

**The Mammalian Type II
Gonadotropin-releasing Hormone Receptor:
Cloning, Distribution and Role in
Gonadotropin gene expression**



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Declaration

I, Wilma van Biljon, hereby declare that the work on which this dissertation is based is my own original work, except where acknowledgements indicate otherwise, and that I haven't previously, in its entirety or in part, submitted it at any university for a degree.



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Signature

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Date

Abstract

Gonadotropin-releasing hormone (GnRH) is well known as the central regulator of the reproductive system through its stimulation of gonadotropin synthesis and release from the pituitary via binding to its specific receptor, known as the gonadotropin-releasing hormone receptor type I (GnRHR-I). The gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), bind to receptors in the gonads, leading to effects on steroidogenesis and gametogenesis. The recent finding of a second form of the GnRH receptor, known as the type II GnRHR or GnRHR-II, in non-mammalian vertebrates triggered the interest into the possible existence and function of a GnRHR-II in humans. The current study addressed this issue by investigating the presence of transcripts for a GnRHR-II in various human tissues and cells. While it was demonstrated that antisense transcripts for this receptor, containing sequence of only two of the three coding exons, are ubiquitously and abundantly expressed in all tissues examined, potentially full-length (containing all three exons), sense transcripts for a GnRHR-II were detected only in human ejaculate. Further analysis revealed that the subset of cells in the ejaculate expressing these transcripts is mature sperm. These findings, together with the reported role for GnRH in spermatogenesis and reproduction led to the further analysis of the presence of a local GnRH/GnRHR network in human and vervet monkey ejaculate or sperm. Indeed, such a network seems to be present in humans since transcripts for both forms of GnRH present in mammals, as well as transcripts for the GnRHR-I, are expressed in human ejaculate. Furthermore, transcripts for the GnRHR-II are expressed in both human and vervet monkey ejaculate. Thus, it would appear that locally produced GnRH-1 and/or GnRH-2 in the human male reproductive tract might mediate their effects on fertility via a local GnRHR-I, and possibly via GnRHR-II.

Remarkably, in the pituitary, LH and FSH are present in the same gonadotropes, yet they are differentially regulated by GnRH under various physiological conditions. While it is well established that post-transcriptional regulatory mechanisms occur, the contribution of transcriptional regulation to the differential expression of the *LH β* - and *FSH β* -subunit genes is unclear. In this study, the role of GnRH-1 and GnRH-2 via the GnRHR-I and the GnRHR-II in transcriptional regulation of mammalian *LH β* - and *FSH β* genes was determined in the L β T2 mouse pituitary gonadotrope cell-line. It is demonstrated for the first time that GnRH-1 may affect gonadotropin subunit gene

expression via GnRHR-II in addition to GnRHR-I, and that GnRH-2 also has the ability to regulate gonadotropin subunit gene expression via both receptors. Similar to other reports, it is shown that the transcriptional response to GnRH-1 of *LH β* and *FSH β* is low (about 1.4-fold for *bLH β Luc* and 1.2-fold for *oFSH β Luc*). In addition, evidence is supplied for the first time that GnRH-2 transcriptional regulation of the gonadotropin β subunits is also low (about 1.5-fold for *bLH β Luc* and 1.1-fold for *oFSH β Luc*). It is demonstrated that GnRH-1 is a more potent stimulator of *bLH β* promoter activity as compared to GnRH-2 via the GnRHR-I, yet both hormones result in a similar maximum induction of *bLH β* . However, GnRH-2 is a more efficacious stimulator of *bLH β* transcription via the GnRHR-II than GnRH-1. No discriminatory effect of GnRH-1 vs. GnRH-2 was observed for *oFSH β* promoter activity via GnRHR-I or GnRHR-II. By comparison of the ratio of expression of transfected *oFSH β* - and *bLH β* promoter-reporters via GnRH-1 with that of GnRH-2, it is shown that GnRH-2 is a selective regulator of *FSH β* gene transcription. This discriminatory effect of GnRH-2 is specific for GnRHR-I, as it is not observed for GnRHR-II, where GnRH-1 results in a greater *oFSH β* -to-*bLH β* ratio. These opposite selectivities for GnRHR-I and GnRHR-II on the ratios of *oFSH β :bLH β* promoter activity for GnRH-1 vs. GnRH-2 suggest a mechanism for fine control of gonadotropin regulation in the pituitary by variation of relative GnRHR-I vs. GnRHR-II levels. In addition, a concentration-dependent modulatory role for PACAP on GnRH-1- and GnRH-2-mediated regulation of *bLH β* promoter activity, via both GnRHR-I and GnRHR-II, and of *oFSH β* promoter activity, via GnRHR-I, is indicated. The concentration-dependent effects suggest the involvement of two different signalling pathways for the PACAP response. Together these findings suggest that transcription of the gonadotropin genes *in vivo* is under extensive hormonal control that can be fine-tuned in response to varying physiological conditions, which include changing levels of GnRH-1, GnRH-2, GnRHR-I and GnRHR-II as well as PACAP.

Opsomming

Gonadotropien-vrystellingshormoon (GnRH) is bekend as die sentrale reguleerder van die voorplantingsstelsel deur die stimulasie van gonadotropiensintese en -vrystelling vanaf die pituitêre klier via binding aan 'n spesifieke reseptor, die sogenaamde tipe I gonadotropien-vrystellingshormoonreseptor (GnRHR-I). Die gonadotropiene, lutineringshormoon (LH) en follikel-stimuleringshormoon (FSH), bind aan reseptore in die gonades waar dit steroïedogenese en gametogenese beïnvloed. Die onlangse ontdekking van 'n tweede vorm van die GnRH-reseptor, bekend as die tipe II GnRHR of GnRHR-II, in nie-soogdier vertebrate het belangstelling in die moontlike bestaan en funksie van 'n GnRHR-II in die mens gewek. Hierdie kwessie is aangeraak deur die teenwoordigheid van transkripte vir 'n GnRHR-II in verskeie weefsel- en selteipes van die mens te ondersoek. Daar is aangetoon dat nie-sin transkripte vir hierdie reseptor, wat die DNA-opeenvolging van slegs twee van die drie koderende eksone bevat, oormatig uitgedruk word in al die weefselteipes wat ondersoek is. Daarteenoor is potensieel volledige (bevattende al drie eksone) sin transkripte vir 'n GnRHR-II in die mens slegs in semen gevind. Verdere analise het getoon dat dit volwasse sperma binne die semen is wat laasgenoemde transkripte uitdruk. Hierdie bevindinge, tesame met die aangetoonde rol vir GnRH in spermatogenese en reproduksie het gelei tot die verdere analise van die teenwoordigheid van 'n lokale GnRH/GnRHR-netwerk in mens- en blouaapsemen of -sperm. So 'n netwerk blyk om teenwoordig te wees in die mens, aangesien transkripte vir beide vorme van GnRH wat in soogdiere gevind word, asook transkripte vir die GnRHR-I, in menssemen uitgedruk word. Daarbenewens word transkripte vir die GnRHR-II uitgedruk in beide mens- en blouaapsemen. Dit wil dus voorkom asof lokaalgeproduseerde GnRH-1 en/of GnRH-2 in die manlike voortplantingstelsel van die mens hul effek op vrugbaarheid bemiddel via 'n lokale GnRHR-I, en moontlik ook via GnRHR-II.

Dit is opmerklik dat LH en FSH teenwoordig is in dieselfde gonadotroopselle van die pituitêre klier en tog verskillend gereguleer word deur GnRH tydens verskeie fisiologiese kondisies. Terwyl dit bekend is dat post-transkripsionele reguleringsmeganismes teenwoordig is, is die bydrae van transkripsionele regulering tot die differensiële uitdrukking van die *LH β* - en *FSH β* -subeenheidgene minder duidelik. In hierdie studie is die rol van GnRH-1 en GnRH-2 via die GnRHR-I en die GnRHR-II in transkripsionele

regulering van soogdier-*LH β* en -*FSH β* -gene in die L β T2 muis pituïtêre gonadotroopsellyn bepaal. Dit is vir die eerste keer aangetoon dat GnRH-1 'n effek mag hê op gonadotropiensubeenheid-geenuitdrukking via GnRHR-II bykomend tot GnRHR-I, en dat GnRH-2 ook die vermoë besit om gonadotropiensubeenheid-geenuitdrukking via beide reseptore te reguleer. Soos deur ander studies aangetoon is die transkripsionele respons van *LH β* en *FSH β* tot GnRH-1 klein (ongeveer 1.4-voudig vir *bLH β Luc* en 1.2-voudig vir *oFSH β Luc*). Verder is daar vir die eerste keer bewys gelever dat transkripsionele regulering van die gonadotropien β -subeenhede deur GnRH-2 ook gering is (ongeveer 1.5-voudig vir *bLH β Luc* en 1.1-voudig vir *oFSH β Luc*). Daar is aangetoon dat GnRH-1 'n sterker stimuleerder van *bLH β* -promotoraktiwiteit is in vergelyking met GnRH-2 via die GnRHR-I, hoewel beide hormone tot 'n soortgelyke maksimum induksie van *bLH β* lei. GnRH-2 is egter 'n meer effektiewe stimuleerder van *bLH β* -transkripsie as GnRH-1 via die GnRHR-II. Geen verskille is gevind tussen die effekte van GnRH-1 en GnRH-2 op *oFSH β* -promotoraktiwiteit via GnRHR-I of GnRHR-II nie. Wanneer die verhouding van uitdrukking van getransfekteerde *oFSH β* - en *bLH β* -promotor-verslaggewers via GnRH-1 met dié van GnRH-2 vergelyk is, is aangetoon dat GnRH-2 'n selektiewe reguleerder van *FSH β* -geentranskripsie is. Hierdie diskriminasie-effek van GnRH-2 is spesifiek vir GnRHR-I aangesien dit nie vir GnRHR-II waargeneem word nie. GnRH-1 lei tot 'n groter *oFSH β* tot *bLH β* -verhouding via GnRHR-II. Hierdie teenoorgestelde selektiwiteite van GnRHR-I en GnRHR-II op die verhoudings van *oFSH β* tot *bLH β* -promotoraktiwiteit vir GnRH-1 teenoor GnRH-2 suggereer dat daar 'n meganisme bestaan vir die fyn regulering van gonadotropiene in die pituïtêre klier, deurdat die relatiewe vlakke van GnRHR-I teenoor GnRHR-II gevarieer word. Daarbenewens is 'n konsentrasie-afhanklike moduleringsrol vir PACAP op GnRH-1- en GnRH-2-bemiddelde regulering van *bLH β* -promotoraktiwiteit aangetoon, via beide GnRHR-I en GnRHR-II, asook op *oFSH β* -promotoraktiwiteit via GnRHR-I. Hierdie konsentrasie-afhanklike effekte dui op die betrokkenheid van twee verskillende seinpadweë vir die PACAP-respons. Tesame suggereer hierdie bevindinge dat transkripsie van die gonadotropiengene *in vivo* onder ekstensiewe hormonale kontrole is wat verfyn kan word in respons to veranderlike fisiologiese kondisies. Laasgenoemde sluit veranderende vlakke van GnRH-1, GnRH-2, GnRHR-I en GnRHR-II asook PACAP in.

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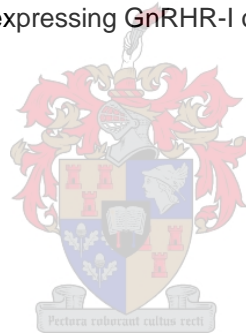
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Thesis structure

This thesis is divided into five chapters. Chapter 1 consists of a general introduction that introduces the reader to the basic relevant background on the structure, function and expression of gonadotropin-releasing hormone receptors (GnRHRs). Some of this background information has been published in an international journal [Hapgood JP *et al.*, 2005] and a copy of the publication is included at the back of the thesis. The general introduction is followed by a summary of the general aims of the study. Chapters 2, 3 and 4 contain the results of the study. Each of the results chapters is written in paper format by having its own background (which supplies more specific and detailed background information), aim, experimental section, results and discussion.

Chapter 2 covers the results of cloning of a mammalian type II GnRHR. The main focus is on the cloning from human ejaculate cells but cloning results from several human, as well as monkey and baboon, tissue types are included in the study. Some of the results shown in Chapter 2 have been published [Van Biljon W *et al.*, 2002] and a copy of the publication is included at the back of the thesis.

Chapter 3 reports on the findings of whether transcripts for the gonadotropin-releasing hormone(s) (GnRHs) as well as the type I GnRHR are found in ejaculate. The study was done in human and monkey ejaculate in parallel.

Chapter 4 contains the results of an extensive study performed in the L β T2 mouse pituitary gonadotrope and COS-1 monkey kidney cell-lines, with the aim being to compare cellular responses to GnRH-1 and GnRH-2 when either the mammalian type I GnRHR or the mammalian type II GnRHR is overexpressed.

A final chapter, Chapter 5, containing a concluding discussion and listing some future prospects, follows the three results chapters. Chapter 5 highlights the main results and conclusions reported in Chapters 2 to 4 and gives insight into the significance of the entire thesis. Thereafter follows a list of all references used throughout the thesis, in alphabetical order. The appendices can be found after the list of references at the back

of the thesis. These include the appendices that are referred to within the chapters as well as copies of publications.

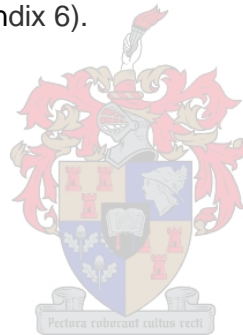
The author of this thesis did most of the work presented here. The human dot blot and *in situ* hybridisations mentioned in Chapter 2 and shown in Appendix 5 were however done by Dr Sonja Scherer (in our laboratory) and Dr Susan Wykes (under the supervision of Prof SA Krawetz), respectively. Brief mention is made of cloning results obtained from human testis tissue and exontrap results within Chapter 2 – these were performed by Ms Emerentia Hutchinson in our laboratory.

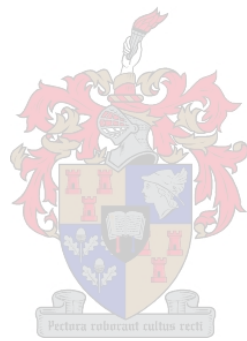


CHAPTER 1

GENERAL INTRODUCTION¹ & GENERAL AIMS

1. Part of the information in this chapter has been published [Hapgood JP, Sadie H, Van Biljon W, Ronacher K. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *Journal of Neuroendocrinology* 2005; **17**: 619-638]. A copy of the review can be found at the back of this thesis (Appendix 6).





General Introduction

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), is a central regulator of the mammalian reproductive system [Matsuo H *et al.*, 1971; Amoss M *et al.*, 1971]. It acts mainly on the anterior pituitary lobe via a specific GnRH receptor (the so-called type I GnRHR or GnRHR-I) on the plasma membrane, where it triggers the synthesis of the gonadotropin hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH in turn stimulate gonadal production of sex steroids. GnRH not only causes *de novo* production of the gonadotropins, but also induces their secretion from pituitary gonadotropes, allowing them to regulate the synchronisation of the reproductive cycle [Cheng CK & Leung PC, 2005]. The hypothalamus, pituitary and gonads together form the reproductive axis, also known as the hypothalamic-pituitary-gonadal (HPG) axis. GnRH was initially known as luteinising hormone-releasing hormone, referring to its stimulatory effect on LH release, but later obtained its current, more general, name [Dubois EA *et al.*, 2002].

The GnRH peptide is synthesised in the hypothalamic region of vertebrate brains but is also distributed in extrahypothalamic tissues such as the midbrain, central and peripheral nervous system, pituitary, and other peripheral tissues and cells (table 1). Interestingly, at least two, and often three, GnRH subtypes are found within a single species [Millar RP, 2003]. Generally, the GnRHs are named after the species in which they were first discovered but their distribution is not limited to that particular species. Humans, for example, express the mammalian GnRH and chicken GnRH-II subtypes [Millar RP, 2003] that will be named GnRH-1 and GnRH-2, respectively, from here onwards. GnRH-2 is regarded as the most conserved member of the GnRH family because it has been found in representative members of every vertebrate class, including from fish to humans [Millar RP *et al.*, 2004]. Its amino acid sequence (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂) only differs from GnRH-1 (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) at positions 5, 7 and 8 [Millar RP *et al.*, 2004].

Table 1: Distribution of GnRH-1 and GnRH-2 in mammals.

Mammalian tissue/cell type		GnRH-1	GnRH-2	Reference
Bone marrow		1	1	[Kakar SS & Jennes L, 1995; White RB <i>et al.</i> , 1998]
Brain	Amygdala	2	1	[Anthony EL <i>et al.</i> , 1984; Hayflick JS <i>et al.</i> , 1989; Stopa EG <i>et al.</i> , 1991; Lescheid DW <i>et al.</i> , 1997; Chen A <i>et al.</i> , 1998; White RB <i>et al.</i> , 1998; Urbanski HF <i>et al.</i> , 1999; Chen A <i>et al.</i> , 2001; Latimer VS <i>et al.</i> , 2001]
	Anterior olfactory area	2	N/D	
	Arcuate nucleus	N/D	1	
	Caudate nucleus	1	1	
	Cerebral cortex	N/D	2	
	Corpus callosum	N/D	1	
	Dentate gyrus	N/D	1	
	Foetal brain	N/D	1	
	Hippocampus	N/D	1	
	Hypothalamus	1	1,2	
	Infundibular stalk	2	N/D	
	Median eminence	2	2	
	Medulla oblongata	N/D	1,2	
	Midbrain	N/D	1,2	
	Neurohypophysis	2	N/D	
	Neuronal cell-lines	1,2	1,2	
	Pons	N/D	1,2	
	Periventricular region	N/D	2	
	Preoptic area	2	2	
	Septal region	2	N/D	
Suprachiasmatic nucleus	N/D	1		
Supraoptic nucleus	N/D	1		
Thalamus	N/D	1		
Breast	Cancer & carcinoma cells	1	1	[Seppälä M & Wahlston T, 1980; Harris N <i>et al.</i> , 1991; Chen A <i>et al.</i> , 2002b]
	Normal tissue	1	1	
Heart		1	N/D	[Kakar SS & Jennes L, 1995]
Immune cells	Normal and Jurkat leukemic T cells		2	[Azad N <i>et al.</i> , 1991; Chen HF <i>et al.</i> , 1999; Chen A <i>et al.</i> , 2002a]
	Peripheral blood mononuclear cells		1	
	Spleen lymphocytes		1	
Kidney		1	1	[Kakar SS & Jennes L, 1995; White RB <i>et al.</i> , 1998]
Liver		1	N/D	[Kakar SS & Jennes L, 1995]
Pituitary: normal & adenoma tissue		1	N/D	[Miller GM <i>et al.</i> , 1996; Sanno N <i>et al.</i> , 1997]
Pituitary stalk		N/D	2	[Chen A <i>et al.</i> , 1998]
Reproductive system	Fallopian tube		1,2	[Casañ EM <i>et al.</i> , 2000]
	Ovary	Carcinoma	1	[Irmer G <i>et al.</i> , 1995; Peng C <i>et al.</i> , 1994; Botté M-C <i>et al.</i> , 1998; Kang SK <i>et al.</i> , 2000; Choi KC <i>et al.</i> , 2001; Kang SK <i>et al.</i> , 2001]
		Granulosa-luteal cells	1	
		Surface epithelium	1	

	Placenta	First trimester	1,2	1,2	[Khodr GS & Siler-Khodr T, 1978; Kelly AC <i>et al.</i> , 1991; Wolfahrt S <i>et al.</i> , 1998; Chou CS <i>et al.</i> , 2004]
		Term	1,2	-	
	Pre-implantation embryos		2	N/D	[Casañ EM <i>et al.</i> , 1999]
	Prostate	Normal tissue	1	1	[Kakar SS & Jennes L, 1995; Limonta P <i>et al.</i> , 1992; White RB <i>et al.</i> , 1998]
		Cancer cells	1	N/D	
	Seminal plasma		2	N/D	[Izumi S-I <i>et al.</i> , 1985; Sokol RZ <i>et al.</i> , 1985]
	Testis	Seminiferous tubular cells	2	N/D	[Bhasin S <i>et al.</i> , 1983; Bahk JY <i>et al.</i> , 1995; Botté M-C <i>et al.</i> , 1998]
		Sertoli cells	1	N/D	
	Uterus	Endometrium and endometrial cancer cell lines	1,2	1	[Irmer G <i>et al.</i> , 1995; Chegini N <i>et al.</i> , 1996; Raga F <i>et al.</i> , 1998; Cheon KW <i>et al.</i> , 2001]
		Isolated epithelial cells	1,2	2	
Isolated stromal cells		1,2	2		
Leiomyomata		1,2	-		
Myometrium		1,2	-		
Skeletal muscle		1	N/D	[Kakar SS & Jennes L, 1995]	
Spinal cord		1,2	N/D	[Dolan S <i>et al.</i> , 2003]	
Sympathetic ganglion		2	N/D	[Jan YN <i>et al.</i> , 1980]	
Key:					
1 Expression indicated on mRNA level.					
2 Expression indicated on protein level.					
- Investigated but found to not be present.					
N/D Not determined to the author's knowledge.					

The secreted, mature hormones exist as decapeptides (the length of GnRH has been conserved) but the precursor or preprohormones are much longer in length, consisting of a signal peptide (21 to 23 amino acids) followed by the mature peptide, a cleavage site (Gly-Lys-Arg or G-K-R) and a GnRH-associated peptide (GAP, 40 to 60 amino acids) [Sherwood NM *et al.*, 1993] (figure 1). The G-K-R sequence serves to signal enzymatic cleavage of the decapeptide from the preprohormone [Cheng CK & Leung PC, 2005]. GAP, on the other hand, is possibly involved in the correct processing and packaging of GnRH [Sherwood NM *et al.*, 1993]. The coding region of the human GnRH-1 cDNA contains an open reading frame of 276 bp encoding a preprohormone of 92 amino acids [Cheng CK & Leung PC, 2005] (figure 1). The reading frame is followed by a 160 bp 3' untranslated sequence (UTR), which contains an AATAAA sequence for polyadenylation shortly upstream of a polyadenylated tail [Cheng CK & Leung PC, 2005] (figure 1). The human GnRH-1 signal peptide consists of 23 amino acids and is separated by the GnRH decapeptide

by two serine (S-S) residues, whereas GAP consists of 56 amino acids [Cheng CK & Leung PC, 2005] (figure 1). The predicted GnRH-2 preprohormone is organised identically to the GnRH-1 precursor [Cheng CK & Leung PC, 2005] (figure 1). GAP is however 50% longer in GnRH-2 than in GnRH-1 (84 vs. 56 amino acids) [White RB *et al.*, 1998] (figure 1).



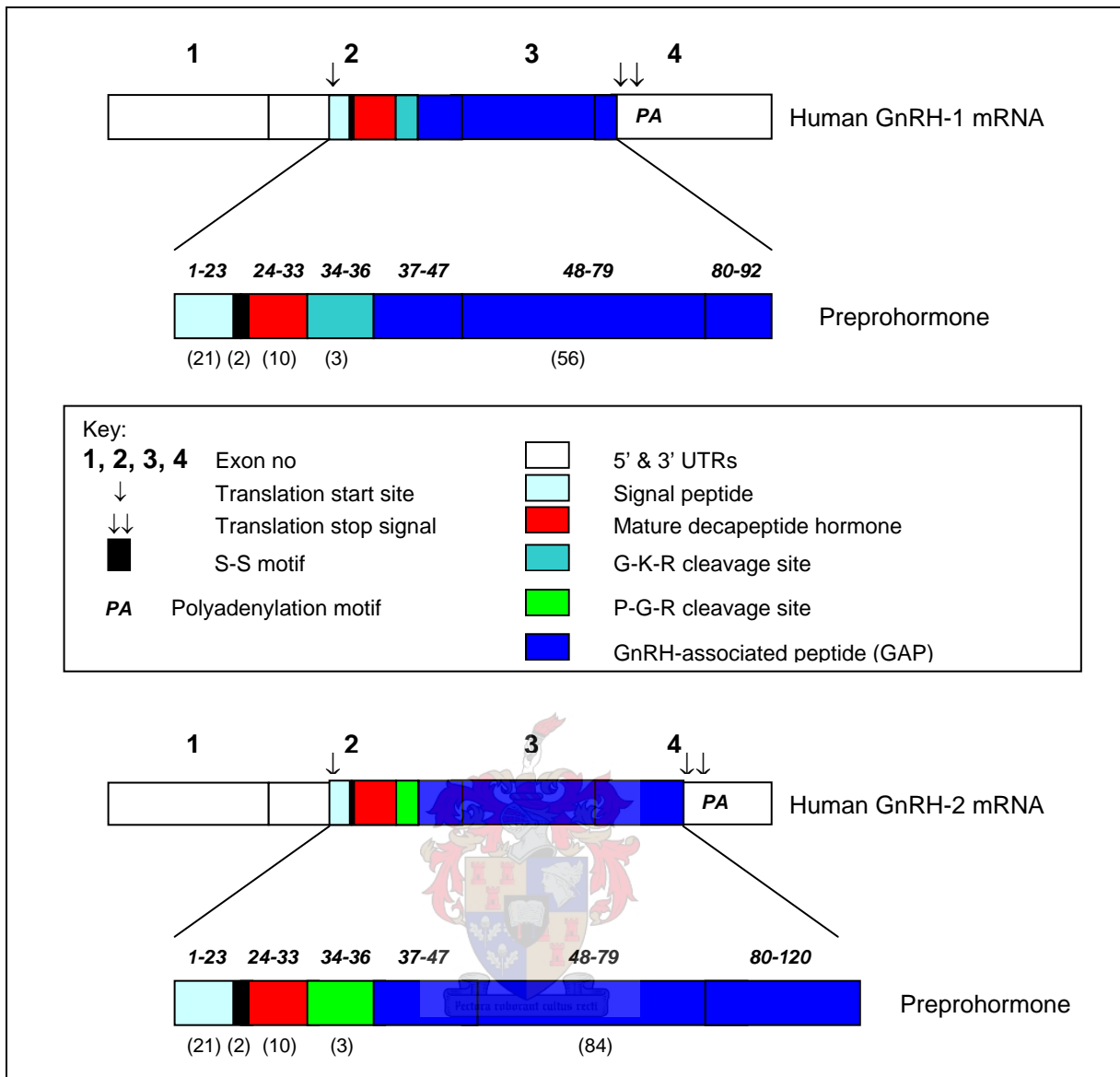


Fig 1. Schematic representation of human GnRH-1 and GnRH-2: mRNAs and preprohormones. Amino acid numbers are indicated above, whereas the number of amino acids of each segment of the preprohormones is indicated underneath the preprohormones. Data taken from Cheng CK & Leung PC [2005]; Seeburg PH & Adelman JP [1984] and White RB *et al.* [1998].

The GnRH genes are organised into four exons separated by three introns. The human *GnRH-1* gene is present as a single gene copy on chromosome 8p11.2-p21 [Cheng CK & Leung PC, 2005]. Exon 1 contains 5' UTR sequence. Exon 2 contains the rest of the 5' UTR as well as the signal peptide, GnRH, the G-K-R processing signal and the first 11 amino acids of GAP. Exon 3 encodes the next 32 GAP amino acids, while exon 4 encodes the remaining amino acids, the translation stop and

polyA⁺ signals and the entire 3' UTR [Seeburg PH & Adelman JP, 1984] (figure 1). The human *GnRH-2* gene has been mapped to chromosome 20p13 and is remarkably short (2.1 kilobases, kb) compared with *GnRH-1* (5.1 kb) primarily because the second and third introns are much larger in *GnRH-1*. The lengths of the various exons differ quite substantially between *GnRH-1* and *GnRH-2* (figure 1). In general, the sequence of exon 2 is the most conserved between the various GnRHs, whereas the other exons show high variability. As a consequence, the signal peptides and the GnRHs are well conserved, but the GAPs show less homology among species [King JA & Millar RP, 1992; King JA & Millar RP, 1997].

GnRH-1 (or hypothalamic GnRH) is classically the regulator of gonadotropin hormone expression in the pituitary. Hypothalamic GnRH is released in pulses from neuronal nerve endings into the hypophysial portal system every 30 to 120 min from where it binds to its receptor on pituitary gonadotropes [Millar RP, 2003]. In addition to its gonadotropin-regulating role, GnRH-1 performs other functions as well by binding to the GnRHR-I (see table 2). In contrast, indications are that the primary role of GnRH-2 is not to stimulate gonadotropin release but rather to act as a neurotransmitter to, for example, coordinate reproductive behaviour with an organism's energetic condition [Temple JL *et al.*, 2003; Kauffman AS & Rissman EF, 2004]. However, recently, GnRH-2 has also been shown to be capable of stimulating LH and FSH release both *in vivo* and in cultured pituitary cells, via activation of the GnRHR-I [Cheng CK & Leung PC, 2005]. Some suggestions are that GnRH-2 preferentially regulates FSH synthesis and release, but this is controversial [Padmanabhan V & McNeilly AS, 2001; Millar RP, 2003]. In addition, GnRH-2 mimics some of the other known actions of GnRH-1, such as its antiproliferative effects on human endometrial and ovarian cancer cells [Gründker C *et al.*, 2002] and its regulatory effect on the secretion of human chorionic gonadotropin (hCG) by human placenta [Siler-Khodr TM & Grayson M, 2001]. It is further possible that GnRH-2 plays a role in modulating pituitary responsiveness to GnRH-1 by competing for binding to GnRHR-I [Densmore VS & Urbanski HF, 2003]. This idea comes from the fact that the GnRHR-I becomes desensitised when exposed to continuous, rather than pulsatile, GnRH-1 [Belchetz PE *et al.*, 1978] and that GnRH-2 has a substantially longer circulating half-life than GnRH-1 [Licht P *et al.*, 1994]. In other words, if GnRH-2 remains bound to the GnRHR-I for a longer period, it is possible that GnRH-2 treatment would result in a more profound desensitisation of the GnRHR-I [Densmore VS & Urbanski HF, 2003].

The GnRHR-I was first identified exclusively in pituitary gonadotropes. However, since the isolation of the GnRHR-I cDNA, the expression of GnRHR-I mRNA has also been detected in several extrapituitary tissues (table 2). Whereas in pituitary cells the GnRHR-I, by binding of GnRH-1 or GnRH-2, seems to be specific for the regulation of LH and FSH synthesis and secretion, it functions as an autocrine and/or paracrine factor in extrapituitary compartments where it regulates steroidogenesis, cell proliferation, apoptosis and embryo implantation as well as a number of other functions (table 2). Thus, the extrapituitary actions of GnRH-1, GnRH-2 and their analogs might be mediated by local receptors or by desensitisation of pituitary receptors followed by decreased serum gonadotropin levels and gonadal steroids, or by both mechanisms [Naor Z, 1997; Cheng CK & Leung PC, 2005].

The first primary structure of a mammalian GnRHR-I was determined by sequencing of a functional receptor cDNA isolated from an immortalised mouse pituitary gonadotrope cell-line (alphaT3-1 or α T3-1) during the early 1990s [Tsutsumi M *et al.*, 1992; Reinhart J *et al.*, 1992]. The cloned cDNA encodes a 327 amino acid receptor protein that consists of seven hydrophobic stretches that are predicted to form transmembrane alpha (α) helices, separated by alternating intracellular- (ICL) and extracellular (ECL) loops, making it a member of the largest group of cell surface receptors, known as the serpentine or seven-transmembrane family of receptors. These receptors transmit their signals mainly through GTP-binding proteins (G proteins) and therefore are known as G protein-coupled receptors (GPCRs). Most of the primary sequence homology among GPCRs and thus among GnRHRs is contained within the transmembrane (TM) domains [Strader CD *et al.*, 1994; Kraus S *et al.*, 2001].

Table 2: Tissue distribution of the mammalian GnRHR-I as well as its tissue-specific function.

Mammalian tissue/cell type		GnRHR-I ^a	Some functions	Reference
Brain		1	Regulates food intake in females. Regulates reproductive behaviour.	[Krsmanovic LZ <i>et al.</i> , 1993; Temple JL <i>et al.</i> , 2003; Kauffman AS & Rissman EF, 2004]
Breast	Cancer tissue and tissue with fibrocystic disease	1,2	Inhibits tumour cell growth.	[Miller WR <i>et al.</i> , 1985; Eidne KA <i>et al.</i> , 1987; Kottler ML <i>et al.</i> , 1997; Mangia A <i>et al.</i> , 2002]
	Cancer cell-lines	1		
	Normal tissue	1,2		
Digestive tract and submaxillary glands		1,2	Secretion of epidermal growth factor (EGF).	[Yao B <i>et al.</i> , 2003]
Pre-implantation embryo		1	Improves blastocyst formation and quality of <i>in vitro</i> synthesised embryos.	[Nam DH <i>et al.</i> , 2005]
Gastric smooth muscle cells		1,2	Inhibits cell proliferation and DNA synthesis.	[Chen L <i>et al.</i> , 2004]
Heart		1		[Kakar SS & Jennes L, 1995; Chen HF <i>et al.</i> , 1999]
Immune cells	Peripheral blood mononuclear cells	1	Cell adhesion. Chemotaxis.	[Azad N <i>et al.</i> , 1993; Kakar SS & Jennes L, 1995; Azad N <i>et al.</i> , 1997; Chen HF <i>et al.</i> , 1999; Chen A <i>et al.</i> , 2002a]
	Peripheral T-lymphocytes	2	Increases cell proliferation.	
Kidney		1		[Kakar SS & Jennes L, 1995; Chen HF <i>et al.</i> , 1999]
Liver (hepatocarcinoma cell-line)		1	Inhibits cell proliferation.	[Kakar SS & Jennes L, 1995; Pati D & Habibi H, 1995; Cheng HYKW <i>et al.</i> , 1998; Yin H <i>et al.</i> , 1998; Chen HF <i>et al.</i> , 1999]
Melanoma cells		1,2	Promotes proliferation.	[Moretti RM <i>et al.</i> , 2002]
Olfactory epithelium		1,2	Triggers axon growth and actin cytoskeleton remodelling. Down-regulates nestin expression.	[Romanelli RG <i>et al.</i> , 2004]

Pituitary	Adenoma tissue	α -subunit/null-cells	2	LH and FSH synthesis and release.	[Kakar SS <i>et al.</i> , 1992; Sanno N <i>et al.</i> , 1997; La Rosa S <i>et al.</i> , 2000; Densmore VS & Urbanski HF, 2003]
		Gonadotropes	1,2		
		Somatotropes	2		
	Normal tissue	α -subunit/null-cells	2		
		Gonadotropes	1,2		
		Somatotropes	2		
		Thyrotropes	2		
	Cell-lines	α T3-1	1		
L β T2		1			
Reproductive system	Decidua		1	Regulates urokinase-type plasminogen activator and its endogenous inhibitor during pregnancy.	[Chou C-S <i>et al.</i> , 2003; Huang HY <i>et al.</i> , 2003]
	Ovary	Cancer cell-lines	1	Inhibits progesterone release. Steroidogenesis. Apoptosis. Follicular maturation, ovulation and atresia. Regulates cell growth.	[Ny T <i>et al.</i> , 1987; Leung PCK & Steele, 1992; Bussenot I <i>et al.</i> , 1993; Emons G & Scally AV, 1994; Irmer G <i>et al.</i> , 1995; Peng C <i>et al.</i> , 1994; Kakar SS <i>et al.</i> , 1995; Whitelaw PF <i>et al.</i> , 1995; Yin H <i>et al.</i> , 1998; Kang SK <i>et al.</i> , 2000; Zhao S <i>et al.</i> , 2000; Choi KC <i>et al.</i> , 2001; Cheng CK <i>et al.</i> , 2002; Siler-Khodr TM <i>et al.</i> , 2003]
		Cancer tissue	1		
		Epithelial carcinoma	1		
		Granulosa-luteal cells	1,2		
		Normal epithelium	1		
	Placenta		1	Human chorionic gonadotropin (hCG) secretion.	[Lin LS <i>et al.</i> , 1995]
	Prostate	Cancer tissue	1	Stimulates/inhibits cell growth.	[Limonta P <i>et al.</i> , 1992; Kakar SS <i>et al.</i> , 1995; Bahk JY <i>et al.</i> , 1998; Limonta P <i>et al.</i> , 1999; Tieva A <i>et al.</i> , 2001; Enomoto M <i>et al.</i> , 2004b]
		Cancer cell-lines	1,2		
		Intraprostatic lymphocytes	2		
Normal tissue		1			

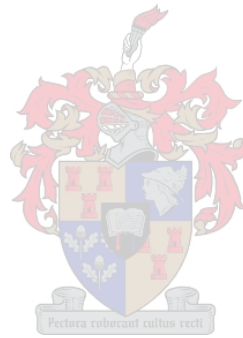
	Sperm		2 (N/C)	Spermatogenesis & sperm maturation. Sperm-egg binding during fertilisation. (refer to Chapter 3).	[Morales P <i>et al.</i> , 1994; Kangasniemi M <i>et al.</i> , 1996; Morales P & Llanos M, 1996; Glander H & Kratzsch J, 1997; Morales P, 1998; Lee CY <i>et al.</i> , 2000]
	Testis		1	Inhibits testosterone production by inhibiting 17 α -hydroxylase and 17,20-desmolase activities.	[Clayton RN <i>et al.</i> , 1980; Hsueh AJW <i>et al.</i> , 1983]
	Uterus	Endometrial carcinoma	1,2	Inhibits endometrial tumour cell growth.	[Imai A <i>et al.</i> , 1994; Chatzaki E <i>et al.</i> , 1996; Chegini N <i>et al.</i> , 1996; Kobayashi Y <i>et al.</i> , 1997]
		Leiomyomal cells	1,2		
		Myometrial cells	1,2		
		Normal endometrial tissue	1		
		Stromal cells	1		
		Cervical cancer cell-line	-		
	Retina		1,2		[Wirsig-Wiechmann CR & Wiechmann AF, 2002]
	Skeletal muscle		1		Kakar SS & Jennes L, 1995; Chen HF <i>et al.</i> , 1999]
	Spinal cord		1	Regulates currents through K ⁺ and Ca ²⁺ channels.	[Jan YN <i>et al.</i> , 1980; Dolan S <i>et al.</i> , 2003]
<p>Key:</p> <p>a Most of this data has been obtained from [Hapgood JP <i>et al.</i>, 2005] (see table 1 of Appendix 6).</p> <p>1 Expression indicated on mRNA level.</p> <p>2 Expression indicated on protein level by immunodetection, not binding studies.</p> <p>- Expression investigated and found not to occur.</p> <p>N/C Expression indicated but results are not convincing.</p>					

GPCRs are integral membrane proteins involved in the transmission of a wide variety of signals from the extracellular environment to the intracellular milieu. The G proteins that are coupled to GnRHRs are heterotrimeric proteins composed of an α subunit ($G\alpha$) that binds guanine nucleotides (GTP or GDP), and a dimer that consists of a β and γ subunit ($G\beta\gamma$). Upon stimulation, $G\alpha$ dissociates from the $G\beta\gamma$ dimer which results in the active GTP-bound form of $G\alpha$ that influences various effector molecules. The $G\beta\gamma$ dimer remains attached to the plasma membrane and can by itself initiate several signalling events. G proteins can be broadly classified according

to the subtype of their α subunit into the four following groups: G_s , G_i , $G_{q/11}$ and $G_{12/13}$ [Kraus S *et al.*, 2001]. G_s mainly exerts its downstream effects via stimulation of adenylyl cyclase, which induces the production of high levels of the second messenger adenosine 3',5'-cyclic monophosphate (cAMP) and activation of PKA [Han XB & Conn PM, 1999; Kraus S *et al.*, 2001]. Unlike G_s , the G_i protein has an inhibitory effect on adenylyl cyclase [Kraus S *et al.*, 2001]. $G_{q/11}$ principally exerts its action by activating membrane-associated phospholipase C (PLC), while $G_{12/13}$ primarily operates by stimulation of protein tyrosine kinases [Kraus S *et al.*, 2001]. Thus, binding of GnRH (-1 and/or -2) to the GnRHR-I activates a signal transduction cascade that eventually directs the synthesis and release of LH and FSH (see figure 2). A single receptor can activate several different pathways in a given cell. Classically, in $\alpha T3-1$ cells, binding of GnRH-1 to the GnRHR-I leads to the stimulation of G_q and/or G_{11} , activating PLC and leading to enhanced phosphoinositide turnover (figure 2). Enhanced phosphoinositide turnover stimulates the production of the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2) [Naor Z, 1997] (figure 2). For this reason, GnRH-induced IP_3 production has been used to assess signal transduction in response to GnRH. IP_3 in turn mobilises Ca^{2+} from intracellular stores, which, combined with DAG and phospholipid, activates various protein kinase C (PKC) subspecies. [Naor Z, 1997] (figure 2). Mobilisation of intracellular Ca^{2+} is followed by an influx of extracellular Ca^{2+} through voltage-gated calcium channels in the plasma membrane. Whereas IP_3 -released Ca^{2+} seems to be critical for gonadotropin secretion, Ca^{2+} influx through the plasma membrane is required mainly for the replenishment of internal stores [Kraus S *et al.*, 2001]. Following a short lag (~1 to 2 min), phospholipase D (PLD) is also activated. It has been suggested that DAG is generated in sequential phases, initially by PLC and later by PLD, permitting selective and sequential activation of various PKC subspecies (figure 2). Ca^{2+} -dependent PKCs may be activated early, whereas the Ca^{2+} -independent PKCs might be activated at a later stage [Naor Z, 1997].

The PKC gene family plays a pivotal role in cell signalling by means of its protein serine/threonine kinase activity. GPCRs are thought to act via PKC-dependent and independent pathways to activate the mitogen-activated protein kinase (MAPK) cascades. MAPK is translocated to the nucleus where it can interact and activate transcription factors. Thus, MAPK provide an important link for the transmission of

signals from the cell surface to the nucleus and play a role in the regulation of gonadotropin gene transcription [Naor Z, 1997; Kraus S *et al.*, 2001].



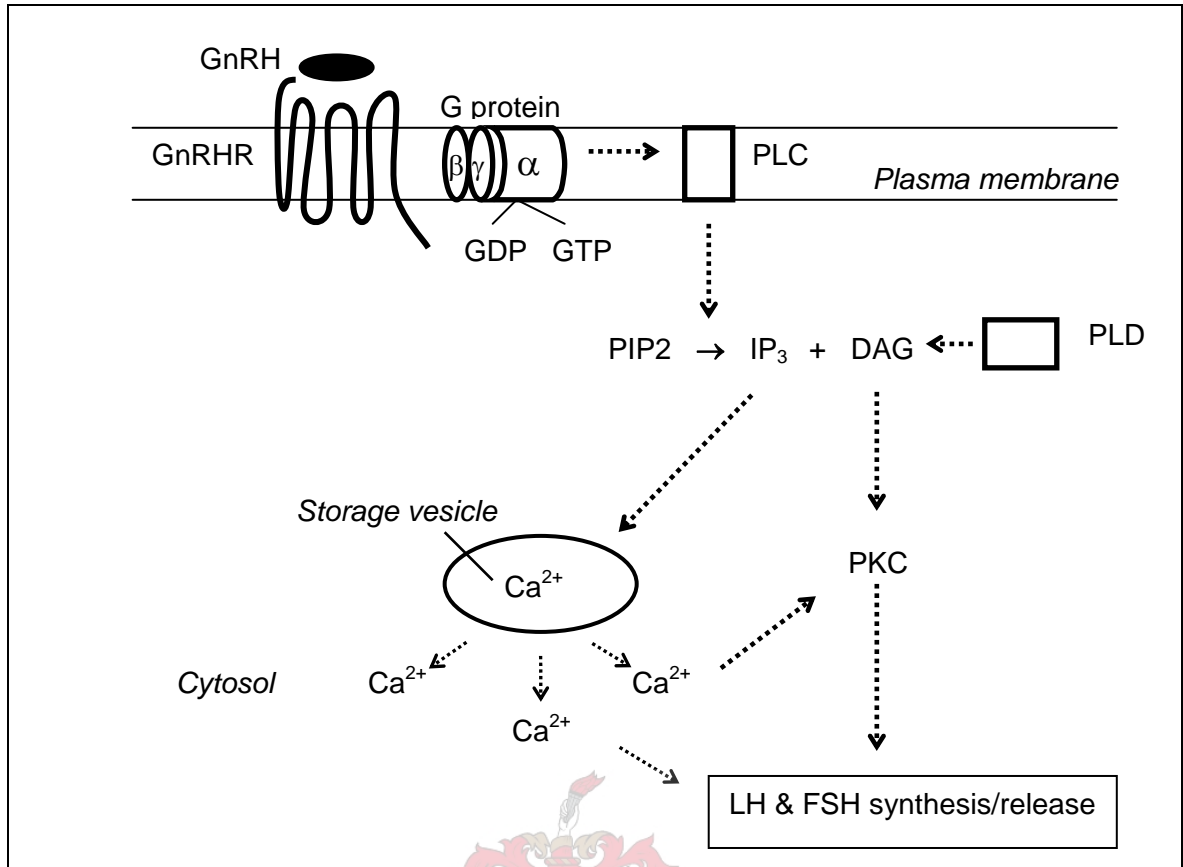


Fig 2. Classical model for GnRH-1 signal transduction via the mammalian GnRHR-I. Data taken from Naor Z [1997]. PLC, phospholipase C; GDP, guanosine diphosphate; GTP, guanosine triphosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; IP₃, inositol-1,4,5-triphosphate; DAG, diacylglycerol; PLD, phospholipase D; PKC, protein kinase C.

In other cell types, the GnRHR-I may couple to different G proteins, which results in different signalling (see table 3). It is evident from table 3 that cell context is extremely important for coupling of the GnRHR to different G proteins and highlights the danger of extrapolating results from one cell type to another [Liu F *et al.*, 2002b]. Each cell type has a different capacity to amplify a specific signalling cascade, probably due to a differential concentration of cellular components required for signalling pathways [Oh DY *et al.*, 2003].

Table 3: G protein coupling by GnRHR-I in different mammalian cell types.

Cell-type	G protein involved in GnRHR signalling	G protein shown not to be involved in GnRHR signalling	Reference
α T3-1 mouse pituitary gonadotrope cells	G _{q/11}		[Naor Z, 1997]
L β T2 mouse pituitary gonadotrope cells	G _{q/11} & G _s	G _{i/0}	[Liu F <i>et al.</i> , 2002b]
COS-7 cells transfected with human GnRHR-I	G _i (primarily) & G _{q/11}		[Grosse R <i>et al.</i> , 2000; Kraus S <i>et al.</i> , 2001]
GGH3 cells (GH3 rat pituitary lactotrope cells stably transfected with rat GnRHR-I)	G _{q/11} , G _s & G _i (only when GnRHR is overexpressed)		[Stanislaus D <i>et al.</i> , 1997]
Human reproductive tract tumours	G _{i/0}		[Imai A <i>et al.</i> , 1996]
LNCaP prostate cancer cells	G _{i/0}		[Kraus S <i>et al.</i> , 2001]
Rat gonadotropes	G _{q/11} , G _s & G _i		[Stanislaus D <i>et al.</i> , 1998]

In contrast to the genes of many other GPCRs, which are intronless, the structural organisation of all mammalian GnRHR genes that have been cloned to date is three exons separated by two introns. The human *GnRHR-I* gene exists as a single copy on chromosome 4q21.2 and it spans more than 20 kilobase pairs (kb) [Cheng CK & Leung PC, 2005] (see figure 3). Exon 1 encodes the 5' UTR and the first 522 nucleotides (nt) of the open reading frame, which encode TMs 1 to 3 and a portion of the 4th TM domain (figure 3). Exon 2 encodes the next 220-nt of the reading frame (nt +523 to +742), which encompass the remainder of TM4, the 5th TM domain, as well as part of ICL3 (figure 3). Exon 3 contains the rest of the coding sequence (nt +743 to +987) and the 3' UTR [Kakar SS, 1997; Cheng CK & Leung PC, 2005] (figure 3).

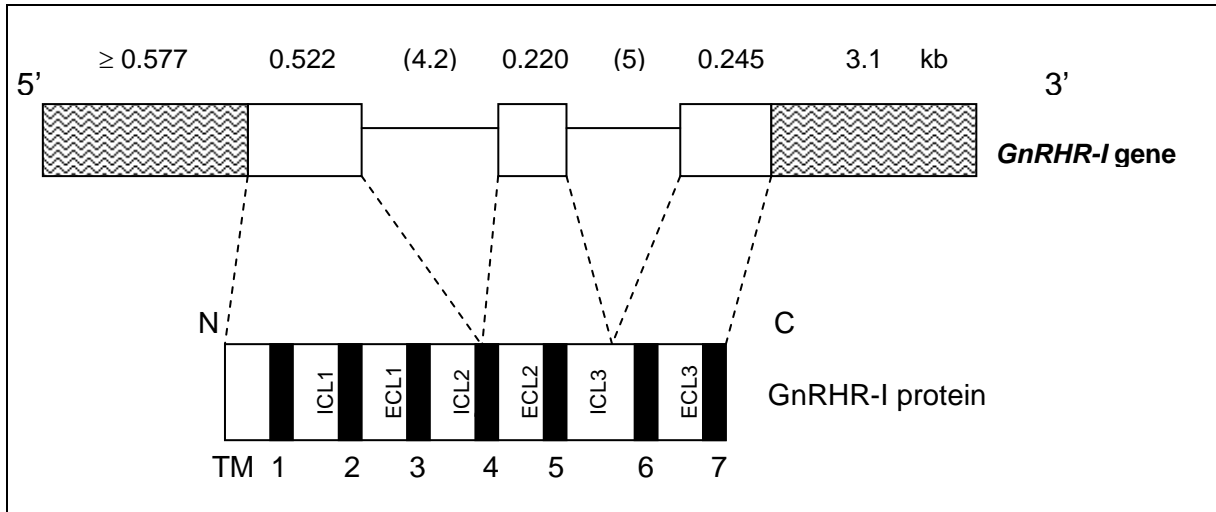


Fig 3. Structural organisation of the human *GnRHR-I* gene. Exons are numbered with Roman numerals and are represented by blocks, with portions of exons containing coding sequences shown as white areas, and untranslated regions shown as shaded areas. Sizes of coding and non-coding portions of each exon are indicated. Introns are represented by solid lines, with sizes as indicated between brackets. All sizes are indicated in kilobase pairs (kb). The size of the 5' UTR is given relative to the most-3' transcription start site as identified by Kakar SS *et al.* [1997] for human pituitary tissue, and the size of the 3' UTR is as established by Fan NC *et al.* [1995] for human brain tissue. The correlation of coding regions with protein structure is indicated, with transmembrane (TM) domains shown as black bars. The figure was adapted from Hapgood JP *et al.* [2005]. Note that the figure is not drawn to scale.

