 kDa molecular weight marker is indicated.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Regulation of pituitary GnRHR numbers plays a central role in the control of mammalian reproductive function, with the responsiveness of the pituitary gonadotropes to GnRH correlating directly with the numbers of GnRHRs on the gonadotrope cell surface (Conn PM *et al*., 1995). Pituitary GnRHR numbers are regulated at both transcriptional and post-transcriptional levels. The aim of this project was to investigate particular aspects of the transcriptional regulation of GnRHR expression in the αT3-1 and LβT2 gonadotrope cell lines. In αT3-1 cells, the mechanism of regulation of GnRHR transcription by PKA was investigated. The results obtained in this cell line were subsequently followed up by investigating the role of PKA and PKC in the GnRH-mediated regulation of GnRHR transcription in LβT2 cells.

5.1 Transcriptional regulation of GnRHR gene expression by GnRH, forskolin and PACAP in αT3-1 cells and LβT2 cells

Whether transcriptional regulation of GnRHR expression occurs by its homologous hormone in gonadotrope cell lines is somewhat controversial. Earlier studies in αT3-1 cells by the group of Sealfon (Tsutsumi M *et al*., 1993; Tsutsumi *et al*., 1995) found that GnRH regulated GnRHR numbers, but not mRNA levels. However, a number of articles have subsequently been published, describing homologous regulation of GnRHR transcription in αT3-1 cells (Norwitz ER *et al*., 1999a; White BR *et al*., 1999), and in transgenic mice (McCue JM *et al*., 1997; Ellsworth BS *et al*., 2003b). In agreement with results published by White *et al.*, 1999 and Norwitz *et al.*, 1999, a -579/+1 GnRHR promoter-reporter construct (pLG) transfected into αT3-1 cells in the present study responded to continuous GnRH stimulation after 4 hours (figure 3.4), confirming that homologous regulation of GnRHR promoter activity does occur in these cells.

Transcriptional regulation of the GnRHR gene in LβT2 cells has not been well characterized. Only one study investigating homologous regulation of mouse GnRHR promoter activity in LβT2 cells has been published to date (Bedecarrats GY and Kaiser UB, 2003). These authors found that pulsatile, but not

continuous treatment with 10 nM GnRH increased the levels of functional GnRHR on the cell surface, but they concluded that this increase could not be fully explained by the modest stimulation of GnRHR promoter activity observed in the same study, and that post-transcriptional mechanisms were primarily responsible for the increased receptor numbers (Bedecarrats GY and Kaiser UB, 2003). In support of this, it has been demonstrated in both LβT2 (Nguyen KA *et al*., 2004) and αT3-1 cells (Sosnowski R *et al*., 2000) that GnRH can stimulate general levels of cap-dependent translation initiation. In the study by Nguyen *et al*., it was shown that continuous GnRH treatment increased LH synthesis and secretion by LβT2 cells after 4 hours, while the transcriptional activity of a transfected rat LHβ promoter-reporter construct remained unchanged, even after 6 hours of continuous GnRH treatment (Nguyen KA *et al*., 2004). It has been postulated that such non-transcriptional mechanisms could contribute to GnRH-mediated acute increases in LHβ subunit during the reproductive cycle (Nguyen KA *et al*., 2004), and it is quite likely that similar non-transcriptional mechanisms are involved in homologous regulation of GnRHR numbers in LβT2 cells.

Since the transcriptional effects of GnRH observed in the abovementioned study in LβT2 cells (Bedecarrats GY and Kaiser UB, 2003) were quite weak (maximum 2-fold increase in GnRHR promoter activity), it was necessary in the present study to optimize the transcriptional response of the transfected pLG construct to GnRH in LβT2 cells, with regards to culture conditions as well as duration of stimulation. The time-course results presented here (figure 4.2) demonstrate that the transcriptional activity of the pLG construct, transfected into LβT2 cells, was significantly upregulated in response to continuous GnRH administration, reaching a maximal response after 8 hours. Supporting the relevance of these results, obtained for a transfected promoter-reporter construct, endogenous GnRHR mRNA levels in these cells were also increased after 8 hours of continuous GnRH treatment (figure 4.4). Interestingly, the magnitude of the GnRH-mediated upregulation of endogenous GnRHR gene expression in LβT2 cells was often greater than the GnRH response of the transfected -579/+1 pLG construct (3- to 6-fold on mRNA level, compared to 2-fold with pLG; compare figures 4.3 and 4.4), suggesting that promoter regions upstream of -579 are involved in facilitating maximal transcriptional activation of the GnRHR gene by GnRH. This idea is supported by findings in transgenic mice, where immunoneutralization of GnRH resulted in approximately 7-fold lower expression levels of a -1900 GnRHR promoter-reporter transgene (Ellsworth BS *et al*., 2003b). However, the mouse GnRHR

promoter has not been characterized in detail upstream of -600 (see figure 1.4), and it is therefore not possible to speculate on which specific upstream promoter elements might be involved.

It is necessary to comment on why continuous GnRH administration was performed in the present study, whereas results obtained with pulsatile GnRH stimulation would have been physiologically more relevant (Fink G, 1988; Conn PM and Crowley WF, 1994; Kaiser UB *et al*., 1997a). In the study performed by Bedecarrats and Kaiser, which compared the effects of continuous and pulsatile GnRH administration on GnRHR numbers and GnRHR promoter activity in LβT2 cells, GnRH pulses were delivered to cultured cells by means of an automated pump system (Bedecarrats GY and Kaiser UB, 2003). Since this type of equipment was not available in the laboratory of the author, it was decided that GnRH would be administered continuously to LβT2 cells in the present study. The results in figure 4.3 and 4.4 show that the magnitude of the transcriptional responses to continuous GnRH administration in the present study (between 2- and 6-fold) is similar and may even be greater than that achieved with pulsatile GnRH stimulation (2-fold) by Bedecarrats and Kaiser (Bedecarrats GY and Kaiser UB, 2003). This results shows that under the conditions of the present study, endogenous GnRHR mRNA and promoter activity are both regulated by continuous treatment with GnRH in LβT2 cells, which enabled further investigation of the mechanisms involved.

It has been shown that, in αT3-1 cells, the GnRHR does not couple to the G_s G-protein, but exclusively couples to G_q (Grosse R *et al*., 2000), whereas in LβT2 cells, the GnRHR can couple to both G_s and G_q (Liu F *et al*., 2002b). It is therefore likely that GnRH binding to the GnRHR cannot activate PKA via G_s in αT3-1 cells, and this is consistent with the observations that GnRH does not increase cAMP levels in these cells (Horn F *et al*., 1991; Kanasaki H *et al*., 2002), and that the GnRH-mediated response of transfected mouse GnRHR promoter-reporter constructs in these cells involves PKC, but not PKA (White BR *et al*., 1999; Norwitz ER *et al*., 1999a). Consistent with these findings, results from the present study show that the transcriptional activity of the transfected pLG construct in αT3-1 cells was upregulated by GnRH after four hours, while four hours of forskolin stimulation had no effect (figure 3.4), suggesting that PKA is not involved in the GnRH response observed here. In contrast, from the results obtained for LβT2 cells in the present study, on both endogenous GnRHR mRNA levels as well as with the transfected pLG construct, it can be concluded that both PKA and

PKC are involved in homologous upregulation of the GnRHR gene in LβT2 cells (figures 4.3 and 4.4), consistent with the GnRHR coupling to both G_s and G_q (Liu F *et al*., 2002b). This result is novel and adds considerably to the understanding of the mechanism of homologous regulation of GnRHR transcription in the more differentiated LβT2 gonadotrope cell line, and is likely to be physiologically relevant.

In contrast to the finding that GnRH does not increase cAMP production in αT3-1 cells (Horn F *et al*., 1991; Kanasaki H *et al*., 2002), GnRH stimulation acutely elevates intracellular cAMP levels in LβT2 cells, consistent with the finding that the GnRHR couples to G_s G-protein in LβT2 cells (Liu F *et al*., 2002b). Earlier experiments in anterior pituitaries harvested from male rats also indicated that GnRH stimulated intracellular cAMP levels and gonadotropin secretion, specifically through the activation of adenylyl cyclase (Borgeat P *et al*., 1972), suggesting that LβT2 cells provide a more accurate representation of *in vivo* gonadotrope function than αT3-1 cells. However, Fowkes *et al.* demonstrated that, while forskolin treatment resulted in comparable increases in intracellular cAMP levels in both αT3-1 and LβT2 cells, a blunted transcriptional response to intracellular cAMP was observed in LβT2 cells, as compared to αT3-1 cells (Fowkes RC *et al*., 2003). It was found that both these cell lines expressed similar levels of the PKA type IIα catalytic subunit and the type Iα regulatory subunit, but that LβT2 cells also expressed the type IIα regulatory subunit, which was not detectable in αT3-1 cells. Moreover, the levels of this latter regulatory subunit tended to increase upon forskolin treatment, and it has therefore been postulated that LβT2 cells might require higher levels of intracellular cAMP to overcome the inhibitory effects of these additional regulatory subunits on the activity of the PKA catalytic subunit (Fowkes RC *et al*., 2003). Consistent with these ideas are results presented here, showing the upregulation of the major GnRHR mRNA transcript by 8-Br-cAMP and forskolin (figure 3.1) and the upregulation of pLG activity by forskolin (figure 3.4) in αT3-1 cells, while the response of pLG to forskolin in LβT2 cells is weak and non-reproducible (figure 4.9B).

The results presented in figure 3.4 show that mouse GnRHR promoter activity is upregulated by PACAP in αT3-1 cells, similar to results obtained for the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I promoters in these cells. The observation that both

forskolin and PACAP caused a significant increase in mouse GnRHR promoter activity in αT3-1 cells after 16 hours (figure 3.4) suggests that the transcriptional effects of PACAP are mediated via PKA, as was demonstrated for the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I promoters. However, unlike the case with forskolin, upregulation of mouse promoter activity by PACAP was also observed at earlier time-points (figure 3.4), indicating that additional pathways are involved in mediating the transcriptional effects of PACAP. Consistent with this idea, PACAP has been shown to activate both adenylyl cyclase and phospholipase C in αT3-1 cells (Schomerus E *et al*., 1994). Interestingly, although some results have been published showing PACAP-mediated upregulation of FSHβ mRNA levels and ovine FSHβ promoter activity in LβT2 cells (Fujii Y *et al*., 2002), a more recent study has demonstrated that the LβT2 cell line expresses such low levels of PACAP receptors that PACAP cannot actually elicit any intracellular response in these cells (Fowkes RC *et al*., 2003). This is consistent with the present observation that PACAP does not stimulate mouse GnRHR promoter activity in these cells, even after 12 hours (figure 4.2).

5.2 Comparison between αT3-1 and LβT2 cells as model systems for studying transcriptional regulation of GnRHR gene expression

In this study, basal GnRHR promoter activity, as well as the regulation of GnRHR promoter activity by GnRH, forskolin and PACAP was investigated in the αT3-1 and LβT2 gonadotrope cell lines. The -244/-236NRS was found to be crucial for basal GnRHR promoter activity in αT3-1 cells (figure 3.6), while this element does not appear to contribute significantly to basal promoter activity in LβT2 cells (figure 4.8). The CRE was found to be indispensable for basal GnRHR promoter activity in LβT2 cells (figure 4.8), and results suggested that the CRE is also involved in the transcriptional response to GnRH (figures 4.9, 4.10 and 4.11). These results are summarized in table 5.1.

	Basal		Stimulated	
Promoter *cis* element	**αT3-1**	**LβT2**	**αT3-1 (FSK/PKA)**	**LβT2 (GnRH)**
-15/-7NRS	No	No	No**	No
-244/-236NRS	Yes	?*	No**	No
CRE	ND	Yes	ND	Yes?***
AP-1	ND	No	ND	Yes

Table 5.1: Comparison of results obtained in the present study in αT3-1 and LβT2 cells, with regards to the respective roles of individual GnRHR promoter *cis* elements in basal and stimulated GnRHR promoter activity. "Yes" indicates where a *cis* element was found to be involved; "No" indicates where a *cis* element was found not to be required. ND = not determined; FSK = forskolin; NRS = nuclear receptor binding site; CRE = cyclic AMP response element; AP-1 = activator protein-1 binding site.
*Mutation of the -244/-236NRS did cause an apparent decrease in basal GnRHR promoter activity (figure 4.8), but the trend was not statistically significant.
**Although these *cis* elements are not required for the transcriptional responses to forskolin or PKA, they are involved in modulating the magnitude of the responses (figures 3.5 and 3.6).
***Results presented in figure 4.9 suggest that the pLGmCRE construct still responds to GnRH, but that the magnitude of the response decreases relative to the wild type construct. However, due to the low transcriptional activity of this construct, relative to experimental error and variability, it was not possible to determine with confidence, even with several repeat experiments, the extent of this apparent decrease in the GnRH response.

GnRH was found to upregulate GnRHR transcription in both cell lines (figures 3.4, 4.3 and 4.4). In αT3-1 cells, GnRH-mediated upregulation of GnRHR transcription is PKC-dependent and PKA-independent (White BR *et al*., 1999; Norwitz ER *et al*., 1999a), whereas in LβT2 cells it is dependent on both PKA and PKC (figures 4.3 and 4.4). The difference in kinase pathways involved is most likely the result of the GnRHR coupling exclusively to G_q in αT3-1 cells (Grosse R *et al*., 2000), versus coupling to G_s and G_q in LβT2 cells (Liu F *et al*., 2002b), and the finding that GnRH elevates intracellular cAMP levels in LβT2 cells (Liu F *et al*., 2002b) but not in αT3-1 cells (Horn F *et al*., 1991). Homologous regulation of GnRHR promoter activity in both cell lines requires the AP-1 element at -336/-330 (White BR *et al*., 1999; Norwitz ER *et al*., 1999a; figure 4.9A). However, while regulation of GnRHR promoter activity via AP-1 was shown to be PKC-dependent in αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a), results presented in figure 4.14 show that, in LβT2 cells, the GnRH-mediated induction of AP-1 protein expression and binding to the AP-1 site *in vitro* is PKC-

independent. While forskolin and PACAP stimulate significant transcriptional responses in αT3-1 cells (figure 3.4), PACAP has no effect on pLG activity in LβT2 cells (figure 4.2), and the stimulatory effect of forskolin on pLG activity is weak and non-reproducible in these cells (figure 4.9B). These findings are summarized in table 5.2.

	αT3-1		**LβT2**	
GnRHR G-protein coupling	G_q (Grosse *et al*., 2000)		G_q, G_s (Liu *et al*., 2002)	
GnRH-mediated elevation of cAMP	No (Horn *et al*., 1991)		Yes (Liu *et al*., 2002)	
	Effect	**Kinase involved**	**Effect**	**Kinase involved**
GnRH	↑	PKC, not PKA (White *et al*., 1999; Norwitz *et al*., 1999)	↑ (figures 4.3, 4.4)	PKA, PKC (figures 4.3, 4.4)
Forskolin	↑	PKA (Cheng *et al*., 2001)	?* (figure 4.9B)	ND
PACAP	↑	PKA and others (Schomerus *et al*., 1994)	- (figure 4.2)	NA

Table 5.2: Summary of published and present data comparing GnRHR G-protein coupling, and regulation of GnRHR transcription by GnRH, forskolin and PACAP between αT3-1 cells and LβT2 cells. ↑ = increase measured; - = no response measured; ND = not determined; NA = not applicable.
*The response of pLG to forskolin in LβT2 cells was weak and non-reproducible.

The -15/-7NRS and -244/-236NRS are not required for the transcriptional response to PKA in αT3-1 cells (figure 3.6), or for the response to GnRH in LβT2 cells (figure 4.9A). However, results from experiments in αT3-1 cells where SF-1 and PKA were overexpressed indicate that SF-1 modulates the magnitude of the PKA response negatively via the -15/-7NRS and positively via the -244/-236NRS (figure 3.6). This effect was not apparent in LβT2 cells (not shown). However, the finding that PKA is not required for the GnRH-mediated downregulation of SF-1 protein expression and *in vitro* DNA binding suggests that SF-1 is probably not involved in modulating the transcriptional response to PKA in LβT2 cells. When considering all the abovementioned results, it can be concluded that the involvement of SF-1 in the regulation of GnRHR transcription differs between these two cell lines.

Results published by Fowkes *et al*. show that SF-1 expression is significantly lower in LβT2 cells than in αT3-1 cells, while the levels of DAX-1 (shown to inhibit SF-1 function) (Ito M *et al*., 1997; Crawford PA *et al*., 1998) are similar in the two cell lines (Fowkes RC *et al*., 2002). The relative SF-1:DAX-1 ratio is therefore lower in LβT2 cells than in αT3-1 cells, which could contribute towards explaining the differences in SF-1 function between the two cell lines.

When comparing the suitability of these two cell lines for studying transcriptional regulation in gonadotropes, the LβT2 cell line is more suitable than the αT3-1 cell line in many regards. LβT2 cells exhibit more characteristics of mature gonadotropes than αT3-1 cells, such as gonadotropin hormone subunit expression (reviewed in section 1.2). In addition, the GnRHR in LβT2 cells can couple to G_s and G_q G-proteins, as has been observed for primary pituitary cells (Liu F *et al*., 2002b). GnRH stimulation can also elevate cAMP production in LβT2 cells, as in primary pituitary culture (Borgeat P *et al*., 1972), while it has no effect on cAMP levels in αT3-1 cells (Horn F *et al*., 1991; Kanasaki H *et al*., 2002). From these findings, it can be concluded that the intracellular signalling pathways and kinases activated by GnRH via the GnRHR in LβT2 cells are similar to those in primary pituitary cultures, indicating that LβT2 cells more closely represents *in vivo* gonadotrope function than αT3-1 cells. However, for studies focusing on the transcriptional effects of PACAP, the LβT2 cell line would not be suitable, since it has been demonstrated that PACAP cannot elicit an intracellular response (Fowkes RC *et al*., 2003) or a transcriptional response (figure 4.2) in these cells, due to extremely low expression levels of PACAP receptors (Fowkes RC *et al*., 2003). These PACAP results suggest that LβT2 cells do not behave like gonadotropes *in vivo* with respect to all responses, since PACAP has been shown to activate PKA in primary pituitary gonadotropes (Garrel G *et al*., 2002). In addition, the blunted transcriptional response to intracellular cAMP observed in LβT2 cells (Fowkes RC *et al*., 2003) might be regarded as a disadvantage of this cell line.

5.3 Mechanisms of transcriptional regulation of GnRHR gene expression in αT3-1 cells and LβT2 cells

While it had been established previously that SF-1 protein binds to the mouse GnRHR -244/-236NRS *in vitro* (Duval DL *et al*., 1997a), the binding of nuclear proteins from gonadotrope cells to the -15/-7NRS has not been reported. The results presented in figures 3.2 and 4.5 show that SF-1 and Nur77 protein from both αT3-1 and LβT2 cells can bind to the -15/-7NRS and the -244/-236NRS *in vitro*. SF-1 and Nur77 proteins have different sequence requirements for binding to these sites, with Nur77 able to recognize a mutated NRS sequence that is not recognized by SF-1 (figure 3.3 and figure 4.5). Interestingly, *in vitro* binding experiments with nuclear extracts from LβT2 cells and from CCRF-CEM cells indicate that Nur77 protein has a lower affinity for the -244/-236NRS than for the -15/-7NRS. Although it is well known that Nur77 protein is expressed in pituitary corticotropes (Okabe T *et al*., 1998; Maruyama K *et al*., 1998), the expression of this protein has not been previously shown in gonadotropes. The presence of Nur77 transcripts, and their upregulation by GnRH, had been reported in LβT2 cells (Wurmbach E *et al*., 2001), but the results presented in figure 4.5 provide evidence of functional Nur77 protein expression in these cells.

5.3.1 Mechanisms of basal and PKA-dependent regulation of GnRHR gene transcription in αT3-1 cells

In these cells, SF-1 binding to the -244/-236NRS in the mouse GnRHR promoter was found to be crucial for basal promoter activity, whereas mutation of the -15/-7NRS did not have any influence on promoter activity (figures 3.5B and 3.6) Given the importance of the -244/-236NRS in basal promoter activity, it is interesting that of overexpression of SF-1 and Nur77 had no effect on basal pLG activity (figure 3.6). The transcriptional activity of the pLG construct was significantly upregulated by forskolin (figure 3.4) and by overexpressed PKA (figure 3.6) in αT3-1 cells. While intact SF-1 binding sites at -15/-7NRS and -244/-236 were not required for the forskolin response or the PKA response of pLG, the results presented in figures 3.5 and 3.6 do suggest that the levels of SF-1 protein and the binding of SF-1 at the proximal and distal NRS sites play a modulatory role in regulating the extent of the PKA response, with positive modulation of the PKA response via the -244/-236NRS, and negative modulation via the -15/-7NRS. It is difficult to envisage how the same protein could have both positive and negative effects on PKA stimulation. Since the sequences of the two NRSs differ, it is possible

that SF-1 might display different binding properties at these sites, which might then also influence the function of SF-1 at the sites. A similar idea has been proposed for the interaction between the estrogen receptor and estrogen response elements (Gruber CJ *et al.,* 2004). It is also possible that PKA-activated SF-1 binding at the -15/-7NRS may recruit a co-repressor, while SF-1 binding at the distal NRS may recruit a co-activator in these cells, due to interaction with different factors at the proximal and distal sites. SF-1 has been shown to interact with both transcriptional repressors, such as N-CoR (recruited to SF-1 by DAX-1) (Crawford PA *et al*., 1998), and activators, such as Sp1 (Liu Z and Simpson ER, 1997) and SRC-1 (Ito M *et al*., 1998). The positioning of the -15/-7NRS in the 5' untranslated region may also contribute to negative modulation of the PKA response, by interfering with components of the basal transcriptional machinery.

Transfection experiments with overexpressed Nur77 clearly showed that Nur77 does not regulate basal pLG activity in αT3-1 cells (figure 3.6). However, results obtained with the pLGM1 and pLGM2 constructs strongly suggest that Nur77 could act as a repressor of the PKA response via the -15/-7NRS, but not via the -244/-236NRS (figure 3.6). The finding that overexpression of Nur77 can completely blunt the SF-1-mediated enhancement of the PKA response of pLG (figure 3.7) is consistent with a negative modulatory role for Nur77 in αT3-1 cells. This is in contrast to the positive role shown for Nur77 in PKA-mediated gene regulation in pituitary corticotropes (Kovalovsky D *et al*., 2002; Maira M *et al*., 2003). Expression of Nur77 is upregulated by forskolin in AtT20 corticotrope cells (Murphy EP and Conneely OM 1997) and by cAMP in Y1 adrenocortical cells (Crawford PA *et al.,* 1995), and it is therefore clear that further experiments would be necessary to determine the effect of PKA activation on Nur77 expression and activity in αT3-1 cells. The findings on Nur77 in the present study are novel, since, although it has been previously shown by microarray analysis that Nur77 mRNA levels are upregulated by GnRH in LβT2 cells (Wurmbach E *et al.,* 2001), no role has previously been defined for Nur77 in the regulation of GnRHR transcription in LβT2 cells.

The finding that the proximal and distal NRSs are not required for the PKA response in αT3-1 cells (figure 3.6) raised the question as to which *cis*-elements and transcription factor(s) are essential for this response. Both the rat (Pincas H *et al*., 2001b) and the human (Cheng KW and Leung PCK, 2001) GnRHR I promoters have been shown to respond to PKA pathway activators via similar imperfect

cAMP response elements in αT3-1 cells. The -15/-7 and -244/-236 nuclear receptor half sites, as well as the imperfect CRE, are conserved in the rat and mouse GnRHR promoters (figure 1.4). It thus seems likely that the imperfect cAMP response element in the mouse GnRHR promoter is essential for the PKA response in αT3-1 cells, and that factor(s) binding there interact directly or indirectly with SF-1 at the proximal and distal NRS sites. However, the role of the CRE in basal and PKA-stimulated mouse GnRHR promoter activity in αT3-1 cells was not investigated in the present study.

5.3.2 Mechanism of homologous regulation of GnRHR gene transcription in LβT2 cells

The results from the present study in LβT2 cells are summarized in tables 5.3 and 5.4. A model for homologous regulation of GnRHR transcription is shown in figure 5.1, and the results will be discussed in the context of the model.

In the absence of GnRH stimulation, the primary transcription factor determining basal GnRHR transcription levels is CREB (figure 5.1, upper panel), as can be deduced from the observation that binding of CREB to the CRE in the mouse GnRHR promoter is essential for promoter activity (figure 4.8, table 5.3). This is likely due to the fact that the proximal mouse GnRHR promoter does not contain a TATA box (figure 1.4), and therefore requires the recruitment of the basal transcriptional machinery by the constitutive activation domain (CAD) of CREB (Kim J *et al*., 2000; Felinski EA *et al*., 2001). Current models for the mechanism of CREB-mediated transcriptional activation hypothesize that, for promoters lacking a TATA box, the CAD facilitates assembly of the basal transcriptional machinery, independent of the phosphorylation status of CREB (figure 5.1, upper panel), while the kinase-inducible domain (KID) mediates hormone-stimulated transcriptional activation in a phosphorylation-dependent manner (Kim J *et al*., 2000; Felinski EA *et al*., 2001).

Promoter *cis* element	Basal	GnRH-stimulated
-15/-7NRS	No	No
-244/-236NRS	?*	No
CRE	Yes	ND
AP-1	No	Yes

Table 5.3: Summary of results obtained in the present study in LβT2 cells, with regards to the respective roles of individual GnRHR promoter *cis* elements in basal and GnRH-stimulated GnRHR promoter activity. "Yes" indicates where a *cis* element was found to be involved; "No" indicates where a *cis* element was found not to be required. ND = not determined; NRS = nuclear receptor binding site; CRE = cAMP response element; AP-1 = activator protein-1 binding site.
*Mutation of the -244/-236NRS did cause an apparent decrease in basal GnRHR promoter activity (figure 4.8), but the trend was not statistically significant.

Interestingly, *in vitro* data (figure 4.10) suggest that CREB is at least 50% phosphorylated under basal conditions in LβT2 cells, and it is therefore possible that the CRE is occupied by phospho-CREB in the absence of hormonal stimulation. Alternatively, since the cells were only incubated in serum-free medium for 30 minutes before nuclear extracts were prepared, it is possible that the relatively high levels of CREB phosphorylation observed in the vehicle-treated samples is due to the residual effects of serum stimulation. Thus in figure 5.1, the CRE is shown to be occupied by CREB under basal conditions, but it is not clear from the present results whether CREB is required to be phosphorylated for basal activity, although the literature suggest that this is not required. It is noteworthy that the rat and human GnRHR I promoters have at least one TATA box each, while the mouse promoter does not (see figure 1.4). While mutation of the mouse GnRHR promoter CRE results in a complete loss of basal promoter activity in LβT2 cells (figure 4.8), mutation of the corresponding CRE in the rat GnRHR I promoter was found to have no significant effect on promoter activity in αT3-1 cells (Pincas H *et al*., 2001b). This finding is consistent with the idea that the role of a CRE in basal transcription is different in the presence of a TATA box, as compared to in the absence of a TATA box (Kim J *et al*., 2000; Felinski EA *et al*., 2001).

	mRNA		Protein		*In vitro* DNA binding	
Gene	**GnRH**	**Kinase**	**GnRH**	**Kinase**	**GnRH**	**Kinase**
GnRHR	↑ (fig. 4.4)	PKA, PKC (fig. 4.4)	↑ (Bedecarrats and Kaiser, 2003)	ND	NA	NA
SF-1	- (Dorn *et al.*, 1999)	ND	↓ (fig. 4.13)	PKC (fig. 4.13)	↓ (fig. 4.12)	PKC (fig. 4.12)
Nur77	↑ (Wurmbach *et al.*, 2001)	ND	ND	ND	- (fig. 4.12)	ND
CREB	ND	ND	- (Duan *et al.*, 1999)	ND	ND	ND
P-CREB	NA	NA	↑ (fig. 4.10)	PKA, PKC (fig. 4.10)	↑ (fig. 4.10)	PKA, PKC (fig. 4.10)
AP-1	↑ (Wurmbach *et al.*, 2001; Kakar *et al.*, 2003)	ND	↑ (fig. 4.14; Coss *et al.*, 2004)	MEK (Coss *et al.*, 2004) Not PKA, PKC (fig. 4.14)	↑ (fig. 4.14)	Not PKA, PKC (fig. 4.14)

Table 5.4: Summary of published and present results showing the effect of GnRH and kinases on the GnRH-mediated regulation of mRNA and protein expression levels of selected genes in LβT2 cells, as well as on binding of the selected proteins to their cognate sites in the GnRHR promoter. ↑ = increase measured; ↓ = decrease measured; - = no difference measured; ND = not determined; NA = not applicable; P-CREB = CREB phosphorylated on Ser-133.

The *in vitro* binding data presented in figures 4.5 and 4.12 suggest that the NRSs are occupied by SF-1 or by Nur77 under basal conditions in LβT2 cells, although this may not be the case *in vivo*, since the NRSs are not required for basal activity in these cells (table 5.3). For this reason, in figure 5.1 (upper panel), the occupancy of the NRSs by these proteins is shown with a question mark. Since the intensity of the SF-1 complexes in the *in vitro* binding experiments with both NRS probes were much higher than that of the Nur77 complexes (figures 4.5 and 4.12), this suggests that, in the absence of GnRH, the NRSs would be preferentially occupied by SF-1 *in vivo*. However, the binding of SF-1 to the NRSs in intact LβT2 cells, and the function of this putative binding, remains to be established.

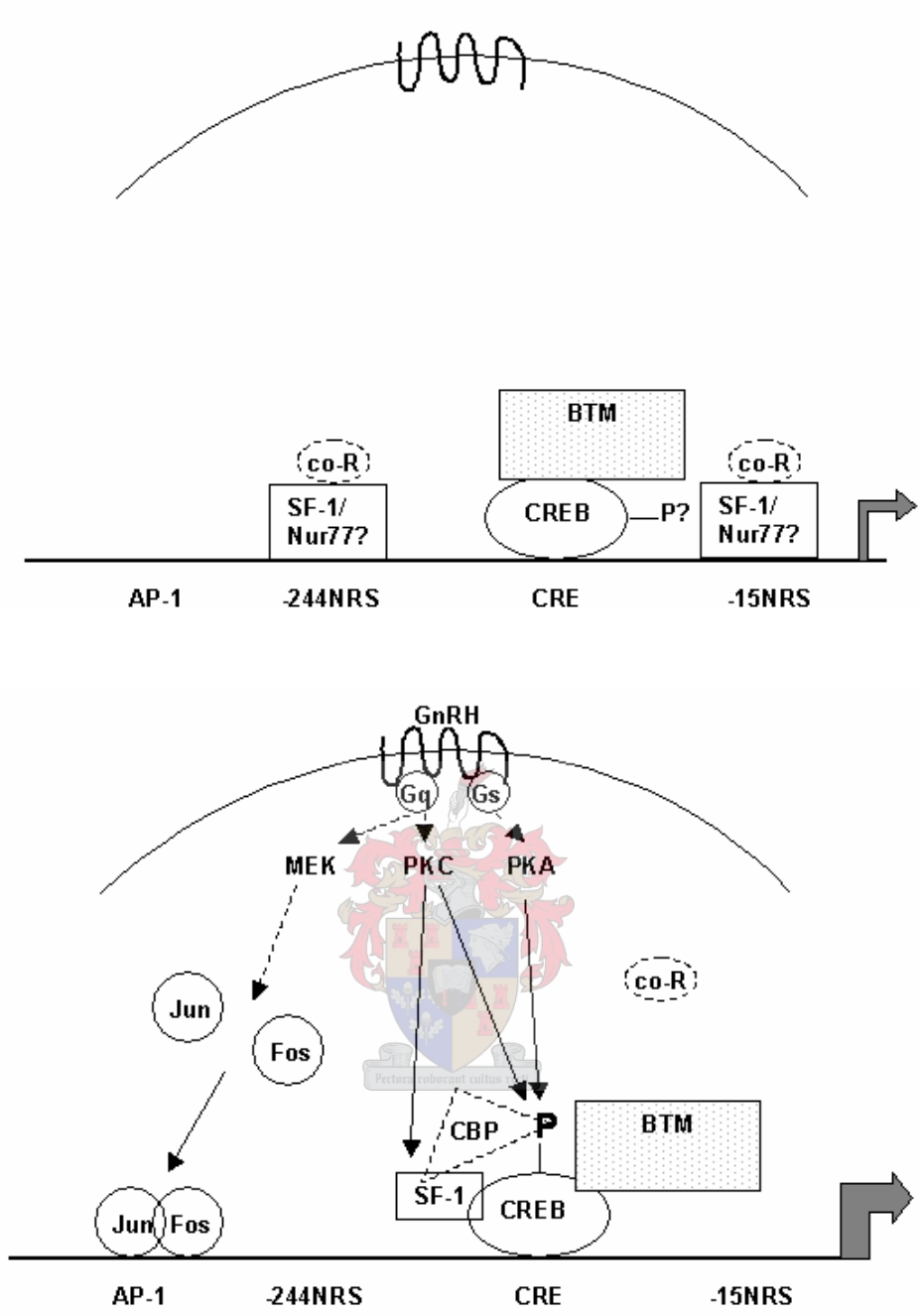


Figure 5.1: A schematic representation of the proposed model for regulation of GnRHR gene transcription in LβT2 cells, under basal (upper panel) and GnRH-stimulated (lower panel) conditions. Broken lines indicate aspects of the model that have not been conclusively demonstrated in this study. The phosphorylation status of CREB bound to the CRE under basal conditions and the occupancy of the NRSs by SF-1 and/or Nur77 under basal conditions has not been established, and is therefore indicated with question marks (upper panel). BTM = basal transcriptional machinery (basal transcription factors plus RNA polymerase II); co-R = putative co-repressor; CRE = cyclic AMP response element; CREB = CRE binding protein; CBP = CREB binding protein; SF-1 = Steroidogenic Factor-1; NRS = nuclear receptor binding site; AP-1 = Activator Protein-1 binding site.

Upon GnRH stimulation, GnRHR transcription is upregulated, requiring the involvement of both PKA and PKC (figures 4.3 and 4.4, also see figure 5.1 (lower panel) and table 5.4). GnRH stimulation results in a PKC-dependent decrease in SF-1 protein expression (figure 4.13) and a concomitant decrease in binding to the NRSs *in vitro* (figure 4.12), and also results in a PKA and/or PKC-dependent increase in phospho-CREB binding to the CRE *in vitro* (figure 4.10, figure 5.1 (lower panel). In addition to these events, GnRH also increases AP-1 protein levels and binding to the AP-1 site *in vitro*, in a PKA- and PKC-independent manner (figures 4.14 and 5.1). The observation that mutation of the AP-1 site caused a significant loss of GnRH responsiveness, while mutation of the CRE appeared to also decrease the GnRH response (figures 4.9A and B), indicates that the response to GnRH requires binding of both AP-1 and CREB to their respective promoter elements. However, the finding that the GnRH response requires both PKA and PKC, even though the increase in AP-1 binding is independent of both kinases, suggest that all of the abovementioned events are required for the response of the GnRHR promoter to GnRH (figure 5.1, lower panel).

The model in figure 5.1 is consistent with results obtained with wild-type and mutated promoter-reporter constructs (figures 4.8 and 4.9A, also see tables 5.3 and 5.4). If one proposes that GnRH reduces the binding of SF-1 to the NRSs in whole cells, as suggested by the *in vitro* results (figure 4.12), it is not surprising that mutation of the NRSs does not affect the GnRH response (figure 4.9A). Moreover, reduced SF-1 binding alone, as would occur for the promoter-reporter constructs with mutated NRSs (pLGM1, pLGM2, pLGM1+2), does not result in increased promoter activity (figure 4.8). This is consistent with the proposed model where decreased SF-1 binding is not sufficient for GnRH-mediated increase in transcription, but that other factors, such as AP-1 and phospho-CREB, are also required. A requirement for an intact CRE for GnRH responsiveness could not be tested with a mutated promoter-reporter construct, but the mutated CRE results in a complete loss of promoter activity under all conditions, consistent with a requirement for an intact CRE for a GnRH response.

The finding that GnRH reduces SF-1 binding to the NRSs (figure 4.12) and concomitantly increases promoter-reporter activity expression, suggests that, in the basal state, SF-1 might recruit a transcriptional co-repressor, as proposed in the model in figure 5.1 (lower panel). As an example, it has been shown that DAX-1 can recruit the nuclear receptor co-repressor N-CoR to SF-1 through the

interaction of DAX-1 with a repressive domain within the C-terminus of SF-1 (Crawford PA *et al*., 1998), Dowregulation of SF-1 protein expression levels and decreased binding to the NRSs could therefore result in diminished recruitment of the co-repressor to the GnRHR promoter, lifting the transcriptional inhibition and resulting in increased GnRHR transcription, as proposed in figure 5.1. Alternatively, it is possible that SF-1 bound to the -15/-7NRS could create a spatial hindrance, interfering with the assembly of the basal transcriptional machinery (figure 5.1, upper panel). A similar mechanism has been postulated for the negative modulation of the PKA response by SF-1 in αT3-1 cells (see section 5.3.1).

As indicated by the results of the two-hybrid experiments (figure 4.11), GnRH also induces an interaction between SF-1 and CREB, which does not require CREB phosphorylation on Ser-133, but is dependent on PKC. It is important to note that the effect of GnRH on the transactivation potential of either pBIND-SF-1 or pACT-CREB alone was not investigated in the two-hybrid experiments, and the results obtained from these experiments should therefore be regarded as preliminary. Nevertheless, these data suggest that GnRH not only results in a PKC-dependent reduction in SF-1 protein levels, but also causes PKC-dependent post-translational modification of SF-1, which promotes interaction with CREB. It is possible that the interaction with CREB further inhibits recruitment of a co-repressor. It is tempting to speculate that the CREB-SF-1 complex recruits the transcriptional co-activator CBP, as hypothesized in figure 5.1 (lower panel). It has been shown that CBP can interact with both CREB and SF-1, via distinct domains (Monte D *et al*., 1998). CBP has been shown to interact specifically with the phosphorylated Ser-133 of CREB (Chrivia JC *et al*., 1993), as indicated in figure 5.1 (lower panel). The finding that the GnRH-induced CREB-SF-1 interaction in the present system does not require phosphorylation of CREB on Ser-133 (figure 4.11) suggests that phosphorylated Ser-133 of CREB is not involved in the interaction with SF-1, and that this CREB site would therefore be available for interaction with CBP. The PACAP-mediated recruitment of CBP by CREB and SF-1 has been postulated for the rat GnRHR I promoter in αT3-1 cells (Pincas H *et al*., 2001b), and since the -244/-236NRS and the CRE are conserved between the rat and mouse GnRHR promoters (see figure 1.4), it is possible that a similar mechanism, ie the recruitment of the transcriptional co-activator CBP by both SF-1 and CREB, contributes to the transcriptional response of the mouse GnRHR promoter to GnRH in LβT2 cells.

Several lines of evidence are presented here to support a role for CREB in the transcriptional response to GnRH. Mutation of the CRE appeared to decrease the response of the transfected GnRHR promoter-reporter construct to GnRH (figure 4.9A and B, table 4.2). In addition, GnRH treatment resulted in increased binding of phospho-CREB (Ser-133) to the GnRHR promoter CRE *in vitro* (figure 4.10, also see figure 5.1 and table 5.4). The GnRH-mediated phosphorylation of CREB could be inhibited by both the PKA and the PKC inhibitors (figure 4.10, also see figure 5.1 (lower panel) and table 5.4), consistent with the inhibition of the GnRH-mediated transcriptional activation of the GnRHR gene by both kinase inhibitors (figures 4.3 and 4.4). In agreement with the upregulation of phospho-CREB levels in LβT2 cells presented here, pituitary phospho-CREB levels in rats appear to be regulated by GnRH and estradiol *in vivo*, as indicated by the finding that pituitary phospho-CREB levels differ between male and female rats, and between different stages of the estrous cycle (Duan WR *et al*., 1999). Taken together, these data suggest that modulation of phospho-CREB levels can form part of the mechanism of GnRH-mediated transcriptional regulation of CREB-regulated genes in gonadotropes and other GnRH-responsive cell types *in vivo.* In support of this idea, CREs have been shown to be important for the basal promoter activity and GnRH responsiveness of two other gonadotrope genes, namely the neuronal nitric oxide synthase (nNOS) gene (Bachir LK *et al*., 2003) and the gonadotropin alpha-subunit gene (Duan WR *et al*., 1999).

Results presented in figure 4.9A and B and in table 4.2 show that the AP-1 site at -336/-330 is involved in the transcriptional response of the GnRHR promoter to GnRH in LβT2 cells. This is consistent with results obtained for a similar promoter fragment in αT3-1 cells (White BR *et al*., 1999). In addition, the finding in intact LβT2 cells that c-Jun protein occupies a region of the mouse GnRHR promoter containing the AP-1 site (Jeong KH *et al*., 2004) further supports the idea that AP-1 proteins are involved in GnRHR transcriptional regulation in these cells. However, while mutation of the AP-1 site resulted in a complete loss of GnRH responsiveness in αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a), the pLGmAP1 construct transfected into LβT2 cells in the present study did retain some responsiveness to GnRH (figure 4.9A). This indicates that, in LβT2 cells, other *cis* elements besides the AP-1 site in the mouse GnRHR promoter are involved in the transcriptional response to GnRH, as proposed in figure 5.1. As already discussed, based on the results presented in figures 4.9A, 4.10 and 4.11, the CRE is likely to play a role in the GnRH response. Studies in αT3-1 cells

have also identified two composite promoter elements, SURG-1 (Norwitz ER *et al*., 1999a; Kam KY *et al*., 2005) and GRAS (Norwitz ER *et al*., 2002b) that contribute to GnRH responsiveness of the mouse GnRHR promoter in these cells. While no reports on the function of these elements in LβT2 cells have been published to date, they are likely to also contribute to GnRH responsiveness of the GnRHR promoter in LβT2 cells.

The results presented in figure 4.14 demonstrate that nuclear extracts from unstimulated LβT2 cells contained no AP-1 binding activity under the EMSA conditions (figure 4.14A), due to the fact that expression of Jun and Fos proteins could not be detected in these extracts (at least not by means of Western blotting (figure 4.14B and 4.14C)). GnRH strongly induced expression of both Jun and Fos proteins, as well as binding of these proteins to the AP-1 element *in vitro* (figure 4.14, also see figure 5.1 (lower panel) and table 5.4). These results provide evidence for a mechanism whereby GnRHR gene transcription is upregulated by increased AP-1 protein levels and increased binding to the AP-1 site in the GnRHR promoter, as proposed in figure 5.1 (lower panel). The *in vitro* data presented here (figure 4.14) are not consistent with chromatin immunoprecipitation (ChIP) assay data from intact LβT2 cells which showed binding of c-Jun to the AP-1 site in the mouse GnRHR promoter in the absence of GnRH stimulation (Jeong KH *et al*., 2004). However, the experiments performed by Jeong KH *et al.* were done in the presence of serum stimulation, while the present results (figure 4.14) were performed after 8 hours of serum starving, and it is therefore likely that the binding of c-Jun to the GnRHR promoter observed by Jeong KH *et al.* was due to induction of c-Jun protein expression by serum factors. In addition, the data from figure 4.14 are consistent with findings published by other groups, showing that GnRH treatment induces the mRNA levels (Wurmbach E *et al*., 2001; Kakar SS *et al*., 2003) and protein expression levels (Coss D *et al*., 2004) of several Jun and Fos proteins in LβT2 cells, and that GnRH stimulates binding of these proteins to the mouse FSHβ promoter in intact LβT2 cells (Coss D *et al*., 2004).

The immunoblotting results also indicate that neither PKA nor PKC is required for the GnRH-mediated induction of Jun and Fos protein expression in LβT2 cells (figure 4.14B and 4.14C). It can therefore be concluded that the GnRH-mediated increase in AP-1 protein expression and increased DNA binding do not form part of the PKA- and PKC-mediated mechanisms of transcriptional upregulation of the

GnRHR gene by GnRH (figure 4.3 and 4.4), as indicated in the model in figure 5.1. In agreement with the findings presented here, GnRH-mediated induction of c-Fos and JunB proteins in LβT2 cells was shown to be MEK (MAPK-kinase)-dependent (Coss D *et al*., 2004) and in the case of c-Fos expression, PKC-independent (Liu F *et al.,* 2002a). These findings have been integrated into the model in figure 5.1 (lower panel). Interestingly, while results from other investigators suggest that the induction of c-Fos expression in LβT2 cells also involves cAMP signaling (Liu F *et al*., 2003), in the present system, inclusion of a PKA inhibitor did not inhibit GnRH-induced Fos protein expression (figure 4.14C, also see figure 5.1 and table 5.4). It is also possible that the AP-1-mediated upregulation of GnRHR expression involves not only increased AP-1 expression, but also activation via phosphorylation by kinases such as JNK and ERK (reviewed in Wisdom R, 1999). The anti-AP-1 antibodies utilized in the present experiments are not phosphoprotein-specific, and therefore the phosphorylation status of AP-1 proteins could not be determined in these experiments. However, results published by Yokoi *et al* demonstrating that GnRH can activate both ERK and JNK in LβT2 cells (Yokoi T *et al*., 2000) indicate that these kinases could be involved in transcriptional regulation via GnRH and AP-1. Although GnRH-mediated JNK activation in LβT2 cells has been found to be PKC-independent (Yokoi T *et al*., 2000), the GnRH-mediated activation (Yokoi T *et al*., 2000) and nuclear translocation of ERK (Liu F *et al*., 2002a) both require PKC signaling. Therefore, while PKC does not play a role in the expression and DNA-binding of AP-1 proteins in LβT2 cells, it is possible that PKC is required for the transcriptional activity of these proteins, thereby contributing to the role of PKC in the GnRH-mediated transcriptional upregulation of the GnRHR gene, as observed in the present study (figure 4.3 and 4.4).

5.4 Future perspectives

An ongoing issue in the field of transcriptional regulation is the relevance of results obtained in immortalized cell lines, since it is possible that these cell lines do not accurately reflect *in vivo* conditions. However, immortalized cell lines greatly facilitate the investigation of cell-specific intracellular signalling and transcriptional regulation. With *in vivo* studies, it is not always possible to determine whether measured effects are direct or not, since other systems or cell-types might be contributing to the final outcome measured. In the case of pituitary function, direct pituitary effects can be measured in primary pituitary cultures, but these cultures typically contain a mixture of different

pituitary cell-types, creating difficulty when trying to determine gonadotrope-specific effects. Performing experiments in immortalized cell lines can therefore be advantageous, provided that results obtained in these cell lines are verified in other systems that are physiologically more relevant, such as transgenic mice. As an example, several investigators have demonstrated that GnRH regulates GnRHR promoter activity via a mechanism requiring a functional AP-1 element, in both αT3-1 cells (White BR *et al*., 1999; Norwitz ER *et al*., 1999a) and in LβT2 cells (figure 4.9A). This result was confirmed in transgenic mice harbouring a mouse GnRHR promoter-reporter transgene, providing strong evidence that similar mechanisms are involved in the homologous regulation of GnRHR expression in these cell lines and *in vivo*. It can therefore be concluded that results obtained in these cell lines are likely to be relevant, supporting their continued use as model systems for directly testing hypotheses on mechanistic pathways regulating gonadotrope function. However, it would be advisable to continue confirming results obtained in these cell lines in primary pituitary cultures or in transgenic mice, to support the relevance of these findings.