The amino (N)-terminal domain of GPCRs is extracellular and often contributes to ligand recognition and binding, while the intracellular carboxyl (C)-terminal domain contributes to effector binding and downstream signalling events. Sustained stimulation of GPCRs typically causes receptor desensitisation and internalisation, which is mediated by phosphorylation, often within the C-terminal tail of the receptor [McArdle CA *et al.*, 2002]. Desensitisation is defined as a waning of response in the presence of a constant, or repeated, stimulus [McArdle CA *et al.*, 2002]. The mammalian GnRHR-Is are unique in that they lack C-terminal tails and apparently do not undergo agonist-induced receptor desensitisation and internalise slowly [McArdle CA *et al.*, 2002]. Although mammalian GnRHR-Is do not desensitise, sustained activation of GnRHR-Is causes desensitisation of gonadotropin secretion, and can

result in down-regulation of IP₃ receptors and desensitisation of Ca²⁺ mobilisation in pituitary cells [McArdle CA *et al.*, 2002]. Non-mammalian GnRHRs do however possess intracellular C-terminal tails [McArdle CA *et al.*, 2002] and can be internalised in small vesicles and recycled [Naor Z, 1997].

One of the reasons for the great interest in the GnRH/GnRHR system is its application in the medical field. Synthetic GnRH and GnRH analogues are being used clinically in applications such as the advancement of puberty in the instance of delayed puberty; as a contraceptive by inhibiting ovulation and spermatogenesis; as a treatment of hormone-dependent diseases such as prostatic and breast cancer; and as a treatment of infertility by inducing ovulation [Millar RP *et al.*, 1993; Millar RP *et al.*, 2004]. Interestingly, at the time when this study was begun, new sequence information became available about a second form of the GnRHR (designated “type II” GnRHR or GnRHR-II) in a number of non-mammalian vertebrates [Troskie B *et al.*, 1997; Troskie B *et al.*, 1998; Illing N *et al.*, 1999] (refer to Chapter 2 for a more detailed description of the discovery of the GnRHR-IIs). The GnRHR-IIs were shown to also bind GnRH-1 but with a lower affinity compared to GnRH-2 [Millar R *et al.*, 2001]. The finding of the existence of more than one GnRHR subtype added to the complexity of the GnRH/GnRHR system. Not only does GnRH exist in multiple forms but it is also able to bind to and signal via distinct GnRH-specific receptors. The existence of a mammalian GnRHR-II was not yet established at the start of this study. Should such a functional GnRHR-II (which possesses a unique tissue distribution and a different ligand selectivity as compared to the GnRHR-I) exist in humans, this would be very significant in the medical field.

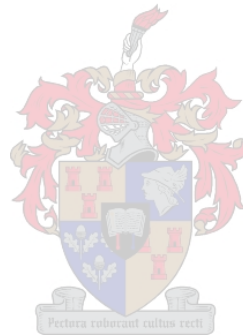
Taken together, when this study was started, there were a number of outstanding questions in the GnRH/GnRHR field. Firstly, the ability of GnRH-1 and GnRH-2 to differentially regulate LH and/or FSH gene transcription by binding to the mammalian GnRHR-I was not yet established satisfactorily. Another issue on the forefront was the question of whether mammals, including humans, express a GnRHR-II. If mammals indeed express a functional GnRHR-II, its role as a putative regulator of the gonadotropin hormones would need to be determined. Finally, while data is accumulating for the extrapituitary actions of GnRH-1 and/or GnRH-2 and that some of these actions are mediated by locally produced GnRH via binding to a local receptor, it has not yet been determined whether a local GnRH/GnRHR system is present in the male reproductive tract where it may affect reproduction directly. This study was designed to investigate the above-mentioned outstanding questions.

General Aims

The general aims of the study were to determine whether

- humans and non-human primates express a functional GnRHR-II cDNA (Chapter 2);
- an autocrine GnRH/GnRHR system is contained within human and/or monkey ejaculate (Chapter 3);
- the *LH β* and *FSH β* genes are differentially regulated by GnRH-1 and GnRH-2 via the GnRHR-I and the GnRHR-II (Chapter 4).

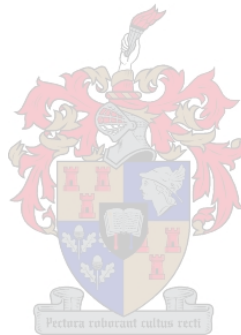
More specific aims are outlined within the relevant chapters.



CHAPTER 2

CLONING AND SEQUENCING OF A MAMMALIAN TYPE II GONADOTROPIN-RELEASING HORMONE RECEPTOR (GnRHR-II) cDNA¹

- 1 Some of the results shown in this chapter have been published [[Van Biljon W, Wykes S, Scherer S, Krawetz SA, Hapgood J. Type II gonadotropin-releasing hormone receptor transcripts in human sperm. *Biology of Reproduction* 2002; 67: 1741-1749](#)]. A copy of the publication can be found at the back of this thesis (Appendix 5).

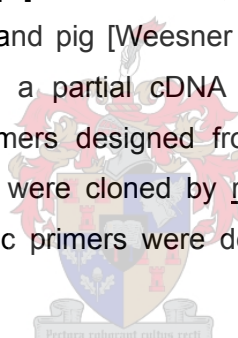




Background

The first GnRHR cloned was the mouse GnRHR-I, from RNA isolated from α T3-1 pituitary gonadotrope cells [Tsutsumi M *et al.*, 1992; Reinhart J *et al.*, 1992]. A reverse transcriptase polymerase chain reaction (RT-PCR)-based cloning strategy was followed, whereby cDNA prepared from α T3-1 cells was used as template, together with degenerate primers designed to bind to conserved regions of GPCRs [Tsutsumi M *et al.*, 1992; Reinhart J *et al.*, 1992]. Functionality of the partial cDNAs obtained was tested in a hybrid-arrest assay in *Xenopus laevis* oocytes whereafter an α T3-1 cDNA library was screened to obtain the full-length mouse GnRHR-I cDNA [Tsutsumi M *et al.*, 1992; Reinhart J *et al.*, 1992].

Subsequently, the cloning of GnRHRs from several mammalian and non-mammalian vertebrates has been described. The cloned mammalian GnRHR-Is include that of rat [Eidne KA *et al.*, 1992], sheep [Brooks J *et al.*, 1993], human [Chi L *et al.*, 1993], bovine [Kakar SS *et al.*, 1993] and pig [Weesner GD *et al.*, 1994]. Generally, the cloning strategy was to obtain a partial cDNA sequence by PCR amplification performed with degenerate primers designed from conserved regions of known GnRHRs. The 5' and 3' ends were cloned by rapid amplification of cDNA ends (RACE) whereafter gene-specific primers were designed to amplify the full-length cDNAs.



The presence of more than one form of GnRH within a single vertebrate species indicated the probable existence of multiple GnRHR subtypes. With the use of a series of pairs of degenerate oligonucleotides to the mammalian GnRHR-I, short sequences encoding ECL3, which suggested the presence of at least two distinct GnRHR genes, were cloned from genomic DNA of species of amphibian, fish, reptile and bird [Troskie B *et al.*, 1998]. One of these ECL3 sequences was most similar to the mammalian pituitary GnRHR-I [Troskie B *et al.*, 1998]. The other was different and was designated "GnRHR-II" [Troskie B *et al.*, 1998].

The full-length cDNAs for two goldfish GnRHR-IIs, called GfA and GfB, were cloned from pituitary and brain respectively [Illing N *et al.*, 1999; Lethimonier C *et al.*, 2004]. It was found that GfA has a greater preference for GnRH-2 and a lesser preference for the other natural GnRHs [Illing N *et al.*, 1999]. Furthermore, in amphibian sympathetic ganglia the presence of a GnRH-2-selective receptor was indicated by

receptor binding studies [Troskie B *et al.*, 1997]. The existence of a receptor selective for GnRH-2 in these non-mammalian vertebrates, together with the presence of GnRH-2 in all vertebrates from jawed fish to humans [Millar RP, 2003] suggested the existence of a GnRH-2-selective GnRHR in mammals. At the onset of the present study, no mammalian GnRHR-II was yet identified.

Millar R *et al.* [1999] used sequence information of ECL3 of a putative reptile GnRHR-II to search for a human GnRHR-II homolog in a human expressed sequence tag (EST) database. The use of sequence information of ECL3 to search for novel GnRHR-IIs, as described in many instances above, was based on the indication that ECL3 plays a role in determining differential ligand selectivity [Li JH *et al.*, 2005] and hence that ECL3 is degenerate between different GnRHRs. The gene sequence of a putative human GnRHR-II was not available at that time because the human genome project was in progress but not yet completed. Several GnRHR-like ESTs were obtained from the EST database. A consensus sequence was derived that contained nucleotide sequence corresponding to exon 2 (ECL2 to ICL3) and exon 3 (ICL3 to the end of TM7) of the human GnRHR-I [Millar R *et al.*, 1999]. The overall amino acid identity between this region of the human GnRHR-I and the EST-derived putative human GnRHR-II was 42%, which was much higher than the percentage homology to any other GPCR [Millar R *et al.*, 1999]. The homology of the human GnRHR-II homolog to ECL3 of the reptile GnRHR-II was 80% [Millar R *et al.*, 1999]. Surprisingly, all EST transcripts detected matched the GnRHR-I in a reverse, or antisense, orientation (figure 4). PCR performed on cDNA from a wide range of human tissues (refer to table 4 within the Discussion of this chapter) revealed that intronic sequence equivalent to intron 2 of the GnRHR-I was retained. The failure to splice out putative intron sequences in transcripts which spanned exon-intron boundaries is expected in antisense transcripts, as candidate donor and acceptor sites are only present in the gene when transcribed in the orientation encoding the GnRHR homolog (figure 4). No transcripts extended 5' to the sequence corresponding to exon 2 of the GnRHR-I as the antisense transcripts terminated in polyA due to the presence of a polyadenylation signal sequence in the putative intron 1 when transcribed in the antisense orientation (figure 4).

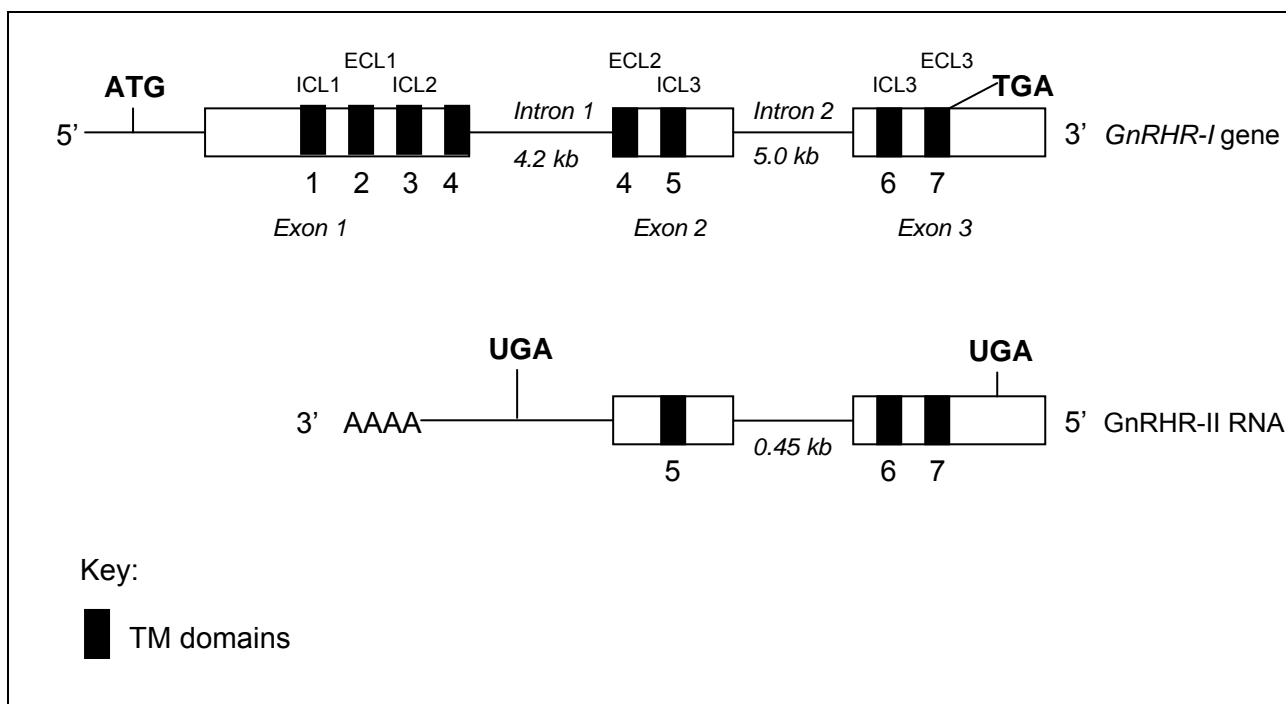


Fig 4. Comparison of the human GnRHR-II antisense transcript and the *GnRHR-I* gene (adapted from Millar R *et al.* [1999]). Positions of intracellular (ICL) and extracellular (ECL) loops, as well as transmembrane (TM) domains (dark blocks) are indicated. The polyA tail (AAAA) at the position of putative intron 1 is indicated for the GnRHR-II antisense transcript.

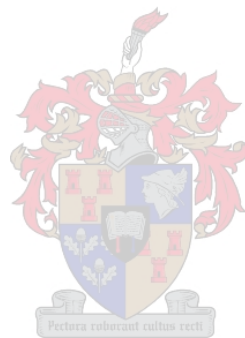
Thus, the only information available for a putative human GnRHR-II at the start of this project was partial sequence information of exons 2 and 3, obtained from an EST database, as well as the fact that an antisense transcript is abundantly expressed in a wide variety of tissues. In addition, prior results obtained in our laboratory using an exon 2-3-specific primer pair in RT-PCR revealed the presence of an intronless transcript, together with the intron 2-containing antisense transcript, in human testis RNA [Hutchinson E, 1997]. The intron-containing transcript was in abundance over the intronless transcript, which seemed to be a minor product of the RT-PCR [Hutchinson E, 1997]. Two overlapping intronless exon 2-3 amplicons were cloned from human testis which, together, formed an amplicon of 628 bp in length, stretching from the coding region for ECL2 in exon 2 (primer S10) to the 3' UTR in exon 3 (primer AS11) [Hutchinson E, 1997] (refer to figure 6, as well as Appendix 1 and Appendix 2 for a description of primer sequences and positions in the *GnRHR-II* gene). 5' RACE attempts on human testis RNA, with the aim to obtain novel human GnRHR-II sequence 5' to exon 2, were unsuccessful [Hutchinson E, 1997]. Subsequently, some human GnRHR-II exon 1 sequence information, obtained from

an EST database, as well as the sequences of three sense and three antisense primers within this region, was obtained from Dr A Katz (Department of Medical Biochemistry, University of Cape Town, Cape Town, South Africa). The six exon 1-specific primers were S4, S5, S6, AS3, AS4 and AS5. Of these, S4 was the most 5' primer and was designed to the coding region of the extracellular N-terminus. Primer AS5, which was the most 3' exon 1-specific primer, was designed to a region encoding part of TM4 (refer to figure 6 and Appendices 1 to 3). In addition, human P1 genomic clones containing *GnRHR-II* gene sequence were obtained from Dr A Katz. These had been purchased from Genome Systems, St Louis, USA.

Based on the results in the literature as well as results obtained in our laboratory, it was apparent that expression levels of the intronless, sense transcript for a putative human GnRHR-II was very low compared to that of the antisense transcript. This was strengthened by results obtained in our laboratory of low-level expression (compared to the intron-containing transcript) of the intronless transcript in human testis. Since the antisense transcript would not contain exon 1 due to the presence of the polyadenylation signal within putative intron 1 (figure 4), the focus of the present study was to find a human tissue- or cell type that expresses exon 1-containing GnRHR-II transcripts. Therefore, human tissues and cells that were available were screened for the presence of exon 1-containing GnRHR-II transcripts using RT-PCR, *in situ* hybridisation and dot blotting. Human ejaculate was included in the study based on the prior results obtained in human testis RNA. The scope of the study was subsequently broadened to include tissues and cells from two other mammalian primate species, namely vervet monkey (also known as the African green monkey) and baboon, in an attempt to clone a mammalian GnRHR-II.

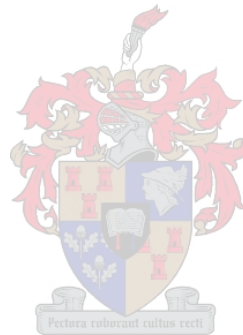
A central question is what the significance of a GnRHR-II in humans would be. One possibility is that the presence of a human GnRHR-II may explain why, in some instances, clinically used GnRH-1 agonists were found to act as antagonists *in vivo* [Morgan JE *et al.*, 1986; Rivier J *et al.*, 1996]. Indications are that the GnRHR-IIs are selective for GnRH-2 [Troskie B *et al.*, 1997; Illing N *et al.*, 1999] and thus have a different ligand selectivity compared to the GnRHR-I. It is therefore possible that agonists for the human GnRHR-I could act as antagonists for a human GnRHR-II, if humans express a functional GnRHR-II. Considering that GnRH-2 is highly conserved between species and widely distributed in mammalian tissues, it most likely has important functions [Millar RP, 2003]. The cloning of a human GnRHR-II would open up the opportunity to elucidate the GnRH-2-specific functions and to

develop GnRH-2 analogues for specific use as therapeutic agents. A better understanding of the characteristics of such a putative receptor would open up doors to predict the outcome of GnRH agonist and antagonist treatments.



Aim

The aim of this study was to clone a full-length cDNA, originating from the sense RNA transcript, for a GnRHR-II in humans. In addition, an attempt was made to clone a functional GnRHR-II cDNA from a non-human primate species.



Experimental

Animals and cells

Human and vervet monkey ejaculate were freshly obtained from the Andrology Department, Groote Schuur hospital (Cape Town, South Africa) and from the MRC at Tygerberg (Bellville, South Africa) (courtesy of Dr J Zyer), respectively, kept at room temperature (rt) and used within a few hours for RNA isolation. Each ejaculate sample was pooled from several male donors. Adult human tissues were obtained from the Salt River Mortuary (Cape Town, South Africa) after approval from the Medical Ethics Committee at the University of Cape Town Medical School (Cape Town, South Africa). The tissues were snap frozen in liquid nitrogen and stored at -80°C until later use. Vervet monkey and baboon tissues were obtained from the MRC at Tygerberg and from the Department of Dental Science at the University of Stellenbosch Medical School (Bellville, South Africa) (courtesy of Prof N Louw), respectively. Monkey and baboon tissues were extracted from animals immediately after mortalisation and were either homogenised on the spot in TRI-reagent (Sigma-Aldrich, Saint Louis, Missouri) ($\sim 10 \times$ the tissue volume) or in tissue guanidinium solution (5 M Guanidinium isothiocyanate, 0.05 M Tris-HCl pH 7.5, 0.01 M Na_2EDTA pH 8.0, 5% v/v mercapto-ethanol) with the use of an Ultra-turrax or stored whole in RNA*later*® (Ambion, Inc., Austin, USA) ($\sim 4 \times$ the tissue volume), snap frozen in liquid nitrogen and transferred to a -80°C freezer for later use. COS-1 cells were kept in culture at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1% v/v PenStrep and supplemented with 10% foetal bovine serum (FBS) (Highveld Biological, Kelvin, South Africa), under 5% CO_2 .

RNA preparation and cDNA synthesis

Total RNA was isolated from human and vervet monkey ejaculate by cesium chloride (CsCl)-guanidinium isothiocyanate ultracentrifugation after pelleting the ejaculate cells for 10 min at $2000 \times g$, as described by Ausubel FM *et al.* [1996a]. Total RNA from vervet monkey and baboon tissues was isolated by CsCl-guanidinium isothiocyanate ultracentrifugation after pelleting the homogenised tissue for 10 min at $12000 \times g$ using the protocol described by Ausubel FM *et al.* [1996a] or according to the TRI reagent™ protocol (Sigma-Aldrich). Tissue samples that had been stored frozen in RNA*later*® were thawed on ice, rinsed in diethyl pyrocarbonate (DEPC)-treated water and homogenised in tissue guanidinium solution. Total RNA from COS-1 cells was isolated according to the TRI reagent™ protocol, with the use of 1

m ℓ TRI-reagent per confluent T75 flask (surface area 75 cm²). Total RNA from other human tissues and cells was isolated with the use of TRI reagentTM. All RNA samples, except the ultracentrifuged ultra pure preparations, underwent a DNaseI treatment, as described in Ausubel FM *et al.* [1996b] to minimise the possibility of genomic DNA contamination prior to cDNA preparation. This was to ensure that RT-PCR products are the result of priming of cDNA only and not contaminating genomic DNA. The quality of the total RNA preparations was determined by gel electrophoresis on a 1% denaturing formaldehyde agarose gel according to the protocol described by Sambrook J *et al.* [1989a]. Three (3) to 5 μ g of each total RNA sample was loaded. Interestingly, it was found that the ejaculate total RNA preparations were intact only when using the CsCl-guanidinium isothiocyanate ultracentrifugation method of isolation but not when using TRI reagentTM (not shown). All RNA isolations from human and vervet monkey ejaculate used for cDNA synthesis were thus performed according to the CsCl-ultracentrifugation method described above.

cDNAs used as templates for RT-PCR were prepared from 1 μ g of denatured total RNA using 200 ng random hexamer primers (Promega, Madison, WI) and 200 U Superscript II reverse transcriptase (GibcoBRL/Invitrogen, California, USA) in a 20 μ l reaction volume with incubation at 42°C for 60 min. Double-stranded Marathon-ready cDNA for 5' RACE was prepared from human ejaculate total RNA with the use of the MarathonTM cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA). One (1) μ g of total RNA was primed with hexanucleotide primers (5 mM, Promega) for first-strand cDNA synthesis using AMV reverse transcriptase and all other components of the MarathonTM cDNA amplification kit. Adaptor-ligated double-stranded cDNA was diluted 1:25 in Tricine-EDTA buffer, which was supplied with the Marathon kit. Vervet monkey and baboon single-stranded cDNAs used for 5' and 3' RACE were synthesised from 1.5 to 3.5 μ g of total RNA using the SMARTTM RACE cDNA amplification kit (BD Biosciences Clontech) according to the manufacturer's instructions. Powerscript reverse transcriptase (BD Biosciences Clontech) was utilised in a total volume of 10 μ l for the cDNA synthesis reaction, at 42°C for 90 min. Single-stranded SMARTTM RACE-ready cDNA was diluted by adding 100 μ l Tricine-EDTA buffer (BD Biosciences Clontech).

RT-PCR and RACE

RT-PCR reactions were performed using 10 μl cDNA, 5 U *Taq* DNA polymerase in Storage Buffer A (Promega), 20 pmoles of each primer, 0.2 mM of each dNTP, 1.7 mM MgCl_2 and 1 X PCR reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100) in a 50 μl reaction volume. Five per cent (5%) Dimethylsulfoxide (DMSO) was included in the RT-PCR reaction make-up for vervet monkey and baboon RNAs. Primer sets were designed such that the PCR extended over more than one exon. Refer to Appendices 1 to 3 for a list of the primers that were used in RT-PCR and Southern blotting as well as the primer sequences, relative positions with regards to the relevant genes and expected sizes of the RT-PCR products. A schematic representation of the GnRHR-II cDNA, with the relative positions of the primers indicated, are shown in the Results section of this chapter (see figure 6). Seeing that no vervet monkey and baboon genomic DNA sequences for a GnRHR-II were available, human-specific primers were used in the vervet monkey and baboon RT-PCRs and in RACE.

Cycle conditions for PCR were as follows: 93°C for 2.5 min, 35 to 40 cycles of 93°C for 1 min, annealing as indicated in Appendix 2 (ranging from 52°C to 60°C) for 1 min and extension at 72°C for 1 min, followed by a final 10 min extension time at 72°C. Annealing temperature was chosen to be approximately 5°C below the lowest melting temperature (T_m) of the primer combination used (refer to Appendix 2 for a list of annealing temperatures used in PCR). A control PCR reaction, to test for efficiency of the RT-reaction, was routinely performed using primers designed to the mouse beta (β)-actin housekeeping gene. The β -actin primer set was designed such to cross exon-intron boundaries.

5' and 3' RACE were performed according to the protocols described in the Marathon™ (for human ejaculate) and SMART™ (for all monkey and baboon templates) RACE cDNA amplification kits. The Marathon™ RACE protocol utilises double-stranded cDNA whereas the SMART™ RACE technology makes use of first-strand (single-stranded) cDNA (see figure 5). The switch from the Marathon™ to the SMART™ RACE protocol was recommended by the manufacturer of the RACE kits when total RNA is used as starting material for RACE, as was the case in the present study. This was an attempt to increase efficiency of the cDNA synthesis reactions, using the improved technology of the SMART™ kit.

See Figure 5 in "Separate Figures" folder on CD



For the Marathon™ RACE PCR a 5 µl aliquot of RACE-ready human ejaculate cDNA and 10 picomoles of each primer were used per 50 µl reaction. Primary 5' RACE was performed with the adaptor-specific AP1 primer (BD Biosciences Clontech) in conjunction with exon 1-specific antisense oligo AS5. Nested 5' RACE was performed using 5 µl of the primary reaction, adaptor-specific AP2 primer (BD Biosciences Clontech) and antisense gene-specific primer AS3, in a final volume of 50 µl. The Advantage™ cDNA polymerase mix (BD Biosciences Clontech) was used in the Marathon™ RACE reactions, together with Advantage PCR buffer (BD Biosciences Clontech). The Advantage™ cDNA polymerase mix contained KlenTaq-1 DNA polymerase (containing an N-terminal deletion of *Taq* DNA polymerase) as the primary polymerase and a minor amount of a 3'→5' proofreading polymerase, as well as TaqStart™ antibody to provide automatic hot start PCR. Touchdown PCR was performed according to the manufacturer's instructions using 36 cycles for the primary and 30 cycles for the secondary PCR reactions, respectively.

For the SMART™ 5' RACE PCR a 2.5 µl aliquot of SMART™ RACE-ready COS-1, vervet monkey or baboon cDNA, 1 X universal primer mix (UPM) (BD Biosciences Clontech) and 10 picomoles of a *GnRHR-II*-specific antisense oligo (COS-1, AS2; vervet monkey and baboon, AS8), were used in the primary reaction in a 50 µl reaction volume. Nested 5' RACE was performed using 5 µl of a 1:49 tricine-EDTA dilution of the primary reaction, 10 picomoles each of the nested universal primer (NUP) (BD Biosciences Clontech) and an antisense gene-specific oligo which is internally nested to the primary gene-specific oligo (COS-1, AS1; vervet monkey and baboon, AS7), in a final volume of 50 µl. 3' SMART RACE was performed on vervet monkey and baboon RNA using the same conditions as described for 5' SMART RACE, except for the use of sense gene-specific primers S7 and S8 for the primary and secondary reactions, respectively. DMSO (5%) was included in the reaction make-up of both the primary and the secondary SMART RACE reactions. The Advantage™2 cDNA polymerase mix was used in the SMART RACE reactions, together with Advantage™2 PCR buffer (polymerase mix and PCR buffer both from BD Biosciences Clontech). This polymerase mix contained Titanium *Taq* DNA polymerase, which provides higher yields than *Taq* and contains the TaqStart™ antibody for an integrated hot start, as well as a proofreading polymerase. Cycling conditions for PCR were according to the manufacturer's instructions, using 40 cycles for both the primary and the secondary PCR reactions.

Southern blot analysis

RT-PCR and RACE products were separated by electrophoresis in 1 X TAE [Sambrook J *et al.*, 1989b] on an agarose gel containing ethidium bromide (EthBr) (0.5 µg/ml). Specificity of the RT-PCR and RACE reactions for *GnRHR-II* was confirmed by Southern blot analysis. After resolution of the RT-PCR products by electrophoresis, the gel was transferred to a UV-light box and photographed. DNA was denatured by soaking the agarose gel with shaking in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min at rt. This was followed by a brief rinse in dH₂O. The denaturant was neutralised by washing twice for 15 min at rt in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA pH 8.0), with dH₂O washes in between and afterwards. DNA was transferred onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by means of capillary transfer. The transfer was performed overnight in 20 X SSC pH 7.0 (3 M NaCl, 0.3 M Na₃-citrate). Complete transfer of the DNA was verified by staining the gel with EthBr (0.5 µg/ml). The membrane was washed in 2 X SSC pH 7.0 (0.3 M NaCl, 0.03 M Na₃-citrate) for 1 min at rt and air-dried. Thereafter DNA was covalently linked to the nylon membrane by UV irradiation in a UV crosslinker (Amersham Pharmacia Biotech) at 70000 µJ/cm² for 15 s. A DNA oligonucleotide probe specific to a sequence between the amplification primers was labelled with fluorescein-11-dUTP (10 µl/100 pmoles oligonucleotide) using the Gene Images 3'-oligolabelling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's specifications. Fluorescein-11-dUTP is supplied with the Gene Images 3'-oligolabelling system. Labelling was performed for 90 min at 37°C. The membrane was pre-hybridised in hybridisation solution (5 X SSC, 0.02% w/v SDS, 0.1% w/v hybridisation buffer component supplied, 1/20 dilution liquid block supplied) at 0.25 ml/cm² of membrane for 30 min at 42°C. A total of 10 ng/ml of labelled DNA oligo probe was added to the hybridisation solution. Hybridisation was carried out overnight at 5 to 10°C below the T_m of the probe. The membrane was washed twice in 5 X SSC, 0.1% w/v SDS for a total of 5 min at rt. This was followed by two washes in 1 X SSC, 0.1% w/v SDS for 15 min at 60°C. The membrane was rinsed in Buffer 1 (0.15 M NaCl, 0.1 M Tris-HCl pH 7.5) for 1 min at rt. Membrane blocking was performed in block solution (supplied with the Gene Images 3'-oligolabelling system) for 5 min at rt, followed by a 1 min wash in Buffer 1 at rt. Anti-fluorescein antibody conjugate was prepared as instructed. The membrane was incubated with the antibody for 30 min at rt and washed four times in Buffer 2 (0.4 M NaCl, 0.1 M Tris-HCl pH 7.5) for 5 min at rt. Signal generation was carried out by incubating the

membrane with a mixture of equal volumes of the ECL detection reagents (Amersham Pharmacia Biotech) to a total volume of 0.125 ml/cm² of membrane for 1 min at rt. The membrane was exposed to hyperfilm (Amersham Pharmacia Biotech) for 1 to 60 min at rt.

Cloning of the relevant cDNAs

RT-PCR products of the correct size and which were found to be *GnRHR-II*-specific by Southern blotting were purified from the PCR components for cloning purposes by gel electrophoresis. The RT-PCR products were separated on a 2% low melting point agarose (Promega) gel in 1 X TAE. A slice containing the relevant band was excised from the gel with a sterile blade. cDNA was extracted from the gel slice with the use of the GenElute gel purification system (Sigma-Aldrich) according to the manufacturer's instructions. Purified cDNA was eluted from the GenElute column in 30 µl elution buffer (10 mM Tris-HCl pH 8.0). Of this, 7 µl was visualised on 1% agarose together with λ DNA standards (Roche Molecular Biochemicals, Indianapolis, IN) to estimate the concentration of the purified DNA.

The cDNA was subsequently ligated to the pMOS vector and used to transform *MOSBlue* cells with the use of the pMOSBlue blunt ended cloning kit (Amersham Pharmacia Biotech), according to the protocol described by the manufacturer. The amount of cDNA used in the ligation reaction was calculated at a vector:insert molar ratio of 1:2.5. Transformants were plated onto Luria Bertani (LB) agar plates containing ampicillin (Amp, 50 µg/ml) and tetracyclin (Tet, 15 µg/ml) which had been freshly coated with a mixture of 1 M Isopropyl β-D-thiogalactopyranoside (IPTG, 2 µl) and 25 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal, 70 µl) and grown overnight at 37°C. White (insert-containing) colonies were subjected to PCR screening with the T7 promoter primer and U19-mer primer pair, as follows: A bacterial suspension was prepared by inoculating 20 µl ddH₂O with a white colony that had been picked off the plate with a sterile toothpick. Of this, 5 µl was added to a 20 µl mix containing 0.625 U *Taq* DNA polymerase (Promega), 25 pmoles each of the T7 and U19-mer primers, dNTPs to a final concentration of 0.2 mM, MgCl₂ to a final concentration of 1.2 mM and 1 X PCR reaction buffer (Promega). The rest of the bacterial suspension in H₂O (15 µl) was added to 385 µl LB/Amp broth and incubated for 5 to 8 h at 37°C with shaking, before addition of 80% glycerol (100 µl) to make glycerol stocks. Cycle conditions for the colony screen PCR were as follows: 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min followed by a

final 10 min extension time at 72°C. Products of the PCR screening were visualised on 1% agarose and subjected to Southern blot analysis as described above, using the same oligonucleotide probe that had been used in the initial blot. Colonies containing pMOS plasmids with the right size insert were selected for plasmid DNA isolation.

Plasmid DNA preparation

Plasmid DNA containing the cloned cDNAs was isolated using the Wizard® *Plus* SV Miniprep DNA purification system (Promega). Briefly, 9 to 10 ml LB/Amp broth was inoculated with the glycerol stock using an eye needle that had been sterilised over a flame. Cultures were grown overnight at 37°C with shaking; whereafter plasmid DNA was isolated using a spin protocol according to the specifications described in the Wizard® *Plus* SV Miniprep manual. Purified plasmid DNA was eluted in 60 to 100 µl nuclease-free water. Plasmid DNA concentrations were determined spectrophotometrically by measuring A_{260} . Integrity of the plasmid DNA was determined by agarose gel electrophoresis; 200 ng of plasmid DNA was loaded onto the gel.

Sequencing and sequence analyses of cloned cDNAs

Sequencing was done at the Core DNA Sequencing facility at the Department of Genetics, University of Stellenbosch (South Africa). Several clones originating from independent RNA or cDNA batches were sequenced from both sides (T7 as well as U19-mer) to be able to correct for PCR and sequencing errors and create consensus sequences. Sequencing results were analysed with the use of a Windows-based program namely "BioEdit sequence alignment editor". Other applications that were used can be found on the Internet at www.ch.embnet.org/software/LALIGN_form.html (for local alignment of two DNA sequences) and www.expasy.ch/tools/dna.html (for translation of DNA sequence to amino acid sequence).

Cloning and sequencing results from human, vervet monkey and baboon are summarised in tables 6 and 7 at the end of this chapter. Figure 23, also found at the end of this chapter, gives a schematic representation of all cloned sequences.

Results

Distribution of human GnRHR-II transcripts

Multiple strategies were used to examine the distribution of exon 1-containing GnRHR-II transcripts in human tissues and cells, due to the abundance and wide expression of the antisense transcript. The rationale for this approach was that antisense transcripts would not contain exon 1 because of the presence of the polyadenylation signal when the *GnRHR-II* gene is transcribed in the antisense orientation.

In a previous study using an exon 1-specific DNA probe in dot blot analysis of multiple human tissue polyA⁺ RNAs, positive signals were detected for a number of brain parts and peripheral tissues (refer to Appendix 5). These included putamen, caudate nucleus, cerebellum, occipital lobe, adult heart, testis, salivary gland, peripheral leukocyte and lymph node (see Appendix 5 figure 1, A & B).

The fidelity of the positive signals was assessed in this study by RT-PCR on total RNA from human tissues, cells, or cell-lines that were available. The human tissues were adult cerebellum, cortex, hypothalamus, kidney, medulla, midbrain, pituitary, pons, testis, thyroid and uterus, as well as foetal adrenals, cerebellum, frontal lobe, hypothalamus, medulla, midbrain, lumbar sympathetic chain, olfactory bulb, pituitary, pons, and retina. The human cells were total ejaculate as well as HepG2 hepatocyte carcinoma cells. Several sets of *GnRHR-II*-specific PCR primers were utilised. These included the exon 1-specific primer S5 (designed to part of the coding region of ECL1) in combination with primers to either exon 2 (AS6, designed to part of the coding region of ECL2, that would yield a 319 bp intronless amplicon) or exon 3 (AS10, designed to part of the coding region of TM6, that would yield a 542 bp intronless amplicon), as well as the exon 2-3 primer pair (S10 & AS10, that would yield a 660 bp or 250 bp amplicon, respectively, if an intron was present or absent). S10 is designed to part of the coding region of ECL2. The relative positions of the primers used are indicated in figure 6.

See Figure 6 in “Separate Figures” folder on CD



Expression of exon 1-containing, sense GnRHR-II transcripts in human ejaculate

As expected, the intron 2-containing antisense 660 bp product was amplified from almost all human RNA samples with the use of the exon 2-3 primer pair S10 & AS10 (not shown). This product was also present in human testis and ejaculate (figure 7, lane 2 and figure 8, lane 3, respectively). In contrast, the processed 250 bp exon 2-3 product, arising from transcription of the *GnRHR-II* gene in the sense orientation, was detected only in testis and ejaculate (figure 7, lane 2 and figure 8, lane 3, respectively). In testis and ejaculate RNA, the antisense 660 bp exon 2-3 product was in abundance over the processed 250 bp product (figure 7, lane 2 and figure 8, lane 3). Furthermore, human GnRHR-II transcripts containing exon 1 were detected only in ejaculate RNA (figure 8, lanes 1 and 2). The expected 319 bp (exon 1-2, encoding part of ECL1 to part of ECL2) and 542 bp (exon 1-3, encoding part of ECL1 to part of TM6) intronless amplicons were faintly visible on an agarose gel (figure 8, lanes 1 and 2) but clearly visible on a Southern blot using an internally nested exon 1-specific probe, AS3 (figure 9, lanes 1 and 2). In addition to the expected size intronless amplicons, RT-PCR products of other sizes were also obtained from human ejaculate. For example, the presence of a 419 bp exon 1-2 amplicon, in addition to the expected 319 bp amplicon, was visible on an agarose gel (figure 8, lane 1) and confirmed by Southern blot analysis (figure 9, lane 1). Similarly, a ~1 kb exon 1-3 amplicon was obtained from human ejaculate RNA apart from the expected 542 bp amplicon (figure 8, lane 2) but the ~1 kb band was not positive on the Southern blot (figure 9, lane 2). Another RT-PCR product of unexpected size in human ejaculate RNA, arising from amplification with the exon 2-3 primer pair S10 & AS10, was visible on an agarose gel together with the intronless 250 bp and intron-containing 660 bp products (~600 bp; figure 8, lane 3). Specificity of this ~600 bp amplicon was not determined by Southern blot analysis.

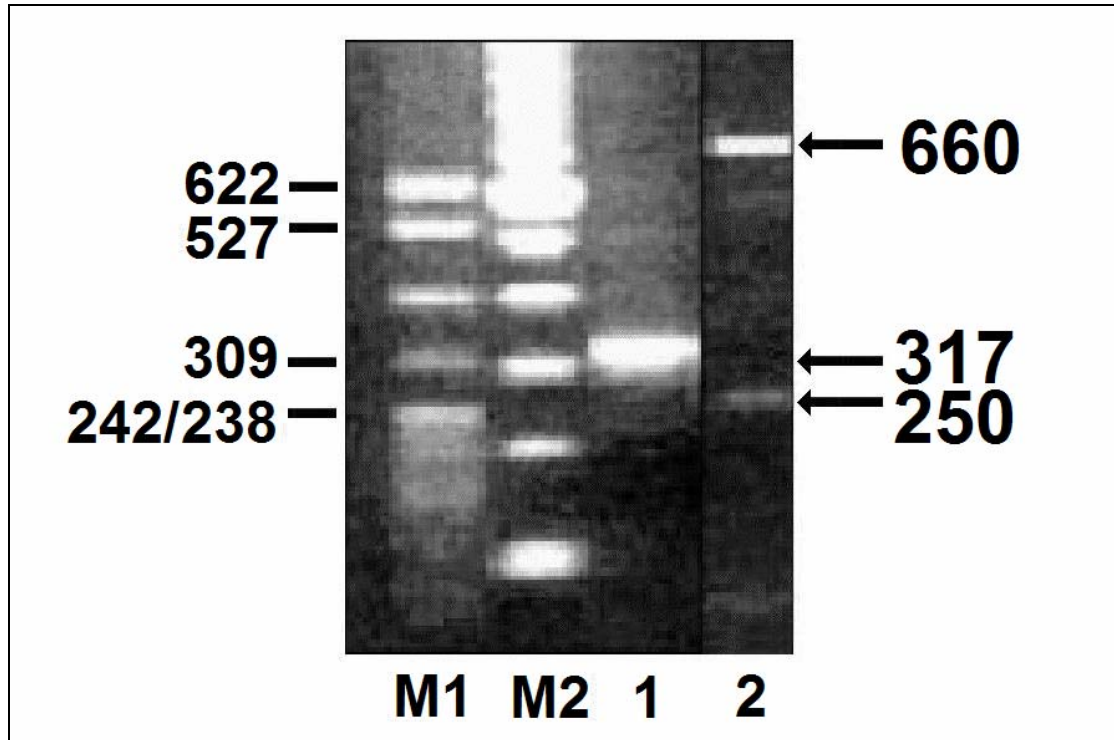


Fig 7. Agarose gel visualisation of RT-PCR results on human testis RNA: indication of expression of a sense transcript for the GnRHR-II. The lanes are marked as follows: M1, pBR322*HapII* molecular weight markers; M2, 100 bp ladder molecular weight markers (Promega); 1, 317 bp product obtained with the β -actin control primers; 2, 250 bp and 660 bp product obtained with the *GnRHR-II* exon 2-exon 3 primer pair S10 & AS10. The positions and sizes of some of the marker bands are indicated to the left whereas the positions and sizes of the appropriate amplicons are indicated to the right of the photographed gel picture.

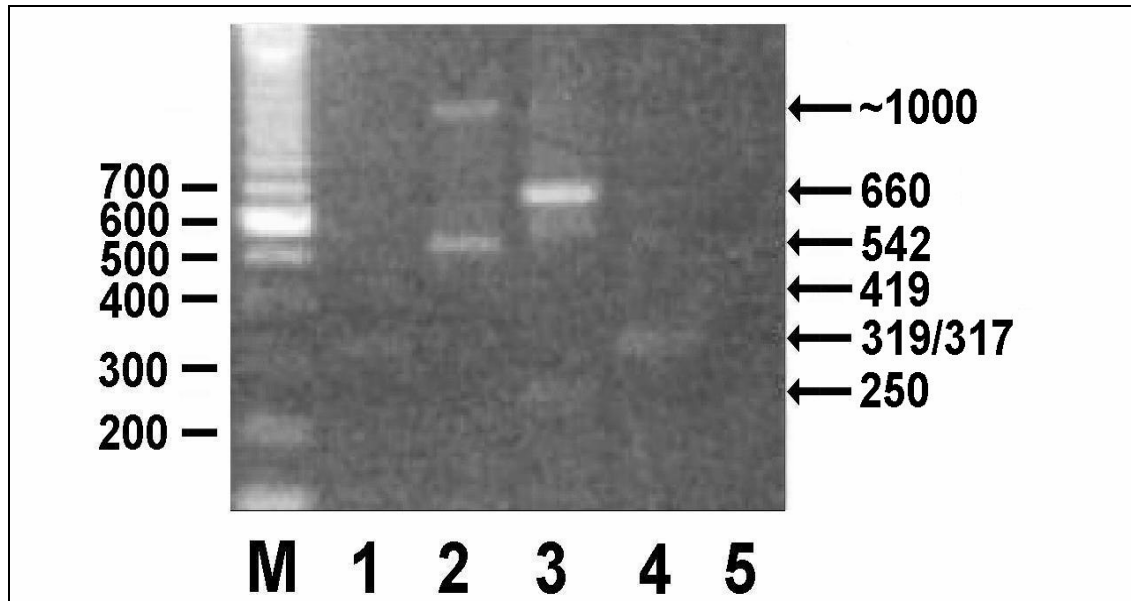


Fig 8. Agarose gel visualisation of RT-PCR results on human ejaculate RNA: amplification of exon 1-containing, sense GnRHR-II transcripts. The lanes are marked as follows: M, 100 bp ladder molecular weight markers (Promega); 1, 319 bp and 419 bp products obtained with the exon 1-exon 2 primer pair S5 & AS6; 2, 542 bp and ~1 kb product obtained with the exon 1-exon 3 primer pair S5 & AS10; 3, 250 bp, ~600 bp and 660 bp products obtained with the exon 2-exon 3 primer pair S10 & AS10; 4, 317 bp product obtained with the β -actin control primers; 5, negative control. The positions and sizes of some of the marker bands are indicated to the left whereas the positions and sizes of the appropriate amplicons are indicated to the right of the photographed gel picture.

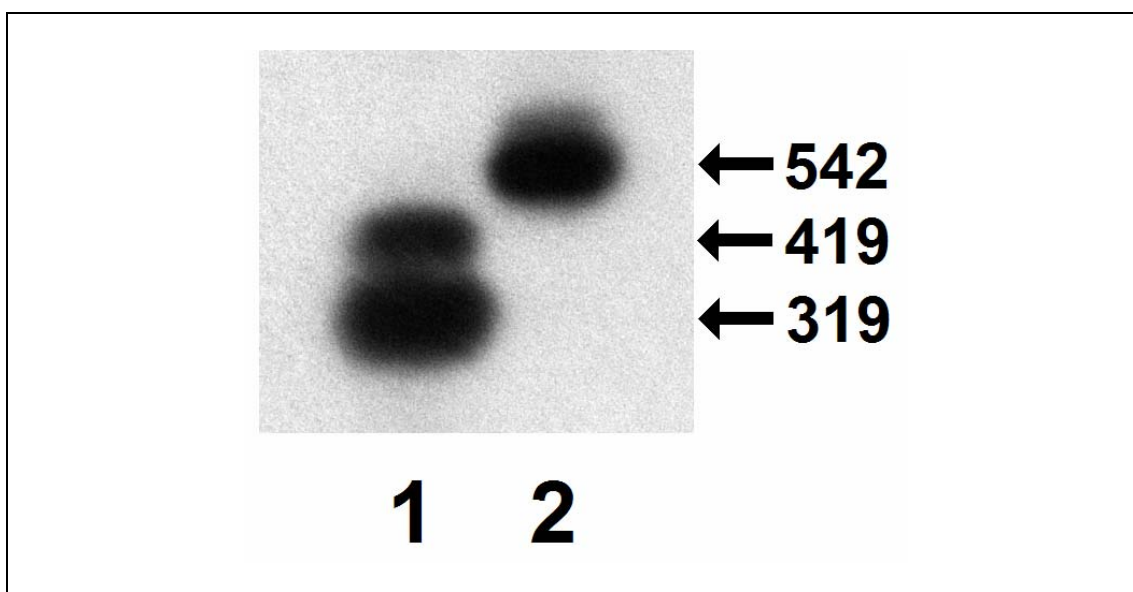


Fig 9. Autoradiogram of the Southern blot of the gel shown in figure 8. The blot was probed with exon 1-specific oligo AS3. All six lanes, including the marker lane, were probed. Results are only shown for lanes 1 and 2 since no signals were obtained for the other lanes with the exon 1 probe. The positions and sizes of the appropriate amplicons are indicated to the right of the photographed autoradiogram.

Sequence identity of the human ejaculate GnRHR-II transcripts

The human ejaculate 319 bp, 419 bp and 542 bp exon 1-containing GnRHR-II transcripts were cloned and sequenced (see Sequence no 1 in Appendix 4). The sequence of the 319 bp exon 1-2 amplicon (S5 & AS6) is contained within the 542 bp sequence of exon 1-3 (S5 & AS10). Surprisingly, within exon 2 and in the correct reading frame, a TGA translation stop signal was found in the human ejaculate cDNA sequence. Subsequent completion of the human genome project confirmed that the TGA is contained within the human *GnRHR-II* gene (accession AL160282). The sequence of the 419 bp amplicon (figure 8, lane 1) corresponded to that of the 319 bp amplicon (including the TGA translation stop signal), except that it contained additional sequence of a 100 bp between exons 1 and 2 (sequence data not shown). This insert resulted in the shift in the reading frame between the two exons. The sequence of this 100 bp insert in the human ejaculate transcript was compared to the database sequence of the human *GnRHR-II* gene (accession AL160282) and found to be a part of intron 1, which is situated between exons 1 and 2 and is ~4.3 kb in length. Hence, this 419 bp amplicon is likely to be the result of amplification of an

incompletely processed mRNA. Intriguingly, the 100 bp retained intronic sequence did not follow immediately 3' to exon 1 nor did it immediately precede exon 2 of the human *GnRHR-II* gene (accession AL160282). Rather, it was located at ~2.6 kb downstream of the 3' end of exon 1 and ended ~1.6 kb upstream of the 5' end of exon 2. Thus, this additional 100 bp sequence was most likely the result of an additional splicing event, where exon 1 was spliced onto a part of intron 1 before splicing onto exon 2. Sequencing of the ~1 kb exon 1-3 amplicon (figure 8, lane 2) was not pursued because this product did not appear on the Southern blot (figure 9, lane 2). The ~600 bp amplicon obtained with the exon 2-3 primer pair (figure 8, lane 3) was not sequenced because the focus was on exon 1-containing GnRHR-II transcripts.

By 5' and 3' RACE, attempts were made to obtain sequence information of the 5' and 3' ends of the human ejaculate GnRHR-II cDNA because of the unavailability of the human *GnRHR-II* gene sequence at the time. The primary 5' RACE reaction, using exon 1-specific oligo AS5 (designed to part of the coding region of TM4) in combination with the Marathon adaptor primer AP1 produced no bands on an agarose gel (results not shown). Secondary 5' RACE with exon 1-specific primer AS3 (designed to part of the coding region of ECL1 and which is internally nested to AS5) in combination with the Marathon nested adaptor primer AP2 produced a broad band on an agarose gel, with products ranging in size between 622- and 810 bp, as well as a range of minor smaller products (figure 10, A, lane 1, indicated with an arrow). The finding of multiple products is a typical result of the RACE technique. Southern blot analysis using labelled exon 1-specific oligo S5 confirmed specificity of all these secondary 5' RACE products (figure 10, B, lane 1). A gel slice containing the broad band was cut out for subsequent cloning and sequencing. Sequence analysis of this secondary 5' RACE product revealed a consensus sequence of 751 bp (706 bp excluding the AS3 and AP2 primer sequences, 5 clones sequenced), which included a novel 5' sequence of 481 bp as well as the putative translation start site (see Sequence no 1 in Appendix 4).

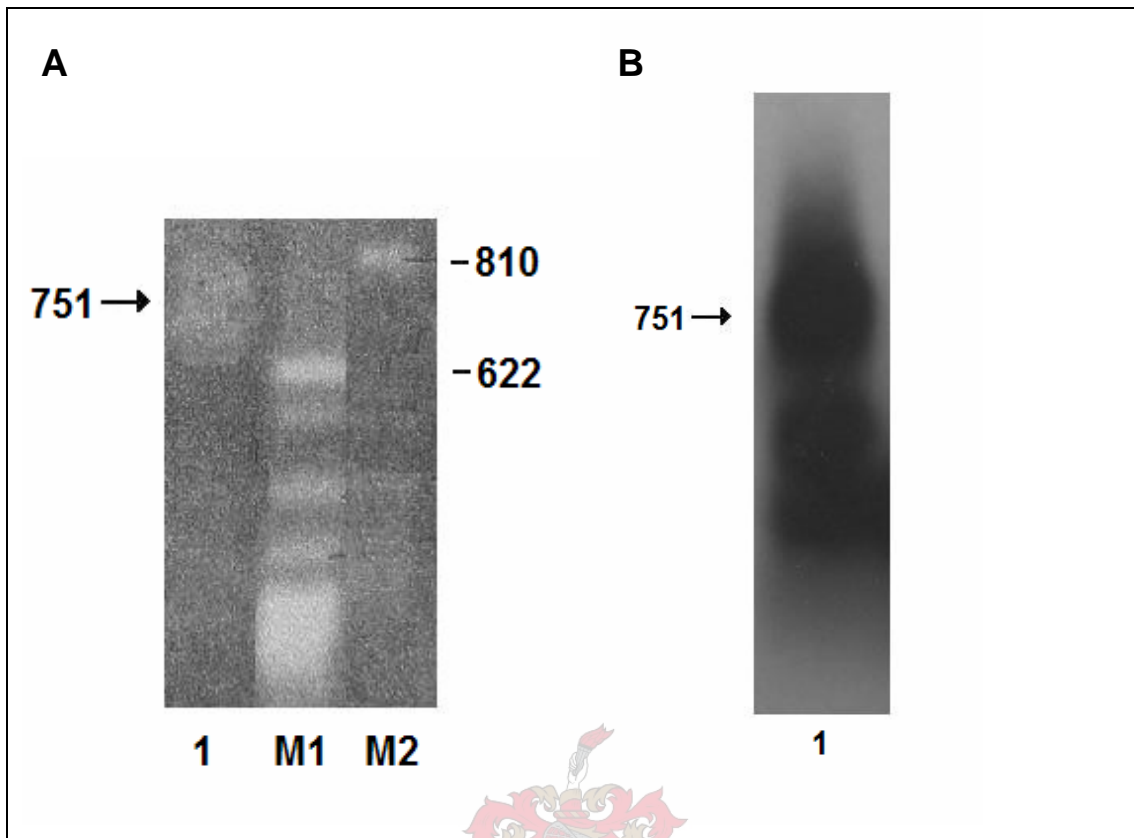


Fig 10. Results of secondary 5' RACE on human ejaculate Marathon RACE-ready cDNA: amplification of the 5' end of the human GnRHR-II cDNA. The lanes are marked as follows: 1, Broad band obtained with primer pair AP2 & AS3 from which a 751 bp product was sequenced; M1, pBR322*HapII* molecular weight markers (1 μ g); M2, λ *PstI* molecular weight markers (1 μ g).

- A. Agarose gel visualisation. The positions and sizes of the appropriate marker bands are indicated to the right of the photographed gel picture.
- B. Autoradiogram of the Southern blot of the gel shown in A. The blot was probed with exon 1-specific oligo S5. All lanes, including the marker lanes, were probed. The very dark dots are due to overexposure of the blot.

All GnRHR-II sequences obtained from human ejaculate RNA, by RT-PCR as well as by 5' RACE, were assembled to create a 1227 bp contiguous consensus sequence from overlapping clones (see Sequence no 1 in Appendix 4). The assembled human ejaculate sequence contained almost the full putative coding region of the GnRHR-II cDNA, stretching from -392 to +835 (numbering relative to the putative ATG

translation start site), until 302 bp 5' to the translation stop signal. This sequence would encode a GnRHR-II protein containing TM1 to almost the end of TM6. Attempts to obtain the translation stop and polyadenylation signals, using 3' RACE on human ejaculate RNA, were unsuccessful. When the primary and the secondary 3' RACE products were visualised on an agarose gel, only smears, and no distinct bands, were seen, indicating the generation of multiple amplicons of varying length (not shown).

Localisation of GnRHR-II transcripts to human sperm cells

Subsequent to this study, *in situ* hybridisation analyses were performed using both sense and antisense GnRHR-II exon 1-specific riboprobes to confirm the presence and further examine the distribution of the human GnRHR-II transcripts in testis and mature sperm. Bright-field photomicrographs summarising these results are shown in figures 4 and 5 of Appendix 5. In human testis the presence of exon 1-containing GnRHR-II sense transcripts was indicated within the adluminal region of the seminiferous epithelium, in association with various types of differentiating haploid spermatids (see Appendix 5, figure 4, A). This was consistent with the view that the *GnRHR-II* gene is transcribed during the haploid phase of spermatogenesis. *In situ* hybridisation analysis also revealed the persistence of human exon 1-containing sense GnRHR-II transcripts in mature sperm (see Appendix 5, figure 5, A). These results indicate that the GnRHR-II transcripts obtained in this study by RT-PCR and 5' RACE in human ejaculate RNA result from mature sperm within the ejaculate.

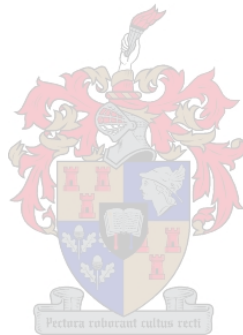
Distribution of non-human primate GnRHR-II transcripts

Expression of GnRHR-II transcripts in vervet monkey

While performing exontrapping with human P1 genomic DNA clones in COS-1 cells, a GnRHR-II sequence was cloned that was clearly not human. This led to the conclusion that the sequence must have come from COS-1 RNA. For that reason, the presence of a monkey GnRHR-II was further investigated by RT-PCR and RACE, using RNA isolated from COS-1 cells. In addition, the cloning of a GnRHR-II cDNA was attempted from RNA isolated from vervet monkey cerebellum, hypothalamus, kidney, occipital lobe, pituitary, testis and total ejaculate.

- *COS-1 GnRHR-II*

A number of overlapping GnRHR-II transcripts were amplified by RT-PCR from COS-1 RNA, including a 542 bp exon 1-3 amplicon obtained with PCR primer pair S5 & AS10 (figure 11, lane 1) that was cloned and sequenced. The 542 bp amplicon stretched from the coding region for part of ECL1 to the coding region for part of TM6 (see Sequence no 2 in Appendix 4), similar to what was found in human ejaculate RNA. Apart from the expected 542 bp amplicon, RT-PCR products of other sizes were also obtained (see figure 11, lane 1 and figure 12, lane 1, showing two additional bands at ~1000 bp and ~350 bp).



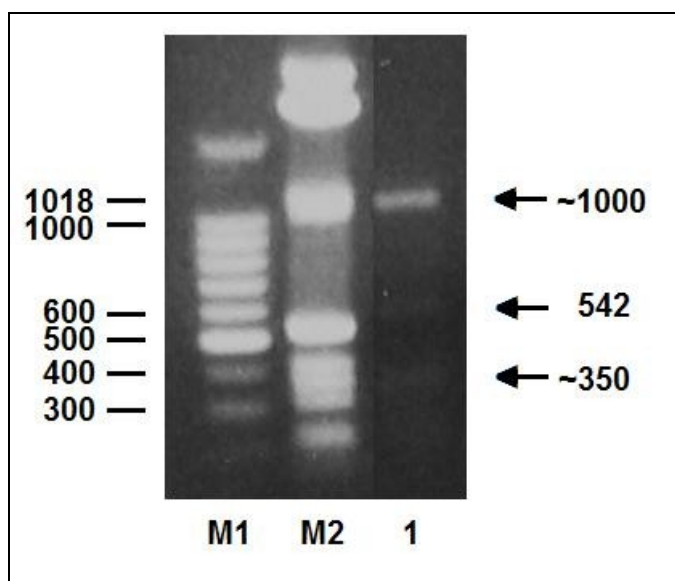


Fig 11. Agarose gel visualisation of RT-PCR results on COS-1 vervet monkey kidney cell RNA: amplification of exon 1-containing, sense GnRHR-II transcripts. The lanes are marked as follows: M1, 100 bp ladder molecular weight marker (6 μ l, Promega); M2, 1 kb molecular weight marker (2.2 μ g, GibcoBRL/Invitrogen); 1, ~350 bp, 542 bp and ~1 kb products obtained with the exon 1-exon 3 primer pair S5 & AS10. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the positions and sizes of the appropriate amplicons are indicated to the right of the photographed gel picture.

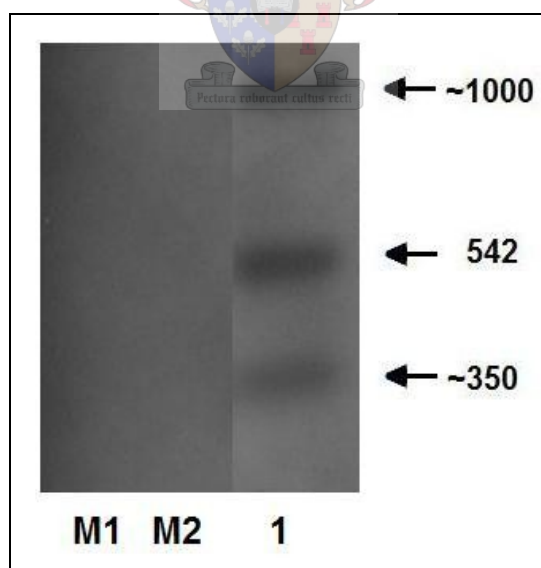


Fig 12. Autoradiogram of the Southern blot of the gel shown in figure 11. The blot was probed with exon 1-specific oligo AS3. All lanes, including the marker lanes, were probed. The positions and sizes of the exon 1-containing amplicons that were visible on the blot are indicated to the right of the photographed autoradiogram.

5' SMARTTM RACE was performed on SMARTTM RACE-ready cDNA prepared from COS-1 RNA, using primer pair UPM & AS2 (exon 1, TM2) in the primary and NUP & AS1 (exon 1, TM1) in the secondary reactions. Multiple products were obtained in both reactions (figure 13). A gel slice containing a distinct band at approximately 600- to 650 bp was extracted for subsequent DNA purification, cloning and sequencing of the secondary 5' SMARTTM RACE reaction product (figure 13, lane 2). A consensus sequence, 290 bp in length including the sequences of both primers, was derived from the sequences of four clones (see Sequence no 2 in Appendix 4). This 290 bp COS-1 5' SMARTTM RACE sequence contained 83 bp of 5' UTR sequence, including the putative translation start site (see Sequence no 2 in Appendix 4). Interestingly, the COS-1 GnRHR-II transcript was found to utilise an ACG threonine translation start codon instead of the universal AUG that encodes methionine.

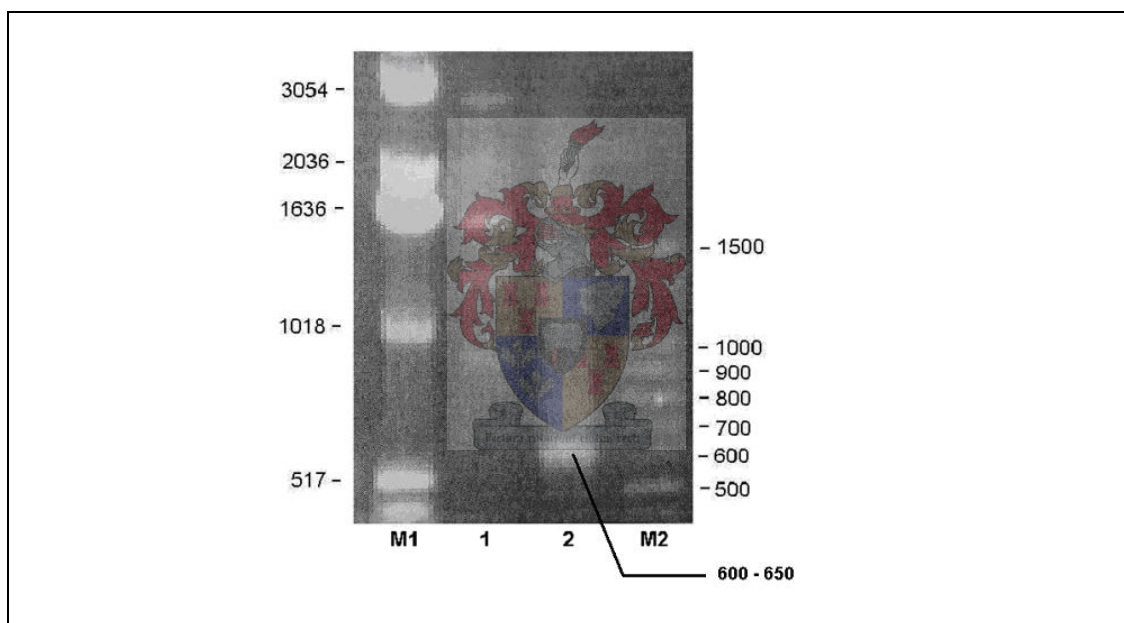


Fig 13. Agarose gel visualisation of 5' RACE results on COS-1 SMARTTM RACE-ready cDNA: amplification of the 5' end of the monkey GnRHR-II cDNA. The lanes are marked as follows: M1, 1 kb molecular weight marker (3.5 μ g, GibcoBRL/Invitrogen); 1, primary 5' SMARTTM RACE using UPM & AS2; 2, secondary 5' SMARTTM RACE using NUP & AS1; M2, 100 bp ladder molecular weight marker (0.65 μ g, Promega). Some of the positions and sizes of the molecular weight markers are indicated to the left and to the right of the photographed gel picture. The position of the band containing secondary 5' SMARTTM RACE products of ~600- to ~650 bp, which was extracted for sequence analysis, are indicated to the right of the photographed gel picture. Southern blot analysis of this particular gel was not performed. Southern blot analysis of a similar gel probed with exon 1-specific oligo probe S1 revealed that all bands obtained by 5' SMARTTM RACE on COS-1 cDNA were *GnRHR-II*-specific.

3' RACE was not performed on COS-1 RNA. An attempt was made to amplify a full-length cDNA containing the full coding region of the vervet monkey *GnRHR-II* from COS-1 RNA, using primer sets S1 & AS12 as well as S2 & AS12 in RT-PCR. These primer pairs are designed such to amplify a 1295 bp amplicon, stretching from -48 relative to the translation start within the 5' UTR to 82 nt downstream of the translation stop within the 3' UTR (see Appendices 1 to 3). Primers S1 and S2 are designed to identical gene positions, but differ in their species-specificity. The sequence of primer S1 is 100% human-specific and 92.6% vervet monkey-specific, whereas the sequence of primer S2 is 100% vervet monkey-specific and 92.6% human-specific. Primer AS12 was designed based on the human *GnRHR-II* gene sequence. Numerous RT-PCR attempts using these primer pairs resulted in the expected 1295 bp amplicon (not shown) but Southern blot analysis using labelled exon 1-specific oligo probe AS3 indicated that the obtained product was not GnRHR-II-specific. Furthermore, subsequent cloning and sequence analysis of the S1 & AS12 and S2 & AS12 amplicons confirmed that they did not contain GnRHR-II sequence. It later became evident, when 3' RACE results were obtained from vervet monkey occipital lobe RNA (see below), that the sequence of primer AS12 is not present in the vervet monkey cDNA at the expected position, which would explain why cloning attempts with this primer had failed. In summary, from two overlapping clones obtained with 5' RACE and RT-PCR, respectively, on COS-1 RNA, a GnRHR-II cDNA sequence was derived that contained a total sequence length of 918 bp, stretching from -82 to +836 (numbering relative to the translation start site), within TM6 in exon 3 (see Sequence no 2 in Appendix 4).

- *Expression of GnRHR-II transcripts in other vervet monkey tissues and cells*
RNA isolated from a number of vervet monkey tissues (refer to list above) was initially screened by RT-PCR for the expression of intronless, sense, GnRHR-II transcripts using the exon 2-3 primer pair S10 & AS10. Some of the results obtained are shown in figure 14, lanes 1 to 4. Primer pair S10 & AS10 should theoretically yield a 660 bp intron-containing or 250 bp intronless exon 2-3 (ECL2 to TM6) amplicon. As expected, the antisense 660 bp amplicon was obtained from all vervet monkey RNAs (see figure 14, lanes 1 to 4, for examples). In addition, the 250 bp intronless amplicon was obtained from vervet monkey testis and occipital lobe RNA (figure 14, lanes 3 and 4, respectively).

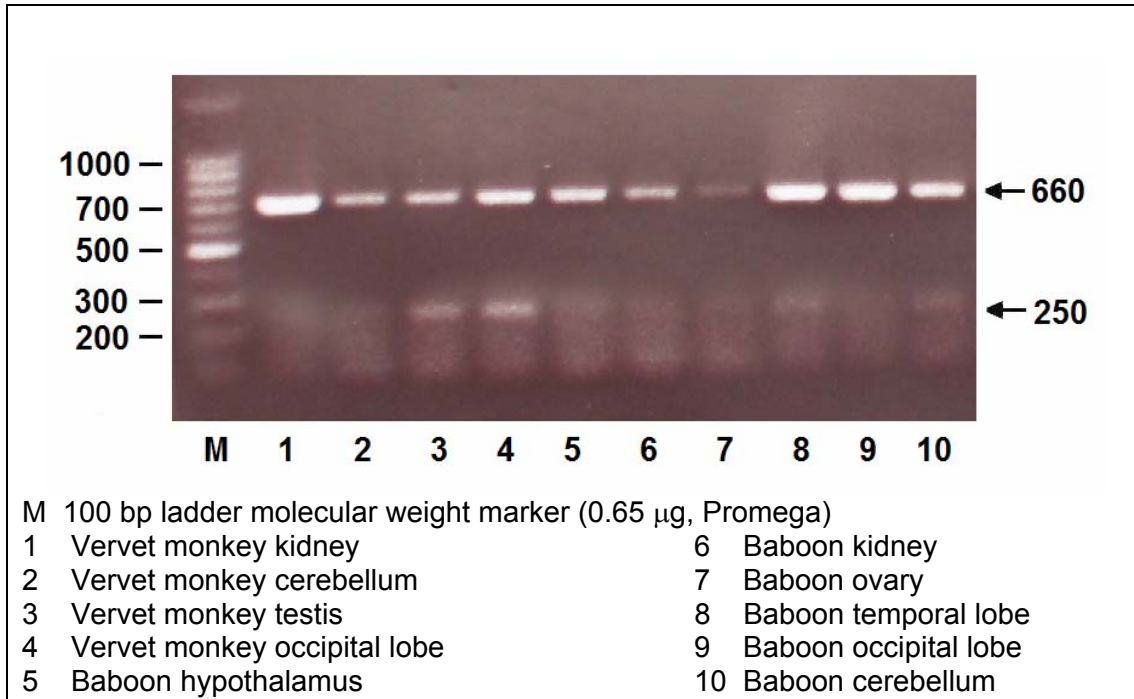


Fig 14. Agarose gel visualisation of RT-PCR results on vervet monkey and baboon tissue RNAs: attempts to amplify exon 2-3 intronless, sense GnRHR-II transcripts. Primer pair S10 & AS10, for the amplification of a 250 bp intronless sense amplicon or a 660 bp intron-containing antisense amplicon (ECL2 to TM6), was utilised in the RT-PCRs. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the positions and sizes of the appropriate amplicons are indicated to the right of the photographed gel picture.

Furthermore, RT-PCR using the exon 1-3 primer pair S5 & AS10, designed to amplify a 542 bp intronless sense GnRHR-II amplicon (ECL1 to TM6), was performed on the same vervet monkey tissue RNAs. The expected 542 bp amplicon was detected in RNA from vervet monkey cerebellum and occipital lobe (figure 15, lanes 2 and 4, respectively), as well as from total ejaculate (not shown). These results suggested that vervet monkey cerebellum, occipital lobe and ejaculate RNAs were good candidates for the cloning of a full-length mammalian GnRHR-II cDNA. The absence of the 542 bp product on a Southern blot (figure 16, lanes 2 and 4) is hard to explain since sequence analysis of this 542 bp amplicon obtained from vervet monkey occipital lobe RNA revealed that it contained GnRHR-II sequence (see Sequence no 3 in Appendix 4). Surprisingly, a 331 bp S5 & AS10 amplicon was detected in all vervet monkey RNAs and was also present on a Southern blot (figure 15 and figure 16, lanes 2 to 10).

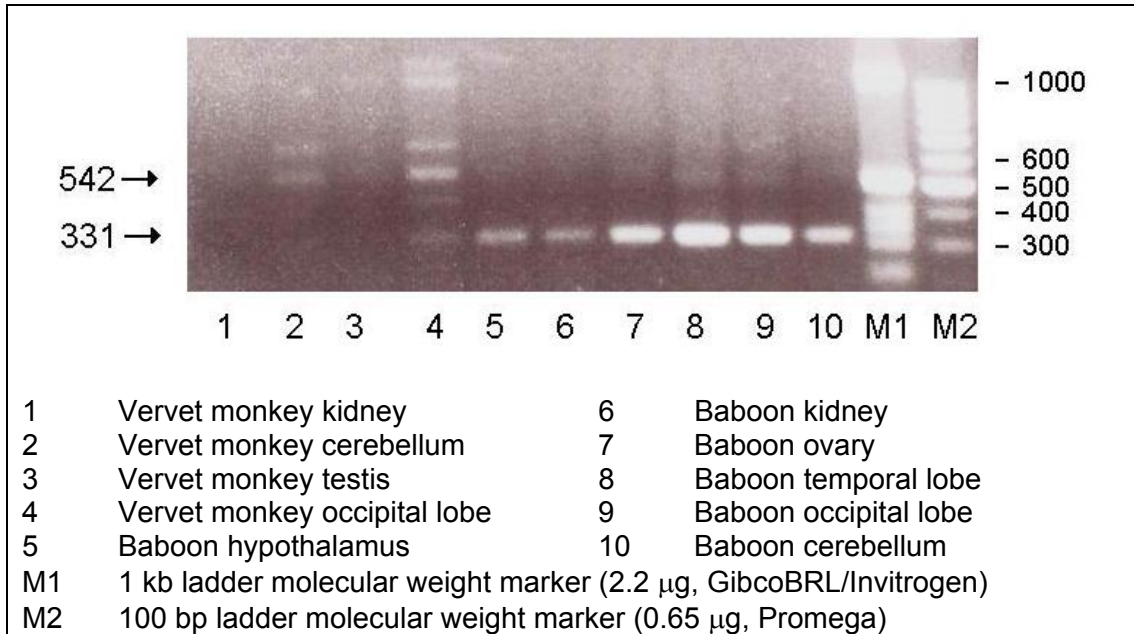


Fig 15. Agarose gel visualisation of RT-PCR results on monkey and baboon tissue RNAs: attempts to amplify GnrHR-II transcripts containing part of exon 1, the full exon 2 and part of exon 3 (ECL1 to TM6). Exon 1-3 primer pair S5 & AS10, for the amplification of a 542 bp amplicon, was utilised in the RT-PCRs. The positions and sizes of the appropriate amplicons are indicated to the left whereas some of the positions and sizes of the molecular weight markers are indicated to the right of the photographed gel picture. Results were confirmed by Southern blot analysis using labelled exon 1-specific and internally nested oligo AS3.

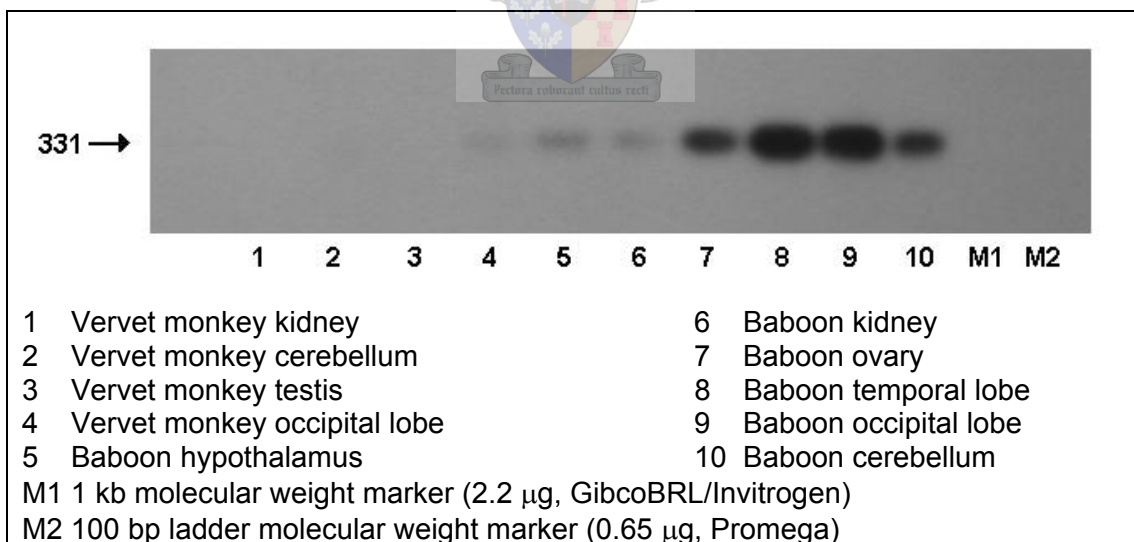


Fig 16. Autoradiogram of the Southern blot of the gel shown in figure 15. The blot was probed with exon 1-specific oligo AS3. All twelve lanes, including the marker lanes, were probed. The position and size of an abundantly expressed 331 bp amplicon is indicated to the left of the photographed autoradiogram.

RNA isolated from vervet monkey occipital lobe and from ejaculate was subjected to further RT-PCR and RACE analyses. Primer pair UPM & AS8 (designed to the exon 2-3 barrier, in a region encoding part of ICL3) was used in the primary 5' RACE reactions. The choice of oligo AS8 for 5' RACE was based on the presence of a ubiquitous 331 bp exon 1-3 amplicon in the non-human primate RNAs, as described above (figure 15 & figure 16). Prior sequence analysis of this unusually sized amplicon (sequenced from baboon cerebellum, see Sequence no 4 in Appendix 4) indicated that it contained part of exon 1 and part of exon 3 of the *GnRHR-II*, but lacked exon 2. The omission of exon 2 from this transcript resulted in a shift in the reading frame between exons 1 and 3 (Sequence no 4 in Appendix 4). Primer AS8 contains partly exon 2 and partly exon 3 sequence and hence would facilitate the amplification of transcripts containing both exons. Secondary 5' RACE was performed with primer pair NUP & AS7 (designed to exon 2, in a region encoding part of TM5). By 5' RACE on vervet monkey occipital lobe RNA, novel 5' sequence of the vervet monkey *GnRHR-II* cDNA was obtained. Sequence analysis of the secondary 5' RACE reaction product (958 bp including primer sequences; figure 17, lane 4 and figure 18, lane 4) revealed that it contained most of exon 2 plus most of exon 1, including the putative translation start and 141 bp of 5' UTR sequence (Sequence no 5 in Appendix 4). Similar to what was found for the COS-1 *GnRHR-II*, the vervet monkey occipital lobe transcript contained an ACG threonine codon at the translation start position (Sequence no 5 in Appendix 4). Interestingly, a sequence of 116 bp, resulting in a shift in the reading frame, was inserted between exons 1 and 2 of the occipital lobe sequence. A second product was obtained by 5' RACE on vervet monkey occipital lobe RNA, which contained most of exon 2 but did not include exon 1 sequence (sequence data not shown). The presence of this exon 1-less 5' RACE product suggested that the vervet monkey *GnRHR-II* gene is, similar to its human counterpart, also transcribed in the antisense orientation.

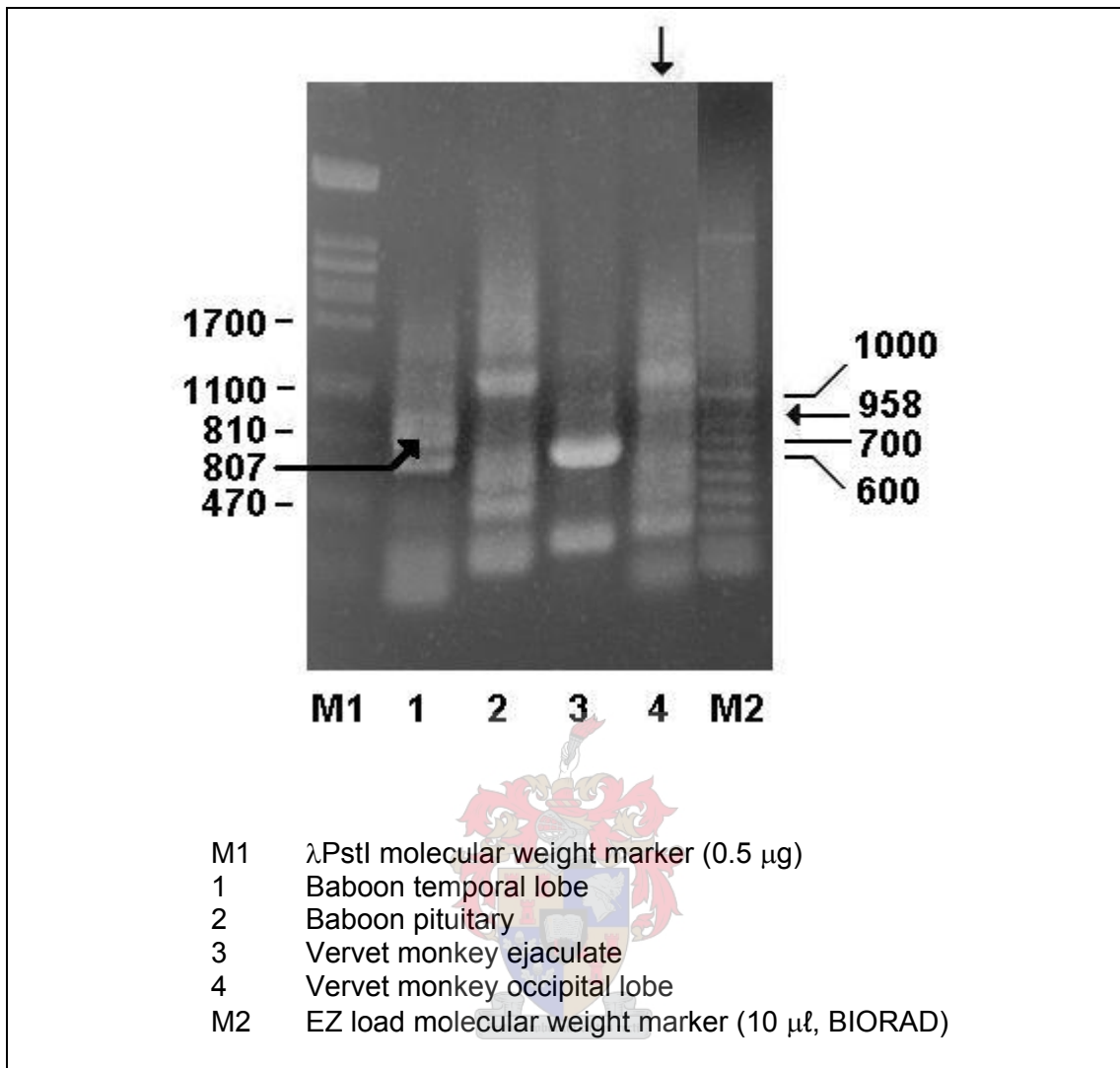


Fig 17. Agarose gel visualisation of results of 5' SMARTTM RACE performed on RNA from vervet monkey and baboon tissues: attempts to obtain the 5' end of the mammalian GnRHR-II cDNA. The primary reactions were performed with primer pair UPM & AS8. Results of the secondary reactions, using primer pair NUP & AS7, are shown here. The positions and sizes of some of the molecular weight markers are indicated to the right of the photographed gel picture. Specificity of the RACE products was confirmed by Southern blotting with labelled GnRHR-II exon 2-specific primer S9.

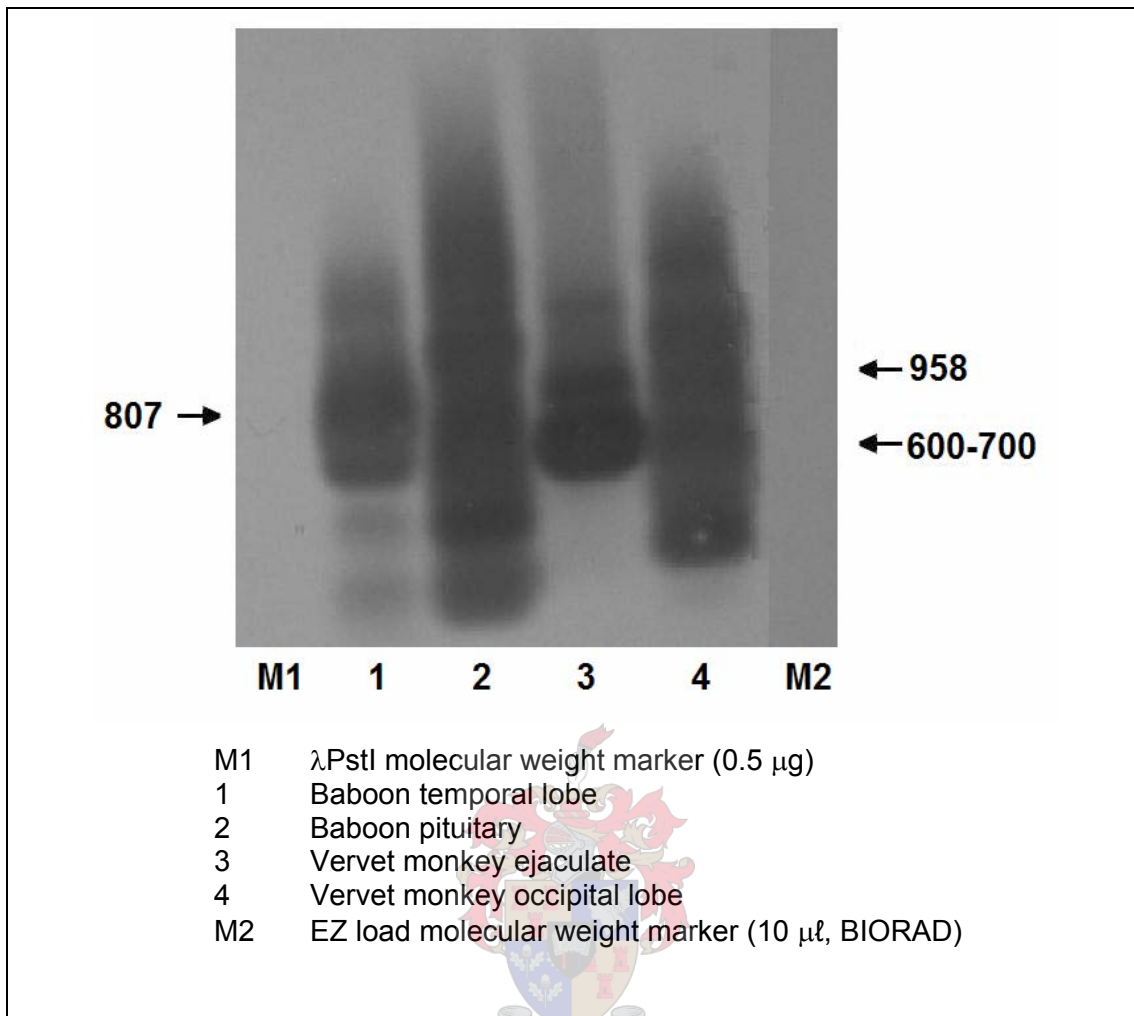


Fig 18. Autoradiogram of the Southern blot of the gel shown in figure 17. The blot was probed with exon 2-specific oligo S9. All six lanes, including the marker lanes, were probed. The positions and sizes of some of the relevant bands are indicated on either side of the photographed autoradiogram.

By 3' RACE, using primer pair S7 & UPM in the primary and S8 & NUP in the secondary reaction, respectively, a GnRHR-II amplicon of 727 bp was obtained from vervet monkey occipital lobe RNA (figure 19, lane 4 and figure 20, lane 4). Gene-specific primer S7 is designed such to bind to the exon 1-2 barrier, within a region encoding the 3' end of TM4. The use of S7 in the primary 3' RACE reaction would therefore facilitate the selection of transcripts that contained exon 1 as well as exon 2, which are likely to contain the full-length GnRHR-II sequence in the sense orientation. Primer S8 is designed to a region within exon 2 that encodes the 5' end of ECL2. Amplicons with a length greater than 592 bp (refer to Appendix 2) would contain novel 3' sequence. The vervet monkey occipital

lobe 727 bp amplicon contained most of exon 2 plus part of exon 3 of the GnRHR-II, stretching from ECL2 to TM6, but did not include the translation stop signal or novel 3' sequence (sequence data not shown). Furthermore, an insert of 447 bp was present between exons 2 and 3 of the 3' RACE product.

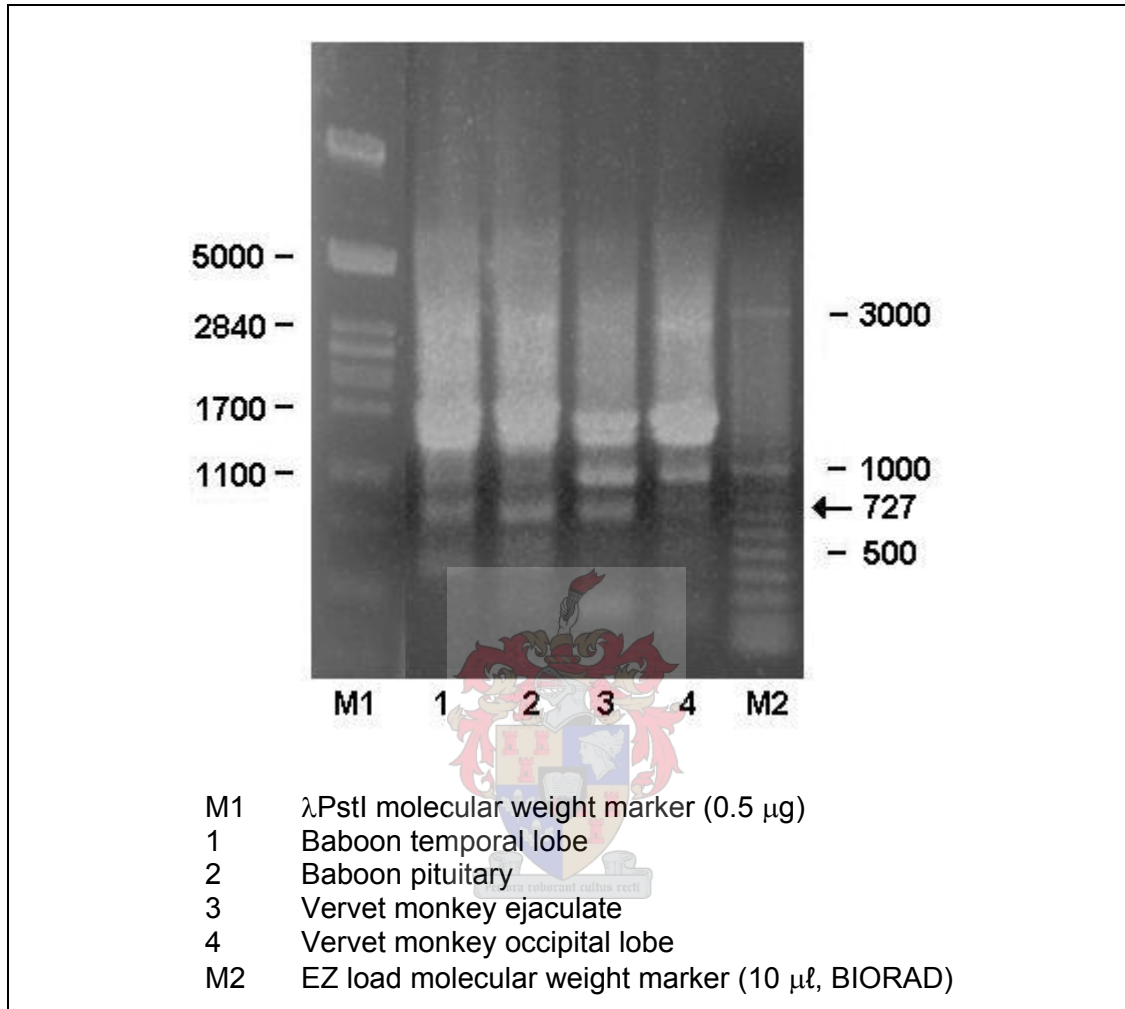


Fig 19. Agarose gel visualisation of results of 3' SMARTTM RACE performed on RNA from vervet monkey and baboon tissues: attempts to obtain novel 3' UTR sequence including the polyadenylation signal. The primary reactions were performed with primer pair S7 & UPM. Results of the secondary reactions, using primer pair S8 & NUP, are shown here. The positions and sizes of some of the molecular weight markers are indicated to the left and to the right of the photographed gel picture. Specificity of the RACE products was confirmed by Southern blot analysis with labelled GnRHR-II exon 2-specific primer S9.

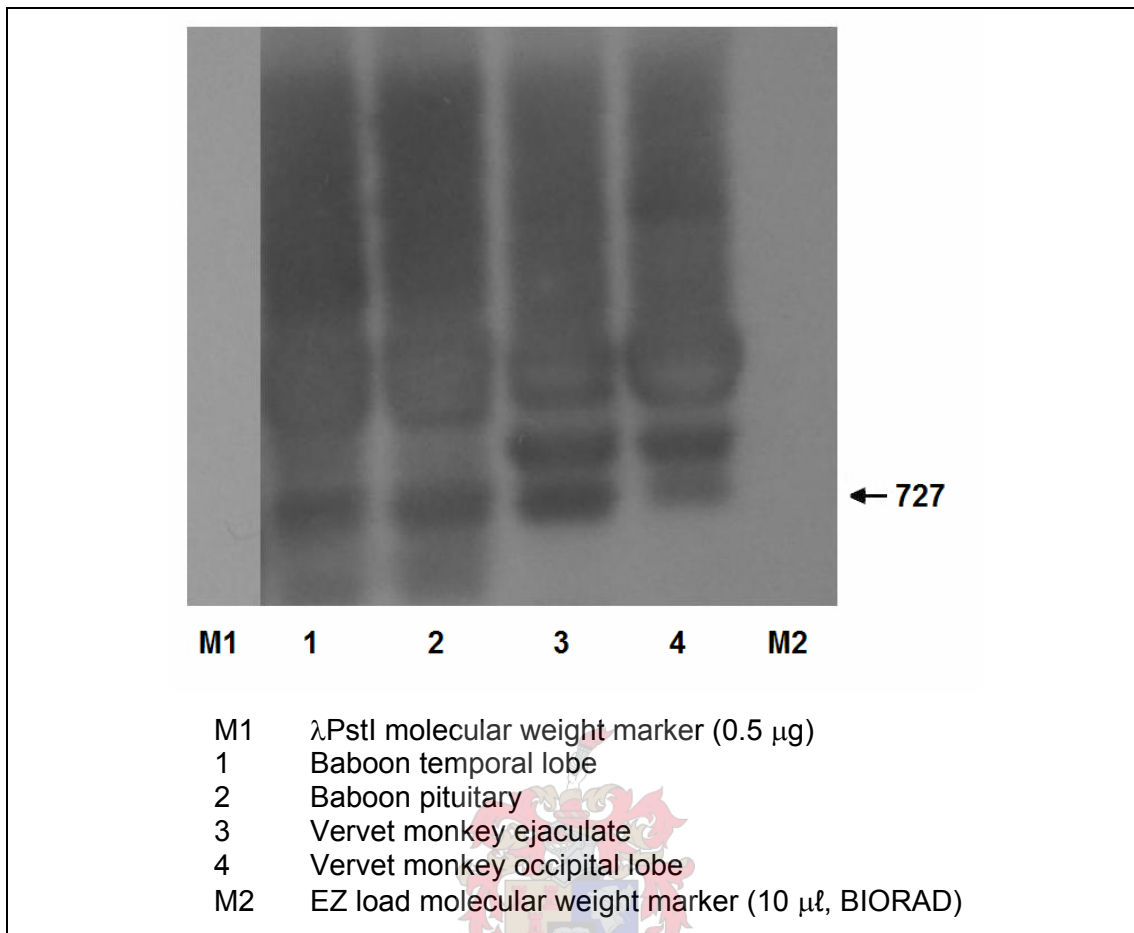


Fig 20. Autoradiogram of the Southern blot of the gel shown in figure 19. The blot was probed with exon 2-specific oligo S9. All six lanes, including the marker lanes, were probed. The position and size of the relevant band is indicated to the right of the photographed autoradiogram.

5' RACE was also performed on vervet monkey ejaculate SMARTTM RACE-ready cDNA using the same primer pairs that were used for 5' RACE on occipital lobe RACE-ready cDNA (1^o: UPM & AS8; 2^o: NUP & AS7). However, the obtained vervet monkey ejaculate secondary 5' RACE product was not selected for cloning and sequencing due to its size, which ranged between 600 bp to 700 bp (figure 17, lane 3). An amplicon of at least 750 bp was expected (refer to Appendix 2). Only amplicons with a length greater than 750 bp would most likely contain novel 5' sequence. 3' RACE on monkey ejaculate cDNA (figure 19, lane 3 and figure 20, lane 3) did not produce novel 3' sequence of the monkey GnRHR-II. A GnRHR-II amplicon containing the full coding region, including part of the 5' and 3' UTRs, was obtained from vervet monkey ejaculate RNA. This 1729 bp

amplicon was amplified using primer pair S3 & AS13 in RT-PCR (Sequence no 6 in Appendix 4). The 5' end of primer S3 is at -37 relative to the putative translation start whereas the 3' end of AS13 ends at 86 bp downstream of the translation stop signal. Similar to results obtained with 3' RACE on occipital lobe RNA (see above), the vervet monkey ejaculate 1729 bp amplicon contained an insert of 447 bp between exons 2 and 3. This insert did not change the reading frame between the two exons but it did result in the incorporation of a number of in-frame premature translation stop signals (Sequence no 6 in Appendix 4).

Thus, to summarise, an amplicon containing exons 1 and 3 but lacking exon 2 was found to be ubiquitously expressed in monkey (and baboon) tissue RNAs. By 5' RACE on vervet monkey occipital RNA a GnRHR-II transcript was obtained that stretched from -141 relative to the putative ACG translation start in exon 1 and included most of exon 2, and which had an insert sequence of 116 bp between the two exons. A second vervet monkey occipital lobe 5' RACE product contained exon 2 sequence but not exon 1. By 3' RACE a GnRHR-II amplicon was obtained from vervet monkey occipital lobe RNA that contained part of exon 2 plus part of exon 3, with an additional insert of 447 bp between the two exons in-frame. Furthermore, 5' and 3' RACE on vervet monkey ejaculate RNA failed to produce novel 5' or 3' sequence of the monkey GnRHR-II. However, a GnRHR-II amplicon containing the full coding region, including part of the 5' and 3' UTRs, was obtained from vervet monkey ejaculate RNA by RT-PCR. This amplicon did however contain an insert of 447 bp between exons 2 and 3, similar to what was found for vervet monkey occipital lobe RNA.

Expression of GnRHR-II transcripts in baboon

The presence of transcripts for a GnRHR-II was investigated in RNA isolated from baboon cerebellum, heart, hypothalamus, kidney, occipital lobe, ovary, pituitary and temporal lobe. An initial screen using the exon 2-3 primer pair S10 & AS10 in RT-PCR revealed that in baboon, similar to human and vervet monkey, an antisense GnRHR-II transcript with intron 2 retained is ubiquitously expressed, as was evident from the presence of the 660 bp amplicon in all baboon tissue RNAs examined (for example, see figure 14, lanes 5 to 10). Furthermore, the 250 bp intronless exon 2-3 amplicon was faintly visible in baboon temporal lobe and cerebellum RNAs (figure 14, lanes 8 and 10, respectively). With the use of the exon 1-3 primer pair S5 & AS10 in RT-PCR to detect an intronless GnRHR-II transcript of 542 bp, faint bands of the right size were obtained in RNA from baboon temporal lobe and baboon occipital

lobe (figure 15, lanes 8 and 9, respectively), but these bands did not light up on a Southern blot (figure 16, lanes 8 and 9). Based on these findings, baboon temporal lobe RNA was selected for further 5' RACE analysis.

5' SMARTTM RACE was performed on baboon temporal lobe RNA using the same primer sets in the primary and secondary reactions as utilised in 5' RACE on vervet monkey occipital lobe RNA, namely UPM & AS8 and NUP & AS7, respectively. A product of 807 bp was obtained from baboon temporal lobe in the nested 5' RACE reaction (figure 17, lane 1 and figure 18, lane 1) that contained most of exon 2 (from AS7, within TM5, until the 5' end of exon 2, within TM4) but exon 1 was excluded (sequence data not shown). Thus, no additional 5' sequence of the baboon GnRHR-II was obtained with the use of 5' RACE on temporal lobe RNA. Nevertheless, these 5' RACE results in baboon temporal lobe revealed the presence of a baboon GnRHR-II transcript that contained exon 2 but lacked exon 1 sequence.

From baboon pituitary RNA a transcript containing exons 1 and 3 but lacking exon 2 was amplified by RT-PCR, using primer pairs S0 & AS13 in the primary and S1 & AS12 in the secondary reactions, respectively. These primer sets are designed to amplify a GnRHR-II transcript containing the full coding region, stretching from the 5' UTR to the 3' UTR. The primary reaction using S0 & AS13 yielded no visible bands on an agarose gel (not shown) and was therefore subjected to a secondary reaction with primer pair S1 & AS12. S1 and AS12 is designed such to amplify a 1295 bp amplicon containing the full coding region of the GnRHR-II. Priming of the primary reaction with this primer set produced multiple bands of various sizes (figure 21, lane 1), including a band of the expected size of 1295 bp. Specificity of the RT-PCR results was confirmed by Southern blotting using the labelled exon 1-specific and internally nested oligo AS5 (not shown). A number of bands were positive on the Southern blot. Of these, the largest product was the 1295 bp amplicon. Sequence analysis of this amplicon revealed that it contained only 1084 bp, corresponding to exons 1 and 3 of the baboon GnRHR cDNA, but lacking the 211 bp of exon 2 (see Sequence no 7 in Appendix 4). Thus, exon 3 followed directly after exon 1. Furthermore, exon 2 skipping resulted in a shift in the reading frame between the two exons (Sequence no 7 in Appendix 4). The failure to clone the full-length 1295 bp product from baboon pituitary RNA can be explained by the presence of multiple RT-PCR products visible on an agarose gel as a result of S1 & AS12 priming (figure 21, lane 1). It is possible that the gel slice containing the 1295 bp amplicon that was excised was contaminated with the 1084 bp amplicon because the bands were so

closely positioned (figure 21, lane 1). The existence of multiple GnRHR-II fragments adds to the complexity of cloning of a full-length mammalian GnRHR-II cDNA containing all three exons, as was evident from these results in baboon pituitary RNA. Although such a full-length cDNA could not be cloned from baboon pituitary RNA, another feature of the baboon GnRHR-II cDNA worth mentioning, was noted. Whereas the vervet monkey GnRHR-II transcript contains an ACG threonine codon at the translation start, the baboon transcript was found to contain an AUG methionine codon at that position, similar to what was found in humans.