An issue that is closely related is the relevance of data obtained with transfected promoter-reporter constructs. These constructs are handy tools for studying the detailed mechanisms of transcriptional regulation, but since they usually only contain a promoter fragment, cloned into a plasmid, it is quite possible that the activity of multiple promoter-reporter constructs might not be regulated by the same mechanisms as a single copy of a full-length native promoter in the context of the native chromatin structure. It is therefore important to demonstrate the relevance of results obtained with transfected promoter-reporter constructs by verifying these results on the endogenous promoter. In the present study, GnRH upregulated endogenous GnRHR transcript levels (figure 4.4) and pLG activity (figure 4.3) in LβT2 cells after 8 hours, and for both the endogenous and the transfected promoter, this upregulation required PKA and PKC activity. The finding that homologous upregulation of endogenous GnRHR transcription occurred at the same time-point and required the same kinases as pLG provides strong evidence that the transcriptional activity of pLG is regulated by mechanisms similar to that of the endogenous promoter. This increases the likelihood that data obtained with promoter-reporter constructs regarding the regulation of GnRHR transcription in LβT2 cells would also be relevant for the transcriptional regulation of the endogenous GnRHR gene in these cells.

Similar to the issues discussed above, it is also necessary to correlate *in vitro* protein-DNA binding data, such as obtained by EMSA, with data obtained in living cells, such as with chromatin immunoprecipitation (ChIP) assays. By means of these assays, investigators can determine the effects of different experimental conditions on the identity and relative levels of transcription factors bound to a specific promoter region (either the native promoter or a transfected promoter construct) in intact cells. It is clear that significant amounts of *in vivo* data would be required to fully elucidate the model proposed in fig 5.1 for the mechanism of GnRH-mediated transcriptional regulation of the GnRHR gene in LβT2 cells, and ChIP assays will definitely be a valuable tool in gathering such data. In particular, since the data presented in this thesis strongly indicate that several *cis* elements (CRE, AP-1 site, NRSs) and transcription factors (CREB, AP-1, SF-1 and possibly Nur77) are involved in the GnRH response of the GnRHR promoter, the interplay between the factors bound at these *cis* elements could be investigated on the full-length promoter. Specific issues that could be clarified by means of ChIP assays would include the interaction between SF-1 and CREB, the relative occupancy of the NRSs by SF-1 and Nur77, and the binding of AP-1 proteins to the AP-1 element in the absence and presence of GnRH stimulation. Another possible approach to gather more *in vivo* data is to knock down protein expression by means of small interfering RNA (siRNA) studies. These experiments would be especially helpful in delineating the specific functions of individual transcription factors, such as CREB, SF-1 and Nur77, in the mechanisms of transcriptional regulation in these cells.

When considering the results obtained in LβT2 cells (chapter 4), it is evident that in most cases, responses were quite small, with substantial statistical errors. In many instances, these large errors resulted in apparent differences in responses being statistically insignificant, even if these trends were reproducible. This variability in responsiveness appears to be an inherent characteristic of the LβT2 cell system, rather than simply careless execution of experimental procedures. Other groups using these cells as their model system have also reported small responses, especially with transfected promoter-reporter constructs (Kaiser UB *et al*., 2000; Pernasetti F *et al*., 2001; Vasilyev VV *et al*., 2002; Coss D *et al*., 2004). Since these are neuroendocrine cells, it is likely that they are exquisitely sensitive to their culture environment, with small changes in experimental conditions affecting their behaviour. In addition, it has been observed that any given population of LβT2 cells will consist of responsive and non-responsive cells, and the final measured response will depend on the proportion

of responsive cells (Dr Stuart Sealfon, Mount Sinai Medical School, New York, USA, personal communication). It is therefore essential to control the cell culture environment and the experimental conditions, such as cell density and passage number, as closely as possible, in order to generate reproducible, statistically relevant results in this system. It is also possible that pituitary cells might display diurnal rhythms (as they would *in vivo*), and therefore it might even be necessary to perform experiments in gonadotrope cell lines at exactly the same time of day, in order to observe reproducible responses. In the future, it might also be advantageous to measure responses in individual cells, to limit the effect of variable responses between cells on the final response measured (Dr Stuart Sealfon, personal communication).

5.5 Conclusion

In summary, the results presented in this thesis show that the transcriptional activity of the mouse GnRHR promoter can be upregulated by activators of the PKA pathway, and by PKA itself, in αT3-1 cells. This is similar to what was found for the rat (Pincas H *et al*., 2001b) and human (Cheng KW and Leung PCK, 2001) GnRHR I promoters in these cells. These results demonstrate that, although GnRH does not stimulate PKA activity in αT3-1 cells (Horn F *et al*., 1991; Kanasaki H *et al*., 2002), the downstream mechanisms for cAMP/PKA-mediated regulation of GnRHR gene transcription are still functional in these cells. Given that there is strong evidence that both GnRH (Borgeat P *et al*., 1972) and PACAP (Garrel G *et al*., 2002) can activate PKA in primary pituitary gonadotropes, these results therefore most likely reflect a physiologically relevant mechanism for the transcriptional effects of both of these hormones *in vivo.* In addition, these results raise the possibility that other signals that activate the PKA pathway in pituitary gonadotropes *in vivo* could regulate expression of the GnRHR gene. The results presented here also demonstrate homologous upregulation of mouse GnRHR promoter activity in both αT3-1 and LβT2 cells, and homologous upregulation of endogenous GnRHR transcription in LβT2 cells. Taken together, these results confirm that GnRHR numbers are regulated at the level of transcription, by GnRH as well as by other physiological factors. While it is well established that GnRHR numbers are regulated by post-transcriptional mechanisms, it is possible that transcriptional regulation of GnRHR expression *in vivo* is involved in long-term changes in receptor levels in response to differences in hypothalamic GnRH secretion during the estrous cycle, and also during fetal development, sexual development and puberty.

ADDENDUM A

LIGHTCYCLER® REAL-TIME QUANTITATIVE RT-PCR

A.1 Introduction

The most powerful tool that is currently available to amplify and quantify small amounts of mRNA is reverse transcription, followed by polymerase chain reaction (PCR) (Pfaffl MW, 2001; Marino JH *et al*., 2003). During real-time PCR, data is collected throughout the PCR process as it occurs, thereby combining amplification and detection/quantification into a single step. This is achieved by using any one of a wide variety of fluorescence chemistries that correlate the concentration of the PCR product to measured fluorescence intensity (reviewed in Wong ML and Medrano JF, 2005).

Real-time RT-PCR is highly sensitive and allows detection and quantification of rare (low-abundance) transcripts and small changes in gene expression (Pfaffl MW, 2001). It can have an accurate dynamic range of several log orders of magnitude (Morrison TB *et al*., 1998). It is 1000-fold to 100 000-fold more sensitive than other conventional assays, such as RNAse protection assays and dot-blot hybridisation. In addition, real-time PCR assays have a lower variance coefficient, making it possible to detect smaller differences in expression between samples than with other methods, and it requires much smaller amounts of RNA template (reviewed in Wong ML and Medrano JF, 2005).

The simplest detection method for PCR products in real-time PCR utilizes the fluorescent dye SYBR Green I, which binds to the minor groove of double-stranded DNA in a sequence-independent way, and has 100-fold higher fluorescence emission when bound to DNA (Lekanne Deprez RH *et al*., 2002). The use of fluorescence dyes to monitor PCR amplification in real-time allows for the combination of amplification, product detection, quantification and verification (Pfaffl MW, 2001).

A.2 General principles and experimental considerations

A2.1 One-step vs two-step RT-PCR

Quantifying mRNA by real-time RT-PCR can either be performed as a one-step reaction, where the reverse transcriptase (RT) reaction and the PCR is performed in a single tube, or as a two-step

reaction, where the two reactions are separated. Both methods have their advantages and disadvantages. One-step RT-PCR is thought to minimize experimental variation, because all steps are performed in a single tube. However, one-step RT-PCR uses RNA as starting material, and can therefore be jeopardized by RNA degradation. It is also thought to be less sensitive than two-step RT-PCR. Since two-step RT-PCR separates the cDNA synthesis (RT) from the PCR assay, this allows for several different PCR assays to be performed on different dilutions of a single cDNA preparation. The efficiency of the reverse transcription reaction is highly variable, and therefore it is crucial to use dilutions of the same cDNA preparation, to ensure that all assays have the same amount of template. However, two-step protocols allow for more opportunities of DNA contamination of PCR samples, as well as experimental (pipetting) error (reviewed in Wong ML and Medrano JF, 2005).

A2.2 Quantification

PCR amplification consists of four major phases: linear ground phase, early exponential phase, log-linear (exponential) phase and plateau phase. During the linear ground phase (up to approximately cycle 20), the fluorescence emission measured at the end of each cycle has not yet emerged from background fluorescence, and this is referred to as baseline fluorescence. During the early exponential phase, the amount of fluorescence becomes significantly higher than the background/baseline. The cycle number at which this occurs is called the crossing point (CP) in LightCycler software, and is representative of the target copy number in the original sample. Therefore, the higher the concentration of target template in the starting material, the faster this threshold will be reached, resulting in a lower CP (Liu WL and Saint DA, 2002; reviewed in Wong ML and Medrano JF, 2005). The log-linear phase is the optimal amplification period, and the plateau phase is where reaction components become limiting, causing the fluorescence emission to reach a plateau (Pfaffl MW, 2001; reviewed in Wong ML and Medrano JF, 2005).

Two types of quantification are possible with real-time RT-PCR: absolute quantification and relative quantification. Absolute quantification requires the use of an internal or external standard curve, and gives an absolute number of mRNA transcripts per sample. The standard curve is generated by serial dilutions of a sample of known concentration, and provides a linear relationship between CP and initial target concentration in the starting material. It is then used to quantify the levels of the target transcript

in the unknown samples, and results are expressed as a concentration or as an absolute number of target transcript copies (reviewed in Wong ML and Medrano JF, 2005). For relative quantification, changes in target gene expression between different experimental conditions (eg. basal vs hormone-stimulated conditions) are expressed relative to changes in expression levels of a reference gene under the same conditions. No absolute measurements are taken, and therefore relative quantification does not require the use of a standard curve (Pfaffl MW, 2001).

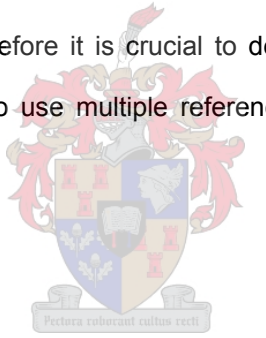
When performing relative quantification, it is crucial to determine the amplification efficiency of each target transcript. Under ideal reaction conditions, the efficiency would be 2, in other words, the amount of product in the PCR sample would double after every cycle during the exponential phase, but many PCR samples do not have ideal amplification efficiency. Therefore, assuming an efficiency of 2 might overestimate the amount of target transcript in the starting material, and could yield erroneous results (reviewed in Wong ML and Medrano JF, 2005). Traditionally, amplification efficiency is calculated by using the slope of a dilution series curve, generated by plotting CP against fluorescence for serial dilutions of a standard sample (Pfaffl MW, 2001). This value can then be used for subsequent experiments. There are also several other methods of determining amplification efficiency, using raw data generated during the actual PCR (reviewed in Wong ML and Medrano JF, 2005), and these methods are believed to be more accurate than the dilution series method. However, one study found that the accuracy of the “dilution series” method and the “raw data” method is comparable (Liu WL and Saint DA, 2002).

Both absolute and relative quantification methods have advantages and disadvantages (discussed in Wong ML and Medrano JF, 2005). However, it has been established that both methods yield comparable results when determining fold changes in target gene expression (Peirson SN *et al*., 2003).

A2.3 Normalizing target gene expression

When measuring mRNA expression levels by RT-PCR, it is essential to correct for experimental error by normalizing target gene expression. Target transcript levels can be normalized against several factors, such as sample size (for example tissue weight or cell number), total RNA quantitiy, cDNA

concentration or reference gene transcript levels (reviewed in Huggett J *et al*., 2005). Reference genes are non-regulated under experimental conditions, and are often housekeeping genes, so named because they are present in all cell types and are required for basic cell survival. Examples of such housekeeping genes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH or G3PDH), actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA (Thellin O *et al*., 1999). The mRNA levels of these housekeeping genes are considered to remain stable, even under experimental conditions. However, it has been shown in many systems that housekeeping gene expression levels can vary under different types of experimental conditions (Pfaffl MW, 2001; Thellin O *et al*., 1999). This could at least in part be due to housekeeping proteins also being involved in functions other than basal cell metabolism (reviewed in Thellin O *et al*., 1999). Consequently, the choice of housekeeping gene to use as internal control must be considered carefully for each cell type and each type of experimental manipulation, otherwise this could yield erroneous results (Thellin O *et al*., 1999). No single gene can meet all the criteria of a universal reference gene. No gene is constitutively expressed in all cell types under all experimental conditions. Therefore it is crucial to demonstrate the appropriateness of the specific choice of reference gene, or to use multiple reference genes for normalizing (reviewed in Bustin SA *et al*., 2005).



A2.4 Causes of variation

Due to the high sensitivity of real-time RT-PCR, several factors can potentially have a significant effect on the final result obtained. The quality of the cDNA synthesized can greatly affect the amplification efficiency and the overall results. During one-step RT-PCR, all factors interfering with the RT reaction will influence the amplification of all target and reference transcripts. With two-step RT-PCR, the concentration of the cDNA is determined by measuring the optical density at 260 nm, and equal amounts of template is subsequently added to each sample. Therefore, with two-step RT-PCR, when the amount of cDNA template is constant for all PCR samples, variability will be decreased, and reproducibility increased (Pfaffl MW, 2001). Both reverse transcriptase and DTT (present in the RT reaction buffer) can inhibit the PCR reaction (Lekanne Deprez RH *et al*., 2002), and therefore it is advisable, when using non-purified cDNA as PCR template, to dilute the cDNA and to lower the volume of cDNA added to each PCR sample, as suggested by the Roche LightCycler protocol.

Alternatively, the cDNA can be purified by column purification methods or by ethanol precipitation, to remove RT reaction components before performing the PCR reaction.

The amount and quality of cDNA yielded by the RT reaction will be influenced by the type of oligonucleotide used for the RT priming. RT reactions can be performed on either total RNA or on purified mRNA. Gene-specific primers will yield the most efficient reaction; however, this means that one cannot assay different target and/or reference genes from the same cDNA preparation. Oligo(dT) priming has an intermediate efficiency, and since it anneals to the poly-A) tail of mRNA species, this is the best method to get an accurate representation of the mRNA population in a total RNA preparation. However, since this method selects only for mRNA species, it therefore excludes the use of rRNA species as reference genes. In addition, fragmented RNA will not be effectively transcribed with this method, and the possibility exists that the 5' end of long mRNA transcripts may not be efficiently transcribed (reviewed in Bustin SA *et al.,* 2005). Random primers have the lowest priming efficiency (Lekanne Deprez RH *et al*., 2002; reviewed in Wong ML and Medrano JF, 2005). Since these oligonucleotides prime the RT reaction at multiple origins along the RNA template strand, more than one cDNA copy can be yielded per original RNA. Also, the majority of cDNA synthesized from total RNA in this way will be ribosomal RNA-derived, and this can create problems for detection and quantification of low-abundance mRNA species (reviewed in Bustin SA *et al*., 2005).

Secondary structure in the RNA template can cause great variation during cDNA synthesis, with the RT enzyme pausing, skipping over looped RNA regions, or dissociating from the template. This effect can be reduced by increasing the RT reaction temperature, or by pre-incubating the RNA template at a high temperature to denature secondary structure, as recommended for Promega M-MLV Reverse Transcriptase (RNase H minus) (reviewed in Wong ML and Medrano JF, 2005).

A2.5 The melting curve analysis

It is important to remember that SYBR Green I complexes with all double-stranded DNA products in the PCR reaction. The fluorescence measured during the real-time PCR protocol is therefore the sum of the relative levels of all the products present. For this reason, it is necessary to perform a melting curve analysis at the end of the PCR reaction. This is achieved by slowly increasing the temperature

of the samples (0.2 °C per second) to a maximum of 95 °C. A gradual loss of fluorescence will be observed throughout the melting process, with a fast loss of fluorescence at the melting temperature of the amplicon (Lekanne Deprez RH *et al*., 2002). The shape of the melting curve and the position of the melting peak of any given PCR product are determined by GC/AT ratio, size and sequence of the product (Ririe KM *et al*., 1997), and is therefore a unique feature of each product. The melting curve can therefore be used to distinguish between different amplification products separated by as little as 2 °C (Ririe KM *et al*., 1997).

Ideally, the melting curve should only contain one sharp peak of relatively high amplitude. Additional peaks, and "shoulders" on either side of the main peak, indicate the presence of additional PCR products, such as fortuitous non-specific products and primer-dimers. These will contribute to the measurable fluorescence in the sample, and will interfere with quantification of the specific product. The size of the PCR product can be confirmed by analysing the PCR samples by agarose gel electrophoresis.

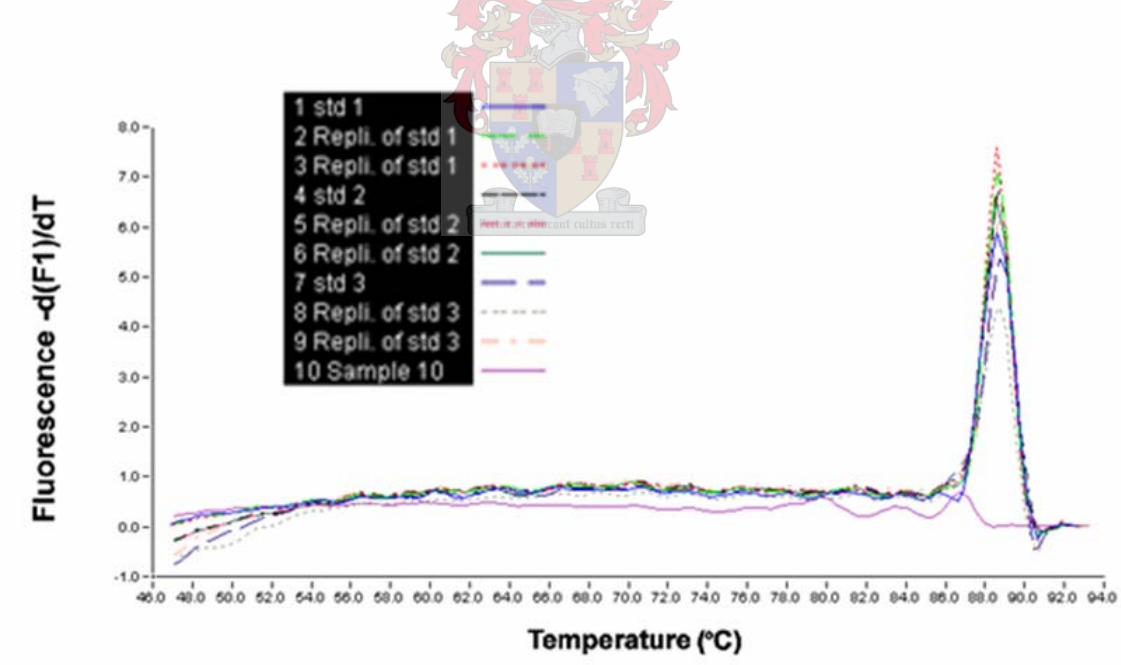


Figure A1: Example of melting curve analysis generated by the LightCycler software for β-actin amplification products. PCR conditions were as described for the β-actin primer set in section 2.9.2. For this experiment, a 5-fold dilution series of a control cDNA sample was prepared and amplification of each dilution was performed in triplicate. Std 1 = 0.4 µg cDNA template, std 2 = 0.08 µg cDNA, std 3 = 0.016 µg cDNA. Sample 10 (purple curve at the bottom) contained no template, and therefore served as negative control.

A2.6 Important considerations when designing a RT-PCR protocol:

1. **RNA integrity:** confirm integrity of RNA on a denaturing agarose gel before reverse transcription.
2. **Primer purity:** Highly purified salt-free (HPSF) or HPLC-purified primers are recommended.
3. **Primer sequence:** Check different primer sets for the formation of primer dimers and non-specific products. Analyse PCR products by melting curve analysis, agarose gel electrophoresis and DNA sequencing.
4. **Annealing temperature:** Usually 5 degrees below the melting temperature (T_m) of the primer.
5. **Extension time:** For LightCycler real-time PCR, an elongation time of 1 second per 25 base-pairs of amplicon is recommended.
6. **Product size:** A product size between 150 and 750 base-pairs is recommended.
7. **$MgCl_2$ concentration:** As with any PCR protocol, this needs to be optimised for the specific template and primer set. However, with the LightCycler FastStart DNA Master PLUS SYBR Green I kit, no optimisation of $MgCl_2$ is necessary.
8. **Template concentration:** Too high concentrations of cDNA template can lead to "template inhibition", where the plateau phase is reached too early during the amplification. On the other hand, low template concentrations tend to have higher intra-assay variability (duplicate amplifications of the same sample) (Wong ML and Medrano JF, 2005; personal observation). The optimal template concentration should therefore be determined for each template/primer set, by means of a template dilution series. In the present study, 0.4 µg cDNA was used in each PCR reaction.
9. **Inter-assay variation:** It was established by Marino JH *et al*. that data collected from the same samples on different days varied significantly. Thus, for maximum accuracy, it is recommended that all data for a specific amplicon must be obtained on the same day, using the same reaction mix (in this case the LightCycler Master Mix) (Marino JH *et al*., 2003).

A2.7 A typical LightCycler real-time PCR protocol

The Roche FastStart Taq polymerase is complexed with blocking groups that can be removed by pre-incubating the PCR samples at 95 °C for 10 minutes. During this step, no fluorescence is measured and no analysis is performed. For the amplification cycles, denaturation takes place at 95 °C, the annealing temperature is determined by the primers (see above) and the extension temperature is typically 72 °C. A single fluorescence measurement is taken at the end of each extension step. Throughout the PCR protocol, the temperature transition rate is set at 20 °C per second, except during the melting curve analysis, when it is set at 0.2 °C per second. Fluorescence is measured throughout the melting step, to generate the melting curve. (The LightCycler FastStart DNA Master PLUS SYBR Green I kit instruction manual gives a detailed example of how to set up a typical LightCycler PCR protocol.)

A3 Relative quantification of RT-PCR products

A3.1 Calculation of amplification efficiency

For relative quantification, the amplification efficiency of each template/primer set has to be determined over a broad range of template concentrations. This is done by generating serial dilutions of a single cDNA sample, reverse transcribed from total RNA isolated from unstimulated cells. Data obtained for the dilution series can then be analysed with Roche LightCycler software, using the "Fit Point Method". A dilution curve is generated by plotting crossing points (Y-axis) against log of cDNA concentration (X-axis). The slope of the curve, as calculated by the LightCycler software, can be used to calculate the PCR efficiency, by means of the following equation (Pfaffl MW, 2001):

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

Efficiency determination via a dilution curve assumes that a given amplicon will always amplify at the same efficiency as determined by the dilution analysis, in other words that the efficiency will not be influenced by the experimental conditions. However, this might not always be the case (discussed in Marino JH *et al*., 2003). For this reason, it would be ideal to generate the dilution curve, as well as all the data to be analysed, on the same day (the so-called "day effect") (Marino JH *et al*., 2003).

However, this is clearly not always practical, and so it is recommended that the PCR amplification efficiency at least be measured on different days, using independent cDNA samples.

A3.2 Calculation of relative expression levels

The relative expression of a target gene can be determined by the amplification efficiency of the target transcript, and the change in CP between an unknown sample and a control sample. This is expressed relative to the levels of a reference transcript in the same sample. For relative quantification using the LightCycler software, the "Fit Point Method" is used, measuring crossing points at a constant fluorescence level. With this method, the threshold fluorescence, and therefore the total amount of double-stranded DNA in the sample, is identical for all samples (Pfaffl MW, 2001).

The relative expression (*R*) of a target gene can be calculated by using the following equation:

$$R = \frac{(E_t)^{\Delta CP\ (control\ -\ sample)}}{(E_r)^{\Delta CP\ (control\ -\ sample)}} \qquad (2)$$

E_t is the amplification efficiency of the target transcript, and E_r the efficiency of the reference transcript. The difference in crossing points (ΔCP) between the control and the sample (in these experiments, the unstimulated and the stimulated samples, respectively) is determined for the target transcript and the reference transcript (Pfaffl MW, 2001).

A4 Measuring GnRHR mRNA levels in LβT2 cells by LightCycler real-time RT-PCR

A4.1 GnRHR primer design

G-protein coupled receptors have highly conserved amino acid sequences in their transmembrane domains, with more receptor-specific sequences in the intra- and extracellular loop regions (Probst WC *et al*., 1992). Therefore, in order to detect mouse GnRHR mRNA, primers had to be complementary to loop region sequences, to minimize the risk of amplifying non-GnRHR transcripts. Furthermore, in principle, primers used in RT-PCR should ideally span exon-exon boundaries, in order to exclude the amplification of non-spliced transcripts or genomic DNA.

Five possible GnRHR primer combinations were considered:

1) S1/AS2, with a product size of 212 bp, spanning exon1/exon 2;
2) S2/AS1, product size 139 bp, spanning exon 1/exon 2;
3) S4/AS4, product size 192 bp, spanning exon2/exon 3;
4) S5/AS4, product size 85 bp, spanning exon 2/exon 3;
5) S5/AS5, product size 205 bp, spanning exon 2/exon 3.

It is important to note that quantification of mouse GnRHR mRNA levels by means of real-time RT-PCR has not been reported in the literature. For this reason, GnRHR gene-specific primers had to be specially designed for the present study. Primer sequences were obtained from W van Biljon, previously from the same research group as the present author (personal communication).

Primer set S2/AS1 was excluded, due to a very low GC content of individual primers (45.8% and 50%, respectively). Primer set S5/AS4 was also excluded, since the PCR product was too small (the recommended LightCycler PCR product size is 150-750 base-pairs). Primer set S4/AS4 was finally chosen, because of the high GC content of the individual primers, the similarity in the melting points of the individual primers and the suitable size of the PCR product.

A4.2 Reference gene

As already explained, the expression levels of a target transcript are related to the expression levels of a reference (unregulated) transcript in the same sample. The housekeeping gene β-actin was chosen as reference gene, since it has been demonstrated by others that β-actin transcript levels are not regulated by GnRH in LβT2 cells (Kakar SS *et al*., 2003). The β-actin primer sequences were obtained from Herbert DR *et al*., 2004.

Primer name	Primer sequences (5' – 3')
mGnRHR S4	CCACAGTGGTGGCATCAGGCCTTC
mGnRHR AS4	TAGCGTTCTCAGCCGAGCTCTTGG
β-actin (s)	TGGAATCCTGTGGCATCCAGAAAC
β-actin (a)	TAAAACGCAGCTCAGTAACAGTCCG

Table A.1: Sequences of gene-specific primers used in RT-PCR.

A4.3 Reverse transcription

Total RNA was isolated with Tri-reagent (Sigma) (Chomczymski P, 1993), and the final pellet was dissolved in RNAse-free H_2O, since Formazol inhibits reverse transcriptase activity. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis. Synthesis of cDNA was performed with M-MLV Reverse Transcriptase (RNase H minus)(Promega), according the product protocol. One microgram of total RNA was mixed with 1 µg of oligo(dT) (20-mer) and RNAse-free H_2O, in a final volume of 14 µl. This was incubated for 5 minutes at 70 °C, to denature secondary structures in the RNA. Afterwards, 5 µl dNTP mix (10 mM; Sigma), 5 µl M-MLV RT 5X reaction buffer (Promega) and 1 µl (200 units) enzyme was added, and the reaction mix incubated at 42 °C for 60 minutes. Samples were stored at -20 °C.

A4.4 Optimization of LightCycler PCR conditions

Note: all primers were used at a final concentration of 0.5 µM, as recommended by the LightCycler FastStart SYBR Green I kit instruction manual. Annealing temperatures were calculated as 5 degrees Celsius below the melting temperature (T_m) of the primers. Elongation times were calculated as (product size in bp)/ 25 = elongation time in seconds, as recommended by Roche.

As already stated in the previous section, the amplification efficiency of each template/primer set had to be determined. A 5-fold cDNA dilution series was generated, ranging from 1 µg/µl to 0.008 µg/µl. **In order to minimize dilution errors being transferred to subsequent dilutions, each diluted sample was equilibrated at room temperature for 20 minutes, before the next dilution was made.**

Each PCR reaction contained the following, in a total volume of 20 µl:

cDNA template	2 µl
sense primer (5 µM)	2 µl
antisense primer (5 µM)	2 µl
LightCycler SYBR Green I Master Mix	4 µl
PCR H_2O	10 µl

The SYBR Green I Master mix was prepared by adding 13 µl FastStart Taq polymerase to a thawed vial of SYBR Green I. **(NB This has to be protected from light.)** Capillary tubes were chilled inside centrifuge adapters, on an aluminium cooling block (Roche). The PCR reaction components, excluding the cDNA, were mixed and 18 µl was pipetted into each capillary tube (Roche). Subsequently, 2 µl of the relevant cDNA sample was pipetted into each capillary. For the dilution series, the final amount of template in the reactions was therefore 2 µg, 0.4 µg, 0.08 µg and 0.016 µg, respectively. Capillaries were capped and centrifuged for 5 seconds at 3000 rpm, to collect the samples in the bottom of the capillaries.

For the GnRHR S4/AS4 primer set, the slope of the dilution series curve, as determined in two independent experiments, was -3.477 and -3.558, respectively. When substituted into equation (1) of section 3.4.1.2, this translated to PCR amplification efficiency of 1.93 and 1.91, respectively. For all subsequent experiments, an amplification efficiency of 1.92 was assumed. It was also determined that, for this primer set, 0.4 µg of cDNA template yielded the best results, generating high levels of specific product without “template inhibition” (inhibition of amplification by excessively high template concentrations).

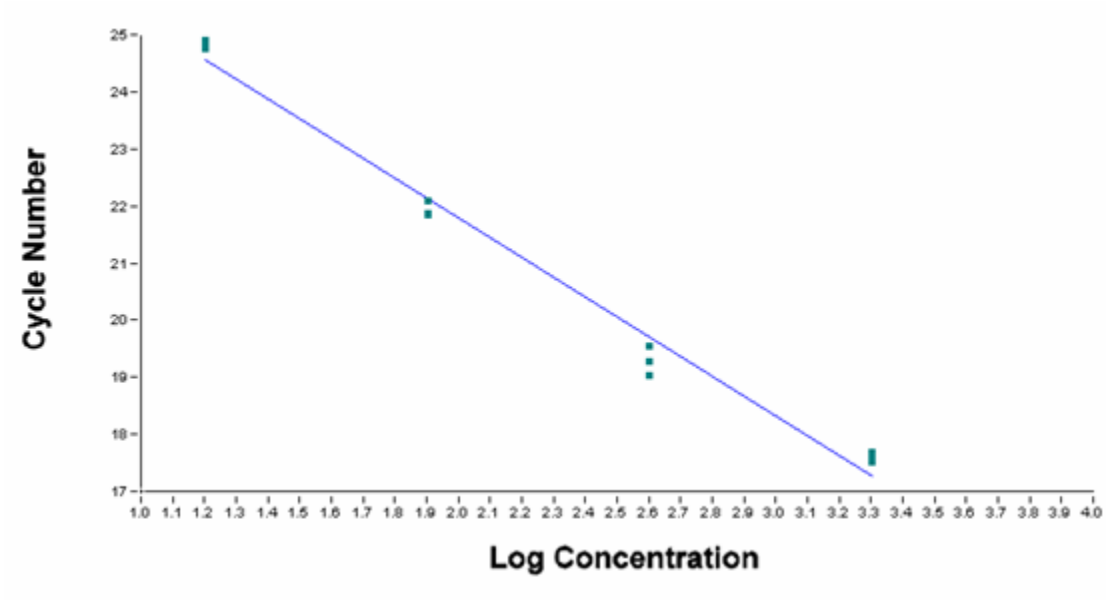


Figure A.2: Example of dilution series data generated by LightCycler software for GnRHR amplification products. A 5-fold dilution series of cDNA template (2; 0.4; 0.08; 0.016 µg) was amplified using the GnRHR S4/AS4 primer set. The PCR conditions were as described in section 2.9.2. The slope of the curve (-3.477) was used to calculate the amplification efficiency with equation (1).

The amplification efficiency with the β-actin primer set was determined using the method described above, and was found to be 1.92.

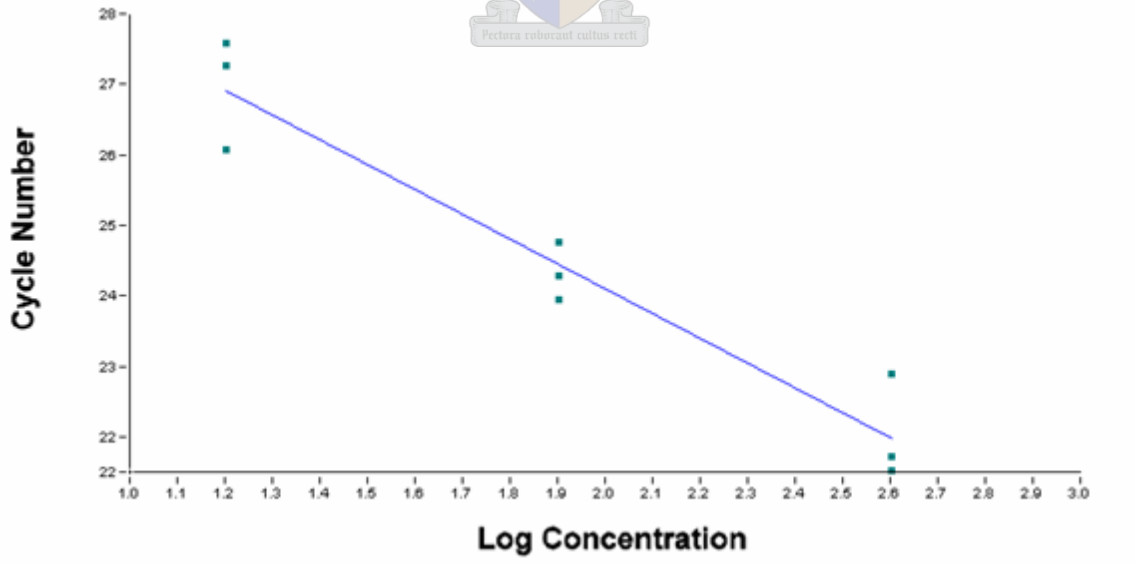
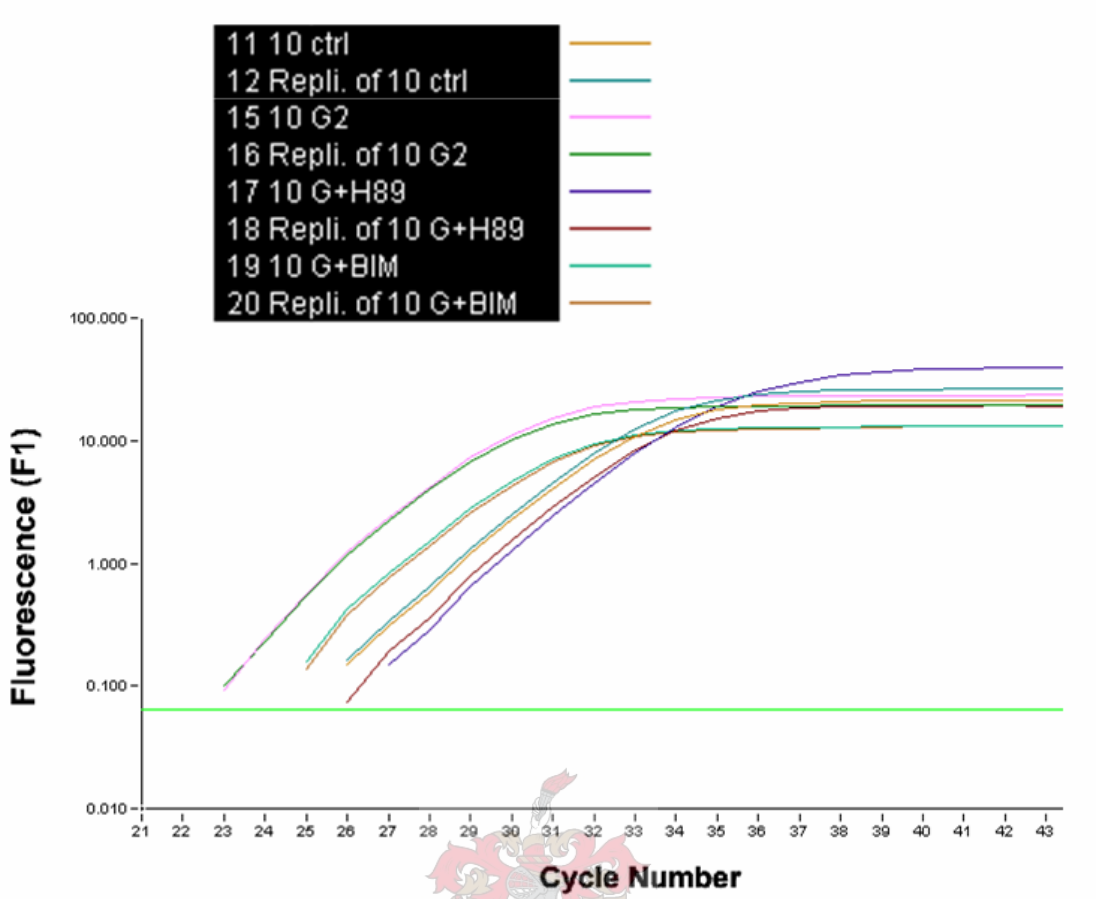


Figure A.4: Example of dilution series data generated by LightCycler software for β-actin amplification products. A 5-fold dilution series of cDNA template (0.4; 0.08; 0.016 µg) was amplified using the β-actin s/a primer set. The slope of the curve (-3.530) was used to calculate the amplification efficiency with equation (1). PCR conditions were as described in section 2.9.2.



Conditions	GnRHR	β-actin
Vehicle	24.86; 24.76	16.85; 20.67
GnRH	22.63; 22.48	21.54; 21.46
GnRH+H89	25.67; 25.87	20.31; 20.25
GnRH+BIM	24.12; 24.29	21.56; 21.84

Figure A.5: Example of data obtained by LightCycler PCR. Graph shows typical amplification curves generated by the LightCycler software with the GnRHR S4/AS4 primer set. Crossing points measured by the LightCycler software are shown in the table. Crossing points measured with the β-actin primer set for the same cDNA samples are also shown. All samples were amplified in duplicate; these duplicate values are grouped in the table. The error between duplicates for the same sample should ideally not be more than 0.5. PCR conditions were as described in section 2.9.2. Samples were as follows: ctrl = vehicle-treated; G2 = 100 nM GnRH; G+H89 = 100 nM GnRH plus 1 μM H89; G+BIM

= 100 nM GnRH plus 100 nM BIM. All samples were treated for 8 hours.The final results of this experiment can be seen in figure A.6.

The same cDNA dilution samples (0.2 µg/µl) were used for determining transcript levels of both the target gene (GnRHR) and the reference gene (β-actin), so that the reference gene could serve as a true internal control.

From the data in figure A.5, relative expression can be calculated as follows:

For instance, to calculate the effect of the PKA inhibitor H89 on the GnRH-mediated increase in GnRHR transcript levels, the average crossing points for each condition are used (these averages are calculated by the LightCycler software, but the calculations are shown below for clarity):

GnRH-treated: average CP (GnRHR) = (22.63 + 22.48)/2 = 22.56

average CP (β-actin) = (21.54 + 21.46)/2 = 21.50

GnRH + H89: average CP (GnRHR) = (25.67 + 25.87)/2 = 25.77

average CP (β-actin) = (20.31 + 20.25)/2 = 20.28

To calculate fold differences in GnRHR transcript levels, the GnRH-treated sample would be the "sample", and the sample treated with GnRH plus H89 would be the "control" (these calculations can be performed for any control/sample set):

ΔCP_{GnRHR} (control – sample) = 25.77 – 22.56 = 3.21

$\Delta CP_{\beta\text{-actin}}$ (control – sample) = 20.28 – 21.50 = -1.22

The amplification efficiency for both the GnRHR and β-actin primer sets was determined to be 1.92. When all these values are substituted into equation (2),

$$R = \frac{(E_t)^{\Delta CP\ (control\ -\ sample)}}{(E_r)^{\Delta CP\ (control\ -\ sample)}} \qquad (2)$$

then $R = (1.92^{3.21}) / (1.92^{-1.22}) = 8.117 / 0.451 = 17.998$

This means that the presence of H89 resulted in a 18-fold reduction of GnRHR transcript levels, relative to the change in β-actin levels. When the results of the whole experiment are graphically represented (figure A.6), it can be seen that GnRH caused a ~7.5-fold increase in GnRHR transcript levels. In the sample treated with GnRH plus H89, GnRHR transcript levels were ~0.4, relative to the control, which translates to a 18-fold decrease in levels between the GnRH-treated sample and the sample treated with GnRH plus H89.

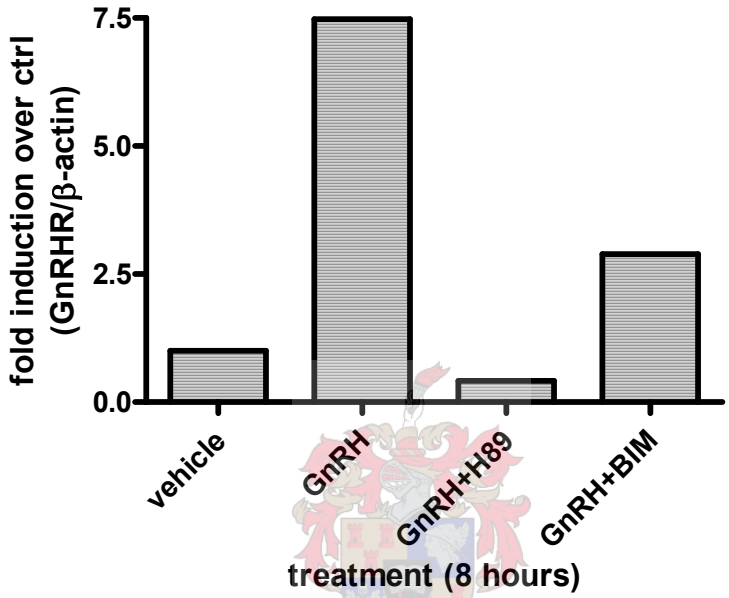


Figure A.6: Final results calculated from the data in figure A.5. For all samples, GnRHR transcript levels (normalized to β-actin levels) were expressed relative to GnRHR transcript levels in the vehicle-treated sample, which was set as 1. These results were obtained from single samples, and were combined with data from other independent experiments, as presented in figure 4.4.

ADDENDUM B

OLIGONUCLEOTIDE AND PRIMER SEQUENCES

All sequences are given in the 5'-3' orientation. For each oligonucleotide, "s" denotes the sense strand, and "a" the antisense strand. All numbering refers to the position relative to the translation start site.

B1: Oligonucleotide probes used in electrophoretic mobility shift assays

Transcription factor consensus recognition sequences are underlined, and mutated bases are indicated in lowercase. Some oligonucleotides contained a 5' GG overhang to improve radioactive labeling efficiency; these are also indicated in lowercase.

-15/-7NRSs	GAAGCCTGTCCTTGGAGAAA
-15/-7NRSa	ggTTTCTCCAAGGACAGGCTTC
-15/-7NRSms	GAAGCCTGTttTTGGAGAAA
-15/-7NRSma	ggTTTCTCCAAaaACAGGCTTC
-244/-236NRSs	ggTACACTTGGCCTTCAGGAGG
-244/-236NRSa	ggCCTCCTGAAGGCCAAGTGTA
-244/-236NRSms	TACACTgcagCTTCAGGAGG
-244/-236NRSma	ggCCTCCTGAAGctgcAGTGTA
NBREs	CGCGGGACTTTGACCTTCCCGGCA
NBREa	TGCCGGGAAGGTCAAAGTCCCGCG
mgCREs	ggACCTGTGACGTTTCCATCTA
mgCREa	TAGATGGAAACGTCACAGGT
AP1s	ggGATATTATGAGTCACTTTCGA
AP1a	TCGAAAGTGACTCATAATATC

B2: Primers used in PCR protocols

Mutated bases are indicated in lowercase. Unless indicated otherwise, transcription factor consensus recognition sequences are underlined. Please refer to footnotes for additional primer information.

Primers used for PCR mutagenesis

1S[1]	CTTGGAGAAAG*ATC*TCTAACAATGCATC
2S[1]	GAAGCCTGTttTTGAGAAAG*ATC*TCTAACAATGC
GL1[2]	TGTATCTTATGGTACTGTAACTG
GL2[2]	CTTTATGTTTTTGGCGTCTTCCA
-244/-236mps[3]	AGGGGCTACGGTTACACTgcagCTTCAGGAGGGCTTGGC
-244/-236mpa[3]	GCCAAGCCCTCCTGAAGctgcAGTGTAACCGTAGCCCCT
PCRmcres[4]	AAATGCTAACCTGgaAttcgcCCATCTAAAGGAGG
PCRmcrea[4]	CCTCCTTTAGATGGgcgaaTtcCAGGTTAGCATTT
PCRmAP1s[5]	AATTGGATGATATTAgGAaTtcCTTTCGACATCAG
PCRmAP1a[5]	CTGATGTCGAAAGgaAtTCcTAATATCATCCAATT

Primers used for LightCycler real-time RT-PCR

GnRHR S4	CCACAGTGGTGGCATCAGGCCTTC
GnRHR AS4	AGCGTTCTCAGCCGAGCTCTTGG
β-actins	TGGAATCCTGTGGCATCCAGAAAC
β-actina	TAAAACGCAGCTCAGTAACAGTCCG

[1] The mutated bases replacing the original start codon are in italics

[2] Promega Corporation pGL2-basic vector-specific primers

[3] Primer position: -262 to -223

[4] Primer position: -120 to -86

[5] Primer position: -353 to -319

ADDENDUM C

BUFFERS AND MIXES

Note that components in *italics* were added to buffers immediately before use

C1: Buffers used in preparation of nuclear extracts

Buffer A (10 ml):

	[final]	volume added	[stock]
HEPES	10 mM	400 µl	0.25 M (pH 7.9)
KCl	10 mM	50 µl	2 M
EDTA	0.1 mM	2 µl	0.5 M (pH 8.0)
EGTA	0.1 mM	10 µl	0.1 M
H_2O		9088 µl	
PMSF	*0.5 mM*		
DTT	*1 mM*		
Glycerol-phosphate	*1 mM*		
Na_3VO_4	*1 mM*		
NaF	*10 mM*		

This buffer, minus the components in italics, was stored at 4 °C. For 2 ml complete Buffer A, the following components were mixed just before use (stock concentrations are indicated in brackets):

1910 µl buffer mix

10 µl PMSF (0.1 M)

20 µl DTT (0.1 M)

20 µl glycerol-phosphate (0.1 M)

20 µl Na_3VO_4 (0.1 M)

20 µl NaF (1 M)

Buffer C (10 ml):

	[final]	volume added	[stock]
HEPES	20 mM	800 µl	0.25 M (pH 7.9)
NaCl	400 mM	800 µl	5 M
EDTA	1 mM	20 µl	0.5 M (pH 8.0)
EGTA	1 mM	100 µl	0.1 M
H_2O		7780 µl	
DTT	*1 mM*		
PMSF	*1 mM*		
Glycerol-phosphate	*1 mM*		
Na_3VO_4	*1 mM*		
NaF	*10 mM*		

This buffer, minus the components in italics, was stored at 4 °C. For 500 µl complete Buffer C, the following components were mixed just before use (stock concentrations are indicated in brackets):

475 µl buffer

5 µl DTT (0.1 M)

5 µl PMSF (0.1 M)

5 µl glycerol-phosphate (0.1 M)

5 µl Na_3VO_4 (0.1 M)

5 µl NaF (1 M)

Tris buffered saline (TBS) (100 ml):

	[final]	volume added	[stock]
Tris	25 mM	2.5 ml	1 M (pH 7.4)
NaCl	137 mM	2.74 ml	5 M
KCl	2.7 mM	0.135 ml	2 M
H_2O		94.625 ml	

Phosphatase inhibitors:

NaF: Make up to 1 M in H_2O, aliquot, store at -20 °C.