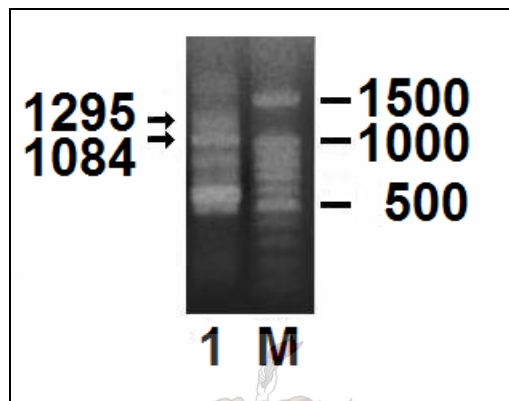
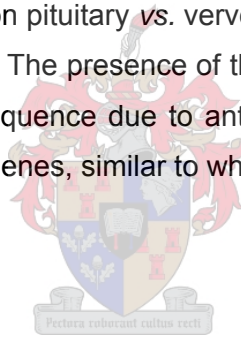


Fig 21. Agarose gel visualisation of RT-PCR results on baboon pituitary RNA: attempts to amplify exons 1, 2 and 3 of the baboon GnRHR-II cDNA. The lanes are marked as follows: 1, 1084 bp & 1295 bp products amongst other products obtained in the nested RT-PCR reaction on 1 with primer pair S1 & AS12; M, 100 bp ladder molecular weight marker (0.65 μ g, Promega). The position and size of the appropriate amplicons are indicated to the left whereas some of the positions and sizes of the molecular weight markers are indicated to the right of the photographed gel picture. Specificity of the RT-PCR results was confirmed by Southern blotting using the labelled exon 1-specific and internally nested oligo AS5.

5' and 3' SMARTTM RACE was performed on baboon pituitary RNA, using the primer pairs described above (5' RACE, figure 17, lane 2 and figure 18, lane 2; 3' RACE, figure 19, lane 2 and figure 20, lane 2). However, no novel 5' or 3' sequence was obtained by 5' or 3' RACE, respectively (sequence data not shown). 5' RACE analysis confirmed the presence of a transcript in baboons that contains exon 2 but lacks exon 1, possibly resulting from antisense transcription of the baboon *GnRHR-II* gene (not shown). In addition, a second 5' RACE product was obtained from baboon pituitary RNA, which contained exon 2 sequence as well as the last 223 bp of exon 1

immediately upstream of exon 2 (not shown). By 3' RACE on baboon pituitary RNA a product was obtained that contained exon 2 as well as part of exon 3 until the end of TM6 (not shown). Furthermore, this baboon pituitary 3' RACE product contained an insert of 448 bp between exons 2 and 3, similar to the 447 bp insert found between exons 2 and 3 of the vervet monkey occipital lobe 3' RACE and ejaculate RT-PCR products (see above). Comparison of the sequences of the inserts between exons 2 and 3 of the baboon pituitary, vervet monkey occipital lobe and vervet monkey ejaculate *GnRHR-II* amplicons revealed that they were the same except for a few nt differences (see Sequence no 8 in Appendix 4). The percentage sequence identity of the insert present in the vervet monkey occipital lobe 3' SMART™ RACE amplicon and the insert present in the vervet monkey ejaculate RT-PCR product was 99.3%, with 3 nt differences over 447 bp. Likewise, the insert sequences in the baboon pituitary 3' RACE product differed in seven and four nt positions from the insert sequences of vervet monkey occipital lobe and vervet monkey ejaculate, respectively, which is an identity of 98.4% (baboon pituitary vs. vervet monkey occipital lobe) and 99.1% (baboon pituitary vs. vervet monkey ejaculate), respectively (Sequence no 8 in Appendix 4). The presence of the insert sequences is likely to be the result of retained intronic sequence due to antisense transcription of the vervet monkey and baboon *GnRHR-II* genes, similar to what was found in humans.



Discussion

There has been an increasing interest in a mammalian GnRHR-II since initial evidence for the presence of a GnRH-2-selective receptor in amphibian [Troskie B *et al.*, 1997] and the subsequent cloning of a GnRHR-II from goldfish [Illing N *et al.*, 1999]. Numerous laboratories have attempted to clone a functional cDNA for this novel receptor from humans, but, to our knowledge, all attempts were unsuccessful. The finding of a full-length human GnRHR-II transcript (containing all three exons including the translation start and stop signals) has been hampered by the abundant and ubiquitous expression of an antisense GnRHR-II transcript that lacks exon 1 and has intron 2 retained [Millar R *et al.*, 1999]. The aim of this study was to find a tissue and/or cell source of a full-length, sense GnRHR-II transcript in humans. In addition, the cloning of a GnRHR-II from two non-human primate species namely vervet monkey and baboon was pursued.

GnRHR-II genes in the human genome

While this study was ongoing, completion of the human genome project revealed the existence of two putative *GnRHR-II* genes in the human genome, one on chromosome 1 and one on chromosome 14 [Neill JD, 2002a]. The *GnRHR-II* gene on chromosome 14 possesses exons 2 and 3 with a sequence identity of 40% to exons 2 and 3 of the human *GnRHR-I* gene on chromosome 4 [Neill JD, 2002a]. This gene is encoded on the antisense DNA strand of the 3' UTR region of a RNA-binding motif protein-8 pseudogene, termed *RBM8B* [Faurholm B *et al.*, 2001; Neill JD, 2002a]. The chromosome 14 *GnRHR-II* gene lacks exon 1 and is therefore considered to be sterile [Faurholm B *et al.*, 2001; Neill JD, 2002a]. Antisense GnRHR-II transcripts, containing exons 2 and 3 and with intron 2 retained and which are abundantly transcribed in a wide variety of tissues, are a product of transcription of the chromosome 14 gene. The chromosome 1 gene possesses three exons, similar to the human *GnRHR-I* gene, and has a 40% sequence identity with the human *GnRHR-I* gene (accession AL160282) [Neill JD, 2002a]. This *GnRHR-II* gene is transcribed in the sense orientation and encodes the full-length GnRHR-II. The use of exon 1-specific primers or probes in RT-PCR, RACE and hybridisation analyses in this study was an attempt to select GnRHR-II transcripts derived from transcription of the chromosome 1 gene.

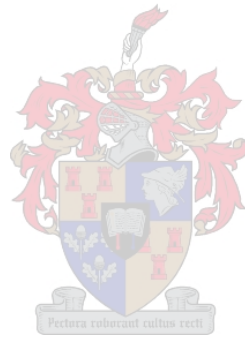
Human chromosome 1 GnRHR-II transcripts in mature sperm

Prior results obtained with the human dot blot indicated that the exon 1-containing, chromosome 1 GnRHR-II transcripts are neither abundantly nor widely expressed, in contrast to the expression of the exon 2-3 chromosome 14 gene (see Appendix 5). Of the tissues that produced positive signals on the dot blot, only cerebellum (adult as well as foetal) and testis were available for further RT-PCR analysis in this study, as well as a number of other human tissues and cells (summarised in table 4). GnRHR-II exon 1-specific primers were utilised in RT-PCR in combination with exon 2- or exon 3-specific primers to screen the human RNAs for the presence of intronless transcripts containing all three exons. Human ejaculate was the only source where a potential full-length (containing all three exons) intronless transcript was detected (table 4). The failure to detect exon 1-containing GnRHR-II transcripts in testis total RNA by RT-PCR may have been due to their degradation post-mortem and/or during the RNA isolation procedure from the testis and/or the low abundance of exon 1-containing transcripts. The RT-PCR results supported the findings that exon 2-3 amplicons, resulting from transcription of the chromosome 14 gene, are abundant for most of the human RNAs, whereas exon 1-containing amplicons are weakly detected. Subsequent to this study, *in situ* hybridisation analyses were performed on human testis tissue and on mature sperm to investigate the distribution of exon 1-containing GnRHR-II transcripts (see Appendix 5). The *in situ* localisation of GnRHR-II transcripts to the adluminal region of the seminiferous epithelium in testis suggests that the human *GnRHR-II* gene is post-meiotically expressed in round and elongating spermatids (Appendix 5). Furthermore, *in situ* hybridisation confirmed the presence of exon 1-containing transcripts in mature sperm (Appendix 5). Hence, the source of exon 1-containing, sense GnRHR-II transcripts in ejaculate appears to be mature sperm. A human sperm GnRHR-II cDNA sequence that stretched from -392 relative to the translation start within exon 1 until almost the end of TM6 in exon 3 was assembled from results of 5' RACE and RT-PCR. 3' RACE attempts to obtain the translation stop and polyadenylation signals were not successful, possibly due to the amplification of multiple GnRHR-II transcripts that were heterologous in the length of their 3' ends. Interestingly, the human sperm GnRHR-II sequence contained a nt deletion causing a frame shift within the coding region in exon 1, as well as a premature TGA translation stop signal in exon 2. The requirement for a nt insertion in exon 1 and the premature translation stop signal in exon 2 were also present in the *GnRHR-II* gene on chromosome 1 (Accession AL160282). These results are novel and interesting, since they are the first report of the finding of GnRHR-II transcripts containing all three exons in any human tissue or cell type.

See Table 4 in "Separate Figures" folder on CD



See Table 4 in "Separate Figures" folder on CD



Functionality of the human sperm GnRHR-II

A central query to be resolved is whether the GnRHR-II transcript is functional in human sperm. In light of the nt deletion in exon 1 and the premature translation stop signal in exon 2, the significance of the obtained human sperm transcripts is presently unknown. One possibility is that the human *GnRHR-II* gene on chromosome 1 is a pseudogene and that the sperm transcripts are the result of transcription of this pseudogene (see Appendix 5 for a detailed discussion). However, the expression of a *GnRHR-II* pseudogene that is on a different chromosome as the paralogous functional *GnRHR-I* gene would appear to be a rare event (Appendix 5). Nevertheless, it is difficult to envisage how transcripts from a gene containing a premature translation stop and a frame shift within the coding region could result in a full-length, functional GPCR. There is however support in the literature for the possible functionality of such transcripts. For example, it has been shown that 5-TM GPCRs, lacking TM helices 1 and 2, are expressed on the cell surface and retained their function [Ling K *et al.*, 1999]. Furthermore, a truncated form of another GPCR, containing only a single TM region, was shown to be as active as the wild type [Sugita S *et al.*, 1998]. Thus, one possibility is that a functional truncated protein, containing TMs 3 to 7, is expressed. This could occur if translation begins at the second AUG, situated at the end of TM2, which would cancel out the effect of the nt deletion in exon 1 (Sequence no 1 in Appendix 4), were it not for the stop codon within ECL2. Even so, there exist a number of possibilities whereby the human sperm GnRHR-II transcript could translate to a full-length functional GnRHR protein. These include RNA editing, whereby a single base insertion within exon 1 could result in the generation of a functional truncated protein (Appendix 5). Another possibility is that a full-length functional protein could be generated by an additional event that involves transition editing of the translation stop in exon 2 (Appendix 5). The presence of a premature stop signal within the human sperm RNA may represent a mechanism of translational control of the GnRHR-II during spermatogenesis, whereby transcripts produced during one stage are subsequently stored as inactive RNAs prior to their editing and translation at a later stage (Appendix 5). There is a possibility that another mechanism may be involved in the production of a functional GnRHR-II ([Faurholm B *et al.*, 2001] and Appendix 5). This would entail the incorporation of an unusual amino acid, selenocysteine, at the position of the translation stop to create an extended open reading frame ([Faurholm B *et al.*, 2001] and Appendix 5). This possibility is strengthened by the fact that selenium, which is incorporated into selenocysteine, is supplied to the testis with an apparent priority over other tissues [Behne D *et al.*, 1982]. Furthermore, it

has been established that selenium plays an important role during spermatogenesis and that the uptake thereof is under gonadotropin control [Wu ASH *et al.*, 1979]. Therefore, the UGA codon in the human GnRHR-II transcript may code for selenocysteine. Although the functionality of the various human GnRHR-II transcripts remains to be tested, there is substantial evidence in the literature for a functional role for a GnRHR in human sperm [Morales P *et al.*, 1994; Morales P & Llanos M, 1996; Morales P, 1998] (further discussed in Chapter 3). The expression of functional GnRHR-II transcripts in human sperm could be part of the existing network of intratesticular or neuroendocrine hormonal regulation governing spermatogenesis [Dimeglio LA *et al.*, 1998]. Although some of the above-mentioned functions could be mediated by the GnRHR-I, the expression of a functional GnRHR-II protein in the testis and sperm would be consistent with these reports. Recent results obtained by Maiti and co-workers further strengthened the case for the expression of a functional human GnRHR-II in reproductive tissue [Maiti K *et al.*, 2005]. Their findings suggested the existence of a GnRH-2 binding protein, in addition to the conventional GnRHR-I, in prostate cancer cells [Maiti K *et al.*, 2005]. Another study indicated that the human GnRHR-II plays a role in cell proliferation [Enomoto M & Park MK, 2004]. Thus, if the chromosome 1 *GnRHR-II* gene is not a pseudogene, transcripts of this gene could possibly be translated as a truncated, immunoreactive protein or edited to result in translation of a full-length protein, possibly containing selenocysteine ([Faurholm B *et al.*, 2001] and Appendix 5). Further experiments using specific antibodies directed against domains encoded by sequences both 5' and 3' to the stop codon would be necessary to clarify whether a full-length or truncated GnRHR-II protein is expressed in sperm.

Other reported findings of human GnRHR-II transcripts

Subsequent to the start of this study, human GnRHR-II transcripts have been detected by others using either dot blotting with an exon 3-specific riboprobe for the sense transcript [Neill JD *et al.*, 2001] or by Northern blotting using an exon-1 specific double-stranded DNA probe [Millar R *et al.*, 2001] on selected polyA⁺ RNA tissue arrays. Interestingly, the reported tissue distribution patterns differed, which may reflect the use of different probes. Similar to this report, Millar R *et al.* [2001] have observed signals for exon 1 of the GnRHR-II in putamen, occipital lobe, cerebellum, caudate nucleus, and heart, amongst other tissues including other brain parts (see table 4).

Finding of GnRHR-II transcripts in vervet monkey and baboon

The presence of RNA for a GnRHR-II in various vervet monkey and baboon tissues and cells has been demonstrated in this study, indicating the existence of genes for vervet monkey and baboon GnRHR-IIs. However, a transcript containing the full coding region of a mammalian GnRHR-II could not be cloned. While busy with these cloning attempts from vervet monkey RNAs including from COS-1 RNA, the finding of a cDNA containing all coding sequences of a mammalian GnRHR-II was reported by two independent laboratories, from rhesus monkey pituitary tissue and COS-1 cells [Neill JD *et al.*, 2001; Accession AF353988] as well as from marmoset monkey pituitary and brain [Millar R *et al.*, 2001; Accession AF368286]. One of these laboratories used the same antisense primer AS12 in their cloning attempts that was utilised in this study [Neill JD *et al.*, 2001]. The inability to obtain the full-length cDNA from COS-1 RNA with AS12 in this study is therefore hard to explain, although, with the use of 3' RACE on vervet monkey occipital lobe RNA it was indicated that the sequence of primer AS12 is not present in the vervet monkey cDNA at the expected position. In addition, in this study, using 5' RACE analysis on COS-1 and vervet monkey occipital lobe RNAs, novel 5' sequence was obtained compared to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988). From COS-1 and vervet monkey occipital lobe RNA, 34- and 92 bp novel 5' UTR sequence was obtained compared to the published vervet monkey GnRHR-II cDNA sequence, respectively (Sequence no 2 & 5 in Appendix 4). Furthermore, the sequence of the vervet monkey occipital lobe GnRHR-II transcript corresponded to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988) except for two nt differences, one in TM3 and the other in ECL2 (Sequence no 3 in Appendix 4). Since the occipital lobe and published COS-1 sequences were both derived from vervet monkey, these nt differences most likely indicated differences between individuals, i.e. between the GnRHR-II expressed in occipital lobe (this study) and in kidney cells (published sequence, Accession AF353988). However, the possibility that these single nt differences originated from errors incorporated during the cDNA synthesis reaction cannot be excluded. Both nt differences would result in the incorporation of a different amino acid compared to the published vervet monkey sequence if the occipital lobe sequence is translated.

Characteristics of the monkey GnRHR-II

In contrast to the mammalian GnRHR-Is, the monkey GnRHR-IIs possess C-terminal tails (similar to non-mammalian GnRHRs) and showed rapid desensitisation and internalisation, with concomitant receptor phosphorylation within the tails [McArdle

CA *et al.*, 2002]. Similar to results obtained in this study, it was shown that the vervet monkey GnRHR-II mRNA contains a CGA arginine codon at the position where the human sequence contains a TGA stop signal within exon 2 (Accession AF353988, and results presented here), creating an extended open reading frame. Furthermore, translation of the vervet monkey GnRHR-II mRNA is initiated by an unusual ACG threonine codon. There are indeed reports of several non-AUG initiation codons, such as CUG, GUG, ACG, and AUU that are functionally active in viral and eukaryotic mRNAs [Sprengrart ML & Porter AG, 1997]. Purines almost always occupy the -3 and/or +4 positions of these initiation sites. In vertebrates, initiation at non-AUG sites of some mRNAs can also be stimulated in the presence of specific nucleotides located downstream of the initiation codon, preferentially G at +4, A or C at +5 and U at +6 [Sprengrart ML & Porter AG, 1997]. The vervet monkey COS-1, occipital lobe and ejaculate GnRHR-II mRNAs indeed possess purines at -3 (COS-1 has an A, occipital lobe has a G, and ejaculate has an A), C at +5 and U at +6 (Sequence numbers 2, 5 and 6 in Appendix 4). The published vervet monkey GnRHR-II cDNA sequence (Accession AF353988) was used to predict the site of initiation of translation in the human sperm cDNA sequence. Interestingly, translation of the human cDNA would be predicted to initiate at an AUG methionine codon like most eukaryotic cDNAs, but the human start codon occurs in a different reading frame ([Faurholm B *et al.*, 2001] and Sequence no 1 & 7 in Appendix 4). Similarly, sequence analysis of the baboon pituitary RT-PCR product revealed that, unlike the vervet monkey GnRHR-II, translation of the putative baboon GnRHR-II most likely initiates with an AUG methionine codon, in the correct frame (Sequence no 7 in Appendix 4). Overall, there is a 96.5% identity between the coding regions of the human and vervet monkey GnRHR-II cDNAs and a 92.9% identity at the amino acid level (Sequence no 1 in Appendix 4).

Multiple GnRHR-II transcripts in vervet monkey and baboon

In vervet monkey occipital lobe RNA the presence of multiple GnRHR-II amplicons was indicated. These include an amplicon containing exons 1 and 2 as well as a 116 bp insert between the two exons, with a resultant shift in the reading frame (Sequence no 4 in Appendix 4). Another amplicon, containing exons 2 and 3 with a 447 bp insert between the two exons in-frame, was obtained from vervet monkey occipital lobe RNA. A third amplicon, containing exon 2 but lacking exon 1, was also found. The finding of this exon 1-less transcript indicated that the vervet monkey *GnRHR-II* gene is, like its human counterpart, transcribed in the antisense orientation. From vervet monkey ejaculate RNA a GnRHR-II amplicon containing the

full coding region, including the translation start and stop as well as partial sequence of the 5' and 3' UTRs, was cloned (Sequence no 6 in Appendix 4). This amplicon contained an insert of 447 bp between exons 2 and 3 in frame, similar to what was found in vervet monkey occipital lobe RNA. The length of the 447 bp insert between exons 2 and 3 of the vervet monkey ejaculate and occipital lobe transcripts as well as the 448 bp insert between exons 2 and 3 of the baboon pituitary transcript would correspond to the size of intron 2 of the human *GnRHR-II* gene ([Millar RP *et al.*, 1999] and results presented here).

From baboon cerebellum and pituitary RNAs, RT-PCR products were amplified that contained exons 1 and 3 of a putative GnRHR-II but lacked exon 2, with a resultant shift in the reading frame between the two exons (Sequence numbers 4 & 7 in Appendix 4). The omitted exon 2 would translate to ECL2 and TM5. The exon 2-less variant seemed to be abundantly and ubiquitously expressed and may mask the amplification of the 3-exon fully processed transcript if the latter exists in baboon. Where the baboon transcripts did contain exon 2, exon 1 was absent and there was an additional insert between exons 2 and 3, as demonstrated in baboon pituitary RNA (Sequence no 8 in Appendix 4). Furthermore, from baboon temporal lobe RNA a GnRHR-II transcript was cloned that contained exon 2 but lacked exon 1. Taken together, the presence of numerous GnRHR-II transcripts is indicated in vervet monkey and baboon RNAs although a GnRHR-II cDNA containing the full three exons and which has been fully processed could not be isolated from the tissues and cells that were examined. To the best knowledge of the author, this is the only report of an extensive study of the various GnRHR-II transcripts expressed in mammals, and also the only reported finding of GnRHR-II transcripts in baboon.

The presence of vervet monkey and baboon transcripts containing inserts between exons 2 and 3 suggests that the vervet monkey and baboon *GnRHR-II* genes are transcribed in the antisense orientation, similar to what was found in humans. Whether they are indeed the results of antisense transcription or rather originating from pre-mRNAs containing intron 2, in the sense orientation, remains to be shown. Seeing that the insert between exons 2 and 3 lies within, or disrupts, ICL3 (Sequence numbers 5 & 8 in Appendix 4), it is possible that it possesses an important function in GnRHR-II signalling. According to Bockaert & Pin the function of splicing with insertions localised at ICL3 is to modify or regulate the specificity and intensity of GPCR coupling to G proteins [Bockaert J & Pin JP, 1999]. This would suggest that GnRHR-II transcripts (in the sense orientation) containing an insert between exons 2

and 3 are functional. It is possible that functionality of insert-containing transcripts depends on the presence of exon 1. Hence, the vervet monkey ejaculate transcript was particularly interesting because of the presence of exon 1.

Whether the exon 2-less GnRHR-II transcripts found in baboon pituitary and cerebellum RNAs encode functional GnRHR-II proteins that are capable of responding to GnRH stimulation is questionable, especially in view of the fact that the domain namely ECL2 that is responsible for receptor stabilisation is absent. ECL2 has been shown to be involved in the formation of disulfide bonds between cysteine residues in the N terminus and/or TM1 of the folded GnRHR protein [Davidson JS *et al.*, 1997]. However, a deletion in ECL2 does not affect the human GnRHR-I function [Ott TR *et al.*, 2002] or that of the *Xenopus laevis* GnRHR-II [Gault PM *et al.*, 2004; Accession AF257320]. There are also reports of GPCRs other than the GnRHR that consist of “split” fragments i.e. complimentary TMs from different proteins that are constituted together to form a functional receptor that can efficiently couple to G proteins [Gudermann T *et al.*, 1997; Bockaert J & Pin JP, 1999]. Furthermore, polypeptide sequences encoded by two different reading frames of the mRNA may be fused by frame shifting. Examples have been found in MS2 bacteriophage RNA as well as retroviral mRNAs such as HIV-1 [Melcher U, 2001]. Thus, there is a possibility that the above-mentioned baboon GnRHR-II fragments are indeed translated into functional membrane receptors. GnRHR transcripts of various lengths other than full-length or transcripts that are not fully processed are not uncommon and have been reported in numerous species, for both the GnRHR-I and the GnRHR-II (table 5).

Table 5. Examples of transcripts for the GnRHR other than full-length, fully processed in various species.

Species	GnRHR subtype	Transcript identity	Reference
Mouse	GnRHR-I	<ul style="list-style-type: none"> Exons 1 & 3 but lacks exon 2, resulting in a shift in the reading frame between the two exons. Exons 1 & 2 but lacks exon 3 and with a 700 bp insert past the exon 2 splice donor. 	[Zhou W & Sealon SC, 1994].
Bull frog	GnRHR-I, -II and -III	<ul style="list-style-type: none"> Partial intron retention or partial exon skipping with a resultant shift in the reading frame and the introduction of a premature stop signal. 	[Wang L <i>et al.</i> , 2001b].
Rainbow trout	GnRHR-I	<ul style="list-style-type: none"> Exons 1 & 2, which may encode a truncated protein corresponding to the N-terminus plus TMs 1 to 5. 	[Madigou T <i>et al.</i> , 2000].
Rat	GnRHR-I	<ul style="list-style-type: none"> Intron 1 retained. 	[Botté M-C <i>et al.</i> , 1998].
Human	GnRHR-I	<ul style="list-style-type: none"> Exons 1 & 3 but lacks exon 2, resulting in a frame shift as well as a premature stop signal. Intron 1 retained. Partial deletion of exon 2 (128 bp or 220 bp deleted). 	[Silveira LFG <i>et al.</i> , 2002; Seeburg PH & Adelman JP, 1984; Dong KW <i>et al.</i> , 1993; Dong KW <i>et al.</i> , 1997; Grosse R <i>et al.</i> , 1997].
Human	GnRHR-II	<ul style="list-style-type: none"> Partial deletion of exon 1. 	[Morgan K <i>et al.</i> , 2003].

Two important questions arise namely what the origin of these alternative GnRHR transcripts is and what their possible function(s) are. One major contributing factor is the fact that GnRHR genes contain introns, which allow for alternative splicing. Furthermore, the existence of multiple transcription initiation sites and polyadenylation signals in the 5' and 3' ends of the GnRHR genes, respectively, facilitates the production of various transcripts with different lengths [Fan NC *et al.*, 1995]. In general, one of the functions of alternative splicing of mRNA transcripts is to expand the range of protein products from a single gene locus [Burgess HA & Reiner O, 2002]. Different combinations of exons can be spliced together to produce different mRNA isoforms of a gene, encoding structurally and functionally different proteins [Gilbert W, 1978]. Thus, it is possible that the alternative human, baboon and vervet monkey GnRHR-II transcripts reported on in this study encode functional receptors possessing alternative functions. Another possible result of alternative processing of GnRHR mRNAs is the production of functional truncated proteins, as described above [Ling K *et al.*, 1999; Sugita S *et al.*, 1998]. It is also possible that the truncated receptors interfere with the function of the wild type receptor. In fact, alternative mRNA splicing is a commonly used strategy to create a functionally diverse pool of gene products derived from a single gene, which is recognised as an

important mechanism for the regulation of the wild type receptor function [Rueter SM *et al.*, 1999; Belaguli NS *et al.*, 1999]. One example is the human pituitary GnRHR-I splice variant that contains a 128 bp deletion between nucleotide positions 522 and 651, which forms part of exon 2, resulting in the interference of GnRH-1 binding to the wild type receptor and thereby inhibiting signalling through the wild type [Grosse R *et al.*, 1997]. Furthermore, when co-expressed with the wild type receptor, this truncated protein caused impaired insertion of the wild type into the plasma membrane [Grosse R *et al.*, 1997]. Similarly, in the bull frog, GnRHR transcripts that contain intronic sequence are expressed at levels comparable to the wild type receptor but exhibited a very low binding affinity to GnRH and did not induce signal transduction in response to GnRH treatment [Wang L *et al.*, 2001b]. Co-transfection of the bull frog variant GnRHR transcripts with the wild type receptor lead to the inhibition of wild type receptor-mediated signalling [Wang L *et al.*, 2001b]. It has been suggested that the variant proteins interact physically with the wild type receptor protein [Wang L *et al.*, 2001b].

Indeed, new data from Pawson AJ *et al.* [2005] indicated a role for a human chromosome 1 GnRHR-II fragment as inhibitor of GnRHR-I function. These authors expressed a protein corresponding to the domains from the cytoplasmic end of TM5, ICL3, TM6, ECL3, TM7 and the C-terminal tail of the putative human GnRHR-II in COS-7 cells. This protein (designated as the GnRHR-II-reliquum) is the result of usage of a putative translational start codon 117 bp downstream of the premature stop signal in exon 2 (AUG, at +654, see Sequence no 1 in Appendix 4 and figure 22) [Pawson AJ *et al.*, 2005]. The expressed GnRHR-II-reliquum was localised throughout the cytoplasm but appeared not to be significantly inserted into the cell plasma membrane. Co-expression of the GnRHR-I and the GnRHR-II-reliquum in COS-7 cells resulted in reduced expression of the GnRHR-I as well as impaired signalling via the GnRHR-I (as revealed by reduction of GnRH-induced IP accumulation) [Pawson AJ *et al.*, 2005]. This inhibitory effect was found to be specific for the tail-less GnRHR-I since the GnRHR-II-reliquum exerted no inhibitory action on the chicken GnRHR-I that possesses a C-terminal tail [Pawson AJ *et al.*, 2005]. The reduction in GnRHR-I numbers is thought to be exerted at the endoplasmic reticulum or Golgi apparatus level, possibly by perturbing the normal processing of GnRHR-I from these sites or enhancing GnRHR-I degradation [Pawson AJ *et al.*, 2005]. These results would thus be consistent with the view that the GnRHR-II-reliquum plays a modulatory role in GnRHR-I expression. Pawson AJ *et al.* [2005] proposed a mechanism whereby expression of the GnRHR-I is inhibited

by the GnRHR-II-remainder, based on the findings of Grosse R *et al.* [1997] that a C-terminal protein fragment, corresponding to TM6 and TM7, was able to interact with the TM1 through TM5 fragment to produce a functional receptor. They suggested that the GnRHR-II-remainder may similarly interact, via interhelical interactions, with the GnRHR-I and lead to an unstable or misfolded receptor complex that would be prevented from onward processing and/or undergo defective intracellular transport from the endoplasmic reticulum, or enhanced degradation [Pawson AJ *et al.*, 2005]. Although expression of the GnRHR-II-remainder has not been detected *in vivo*, the presence of a putative start codon downstream of the premature stop would suggest that synthesis of a GnRHR-II-remainder is possible, should this be used as a translational start site. Indeed, any mRNA transcript derived from the chromosome 1 *GnRHR-II* gene, in which intron 2 has been spliced out, would lead to translation and synthesis of the GnRHR-II-remainder, should the downstream start site be used by the translation machinery. Such transcripts (i.e. in which intron 2 has been spliced out) are present in humans as have been demonstrated in the present study and by others [Morgan K *et al.*, 2003].

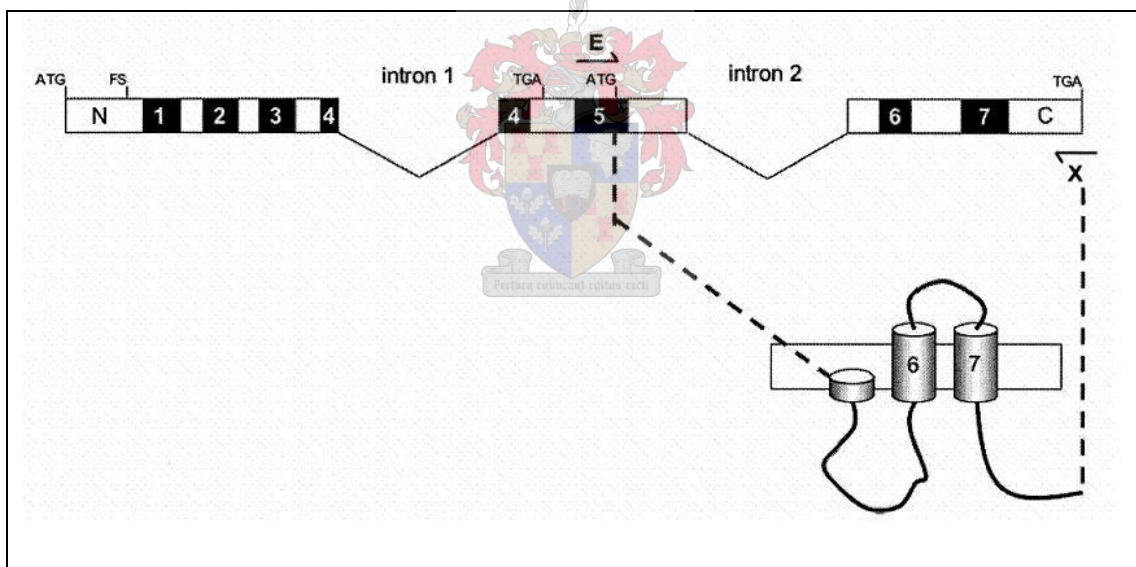


Fig 22. Generation of the GnRHR-II-remainder as demonstrated by Pawson AJ *et al.* [2005]. The structure of the full-length coding region of the *GnRHR-II* gene on chromosome 1 is shown. Exons are indicated as boxes. TMs 1 to 7 (numbered black boxes) and the extracellular N-terminus and cytoplasmic C-terminal tail of the putative GnRHR-II are indicated. The position of the frame shift (FS), in-frame premature translation stop signal (TGA between TM4 and TM5) and putative translation start site (ATG in TM5, 117 bp downstream of the premature stop) are indicated. The GnRHR-II-remainder open reading frame was PCR amplified using primer pair E & X and subcloned into a FLAG-tagged mammalian expression vector. The corresponding GnRHR-II-remainder protein domains are depicted schematically (taken from Pawson AJ *et al.* [2005]).

The variation in mRNA splicing is likely to be both species- and tissue-specific. In human pituitary, for example, the full-length GnRHR-I as well as two variant transcripts with internal deletions are expressed, whereas in the testis the full-length and one of the two shortened transcripts are expressed but only the full-length transcript is found in breast tissue [Kottler ML *et al.*, 1999]. Interestingly, the number of tissue-specific alternative splice forms has been found to be the highest in the brain [Xu Q *et al.*, 2002]. Physiological conditions may also play a role in the differential expression of GnRHR splice variants, as is indicated for the bull frog GnRHR where the ratio between different splice variants change together with a change of season [Wang L *et al.*, 2001a]. Taken together, it seems that regulation of mRNA splicing may introduce an additional level of control of GnRHR expression.

Alternative splicing may occur in a very large fraction of human genes (35% to 59%), suggesting a major role for alternative splicing in the production of functional complexity in the human genome [Xu Q *et al.*, 2002]. This implies extensive regulation of alternative splicing, so that it displays strong specificity to a particular tissue or developmental stage, modulating the functional characteristics of protein isoforms in specific tissues. It seems as if this is indeed the case for the GnRHR (-I and -II). While the current study as well as studies performed by a number of others showed the presence of various alternative human GnRHR-II transcripts, a transcript that does not need some kind of alteration or editing to encode a full-length functional protein has not been found. It is possible that such a transcript exists only under certain physiological conditions, which are presently unknown, or does not exist at all.

The study of GnRHRs has been hampered by the unavailability of antibodies that discriminate between the GnRHR-I and GnRHR-II subtypes. Such antibodies would be especially helpful to detect whether humans express a functional GnRHR-II protein, and, if they do, in which tissues or cells. This could contribute greatly to the eventual understanding of the physiology of the two receptor subtypes and lead to the development of drugs to target GnRHR-related ailments specifically and with greater success. However, the fact that most of the GnRHR protein is buried in the cell membrane makes it difficult to purify the receptor from the membrane in an active form for production of antibodies to the native protein.

See Table 6 in "Separate Figures" folder on CD



See Table 7 in "Separate Figures" folder on CD

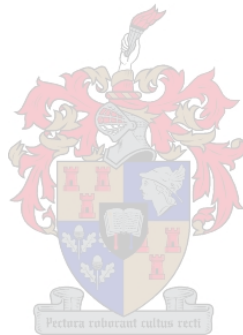


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CHAPTER 3

DISTRIBUTION OF TRANSCRIPTS FOR GnRH-1, GnRH-2, THE GnRHR-I AND THE GnRHR-II IN HUMAN AND MONKEY EJACULATE





Background

Substantial evidence exists for extrapituitary functions of GnRH in addition to its well-known gonadotropin-releasing role in the pituitary [Cheng CK & Leung PC, 2005]. Many of the extrapituitary actions of GnRH involve tissues and cells of the reproductive system, such as the ovary, uterus, placenta, testis as well as developing and mature sperm (see table 2 in Chapter 1). Furthermore, by comparison of tables 1 and 2 (Chapter 1) it is evident that the GnRHR-I is often co-expressed with GnRH-1 and/or GnRH-2, or expressed by adjacent tissues or cells. This suggests local actions of GnRH-1 and GnRH-2 as autocrine and/or paracrine regulators in extrapituitary tissues.

Evidence exists for a direct role of GnRH-1 in spermatogenesis, sperm maturation and fertilisation [Kangasniemi M *et al.*, 1996; Morales P, 1998; Morales P *et al.*, 2000]. For example, when human sperm aliquots were incubated with the zona pellucida (ZP), which is the extracellular coat of an egg, it was found that the number of sperm that bound to the ZP increased three-fold in the presence of GnRH-1 (20 nM) compared to a saline control [Morales P, 1998; Morales P *et al.*, 2000]. Furthermore, the effects of GnRH-1 upon sperm-zona binding could be inhibited by GnRH-1 antagonists [Morales P *et al.*, 2000], indicating that a GnRH-1 receptor is involved. Likewise, a GnRH-1 antagonist was found to be able to suppress mouse spermatogenesis *in vivo* [Kangasniemi M *et al.*, 1996].

These observations imply that human and mouse sperm express receptors for GnRH-1 on their cell surface. Since GnRH-1 can bind to and signal via both the GnRHR-I and the GnRHR-II [McArdle CA *et al.*, 2002], any one of the two receptor subtypes or both may be expressed in mature mammalian sperm. A single immunohistochemical study has indicated that the GnRHR-I protein is localised in the acrosomal region of human sperm [Lee CY *et al.*, 2000]. Results of the present study revealed that GnRHR-II transcripts are present in human sperm and in monkey ejaculate (Chapter 2). Others have demonstrated the presence of the GnRHR-I in human testis by RT-PCR [Moumni M *et al.*, 1994; Petersson F *et al.*, 1989; Clayton RN *et al.*, 1980; Sharpe RM & Fraser HM, 1980]. Further support for the actions of a local GnRHR by binding of locally produced GnRH in mammalian sperm comes from the fact that the levels of hypothalamic GnRH in circulation are too low to have effects on extrapituitary tissues [Nett TM *et al.*, 1974]. Besides this, GnRH has a short half-life [Eskay RL *et al.*, 1997; Hsueh AJW & Jones PBC, 1981]. Therefore,

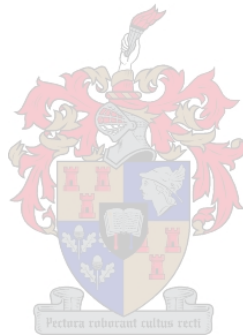
GnRH produced locally and present in the fallopian tube, where sperm and oocytes are deposited to form zygotes [Casañ EM *et al.*, 2000], might be responsible for increased sperm-ZP binding during fertilisation *in vivo*. Similarly, GnRH produced locally in the testis or sperm may possibly orchestrate the effects of GnRH in spermatogenesis and sperm maturation. Indeed this is supported by the detection a number of years ago of GnRH-1 and GnRH-like molecules in human seminal plasma [Izumi S-I *et al.*, 1985; Sokol RZ *et al.*, 1985].

Taken together, it appears likely that human sperm expresses a local GnRH/GnRHR system. Whereas data from this and other studies have suggested the presence of GnRH-1, the GnRHR-I and the GnRHR-II in human sperm, it has not been established whether GnRH-2 is expressed in mammalian sperm. Also, to the author's knowledge there are no reports of the presence of GnRHR-I mRNA as detected by RT-PCR amplification in mammalian sperm. Furthermore these observations of the presence of GnRH-1 and the GnRHR (-I and/or -II) in human sperm were made at different times and in independent laboratories. Therefore, the focus of the present study was to determine in a single study, by RT-PCR, whether transcripts for GnRH-1 and/or GnRH-2 and the two GnRHR subtypes are found in human and vervet monkey ejaculate. The results of such a study could be very significant in the reproductive field, especially since a role for GnRH has been indicated in mammalian fertilisation.



Aim

To determine within a single study whether transcripts for GnRH-1, GnRH-2, the GnRHR-I and the GnRHR-II are expressed in human and/or monkey ejaculate, with the use of the techniques of RT-PCR, Southern blotting and sequencing.



Experimental

Animals and cells

Human and vervet monkey ejaculate were freshly obtained from the Andrology Department, Groote Schuur hospital (Cape Town, South Africa) and from the MRC at Tygerberg (Bellville, South Africa), respectively, kept at rt and used within a few hours for RNA isolation. Each ejaculate sample was pooled from several male donors. Adult human hypothalamic and pituitary tissue was obtained from the Salt River Mortuary (Cape Town, South Africa) after approval from the Medical Ethics Committee at the University of Cape Town Medical School (Cape Town, South Africa). The tissues were snap frozen in liquid nitrogen and stored at -80°C until later use. MCF-7 human breast cancer and COS-1 monkey kidney cells were kept in culture at 37°C in DMEM containing 1% v/v PenStrep and supplemented with 10% FBS, under 5% CO₂.

RNA preparation and cDNA synthesis

Total RNA was isolated from human and monkey ejaculate by CsCl-guanidinium isothiocyanate ultracentrifugation and from human pituitary, human hypothalamus, and MCF-7, L β T2 and COS-1 cells according to the TRI-reagentTM protocol and subjected to a DNaseI treatment as previously described (Chapter 2). cDNAs used as templates for RT-PCR were prepared from 1 μ g of denatured total RNA using 500 ng random hexamer primers (Promega) and 1 μ l ImProm-IITM reverse transcriptase (Promega) in a 20 μ l reaction volume with incubation at 42°C for 60 min.

RT-PCR, Southern blot and sequence analyses

RT-PCR reactions were performed using 10 μ l cDNA in a 50 μ l reaction volume as previously described (Chapter 2), with the inclusion of 5% DMSO in the reaction make-up. Primer sets were designed such that the PCR extended over more than one exon. Primers were mainly human-specific but in some instances corresponded to the mouse or vervet monkey gene sequence(s) (refer to Appendices 1 to 3). Schematic representations of the GnRH-1, GnRH-2, GnRHR-I and GnRHR-II cDNAs, with the relative positions of the primers indicated, are shown within the Results section of this chapter (figures 25 & 26). A control PCR reaction was routinely performed using primers designed to the mouse β -actin housekeeping gene, as previously described (Chapter 2). The cycle conditions for PCR were the same as previously described (Chapter 2), with annealing temperatures ranging between 50°C

and 60°C (Appendix 2). RT-PCR products were separated by electrophoresis, subjected to Southern blot analysis, cloning and sequencing as described previously (Chapter 2).



Results

Total RNA was isolated from human and vervet monkey ejaculate and used in RT-PCR with a series of gene-specific primers for *GnRH-1*, *GnRH-2*, the *GnRHR-I* and the *GnRHR-II*. Integrity of total RNA preparations was confirmed by gel electrophoresis (not shown) as previously described (Chapter 2). Quality of cDNA preparations was determined by a control PCR using primers specific for the *β -actin* gene to amplify a 317 bp exon 2-3 fragment (see figure 24). Parallel positive control RT-PCRs were performed on RNAs isolated from tissues that had previously been shown to express the relevant genes. In addition, negative control RT-PCRs were performed where indicated on RNAs isolated from tissues that do not express the relevant genes. The use of primers spanning introns was used to control for genomic DNA contamination. Furthermore, the use of a laminar flow cabinet and dedicated PCR pipettes for setting up of reactions would make contamination of cDNA templates or reagents by plasmids an unlikely event. This is supported by the finding that, of the samples analysed in parallel for the presence of intronless GnRH or GnRHR transcripts, using the same reagents, most did not show positive signals (figures 27, 29, 31 and results not shown). The specificity of RT-PCR products of the expected size was confirmed by Southern blot analysis followed by isolation and sequencing to finally confirm the identity of the amplicons.



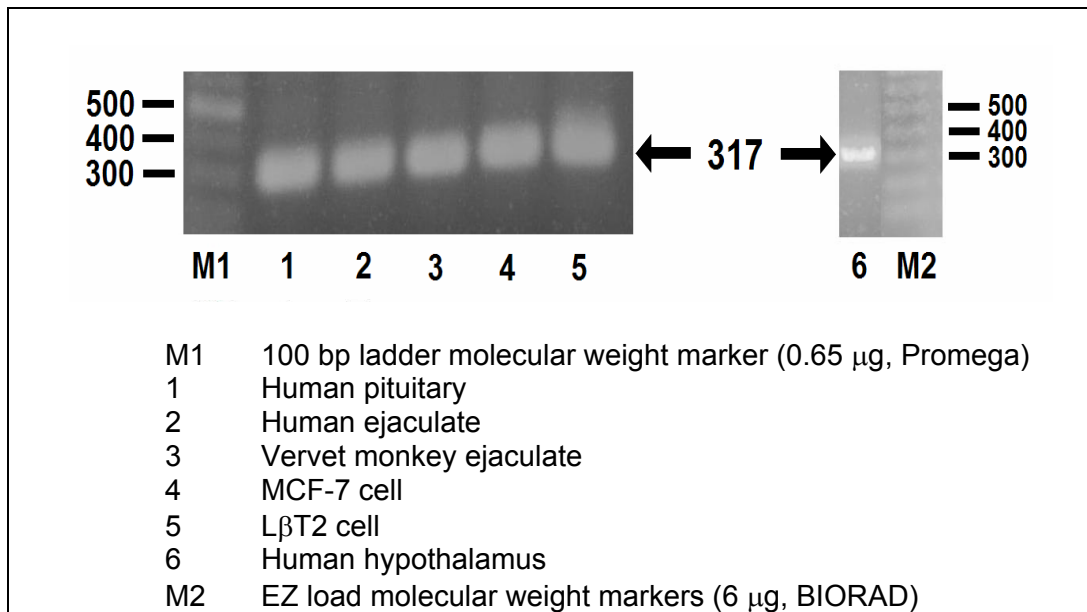
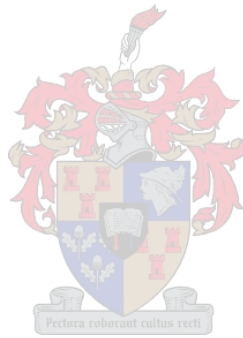


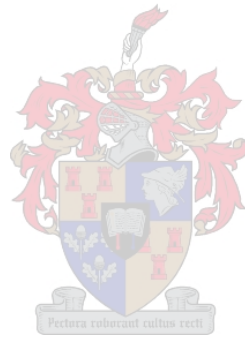
Fig 24. Agarose gel visualisation of RT-PCR results on human and vervet monkey ejaculate RNA and control RNAs using *β -actin* primers: indications that the cDNA synthesis reactions were successful. The position and size of the appropriate amplicon as well as some of the positions and sizes of the molecular weight markers are indicated.

To be able to better understand the strategy of and the results obtained in the GnRH and GnRHR RT-PCRs, two schematic diagrams are included that show the relevant positions of the primers utilised (figures 25 & 26). Furthermore, a detailed description of the primer positions and design can be found in Appendices 1 to 3.

See Figure 25 in “Separate Figures” folder on CD



See Figure 26 in "Separate Figures" folder on CD



Distribution of transcripts for GnRH-1 and GnRH-2 in human and vervet monkey ejaculate RNA

The distribution of GnRH-1 transcripts in human and vervet monkey ejaculate RNA was investigated using *GnRH-1*-specific primers H1S1 & H1AS1 (figure 25). RNA isolated from human hypothalamus tissue was included as a positive control. The expected size of a H1S1-H1AS1 amplicon is 387 bp if the mRNA is fully processed. In human ejaculate RNA, the presence of transcripts for GnRH-1 was revealed by the successful amplification of the 387 bp H1S1 & H1AS1 amplicon (figure 27, lane 2). Furthermore, specificity of this 387 bp product was confirmed by Southern blot analysis using an internally nested and exon 2-specific oligonucleotide, H1S2 (figure 28, lane 2). In monkey ejaculate RNA the 387 bp product was not visible in the photograph of the agarose gel (figure 27, lane 3) yet a very faint band could be seen on the Southern blot at the expected position (figure 28, lane 3). These results suggest that *GnRH-1* is also expressed in vervet monkey ejaculate. The absence of a band on the agarose gel may be due to a low efficiency of the reaction due to the use of the human-specific primer pair H1S1 & H1AS1 that could have a low sequence homology to the vervet monkey GnRH-1 cDNA. Unfortunately, the vervet monkey *GnRH-1* gene or mRNA sequences have not been published, but the *GnRH-1* gene sequence of the rhesus monkey is known (Accession X88795). Comparison of the sequences of the human-specific GnRH-1 primers that were used with that of the rhesus monkey gene sequence revealed a 47.8% identity to one of the primers, H1S1 (used as the sense primer in RT-PCR). The sequences of the other two primers, H1AS1 and H1S2, used as antisense primer in RT-PCR and as a probe in Southern blotting respectively, could not be recognised in the rhesus monkey gene.

The human ejaculate 387 bp GnRH-1 amplicon was cloned and sequenced (Sequence no 9 in Appendix 4). In parallel, partial sequence information was obtained from sequencing of the human hypothalamic positive control GnRH-1 amplicon. Comparison of the human ejaculate GnRH-1 sequence to the published GnRH-1 cDNA sequence (Accession X15215) revealed three nt differences between the two sequences (Sequence no 9 in Appendix 4). One of the three nt differences, namely a “G” instead of a “C”, resulting in translation to a tryptophan (W) instead of a serine (S) in the human ejaculate sequence was also found in the human hypothalamus sequence (Sequence no 9 in Appendix 4). This C-for-G substitution formed part of the signal peptide. The other two alternative nucleotides formed part of GAP, and fell out of the frame of the obtained hypothalamic sequence. Thus, the

human ejaculate and hypothalamus sequences were identical in the overlapping parts but differed from the published GnRH-1 cDNA sequence (Accession X15215).