Na_3VO_4: Make up to 0.1 M in H_2O. Adjust pH to 10 with HCl (dropwise), solution will turn yellow. Place in a boiling water bath for 10 minutes, until solution becomes clear. Aliquot, store at -20 °C (Gordon JA, 1991).

Glycerol-phosphate (alternative substrate for phosphatases)**:** Make up to 0.1 M in H_2O, aliquot, store at -20 °C.

C2: EMSA sample buffers, reaction mix and poly-acrylamide gel mix

For sample buffers, the calculations were as follows: The nuclear extracts (in buffer C) and the sample buffer contribute to the final buffer conditions in the EMSA sample. 1.5 µl nuclear extracts are added to a final sample volume of 5 µl, therefore the concentrations of the components are diluted 1.5 / 5. The sample buffer (SB) has to contribute the remainder of the final buffer components, but since only 0.7 µl of this buffer is added in a final volume of 5 µl, the concentrations of the components of the sample buffer have to be multiplied by 5 / 0.7.

Nuclear receptor (SF-1 / Nur77) binding (adapted from Horn F *et al.*, 1992):

[final]	Nuclear extracts (buffer C)	1.5 / 5	SB	5 / 0.7	Volume added (2 ml)	[stock]
Hepes 20 mM	20 mM	6 mM	14 mM	100 mM	800 µl	0.25 M
NaCl 120 mM	400 mM	120 mM	-	-	-	-
EDTA 1 mM	1 mM	0.3 mM	0.7 mM	5 mM	20 µl	0.5 M
EGTA 0.3 mM	1 mM	0.3 mM	-	-	-	-
$MgCl_2$ 1.5 mM	-	-	1.5 mM	10.7 mM	21.42 µl	1 M
KCl 20 mM	-	-	20 mM	142.8 mM	142.8 µl	2 M
Glycerol 5.6%	-	-	5.6%	40%	1005.78 µl	80%
DTT 3.5 mM	*1 mM*	*0.3 mM*	*3.2 mM*	*22.9 mM*	*45.8 µl*	*1 M*
PMSF 0.68 mM	*1 mM*	*0.3 mM*	*0.38 mM*	*2.7 mM*	*54 µl*	*0.1 M*

Add 0.5 µl DTT (1M) and 0.5 µl PMSF (0.1 M) to 19 µl sample buffer just before use.

CREB binding (adapted from Cheng KW and Leung PCK, 2001):

[final]	Nuclear extracts (buffer C)	1.5 / 5	SB	5 / 0.7	Volume added (2 ml)	[stock]
Hepes 20 mM	20 mM	6 mM	14 mM	100 mM	800 µl	0.25 M
NaCl 120 mM	400 mM	120 mM	-	-	-	-
EDTA 1 mM	1 mM	0.3 mM	0.7 mM	5 mM	20 µl	0.5 M
EGTA 0.3 mM	1 mM	0.3 mM	-	-	-	-
$MgCl_2$ 1.5 mM	-	-	1.5 mM	10.7 mM	21.42 µl	1 M
KCl 20 mM	-	-	20 mM	142.8 mM	142.8 µl	2 M
Glycerol 5.6%	-	-	5.6%	40%	1005.78 µl	80%
DTT 1 mM	*1 mM*	*0.3 mM*	*0.7 mM*	*5 mM*	*10 µl*	*1 M*
PMSF 0.3 mM	1 mM	0.3 mM	-	-	-	-

Add 0.1 µl DTT (1M) to 20 µl sample buffer just before use

AP-1 binding (adapted from White BR *et al.*, 1999):

[final]	Nuclear extracts (buffer C)	1.5 / 5	SB	5 / 0.7	Volume added (2 ml)	[stock]
Hepes 20 mM	20 mM	6 mM	14 mM	100 mM	800 µl	0.25 M
NaCl 120 mM	400 mM	120 mM	-	-	-	-
EDTA 0.3 mM	1 mM	0.3 mM	-	-	-	-
EGTA 0.3 mM	1 mM	0.3 mM	-	-	-	-
Glycerol 5%	-	-	5%	35.7%	893 µl	80%
DTT 0.5 mM	*1 mM*	*0.3 mM*	*0.2 mM*	*1.5 mM*	*30 µl*	*0.1 M*
PMSF 0.3 mM	1 mM	0.3 mM	-	-	-	-
H_2O					277 µl	

Add 0.3 µl DTT (0.1 M) to 19.7 µl buffer just before use.

Sample mix: 0.7 µl sample buffer

0.5 µl poly-d[I-C] (1 µg/µl)

0.3 µl BSA (20 µg/µl)

1 µl radiolabelled probe (0.1 pmol doublestranded oligonucleotide)

1.5 µl nuclear extracts

1 µl H_2O / competitor oligo / antibody

NB: The sample buffer, poly-d[I-C] and BSA were mixed as a cocktail (enough for the number of samples plus 2 extra) and 1.5 µl was added to each sample.

Poly-acrylamide gel mix (5%) (40 ml): 6,7 ml acrylamide: bisacrylamide (29:1)

32.5 ml H_2O

400 µl 50X TAE

70 µl TEMED

400 µl 10% AMPS

C3: Buffers used for extraction and analysis of RNA

10X Morpholinopropanesulfonic acid (MOPS) buffer: 0.4 M MOPS, pH 7.0

0.1 M sodium acetate

0.01 M EDTA, pH 8.0

To prepare 1 L: dissolve 83.71 g MOPS salt in 700 ml DEPC-treated H_2O. Add 33.4 ml 3M sodium acetate and 20 ml 0.5M EDTA. Make up to 800 ml in DEPC-treated H_2O, adjust pH to 7.0 with 10M NaOH. Adjust volume to 1 L, wrap bottle in foil and autoclave.

Denaturing formaldehyde gel mix (1%, 50 ml):

Dissolve 0.5 g agarose in 30 ml DEPC-treated H_2O by boiling in a microwave. Mix 5 ml 10X MOPS buffer, 2.79 ml formaldehyde and 12.21 ml DEPC-treated H_2O, and add to agarose solution. Mix well and pour.

Sample loading dye: 1 mM EDTA, pH 8.0

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

50% (v/v) glycerol

Sample loading buffer (for RNA dissolved in H_2O; mix and add 15 µl per sample):

2.5 µl 10X MOPS buffer

4 µl formaldehyde

12.5 µl formamide

Incubate samples in buffer at 65 °C for 10 minutes, cool on ice for 5 minutes. Add 0.5 µl 10mg/ml ethidium bromide and 2.5 µl loading dye to each sample before loading.

C4: Buffers used for SDS-PAGE and Western blotting

Sample buffer (5X): 5 ml 10% SDS

2ml 80% glycerol

1 ml 1 M Tris (pH 6.8)

0.5 ml beta-mercapto-ethanol

1.5 ml H_2O

Running/blotting buffer (2 litre 10X stock solution): Dissolve 60.6 g Tris base and 288 g glycine in H_2O to a final volume of 2 L.

SDS-PAGE electrophoresis buffer: Mix 200 ml 10X stock solution, 20 ml 10% SDS and H_2O to a final volume of 2 L.

Electroblotting buffer: Mix 200 ml 10X stock solution, 200 ml methanol and H_2O to a final volume of 2 L.

Casein buffer (500 ml): 4,5 g NaCl

2.5 g casein (BDH biochemical grade)

0.6 g Tris base

0.1 g thiomersal (preservative agent) (BDH)

Dissolve the components, excluding the casein, in H_2O. Set the pH at 7.6, add casein and stir overnight at 4 °C.

ADDENDUM D

SITE-DIRECTED PCR MUTAGENESIS

D1: General method

Site-directed PCR mutagenesis utilizes primers with mutated sequences to generate a mutation at a particular site in a DNA sequence. For the mutagenesis procedures performed in the present study, mutations were generated at particular sites in the GnRHR promoter. An existing GnRHR promoter-reporter construct plasmid, i.e. pLG, the wild-type promoter fragment cloned into the *Bgl*II site of the pGL2-basic vector (Pheiffer CP, 1998; also see section 2.2), was used as template (see figure D1). The pGL2-basic vector-specific primers GL1 (sense) and GL2 (anti-sense), overlapping the multiple cloning site of pGL2-basic, were used in two separate initial PCR reactions in combination with overlapping primers containing the mutated promoter sequence in the anti-sense (MPA) and sense (MPS) orientation. In reaction 1, GL1 was used together with MPA, and in reaction 2, GL2 was used together with MPS (see figure D2, step 1).

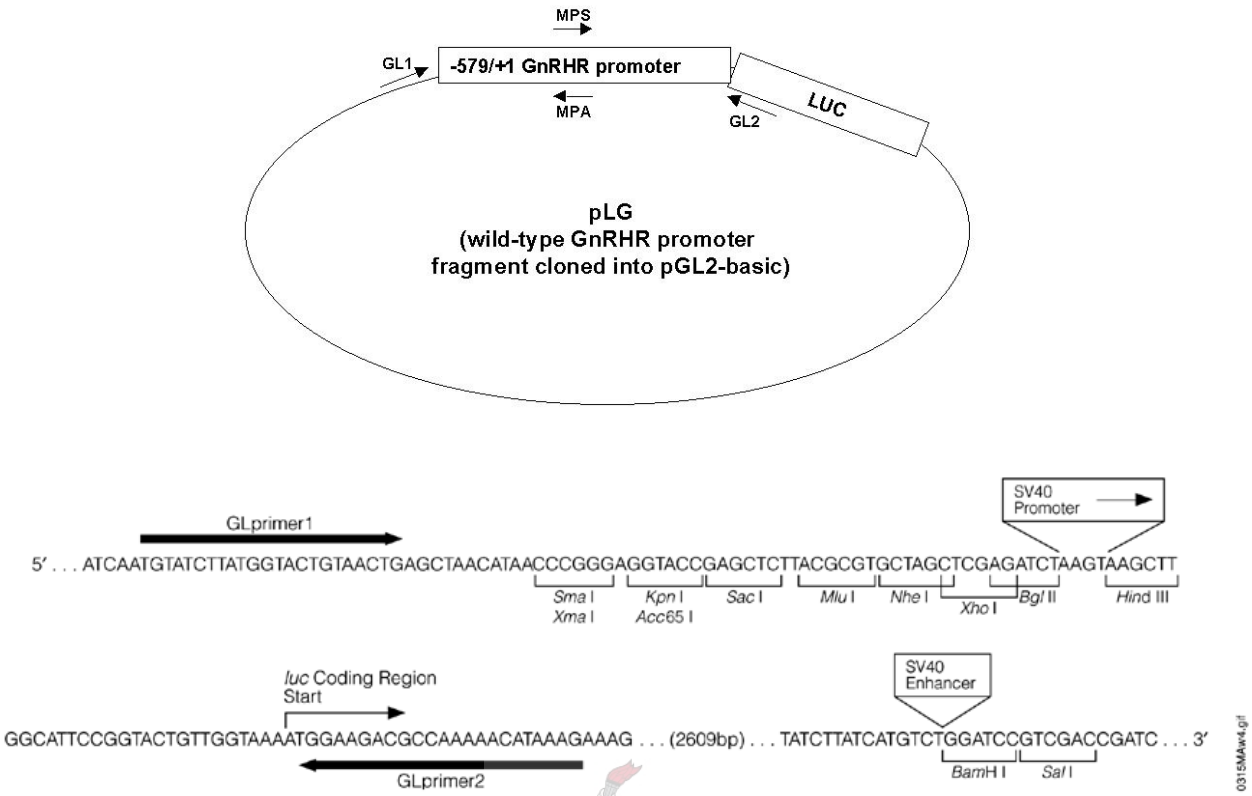


Figure D1: Schematic of the pLG GnRHR promoter-reporter construct. The relative positions of the primers used for PCR site-directed mutagenesis are indicated. GL1 (sense) and GL2 (anti-sense) are pGL2-basic vector-specific primers, located on either side of the multiple cloning site of pGL2-basic. (The detailed sequence of the multiple cloning site and restriction enzyme digestion sites are shown in the lower panel.) MPS (sense) and MPA (anti-sense) are fully overlapping primers containing the mutated sequence that will be incorporated into the promoter.

For example, to generate pLGM2, pLG was used as template in two separate PCR reactions. The first reaction was with the primer set GL1 and -244/-236mpa, generating a PCR product of approximately 300 bp. The second reaction was with the primer set GL2 and -244/-236mps, generating a PCR product of approximately 400 bp (see figure D2, step 1, also see section D2.1 for PCR reaction conditions).

These reactions generated products that overlap in the region of the mutation. The two PCR products were mixed together in a subsequent PCR reaction mix (figure D2, step 2, also see section D2.2 for PCR reaction conditions), along with the GL1 and GL2 vector primers. During the denaturing steps, the single-stranded “short” products annealed at the overlapping mutagenesis site, serving as a

priming step for the elongation of the rest of the strand. The fused PCR product was gel-purified (Macherey-Nagel Nucleospin kit or Sigma GenElute kit), after which the PCR product (insert) (figure D2, step 3A) and the pGL2-basic vector (figure D2, step 3B) were both digested with the same restriction enzymes, i.e.either *Sac*I and *Hind*III, or *Kpn*I and *Bgl*II (see figure D1, lower panel, for position of restriction enzyme digestion sites). The digested vector was treated with calf intestinal alkaline phosphatase (Roche, according to the product protocol), to remove the 5' phosphate groups, so as to prevent vector self-ligation. The insert and vector were gel-purified and ligated with T4 DNA ligase (Promega, according to the product protocol) (figure D2, step 4). The ligation product was transformed into MOSBlue supercompetent cells (AEC Amersham), and the reaction mixture was plated onto LB agar plates containing ampicillin (50 µg/ml) (figure D2, step 5). Single colonies were picked and screened for the presence of the insert by means of PCR colony screening, using primers GL1 and GL2 (see section D2.3 for PCR reaction conditions). The products of these PCR reactions were analysed on a 1% agarose gel to demonstrate the presence of the correct size (~ 700 bp) insert. Clones with the correct size insert were inoculated into liquid LB medium plus ampicillin for plasmid isolation. Isolated plasmids were sequenced to confirm the presence of the correct insert, and to make sure that no other mutations were incorporated during the PCR reactions. Note that, for constructs harbouring one mutation, pLG was used as template. For double mutant constructs, a construct already containing one of the desired mutations was used as template, i.e. for preparation of pLGM1+2, pLGM1 was used as template, and the -244/-236 mutant primers were used to mutate the -244/-236NRS.

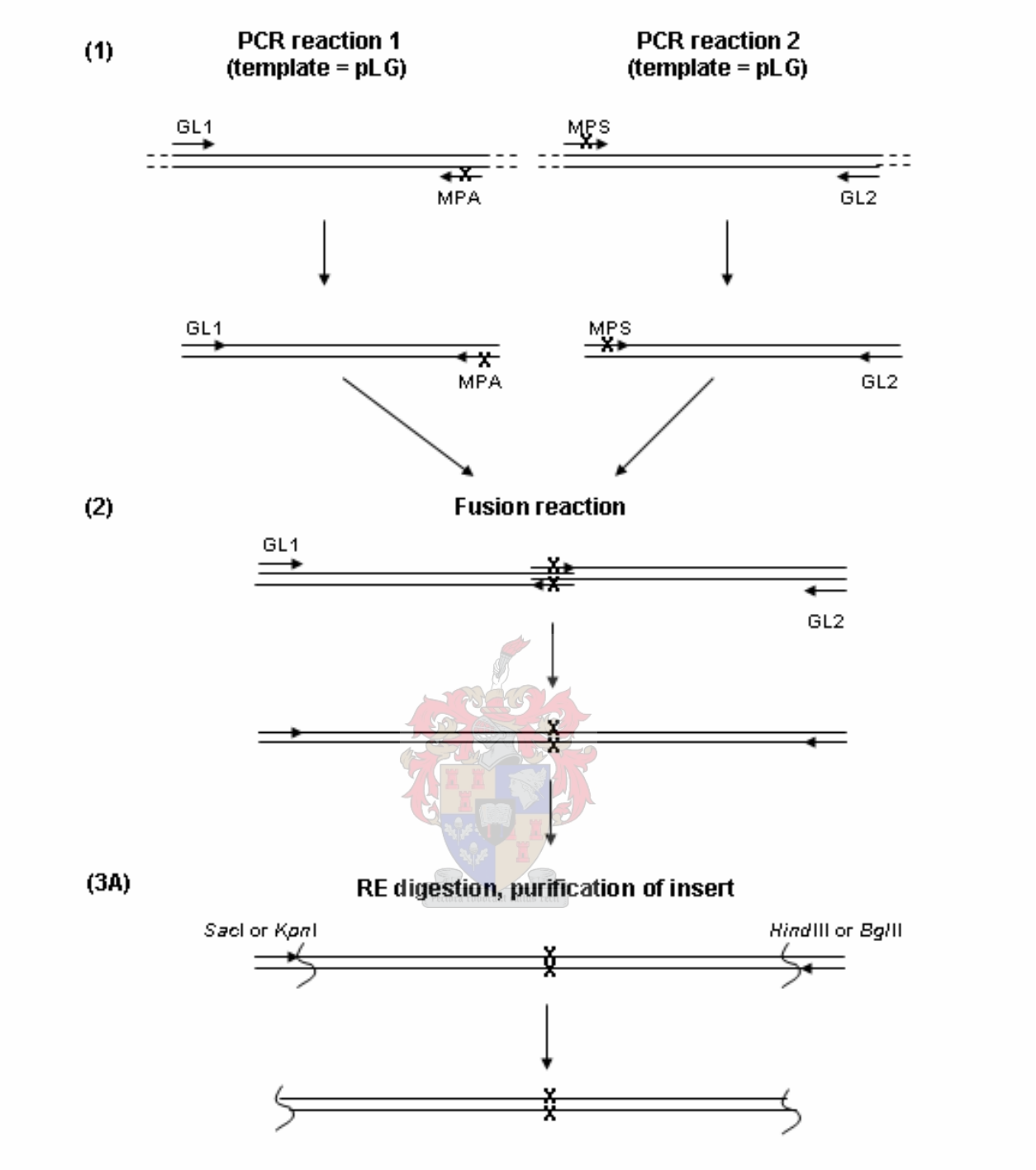
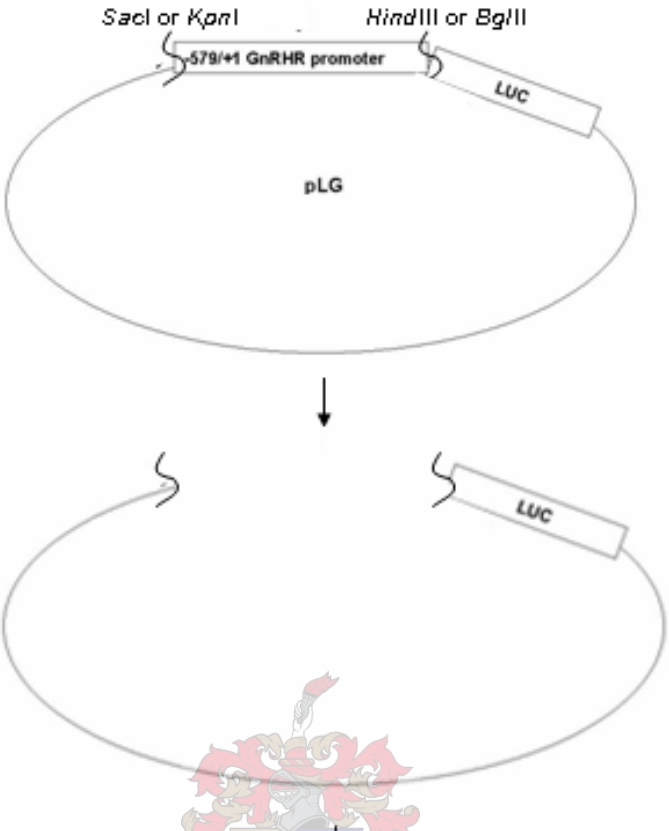
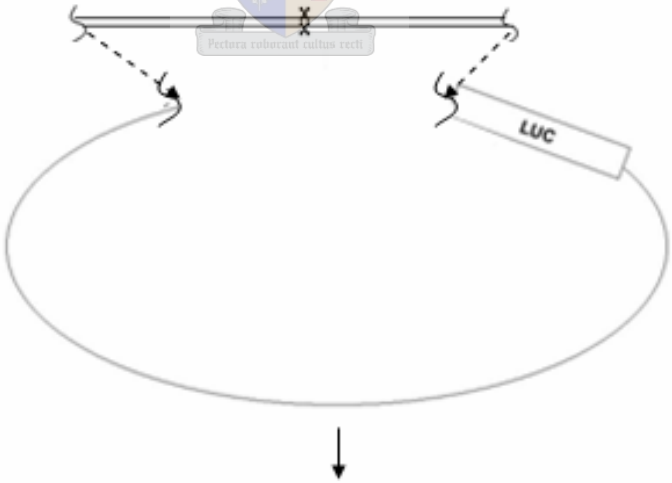


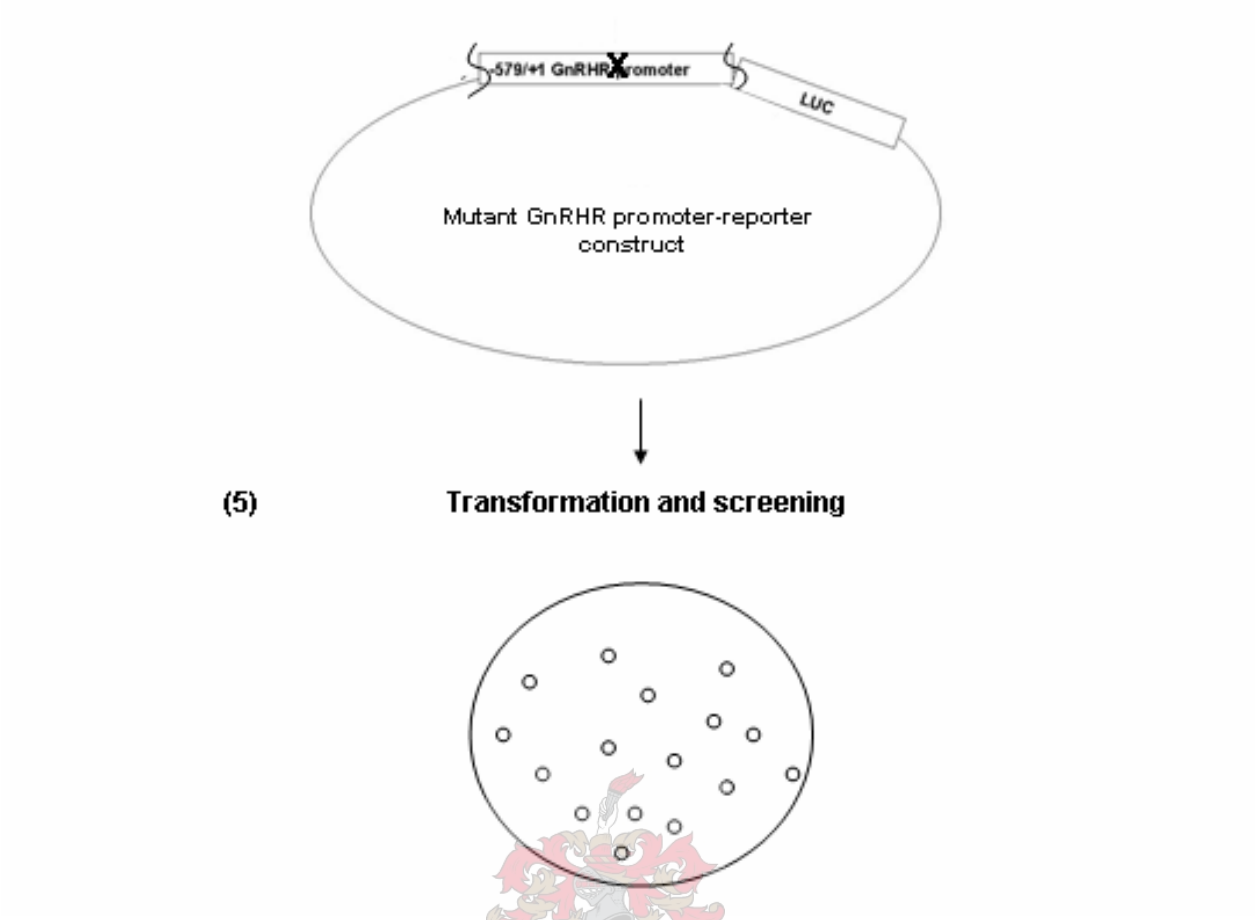
Figure D2: Schematic of PCR mutagenesis protocol (continued on next page).

(3B) **RE digestion, purification of vector**



(4) **Ligation of vector and insert (3A and 3B)**





Legend to figure D2: (1) Two initial separate PCR reactions, each with the pLG as template. Reaction 1 used vector sense primer (GL1) and mutant antisense primer (MPA), while reaction 2 used vector antisense primer (GL2) and mutant sense primer (MPS). Mutated sequences are indicated by **X. (2)** Fusion reaction with products from PCR reaction 1, plus those from PCR reaction 2, using primers GL1 and GL2, followed by gel purification. **(3A)** Restriction digestion and gel purification of fusion PCR product (full-length insert plus GL1 and GL2 sequences, total size ~ 700 bp). **(3B)** Restriction enzyme digestion (to remove existing wild-type insert), alkaline phosphatase treatment and gel purification of the vector. **(4)** Ligation of insert (see 3A) and vector (see 3B). **(5)** Transformation of ligated construct into MOSBlue cells, culturing of transformants on LB agar plates containing ampicillin, and subsequent PCR colony screening using primer GL1 and GL2.

D2: PCR conditions

D2.1 First PCR reactions

400 ng plasmid template (eg pLG)

1 µl sense primer (eg GL1) (20 µM stock solution)

1 µl antisense primer (eg MPA) (20 µM stock solution)

1 µl dNTP mix (10 mM; Sigma)

5 µl 10X Taq polymerase buffer (Promega cat no M190A)

3.4 µl $MgCl_2$ (Promega cat no A351B; stock solution 25 mM, final concentration 1.7 mM)

1 µl Taq polymerase (Promega cat no M186A; 5U/µl)

x µl PCR-grade H_2O

50 µl

Note that 20 µl mineral oil was added to each PCR sample to prevent vaporizing of reaction components.

The PCR program was as follows, after which the samples were frozen:

Temperature (°C)	Time (minutes)	Number of cycles
93	2.5	1
93 55 72	1 1 1	35
72	10	1

The mineral oil was removed from the samples by siphoning the oil off the frozen sample.

D2.2 PCR fusion reaction

10 µl from reaction 1 (D2.1: primer set GL1/MPA)

10 µl reaction 2 (D2.1: primer set GL2/MPS)

1 µl dNTP (10 mM; Sigma)

1 µl GL1 (20 µM stock solution)

1 µl GL2 (20 µM stock solution)

5 µl 10X Taq polymerase buffer (Promega cat no M190A)

3.4 µl $MgCl_2$ (Promega cat no A351B; stock solution 25 mM, final concentration 1.7 mM)

1 µl Taq polymerase (Promega cat no M186A; 5U/µl)

17.6 µl PCR-grade H_2O

50 µl

Note that mineral oil was added, as described in D2.1.

The PCR program was as follows, after which the samples were frozen:

Temperature (°C)	Time (minutes)	Number of cycles
94	0.5	30
72	30	

(Note that this is the PCR protocol that was followed to prepare all the mutant constructs used in chapters 3 and 4. It was subsequently established that the fusion reaction could also be performed by using the PCR program described in D2.1.)

D2.3 Colony screening

Colonies were picked off the agar plates with a sterile tooth-pick and resuspended in 20 µl sterile H_2O. 15 µl bacterial suspension was used to prepare glycerol stocks. Thereafter the following PCR reaction was performed:

5 µl bacterial suspension

9.2 µl PCR-grade H_2O

2 µl 10X buffer Taq polymerase buffer (Promega cat no M190A)

1.2 µl $MgCl_2$ (Promega cat no A351B; stock solution 25 mM, final concentration 1.5 mM)

0.5 µl dNTP (10 mM; Sigma)

1 µl GL1 (20 µM stock)

1 µl GL2 (20 µM stock)

0.125 µl Taq polymerase (Promega cat no M186A; 5U/µl)

20 µl

Mineral oil was added to the samples, as described in D2.1.

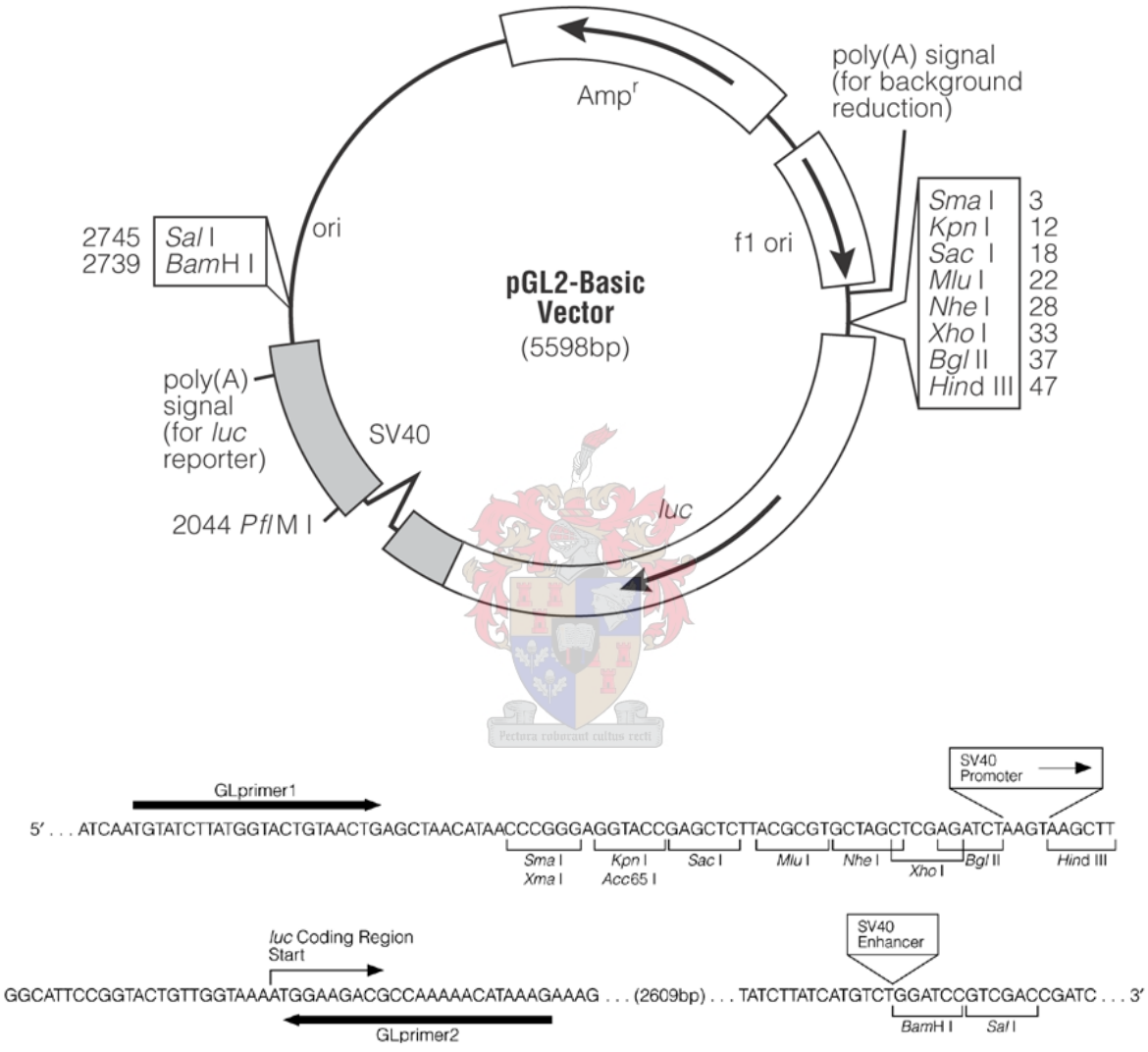
The PCR program was as follows:

Temperature (°C)	Time (minutes)	Number of cycles
94 50 72	1 1 1	35
72	10	1

ADDENDUM E

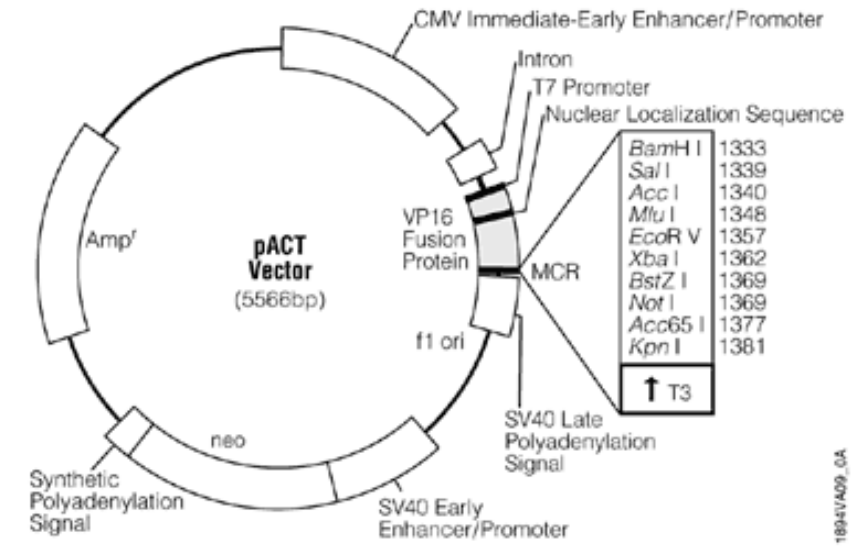
PLASMID MAPS

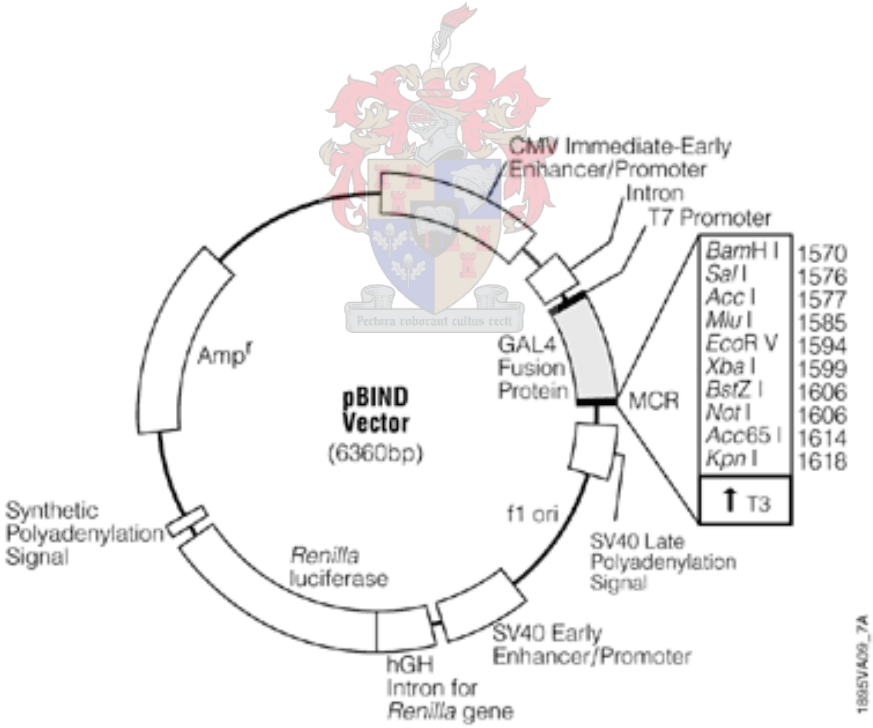
E1: pGL2-Basic vector map and multiple cloning site sequence



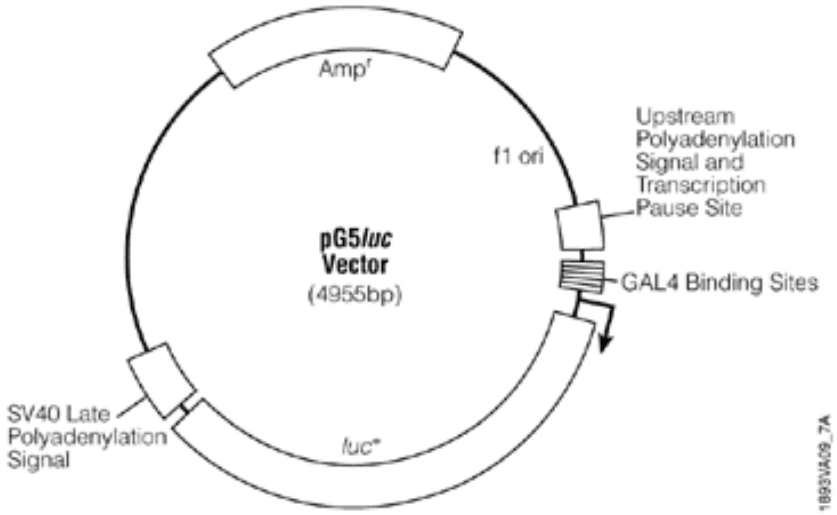
E2: Constructs used in mammalian two-hybrid assays:

E2.1: pACT and pBIND fusion protein expression vectors

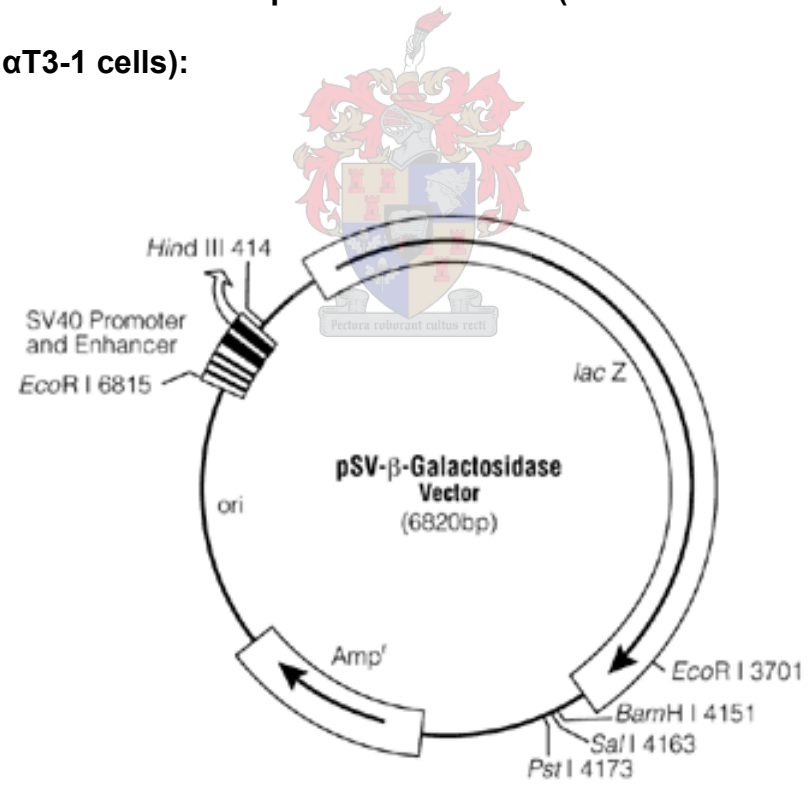




E2.2 pG5*luc* reporter vector



E3: pSV-β-Galactosidase expression vector (for control of transfection efficiency in αT3-1 cells):



E4: pFC-PKA:

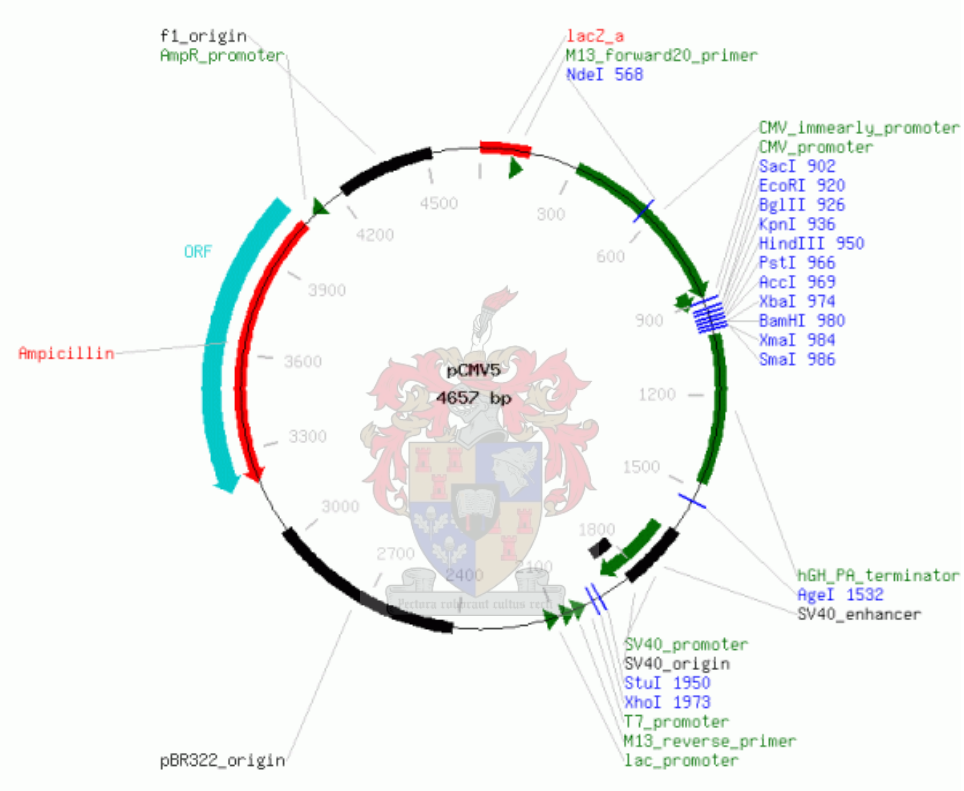
No map was available for this construct. Technical information from Stratagene was as follows:

Size: 7180 bp

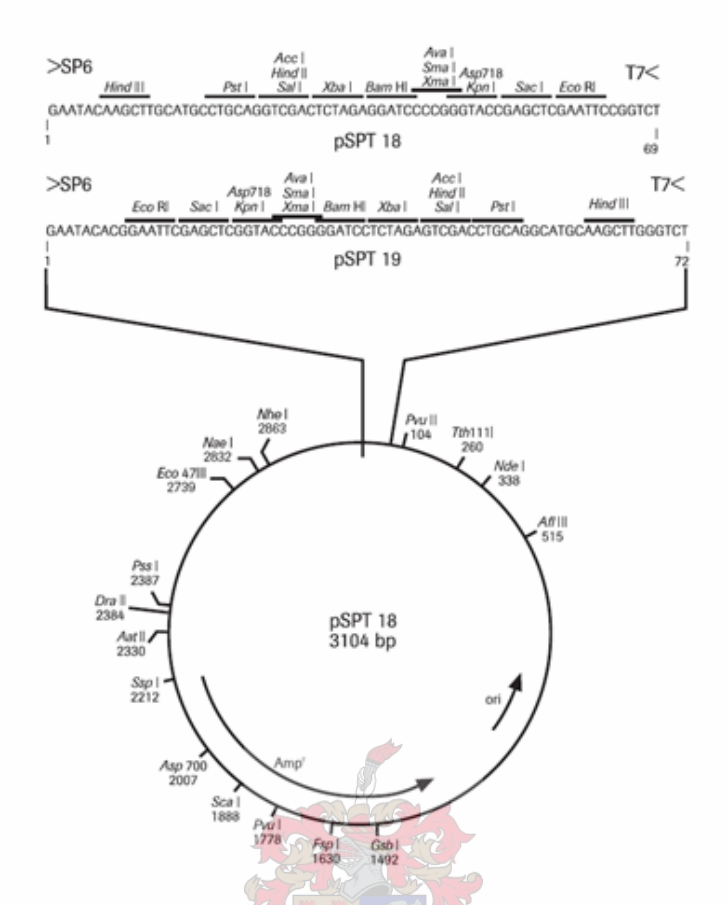
Restriction pattern: *Hind*III, *Xba*I, *Xho*I: 480 bp, 500 bp, 1200 bp, 5000 bp

E5: Other vector backbones used for expression constructs

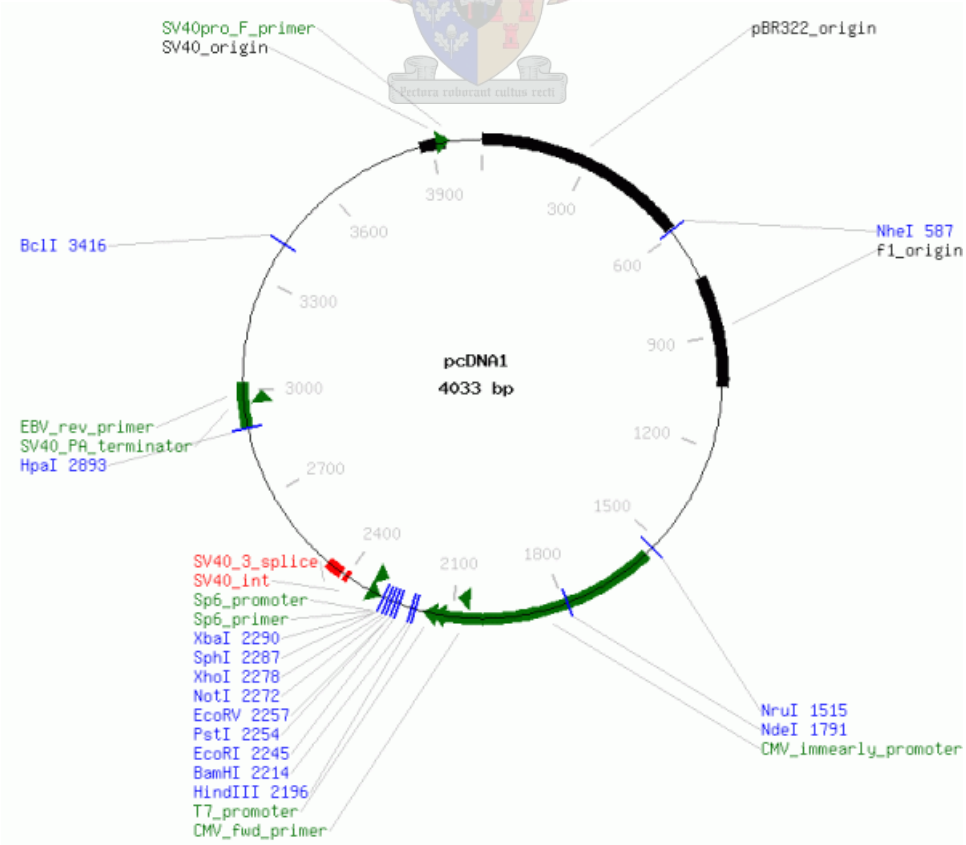
E5.1 pCMV5



E5.2 pSPT 19



E5.3 pcDNA1



BIBLIOGRAPHY

Albarracin CT, Kaiser UB, Chin WW 1994 Isolation and characterization of the 5'-flanking region of the mouse gonadotropin-releasing hormone receptor gene. Endocrinology 135:2300-2306