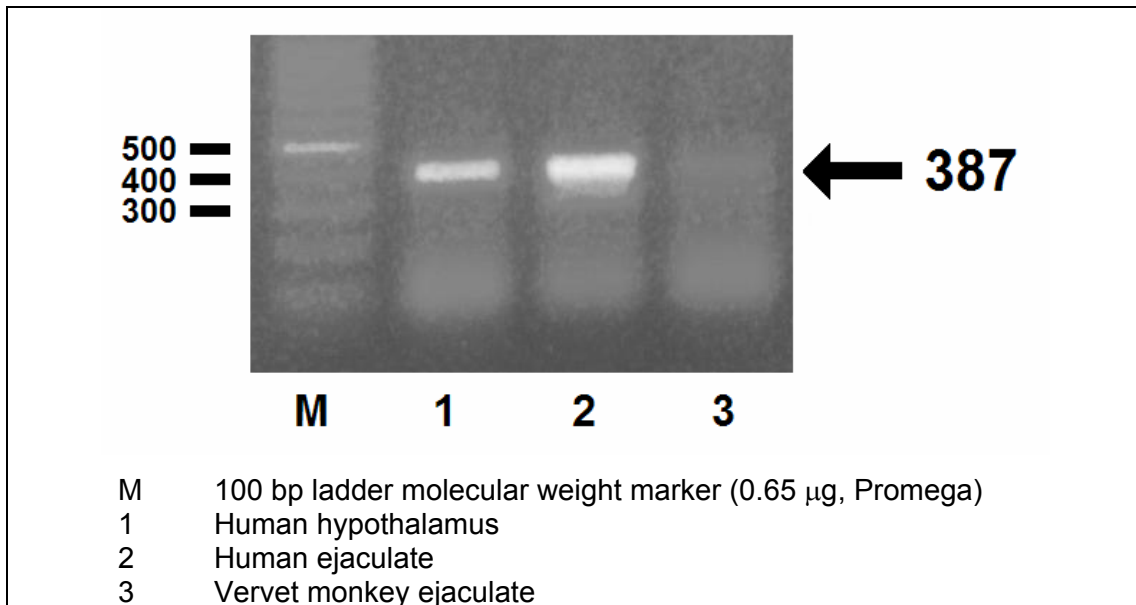


Fig 27. Agarose gel visualisation of RT-PCR results on human and vervet monkey ejaculate RNA: attempts to determine whether transcripts for GnRH-1 are expressed. Human hypothalamic RNA was included as positive control. Primer pair H1S1 & H1AS1, for the amplification of a 387 bp intronless exon 2-4 amplicon was utilised in the RT-PCRs. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the position and size of the appropriate amplicon is indicated to the right of the photographed gel picture.

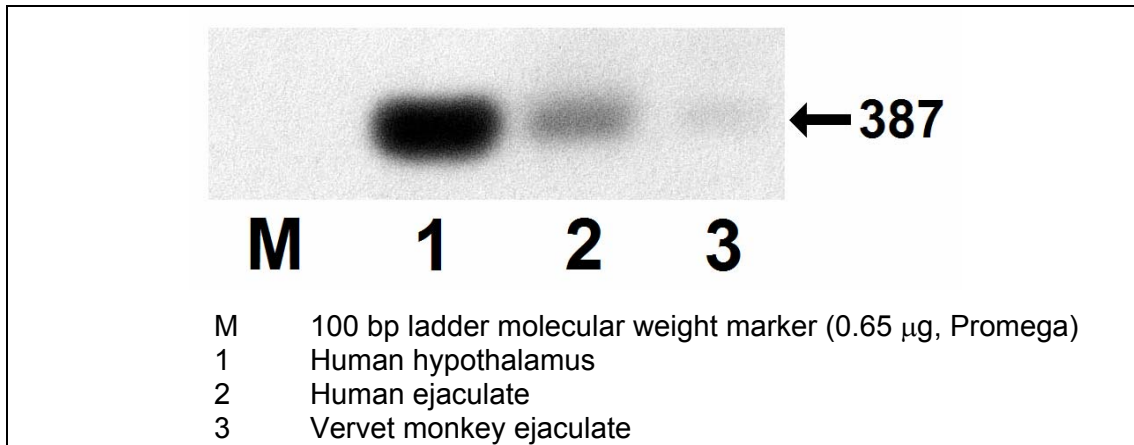


Fig 28. Autoradiogram of the Southern blot of the gel shown in figure 27: demonstration that the human hypothalamus and ejaculate 387 bp amplicons are *GnRH-1*-specific and indication of the presence of GnRH-1 transcripts in vervet monkey ejaculate. The blot was probed with exon 2-specific oligo H1S2. All four lanes, including the marker lane, were probed. The position and size of the appropriate amplicon are indicated to the right of the photographed autoradiogram.

Similarly, the distribution of GnRH-2 transcripts in human and vervet monkey ejaculate RNA was determined by RT-PCR using the primer pair H2S1 & H2AS2 (figure 25) that is expected to produce a processed amplicon of 389 bp, 368 bp or 365 bp from human RNA templates. In human ejaculate RNA, the presence of transcripts for GnRH-2 was revealed by the successful amplification of a 368 bp H2S1 & H2AS2 amplicon (figure 29, lane 1). Furthermore, specificity of this 368 bp product was confirmed by Southern blot analysis using an internally nested and exon 3-specific oligonucleotide, H2AS1 (figure 30, lane 1). Likewise, a GnRH-2 amplicon of the expected size was obtained in vervet monkey ejaculate RNA, as was faintly evident on a Southern blot (figure 30, lane 2) although not visible on an agarose gel (figure 29, lane 2).

The human ejaculate 368 bp GnRH-2 amplicon was cloned and sequenced (Sequence no 10 in Appendix 4). A consensus sequence was derived from three clones, which correlated with the sequence of the published human GnRH-2 variant 3 mRNA (Accession NM_178331). This variant utilises an alternative in-frame splice site compared to the longest variant, variant 1, so that a stretch of 21 nt is excluded from the mRNA. This missing region contains the first 21 nt of exon 3 (see

Sequence no 10 in Appendix 4 for a comparison of the cloned human ejaculate GnRH-2 cDNA sequence with the published GnRH-2 variant 1 sequence (Accession NM_001501)). The missing 21 nt correspond to the last two amino acids of the Pro-Gly-Arg (PGR) processing site of the GnRH-2 preprohormone as well as the first five amino acids of GAP (see figure 1 in Chapter 1 & Sequence no 10 in Appendix 4). The vervet monkey ejaculate GnRH-2 amplicon could not be cloned and subsequently sequenced due to its low yield.

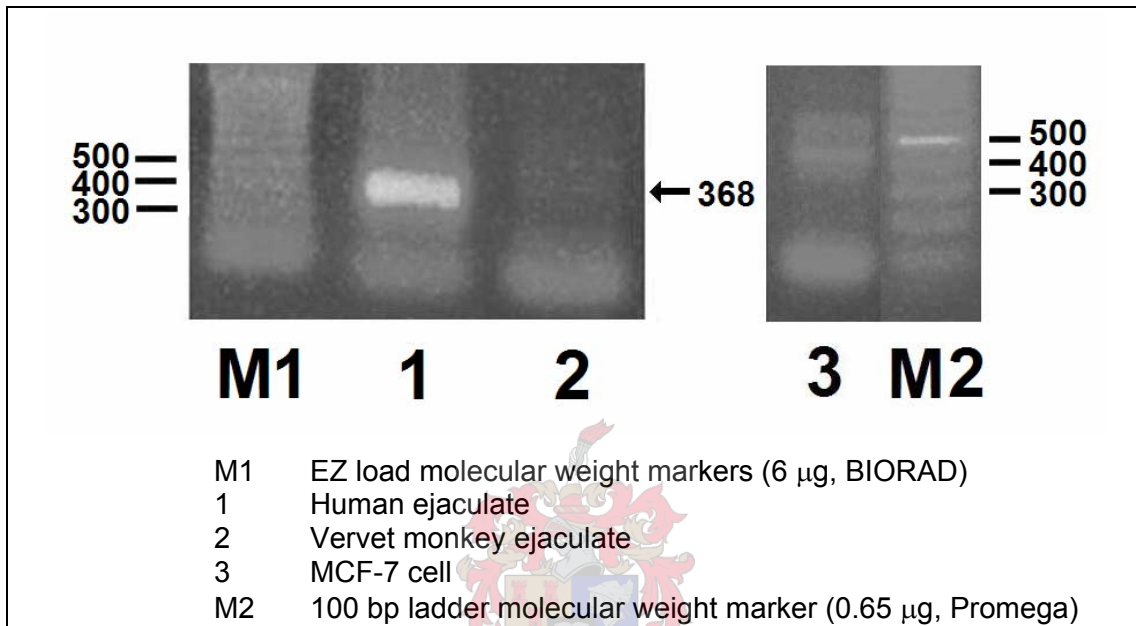


Fig 29. Agarose gel visualisation of RT-PCR results on human and monkey ejaculate RNA: attempts to determine whether transcripts for GnRH-2 are expressed. RNA isolated from MCF-7 human breast cancer cells was included as negative control. Primer pair H2S1 & H2AS2, for the amplification of a 368 bp or 350 bp intronless amplicon from human and vervet monkey, respectively, was utilised in the RT-PCRs. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the position and size of the appropriate amplicon is indicated to the right of the photographed gel picture.

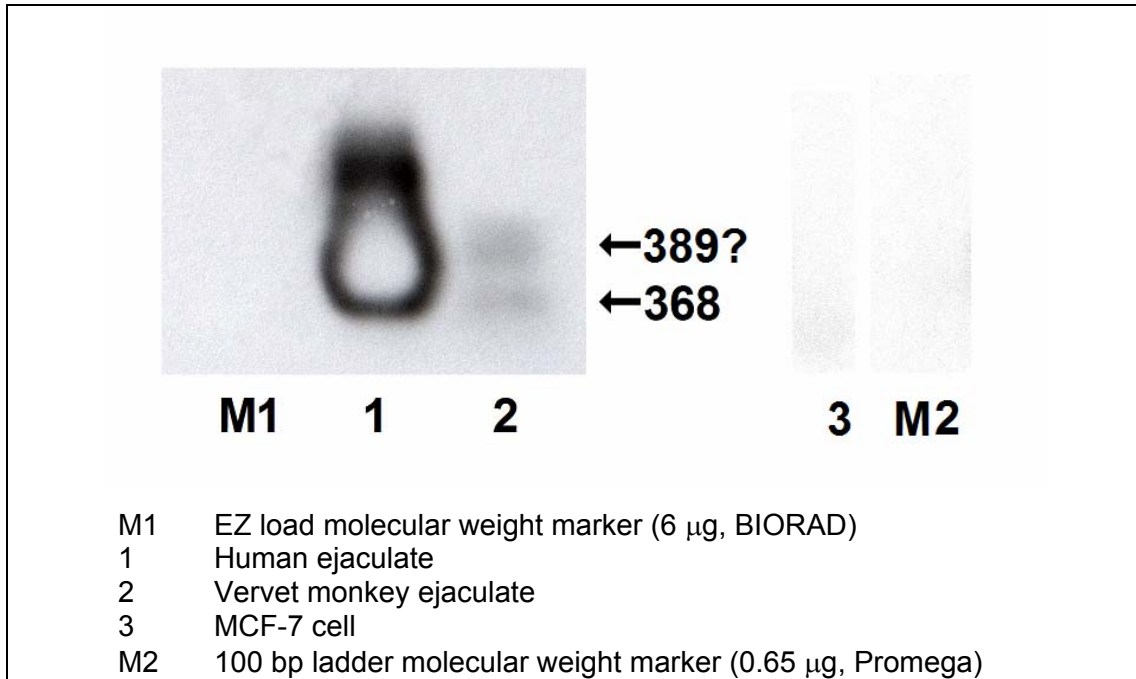


Fig 30. Autoradiogram of the Southern blot of the gel shown in figure 29: demonstration that the human ejaculate 368 bp amplicon is *GnRH-2*-specific and indication of the presence of GnRH-2 amplicons in vervet monkey ejaculate. The blot was probed with exon 3-specific oligo H2AS1. All three lanes, including the marker lane, were probed. The position and size of the appropriate amplicon is indicated to the right of the photographed autoradiogram. The “bubble”-like appearance in figure 30, lane 1 is possibly the result of a trapped air bubble when the membrane was wrapped in cling film.

Distribution of transcripts for GnRHR-I and GnRHR-II in human and vervet monkey ejaculate RNA

Three different sets of primers were used to determine the distribution of GnRHR-I transcripts in human and vervet monkey ejaculate RNA, namely R1S2 & R1AS1; R1S1 & R1AS2 and R1S0 & R1AS2 (figure 26). The expected sizes of GnRHR-I amplicons using these primer pairs are 396 bp (R1S2-R1AS1, exon 1-2), 909 bp (R1S1-R1AS2, exons 1-3) and 948 bp (R1S0-R1AS2, exon 1-3) if the mRNA is fully processed. RNA isolated from human pituitary tissue served as a positive control for the GnRHR-I RT-PCR. Photographs of the agarose gels containing results of the R1S2 & R1AS1 (396 bp) and R1S1 & R1AS2 (909 bp) RT-PCRs are shown. As expected, the appropriate size amplicons were obtained from human pituitary RNA (figure 31, A & B, lane 1) and confirmed to be *GnRHR-I*-specific by Southern blot

analysis using internally nested exon 1-specific oligo R1S3 (figure 32, A & B, lane 1). In human ejaculate RNA, the presence of transcripts for the GnRHR-I was revealed by the successful amplification of the 396 bp exon 1-2 amplicon using primer pair R1S2 & R1AS1 (figure 31, A, lane 2), which was confirmed to be *GnRHR-I*-specific by Southern blot analysis (figure 32, A, lane 2) and by sequencing (Sequence no 11 in Appendix 4). Surprisingly, none of the other two *GnRHR-I*-specific primer pairs designed to amplify an almost full-length transcript produced bands of the expected size in human ejaculate RNA as visualised on an agarose gel (figure 31, B, lane 2 and results not shown). However, an extremely faint exon 1-3 R1S1 & R1AS2 band (909 bp) was visible on the Southern blot at the expected position (figure 32, B, lane 2). Cloning of this larger human ejaculate product was not pursued due to low abundance (it could not be seen on the agarose gel). In monkey ejaculate RNA an RT-PCR product of the expected size (396 bp) using the exon 1-2 *GnRHR-I*-specific primer pair R1S2 & R1AS1 could not be seen on an agarose gel (figure 31, A, lane 3). However, an extremely faint band was visible on the Southern blot at the expected position (figure 32, A, lane 3). Sequence analysis revealed that this amplicon did not contain *GnRHR-I* sequence (sequencing data not shown), which suggests that the GnRHR-I is not expressed in vervet monkey ejaculate. However, it could be that the primers utilised are not homologous enough to the vervet monkey *GnRHR-I*, since, although the sequences of both primer R1S2 and primer R1AS1 are identical to the published bonnet monkey pituitary GnRHR-I mRNA sequence (Accession AF156930), they may differ from the (as yet unpublished) vervet monkey *GnRHR-I* sequence. None of the other *GnRHR-I* primer combinations produced RT-PCR products that were visible on an agarose gel or on a Southern blot from vervet monkey ejaculate RNA (for example, see figures 31 & 32, B, lane 3). Interestingly, no product was obtained from L β T2 mouse pituitary gonadotrope RNA using primer pair R1S1 & R1AS2 (figures 31 & 32, B, lane 4). The inability to amplify GnRHR-I fragments from RNA isolated from these cells strengthens the case for a compatibility problem of the human primers across other species, since it is well known that these cells do express the mouse *GnRHR-I* [Thomas P *et al.*, 1996; Turgeon JL *et al.*, 1996; Alarid ET *et al.*, 1996].

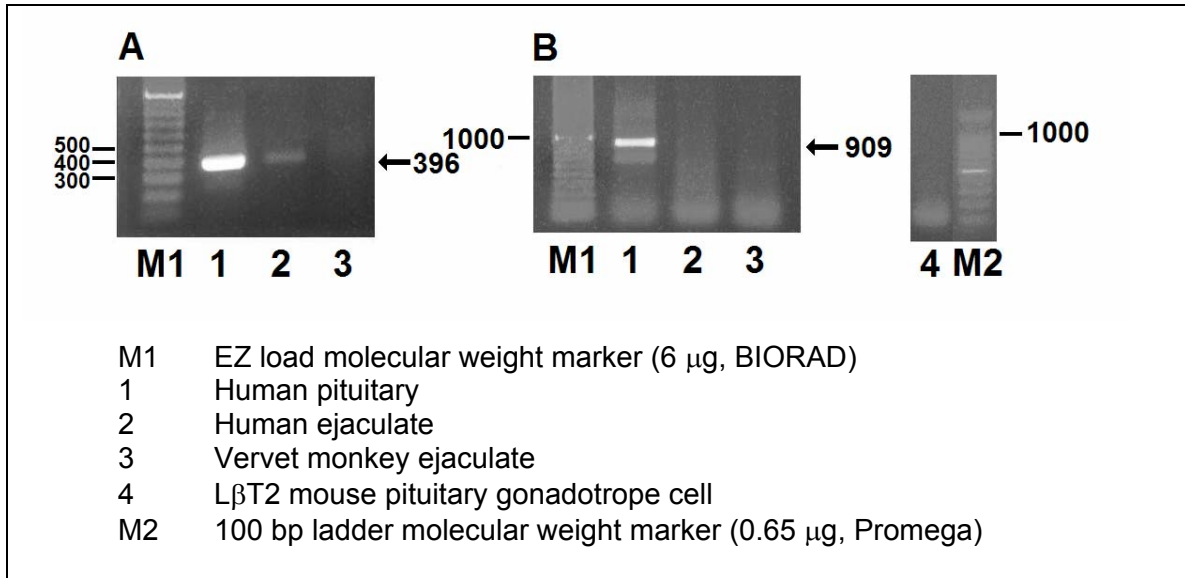


Fig 31. Agarose gel visualisation of RT-PCR results on human and vervet monkey ejaculate RNA: attempts to determine whether transcripts for GnRHR-I are expressed. Human pituitary RNA was included as positive control. Primer pairs R1S2 & R1AS1 (A) and R1S1 & R1AS2 (B), for the amplification of a 396 bp exon 1-2 or 909 bp exon 1-2-3 intronless amplicon, respectively, were utilised in the RT-PCRs. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the position and size of the appropriate amplicon is indicated to the right of the photographed gel picture.

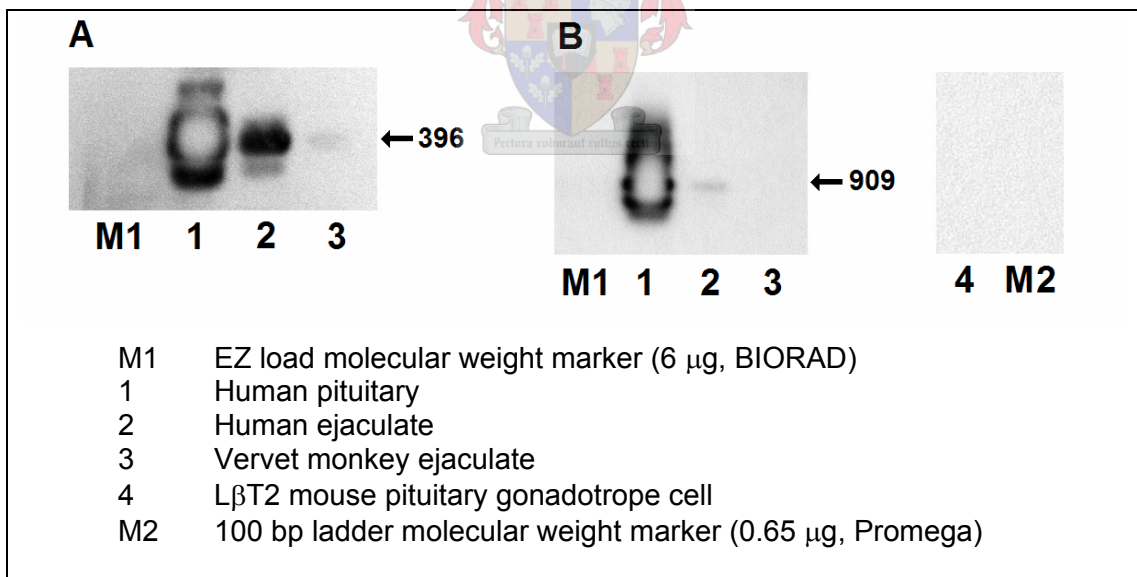


Fig 32. Autoradiogram of the Southern blot of the gel shown in figure 31: demonstration of the specificity of the GnRHR-I RT-PCRs. The blots were probed with exon 1-specific oligo R1S3. All four lanes of both blots, including the marker lanes, were probed. The positions and sizes of the appropriate amplicons are indicated to the right of the photographed autoradiograms. The "bubble"-like appearance in figure 32, A, lane 1 is possibly the result of a trapped air bubble when the membrane was wrapped in cling film.

An extensive study of the type and distribution of GnRHR-II transcripts in human and vervet monkey ejaculate RNA was performed and the results are discussed in full in Chapter 2 of this thesis. However, some of the GnRHR-II RT-PCRs were repeated on the ejaculate RNAs in order to include them in a single study with GnRH-1, GnRH-2 and the GnRHR-I altogether, and the results obtained are briefly summarised here. One of two GnRHR-II primer sets were used, namely S5 & AS10 (542 bp exon 1-3, ECL1 to TM6) (human and vervet monkey ejaculate RNA) and/or S3 & AS13 (1262 bp exon 1-3, 5' UTR to 3' UTR) (vervet monkey ejaculate RNA) (figure 26). In addition, parallel amplification using primer pair S5 & AS10 in RT-PCR on COS-1 cell RNA served as a positive control. At the time when the initial study of the expression of GnRHR-II transcripts in human and vervet monkey ejaculate was performed (Chapter 2), a monkey GnRHR-II cDNA sequence was not yet published. However, in the mean time, GnRHR-II cDNAs were cloned from several monkey RNA sources, including from COS-1 cells and from rhesus and marmoset monkey brain and pituitary [Neill JD *et al.*, 2001; Millar R *et al.*, 2001]. As a result, when the study of the distribution of GnRH and GnRHR transcripts in human and vervet monkey ejaculate was performed and some of the GnRHR-II RT-PCRs were repeated (results presented in this Chapter), RNA isolated from COS-1 cells could serve as a positive control (figures 33 & 34, lane 1). The expected size of a fully processed exon 2-3 S5-AS10 amplicon is 542 bp, whereas the expected size of a fully processed vervet monkey S3-AS13 amplicon, which would contain the full coding region plus parts of the 5' and 3' UTRs, is 1262 bp. In human ejaculate RNA the presence of a fully processed 542 bp S5-AS10 GnRHR-II amplicon was shown (figure 33, lane 2). The specificity of this 542 bp amplicon was confirmed by Southern blot analysis with an internally nested exon 2-specific oligo S9 (figure 34, lane 2). Sequence analysis of this human ejaculate GnRHR-II transcript revealed a single nt deletion within exon 1 as well as an in-frame translation stop signal within exon 2, and thereby questioning the functionality of the human transcript (Sequence no 1 in Appendix 4) (refer to Chapter 2 for a detailed discussion). In vervet monkey ejaculate RNA the S5 & AS10 primer combination resulted in a band of the expected size (542 bp) that was faintly visible on an agarose gel (figure 33 lane 3) but clearly visible on the Southern blot (figure 34, lane 3). Surprisingly, subsequent sequencing of this vervet monkey ejaculate 542 bp product revealed that it did not contain *GnRHR-II*-specific sequence. The failure to obtain a vervet monkey ejaculate S5-AS10 sequence could however be explained by the faintness of the vervet monkey amplicon on the agarose gel (figure 33, lane 3). Background smearing is visible in the same lane (figure 33, lane 3), which could have hindered the purification of the

542 bp product from the agarose gel. Nevertheless, a *GnRHR-II*-specific product of 1729 bp was amplified from vervet monkey ejaculate RNA with the use of primer pair S3 & AS13 (not shown). Sequence analysis of this 1729 bp amplicon revealed that an additional 447 nt, possibly retained intronic sequence, is inserted between exons 2 and 3 (Sequence no 5 in Appendix 4). These extra nucleotides do not change the reading frame but contain a number of in-frame premature translation stop signals (refer to Chapter 2 for a more detailed discussion). Furthermore, larger size products (~1000 bp), possibly containing additional insert sequence between two exons, were obtained with S5 & AS10 apart from the expected 542 bp amplicon, as was evident on the agarose gel (figure 33, lane 1) and on a Southern blot (figure 34, lanes 1 & 3).



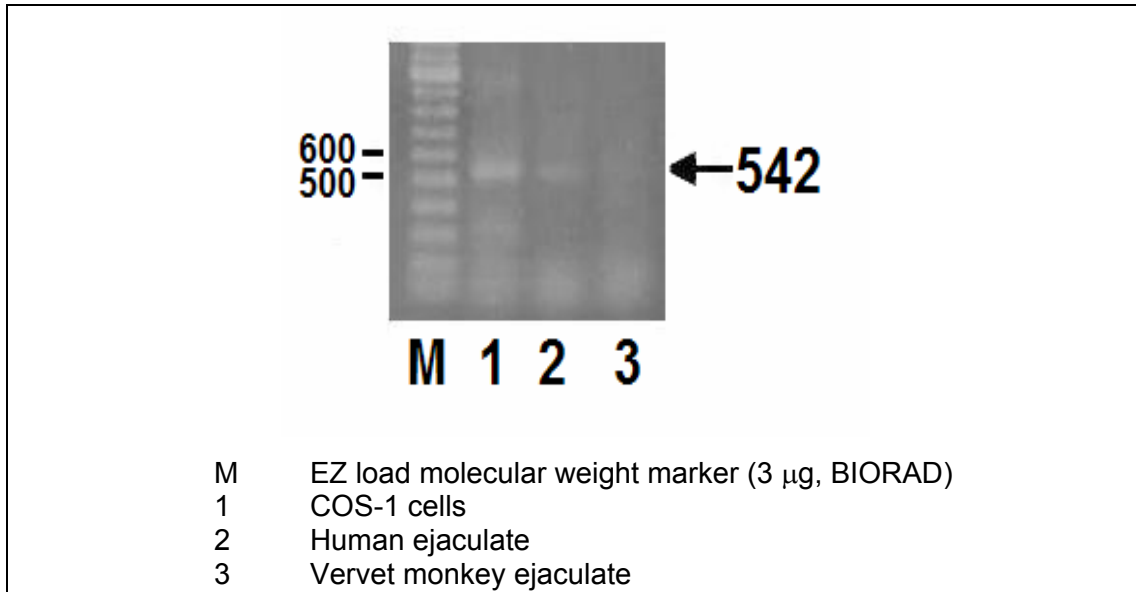


Fig 33. Agarose gel visualisation of RT-PCR results on human and vervet monkey ejaculate RNA: attempts to detect the expression of transcripts for GnRHR-II within a single experiment together with GnRH-1, GnRH-2 and the GnRHR-I. COS-1 RNA was included as positive control. Primer pair S5 & AS10, for the amplification of an exon 1-2-3 542 bp intronless amplicon containing TMs 1 to 6, was utilised in the RT-PCRs. Some of the positions and sizes of the molecular weight markers are indicated to the left whereas the position and size of the appropriate amplicon as well as that of a larger amplicon are indicated to the right of the photographed gel picture.

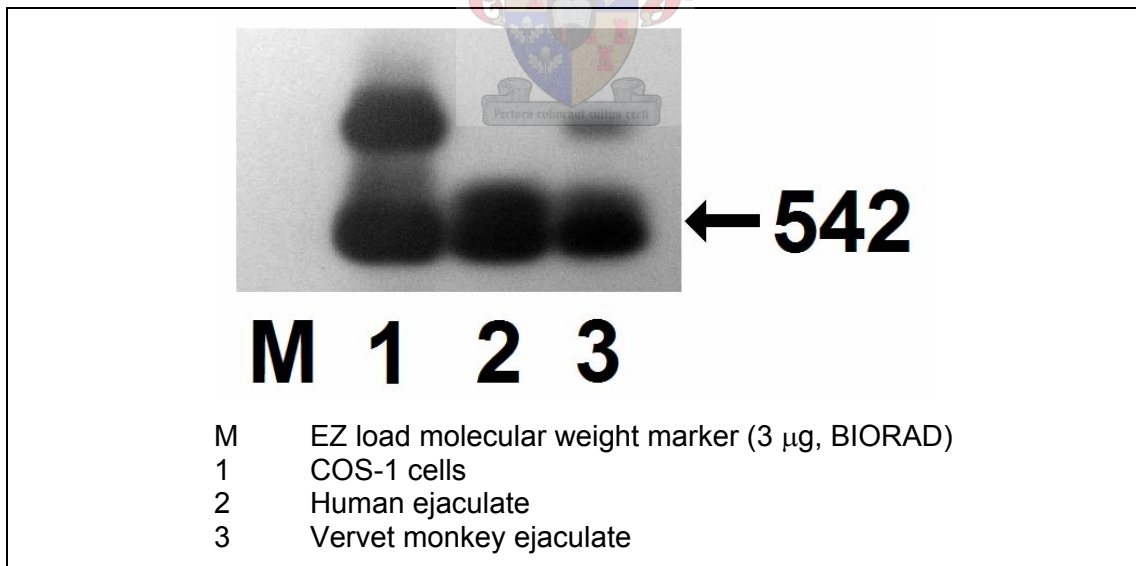


Fig 34. Autoradiogram of the Southern blot of the gel shown in figure 33: demonstration that the human and vervet monkey ejaculate exon 1-2-3 542 bp amplicons are *GnRHR-II*-specific. The blot was probed with exon 2-specific oligo S9. All four lanes, including the marker lane, were probed. The position and size of the appropriate amplicon as well as that of a larger amplicon are indicated to the right of the photographed autoradiogram.

Discussion

To the best knowledge of the author, this thesis reports on the first investigation into the distribution of transcripts for GnRH-1, GnRH-2, the GnRHR-I and the GnRHR-II in human and vervet monkey ejaculate.

Expression of GnRH-1 and GnRH-2 in human and vervet monkey ejaculate

It was shown by RT-PCR and sequencing that human ejaculate expresses transcripts for GnRH-1. Although the full-length cDNA was not cloned, an amplicon was obtained from human ejaculate RNA that included sequence stretching from the signal peptide (exon 2) until the 3' UTR (exon 4), also containing the coding sequence of the mature peptide, QHWSYGLRPG. A single nt difference as compared to the published human cDNA sequence (Accession X15215) was found in the human ejaculate GnRH-1 amplicon within the signal peptide sequence. This same nt difference, which would result in the incorporation of a Trp (W), with its bulky aromatic side-chain, instead of a polar uncharged Ser (S) residue upon translation, was also evident in the sequence obtained from the human hypothalamus positive control RNA (Sequence no 9 in Appendix 4), suggesting that it is not the result of PCR or sequencing error(s). Furthermore, two human ejaculate clones were sequenced and both contained the nt difference. Two other nt differences, compared to the published sequence, were found within the GAP region of the human ejaculate GnRH-1 amplicon. One of these would result in the incorporation of a different amino acid when the mRNA is translated, namely an uncharged Gly (G) instead of a negatively charged Glu (E), within the GAP sequence (Sequence no 9 in Appendix 4, at +170 relative to translation start). The presence of three nt differences compared to the published sequence, which would result in the incorporation of two alternative amino acids in the human ejaculate GnRH-1 preprohormone, is likely due to variations between individuals. Be that as it may, the incorporation of alternative amino acids in the GnRH-1 preprohormone does not affect the sequence of the mature decapeptide.

Human ejaculate was also shown to also express transcripts for GnRH-2. The obtained human ejaculate GnRH-2 amplicon was found to be 21 nt shorter in length compared to the sequence that had originally been deposited in GenBank (Accession NM_001501). Human hypothalamus was also shown to express the same shorter variant transcript. These results correspond to the findings of White RB *et al.* [1998] who, with the use of a different set of *GnRH-2*-specific primers, indicated the

existence of two transcripts, which differed in length by 21 nt, for GnRH-2 in humans. Furthermore, White RB *et al.* [1998] reported that both the shorter transcript and the longer variant that is extended at the 5'-end of exon 3 are expressed in human thalamus and foetal brain, but that human kidney only expresses the shorter GnRH-2 variant. This 5' deletion of exon 3 results in a reduction in the predicted length of GAP from 84- to 77 amino acids [White RB *et al.*, 1998]. The identity of the mature GnRH-2 peptide is however not affected. It later became evident that three transcript variants that encode unique preprohormones but the same mature peptide hormone exist for GnRH-2 (Accession NM_001501, NM_178332 and NM_178331, respectively). Of these, GnRH-2 variant 1 is the longest transcript and encodes the longest isoform "a", whereas variants 2 and 3 both utilise an alternative in-frame splice site compared to variant 1, resulting in shorter isoforms "b" and "c", respectively, compared to isoform "a". Splice variant 2 is 24 nt shorter and splice variant 3 is 21 nt shorter than splice variant 1. Interestingly, the reading frame is the same for all 3 splice variants (see figure 35 for a comparison of the mRNA sequences of the three GnRH-2 variants). The results presented here indicated that splice variant 3 is expressed in human ejaculate and hypothalamus. The human ejaculate and hypothalamic sequences obtained were aligned to the database sequence of the longest GnRH-2 variant, variant 1, to indicate the position of the alternative splice junction that are utilised in ejaculate and hypothalamus (Sequence no 10 in Appendix 4). The significance of the existence of three different GnRH-2 preprohormones is presently unknown. A possible explanation for their occurrence is the utilisation of different splice junctions in different tissues, due to the presence of tissue-specific factors. In other words, there are three different ways in which exon 2 can be spliced onto exon 3 (figure 35). Statistically, all three GnRH-2 variants should be expressed at near-equal frequencies (figure 35). It is however difficult to distinguish between amplicons that differ in only 21- or 24 bp from one another on an agarose gel. From the Southern blot result showing a second band at a higher position (figure 30, lane 1) it would appear that variant 1 is also present in human ejaculate, but subsequent sequence analysis revealed that, in this study, transcripts containing variants 1 and 2 were not obtained from human hypothalamus and ejaculate RNAs.



Fig 35. Comparison of the three GnRH-2 mRNA variants. Accession numbers and lengths of the transcripts are given. Nucleotides are numbered from the transcription start and are colour-coded to indicate the result of alternative splicing of the pre-mRNA. According to Padgett RA *et al.* [1986], vertebrate genes always have introns ending with "AG". The two alternative "AG" recognition sites that are utilised to create variants 2 and 3 are underlined in the variant 1 sequence.

There are three different ways in which exon 2 can be spliced onto exon 3:

- (1) splicing of "CAG" onto "GAA" to create variant 1; or
- (2) splicing of "CAG" onto "GCA" to create variant 2; or
- (3) splicing of "CAG" onto "CAG" to create variant 3.

The identities of the splice acceptor sites that are utilised are thus GA, GC or CA. Fifty-five per cent (55%) of all vertebrate splice acceptors contain a "G" in the first position whereas 17% contain a "C". Twenty one per cent (21%) of all vertebrate splice acceptors contain an "A" in the second position whereas 20% contain a "C" [Padgett RA *et al.*, 1986]. Thus, statistically, one would expect that the frequency of occurrence of the above-mentioned splice acceptor sites would be

GA: 20% X 21% = 4.2%

GC: 20% X 20% = 4.0%

CA: 17% X 21% = 3.6%

Although it could not be confirmed by sequencing analysis that GnRH-1 transcripts are present in vervet monkey ejaculate, the presence of a signal at the expected position on a Southern blot suggests that *GnRH-1* is also expressed in monkey ejaculate. One possible explanation for the inability to detect GnRH-1 amplicons on an agarose gel for subsequent cloning and sequencing analyses in this study may be a low efficiency PCR reaction with the human-specific GnRH-1 primers. Alternatively, another possibility could be that vervet monkey ejaculate, in contrast to human ejaculate, does not express *GnRH-1*. Likewise, a signal was detected in vervet monkey ejaculate RNA for GnRH-2 at the expected position on a Southern blot but amplified GnRH-2 cDNA could not be cloned and sequenced due to a very low yield. The vervet monkey *GnRH-2* gene is unpublished and therefore the percentage homology to the vervet monkey *GnRH-2* gene of the primers utilised could not be calculated.

Expression of GnRHR-I and GnRHR-II in human and vervet monkey ejaculate

Interestingly, it was found that human ejaculate expresses part of the *GnRHR-I* gene, as indicated by the successful amplification of a 396 bp exon 1-2 amplicon using primer pair R1S2 & R1AS1 as well as Southern blot confirmation. Sequence analysis of the human ejaculate exon 1-2 transcript revealed that the human ejaculate GnRHR-I transcript is identical to the pituitary GnRHR-I transcript within the amplified region, as well as to the published human GnRHR-I cDNA sequence (Accession L07949). This corresponds to findings that the full-length GnRHR-I cDNAs cloned from placenta, ovary, endometrium and breast are identical to that of pituitary [Cheng CK & Leung PC, 2005]. With the use of primer pair R1S1 & R1AS2 designed to amplify 909 bp of the 986 bp GnRHR-I coding region (spanning most of exons 1 to 3), no amplification products were obtained as evidenced by the fact that no bands could be seen on the agarose gel, from both human and vervet monkey ejaculate RNA. This, together with the failure to clone and sequence a GnRHR-I exon 1-2 amplicon from vervet monkey ejaculate RNA suggests that the *GnRHR-I* gene is not expressed in vervet monkey ejaculate. However, the possibility that the use of human-specific primers precluded the detection of the transcripts cannot be excluded. Nevertheless, it is likely that a full-length GnRHR-I transcript is produced in human ejaculate because a Southern blot of the R1S1 & R1AS2 RT-PCR produced a band at the correct position, albeit very faint. Moreover, parallel RT-PCRs performed on RNA isolated from MCF-7 human breast cancer cells produced similar results i.e. the presence of an exon 1-2 GnRHR-I amplicon with primer pair R1S2 & R1S1 but failure to detect a longer GnRHR-I transcript using primer pair

R1S1 & R1AS2 (results not shown). This is interesting because others have indicated high-affinity binding sites for GnRH-1 in MCF-7 cells [Cheng CK & Leung PC, 2005], which would suggest the expression of a functional GnRHR-I in these cells. Also, the presence of GnRHR immunoreactivity and mRNA with sequence identical to the pituitary have been demonstrated in both normal and malignant breast tissue [Cheng CK & Leung PC, 2005].

A detailed discussion of results of the GnRHR-II RT-PCR on human and vervet monkey ejaculate RNAs is given in Chapter 2. In brief, transcripts for the GnRHR-II containing all three exons were found to be expressed in both human and vervet monkey ejaculate, but the functionality of these transcripts is unknown. In human ejaculate, a GnRHR-II transcript that contains a single nt deletion in exon 1 and a premature translation stop signal in exon 2 is expressed. Both the nt deletion and translation stop signal are also present in the putative human *GnRHR-II* gene on chromosome 1 (Accession AL160282). In vervet monkey ejaculate a GnRHR-II transcript is expressed that contains a 447 bp insertion between exons 2 and 3. This insertion is possibly the result of retained intronic sequence and it leads to the incorporation of a number of premature in-frame translation stop signals in the vervet monkey ejaculate GnRHR-II transcript. The physiological function of a vervet monkey ejaculate GnRHR-II transcript possessing a single retained intronic sequence is unclear. This could possibly be a mechanism for storage of a non-functional transcript, that could facilitate the subsequent rapid generation of a functional transcript when needed by the cell, requiring the removal of only this single intron. This may occur, for example, during a specific stage of development or during the process of fertilisation.

Taken together, the results presented here reveal that transcripts for GnRH-1, GnRH-2 and the GnRHR-I, in addition to transcripts for the GnRHR-II, are expressed in human ejaculate. Furthermore results of the Southern blot analyses would suggest a possibility that *GnRH-1*, *GnRH-2* and *GnRHR-I* are also expressed in vervet monkey ejaculate. The vervet monkey results are however inconclusive due to the utilisation of primers that are 100% homologous to the human but possibly not to the vervet monkey gene sequences. The relatively strong signal obtained in the RT-PCR using the *β-actin* primers (figure 24, lane 3) demonstrates that the RNA prepared from vervet monkey ejaculate is not significantly degraded. It would be useful to repeat these studies with the use of vervet monkey-specific primers,

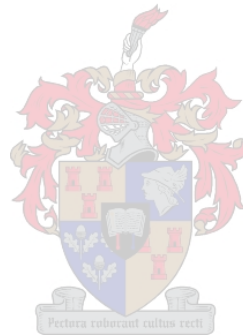
however, the sequences of the vervet monkey *GnRH-1*, *GnRH-2* and *GnRHR-I* genes or mRNAs have not been published yet.

Thus, it seems likely that a local GnRH/GnRHR network is present in human, and possibly in vervet monkey, ejaculate. This suggests paracrine/autocrine actions of GnRH within ejaculate, most likely in mature sperm, based on the results of *in situ* hybridisation analysis presented in Chapter 2 of this thesis. Given the functional evidence for a role for GnRH in mature sperm, it seems likely that such a local GnRH/GnRHR network would have the capacity to modify sperm-egg interactions and could therefore alter the probability of conception.

It is interesting to speculate what the possible mechanism could be whereby GnRH affects sperm-ZP binding. Factors that affect sperm-ZP binding may be present in the fluids that bathe the sperm or the egg before they meet and/or in the fallopian tube, where fertilisation takes place *in vivo*. Interestingly, mature sperm are not able to fertilise an egg upon ejaculation but have to first undergo a final stage of maturation when in contact with the secretions of the female genital tract [Morales P & Llanos M, 1996; Lawrence E, 1995]. Furthermore, mature sperm have an organelle at their apex, known as the acrosome, which contains hydrolytic enzymes to digest the ZP coating and thereby enabling sperm to penetrate the egg [Lawrence E, 1995; Burgos M & Fawcett D, 1955]. The acrosome reaction (AR) is the collective term for the release of hydrolytic enzymes from the acrosome of the sperm when they contact the egg [Lawrence E, 1995]. The AR is possibly induced by some components of the ZP [Cross NL *et al.*, 1988] but Ca^{2+} stores within the sperm may also play a role since the AR is a Ca^{2+} -dependent process [Morales P & Llanos M, 1996; Yanagimachi R, 1994]. GnRH signalling in sperm leads to a Ca^{2+} influx through T-type, voltage-operated channels and subsequent release from intracellular stores, resulting in an elevation of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in mature sperm [Morales P *et al.*, 2000; Stojilkovic SS *et al.*, 1994]. This indicates that the result of GnRH binding to its receptor on sperm cells is probably not the secretion of LH and FSH, but rather the release of Ca^{2+} as intracellular second messenger [Morales P *et al.*, 2000; Stojilkovic SS *et al.*, 1994]. Furthermore, IP_3 receptors are present in the sperm acrosome, as well as $\text{G}\alpha_{q/11}$ and phospholipase $\text{C}\beta 1$ [Morales P & Llanos M, 1996]. Similar to GnRH signalling in the pituitary, GnRHR activation in mature sperm could result in the activation of $\text{G}\alpha_{q/11}$, which could subsequently lead to the activation of phospholipase $\text{C}\beta 1$ and the generation of IP_3 and DAG [Morales P

& Llanos M, 1996]. It is possible that binding of IP_3 to IP_3 receptors localised in the outer acrosomal membrane could induce the release of acrosomal Ca^{2+} , and, subsequently, the sperm AR [Morales P & Llanos M, 1996]. The high $[Ca^{2+}]_i$ together with DAG production are required for molecular events leading to membrane fusion and finally for acrosomal exocytosis and fertilisation [Morales P & Llanos M, 1996]. Thus, it seems likely that mature sperm expresses a local GnRH/GnRHR system that functions to affect the sperm AR and to increase sperm-ZP binding, via elevation of $[Ca^{2+}]_i$.

Other local actions of GnRH in sperm would include a role in sperm development during spermatogenesis and spermiogenesis. Indeed, the results presented here would suggest that GnRH-1 and GnRH-2 as well as the GnRHR-I and/or the GnRHR-II are expressed in human ejaculate to form part of the intratesticular network of hormones that can function in an autocrine manner.



CHAPTER 4

**DIFFERENTIAL EXPRESSION OF THE BETA SUBUNITS OF
LUTEINISING HORMONE (LH)
&
FOLLICLE-STIMULATING HORMONE (FSH)
VIA THE GnRHR-I & THE GnRHR-II
IN L β T2 MOUSE PITUITARY GONADOTROPE
AND COS-1 MONKEY KIDNEY CELL-LINES**





Background

The pituitary gonadotropins, LH and FSH, are members of the glycoprotein hormone family. In mammals, LH regulates steroidogenesis in the gonads and induces ovulation in the ovary [Albanese C *et al.*, 1996]. FSH is required for initiation and regulation of spermatogenesis in the male and for ovulation and follicular development in the female [Baccetti B *et al.*, 1998]. Thus, the combination of LH and FSH plays an essential role in sexual differentiation and fertility [Albanese C *et al.*, 1996]. The glycoprotein hormones are composed of two subunits, namely a common or shared alpha (α)- and a hormone-specific beta (β)-subunit that together form a heterodimer [Albanese C *et al.*, 1996; Maurer RA *et al.*, 1999]. The α -subunit gene is expressed in gonadotropes and thyrotropes of the pituitary [Albanese C *et al.*, 1996]. By contrast, expression of the β genes is limited to gonadotropes, where LH and FSH are synthesised [Pierce JG & Parsons TF, 1981].

The synthesis and secretion of the gonadotropins are regulated by positive and negative factors that act at the brain, pituitary and gonad levels [Gharib SD *et al.*, 1990]. These include GnRH as well as a number of steroid hormones (progesterone, estradiol and testosterone) and gonadal peptides (activin, inhibin and follistatin). The different types of steroid hormones exert different regulatory effects on gonadotropin secretion and subunit synthesis at the pre-translational level [Gharib SD *et al.*, 1990]. They may act directly at the pituitary level or indirectly at the hypothalamus to alter GnRH pulses, and they can have positive or negative actions [Shupnik MA, 1996]. The gonadal peptides regulate the secretion of gonadotropins from the pituitary. Activin and inhibin have stimulatory and inhibitory effects, respectively, on FSH secretion [Gharib SD *et al.*, 1990]. Furthermore, activin stimulates $LH\beta$ transcription [Otsuka F & Shimasaki S, 2002] and enhances GnRH-induced LH secretion [Nicol L *et al.*, 2004]. Follistatin has the capacity to suppress FSH secretion from the pituitary without affecting LH secretion [Gharib SD *et al.*, 1990; De Kretser DM *et al.*, 2002]. GnRH serves as a principal mediator of neuroendocrine control of reproductive function [Ando H *et al.*, 2001]. GnRH is released from the hypothalamus in a pulsatile fashion to stimulate parallel pulsatile release of LH and FSH [Gharib SD *et al.*, 1990]. Moreover, GnRH desensitises the pituitary gonadotropes unless it is presented in a pulsatile fashion. Continuous exposure to long-acting GnRH analogs or exposure to supra-physiologic concentrations of GnRH causes down-regulation of the GnRHRs on the pituitary cells and a decrease in *GnRHR-I* gene expression,

leading to a profound decrease in the secretion of LH and FSH [Belchetz PE *et al.*, 1978; Shupnik MA & Fallest PC, 1994; Chedrese PJ *et al.*, 1994; Schally AV *et al.*, 1995; Pinski J *et al.*, 1996; Halmos G *et al.*, 1996].

In vivo, the frequency and amplitude of the GnRH pulses vary physiologically as a function of hormonal status and stage of the reproductive cycle [Crowley Jr WF *et al.*, 1985]. These variations in GnRH pulse pattern are associated with differential LH and FSH release. Intriguingly, whereas LH pulses always coincide with, or follow, GnRH pulses, both GnRH-associated and non-GnRH-associated pulses of FSH exist [Padmanabhan V & McNeilly AS, 2001]. Thus, LH, but not FSH secretion, is absolutely dependent upon GnRH pulsatility [Padmanabhan V & McNeilly AS, 2001].

The existence of FSH pulses that are not associated with GnRH pulses would indicate the presence of a selective FSH-releasing factor that is released from the hypothalamus and may stimulate FSH in the absence of LH [Padmanabhan V *et al.*, 2002]. Such a factor must be capable of selectively stimulating FSH over LH or at least be a more potent releaser of FSH than of LH to be categorised as an FSH-releasing factor [Padmanabhan V & McNeilly AS, 2001]. In this regard, the finding of GnRH-2 in the hypothalamus and identification of a second GnRHR (the GnRHR-II) in the pituitary open up the possibility of GnRH-2 being the FSH-releasing factor [Padmanabhan V *et al.*, 2002]. Whereas there are some indications that GnRH-2 is indeed a selective regulator of FSH synthesis and release from pituitary gonadotropes, this issue is still controversial [Millar RP, 2003]. For example, at the level of gonadotropin hormone release, several studies have determined that GnRH-2 can promote LH and FSH secretion, but it does so with a much lower potency than GnRH-1 [Hasegawa Y *et al.*, 1984; Millar RP & King JA, 1983; Millar RP *et al.*, 1986; Millar R *et al.*, 2001; Neill JD, 2002a; Okada Y *et al.*, 2003]. Even so, maximum LH and FSH secretion with GnRH-1 and GnRH-2 is similar at high doses of hormone [Montaner AD *et al.*, 2001; Okada Y *et al.*, 2003]. However, an *in vivo* study in rhesus monkeys demonstrated similar plasma LH and FSH concentrations after intravenous injections with either GnRH-1 or GnRH-2 [Densmore VS & Urbanski HF, 2003]. Similarly, in rat pituitary cells GnRH-2 was able to induce LH and FSH release in a GnRH-1-like manner [Montaner AD *et al.*, 2001; Mongiat LA *et al.*, 2004]. Taken together, data from the literature would suggest that GnRH-2 could stimulate LH and FSH release *in vivo* and *in vitro*, and that its efficacy to do so is lower than, or equals, that of GnRH-1.

However, it is unclear whether the gonadotropin-releasing abilities of GnRH-2 have physiological relevance [Densmore VS & Urbanski HF, 2003]. Indications are that the role of GnRH-2 in the differential secretion of FSH (and LH) is only evident when comparing FSH to LH ratios [Millar RP *et al.*, 1986; Millar R *et al.*, 2001]. In rams, for example, the ratio of FSH to LH secretion was higher following intravenous injection with GnRH-2 than with GnRH-1 although GnRH-2 was a less effective stimulator of LH and FSH secretion as compared to GnRH-1 [Millar R *et al.*, 2001]. Furthermore, in early studies of pituitary cells from mature hens, GnRH-2 was found to have a 2-fold greater potency to release FSH vs. LH when compared to stimulation by chicken GnRH-1 [Millar RP *et al.*, 1986]. With rat hemipituitaries on the other hand, GnRH-2 was no more potent than GnRH-1 as a stimulator of FSH when compared to LH [Yu WH *et al.*, 1997].