An BS, Choi JH, Choi KC, Leung PC 2005 Differential role of progesterone receptor isoforms in the transcriptional regulation of human gonadotropin-releasing hormone I (GnRH I) receptor, GnRH I, and GnRH II. J Clin Endocrinol Metab 90:1106-1113

Anderson RA, Baird DT 2002 Male contraception. Endocr Rev 23:735-762

Bachir LK, Garrel G, Lozach A, Laverriere J-N, Counis R 2003 The rat pituitary promoter of the neuronal nitric oxide synthase gene contains an Sp1-, LIM homeodomain-dependent enhancer and a distinct bipartite gonadotropin-releasing hormone-responsive region. Endocrinology 144:3995-4007

Barnhart KM, Mellon PL 1994 The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. Mol Endocrinol 8:878-885

Bauer-Dantoin AC, Hollenberg AN, Jameson JL 1993 Dynamic regulation of gonadotropin-releasing hormone receptor mRNA levels in the anterior pituitary gland during the rat estrous cycle. Endocrinology 133:1911-1914

Bedecarrats GY, Kaiser UB 2003 Differential regulation of gonadotropin subunit gene promoter activity by pulsatile gonadotropin-releasing hormone (GnRH) in perifused LβT2 cells: role of GnRH receptor concentration. Endocrinology 144:1802-1811

Benbrook DM, Jones NC 1990 Heterodimer formation between CREB and JUN proteins. Oncogene 5:295–302

Bockaert J, Pin JP 1999 Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO 18:1723-1729

Bonfil D, Chuderland D, Kraus S, Shahbazian D, Friedberg I, Seger R, Naor Z 2004 Extracellular signal-regulated Kinase, Jun N-terminal Kinase, p38, and c-Src are involved in gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone follicle-stimulating hormone β-subunit promoter. Endocrinology 145:2228-2244

Borgeat P, Chavancy G, Dupont A, Labrie F, Arimura A, Schally AV 1972 Stimulation of adenosine 3':5'-cyclic monophosphate accumulation in anterior pituitary gland *in vitro* by synthetic luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 69:2677-2681

Bouvier C, Lagace G, Collu R 1991 G protein modulation by estrogens. Mol Cell Endocrinol 79:65-73

Braden TD, Conn PM 1992 Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors. Endocrinology 130:2101-2105

Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248-54

Brothers SP, Cornea A, Janovick JA, Conn PM 2004 Human loss-of-function gonadotropin-releasing hormone receptor mutants retain wild-type receptors in the endoplasmic reticulum: molecular basis of the dominant-negative effect. Mol Endocrinol 18:1787-1797

Bustin SA, Benes V, Nolan T, Pfaffl MW 2005 Quantitative real-time RT-PCR - a perspective. J Mol Endocrinol 34:597-601

Campion CE, Turzillo AM, Clay CM 1996 The gene encoding the ovine gonadotropin-releasing hormone (GnRH) receptor: cloning and initial characterization. Gene 170:277-280

Carlone DL, Richards JS 1997 Functional interactions, phosphorylation, and levels of 3',5'-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. Mol Endocrinol 11:292-304

Caunt CJ, Hislop JN, Kelly E, Matharu AL, Green LD, Sedgley KR, Finch AR, McArdle CA 2004 Regulation of gonadotropin-releasing hormone receptors by Protein Kinase C: inside out signalling and evidence for multiple active conformations. Endocrinology 145:3594-3602

Cesnjaj M, Catt KJ, Stojilkovic SS 1994 Coordinate actions of calcium and protein kinase-C in the expression of primary response genes in pituitary gonadotrophs. Endocrinology 135:692-701

Chen A, Ganor Y, Rahimipour S, Ben Aroya N, Koch Y, Levite M 2002 The neuropeptides GnRH-II and GnRH-I are produced by human T cells and trigger laminin receptor gene expression, adhesion, chemotaxis and homing to specific organs. Nat Med 8:1421-1426

Chen HF, Jeung EB, Stephenson M, Leung PCK 1999 Human peripheral blood mononuclear cells express gonadotropin-releasing hormone (GnRH), GnRH receptor, and interleukin-2 receptor γ-chain messenger ribonucleic acids that are regulated by GnRH *in vitro*. J Clin Endocrinol Metab 84:743-750

Chen L, Sun XD, Zhao J, Yang AG, Huang WQ 2005 Distribution, cloning and sequencing of GnRH, its receptor, and effects of gastric acid secretion of GnRH analogue in gastric parietal cells of rats. Life Sciences 76:1351-1365

Cheng CK, Chow BKC, Leung PCK 2003 An Activator Protein 1-like motif mediates 17β-estradiol repression of gonadotropin-releasing hormone receptor promoter via an estrogen receptor α-dependent mechanism in ovarian and breast cancer cells. Mol Endocrinol 17:2613-2629

Cheng CK, Leung PCK 2005 Molecular Biology of Gonadotropin-Releasing Hormone (GnRH)-I, GnRH-II and Their Receptors in Humans. Endocr Rev 26:283-306

Cheng KW, Chow BKC, Leung PCK 2001 Functional mapping of a placenta-specific upstream promoter for human gonadotropin-releasing hormone receptor gene. Endocrinology 142:1506-1516

Cheng KW, Leung PCK 2001 Human gonadotropin-releasing hormone receptor gene transcription: up-regulation by 3',5'-cyclic adenosine monophosphate/protein kinase A pathway. Mol Cell Endocrinol 181:15-26

Cherrington BD, Farmerie TA, Lents CA, Cantlon JD, Roberson MS, Clay CM 2005 Activin responsiveness of the murine gonadotropin-releasing hormone receptor gene is mediated by a composite enhancer containing spatially distinct regulatory elements. Mol Endocrinol 19:898-912

Cheung TC, Hearn JP 2003 Developmental expression and subcellular localization of wallaby gonadotropin-releasing hormone receptor and its splice variants. Gen Comp Endocrinol 133:88-99

Childs GV, Unabia G, Tibolt R, Lloyd JM 1987 Cytological factors that support nonparallel secretion of LH and FSH during the estrous cycle. Endocrinology 121:1801-1813

Chomczymski P 1993 A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 15:532-535

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH 1993 Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855-859

Clay CM, Nelson SE, DiGregorio GB, Campion CE, Wiedemann AL, Nett RJ 1995 Cell-specific expression of the mouse gonadotropin-releasing hormone (GnRH) receptor gene is conferred by elements residing within 500 bp of proximal 5' flanking region. Endocrine 3:615-622

Cohen DR, Ferreira PC, Gentz R, Franza BR Jr, Curran T 1989 The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. Genes Dev 3:173-184

Conn PM, Crowley WF Jr 1994 Gonadotropin-releasing hormone and its analogs. Annu Rev Med 45:391-405

Conn PM, Janovick JA, Stanislaus D, Kuphal D, Jennes L 1995 Molecular and cellular basis of gonadotropin-releasing hormone action in the pituitary and central nervous system. In: Litwack G, ed. Vitamins and Hormones. New York: Academic Press; vol 50:151-214

Coss D, Jacobs SBR, Bender CE, Mellon PL 2004 A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone-β gene by gonadotropin-releasing hormone. J Biol Chem 279:152-62

Crawford PA, Dorn C, Sadovsky Y, Milbrandt J 1998 Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to Steroidogenic Factor 1. Mol Cell Biol 18:2949-2956

Crawford PA, Sadovsky Y, Woodson K, Lee SL, Milbrandt J 1995 Adrenocortical function and regulation of the steroid hydroxylase gene in NGFI-B-deficient mice. Mol Cell Biol 15:4331-4336

Curtin D, Jenkins S, Farmer N, Anderson AC, Haisenleder DJ, Rissman E, Wilson EM, Shupnik MA 2001 Androgen suppression of GnRH-stimulated rat LHβ gene transcription occurs through Sp1 sites in the distal GnRH-responsive promoter region. Mol Endocrinol 15:1906-1917

Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475-1489

Duan WR, Shin JL, Jameson JL 1999 Estradiol suppresses phosphorylation of cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) in the pituitary: evidence for indirect action via gonadotropin-releasing hormone. Mol Endocrinol 13:1338-1352

Duval DL, Ellsworth BS, Clay CM 1999 Is gonadotrope expression of the gonadotropin-releasing hormone receptor gene mediated by autocrine/paracrine stimulation of an activin response element? Endocrinology 140:1949-1952

Duval DL, Nelson SE, Clay CM 1997a A binding site for steroidogenic factor-1 is part of a complex enhancer that mediates expression of the murine gonadotropin-releasing hormone receptor gene. Biol Reprod 56:160-168

Duval DL, Nelson SE, Clay CM 1997b The tripartite basal enhancer of the gonadotropin-releasing hormone (GnRH) receptor gene promoter regulates cell-specific expression through a novel GnRH receptor activating sequence. Mol Endocrinol 11:1814-1821

Dwarki VJ, Montminy M, Verma IM 1990 Both the basic region and the 'leucine zipper' domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J 9:225-232

Eidne KA 1994 Exploring up-stream of the gonadotropin-releasing hormone receptor gene. Endocrinology 135:2297-2299

Eidne KA, Sellar RE, Couper G, Anderson L, Taylor PL 1992 Molecular cloning and characterization of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol Cell Endocrinol 90:R5-9

Ellsworth BS, Burns AT, Escudero KW, Duval DL, Nelson SE, Clay CM 2003a The gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. Mol Cell Endocrinol 206:93-111

Ellsworth BS, White BR, Burns AT, Cherrington BD, Otis AM, Clay CM 2003b c-Jun N-terminal Kinase activation of Activator Protein-1 underlies homologous regulation of the gonadotropin-releasing hormone receptor gene in αT3-1 cells. Endocrinology 144:839-849

Enomoto M, Endo D, Kawashima S, Park MK 2004 Human type II GnRH receptor mediates effects of GnRH on cell proliferation. Zoolog Sci 21:763-770

Fan NC, Jeung EB, Peng C, Olofsson JI, Krisinger J, Leung PC 1994 The human gonadotropin-releasing hormone (GnRH) receptor gene: cloning, genomic organization and chromosomal assignment. Mol Cell Endocrinol 103:R1-R6

Fan NC, Peng C, Krisinger J, Leung PCK 1995 The human gonadotropin-releasing hormone receptor gene: complete structure including multiple promoters, transcription initiation sites, and polyadenylation signals. Mol Cell Endocrinol 107:R1-R8

Faurholm B, Millar RP, Katz AA 2001 The genes encoding the type II gonadotropin-releasing hormone receptor and the ribonucleoprotein RBM8A in humans overlap in two genomic loci. Genomics 78:15-18

Felinski EA, Kim J, Lu J, Quinn PG 2001 Recruitment of an RNA polymerase II complex is mediated by the constitutive activation domain in CREB, independently of CREB phosphorylation. Mol Cell Biol 21:1001-1010

Fernandez-Vazquez G, Kaiser UB, Albarracin CT, Chin WW 1996 Transcriptional activation of the gonadotropin-releasing hormone receptor gene by activin A. Mol Endocrinol 10:356-366

Finch AR, Green L, Hislop JN, Kelly E, McArdle CA 2004 Signaling and antiproliferative effects of type I and II gonadotropin-releasing hormone receptors in breast cancer cells. J Clin Endocrinol Metab 89:1823-1832

Fink G 1988 Gonadotropin secretion and its control. In: Knobil E, Neill J, eds. The Physiology of Reproduction. New York: Raven Press; p. 1349-1377

Fowkes RC, King P, Burrin JM 2002 Regulation of human glycoprotein hormone α-subunit gene transcription in LβT2 gonadotropes by Protein Kinase C and extracellular signal-regulated kinase 1/2. Biol Reprod 67:725-34

Fowkes RC, Sidhu KK, Sosabowski JK, King P, Burrin JM 2003 Absence of pituitary adenylate cyclase-activating polypeptide-stimulated transcription of the human glycoprotein alpha-subunit gene in LβT2 gonadotrophs reveals disrupted cAMP-mediated gene transcription. J Mol Endocrinol 31:263-278

Franklin J, Hislop J, Flynn A, McArdle CA 2003 Signalling and anti-proliferative effects mediated by gonadotrophin-releasing hormone receptors after expression in prostate cancer cells using recombinant adenovirus. J Endocrinol 176:275-284

Freshney RI 1987 Culture of animal cells: a manual of basic technique. New York: John Wiley and Sons; p. 183-185

Fujii Y, Okada Y, Moore JP Jr, Dalkin AC, Winters SJ 2002 Evidence that PACAP and GnRH down-regulate follicle-stimulating hormone-β mRNA levels by stimulating follistatin gene expression: effects on folliculostellate cells, gonadotrophs and LβT2 gonadotroph cells. Mol Cell Endocrinol 192:55-64

Gaetje R 1994 Influence of gonadotropin releasing hormone (GnRH) and a GnRH-agonist on granulosa cell steroidogenesis. Clin Exp Obstet Gynecol 21:164-169

Garrel G, Lozach A, Bachir LK, Laverriere JN, Counis R 2002 Pituitary adenylate cyclase-activating polypeptide stimulates nitric-oxide synthase type I expression and potentiates the cGMP response to gonadotropin-releasing hormone of rat pituitary gonadotrophs. J Biol Chem 277:46391-46401

Gentz R, Rauscher FJ, Abate C, Curran T 1989 Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science 243:1695-1699

Gonzalez GA, Montminy MR 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-680

Gordon JA 1991 Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Meth Enzymol 201:477-482

Grosse R, Schmid A, Schöneberg T, Herrlich A, Muhn P, Schultz G, Gudermann T 2000 Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to $G_{q/11}$ proteins. J Biol Chem 275:9193-9200

Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC 2004 Anatomy of the estrogen response element. Trends Endocrinol Metab 15:73-78

Grundker C, Gunthert AR, Millar RP, Emons G 2002 Expression of gonadotropin-releasing hormone II (GnRH-II) receptor in human endometrial and ovarian cancer cells and effects of GnRH-II on tumor cell proliferation. J Clin Endocrinol Metab 87:1427-1430

Guerrero HE, Stein P, Asch RH, deFried EP, Tesone M 1993 Effect of a gonadotropin-releasing hormone agonist on luteinizing hormone receptors and steroidogenesis in ovarian cells. Fertil Steril 59:803-808

Gunning P, Ponte P, Okayama H, Engel J, Blau H, Kedes L 1983 Isolation and characterizarion of full-length cDNA clones for human α-, β- and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol Cell Biol 3:787-795

Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy MR 1993 Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol Cell Biol 13:4852-4859

Hai T, Curran T 1991 Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci USA 88:3720-3724

Haisenleder DJ, Cox ME, Parsons SJ, Marshall JC 1998 Gonadotropin-releasing hormone pulses are required to maintain activation of mitogen-activated protein kinase: role in stimulation of gonadotrope gene expression. Endocrinology 139:3104-3111

Haisenleder DJ, Yasin M, Dalkin AC, Gilrain J, Marshall JC 1996 GnRH regulates steroidogenic factor-1 (SF-1) gene expression in the rat pituitary. Endocrinology 137:5719-5722

Halvorson LM, Kaiser UB, Chin WW 1996 Stimulation of luteinizing hormone-β gene promoter activity by the orphan nuclear receptor, Steroidogenic Factor-1. J Biol Chem 271:6645-6650

Hapgood JP, Sadie H, Van Biljon W, Ronacher K 2005 Regulation of expression of mammalian GnRH receptor genes. J Neuroendocrinol 17:619-638

Harrison GS, Wierman ME, Nett TM, Glode LM 2004 Gonadotropin-releasing hormone and its receptor in normal and malignant cells. Endocr Relat Cancer 11:725-748

Hawes BE, Marzen JE, Waters SB, Conn PM 1992 Sodium fluoride provokes gonadotrope desensitization to gonadotropin-releasing hormone (GnRH) and gonadotrope sensitization to A23187: evidence for multiple G proteins in GnRH action. Endocrinology 130:2465-2475

Hazel TG, Nathans D, Lau LF 1988 A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc Natl Acad Sci USA 85:8444-8448

Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, Leeto M, Kirsch R, Hall P, Mossmann H 2004 Alternative macrophage activation is essential for survival

during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity 20:623-635

Hirai SI, Ryseck RP, Mechta F, Bravo R, Yaniv M 1989 Characterization of junD: a new member of the jun proto-oncogene family. EMBO J 8:1433-1439

Horn F, Bilezikjian LM, Perrin MH, Bosma MM, Windle JJ, Huber KS, Blount AL, Hille B, Vale W, Mellon PL 1991 Intracellular responses to gonadotropin-releasing hormone in a clonal cell line of the gonadotrope lineage. Mol Endocrinol 5:347-355

Horn F, Windle JJ, Barnhart KM, Mellon PL 1992 Tissue-specific gene expression in the pituitary: the glycoprotein hormone alpha-subunit gene is regulated by a gonadotrope-specific protein. Mol Cell Biol 12:2143-2153

Huang W, Yao B, Sun L, Pu R, Wang L, Zhang R 2001 Immunohistochemical and *in situ* hybridization studies of gonadotropin releasing hormone (GnRH) and its receptor in rat digestive tract. Life Sciences 68:1727-1734

Huggett J, Dheda K, Bustin S, Zumla A 2005 Real-time RT-PCR normalisation; strategies and considerations. Genes and Immunity 6:279-284

Hyde CL, Childs G, Wahl LM, Naor Z, Catt KJ 1982 Preparation of gonadotroph-enriched cell populations from adult rat anterior pituitary cells by centrifugal elutriation. Endocrinology 111:1421-1423

Ibrahim SN, Moussa SM, Childs GV 1986 Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum leves of gonadotropins. Endocrinology 119:629-637

Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP, Parker KL 1993 Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. Mol Endocrinol 7:852-860

Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, Abbud R, Nilson JH, Parker KL 1994 The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. Genes Dev 8:2302-2312

Iovane A, Aumas C, de Roux N 2004 New insights in the genetics of isolated hypogonadotropic hypogonadism. Eur J Endocrinol 151 Suppl 3:U83-U88

Ismaili N, Blind R, Garabedian MJ 2005 Stabilization of the unliganded glucocorticoid receptor by TSG101. J Biol Chem 280:11120-11126

Ito M, Yu R, Jameson JL 1997 DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. Mol Cell Biol 17:1476-1483

Ito M, Yu RN, Jameson JL 1998 Steroidogenic Factor-1 contains a carboxy-terminal transcriptional activation domain that interacts with Steroid Receptor Coactivator-1. Mol Endocrinol 12:290-301

Jacobson JD, Crofford LJ, Sun L, Wilder RL 1998 Cyclical expression of GnRH and GnRH receptor mRNA in lymphoid organs. Neuroendocrinology 67:117-125

Jeong KH, Chin WW, Kaiser UB 2004 Essential role of the homeodomain for pituitary homeobox 1 activation of mouse gonadotropin-releasing hormone receptor gene expression through interactions with c-Jun and DNA. Mol Cell Biol 24:6127-6139

Jiang Z, Gibson JP, Archibald AL, Haley CS 2001 The porcine gonadotropin-releasing hormone receptor gene (GnRHR): genomic organization, polymorphisms, and association with the number of corpora lutea. Genome 44:7-12

Johannessen M, Delghandi MP, Moens U 2004 What turns CREB on? Cellular Signalling 16: 1211-1227

Kaiser UB, Jakubowiak A, Steinberger A, Chin WW 1993 Regulation of rat pituitary gonadotropin-releasing hormone receptor mRNA levels *in vivo* and *in vitro.* Endocrinology 133:931-934

Kaiser UB, Conn PM, Chin WW 1997a Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. Endocr Rev 18:46-70

Kaiser UB, Halvorson LM, Chen MT 2000 Sp1, steroidogenic factor 1 (SF-1), and early growth response protein 1 (Egr-1) binding sites form a tripartite gonadotropin-releasing hormone response element in the rat luteinizing hormone-β gene promoter: an integral role for SF-1. Mol Endocrinol 14:1235-1245

Kaiser UB, Jakubowiak A, Steinberger A, Chin WW 1997b Differential effects of gonadotropin-releasing hormone (GnRH) pulse frequency on gonadotropin subunit and GnRH receptor messenger ribonucleic acid levels *in vitro*. Endocrinology 138:1224-1231

Kaiser UB, Sabbagh E, Katzenellenbogen RA, Conn PM, Chin WW 1995 A mechanism for the differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone. Proc Natl Acad Sci USA 92:12280-12284

Kaiser UB, Zhao D, Cardona GR, Chin WW 1992 Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. Biochem Biophys Res Commun 189:1645-1652

Kakar SS 1997 Molecular structure of the human gonadotropin-releasing hormone receptor gene Eur J Endocrinol 137:183-192

Kakar SS, Musgrove LC, Devor DC, Sellers JC, Neill JD 1992 Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem Biophys Res Commun 189:289-295

Kakar SS, Winters SJ, Zacharias W, Miller DM, Flynn S 2003 Identification of distinct gene expression profiles associated with treatment of LβT2 cells with gonadotropin-releasing hormone agonist using microarray analysis. Gene 308:67-77

Kam KY, Jeong KH, Norwitz ER, Jorgensen EM, Kaiser UB 2005 Oct-1 and Nuclear Factor Y bind to the SURG-1 element to direct basal and gonadotropin-releasing hormone (GnRH)-stimulated mouse GnRH receptor gene transcription. Mol Endocrinol 19:148-162

Kanasaki H, Yonehara T, Yamada Y, Takahashi K, Hata K, Fujiwaki R, Yamamoto H, Takeuchi Y, Fukunaga K, Miyamoto E, Miyazaki K 2002 Regulation of gonadotropin α-subunit gene expression by dopamine D2 receptor agonist in clonal mouse gonadotroph αT3-1 Cells. Biol Reprod 67:1218-1224

Katt JA, Duncan JA, Herbon L, Barkan A, Marshall JC 1985 The frequency of gonadotropin-releasing hormone stimulation determines the number of pituitary gonadotropin-releasing hormone receptors. Endocrinology 116:2113-2115

Kee BL, Arias J, Montminy MR 1996 Adaptor-mediated recruitment of RNA Polymerase II to a signal-dependent activator. J Biol Chem 271:2373-2375

Kim Jeong, Lu J, Quinn PG 2000 Distinct cAMP response element-binding protein (CREB) domains stimulate different steps in a concerted mechanism of transcription activation. Proc Natl Acad Sci USA 97:11292-11297

Knollman PE, Janovick JA, Brothers SP, Conn PM 2005 Parallel regulation of membrane trafficking and dominant-negative effects by misrouted GnRH receptor mutants. J Biol Chem 280:24506-24514

Kogo H, Fujimoto T, Park MK, Mori T 1999 Gonadotropin-releasing hormone receptor mRNA expression in the ovaries of neonatal and adult rats. Cells Tissues Organs 164:14-22

Kottler ML, Starzec A, Carre MC, Lagarde JP, Martin A, Counis R 1997 The genes for gonadotropin-releasing hormone and its receptor are expressed in human breast with fibrocystic disease and cancer. Int J Cancer 71:595-599

Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E 2002 Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, Protein Kinase A, and MAPK pathways. Mol Endocrinol 16:1638-51

Kraus S, Benard O, Naor Z, Seger R 2003 c-Src is activated by the epidermal growth factor receptor in a pathway that mediates JNK and ERK activation by gonadotropin-releasing hormone in COS7 cells. J Biol Chem 278:32618-32630

Kraus S, Naor Z, Seger R 2001 Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. Arch Med Res 32:499-509

Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene D, Bynum JM, Madauss K, Montana V, Lebedeva L, Suzawa M, Williams JD, Williams SP, Guy RK, Thornton JW, Fletterick RJ, Willson TM, Ingraham HA 2005 Structural Analyses Reveal Phosphatidyl Inositols as Ligands for the NR5 Orphan Receptors SF-1 and LRH-1. Cell 120:343-355

La Rosa S, Celato N, Uccella S, Capella C 2000 Detection of gonadotropin-releasing hormone receptor in normal human pituitary cells and pituitary adenomas using immunohistochemistry. Virchows Arch 437:264-269

Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AFM 2002 Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. Analyt Biochem 307:63-69

Levi NL, Hanoch T, Benard O, Rozenblat M, Harris D, Reiss N, Naor Z, Seger R 1998 Stimulation of Jun N-terminal Kinase (JNK) by gonadotropin-releasing hormone in pituitary αT3-1 cell line is mediated by Protein Kinase C, c-Src, and CDC42. Mol Endocrinol 12:815-824

Li X, Lei ZM, Rao CV 1996 Human chorionic gonadotropin down-regulates the expression of gonadotropin-releasing hormone receptor gene in GT1-7 neurons. Endocrinology 137:899-904

Limonta P, Moretti RM, Marelli MM, Motta M 2003 The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. Front Neuroendocrinol 24:279-295

Lin X, Conn PM 1998 Transcriptional activation of gonadotropin-releasing hormone (GnRH) receptor gene by GnRH and cyclic adenosine monophosphate. Endocrinology 139:3896-3902

Liu F, Austin DA, Mellon PL, Olefsky JM, Webster NJG 2002a GnRH activates ERK1/2 leading to the induction of c-fos and LHβ protein expression in LβT2 Cells. Mol Endocrinol 16:419-434

Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM, Webster NJG 2002b Involvement of both $G_{q/11}$ and G_s proteins in gonadotropin-releasing hormone receptor-mediated signaling in LβT2 cells. J Biol Chem 277:32099-32108

Liu F, Austin DA, Webster NJG 2003 Gonadotropin-releasing hormone-desensitized LβT2 gonadotrope cells are refractory to acute Protein Kinase C, cyclic AMP, and calcium-dependent signaling. Endocrinology 144:4354-4365

Liu WL, Saint DA 2002 A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Analyt Biochem 302:52-59

Liu Z, Simpson ER 1997 Steroidogenic factor 1 (SF-1) and SP1 are required for regulation of bovine CYP11A gene expression in bovine luteal cells and adrenal Y1 cells. Mol Endocrinol 11:127-137

Loumaye E, Catt KJ 1982 Homologous regulation of gonadotropin-releasing hormone receptors in cultured pituitary cells. Science 215:983-985

Loumaye E, Catt KJ 1983 Agonist-induced regulation of pituitary receptors for gonadotropin-releasing hormone. Dissociation of receptor recruitment from hormone release in cultured gonadotrophs. J Biol Chem 258:12002-12009

Luo X, Ikeda Y, Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77:481-490

Macgregor PF, Abate C, Curran T 1990 Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. Oncogene 5:451-458

Maira M, Martens C, Philips A, Drouin J 1999 Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. Mol Cell Biol 19:7549-7557

Maira M, Martens C, Batsche E, Gauthier Y, Drouin J 2003 Dimer-specific potentiation of NGFI-B (Nur77) transcriptional activity by the Protein Kinase A pathway and AF-1-dependent coactivator recruitment. Mol Cell Biol 23:763-776

Mangelsdorf DJ, Evans RM 1995 The RXR heterodimers and orphan receptors. Cell 83:841-850

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839

Manna PR, Eubank DW, Lalli E, Sassone-Corsi P, Stocco DM 2003 Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1. J Mol Endocrinol 30:381-397

Marinissen MJ, Gutkind JS 2001 G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 22:368-376

Marino JH, Cook P, Miller KS 2003 Accurate and statistically verified quantification of relative mRNA abundances using SYBR Green I and real-time RT-PCR. J Immunol Methods 283:291-306

Martinez-Fuentes AJ, Hu L, Krsmanovic LZ, Catt KJ 2004 Gonadotropin-releasing hormone (GnRH) receptor expression and membrane signaling in early embryonic GnRH neurons: role in pulsatile neurosecretion. Mol Endocrinol 18:1808-1817

Maruyama K, Tsukuda T, Ohkura N, Bandoh S, Hosono T, Yamaguchi K 1998 The NGFI-B subfamily of the nuclear receptor superfamily (Review). Int J Oncology 12:1237-1243

Mason DR, Arora KK, Mertz LM, Catt KJ 1994 Homologous down-regulation of gonadotropin-releasing hormone receptor sites and messenger ribonucleic acid transcripts in alphaT3-1 cells. Endocrinology 5:1165-1170

Maya-Nunez G, Conn PM 1999 Transcriptional regulation of the gonadotropin-releasing hormone receptor gene is mediated in part by a putative repressor element and by the cyclic adenosine 3',5'-monophosphate response element. Endocrinology 140:3452-3458

Maya-Nunez G, Conn PM 2003 Transcriptional regulation of the GnRH receptor gene by glucocorticoids. Mol Cell Endocrinol 200:89-98

Mayr B, Montminy M 2001 Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599-609

McArdle CA, Conn PM 1986 Hormone-stimulated redistribution of gonadotrope protein kinase C *in vivo*: dependence on Ca^{2+} influx. Mol Pharmacol 29:570-576

McArdle CA, Franklin J, Green L, Hislop JN 2002 Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. J Endocrinol 173:1-11

McCue JM, Quirk CC, Nelson SE, Bowen RA, Clay CM 1997 Expression of a murine gonadotropin-releasing hormone receptor-luciferase fusion gene in transgenic mice is diminished by immunoneutralization of gonadotropin-releasing hormone. Endocrinology 138:3154-3160

McGillivray SM, Bailey JS, Ramezani R, Kirkwood BJ, Mellon PL 2005 Mouse GnRH receptor gene expression is mediated by the LHX3 homeodomain protein. Endocrinology 146:2180-2185

Meyer TE, Waeber G, Lin J, Beckmann W, Habener JF 1993 The promoter of the gene encoding 3',5'-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription. Endocrinology 132:770-780

Michael MD, Kilgore MW, Morohashi K, Simpson ER 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. J Biol Chem 273:4585-4591

Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R, Faurholm B, Scobie G, Kuestner R, Terasawa E, Katz A 2001 A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proc Natl Acad Sci USA 98:9636-9641

Millar RP 2003 GnRH II and type II GnRH receptors. Trends Endocrinol Metab 14:35-43

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR 2004 Gonadotropin-releasing hormone receptors. Endocr Rev 25:235-275

Monte D, DeWitte F, Hum DW 1998 Regulation of the human P450scc gene by Steroidogenic Factor 1 is mediated by CBP/p300. J Biol Chem 273:4585-4591

Montminy M 1997 Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807-822

Montminy MR, Bilezikjian LM 1987 Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328:175-178

Morales P, Llanos M 1996 Interaction of human spermatozoa with the zona pellucida of oocyte: development of the acrosome reaction. Front Biosci 1:d146-d160

Morales P 1998 Gonadotropin-releasing hormone increases ability of the spermatozoa to bind to the human zona pellucida. Biol Reprod 59:426-430

Morgan K, Conklin D, Pawson AJ, Sellar R, Ott TR, Millar RP 2003 A transcriptionally active human type II gonadotropin-releasing hormone receptor gene homolog overlaps two genes in the antisense orientation on chromosome 1q.12. Endocrinology 144:423-436

Mororohashi K, Honda S, Inomata Y, Handa H, Omura T 1992 A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J Biol Chem 267:17913-17919

Morrison TB, Weis JJ, Wittwer CT 1998 Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques 24:954-962

Mulvaney JM, Roberson MS 2000 Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen-activated protein kinases by gonadotropin-releasing hormone. J Biol Chem 275:14182-14189

Murphy EP, Conneely OM 1997 Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. Mol Endocrinol 11:39-47

Nakabeppu Y, Ryder K, Nathans D 1988 DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell 55:907-915

Navratil AM, Bliss SP, Berghorn KA, Haughian JM, Farmerie TA, Graham JK, Clay CM, Roberson MS 2003 Constitutive localization of the gonadotropin-releasing hormone (GnRH)

receptor to low density membrane microdomains is necessary for GnRH signaling to ERK. J Biol Chem 278:31593-31602

Neill JD 2002 Minireview: GnRH and GnRH Receptor Genes in the Human Genome. Endocrinology 143:737-743

Neill JD, Duck LW, Sellers JC, Musgrove LC 2001 A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates. Biochem Biophys Res Commun 282:1012-1018

Neill JD, Musgrove LC, Duck LW 2004 Newly recognized GnRH receptors: function and relative role. Trends Endocrinol Metab 15:383-392

Ngan ESW, Cheng PKW, Leung PCK, Chow BKC 1999 Steroidogenic Factor-1 interacts with a gonadotrope-specific element within the first exon of the human gonadotropin-releasing hormone receptor gene to mediate gonadotrope-specific expression. Endocrinology 140:2452-2462

Nguyen KA, Santos SJ, Kreidel MK, Diaz AL, Rey R, Lawson MA 2004 Acute regulation of translation initiation by gonadotropin-releasing hormone in the gonadotrope cell line LβT2. Mol Endocrinol 18:1301-1312

Norwitz ER, Cardona GR, Jeong KH, Chin WW 1999a Identification and characterization of the gonadotropin-releasing hormone response elements in the mouse gonadotropin-releasing hormone receptor gene. J Biol Chem 274:867-880

Norwitz ER, Jeong KH, Chin WW 1999b Molecular mechanisms of gonadotropin-releasing hormone receptor gene regulation. J Soc Gynecol Invest 6:169-178

Norwitz ER, Xu S, Jeong KH, Bedecarrats GY, Winebrenner LD, Chin WW, Kaiser UB 2002a Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. Endocrinology 143:985-997

Norwitz ER, Xu S, Xu J, Spiryda LB, Park JS, Jeong KH, McGee EA, Kaiser UB 2002b Direct binding of AP-1 (Fos/Jun) proteins to a SMAD binding element facilitates both gonadotropin-releasing hormone (GnRH)- and activin-mediated transcriptional activation of the mouse GnRH receptor gene. J Biol Chem 277:37469-37478

Okabe T, Takayanagi R, Adachi M, Imasaki K, Nawata H 1998 Nur77, a member of the steroid receptor superfamily, antagonizes negative feedback of ACTH synthesis and secretion by glucocorticoids in pituitary corticotrope cells. J Endocrinol 156:169-175

Ostrom RS, Post SR, Insel PA 2000 Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s). J Pharmacol Exp Ther 294:407-412

Padmanabhan V, Dalkin A, Yasin M, Haisenleder DJ, Marshall JC, Landefeld TD 1995 Are immediate early genes involved in gonadotropin-releasing hormone receptor gene regulation? Characterization of changes in GnRH receptor (GnRH-R), c-fos, and c-jun messenger ribonucleic acids during the ovine estrous cycle. Biol Reprod 53:263-269

Parker KL 1998 The roles of steroidogenic factor 1 in endocrine development and function. Mol Cell Endocrinol 145:15-20

Parkes D, Rivest S, Lee S, Rivier C, Vale W 1993 Corticotropin-releasing factor activates c-fos, NGFI-B, and corticotropin-releasing factor gene expression within the paraventricular nucleus of the rat hypothalamus. Mol Endocrinol 7:1357-1367

Pawson AJ, Morgan K, Maudsley SR, Millar RP 2003 Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology. Reproduction 126:271-278

Peirson SN, Butler JN, Foster RG 2003 Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 31:e73

Peng C, Fan NC, Ligier M, Vaananen J, Leung PC 1994 Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 135:1740-1746

Perlmann T, Jansson L 1995 A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev 9:769-782

Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL 2001 Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model. Endocrinology 142:2284-2295

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Pheiffer C 1998 The role of Steroidogenic Factor 1 (SF-1) in gonadotropin-releasing hormone (GnRH) receptor gene regulation. M.Sc. thesis, Department of Chemical Pathology, Faculty of Medicine, University of Cape Town, Cape Town, South Africa

Philips A, Lesage S, Gingras R, Maira MH, Gauthier Y, Hugo P, Drouin J 1997 Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. Mol Cell Biol 17:5946-5951

Pincas H, Amoyel K, Counis R, Laverriere JN 2001a Proximal *cis*-acting elements, including Steroidogenic Factor 1, mediate the efficiency of a distal enhancer in the promoter of the rat gonadotropin-releasing hormone receptor gene. Mol Endocrinol 15:319-337

Pincas H, Forrai Z, Chauvin S, Laverriere JN, Counis R 1998 Multiple elements in the distal part of the 1.2 kb 5'-flanking region of the rat GnRH receptor gene regulate gonadotrope-specific expression conferred by proximal domain. Mol Cell Endocrinol 144:95-108

Pincas H, Laverriere JN, Counis R 2001b Pituitary adenylate cyclase-activating polypeptide and cyclic adenosine 3',5'-monophosphate stimulate the promoter activity of the rat gonadotropin-releasing hormone receptor gene via a bipartite response element in gonadotrope-derived cells. J Biol Chem 276:23562-23571

Probst WC, Snyder LA, Schuster DI, Brosius J, Sealfon SC 1992 Sequence alignment of the G-protein coupled receptor superfamily. DNA Cell Biol 11:1-20

Raga F, Casan EM, Kruessel JS, Wen Y, Huang HY, Nezhat C, Polan ML 1998 Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. Biol Reprod 59:661-669

Rama S, Rao AJ 2001 Embryo implantation and GnRH antagonists: The search for the human placental GnRH receptor. Hum Reprod 16:201-205

Rawlings SR, Hezareh M 1996 Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. Endocr Rev 17:4-29

Reinhart J, Maertz LM, Catt KJ 1992 Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J Biol Chem 267:21281-21284

Reinhart J, Xiao S, Arora KK, Catt KJ 1997 Structural organization and characterization of the promoter region of the rat gonadotropin-releasing hormone receptor gene. Mol Cell Endocrinol 130:1-12

Reiss N, Llevi LN, Shacham S, Harris D, Seger R, Naor Z 1997 Mechanism of mitogen-activated protein kinase activation by gonadotropin-releasing hormone in the pituitary αT3-1 cell line: differential roles of calcium and Protein Kinase C. Endocrinology 138:1673-1682

Ririe KM, Rasmussen RP, Wittwer CT 1997 Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analyt Biochem 245:154-160

Roberson MS, Zhang T, Li HL, Mulvaney JM 1999 Activation of the p38 mitogen-activated protein kinase pathway by gonadotropin-releasing hormone. Endocrinology 140:1310-1318

Roberts VJ, Peto CA, Vale W, Sawchenko PE 1992 Inhibin/activin subunits are costored with FSH and LH in secretory granules of the rat anterior pituitary gland. Neuroendocrinology 56:214-224

Roelle S, Grosse R, Aigner A, Krell HW, Czubayko F, Gudermann T 2003 Matrix metalloproteinases 2 and 9 mediate epidermal growth factor receptor transactivation by gonadotropin-releasing hormone. J Biol Chem 278:47307-47318

Romanelli RG, Barni T, Maggi M, Luconi M, Failli P, Pezzatini A, Pelo E, Torricelli F, Crescioli C, Ferruzzi P, Salerno R, Marini M, Rotella CM, Vannelli GB 2004 Expression and function of gonadotropin-releasing hormone (GnRH) receptor in human olfactory GnRH-secreting neurons: an autocrine GnRH loop underlies neuronal migration. J Biol Chem 279:117-126

Ruf F, Fink MY, Sealfon SC 2003 Structure of the GnRH receptor-stimulated signaling network: insights from genomics. Front Neuroendocrinol 24:181-199

Ryseck RP, Bravo R 1991 c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene 6: 533-542

Sadie H, Styger G, Hapgood J 2003 Expression of the mouse gonadotropin-releasing hormone receptor gene in αT3-1 gonadotrope cells is stimulated by cyclic 3',5'-adenosine monophosphate and Protein Kinase A, and is modulated by Steroidogenic Factor-1 and Nur77. Endocrinology 144:1958-1971

Sambrook J, Fritsch EF, Maniatis T 1989 Molecular Cloning, a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press.

Sassone-Corsi P, Ransone LJ, Verma IM 1990 Cross-talk in signal transduction: TPA-inducible factor jun/AP-1 activates cAMP-responsive enhancer elements. Oncogene 5:427-431

Schomerus E, Poch A, Bunting R, Mason WT, McArdle CA 1994 Effects of pituitary adenylate cyclase-activating polypeptide in the pituitary: activation of two signal transduction pathways in the gonadotrope-derived αT3-1 cell line. Endocrinology 134:315-323

Schreiber E, Matthias P, Muller MM, Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 17:6419

Seminara SB, Hayes FJ, Crowley WF, Jr. 1994 Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's Syndrome): pathophysiological and genetic considerations. Endocr Rev 19:521-539

Shupnik MA 1996 Gonadotropin gene modulation by steroids and gonadotropin-releasing hormone. Biol Reprod 54:279-286

Sim PJ, Wolbers BW, Mitchell R 1995 Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein. Mol Cell Endocrinol 112:257-263

Song KH, Park JI, Lee MO, Soh J, Lee K, Choi HS 2001 LH induces orphan nuclear receptor Nur77 gene expression in testicular Leydig cells. Endocrinology 142:5116-5123

Sosnowski R, Mellon PL, Lawson MA 2000 Activation of translation in pituitary gonadotrope cells by gonadotropin-releasing hormone. Mol Endocrinol 14:1811-1819

Stanislaus D, Janovick JA, Jennes L, Kaiser UB, Chin WW, Conn PM 1994 Functional and morphological characterization of four cell lines derived from GH3 cells stably transfected with gonadotropin-releasing hormone receptor complementary deoxyribonucleic acid. Endocrinology 135:2220-2227

Stanislaus D, Pinter JH, Janovick JA, Conn PM 1998a Mechanisms mediating multiple physiological responses to gonadotropin-releasing hormone. Mol Cell Endocrinol 144:1-10

Stanislaus D, Ponder S, Ji TH, Conn PM 1998b Gonadotropin-releasing hormone receptor couples to multiple G proteins in rat gonadotrophs and in GGH3 cells: evidence from palmitoylation and overexpression of G proteins. Biol Reprod 59:579-586

Stojilkovic SS, Catt KJ 1995 Expression and signal transduction pathways of gonadotropin-releasing hormone receptors. Rec Prog Horm Res 50:161-205

Stojilkovic SS, Reinhart J, Catt KJ 1994 Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. Endocr Rev 15:462-99

Szende B, Srkalovic G, Timar J, Mulchahey JJ, Neill JD, Lapis K, Csikos A, Szepeshazi K, Schally AV 1991 Localization of receptors for luteinizing hormone-releasing hormone in pancreatic and mammary cancer cells. Proc Natl Acad Sci USA 88:4153-4156

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E 1999 Housekeeping genes as internal standards: use and limits. J Biotechnol 75:291-295

Tsutsumi M, Laws SC, Rodic V, Sealfon SC 1995 Translational regulation of the gonadotropin-releasing hormone receptor in αT3-1 cells. Endocrinology 136:1128-1136

Tsutsumi M, Laws SC, Sealfon SC 1993 Homologous up-regulation of the gonadotropin-releasing hormone receptor in αT3-1 cells is associated with unchanged receptor messenger RNA (mRNA) levels and altered mRNA activity. Mol Endocrinol 7:1625-1633

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol 6:1163-1169

Turgeon JL, Kimura Y, Waring DW, Mellon PL 1996 Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. Mol Endocrinol 10:439-450

Turner R, Tjian R 1989 Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. Science 243:1689-1694

Vasilyev VV, Pernasetti F, Rosenberg SB, Barsoum MJ, Austin DA, Webster NJG, Mellon PL 2002 Transcriptional activation of the ovine follicle-stimulating hormone-β gene by gonadotropin-releasing hormone involves multiple signal transduction pathways. Endocrinology 143:1651-1659

Vizcarra JA, Wettemann RP, Braden TD, Turzillo AM, Nett TM 1997 Effect of gonadotropin-releasing hormone (GnRH) pulse frequency on serum and pituitary concentrations of luteinizing hormone and follicle-stimulating hormone, GnRH receptors, and messenger ribonucleic acid for gonadotropin subunits in cows. Endocrinology 138:594-601

Waeber G, Habener JF 1991 Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB. Mol Endocrinol 5:1431-1438

Weiss J, Cote CR, Jameson JL, Crowley WF, Jr. 1995 Homologous desensitization of gonadotropin-releasing hormone (GnRH)- stimulated luteinizing hormone secretion *in vitro* occurs within the duration of an endogenous GnRH pulse. Endocrinology 136:138-143

White BR, Duval DL, Mulvaney JM, Roberson MS, Clay CM 1999 Homologous regulation of the gonadotropin-releasing hormone receptor gene is partially mediated by Protein Kinase C activation of an Activator Protein-1 element. Mol Endocrinol 13:566-577

Willars GB, Royall JE, Nahorski SR, El Gehani F, Everest H, McArdle CA 2001 Rapid down-regulation of the type I inositol 1,4,5-trisphosphate receptor and desensitization of gonadotropin-releasing hormone-mediated Ca^{2+} responses in αT3-1 gonadotropes. J Biol Chem 276:3123-3129

Wilson TE, Fahrner TJ, Johnston M, Milbrandt J 1991 Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Nature 252:1296-1300

Wilson TE, Fahrner TJ, Milbrandt J 1993a The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 13:5794-5804

Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL 1993b The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. Mol Cell Biol 13:861-868

Windle JJ, Weiner RI, Mellon PL 1990 Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol 4:597-603

Wisdom R 1999 AP-1: One switch for many signals. Exp Cell Res 253:180-185

Wong M, Ramayya MS, Chrousos GP, Driggers PH, Parker KL 1996 Cloning and sequence analysis of the human gene encoding steroidogenic factor 1. J Mol Endocrinol 17:139-147

Wong ML, Medrano JF 2005 Real-time PCR for mRNA quantitation. Biotechniques 39:75-85

Woo MMM, Tai CJ, Kang SK, Nathwani PS, Pang SF, Leung PCK 2001 Direct action of melatonin in human granulosa-luteal cells. J Clin Endocrinol Metab 86:4789-4797

Wu JC, Sealfon SC, Miller WL 1994 Gonadal hormones and gonadotropin-releasing hormone (GnRH) alter messenger ribonucleic acid levels for GnRH receptors in sheep. Endocrinology 134:1846-1850

Wu Q, Li Y, Liu R, Agadir A, Lee MO, Liu Y, Zhang X 1997 Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization. EMBO J 16:1656-1669

Wurmbach E, Yuen T, Ebersole BJ, Sealfon SC 2001 Gonadotropin-releasing hormone receptor-coupled gene network organization. J Biol Chem 276:47195-47201

Yasin M, Dalkin AC, Haisenleder DJ, Kerrigan JR, Marshall JC 1995 Gonadotropin-releasing hormone (GnRH) pulse pattern regulates GnRH receptor gene expression: augmentation by estradiol. Endocrinology 136:1559-1564

Yokoi T, Ohmichi M, Tasaka K, Kimura A, Kanda Y, Hayakawa J, Tahara M, Hisamoto K, Kurachi H, Murata Y 2000 Activation of the luteinizing hormone-β promoter by gonadotropin-releasing hormone requires c-Jun NH2-terminal protein kinase. J Biol Chem 275:21639-21647

Young LS, Naik SI, Clayton RN 1984 Adenosine 3',5'-monophosphate derivatives increase gonadotropin-releasing hormone receptors in cultured pituitary cells. Endocrinology 114:2114-2122

Zapatero-Caballero H, Sanchez-Franco F, Guerra-Perez N, Fernandez-Mendez C, Fernandez-Vazquez G 2003 Gonadotropin-releasing hormone receptor gene expression during pubertal development of male rats. Biol Reprod 68:1764-1770

Zerial M, Toschi L, Ryseck RP, Schuermann M, Muller R, Bravo R 1989 The product of a novel growth factor activated gene, fos B, interacts with JUN proteins enhancing their DNA binding activity. EMBO J 8:805-813

Zhang P, Mellon, SH 1997 Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. Mol Endocrinol 11:891-904

Zhou W, Sealfon SC 1994 Structure of the mouse gonadotropin-releasing hormone receptor gene: variant transcripts generated by alternative processing. DNA Cell Biol 13:605-614

Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L 1994 Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. Mol Pharmacol 46:460-469