Another peptide hormone that has been indicated to play a role in the regulation of secretion of LH and FSH, mainly by synergising with GnRH-1, is pituitary adenylate cyclase-activating polypeptide (PACAP) [Rawlings SR & Hezareh M, 1996]. PACAP is a ubiquitously expressed neuropeptide that was originally isolated from sheep hypothalamus and was named for its ability to stimulate cAMP production in rat anterior pituitary cells [Kimura C *et al.*, 1990]. The major form of PACAP is a C-terminal amidated 38-amino acid polypeptide, but a shorter form, PACAP27, corresponding to the N-terminal 27 residues of PACAP38, is also found in the hypothalamus [Arimura A *et al.*, 1991]. PACAP regulates the secretion of GnRH and sensitises the pituitary for the release of the gonadotropin hormones through changes in pituitary GnRHR levels, either by directly activating the *GnRHR* promoter [Cheng KW & Leung PC, 2001; Ngan ES *et al.*, 2001; Pincas H *et al.*, 2001] or through modulation of the follistatin/activin system [Norwitz ER *et al.*, 2002]. While some evidence exists for a role for PACAP in transcriptional regulation of the gonadotropin subunit genes, either alone or by modulating the effects of GnRH-1 (see Discussion of this chapter), no data is available for the combined effects of PACAP and GnRH-2.

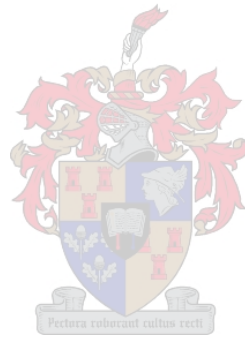
All of the studies on the ability of GnRH-2 to regulate the gonadotropins performed thus far have focused on protein level, by determining its ability to stimulate LH and/or FSH release. Although there are numerous reports in the literature on the transcriptional regulation of the gonadotropin α -, $LH\beta$ - and $FSH\beta$ -subunit genes by GnRH-1 or GnRH-1 analogues, none has described the role of GnRH-2 in this regard. Moreover, the relative abilities of GnRH-1 and GnRH-2 to regulate

gonadotropin subunit gene transcription, either with transfected promoter reporter constructs or of endogenous mRNA, have not been compared prior to the present study. Furthermore, there are no reports in the literature on the transcriptional regulation of the *LH β* or *FSH β* genes via GnRH-1 or GnRH-2 in the presence of expressed GnRHR-II *in vivo* or *in vitro*. While a role for GnRHR-II in gonadotropin regulation in mammalian pituitary cells has not been established, the literature suggests that, at least for marmoset [Millar R *et al.*, 2001] and rhesus monkey [Neill JD *et al.*, 2001], the GnRHR-II is expressed in some mammalian pituitary cells. Given that GnRH-1 is an agonist for GnRHR-II [Millar R *et al.*, 2001; Neill JD *et al.*, 2001], the potential thus exists for GnRHR-II to regulate gonadotropin gene expression in some mammalian pituitary cells.

Taken together, there seem to be a number of, as yet, unanswered questions regarding the differential regulation of *LH β* and *FSH β* -subunit gene transcription by GnRH-1, GnRH-2 and PACAP in mammalian pituitary gonadotropes. In the current study, L β T2 mouse pituitary gonadotrope cells were transiently transfected with mammalian *LH β* and *FSH β* -subunit promoter-reporter constructs and stimulated with GnRH-1 or GnRH-2 and/or PACAP. Co-transfections with exogenous mammalian GnRHR-I or GnRHR-II expression vectors were also performed. Since the α -subunit is in abundance and common to all glycoprotein hormones, and because synthesis of the *LH β* and *FSH β* -subunits is the limiting factor in LH and FSH synthesis respectively [Bhasin S & Swerdloff RS, 1995], the measurement of β -subunit promoter-reporter activity poses a suitable strategy to assess the transcriptional regulation of LH and FSH. Specifically, the focus of the study was to compare the capabilities of GnRH-1 and GnRH-2 to differentially regulate *LH β* and *FSH β* gene transcription in the presence of the GnRHR-I and the GnRHR-II. In addition, the role of GnRH concentration and method of administration as well as PACAP in the regulation of β -subunit gene transcription was investigated. Understanding the molecular control of gonadotropin biosynthesis is relevant both for the clinical treatment of a variety of reproductive disorders and for advancing the basic knowledge of regulation of gene expression.

The L β T2 cell-line seems well suited to study the regulation of these transfected promoters because they express the α -, *LH β* and *FSH β* -subunit genes endogenously in addition to the *GnRHR-I* and are thus representative of mature

pituitary gonadotropes [Thomas P *et al.*, 1996; Turgeon JL *et al.*, 1996; Alarid ET *et al.*, 1996].

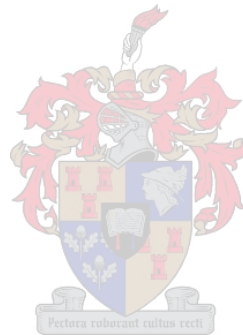


Aim

To investigate the capabilities of GnRH-1 and GnRH-2 to differentially regulate transcription of the *LH β* and *FSH β* -subunit genes, via binding to the mammalian GnRHR-I or GnRHR-II. In particular, the focus of this study was to

- compare the ability of GnRH-1 vs. GnRH-2 to stimulate *LH β* and *FSH β* promoter-reporter activity, via both GnRHR-I and GnRHR-II;
- compare the ability of a single GnRH ligand (GnRH-1 or GnRH-2, respectively) to induce *LH β* vs. *FSH β* promoter-reporter activity (to, accordingly, determine whether GnRH-1 and/or GnRH-2 is a selective stimulator of LH and/or FSH); and, in addition
- investigate the synergistic effect of PACAP on GnRH-1- and GnRH-2-mediated gonadotropin gene expression,

in a cell-line, L β T2, that expresses the GnRHR-I endogenously (but not the GnRHR-II,) as well as in a cell-line, COS-1, that expresses the GnRHR-II endogenously (but not the GnRHR-I).



Experimental

Cells

L β T2 mouse pituitary gonadotrope cells were gifts from Dr P Mellon (University of California, San Diego, CA) and were maintained in monolayer cultures in DMEM supplemented with 10% v/v FBS and PenStrep (1% v/v) in humidified 5% CO₂ at 37°C. COS-1 monkey kidney cells were kept in culture at 37°C in culture media (DMEM containing 1% v/v PenStrep and supplemented with 10% FBS), under 5% CO₂.

Reporter plasmids, expression vectors and vectors used for probe synthesis

All plasmid DNAs used for promoter-reporter studies were prepared from overnight bacterial cultures using QIAGEN DNA plasmid maxi columns according to the manufacturer's protocol (QIAGEN, Chatsworth, CA). Wild-type GnRHR-I expression vector (R10) consists of the full-length mouse GnRHR-I cDNA (1.2 kb) fused to the cytomegalovirus (CMV) promoter in pcDNA1 [Tsutsumi M *et al.*, 1992], and was obtained from Dr SC Sealfon (Mt Sinai Medical School, New York, USA). The marmoset GnRHR-II expression vector (pR-II), containing the full-length marmoset GnRHR-II cDNA (1465 bp) in pcDNA3.1+ [Millar R *et al.*, 2001], was kindly donated by Dr A Katz (Department of Medical Biochemistry, University of Cape Town, Cape Town, South Africa). Luciferase reporter plasmids of LH β and FSH β were generously provided by Dr John H Nilson (Department of Pharmacology, Case Western Reserve School of Medicine, Cleveland, Ohio) and Dr William L Miller (North Carolina State University, Raleigh, NC), respectively. The LH β luciferase reporter construct consists of 779 bp of the bovine LH β (*bLH β*) gene 5'-flank plus 10 bp 3' to the +1 transcription start site of the bovine LH β gene in the pGL2 vector which contains the coding sequence for *luciferase* (construction of this vector is described by Keri RA & Nilson JH [1996] and Quirk CC *et al.* [2001]). This vector was named bLH β Luc. The FSH β luciferase reporter construct contains a 5.5 kb region of the ovine FSH β (*oFSH β*) gene encompassing 4741 bp of the 5'-flanking region plus 759 bp downstream from the +1 transcription start site, which includes exon 1 (63 nt), intron 1 and 62 nt of exon 2 of the *oFSH β* gene, in pGL3 vector which contains the coding sequence for *luciferase* [Huang H *et al.*, 2001]. The β -galactosidase (β gal) reporter plasmid (pSV40 β gal) contained the coding region of β -galactosidase under control of the SV40 viral promoter.

All plasmid DNAs used for Northern blot analyses were prepared from overnight bacterial cultures using the Wizard® Plus SV Miniprep DNA purification system (Promega) according to the manufacturer's protocol. Plasmids containing the full-length cDNAs for mouse α -subunit, rat LH β or human β -actin or part of the coding sequence of the rat FSH β -subunit gene were used to generate DNA fragments used as probes in Northern blot analyses (table 8). Plasmid containing mouse α -subunit cDNA (640 bp) in pGEM3Zf⁺ [Chin WW *et al.*, 1981] was obtained from Dr DF Gordon (Division of Endocrinology, Metabolism and Diabetes, University of Colorado, Health Science Center, Denver Colorado, USA). Rat LH β -subunit cDNA (426 bp) in pGEM2 [Chin WW *et al.*, 1983] was kindly donated by Dr WW Chin (Eli Lilly and Company, Indianapolis, USA). Rat FSH β gDNA (1 kb) in pGEM2, of which part of segment 2 was excised for labeling, was constructed by Dr WW Chin and is described in [Gharib SD *et al.*, 1989]. Human fibroblast cytoplasmic β -actin cDNA (2.1 kb) in the Okayama-Berg expression vector, pSPT19, was from Prof MI Parker (Department of Medical Biochemistry, University of Cape Town, Cape Town, South Africa).

Reagent make-up

Luteinising hormone-releasing hormone (LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂, acetate salt) (GnRH-1) and LHRH II (Pyr-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂, trifluoroacetate salt) (GnRH-2) were purchased from Sigma-Aldrich and Bachem (Bubendorf, Switzerland), respectively, and prepared as stock solutions (5 mM) in water. Working stock solutions (100 μ M) were prepared by further dilution with water and kept in small volumes at -20°C until use. PACAP27 (Sigma-Aldrich) was prepared as a stock solution of 100 μ M in water, diluted to 20 μ M and 3 μ M working concentrations and stored at -20°C until use. For all inductions the compound working stock solution was diluted 1/1000 in DMEM containing 10% FBS, just before use. Control incubations in the absence of hormone were performed with a similar 1/1000 addition of water to induction media. Where inductions were performed with more than one compound simultaneously, i.e. GnRH-1 or GnRH-2 plus PACAP, each compound was diluted 1/1000 into induction media. Accordingly, control incubations were performed in the presence of media containing a 2/1000 addition of water.

Transient transfections

Twenty-four (24) h before transfection, 1×10^5 L β T2 or 5×10^4 COS-1 cells were plated per 15.5 mm well in 24-well plates, in 500 μ l culture media. Cells were transfected with the indicated DNAs using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's guidelines. The FuGENE 6:DNA ratio was 2:1 (2 μ l of FuGENE 6 reagent to 1 μ g of DNA). Luciferase promoter-reporter constructs (*bLH β Luc* or *oFSH β Luc*, 0.125 μ g/well) were co-transfected with GnRHR expression vectors (R10 or pR-II, 6 ng/well) or promoterless pGL2-basic vector (to keep the amount of transfected DNA constant) (6 ng/well) as indicated, as well as pSV40 β gal vector (25 ng/well) to correct for differences in transfection efficiencies between wells. In addition, control transfections using pGL2-basic vector DNA (0.156 μ g) only were performed to determine background luminescence of cells not transfected with a luciferase- or β gal reporter. Some earlier experiments were performed using Lipofectamine 2000 transfection reagent (GibcoBRL/Invitrogen) and not FuGENE 6. Where results of experiments using Lipofectamine 2000 are shown, it is indicated in the legend of the relevant figures in the Results section. Otherwise, all results are from transfections performed using FuGENE 6. Transfection with Lipofectamine 2000 was performed according to the manufacturer's guidelines, using double the amount of DNA specified above and a Lipofectamine 2000:DNA ratio of 2:1 (2 μ l of Lipofectamine 2000 reagent to 1 μ g of DNA).

Continuous and pulsatile treatment of cells with GnRH-1, GnRH-2 and PACAP

The FuGENE 6/DNA solution was replaced with complete medium (DMEM/10% v/v FBS) (500 μ l/well) containing the various treatments after 24 h. Each treatment was applied to triplicate cultures post-transfection. Treatments included GnRH-1 (1 nM, 10 nM or 100 nM), GnRH-2 (1 nM, 10 nM or 100 nM), PACAP (3 nM or 20 nM) or vehicle (water). Pulsatile GnRH treatment was performed as follows: GnRH-1 or GnRH-2 (10 nM) or vehicle in complete medium was added to cells for 15 min. After the 15 min pulse, medium containing the treatment was removed by aspiration and replaced with complete medium alone. For 1 pulse/30 min, medium was replaced with treatment medium 15 min later. For 1 pulse/2 h, medium was replaced with treatment medium after 1 h and 45 min. Cells were lysed 20 min after the last pulse. After incubation (6-, 12-, 18- or 24 h, as indicated), the cells were rinsed with cold phosphate-buffered saline (PBS) (500 μ l/well), air-dried for 5 min and lysed using 50 μ l 1 X reporter lysis buffer (Promega). The cells were then incubated for 15 min at rt with shaking and transferred to -20°C to undergo at least one freeze-thaw cycle

before collection of the lysates. Lysates were scraped from wells, transferred to Eppendorf tubes and centrifuged for 45 sec at 12200 X g to precipitate cell debris. Luciferase activity and β gal activity of the cell lysates were measured as described below.

Luciferase and β gal assays

For both luciferase and β gal assays, 10 μ l of cell lysate was pipetted into black 96-well plates. The luciferase activity was measured for 5 sec using a Luminoskan RS luminometer (Labsystems, Chicago, Illinois) after injection of prepared luciferase assay substrate (Luciferase assay system, Promega) (50 μ l/well). For the β gal assay, Tropix Galacto-StarTM β -galactosidase assay substrate (Applied Biosystems, Bedford, Massachusetts) (50 μ l/well) was injected and the β gal activity was counted for 1 sec after a 60 min incubation using the luminometer described above.

Normalisation and statistical analysis of the transient transfection data

Luciferase activity was first normalised to the level of the matching β gal activity to correct for variation in transfection efficiency between wells. This was done by dividing the luciferase value with the relevant value for β gal. The average luciferase to β gal (Luc/ β gal) ratio for control transfections (using pGL2-basic DNA only) was subtracted from all Luc/ β gal ratios to correct for background luminescence of L β T2 cells. This was not done with values derived from COS-1 cells since background luminescence in these cells were negligible. Thereafter each Luc/ β gal value was multiplied with a common factor so that, within a specific experiment, the average value for a specific treatment group (often, this was the no hormone control group with either endogenous GnRHR or overexpressed GnRHR-I) equalled 1. To calculate fold induction relative to expression in the presence of the control treatment of a specific luciferase reporter, results of the different GnRHR subtypes (endogenous, overexpressed GnRHR-I or overexpressed GnRHR-II) were analysed separately. Values for a specific GnRHR subtype were multiplied by a common factor so that the mean value for the water control group equalled 1.

Each experiment represents a pool of cells from a separate passage. The number of times an experiment was repeated (n) is indicated. Although experiments were repeated a number of times, data for each replicate experiment were analysed independently because of great variation between experiments in fold induction obtained. This variation in fold stimulation of *bLH β* and of *oFSH β* promoter-reporter

activity obtained is possibly due to differences in the status of the cells (growth stage or passage number) as well as in transfection efficiency and GnRHR expression levels between experiments. Therefore, typical results from individual experiments, rather than results from pooled experiments, are shown. Data are shown as the mean Luc/ β gal ratio \pm standard deviation (STDEV) and represent single experiments with each point run in triplicate. Differences between groups were determined by one-way ANOVA and Bonferroni's post-test, which compares all groups to each other, using the software package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). Differences were considered significant if $P \leq 0.05$ and groups were then assigned different letters of the alphabet (a, b, c etc.), such that if one group has the same letter as another, the two groups are not statistically significantly different to each other, while if two groups have different letters, then they are.

RNA preparation and Northern blot analysis

Northern blots were performed in two separate experiments, each performed in duplicate. L β T2 cells were plated at 2×10^6 cells per 100 mm Petri dish in 10 ml complete medium and grown for 2 days. Growth medium was then replaced by treatment media. Cells were challenged continuously with GnRH-1 (100 nM), GnRH-2 (100 nM) or PACAP (3 nM or 20 nM) alone or with a combination of GnRH-1 or GnRH-2 (100 nM) and PACAP (3 nM or 20 nM) in 10 ml complete medium. Control treatments were performed with vehicle (water). Treatment durations were 6- or 24 h. Another control, with cells lysed at the time of treatment (i.e. non-treated) was also included to compare mRNA levels at $t = 0$. After treatment, cells were washed once with 1 X PBS and air-dried. Total RNA was extracted by the TRI reagentTM (Sigma-Aldrich) procedure. One (1) ml of TRI reagentTM was added per dish. Isolated RNA was dissolved in 40 μ l FORMAZol[®] (Molecular Research Center, Inc., Cincinnati, OH) and stored at -20°C until use. The concentration of total RNA was determined spectrophotometrically at 260 nm. Total RNA (15 μ g in 6 μ l DEPC-treated water), together with appropriate RNA molecular weight markers (0.28–6.58 kb, Promega, 5 μ l), was separated in 1 X morpholinopropanesulfonic acid (MOPS) buffer pH 7.0 (0.04 M MOPS, 0.01 M Na-acetate, 0.001 M EDTA) on a 1% agarose gel containing formaldehyde (0.7 M) and 1 X MOPS. Duplicate treatments were run in adjacent lanes. For sample preparation, a loading buffer was prepared using 2.5 μ l 10 X MOPS, 4 μ l 37% formaldehyde and 12.5 μ l formamide per RNA sample. Of this, 15 μ l was added to each sample and the loading buffer/RNA mixture incubated

for 10 min at 65°C to denature secondary structures. Samples were placed on ice for 5 min before addition of 2.5 µl loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol). In addition, 0.5 µl EthBr (10 mg/ml) was added to the markers. After resolution of the RNA by electrophoresis, the marker lane was excised from the gel and photographed on a UV-light box. The gel containing the RNA samples was washed once in DEPC-treated water. RNA was transferred onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) by means of capillary transfer. The transfer was performed overnight in 20 X SSC pH 7.0 (3 M NaCl, 0.3 M Na₃-citrate). Complete transfer of the RNA was verified by staining the gel with EthBr (0.5 µg/ml). The membrane was washed in 2 X SSC pH 7.0 (0.3 M NaCl, 0.03 M Na₃-citrate) for 1 min at rt and air-dried. Thereafter RNA was covalently linked to the nylon membrane by UV irradiation in a UV crosslinker (Amersham Pharmacia Biotech) at 70000 µJ/cm² for 15 s. Membranes were wrapped in cling film and stored at 4°C. α-subunit, LHβ, FSHβ and β-actin mRNA levels were determined by Northern blot analysis as follows: ³²P-labelled DNA probes were generated by random priming from DNA fragments for α-, LHβ- and FSHβ-subunit as well as β-actin (see above). The fragments were prepared from the relevant vectors by restriction enzyme digestion (table 8) and purification from a 2% low melting point agarose gel using the Wizard® SV gel and PCR clean-up system (Promega) as described by the manufacturer. Sizes of the probes are indicated in table 8. Probes were labelled with α-³²P-dCTP (50 µCi/100 ng DNA) using the Fermentas DecaLabel™ DNA labelling kit (Inqaba Biotechnical Industries, Pretoria, South Africa) according to the manufacturer's specifications. One-hundred (100) ng of DNA was labelled per two 150 cm² membranes. Labelling was performed for 5 min at 37°C. Forty-five (45) µl TE buffer pH 8.0 (10 mM M Tris-HCl, 1 mM EDTA) was added to the labelled probe to increase the volume to 100 µl. Labelled probes were purified on a G-50 Sephadex spin column to remove unincorporated α-³²P-dCTP. The percentage incorporation of ³²P as well as specific activity of each probe were calculated (table 8). Membranes were pre-hybridised in DIG Easy Hyb hybridisation solution (Roche Molecular Biochemicals) (20 ml/150 cm² membrane) for at least 30 min at 50°C. Pre-hybridisation solution was replaced with fresh DIG Easy Hyb solution (15 ml/150 cm² membrane). Labelled probes were denatured by incubation in a heat block for 5 min at 95°C and chilled on ice for 5 min before addition to the membrane. A single labelling was divided in two and both membranes were hybridised simultaneously, each with half of the labelling reaction. Hybridisations were performed overnight at 50°C. Membranes were washed twice in

2 X SSC, 0.1% w/v SDS for a total of 5 min at rt. One or two additional washes in 0.1 X SSC, 0.1% w/v SDS were performed for 15 min at 50°C. Membranes were exposed in a Phospho Imager at the University of Cape Town (Cape Town, South Africa) (courtesy of Mr Dave Woolley) and analysed densitometrically using the software installed on the imager computer. Between hybridisations with the various probes, membranes were stripped by pouring boiling SDS (0.5% w/v) over membranes and shaking at rt for 2 h. Membranes were rinsed in 2 X SSC pH 7.0 for 1 min at rt and either stored at 4°C or subjected to the next hybridisation.

Table 8: Size, percentage incorporation of ^{32}P and specific activity of labelled DNA probes used in Northern blot analysis.

Plasmid name	Restriction enzyme used to generate fragment used for labelling	Probe identity	Size of labelled fragment	Percentage incorporation of ^{32}P (%)	Specific activity (cpm/ μg DNA)
α -pGEM3Zf ⁺	<i>Pst</i> I	Mouse α -subunit	460 bp	43.1	7.8×10^8
LH β -pGEM2	<i>Pst</i> I	Rat LH β -subunit	350 bp	48.7	2.3×10^8
FSH β -pGEM2	<i>A</i> l <i>w</i> M	Rat FSH β -subunit	202 bp	36.1	2.6×10^8
β -actin-pSPT19	<i>B</i> am <i>H</i> I	Human β -actin	1.9 kb	31.3	1.7×10^8

Normalisation and statistical analysis of Northern blot data

Northern blot analysis was performed twice with each point run in duplicate. Imager values obtained with Northern analysis (representing the number of counts) were divided by the values for β -actin mRNA in the same lane to correct for loading differences. The corrected values were normalised to the water control (average water control = 1) to obtain fold changes in endogenous mRNA levels. Data were analysed by one-way ANOVA followed by Dunnett's post-test to compare treatment groups to the control group with the use of the GraphPad Prism software package described above; $P \leq 0.05$ was considered significant. Data were also analysed using Bonferroni's post-test to compare all groups to one another. All given values are the mean \pm STDEV.

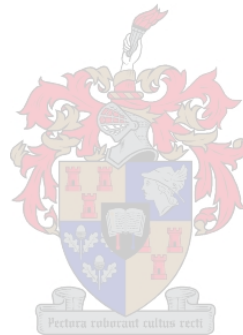
Homologous competition binding

For homologous competition binding analysis, 2×10^5 L β T2 cells were plated per 22 mm well in 12-well plates, in 1 ml complete medium. Cells were transfected as described above, but using double the amount of DNA per well to have the same concentration of DNA per cell and per media volume as for promoter-reporter assays. Twenty-four (24) h after transfection, transfection medium was replaced with 1 ml complete medium and cells incubated for another 24 h at 37°C prior to subsection to homologous competition binding analyses. Whole cell binding experiments were performed in the laboratory of Dr A Katz (Department of Medical Biochemistry, University of Cape Town, Cape Town, South Africa). Binding studies were performed in two separate experiments, each performed in duplicate. Labelled ^{125}I -[His⁵,D-Tyr⁶]GnRH (1350 $\mu\text{Ci}/\mu\text{g}$, MW 1604 [Flanagan CA *et al.*, 1998]) and unlabelled competitor peptide, [His⁵,D-Tyr⁶]GnRH, were kindly donated by Dr A Katz. Plates were placed on ice during the course of the binding experiment, to stabilise ^{125}I -[His⁵,D-Tyr⁶]GnRH. Cells were washed once with ice cold assay medium (DMEM buffered with 10 mM HEPES, pH 7.2) (500 $\mu\text{l}/\text{well}$). Displacement curves were generated by incubating cells at 4°C with ^{125}I -[His⁵,D-Tyr⁶]GnRH (binding experiment no 1, 1.0×10^5 cpm/well or 0.057 nM; binding experiment no 2, 4.7×10^5 cpm/well or 0.26 nM) and increasing concentrations of unlabelled competitor, [His⁵,D-Tyr⁶]GnRH, in assay medium (500 $\mu\text{l}/\text{well}$). The concentration of [His⁵,D-Tyr⁶]GnRH ranged from 10^{-11} M to 10^{-6} M. Non-specific binding was estimated in the presence of 10^{-6} M unlabelled competitor. Plates were incubated for 4.5- to 5 h. Thereafter, assay medium was aspirated and cells washed twice with ice cold PBS (500 $\mu\text{l}/\text{well}$). Cells were lysed by addition of ice cold NaOH (1 N, 500 $\mu\text{l}/\text{well}$) and cell lysates transferred to plastic tubes for determination of the amount of radioactivity.

Normalisation and statistical analysis of binding data

Homologous competition binding experiments were performed twice with each point run in duplicate. When analysing results of the binding experiments the assumption was made that the labelled (^{125}I -[His⁵,D-Tyr⁶]GnRH) and unlabelled ([His⁵,D-Tyr⁶]GnRH) ligands have similar affinities at the expressed GnRHRs. Previous studies showed that the affinities of iodinated GnRH analogs were the same as those of equivalent unlabelled peptides in rat pituitary membranes [Clayton RN *et al.*, 1979, Perrin MH *et al.*, 1983]. However, Flanagan CA *et al.* [1998] have established that ^{125}I -[His⁵,D-Tyr⁶]GnRH has a higher affinity as compared to [His⁵,D-Tyr⁶]GnRH at the human GnRHR-I, but the difference is only ~2-fold. For analysis of binding data,

non-linear curve fitting was performed using a one-site homologous competitive binding equation {Total binding (Y) = (Bmax X [labelled])/([labelled] + [unlabelled] + K_d) + Non-specific binding} (GraphPad Prism version 4.00). From the homologous competition binding curve, the concentration of ^{125}I -[His⁵,D-Tyr⁶]GnRH and the values for K_d , EC_{50} and Bmax, the number of GnRHRs on the cell surface of L β T2 cells co-transfected with either GnRHR-I or GnRHR-II were calculated. All given values are the mean \pm standard error of the mean (SE).



Results

L β T2 and COS-1 cells, expressing endogenous GnRHR-I and GnRHR-II, respectively, were transfected with constructs containing the *bLH β* - or *oFSH β* promoter controlling a *luciferase* reporter gene, as well as with a β gal expression vector. Parallel experiments were performed with cells co-transfected with either a GnRHR-I or a GnRHR-II expression vector, to compare results in the presence of endogenous GnRHR with that of overexpressed GnRHR (figure 36). After transfection, cells were grown for one day before treatment with GnRH-1 or GnRH-2 and/or PACAP or vehicle. Cells were lysed at the appropriate time and luciferase and β -galactosidase protein levels measured. The ratio of luciferase to β -galactosidase was determined for each treatment group. As luciferase expression should reflect transcriptional activity, the calculated Luc/ β gal ratios would be an indication of *bLH β* - and *oFSH β* promoter activity, i.e. regulation at the transcriptional level.

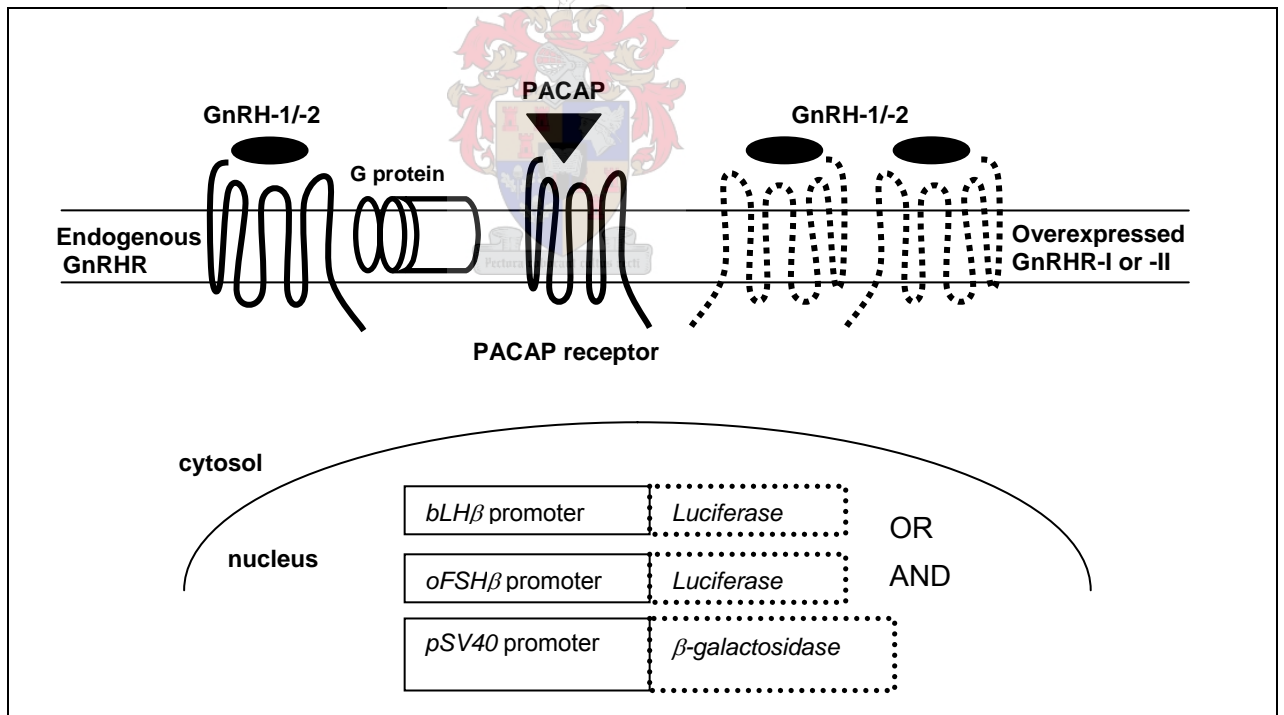


Fig 36. Schematic representation of the cell context after transient transfection with *bLH β Luc* or *oFSH β Luc* in combination with either GnRHR-I or GnRHR-II as well as *pSV40Bgal*. Endogenous GnRH- and PACAP receptors are printed with solid lines whereas overexpressed mammalian GnRHRs are printed with dashed lines.

All figures showing the results of this study and referred to within the text (i.e. figures 37 to 51) are grouped together at the end of the Results section of this chapter, from page number 137 onwards. This was done because each figure represents an experiment where various aspects are compared with one another, and, for this reason, the text often refers to more than one figure at a time in no specific order. The specific issues are however addressed within the text sequentially.

Regulation of $bLH\beta$ - and $oFSH\beta$ promoter-reporter activities by GnRH-1 and GnRH-2 via endogenous GnRHR-I in $L\beta T2$ cells

To investigate whether GnRH-1 and GnRH-2 are able to induce $LH\beta$ - and $FSH\beta$ promoter-reporter activity in $L\beta T2$ cells via endogenous GnRHR-I, cells were transiently transfected with $bLH\beta$ - or $oFSH\beta Luc$ and stimulated continuously for 6 h with 100 nM hormone (n = 8). No significant stimulation of $bLH\beta$ - or $oFSH\beta$ promoter-reporter activity with GnRH-1 or GnRH-2 was observed for most of the experiments performed. Typical fold inductions obtained ranged between 0.7 ± 0.3 - and 1.5 ± 0.4 -fold with $bLH\beta Luc$ and 0.7 ± 0.1 - and 1.4 ± 0.5 -fold with $oFSH\beta Luc$ (for example, see figures 40 & 41, showing no significant stimulation of $bLH\beta$ - or $oFSH\beta$ promoter-reporter activity with 100 nM GnRH-1 or with 100 nM GnRH-2 in the presence of endogenous GnRHR-I after 6 h continuous stimulation). Similar results of no response to GnRH-1 and GnRH-2 by $bLH\beta$ - and $oFSH\beta Luc$ were obtained with different concentrations of hormone (1 nM and 10 nM), a longer treatment duration (18 h) or with pulsatile stimulation (see figures 42 & 43, showing results of 6 h continuous and 6 h pulsatile stimulation at a pulse frequency of 1 pulse/2 h with 10 nM hormone, respectively, and figure 44, showing results of 18 h continuous stimulation with 100 nM hormone). Co-treatment with GnRH-1 or GnRH-2 and PACAP also did not result in an increase in either $bLH\beta$ - or $oFSH\beta$ promoter-reporter activity via endogenous GnRHR-I (figure 46).

Regulation of endogenous α -, $LH\beta$ -, and $FSH\beta$ -subunit mRNA levels in $L\beta T2$ cells: effects of GnRH-1 vs. GnRH-2 and PACAP

An important question is whether the absence of an effect on the transfected $bLH\beta$ - and $oFSH\beta$ promoter-reporter activities with GnRH-1, GnRH-2 or PACAP in $L\beta T2$ cells is a true reflection of the responses of the endogenous $LH\beta$ - and $FSH\beta$ -subunit promoters in these cells. Therefore, Northern blot analysis was performed on RNA isolated from $L\beta T2$ cells to determine endogenous $LH\beta$ - and $FSH\beta$ -subunit mRNA levels. In addition, endogenous α -subunit mRNA levels were also determined. Cells

were plated and incubated for 2 days prior to continuous treatment with GnRH-1 (100 nM), GnRH-2 (100 nM) and PACAP (3 nM or 20 nM) alone or with a combination of GnRH-1 or GnRH-2 plus PACAP. Treatments were performed in duplicate for 6- or 24 h.

α -subunit

A 6 h stimulation with 100 nM GnRH-1 or GnRH-2 resulted in slight (not significant) increases in endogenous α -subunit mRNA levels compared to levels in the absence of hormone (see figure 37, A, showing a 1.14 ± 0.22 -fold increase with 100 nM GnRH-1 and a 1.22 ± 0.02 -fold increase with 100 nM GnRH-2). Interestingly, PACAP (20 nM) alone increased α -subunit mRNA levels 2.04 ± 0.18 -fold after 6 h (figure 37, A, bar no 5), but this was not statistically significant compared to control incubation in the absence of hormone (figure 37, A, bar no 2). The combination of PACAP (20 nM) plus GnRH-1 or GnRH-2 (100 nM) resulted in significant increases in α -subunit mRNA levels compared to control incubation in the absence of hormone (see figure 37, A, showing 2.85 ± 0.18 -fold with PACAP/GnRH-1 (bar no 6), $P < 0.01$ relative to control, and 2.22 ± 0.79 -fold with PACAP/GnRH-2 (bar no 7), $P < 0.05$ relative to control). Based on these observations, it would appear that the short-term (6 h) effects of continuous GnRH-1 and PACAP on α -subunit mRNA are synergistic (see figure 37, A, compare bar no 6 with bars no 3 & 5), whereas increases in α -subunit mRNA levels with GnRH-2 plus PACAP seem to be additive with these conditions of continuous 6 h incubation (see figure 37, A, compare bar no 7 with bars no 4 & 5). However, differences in α -subunit mRNA levels were not observed between PACAP alone (figure 37, A, bar no 5) and PACAP plus GnRH-1 or PACAP plus GnRH-2 (figure 37, A, bar no 6 & 7, respectively). The only difference between groups was observed for α -subunit mRNA levels when stimulating cells with a combination of PACAP plus GnRH-1 (figure 37, A, bar no 6) as compared to α -subunit mRNA levels obtained when stimulating cells with GnRH-1 or GnRH-2 alone (figure 37, A, bar no 3 & 4, respectively). No significant differences were observed in α -subunit mRNA levels after a 24 h stimulation using any treatment, although slight decreases were observed in the presence of GnRH-2 (100 nM) alone, PACAP (20 nM) alone, and PACAP (20 nM) in combination with either GnRH-1 (100 nM) or GnRH-2 (figure 37, B). Interestingly, α -subunit mRNA levels at $t = 0$ (figure 37, B, bar no 1) were significantly lower compared to levels observed after 24 h incubation in absence of hormone (figure 37, B, bar no 2, $P < 0.05$ relative to $t = 0$), indicating a

hormone-independent increase in α -subunit mRNA levels in culture over time. These differences were not apparent when using Bonferroni's post-test.

LH β

All treatments resulted in slight increases in LH β -subunit mRNA levels after 6 h. However, the fold induction observed with 100 nM GnRH-1 or GnRH-2 alone, 20 nM PACAP alone or 20 nM PACAP plus 100 nM GnRH-1 was not significant, ranging from 1.16 ± 0.06 - to 1.28 ± 0.13 -fold (figure 38, A). A combination of 20 nM PACAP plus 100 nM GnRH-2 increased LH β -subunit mRNA levels significantly by 1.37 ± 0.21 -fold compared to control induction in absence of hormone, $P < 0.05$ (figure 38, A, compare bar no 7 with bar no 2). LH β mRNA levels in cells incubated for 24 h were similar to the no hormone control for all groups (figure 38, B). No differences were observed between groups after 6 h or 24 h.

FSH β

FSH β mRNA levels were slightly increased compared to no hormone control after 6 h with all treatments, similar to what was found for LH β . The fold induction observed with 100 nM GnRH-1 or GnRH-2 alone or 20 nM PACAP plus 100 nM GnRH-1 was not significant, ranging from 1.05 ± 0.05 to 1.19 ± 0.12 (figure 39, A). In contrast, PACAP (20 nM) alone or in combination with GnRH-2 (100 nM) resulted in a slight but significant increase in FSH β mRNA (see figure 39, A, showing 1.32 ± 0.03 -fold with PACAP (bar no 5) and 1.34 ± 0.05 -fold with PACAP/GnRH-2 (bar no 7), $P < 0.05$ relative to no hormone control (bar no 2)). Similar to α -subunit, FSH β mRNA levels observed at $t = 0$ were significantly lower compared to levels observed after 24 h incubation in the absence of hormone (see figure 39, B, showing 0.50 ± 0.09 -fold at $t = 0$ (bar no 1), $P < 0.01$ relative to no hormone control). However, similar to LH β , no differences were observed between groups after 6 h or 24 h.

GnRH-1 vs. GnRH-2: Relative effects in regulating LH β - and FSH β promoter-reporter activity, via either overexpressed GnRHR-I or overexpressed GnRHR-II in L β T2 cells

The abilities of GnRH-1 and GnRH-2 to regulate *bLH β* - and *oFSH β* promoter-reporter activities were compared in L β T2 cells overexpressing either GnRHR-I or GnRHR-II to determine whether one of the two hormones is a selective regulator of gonadotropin gene transcription.

GnRH-1 vs. GnRH-2: Relative effects in regulating $bLH\beta$ promoter-reporter activity via overexpressed GnRHR-I

Overexpressed GnRHR-I resulted in a ~50% greater induction of $bLH\beta$ promoter-reporter activity via GnRH-1 as compared to GnRH-2 ($P > 0.05$), when stimulating cells for 6 h with 10 nM hormone, either continuously or in a pulsatile fashion at 1 pulse/2 h (see figure 42, A, showing 5.98 ± 1.57 -fold with 10 nM GnRH-1 (bar no 5) and 3.80 ± 0.97 -fold with 10 nM GnRH-2 (bar no 6); and figure 43, A, showing 4.50 ± 0.68 -fold with 10 nM GnRH-1 (bar no 5) and 3.45 ± 0.02 -fold with 10 nM GnRH-2 (bar no 6)). These differences between GnRH-1 and GnRH-2 were not apparent after 6 h of pulsatile stimulation with 10 nM hormone using a pulse frequency of 1 pulse/0.5 h ($n = 2$) (not shown) or after 6 h continuous stimulation with 100 nM hormone ($n = 6$) (figure 41, A). In contrast, overexpressed GnRHR-I resulted in a ~50% greater induction of $bLH\beta$ promoter-reporter activity via GnRH-2 as compared to GnRH-1 ($P > 0.05$), when stimulating cells continuously for 18 h with 100 nM hormone ($n = 3$) (figure 44, A). A dose-dependent increase in the fold induction obtained of $bLH\beta$ promoter-reporter activity was observed when overexpressing GnRHR-I in L β T2 cells, with both GnRH-1 and GnRH-2. As can be seen in figure 47 and figure 48, a concentration of 1 nM hormone did not stimulate $bLH\beta$ promoter-reporter activity to significant levels. However, in most experiments performed, $bLH\beta$ promoter-reporter activity was induced to significant levels with 10 nM GnRH-1 and GnRH-2, compared to no hormone control. Often, a 100 nM treatment resulted in a further increase in $bLH\beta$ promoter-reporter activity compared to 10 nM, indicating that maximum stimulation of $bLH\beta$ promoter-reporter activity is sometimes reached at 10 nM GnRH-1 or GnRH-2 but often requires concentrations of 100 nM or higher (for example, see figure 47, A, showing 3.51 ± 0.31 -fold with 10 nM (bar no 3) vs. 6.89 ± 0.36 -fold with 100 nM (bar no 4) GnRH-1, $P < 0.001$, and figure 47, B, showing 2.14 ± 0.19 -fold with 10 nM (bar no 3) vs. 5.94 ± 0.59 -fold with 100 nM (bar no 4) GnRH-2, $P < 0.001$ after 6 h continuous stimulation. Also refer to figure 48, A & B). Furthermore, it was found that the fold induction of $bLH\beta$ promoter-reporter activity (compared to no hormone control) increased between 6- and 12 h stimulation with GnRH-1 and GnRH-2 at all concentrations tested, but was decreased at 24 h (see figure 48, A & B, showing a definite trend whereby maximum stimulation of $bLH\beta$ promoter-reporter activity was reached between 6 h and 24 h), indicating that $bLH\beta$ promoter-reporter activity is decreased after prolonged continuous exposure to GnRH-1 or GnRH-2 when overexpressing GnRHR-I. Based on these observations, inductions in subsequent

experiments were performed for 6 h and/or 18 h with 10 nM and/or 100 nM of hormone.

GnRH-1 vs. GnRH-2: Relative effects in regulating $bLH\beta$ promoter-reporter activity via overexpressed GnRHR-II

Unlike the results obtained for overexpressed GnRHR-I, overexpressed GnRHR-II resulted in a markedly greater induction of $bLH\beta$ promoter-reporter activity via GnRH-2 as compared to GnRH-1, when stimulating cells for 6 h with 10 nM hormone, independent of whether GnRH was administered continuously or in a pulsatile fashion (see figure 43, A, showing 3.85 ± 1.13 -fold with 10 nM GnRH-1 (bar no 8) and 5.95 ± 1.00 -fold with 10 nM GnRH-2 (bar no 9), $P < 0.01$. Also see figure 45, A). A similar discriminatory effect was seen after 6 h of continuous administration with 100 nM of hormone (figure 40, $P < 0.001$, and figure 41, A, $P > 0.05$). These differences were not apparent after 18 h of continuous administration ($n = 3$) (figure 49, A).

GnRH-1 vs. GnRH-2: Relative effects in regulating $oFSH\beta$ promoter-reporter activity via overexpressed GnRHR-I

No significant differences were observed in $oFSH\beta$ promoter-reporter activity via GnRH-1 as compared to GnRH-2 upon continuous stimulation, independent of duration of treatment or concentration of hormone used, when overexpressing GnRHR-I (figure 41, B, figure 42, B, figure 43, B, and figure 44, B). Furthermore, a similar dose-dependent increase as observed in $bLH\beta$ promoter-reporter activity was not observed in $oFSH\beta$ promoter-reporter activity at the concentrations tested (not shown).

GnRH-1 vs. GnRH-2: Relative effects in regulating $oFSH\beta$ promoter-reporter activity via overexpressed GnRHR-II

In contrast to the results for GnRHR-I, overexpressed GnRHR-II resulted in a markedly greater induction of $oFSH\beta$ promoter-reporter activity via GnRH-2 as compared to GnRH-1 when stimulating cells in a pulsatile fashion for 6 h with 10 nM, at a frequency of 1 pulse/2 h (see figure 43, B, showing 1.17 ± 0.09 -fold with 10 nM GnRH-1 (bar no 8) and 2.08 ± 0.09 -fold with 10 nM GnRH-2 (bar no 9), $P < 0.01$). The fold induction of $oFSH\beta$ promoter-reporter activity was similar via GnRH-1 as compared to GnRH-2 after 6 h (figure 41, B, and figure 45, B) or 18 h (figure 49, B) of

continuous administration and 6 h of pulsatile administration using 1 pulse/0.5 h (not shown) when overexpressing GnRHR-II.

Relative induction of $oFSH\beta$ - vs. $bLH\beta$ promoter-reporter activity via endogenous GnRHR-I or overexpressed GnRHR-I and GnRHR-II in L β T2 cells

The capabilities of GnRH-1 or GnRH-2 to differentially regulate $bLH\beta$ - and $oFSH\beta$ promoter-reporter activities were determined in L β T2 cells expressing endogenous GnRHR-I or overexpressing either GnRHR-I or GnRHR-II. This was done by dividing the average value for $oFSH\beta Luc$ with the average value for $bLH\beta Luc$ for a specific treatment group, to calculate the $oFSH\beta Luc:bLH\beta Luc$ ratio. The $oFSH\beta Luc:bLH\beta Luc$ ratios obtained with GnRH-1 were compared to the ratios obtained with GnRH-2, in the presence of a specific GnRHR subtype. Some of these ratios, including those calculated for the representative experiments of which results are shown at the end of this section, are shown in table 9.



Table 9: Comparison between *oFSH β Luc:bLH β Luc* ratios obtained with GnRH-1 vs. GnRH-2 in L β T2 cells overexpressing GnRHR-I or GnRHR-II.

Treatment condition	Calculated <i>oFSHβLuc:bLHβLuc</i> ratio relative to the <i>oFSHβLuc:bLHβLuc</i> ratio for no hormone = 1				Relevant figure no
	Overexpressed GnRHR-I		Overexpressed GnRHR-II		
	GnRH-1	GnRH-2	GnRH-1	GnRH-2	
6 h continuous (10 nM)	0.2	0.5			42
	0.3	0.6			Not shown
			0.4	0.2	45
			0.14	0.1	Not shown
6 h pulse 1 per 0.5 h (10 nM)	0.7	0.8	0.7	0.4	Not shown
	0.4	0.5			Not shown
6 h pulse 1 per 2 h (10 nM)	0.4	0.6	0.3	0.3	43
	0.3	0.5			Not shown
6 h continuous (100 nM)	0.3	0.8			Not shown
	0.16	0.23	0.4	0.2	41

Note:

Each row represents one experiment with all conditions shown in that row done in parallel. Ratios from at least two independent experiments are shown unless only one experiment was performed. Ratios were calculated by dividing the average value for *oFSH β Luc* with the average value for *bLH β Luc* for a specific treatment group after normalisation to β gal, each group done with triplicate samples. The *oFSH β Luc:bLH β Luc* ratio for the no hormone group was set at a value of 1 for a specific GnRHR subtype.

When comparing the ratios of *oFSH β Luc:bLH β Luc*, although the absolute values of the ratios differed between experiments, a trend was observed for GnRH-1 vs. GnRH-2 upon 6 h of hormone treatment. When overexpressing the GnRHR-I in L β T2 cells, a greater *oFSH β Luc:bLH β Luc* ratio was obtained via GnRH-2 as compared to GnRH-1 after 6 h, independent of the concentration of hormone used or whether hormones were administered continuously or in a pulsatile fashion (table 9). In contrast to results obtained via overexpressed GnRHR-I, continuous stimulation with GnRH-1 for 6 h resulted in a higher *oFSH β Luc:bLH β Luc* ratio as compared to stimulation with GnRH-2 in cells overexpressing the GnRHR-II (table 9). This observation of a greater *oFSH β Luc:bLH β Luc* ratio with GnRH-1 via overexpressed GnRHR-II was also evident when stimulating cells in a pulsatile fashion at a high (1/0.5 h), but not a low (1/2 h) pulse frequency (table 9). Low pulse frequency

treatment resulted in similar *oFSH* β *Luc*:*bLH* β *Luc* ratios with GnRH-1 vs. GnRH-2 via overexpressed GnRHR-II (table 9). No clear trends were observed after 18 h of hormone treatment (not shown).

PACAP:

Effect on GnRH-1- and GnRH-2-mediated regulation of bLH β - and oFSH β promoter-reporter activity in L β T2 cells

To determine the effect of PACAP on GnRH-1- and GnRH-2-mediated regulation of *bLH* β - and *oFSH* β promoter-reporter activity, L β T2 cells overexpressing GnRHR-I or GnRHR-II were stimulated continuously for 6 h with PACAP (3 nM or 20 nM) alone or in combination with GnRH-1 (100 nM) or GnRH-2 (100 nM).

Regulation of bLH β promoter-reporter activity via overexpressed GnRHR-I in L β T2 cells: effects of PACAP alone or in combination with GnRH-1 or GnRH-2

PACAP alone (3 nM or 20 nM) did not induce *bLH* β promoter-reporter activity significantly in L β T2 cells overexpressing GnRHR-I, after 6 h continuous stimulation (figure 46, A). In contrast, PACAP together with GnRH-1 or GnRH-2 (100 nM) resulted in a significant induction of *bLH* β promoter-reporter activity via overexpressed GnRHR-I, independent of the concentration of PACAP used. GnRH-1 often resulted in a significantly greater fold induction of *bLH* β promoter-reporter activity as compared to GnRH-2, when co-stimulating cells with 3 nM PACAP (see figure 46, A, showing 6.69 ± 1.19 -fold with 3 nM PACAP/100 nM GnRH-1 (bar no 10) and 4.42 ± 0.26 -fold with 3 nM PACAP/100 nM GnRH-2 (bar no 11), $P < 0.05$). This difference between GnRH-1 and GnRH-2 was not observed with the addition of 20 nM PACAP (figure 46, A). These results show that PACAP modulates the response of the *bLH* β promoter to 6 h of continuous stimulation with 100 nM GnRH-1 and/or GnRH-2 via the GnRHR-I, in a dose-dependent fashion. Although inductions with GnRH only were not included in these experiments, the effects seen with PACAP plus GnRH-1 or GnRH-2 can be compared with the relative effects of GnRH-1 vs. GnRH-2 alone observed in separate experiments. As can be seen in figure 41, A, the *bLH* β response to 100 nM GnRH-1 or GnRH-2 alone was similar via overexpressed GnRHR-I. This would suggest that the greater response to 3 nM PACAP plus 100 nM GnRH-1 as compared to 3 nM PACAP plus 100 nM GnRH-2 after 6 h continuous stimulation (figure 46, A) is either the result of an increase in the GnRH-1 response or a decrease in the GnRH-2 response by 3 nM PACAP.

Regulation of $bLH\beta$ promoter-reporter activity via overexpressed GnRHR-II in $L\beta T2$ cells: effects of PACAP alone or in combination with GnRH-1 or GnRH-2

Overexpressed GnRHR-II did not result in induction of $bLH\beta$ promoter-reporter activity via PACAP alone, similar to results obtained with overexpressed GnRHR-I (figure 46, A). A combination of PACAP and 100 nM GnRH-1 or GnRH-2 always increased $bLH\beta$ promoter-reporter activity significantly, and the fold induction obtained was similar independent of the concentration of PACAP used or whether PACAP was combined with either GnRH-1 or GnRH-2 (figure 46, A). Since it was found that for overexpressed GnRHR-II, in the absence of PACAP, 100 nM GnRH-2 resulted in a significantly greater fold induction of $bLH\beta$ promoter-reporter activity as compared to 100 nM GnRH-1 added continuously for 6 h (figures 40 & 41, A), this suggests that PACAP is modulating the response via GnRHR-II to GnRH, either by reducing the relative response to GnRH-2 or increasing the relative response to GnRH-1.

Regulation of $oFSH\beta$ promoter-reporter activity via overexpressed GnRHR-I in $L\beta T2$ cells: effects of PACAP alone or in combination with GnRH-1 or GnRH-2

$oFSH\beta$ promoter-reporter activity was not significantly affected by PACAP alone via overexpressed GnRHR-I, similar to results obtained for $bLH\beta$ (figure 46, B). Interestingly, significant increases, albeit very small, in $oFSH\beta$ promoter-reporter activity were observed with 20 nM PACAP, but not 3 nM PACAP, in the presence of both 100 nM GnRH-1 and GnRH-2 (figure 46, B), as compared to no hormone control. When stimulating cells with GnRH-1 or GnRH-2 (100 nM) alone for 6 h continuously (figure 41, B) a small and similar increase was observed in $oFSH\beta$ promoter-reporter activity for both hormones. Thus while it can be seen that PACAP does not alter the relative effects of GnRH-1 vs. GnRH-2 (figure 46, B), unlike the results obtained for $bLH\beta$, it does appear to modulate the GnRH response via GnRHR-I, in a dose-dependent fashion, by inhibiting the effects of both hormones at 3 nM PACAP, but not at 20 nM PACAP.

Regulation of $oFSH\beta$ promoter-reporter activity via overexpressed GnRHR-II in $L\beta T2$ cells: effects of PACAP alone or in combination with GnRH-1 or GnRH-2

In contrast to overexpressed GnRHR-I, no significant stimulation of $oFSH\beta$ promoter-reporter activity was observed with PACAP alone (both 3 nM and 20 nM) or in combination with GnRH-1 or GnRH-2 via overexpressed GnRHR-II, after 6 h continuous stimulation (figure 46, B). GnRH-1 or GnRH-2 (100 nM) treatment alone

also had no stimulatory effect on *oFSH β* promoter-reporter activity (figure 41, B), and, since this lack in response was not altered upon addition of PACAP, it was evident that PACAP has no modulatory effect on the *oFSH β* response to GnRH via the GnRHR-II.

Tissue-specificity of the regulation of *bLH β* - and *oFSH β* promoter-reporter activity: Comparison of effects in L β T2 vs. COS-1 cells

To determine whether the relative effects of GnRH-1 vs. GnRH-2 in regulating *bLH β* - and *oFSH β* promoter-reporter activities via GnRHR-I and GnRHR-II are specific to pituitary gonadotrope cells, some of the promoter-reporter studies performed in L β T2 cells were repeated in COS-1 kidney cells. COS-1 cells express GnRHR-II endogenously, at very low levels [Neill JD *et al.*, 2001], but do not express GnRHR-I. L β T2 cells do not express endogenous GnRHR-II in addition to GnRHR-I since rodents do not possess a *GnRHR-II* gene [Neill JD, 2002b]. Similar to L β T2 cells, it was found that neither *bLH β* - nor *oFSH β* promoter-reporter activity was significantly increased in response to GnRH-1 or GnRH-2 in the presence of endogenous GnRHR levels in COS-1 cells, independent of the concentration of hormone used or the duration of treatment (figure 50). When overexpressing GnRHR-I or GnRHR-II, *bLH β* but not *oFSH β* promoter-reporter activity was increased in response to hormone treatment in COS-1 cells (figure 50), whereas in L β T2 cells both activities were increased. The maximum fold induction of *bLH β* promoter-reporter activity obtained in COS-1 cells was lower than that observed in L β T2s. These results indicated that GnRH-1 and GnRH-2 are both able to regulate *bLH β* - and *oFSH β* promoter-reporter activity in COS-1 cells, which would suggest that the transcriptional machinery involved in the regulation of the gonadotropin β -subunit genes is not specific to gonadotrope cells. One interesting difference between the two cell-lines was the effect of GnRH treatment duration on *bLH β* - and *oFSH β* promoter-reporter activity. Whereas in L β T2 cells *bLH β* promoter-reporter activity was higher after a short (12 h or less) as compared to a long (more than 12 h) continuous incubation with GnRH-1 and GnRH-2 via overexpressed GnRHR-I or overexpressed GnRHR-II (figure 48, A & B), a similar time dependency of the fold induction of *bLH β* promoter-reporter activity in COS-1 cells was only observed when overexpressing GnRHR-II, but not when overexpressing GnRHR-I (not shown). Furthermore, *oFSH β* promoter-reporter activity was higher after 6 h continuous as compared to 18 h continuous treatment with GnRH-1 or GnRH-2 via overexpressed GnRHR-II (but not via

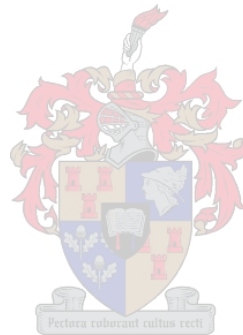
overexpressed GnRHR-I) in L β T2 cells. This time-dependency of the induction of *oFSH β* promoter-reporter activity was not observed in COS-1 cells (not shown). Interestingly, in COS-1 cells there was a significant increase in *bLH β* promoter-reporter activity when stimulating cells continuously with 100 nM as compared to 10 nM GnRH-1, in the presence of overexpressed GnRHR-II (figure 50, A, bar numbers 11 to 13), but not when overexpressing GnRHR-I (figure 50, A, bar numbers 6 to 8). A similar dose-dependent increase in *bLH β* promoter-reporter activity was not observed when stimulating cells with GnRH-2, independent of the subtype of GnRHR overexpressed (figure 50, A, bar numbers 6, 9 to 11, 14 & 15). A similar low-fold, not significant, induction of *oFSH β* promoter-reporter activity was obtained in COS-1 cells (figure 50, B) as observed in L β T2 cells (figure 41, B).

Expression levels of GnRHR-I and GnRHR-II in L β T2 cells

To investigate the possibility that the differences observed in *bLH β Luc*- and/or *oFSH β Luc* activity via GnRHR-I as compared to GnRHR-II are the result of differences in receptor levels, homologous competitive binding assays were performed in L β T2 cells to calculate GnRHR numbers (figure 51). Whole-cell binding was performed to determine cell-surface receptor numbers, i.e. receptors that would be available to respond to GnRH treatment. Cells used for binding analysis were transiently transfected with the same combination and concentration of DNA as used in the promoter-reporter assays to be able to directly compare results of the promoter-reporter assays with binding data. Incubation times of cells were chosen such to mimic incubation times used in the promoter-reporter assays.

Binding of the ^{125}I -[His⁵,D-Tyr⁶]GnRH analogue to the endogenous GnRHR-I in L β T2 cells was negligible (not shown). Analysis of binding data yielded a K_d of 3 nM (LogK_d \pm SE -8.49 \pm 0.02) for ^{125}I -[His⁵,D-Tyr⁶]GnRH in L β T2 cells overexpressing the GnRHR-I and a K_d of 9 nM (LogK_d \pm SE -8.04 \pm 0.12) in cells overexpressing GnRHR-II. These K_d values are in the same range as the K_d determined for ^{125}I -[His⁵,D-Tyr⁶]GnRH at the human GnRHR-I (0.19 nM) with the use of saturation binding assays in COS-1 cells [Flanagan CA *et al.*, 1998]. Using the values for B_{max} (B_{max} \pm SE, overexpressed GnRHR-I: 3.51 X 10⁶ \pm 160500 cpm, overexpressed GnRHR-II: 3.41 X 10⁵ \pm 38400) as determined with the GraphPad Software, as well as the specific activity of ^{125}I -[His⁵,D-Tyr⁶]GnRH (3608 cpm/fmol), the total GnRHR number on the cell surface was calculated. This number for L β T2 cells overexpressing the GnRHR-I was on average 4.87 X 10⁻³ fmol per cell, which is 10.3

times higher compared to the total GnRHR number expressed on the cell surface of cells overexpressing GnRHR-II (4.72×10^{-4} fmol per cell).



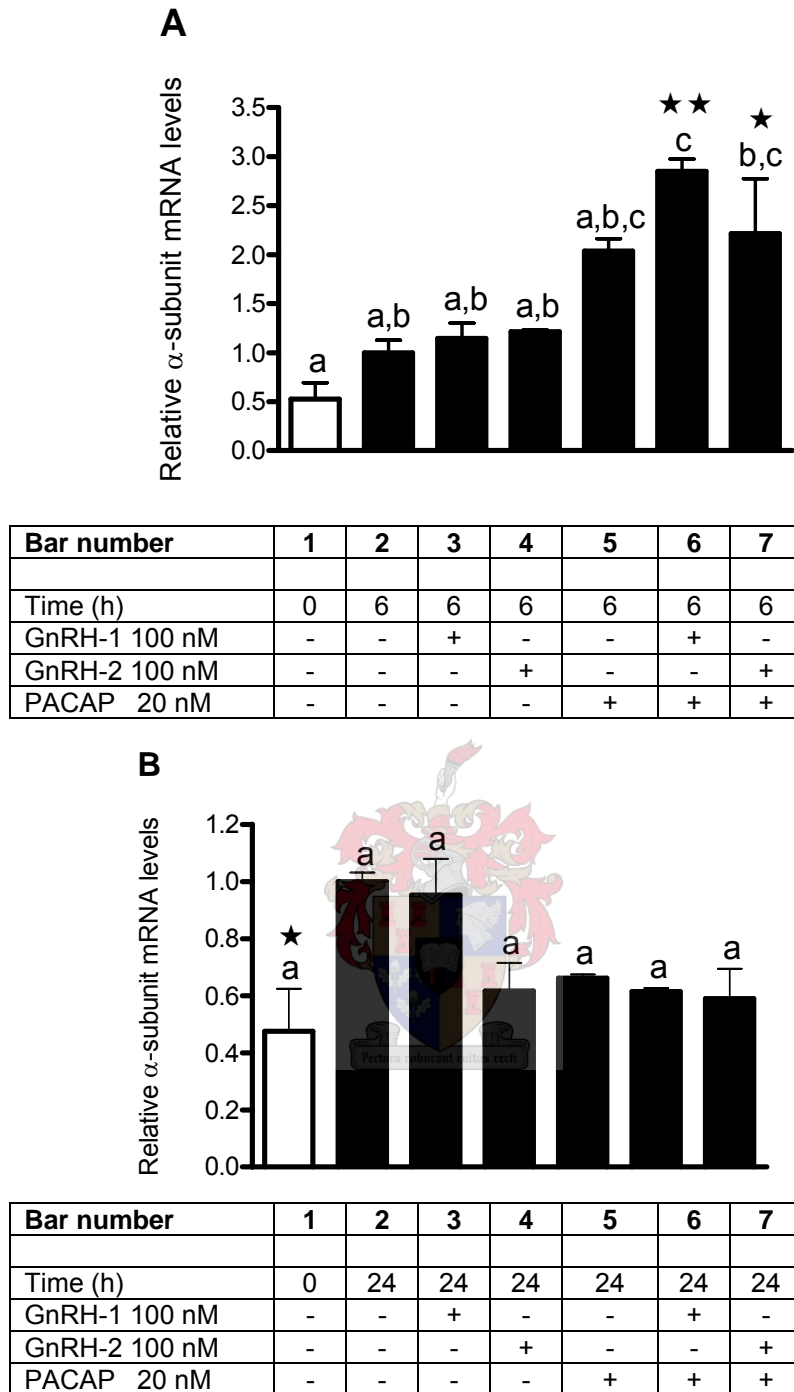


Fig 37. Northern blot analysis of α -subunit mRNA in L β T2 cells after 6- (A) and 24 h (B) stimulation with GnRH-1 (100 nM), GnRH-2 (100 nM) or PACAP (20 nM) alone or a combination of GnRH-1 or GnRH-2 with PACAP: Bar graph representations of the relative mRNA levels of duplicate samples (mean \pm SEM) after normalisation to β -actin mRNA ($n = 1$). Groups were compared to control incubation in absence of hormone (bar no 2) using Dunnet's post-test and were considered significantly different from control if $P \leq 0.05$. Differences to control were indicated with *, $P < 0.05$ or **, $P < 0.01$. Groups were also compared to each other using Bonferroni's post-test and were considered significantly different from another group if $P \leq 0.05$. Differences between groups were indicated with different letters of the alphabet.

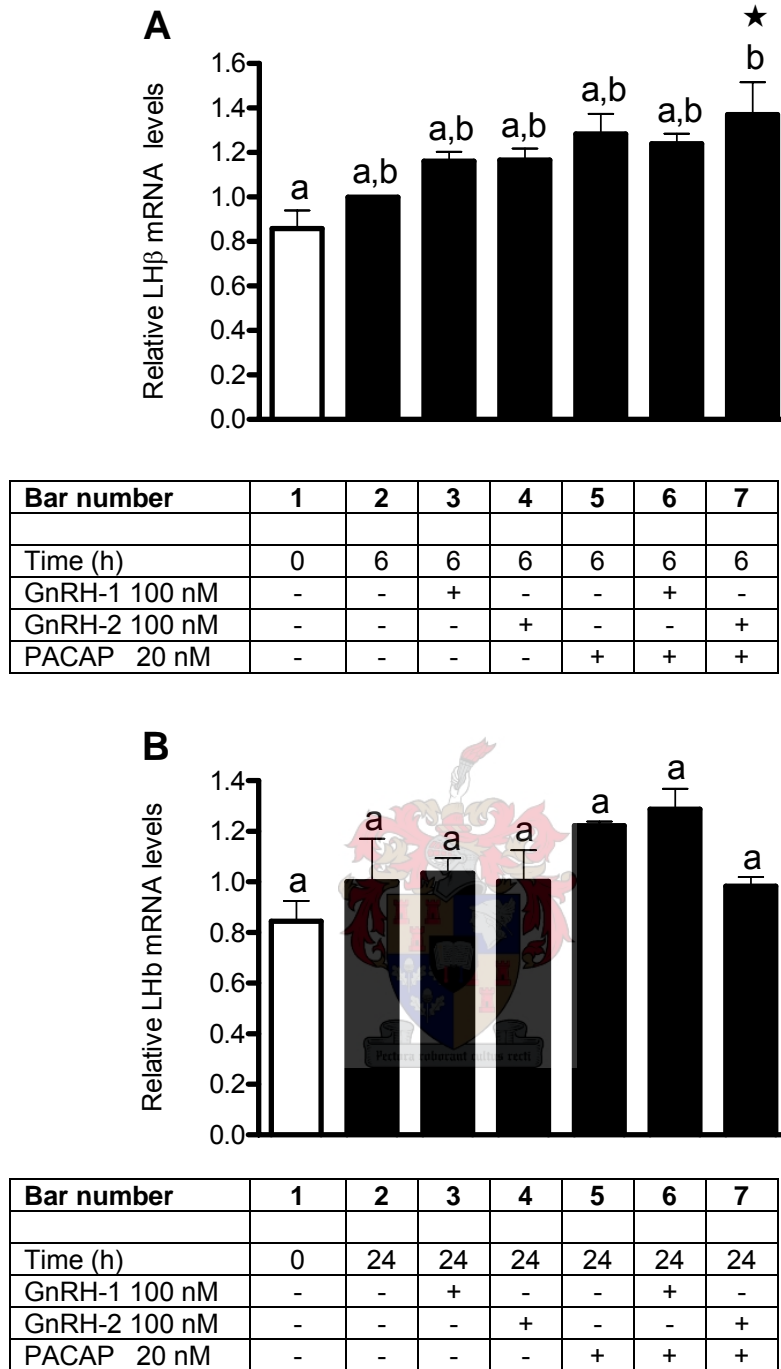


Fig 38. Northern blot analysis of LH β mRNA in L β T2 cells after 6- (A) and 24 h (B) stimulation with GnRH-1 (100 nM), GnRH-2 (100 nM) or PACAP (20 nM) alone or a combination of GnRH-1 or GnRH-2 with PACAP: Bar graph representations of the relative mRNA levels of duplicate samples (mean \pm SEM) after normalisation to β -actin mRNA. Results from one representative experiment out of two independent experiments are shown. Groups were compared to control incubation in absence of hormone (bar no 2) using Dunnet's post-test and were considered significantly different from control if $P \leq 0.05$. Differences to control were indicated with *, $P < 0.05$. Groups were also compared to each other using Bonferroni's post-test and were considered significantly different from another group if $P \leq 0.05$. Differences between groups were indicated with different letters of the alphabet.

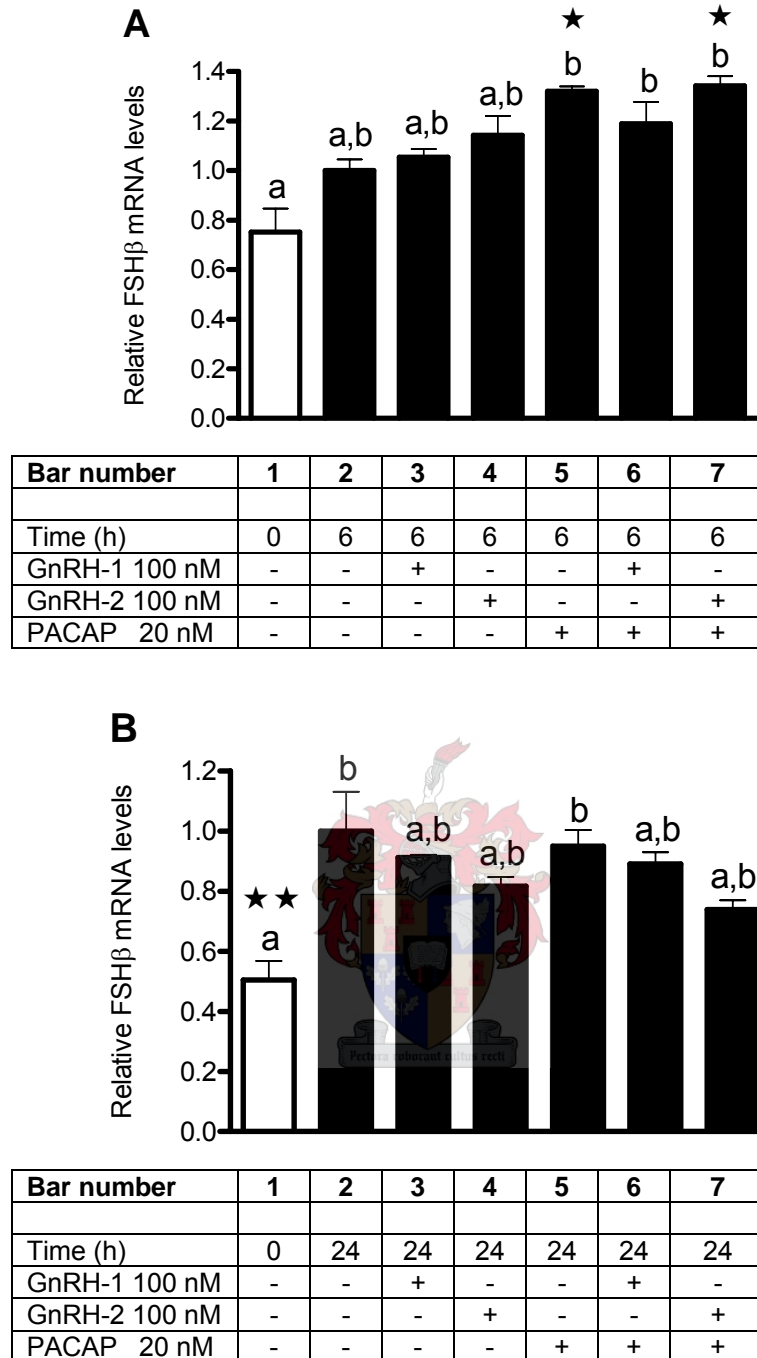
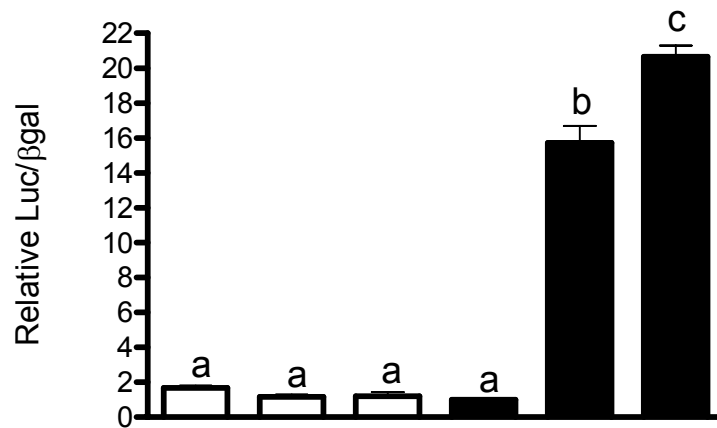
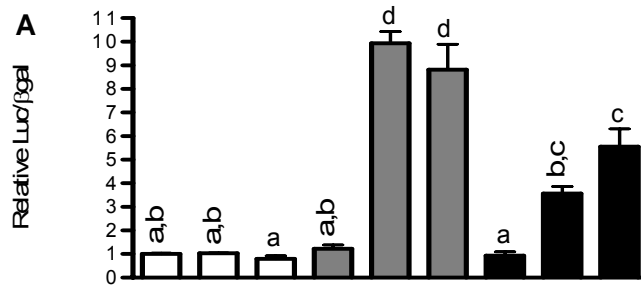


Fig 39. Northern blot analysis of FSH β mRNA in L β T2 cells after 6- (A) and 24 h (B) stimulation with GnRH-1 (100 nM), GnRH-2 (100 nM) or PACAP (20 nM) alone or a combination of GnRH-1 or GnRH-2 with PACAP: Bar graph representations of the relative mRNA levels of duplicate samples (mean \pm SEM) after normalisation to β -actin mRNA. Results from one representative experiment out of two independent experiments are shown. Groups were compared to control incubation in absence of hormone (bar no 2) using Dunnet's post-test and were considered significantly different from control if $P \leq 0.05$. Differences to control were indicated with *, $P < 0.05$ or **, $P < 0.01$. Groups were also compared to each other using Bonferroni's post-test and were considered significantly different from another group if $P \leq 0.05$. Differences between groups were indicated with different letters of the alphabet.

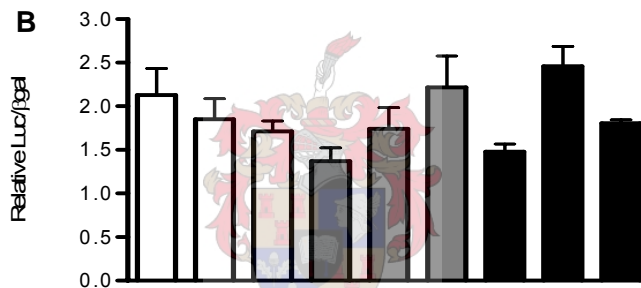


Bar number	1	2	3	4	5	6
<i>bLHβLuc</i>	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+

Fig 40. Induction of *bLHβ* promoter-reporter activity in LβT2 cells overexpressing GnRHR-II, after 6 h continuous stimulation with GnRH-1 or GnRH-2 (100 nM). Results from one representative experiment, with each point done in triplicate, out of three independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.



Bar number	1	2	3	4	5	6	7	8	9
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+	-	-	+



Bar number	1	2	3	4	5	6	7	8	9
<i>oFSHβLuc</i>	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+	-	-	+

Fig 41. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LβT2 cells overexpressing GnRHR-I or GnRHR-II, after 6 h continuous stimulation with GnRH-1 or GnRH-2 (100 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are the same. Results from one representative experiment, with each point done in triplicate, out of six independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$. No significant differences were observed between groups in (B).

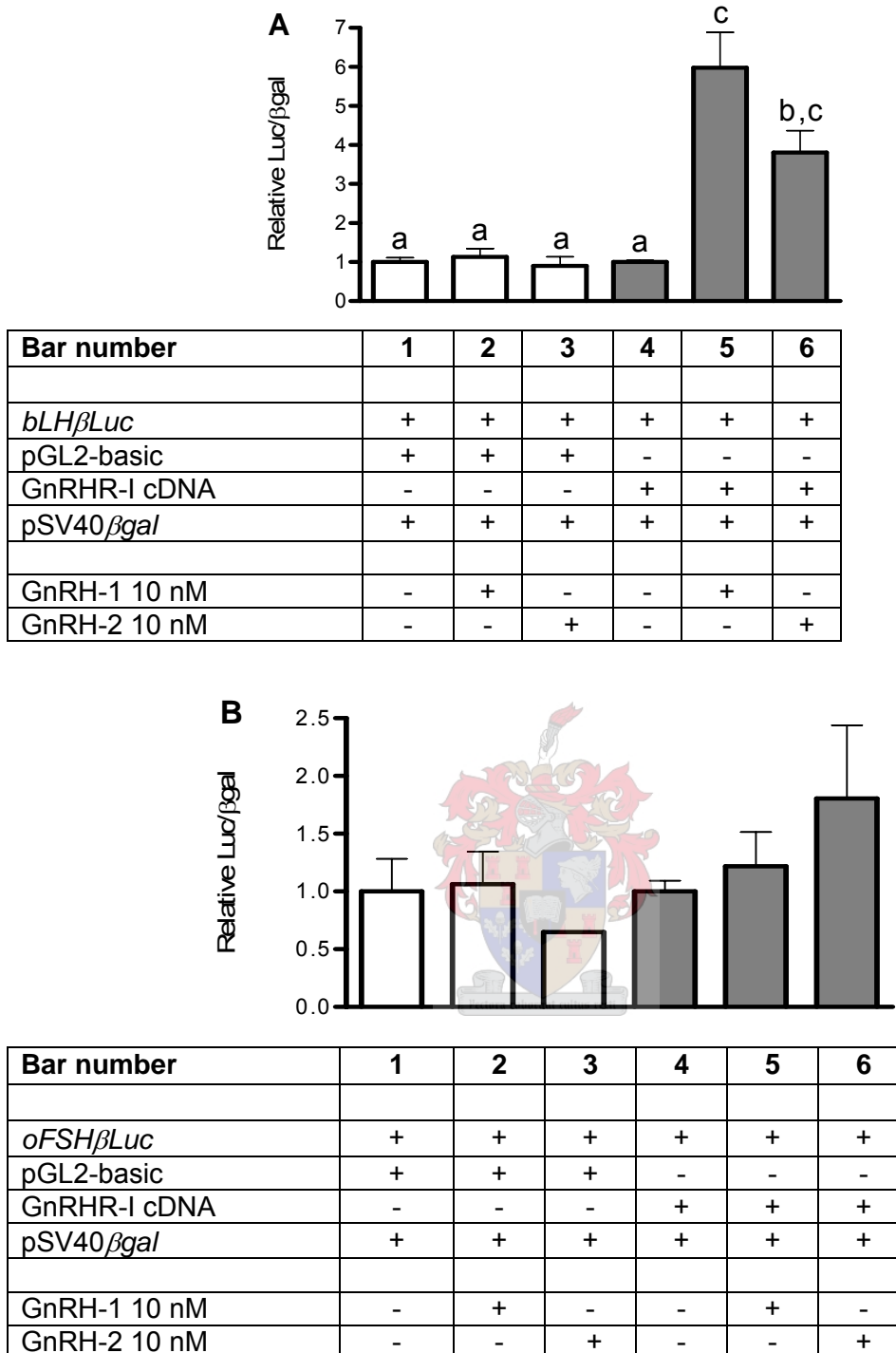
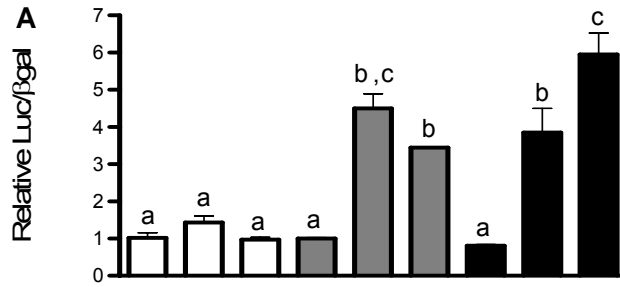
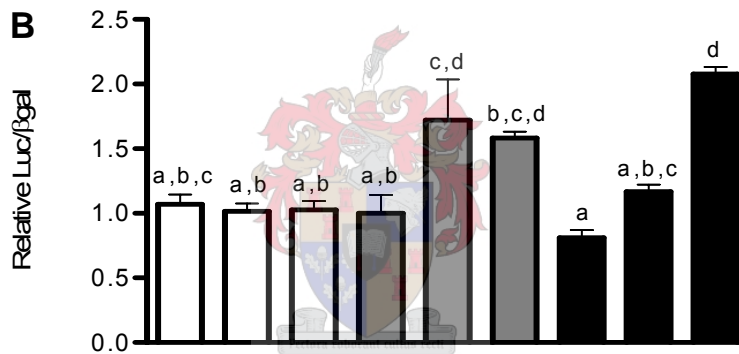


Fig 42. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LBT2 cells overexpressing GnRHR-I, after 6 h continuous stimulation with GnRH-1 or GnRH-2 (10 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of A and B are different, to enable comparison within each panel of the effects of hormone relative to no hormone, which is set at a value of 1 for each panel. The relative Luc/βgal values for (A) vs. (B) are such that a value of 1 for *oFSHβLuc* equals a value of 3.5 for *bLHβLuc*. Results from one representative experiment, with each point done in triplicate, out of four independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$. No significant differences were observed between groups in (B).

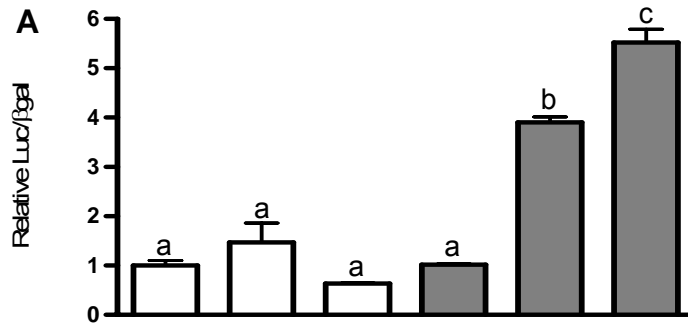


Bar number	1	2	3	4	5	6	7	8	9
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+
GnRH-1 10 nM 1/2 h	-	+	-	-	+	-	-	+	-
GnRH-2 10 nM 1/2 h	-	-	+	-	-	+	-	-	+

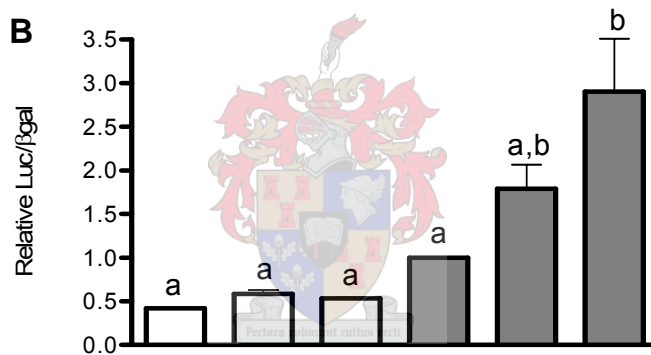


Bar number	1	2	3	4	5	6	7	8	9
<i>oFSHβLuc</i>	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+
GnRH-1 10 nM 1/2 h	-	+	-	-	+	-	-	+	-
GnRH-2 10 nM 1/2 h	-	-	+	-	-	+	-	-	+

Fig 43. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LBT2 cells overexpressing GnRHR-I or GnRHR-II, after 6 h pulsatile stimulation with GnRH-1 or GnRH-2 (10 nM), at a pulse frequency of 1 pulse/2 h. (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are the same. Results from one representative experiment, with each point done in triplicate, out of two independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.

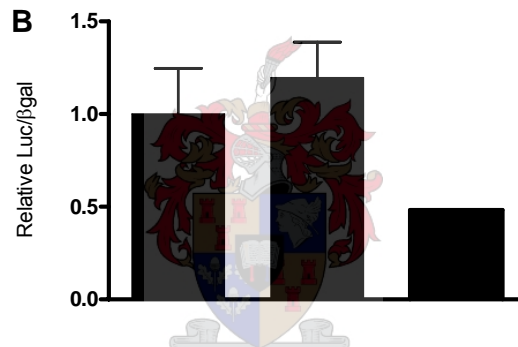
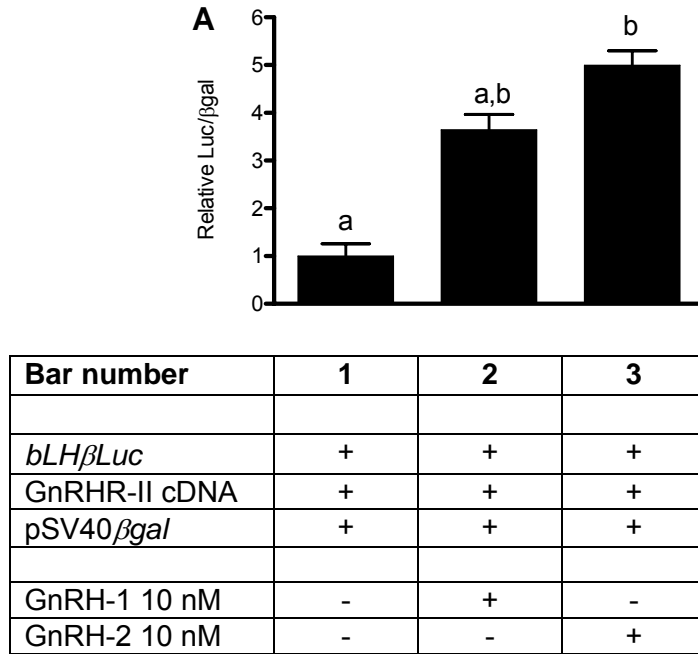


Bar number	1	2	3	4	5	6
<i>bLHβLuc</i>	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+



Bar number	1	2	3	4	5	6
<i>oFSHβLuc</i>	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-
GnRHR-I cDNA	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+

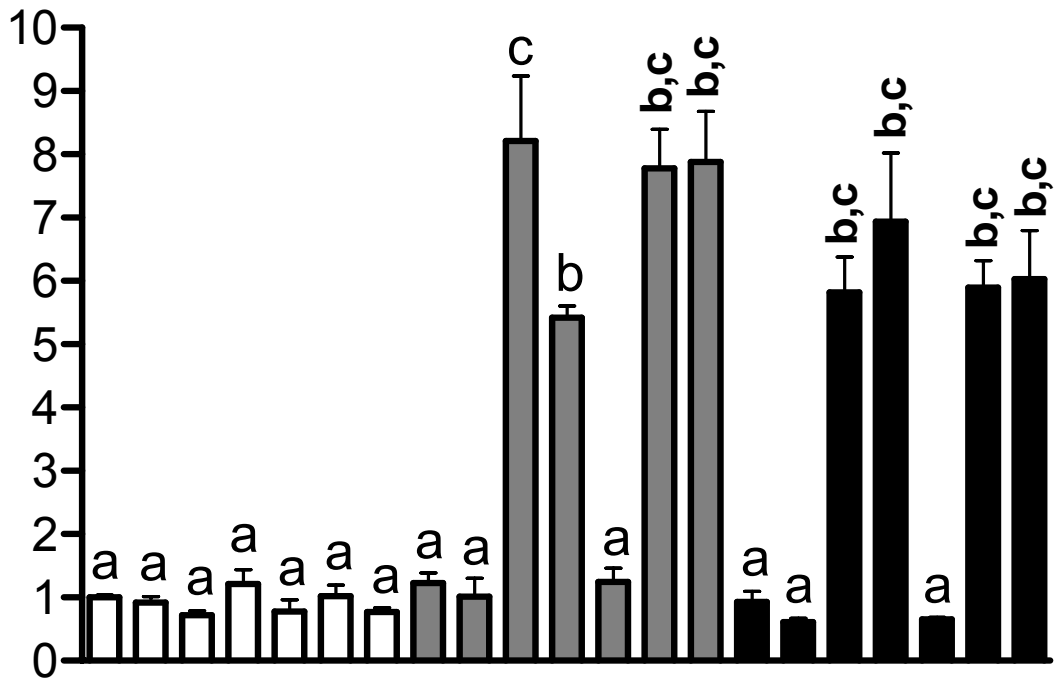
Fig 44. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LβT2 cells overexpressing GnRHR-I, after 18 h continuous stimulation with GnRH-1 or GnRH-2 (100 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are the same. Results from one representative experiment, with each point done in triplicate, out of three independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.



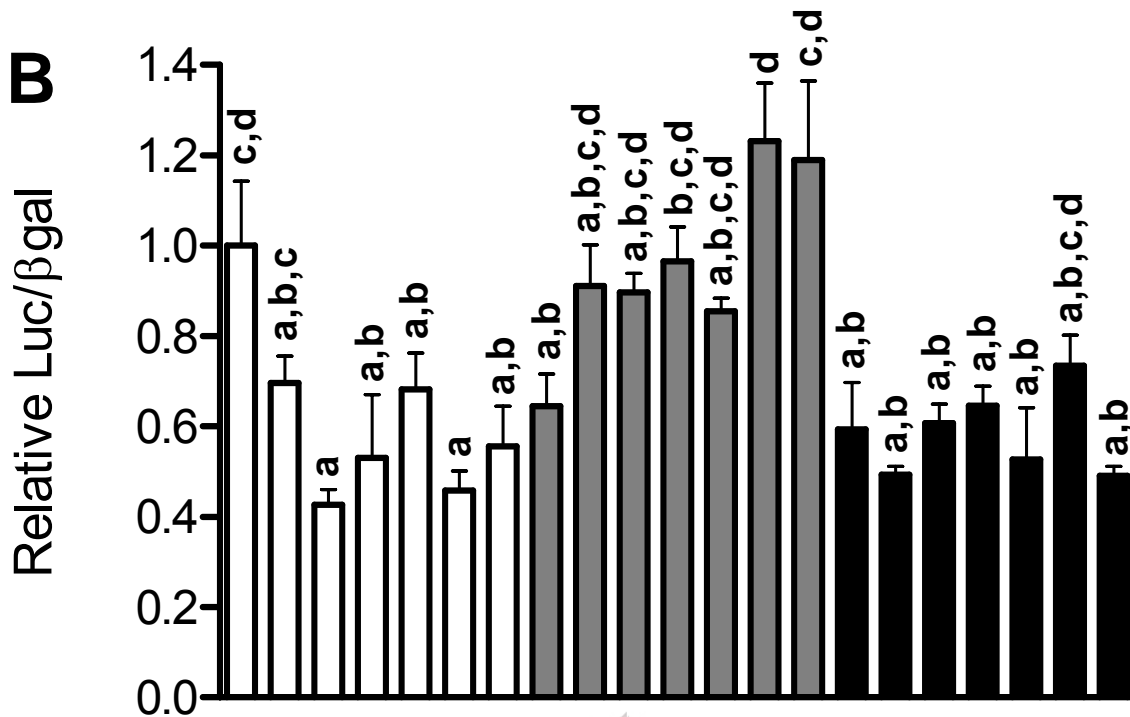
Bar number	1	2	3
<i>oFSHβLuc</i>	+	+	+
GnRHR-II cDNA	+	+	+
pSV40βgal	+	+	+
GnRH-1 10 nM	-	+	-
GnRH-2 10 nM	-	-	+

Fig 45. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LBT2 cells overexpressing GnRHR-II, after 6 h continuous stimulation with GnRH-1 or GnRH-2 (10 nM). (A) and (B) are from the same experiment ($n = 1$), with each point done in triplicate. Note that the scales for the y axes of A and B are different, to enable comparison within each panel of the effects of hormone relative to no hormone, which is set at a value of 1. The relative Luc/βgal values for (A) vs. (B) are such that a value of 1 for *bLHβLuc* equals a value of 2 for *oFSHβLuc*. All groups in (A) were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$. Groups in (B) were not compared to each other since bar no 3 represents only a single value.

A
Relative Luc/ β gal



Bar no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GnRHR-I	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
GnRHR-II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
pSV40 β gal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PACAP 3 nM	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-
PACAP 20 nM	-	-	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+
GnRH-1 100 nM	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-
GnRH-2 100 nM	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+	+



Bar no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
<i>oFSHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PACAP 3 nM	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-
PACAP 20 nM	-	-	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+
GnRH-1 100 nM	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-
GnRH-2 100 nM	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	+	-	-	-	+

Fig 46. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LβT2 cells overexpressing GnRHR-I or GnRHR-II, after 6 h continuous stimulation with PACAP (3 nM or 20 nM) alone or PACAP (3 nM or 20 nM) plus GnRH-1 or GnRH-2 (100 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are different, to enable comparison within each panel of the effects of hormone relative to no hormone in the absence of overexpressed receptor, which is set at a value of 1 for each panel. The relative Luc/βgal values for (A) vs. (B) are such that a value of 1 for *bLHβLuc* equals a value of 2.2 for *oFSHβLuc*. Results from one representative experiment, with each point done in triplicate, out of three independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.

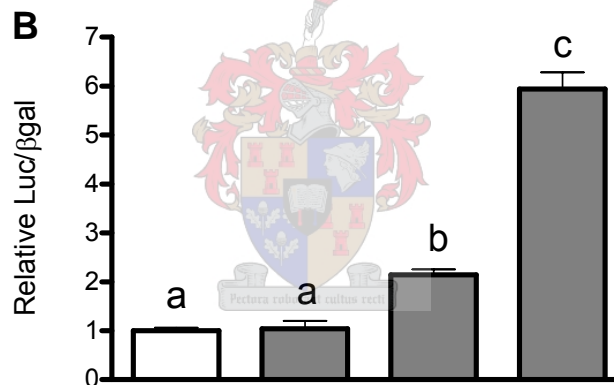
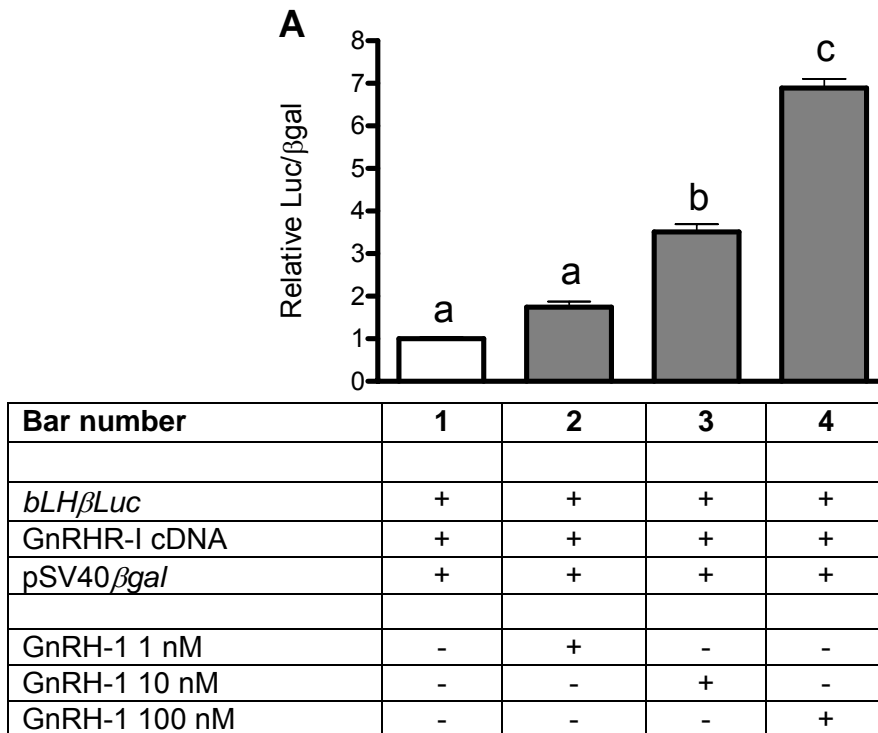
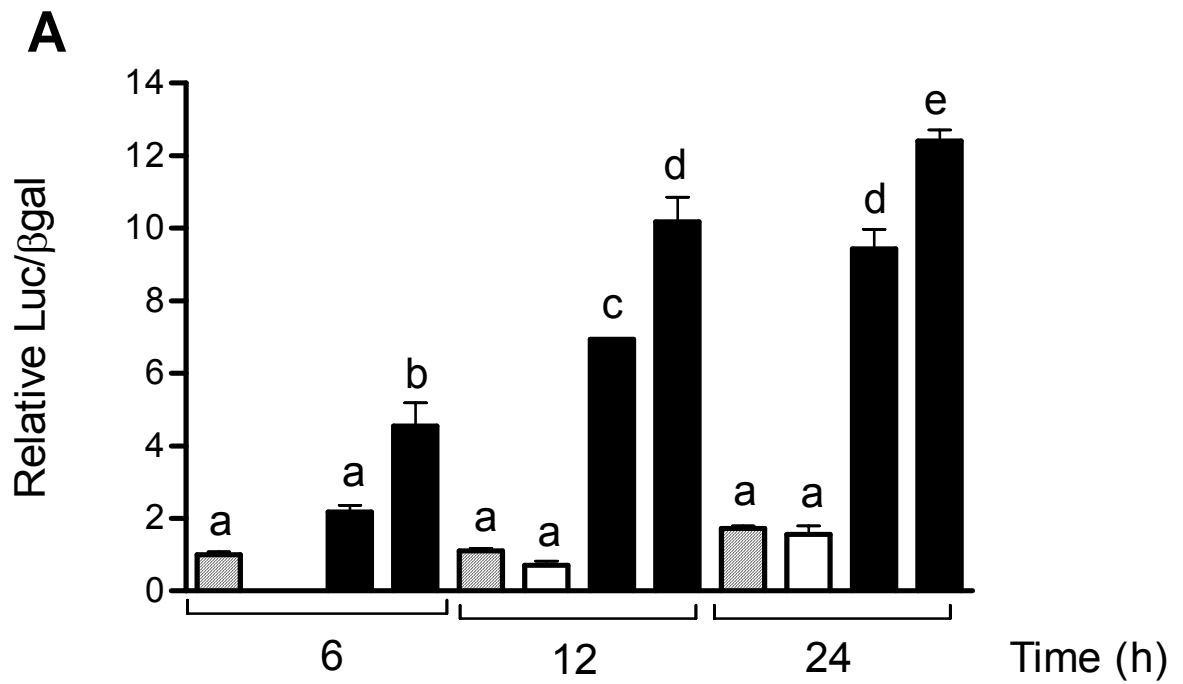
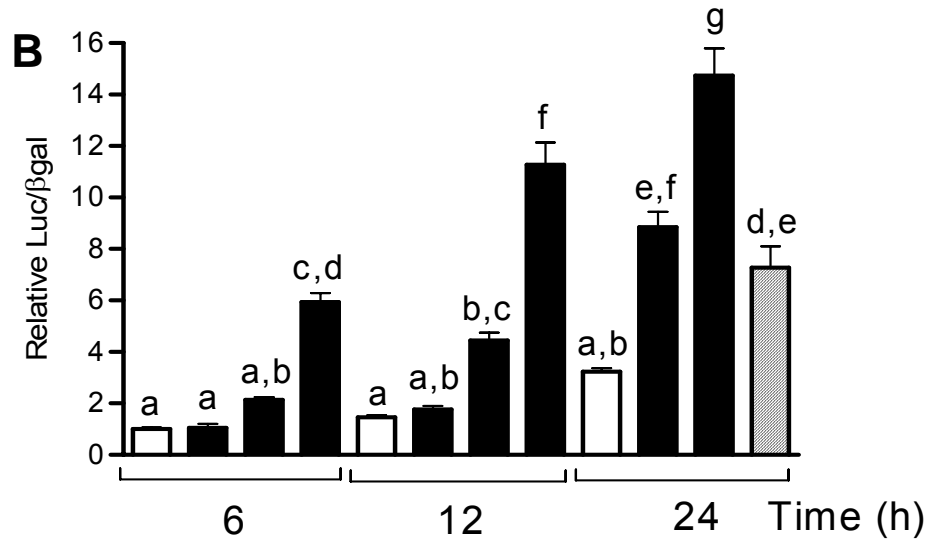


Fig 47. Induction of *bLHβ* promoter-reporter activity in LβT2 cells overexpressing GnRHR-I, after 6 h continuous stimulation with GnRH-1 (A) or GnRH-2 (B) (1, 10 or 100 nM). (A) and (B) are from two independent experiments, each performed once, with each point done in triplicate. Note that (B) represents part of the results of a time course experiment of which the full result is show in figure 48, B. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.



Bar number	1	2	3	4	5	6	7	8	9	10	11	12
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	+	-	-	-	+	-	-	-	+	-	-	-
GnRHR-I cDNA	-	+	+	+	-	+	+	+	-	+	+	+
pSV40 β gal	+	+	+	+	+	+	+	+	+	+	+	+
GnRH-1 10 nM	-	-	+	-	-	-	+	-	-	-	+	-
GnRH-1 100 nM	-	-	-	+	-	-	-	+	-	-	-	+

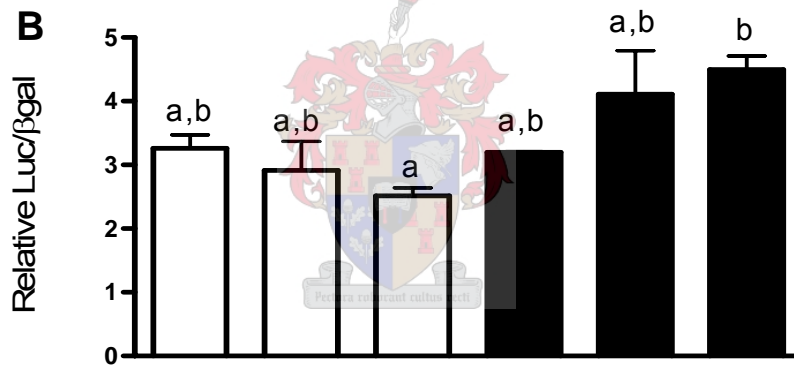


Bar number	1	2	3	4	5	6	7	8	9	10	11	12
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	-	-	-	-	-	-	-	-	-	-	-	+
GnRHR-I cDNA	+	+	+	+	+	+	+	+	+	+	+	-
pSV40βgal	+	+	+	+	+	+	+	+	+	+	+	+
GnRH-2 1 nM	-	+	-	-	-	+	-	-	-	-	-	-
GnRH-2 10 nM	-	-	+	-	-	-	+	-	-	+	-	-
GnRH-2 100 nM	-	-	-	+	-	-	-	+	-	-	+	+

Fig 48. Induction of *bLHβ* promoter-reporter activity in LβT2 cells overexpressing GnRHR-I, after 6-, 12- or 24 h continuous stimulation with GnRH-1 (10 or 100 nM) (A) or GnRH-2 (1, 10 or 100 nM) (B). (A) and (B) are from two independent experiments, each performed once using Lipofectamine 2000 transfection reagent and with each point done in triplicate. Note that samples for the 6 h no hormone group in (A) (bar no 2) were lost, and hence this bar is absent from panel (A). Also, note that in (B) there is no result with 1 nM hormone for 24 h; instead, induction with 100 nM in the presence of only endogenous GnRHR-I was included at this time point. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.

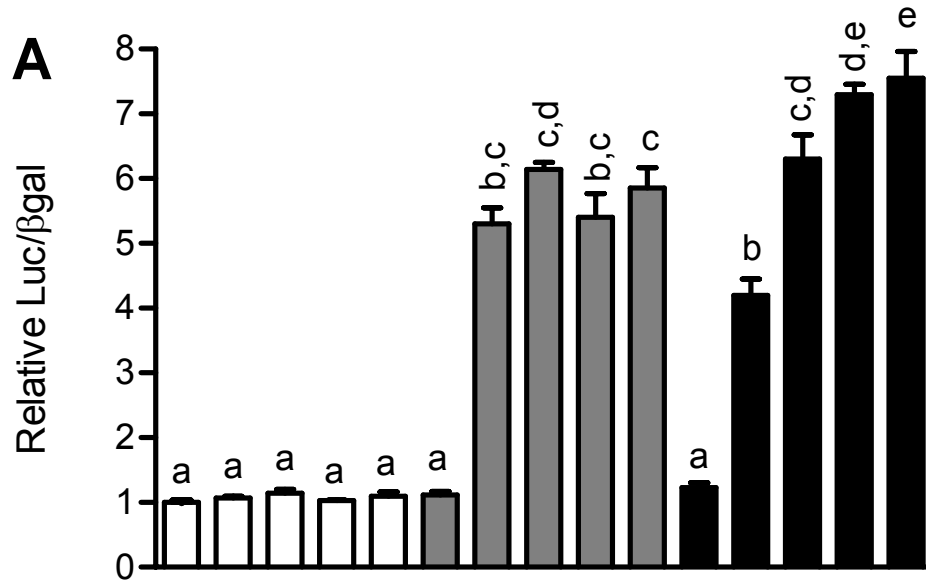


Bar number	1	2	3	4	5	6
<i>bLHβLuc</i>	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+

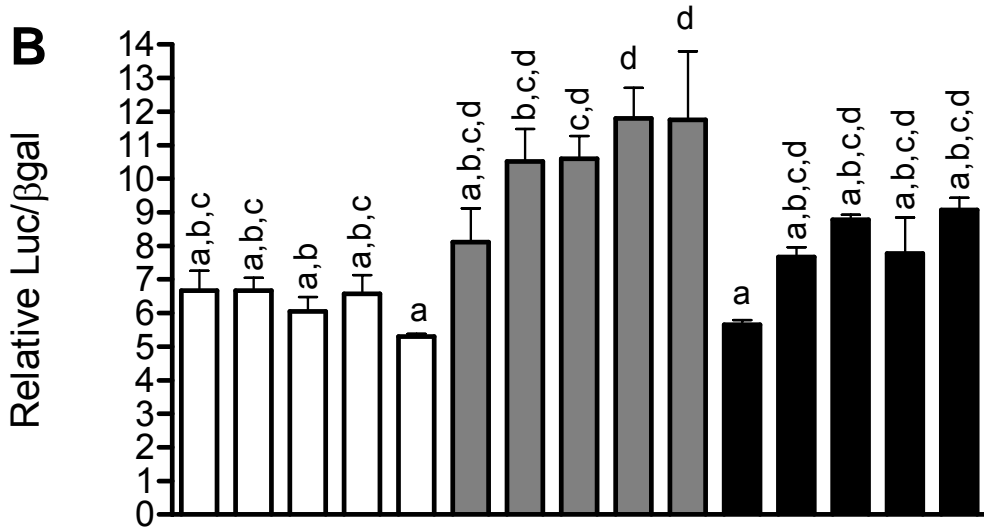


Bar number	1	2	3	4	5	6
<i>oFSHβLuc</i>	+	+	+	+	+	+
pGL2-basic	+	+	+	-	-	-
GnRHR-II cDNA	-	-	-	+	+	+
pSV40βgal	+	+	+	+	+	+
GnRH-1 100 nM	-	+	-	-	+	-
GnRH-2 100 nM	-	-	+	-	-	+

Fig 49. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in LβT2 cells overexpressing GnRHR-II, after 18 h continuous stimulation with GnRH-1 or GnRH-2 (100 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are the same. Results from one representative experiment, with each point done in triplicate, out of three independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.



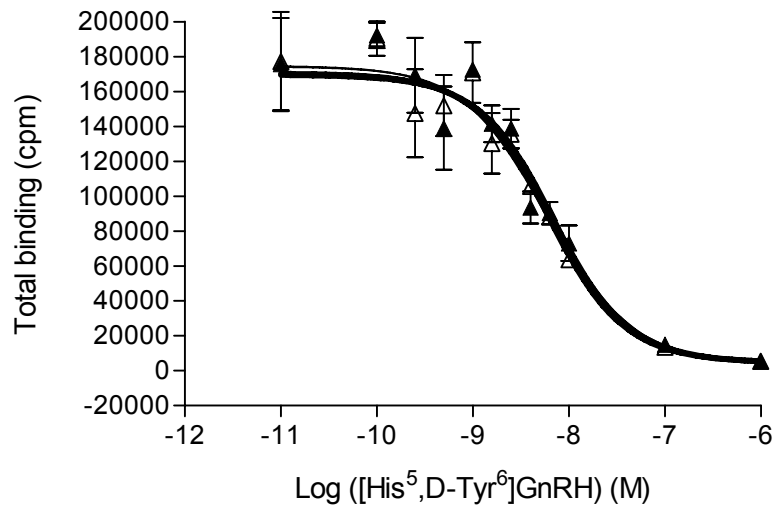
Bar number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>bLHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
pSV40 β gal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GnRH-1 10 nM	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
GnRH-1 100 nM	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
GnRH-2 10 nM	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
GnRH-2 100 nM	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+



Bar number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>oFSHβLuc</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pGL2-basic	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
GnRHR-I cDNA	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
GnRHR-II cDNA	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
pSV40βgal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GnRH-1 10 nM	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
GnRH-1 100 nM	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
GnRH-2 10 nM	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
GnRH-2 100 nM	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

Fig 50. Induction of *bLHβ* (A) and *oFSHβ* (B) promoter-reporter activity in COS-1 cells overexpressing GnRHR-I or GnRHR-II, after 6 h continuous stimulation with GnRH-1 or GnRH-2 (10 or 100 nM). (A) and (B) are from the same experiment. Note that the scales for the y axes of (A) and (B) are the same. Results from one representative experiment, with each point done in triplicate, out of three independent experiments are shown. All groups were compared to each other using Bonferroni's post-test. Differences between groups were considered significant if $P \leq 0.05$.

A. Expressed GnRHR-I



B. Expressed GnRHR-II

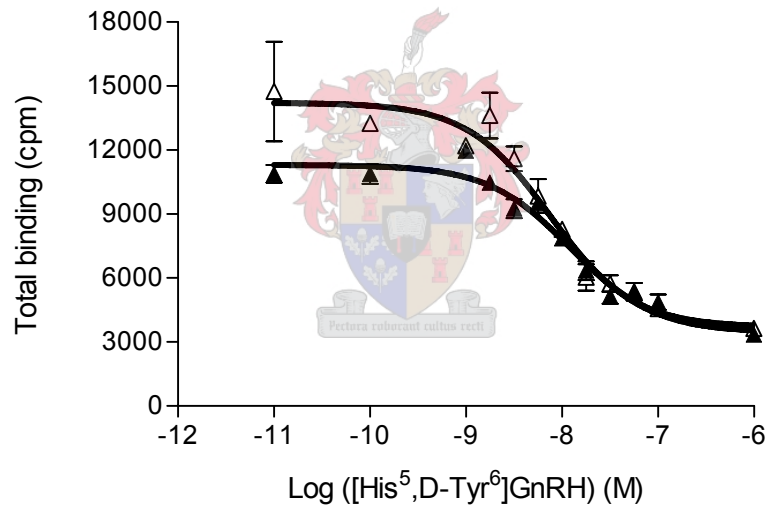


Fig 51. Homologous competition binding curves using ^{125}I -[His⁵,D-Tyr⁶]GnRH in whole L β T2 cells to compare binding to the expressed GnRHR-I (A) and the expressed GnRHR-II (B). Binding was performed in the presence of co-transfected bLH β Luc (Δ) or oFSH β Luc (\blacktriangle) to be able to directly compare binding results with results of promoter-reporter assays. Data points represent the mean \pm SE of duplicate samples. Results from one representative experiment out of two independent experiments are shown.

Discussion

LH and FSH are present in the same gonadotropes, yet their synthesis and expression are differentially regulated by GnRH. There are several possible mechanisms whereby this may occur, both transcriptional and post-transcriptional, the latter including regulation of mRNA and protein turnover, and release of stored LH and FSH from intracellular vesicles. The purpose of the present research is to investigate the role of GnRH-1 and GnRH-2 in transcriptional regulation of transfected *bLH β* and *oFSH β* promoter-reporter constructs in L β T2 and COS-1 cells, and to determine the involvement of endogenous and expressed mammalian GnRHR-I and GnRHR-II therein, as well as possible modulatory effects of PACAP on the responses. Regulation by GnRH-1 and GnRH-2 of endogenous gonadotropin mRNA levels via endogenous GnRHR-I in L β T2 cells and modulatory effects of PACAP thereon, were also investigated.

Transcriptional regulation of *bLH β* - and *oFSH β* -subunit genes via endogenous GnRHR-I in L β T2 cells

When considering the results obtained via endogenous GnRHR-I in L β T2 cells, no statistically significant induction of *bLH β* and *oFSH β* promoter-reporter activity was detected by GnRH-1 or GnRH-2 treatment, independent of the method of hormone administration, concentration or duration of treatment. While it appeared that small GnRH-induced increases (about 1.5-fold) in promoter-reporter activity were sometimes observed, if this was significant this was not detectable within the limits of the experimental system. In accordance with an absence of a transcriptional effect for continuous stimulation with GnRH, Northern blot analysis in L β T2 cells confirmed that LH β and FSH β mRNA levels are not significantly increased after 6- or 24 h of continuous stimulation with GnRH-1 or GnRH-2 (figures 38 & 39). Taken together, these results also show that there is no regulation of LH β and FSH β mRNA turnover in L β T2 cells by continuous stimulation with GnRH-1 or GnRH-2. The promoter-reporter and Northern blot studies reported in the present study for regulation by GnRH-2 of *LH β* and *FSH β* are novel, and as such increase our understanding of the role of continuous GnRH stimulation on transcriptional regulation of these promoters.

LH β regulation by endogenous GnRHR-I upon continuous stimulation with GnRH-1 or GnRH-2

The results obtained for *bLH β* regulation by endogenous GnRHR-I upon continuous stimulation with GnRH-1 are consistent with those in the literature [Wurmbach E *et al.*, 2001; Pernasetti F *et al.*, 2001; Nguyen KA *et al.*, 2004; Kakar SS *et al.*, 2003; Haisenleder DJ *et al.*, 1991]. For example, others have also shown that expression of the *LH β* gene is unaffected after 1-, 3-, 6- or 24 h continuous stimulation with GnRH-1 [Wurmbach E *et al.*, 2001; Pernasetti F *et al.*, 2001; Nguyen KA *et al.*, 2004; Haisenleder DJ *et al.*, 1991]. Likewise, Kakar SS *et al.* [2003], with the use of microarray analysis, also detected no significant change (<1.3 fold) in endogenous mRNA levels for LH β in response to either 1- or 24 h of continuous GnRH-1 agonist (100 nM) treatment. Collectively, these findings that steady-state LH β mRNA levels are not significantly increased in response to continuous GnRH-1 suggest that LH β is not regulated or regulated to a small degree at the level of transcription and/or mRNA turnover by GnRH-1. However, numerous studies in L β T2 cells have reported that LH β protein levels are elevated by short-term continuous GnRH-1 stimulation [Liu F *et al.*, 2002a; Nguyen KA *et al.*, 2004]. In a study by Liu F *et al.* [2002a], increases in LH β protein synthesis in response to a continuous dose of 100 nM GnRH agonist was apparent by 2 h in L β T2 cells and reached a maximum at 8 h, which was maintained for up to 24 h. In a recent study by Nguyen KA *et al.* [2004] using L β T2 cells transfected with a rat *LH β* promoter-reporter construct no significant increase in *LH β* promoter-reporter activity was seen after 6 h of continuous stimulation with 10 nM GnRH-1, whereas a significant increase in LH β subunit (determined by radioimmunoassay) was observed within 4 h in L β T2 cells. Thus, short-term (<6 h) induction of LH synthesis and release by continuous GnRH-1 would appear to be dependent upon new protein synthesis but not new mRNA synthesis, suggesting that the LH response to GnRH-1 treatment is mainly a translational, rather than a transcriptional effect [Nguyen KA *et al.*, 2004], consistent with the results of the present study. There are however two reports in the literature showing acute activation (within 6 h) [Kaiser UB *et al.*, 2000; Vasilyev VV *et al.*, 2002a] and long-term repression (after 24 h) of *LH β* promoter-reporter activity by continuous GnRH-1 agonists in the presence of endogenous GnRHR-I in L β T2 cells [Vasilyev VV *et al.*, 2002a]. These differences in the results as to the effects of continuous GnRH-1 on *LH β* transcriptional regulation might be attributed to the use of different GnRH-1 analogues as well as different promoter-reporter constructs.

As for GnRH-1, the findings of the present study suggest that continuous GnRH-2 does not regulate *LH β* transcription and/or mRNA turnover via endogenous GnRHR-I in L β T2 cells. No previous studies have been reported for regulation of *LH β* gene transcription by GnRH-2 in any system expressing endogenous GnRHR-I, and thus the results presented in this study with GnRH-2 are novel.

FSH β regulation by endogenous GnRHR-I upon continuous stimulation with GnRH-1 or GnRH-2

The results via endogenous GnRHR-I with continuous stimulation with GnRH-1 for *oFSH β* reported here, showing low-fold induction of the transfected *oFSH β* promoter, are consistent with those in the literature. Others have reported similar results of small increases in *oFSH β* promoter-reporter activity using the same promoter-reporter construct as used in this study [Pernasetti F *et al.*, 2001; Vasilyev VV *et al.*, 2002b]. Both Pernasetti F *et al.* [2001] and Vasilyev VV *et al.* [2002b] observed a maximal 2-fold increase in *oFSH β* promoter-reporter activity in L β T2 cells using 1 nM GnRH-1 continuously for 6 h, in the absence of overexpressed GnRHR. In addition, in perfused male rat pituitary cells continuous incubation with 10 nM GnRH-1 for 4 h stimulated FSH β mRNA approximately 2-fold [Besecke LM *et al.*, 1996]. Haisenleder DJ *et al.* [1991], with the use of a nuclear run-off transcription assay in isolated rat pituitaries, determined that a 24 h continuous GnRH-1 infusion did not increase the transcription rate of FSH β mRNA. The finding by others that *FSH β* gene transcription and steady-state mRNA levels are increased by $\leq 200\%$ (2-fold change) in response to continuous GnRH-1 *in vivo* further suggests that FSH β is only partly regulated at the transcriptional level by continuous GnRH-1 [Haisenleder DJ *et al.*, 1991]. No previous studies have been reported for regulation of *FSH β* gene transcription by GnRH-2 in any system expressing endogenous GnRHR-I. Thus, the findings presented here that continuous GnRH-2 does not stimulate *bFSH β* promoter activity or increase *FSH β* mRNA levels significantly are novel and show that, as for GnRH-1, GnRH-2 has no effect on *FSH β* transcriptional regulation in L β T2 cells expressing only endogenous GnRHR-I. Furthermore, by Northern blot analysis it was demonstrated that a hormone-independent increase in endogenous FSH β mRNA levels occurs over time in culture in the absence of hormone (figure 39, B). This finding of stimulation of *FSH β* transcription in the absence of GnRH is a first, and may explain the reported results in the literature indicating that, on protein level, FSH release is not only regulated by GnRH but also by a non-GnRH-associated

pathway [Padmanabhan V & McNeilly AS, 2001] (also discussed in the Introduction of this chapter). Taken together, the results presented here show that *FSH β* promoter activity is either not stimulated or stimulated to very low levels after continuous administration of GnRH-1 and GnRH-2 via endogenous GnRHR-I in L β T2 cells, independent of the duration of treatment or concentration used. When a low-level increase in *FSH β* transcriptional activity is observed, this is possibly due to the effects of a non-GnRH-mediated pathway.

LH β and FSH β regulation by endogenous GnRHR-I upon pulsatile stimulation with GnRH-1 or GnRH-2

It is interesting to compare the effect of the method of administration of GnRH on *LH β* - and *FSH β* transcription and mRNA levels via endogenous GnRHR-I in L β T2 cells. In this study it was found that pulsatile stimulation resulted in a similar lack of response to GnRH-1 and GnRH-2 by *bLH β* - and *oFSH β Luc* as obtained with continuous administration for 6 h. These results are different from reports by Haisenleder DJ *et al.* [1991] and Turgeon JL *et al.* [1996], showing stimulation of *FSH β* - and/or *LH β* transcription by pulsatile GnRH-1 in isolated rat pituitaries as determined by a nuclear run-off transcription assay and in L β T2 cells by Northern blot analysis respectively. GnRH pulses at 30 min intervals elevated *LH β* - and *FSH β* transcription rates 3- to 5-fold vs. control after 1 h in rat pituitaries [Haisenleder DJ *et al.*, 1991]. After 4 h of GnRH pulses, *FSH β* transcription rate was reduced vs. 1 h, but *LH β* mRNA synthesis rate was maintained, whereas, at 24 h, *LH β* - and *FSH β* transcription rates had both fallen to basal levels despite a continuing pulsatile GnRH stimulus [Haisenleder DJ *et al.*, 1991]. By Northern analysis of L β T2 cells after three days in culture it was shown that four GnRH-1 (10 nM) pulses of 15 min duration given every 90 min for three days increased steady-state *LH β* mRNA levels 4- to 5-fold [Turgeon JL *et al.*, 1996]. Furthermore, in perfused L β T2 cells *LH β* promoter activity was found to be preferentially stimulated by a high pulse frequency (1 pulse/30 min) whereas *FSH β* promoter activity is stimulated to the greatest extent at a lower pulse frequency (1 pulse/2 h) after either 10- or 20 h of GnRH stimulation [Bédécarrats GY & Kaiser UB, 2003], similar to an earlier report [Dalkin AC *et al.*, 1989]. However, direct demonstration that pulsatile GnRH affects gonadotropin β -subunit gene transcription *in vivo* has been somewhat elusive, as the action appears to be model-dependent [Turgeon JL *et al.*, 1996]. For example, similar results of increased induction of *LH β* - and *FSH β* mRNA synthesis by pulsatile GnRH have been indicated *in vivo* in the ewe [Hamernik DL & Nett TM, 1988; Molter-Gérard C *et*

et al., 1999] and in male rats [Marshall JC *et al.*, 1991]. In contrast, FSH β -, but not LH β , mRNA synthesis is induced by pulsatile GnRH in adult female or GnRH-deficient female rats [Gajewska A *et al.*, 2000; Weiss J *et al.*, 1990; Haisenleder DJ *et al.*, 1995; Kerrigan JR *et al.*, 1993] although cells from peripubertal females did show an increase in LH β mRNA [Haisenleder DJ *et al.*, 1993].

One possible explanation for the lack of induction of LH β - and FSH β transcription as seen in this and other studies may be due to the method used to generate GnRH pulses. For example, Bédécarrats & Kaiser used a perfusion system that allowed them to replicate the GnRH pulsatility occurring *in vivo* [Bédécarrats GY & Kaiser UB, 2003] whereas, in this study, a manual method was used. Another possibility could be differences in duration of pulsatile treatment. It was shown in one study that 20 h of pulsatile GnRH stimulation was necessary to obtain clear differential regulation of gonadotropin subunit gene promoter activity [Bédécarrats GY & Kaiser UB, 2003]. Also, differences in cell conditions may alter responsiveness, since cells in culture are exquisitely sensitive to conditions of cell growth [Sealfon SC, via personal communication]. Unlike GnRH-1, pulsatile stimulation with GnRH-2 to measure transcription of the gonadotropin β -subunit genes was not done prior to this study. Hence the results presented here for GnRH-2, indicating that GnRH-2 (10 nM) too, like GnRH-1, does not affect *bLH β* - or *oFSH β* promoter activity in L β T2 cells after 6 h pulsatile stimulation, are novel.



Interestingly, recent studies demonstrated a link between the role of GnRH pulse frequency in LH β transcription and the number of GnRHRs on the cell-surface [Kaiser UB *et al.*, 1997a; Bédécarrats GY & Kaiser UB, 2003]. The highest GnRHR numbers occur at those GnRH pulse frequencies that preferentially stimulate LH β gene transcription, with lower levels occurring at slower frequencies that are associated with preferential FSH β gene expression [Kaiser UB *et al.*, 1997b; Bédécarrats GY & Kaiser UB, 2003]. *GnRHR* gene expression is also dependent on GnRH pulse frequency [Bédécarrats GY & Kaiser UB, 2003; Schally AV *et al.*, 1995; Pinski J *et al.*, 1996; Halmos G *et al.*, 1996]. Whereas pulsatile GnRH seems to increase GnRHR number, continuous GnRH stimulation induces a down-regulation of receptor number and a decrease in *GnRHR* gene expression [Bédécarrats GY & Kaiser UB, 2003; Schally AV *et al.*, 1995; Pinski J *et al.*, 1996; Halmos G *et al.*, 1996], which could explain the need for pulsatile stimulation in some of the above-mentioned studies in the absence of co-transfected GnRHR cDNA.

Endogenous GnRHR-I levels in L β T2 cells

Given the lack of response of the *bLH β* - and *oFSH β* promoter-reporters to GnRH-1 and GnRH-2 via the endogenous GnRHR-I in L β T2 cells, even when stimulating cells pulsatile, the question of whether the receptor is expressed and active in the L β T2 cells as cultured in our laboratory, is relevant. Although the results obtained for continuous GnRH-1 stimulation are in agreement with the literature, the results for pulsatile administration differ, raising the question above. The present binding results showing no significant binding of a GnRH analogue in L β T2 cells (not shown) would suggest that, if endogenous GnRHR-I is present, expression levels for this receptor are very low in these cells. However, others have measured GnRH binding in L β T2 cells in the absence of exogenous GnRHR [Bédécarrats GY & Kaiser UB, 2003]. Furthermore, using IP assays, Kakar SS *et al.* [2003] demonstrated that the GnRHRs on the cell membranes of L β T2 cells are high affinity receptors and are biologically active.

Two lines of evidence suggest that GnRHR-I protein is indeed expressed and active in this study in L β T2 cells. Firstly, GnRHR-I mRNA could be detected by Northern blot analysis in these cells (not shown), and, secondly, α -subunit mRNA levels responded to GnRH-1 and GnRH-2 administered together with PACAP, whereas PACAP alone had no effect (figure 37, A), in the absence of transfected exogenous GnRHR cDNA. It may be that endogenous GnRHR-I levels are lower in the cells cultured in our laboratory as compared to those in other labs. This is further strengthened by evidence from the literature that the response of pituitary gonadotropes to GnRH correlates, at least in part, with the density of GnRHRs on the cell surface [Loumaye E & Catt KJ, 1982; Kaiser UB *et al.*, 1995; Bédécarrats GY & Kaiser UB, 2003]. Nevertheless, the findings in the present study of a lack of response of *bLH β Luc* and *oFSH β Luc* or LH β and FSH β mRNA levels with a relatively low level of endogenous GnRHR-I suggest that these low levels of GnRHR-I are insufficient to cause a transcriptional effect on endogenous or on transfected promoters and also make the interpretation of results with transfected GnRHRs easier without a high background due to endogenous receptors. However, it should be noted that low levels of endogenous receptors may possibly modulate the response of transfected receptors, without having an effect on their own.

Expressed GnRHR-I and GnRHR-II levels in L β T2 cells

The calculated number of cell-surface receptors in L β T2 cells overexpressing the GnRHR-I was approximately 10-fold greater than that of cells overexpressing the GnRHR-II. Due to these differences in GnRHR number, it is hard to make a direct comparison of the ability of a specific GnRH ligand (GnRH-1 or GnRH-2) to induce *bLH β* or *oFSH β* promoter-reporter activity via overexpressed GnRHR-I as compared to overexpressed GnRHR-II, i.e. to compare potencies of the two receptor subtypes. This is further complicated by the different affinities of a specific ligand for the two different GnRHR subtypes (GnRH-1: EC₅₀ at human GnRHR-I, 2.81 \pm 0.17 nM and at marmoset and monkey GnRHR-II, 42.6 \pm 3.19 nM and 337 \pm 96 nM, respectively. GnRH-2: EC₅₀ at human GnRHR-I, 26.1 \pm 4 nM and at marmoset and monkey GnRHR-II, 1.07 \pm 0.04 nM and 0.86 \pm 0.18 nM, respectively) [Millar R *et al.*, 2001; Neill JD *et al.*, 2001]). Nevertheless, certain comparisons can be made, as discussed below.

Transcriptional regulation of *bLH β* - and *oFSH β* -subunit genes via overexpressed GnRHR-I or GnRHR-II in L β T2 cells

In the current study the effects of GnRH-1 and GnRH-2 on *bLH β* - and *oFSH β* promoter-reporter activity in the presence of overexpressed GnRHR-I or GnRHR-II were compared in L β T2 cells. Unlike the results obtained for endogenous GnRHR-I in L β T2 cells, both GnRH-1 and GnRH-2 up-regulated *bLH β* - and *oFSH β* promoter-reporter activity via overexpressed GnRHR-I or GnRHR-II in L β T2 cells, under conditions of continuous and pulsatile hormonal administration. The reasons for this difference may be that transcriptional regulation is only obtained at receptor levels higher than those present in the L β T2 cells grown in our lab (discussed above). Furthermore, pulsatile administration of GnRH-1 or GnRH-2 was not required for *bLH β* - or *oFSH β* Luc stimulation in L β T2 cells overexpressing GnRHR-I or GnRHR-II in this study. This may be due in part to the fact that the GnRHR expression plasmids are driven by CMV promoters, which continuously produce GnRHR and are most likely not down-regulated by GnRH.

The physiological significance of the results with overexpressed GnRHRs needs to be interpreted with caution. It is clear that GnRHR-I levels vary *in vivo* during the reproductive cycle [Kaiser UB *et al.*, 1993; Bauer-Dantoin AC & Jameson JL, 1995; Yasin M *et al.*, 1995]. For example, it has been reported that the GnRHR-I number can be as low as 500 per cell in progesterone-treated sheep gonadotropes and as

high as 15000–20000 per cell after treatment with oestradiol, inhibin, or both [Laws SC *et al.*, 1990a; Laws SC *et al.*, 1990b]. The total GnRHR levels reached in the present study in L β T2 cells expressing exogenous GnRHR-I or GnRHR-II were respectively about 10-fold and 100-fold higher than the maximum levels reported above. It is therefore unlikely that the expressed receptor numbers obtained in this study mimic the physiological levels.

Transcriptional regulation of bLH β via overexpressed GnRHR-I

A general trend was observed whereby GnRH-1 resulted in a greater induction of *bLH β* promoter-reporter activity as compared to GnRH-2 when stimulating cells with 10 nM for 6 h, either continuous or at a slow pulse frequency (1 pulse/2 h) (figures 42 & 43), in the presence of overexpressed GnRHR-I. A high pulse frequency treatment (1 pulse/0.5 h) (not shown) or continuous stimulation with 100 nM (figure 41) resulted in similar *bLH β* promoter-reporter activities via GnRH-1 and GnRH-2, showing a clear difference depending on continuous vs. pulsatile administration of hormone.

These results can be interpreted in terms of the greater affinity for GnRH-1 at the GnRHR-I as compared to GnRH-2 (EC_{50} at human GnRHR-I: GnRH-1, 2.81 ± 0.17 nM; GnRH-2, 26.1 ± 4 nM) [Millar R *et al.*, 2001] so that, at a low concentration (10 nM), GnRH-1 is a more potent stimulator of *bLH β* promoter-reporter activity due to greater occupancy of the membrane GnRHR-Is as compared to GnRH-2. However, at a high concentration (100 nM) the GnRHR-I would be saturated by both hormones, with the resulting similar maximal activity suggesting that GnRH-1 and GnRH-2 have the same efficacy (i.e. maximal response) for activation of the *bLH β* promoter-reporter via GnRHR-I.

Interestingly, it was found that GnRH-2 increased *bLH β* promoter-reporter activity to levels approximately 50% higher than those obtained with GnRH-1 after 18 h continuous stimulation using a 100 nM of hormone, via overexpressed GnRHR-I (figure 44, A). Given the result of similar responses at 6 h, one possible explanation would be that GnRH-2 is more stable after 18 h in culture as compared to GnRH-1 due to its longer half-life [Tsai P & Licht P, 1993; Licht P *et al.*, 1994], hence the bioavailability of GnRH-2 after 18 h would exceed that of GnRH-1.

There are no reports in the literature of a comparison of the effects of GnRH-1 and GnRH-2 on *LH β* transcriptional regulation in the presence of overexpressed GnRHR-

In vivo or *in vitro*. Single studies have determined the effect of GnRH-1 on *LHβ* transcriptional activity in cultured pituitary cells transiently [Kaiser UB *et al.*, 1995; Bédécarrats GY & Kaiser UB, 2003] or stably [Saunders BD *et al.*, 1998] transfected with a GnRHR expression vector. For example, in LβT2 cells co-transfected with an *LHβ* promoter-reporter construct and the rat GnRHR-I cDNA, continuous stimulation with GnRH-1 resulted in a significant increase in *LHβ* promoter-reporter activity with no evidence of down-regulation of the response after 10 h [Bédécarrats GY & Kaiser UB, 2003]. A 6 h stimulation with a GnRH-1 agonist (100 nM) resulted in an 8-fold stimulation of *LHβ* promoter-reporter activity in GH₃ cells transfected with a rat GnRHR-I expression vector [Kaiser UB *et al.*, 1995]. Likewise, in GGH3 cells (rat GH3 cells constitutively expressing the rat GnRHR) and transiently transfected with a *luciferase* reporter gene controlled by the *LHβ* gene promoter, a 6 h treatment with GnRH-1 agonist (100 nM) resulted in a ~5-fold increase in *LHβ* promoter-reporter activity as compared to control [Saunders BD *et al.*, 1998]. The fold inductions of *bLHβLuc* observed in the present study using a 100 nM GnRH-1 correspond to that reported in the literature. Results in the present study showing that GnRH-2 is able to regulate *bLHβ* transcription via GnRHR-I are novel.

Transcriptional regulation of bLHβ via overexpressed GnRHR-II

GnRH-2 resulted in a marked greater induction of *bLHβ* promoter-reporter activity compared to GnRH-1 in the presence of overexpressed GnRHR-II after 6 h stimulation, independent of hormone concentration or method of administration (figure 40, and figure 43, A). This result suggests that GnRH-2 is more efficacious than GnRH-1 for activation of the *bLHβ* promoter via GnRHR-II, since at 100 nM hormone, when the receptor would be fully saturated with either hormone, the maximal response for GnRH-2 is greater than that for GnRH-1. This result thus differs for that obtained with GnRHR-I, which does not appear to differ in the maximal response for *bLHβ* promoter regulation via GnRH-1 vs. GnRH-2, as described above.

However, the finding that after 18 h continuous stimulation the obtained fold induction of *bLHβ* promoter activity with GnRH-1 and GnRH-2 via GnRHR-II was similar, is hard to explain. If the half-life of GnRH-2 is greater than that of GnRH-1 [Tsai P & Licht P, 1993; Licht P *et al.*, 1994] and, as proposed above, GnRH-2 is more efficacious for *bLHβ* activation than GnRH-1 via GnRHR-II, one would expect that for longer times, there would be even greater activity for GnRH-2 than for GnRH-1. Possibly time-dependent variation in levels of receptor expressed on the cell surface

may play a role in these time-dependent effects [Bédécarrats GY & Kaiser UB, 2003], or the involvement of different regions of the *bLH β* promoter or other time-dependent signalling pathways [Kaiser UB *et al.*, 1995; Vasilyev VV *et al.*, 2002a].

Whatever the explanation, it is clear that the time, dose and method of hormonal administration can have a significant effect on the relative effects of GnRH-1 vs. GnRH-2 via GnRHR-I and GnRHR-II, on *bLH β* promoter activity. This is likely to be physiologically relevant, and suggests that the response to GnRH hormones can be regulated very finely via changing several parameters, to achieve exquisite control. These results showing a difference in the abilities of GnRH-1 vs. GnRH-2 to stimulate *bLH β* transcriptional activity via GnRHR-II are novel since there are no reports in the literature on the transcriptional regulation of *LH β* via GnRH-1 or GnRH-2 in the presence of overexpressed GnRHR-II *in vivo* or *in vitro*.

Ratio of GnRHR subtypes and likely effects on LH β transcription

One factor that is likely to affect the response of a cell to GnRH-1 vs. GnRH-2, that has not been investigated in the present study, would be the relative levels of GnRHR-I vs. GnRHR-II. If, as shown in this work, GnRH-2 is more efficacious than GnRH-1 in activating *LH β* promoter activity via GnRHR-II, then changes in relative receptor levels would most likely change the *LH β* mRNA levels without changes in hormone levels. For example, in species where both the GnRHR-I and the GnRHR-II are expressed such as monkey [Neill JD *et al.*, 2001; Millar R *et al.*, 2001; Accession AF353988 and AF156930, respectively], the efficacy of GnRH-1 vs. GnRH-2 in *LH β* transcriptional regulation would depend on the ratio between GnRHR-I and GnRHR-II expressed in pituitary gonadotropes.

Transcriptional regulation of oFSH β via overexpressed GnRHR-I or GnRHR-II

In contrast to *bLH β* , results of this study indicated that, in general, GnRH-1 and GnRH-2 possess equal abilities to stimulate *oFSH β* promoter-reporter activity in the presence of overexpressed GnRHR-I and overexpressed GnRHR-II in L β T2 cells (figures 41, B, 42, B, & 49, B). The only exception, where GnRH-2 resulted in a marked greater induction of *oFSH β* promoter-reporter activity as compared to GnRH-1, was seen in L β T2 cells overexpressing the GnRHR-II and stimulated pulsatile with 10 nM for 6 h at a slow pulse frequency (1 pulse/2 h) (figure 43, B). Once again, this result clearly shows that the relative effects of GnRH-1 vs. GnRH-2 can be influenced by the method of hormonal administration, in this case pulse frequency, suggesting a

mechanism for fine control of the pituitary response. No reports are found in the literature where the effects of GnRH-1 and GnRH-2 on *FSH β* gene transcription in the presence of overexpressed GnRHR-I or GnRHR-II are compared. Like *LH β* , single studies have determined the effect of GnRH-1 on *FSH β* transcriptional activity in the presence of co-transfected GnRHR-I [Kaiser UB *et al.*, 1995; Saunders BD *et al.*, 1998; Bédécarrats GY & Kaiser UB, 2003]. Kaiser UB *et al.* [1995], after stimulating cells with continuous GnRH-1 agonist (100 nM) for 6 h, observed a 4-fold stimulation of *FSH β* promoter-reporter activity in GH₃ cells transfected with a rat GnRHR-I expression vector. Furthermore, Saunders BD *et al.* [1998] have demonstrated a 3-fold increase in *luciferase* expression in response to a 6 h continuous treatment with 100 nM GnRH-1 agonist in GGH3 cells transiently transfected with *FSH β Luc*. These values in the literature of the fold induction obtained with GnRH-1 for *FSH β Luc* are consistent with results of this study.

Ratio of oFSH β - to bLH β -subunit promoter-reporter activity via GnRH-1 and GnRH-2 in L β T2 cells

In this study a greater fold induction (actual fold stimulation) of *bLH β* promoter-reporter activity via GnRH-1 or GnRH-2 as compared to the fold induction of *oFSH β* promoter-reporter activity was observed when overexpressing GnRHR-I or GnRHR-II. However, the relevance of this is unclear as this could simply reflect differences in the strength of promoters in the respective *luciferase* expression vectors. Furthermore, basal (in absence of hormone) *oFSH β* promoter-reporter activity was higher as compared to *bLH β* possibly due to stimulation of *oFSH β Luc* in the absence of GnRH. Be that as it may, what is of great interest and where comparisons are relevant and likely to be physiologically significant, is the ratio of *oFSH β* to *bLH β* -subunit promoter-reporter activity via GnRH-1 and GnRH-2.

Ratio of oFSH β - to bLH β -subunit promoter-reporter activity in the presence of overexpressed GnRHR-I in L β T2 cells

When comparing the ratio between *oFSH β* - and *bLH β* promoter-reporter activity obtained with GnRH-2 vs. GnRH-1 via overexpressed GnRHR-I, it was found that the *oFSH β Luc*:*bLH β Luc* ratio was higher with GnRH-2 as compared to the same concentration of GnRH-1 after 6 h, independent of the method of hormonal administration (table 9). These results indicate that induction of *oFSH β* promoter-reporter activity is favoured over *bLH β* promoter-reporter activity by GnRH-2 via

GnRHR-I. In the present study it can be seen that this elevated *oFSH β Luc:bLH β Luc* ratio with GnRH-2 is a direct result of decreased *bLH β* promoter-reporter activity since *oFSH β* promoter-reporter activity was similar via GnRH-2 as compared to GnRH-1 in the presence of overexpressed GnRHR-I. Per definition these results would qualify GnRH-2 as a selective regulator of *FSH β* expression via the GnRHR-I. The demonstration in this study that GnRH-2 is capable of increasing the ratio of *oFSH β* - to *bLH β* transcription after 6 h as compared to GnRH-1 via the GnRHR-I, both when administered continuously or in a pulsatile fashion, is, to the best of our knowledge, the first report showing that GnRH-2 is a selective regulator of *FSH β* transcriptional activity via GnRHR-I. Others have indicated on protein level that GnRH-2 could play a role in the alteration of the FSH-to-LH ratio [Millar R *et al.*, 2001; Millar RP, 2003]. For example, in rams, GnRH-2 was a less effective stimulator of FSH secretion than GnRH-1, yet the ratio of circulating FSH to LH was approximately 2-fold higher following GnRH-2 than with GnRH-1 [Millar R *et al.*, 2001]. Also, in early studies of pituitary cells from mature hens, GnRH-2 was found to have a 2-fold greater potency to release FSH vs. LH when compared to stimulation by chicken GnRH-1 [Millar RP, 2003].

Ratio of oFSH β - to bLH β -subunit promoter-reporter activity in the presence of overexpressed GnRHR-II in L β T2 cells

A general trend was observed whereby GnRH-1 resulted in a greater *oFSH β Luc:bLH β Luc* ratio as compared to GnRH-2 via overexpressed GnRHR-II, when stimulating cells for 6 h, either continuous (10 nM & 100 nM) or at a high pulse frequency (1 pulse/0.5 h) (table 9). In contrast, GnRH-2 resulted in a greater *oFSH β Luc:bLH β Luc* ratio after 6 h treatment at a low pulse frequency (1 pulse/2 h), showing a clear difference in the *oFSH β Luc:bLH β Luc* ratio depending on continuous vs. pulsatile administration of hormone. Similar to results with overexpressed GnRHR-I, these results showing differences in the ratios between FSH β and LH β via GnRH-1 as compared to GnRH-2 on transcriptional level are novel, and add to the understanding of the dynamic interplay between GnRH-1 and GnRH-2 via a specific GnRHR subtype to alter the cellular response.

Collectively, these observations clearly point to the possibility that the differential regulation of gonadotropin subunit gene expression by GnRH observed *in vivo* may be mediated by two different GnRH peptides acting through a single or two different

receptor(s). This provides a physiological mechanism whereby GnRH can differentially regulate the gonadotropin hormones in the same cell.

PACAP

The current study also investigated the possible modulatory role of PACAP on GnRH-1 and GnRH-2 regulation of bLH β - and oFSH β mRNA and reporter activity of the respective transfected promoters in L β T2 cells. L β T2 cells express PACAP-specific type 1 (PAC $_1$) receptors that respond to PACAP via Ca $^{2+}$ mobilisation and activation of PLC and adenylate cyclase [Rawlings SR & Hezareh M, 1996]. It has been demonstrated that low concentrations of PACAP will preferentially stimulate the production of cAMP via the protein kinase A (PKA) pathway (EC $_{50}$ \approx 3 nM) while higher PACAP concentrations will also stimulate the IP production via a PKC pathway (EC $_{50}$ \approx 20 nM) [Schomerus E *et al.*, 1994; Rawlings SR & Hezareh M, 1996]. There is substantial evidence that PACAP alone and in combination with GnRH-1 regulates LH and FSH protein synthesis and release via post-transcriptional mechanisms [Rawlings SR & Hezareh M, 1996]. For example, one report in the literature suggested that PACAP and GnRH are additive in their effects on LH release, but there is significant evidence to indicate that PACAP increases GnRH-stimulated α -, LH β - and FSH β -subunit release in a synergistic manner from both rat gonadotropes and α T3-1 mouse gonadotrope-derived cells [Tsuji T & Winters SJ, 1995; Rawlings SR & Hezareh M, 1996]. Moreover, in rat pituitary cultures PACAP on its own was shown to induce an immediate accumulation of LH and free α -subunit protein in the extracellular medium [Tsuji T *et al.*, 1994]. In addition to post-transcriptional effects, there is evidence for a modulatory role for PACAP at the level of mRNA and transcription [Tsuji T *et al.*, 1995]. For example, one of the actions of PACAP on transcriptional level is to increase α -subunit mRNA concentrations in the pituitary [Schomerus E *et al.*, 1994; Tsuji T *et al.*, 1994], leading to the hypothesis that PACAP is partly responsible for maintaining the high levels of α -subunit peptide, relative to the β -subunits [Tsuji T *et al.*, 1995]. Thus PACAP increases α -subunit gene transcription, although less effectively than GnRH [Tsuji T *et al.*, 1995]. Other indications are that PACAP increases α -subunit mRNA additively with GnRH, and lengthens LH β mRNA, presumably at the polyA tail [Tsuji T *et al.*, 1994]. Furthermore, in L β T2 cells, PACAP increases steady-state levels of FSH β mRNA [Fujii Y *et al.*, 2002]. In contrast to results obtained in L β T2 cells of an increase in FSH β transcription, PACAP reduces FSH β mRNA levels in rat pituitary monolayer cultures [Winters SJ *et al.*, 1997] and in rat pituitary cells perfused with pulses of

GnRH [Tsuji T *et al.*, 1994]. The effect of PACAP together with GnRH-1 on *FSH β* gene transcription is less clear from the literature. Furthermore, the synergistic effects of PACAP on GnRH-2-mediated regulation of gonadotropin subunit gene expression have not been studied thus far. Thus several questions remain unanswered regarding the role of PACAP in modulating effects of GnRH-1 and GnRH-2 on transcriptional regulation of *LH β* and *FSH β* promoters in pituitary gonadotropes.

In this study it was shown that PACAP treatment alone did not affect *bLH β* - or *oFSH β* promoter-reporter activities after 6 h continuous stimulation (figure 46). Northern blot analysis on L β T2 cells stimulated with 20 nM PACAP for 6 h revealed that LH β mRNA levels are also unaffected (figure 38). However, FSH β mRNA was significantly increased (figure 39, A), consistent with the findings of Fujii Y *et al.* [2002]. The discrepancy between results of the promoter-reporter assays and that of Northern analysis may be due to effects on mRNA turnover, or possibly the presence of regulatory elements in the endogenous promoter not contained in the transfected promoter-reporter.

Modulatory effects of PACAP on GnRH-mediated bLH β - and oFSH β promoter-reporter activity in L β T2 cells

While neither *bLH β* nor *oFSH β* promoter-reporter activity was significantly affected by PACAP alone at low or high concentrations, PACAP did modulate the response of the *bLH β* promoter to GnRH via both GnRHR-I and GnRHR-II (figure 46, A), and of the *oFSH β* promoter via GnRHR-I but not via GnRHR-II (figure 46, B). Results presented show that PACAP has a modulatory effect on the GnRH-1 and GnRH-2 response via GnRHR-I of the *bLH β* promoter, which occurs after 6 h of continuous stimulation at 3 nM but not at 20 nM PACAP. The modulatory effect at 3 nM PACAP occurs by either increasing the GnRH-1 response or by decreasing the GnRH-2 response. Since a PACAP effect was observed only at 3 nM, this effect would most likely involve activation of the cAMP pathway by PACAP, and not an alteration in IP production by PACAP. Similarly, PACAP is also shown to have a modulatory role on the GnRH response via GnRHR-II on the *bLH β* promoter activity by, similar to GnRHR-I, reducing the response to GnRH-2 or increasing the response to GnRH-1. This action in the presence of GnRHR-II is however independent upon the dose of PACAP used, suggesting a different mechanism to that which occurs for GnRHR-I, and which can possibly occur via either a PACAP-induced PKA pathway or both PKA

and PKC pathways. PACAP also modulates the response of the *oFSH β* promoter to GnRH via the GnRHR-I in a dose-dependent fashion, but unlike the results obtained for *bLH β* with GnRHR-I, it does not alter the relative effects of GnRH-1 vs. GnRH-2 (figure 46, B). In this case PACAP appears to inhibit the effects of both hormones at 3 nM PACAP, but not at 20 nM PACAP, suggesting an involvement of the PACAP-induced PKA pathway. However, PACAP has no modulatory effect on the *oFSH β* response to both GnRH hormones via the GnRHR-II (figure 46, B).

Results of this study of the modulatory effect of PACAP on GnRH-1- and GnRH-2 transcriptional regulation of *LH β* demonstrate yet another dimension of the complexity of the way in which the gonadotropins are differentially regulated in the same gonadotrope cells. Collectively, these findings indicate that the local pituitary milieu is the means by which changes in GnRH input lead to differential release patterns of LH and FSH [Padmanabhan V & McNeilly AS, 2001], which includes the presence or absence of GnRH-1, GnRH-2, PACAP and any or both GnRHR subtypes, at changing concentrations. This study is the first to show the combined effects of PACAP and GnRH-1 on *FSH β* transcriptional regulation. Furthermore, this study is the first report on the modulatory role of PACAP on GnRH-2 regulation of *LH β* and *FSH β* . Also, for the first time it is shown that differences in PACAP concentration may be involved in selectively increasing or decreasing the GnRH response. Finally, it is demonstrated for the first time that the GnRHR-II might be involved in the modulatory role of PACAP on *LH β* transcriptional regulation.

α -subunit

Northern analysis of α -subunit mRNA in L β T2 cells revealed that α -subunit mRNA levels were unaffected by a 6 h continuous stimulation with GnRH-1 or GnRH-2 alone (figure 37, A). After a 24 h treatment with GnRH-1, α -subunit mRNA levels were similar to basal levels whereas GnRH-2 alone resulted in a decrease in mRNA levels (figure 37, B). Whereas the effect of GnRH-2 on α -subunit mRNA levels has not been described previously, the results of this study showing no increase in α -subunit mRNA are consistent with the reported lack of induction of α -subunit mRNA [Turgeon JL *et al.*, 1996] or protein [Liu F *et al.*, 2002a] by GnRH-1 agonists in L β T2 cells. Nevertheless, stimulation of L β T2 cells with continuous or pulsatile GnRH resulted in an increase in α -subunit promoter activity after 1- to 24 h in several other studies [Haisenleder DJ *et al.*, 1991; Fowkes RC *et al.*, 2002; Bédécarrats GY & Kaiser UB, 2003]. In α T3-1 cells, α -subunit promoter activity was maximally

increased after 4- to 6 h treatment with GnRH-1 agonist, followed by a return to baseline after 24 h [Chedrese PJ *et al.*, 1994; Kay TWH *et al.*, 1994].

Furthermore, in this study α -subunit mRNA was increased by 6 h continuous stimulation with PACAP alone or in combination with GnRH-1 or GnRH-2 (figure 37, A), similar to the findings of Tsujii T *et al.* [1994; 1995]. This stimulatory effect was lost after 24 h (figure 37, B). Thus, α -subunit mRNA levels would appear to be more responsive to a 6 h treatment with PACAP alone or in combination with GnRH-1 or GnRH-2 as compared to LH β - or FSH β mRNA levels in L β T2 cells. While some evidence from the literature indicates that GnRH stabilises α -subunit mRNA [Chedrese PJ *et al.*, 1994], results of the present study would suggest that α -subunit mRNA is insensitive to GnRH-1 and GnRH-2 treatment alone. However, PACAP may modulate the responsiveness of the α -subunit gene promoter to GnRH-1 and GnRH-2. It could be worthwhile to repeat some of the promoter-reporter studies performed on *bLH β* and *oFSH β Luc* with an α -subunit promoter-reporter construct to further investigate this possibility.

With one exception in figure 37, A, when using Bonferroni's post-test to compare all groups to all groups, there were no differences between treatments, other than those detected by Dunnet's post-test between a particular condition and control (figure 37, A, bar no 2). In some cases a difference observed between the non-treatment control and a treatment group using Dunnet's post-test was not observed when using Bonferroni's post-test (eg. figure 37, A, bar no 7 vs. bar no 2, figure 37, B, bar no 1 vs. bar no 2, figure 38, A, bar no 7 vs. bar no 2, and figure 39, A, bar no 5 vs. bar no 2 and bar no 7 vs. bar no 2). A possible explanation for this could be that the Bonferroni post-test is a more stringent test compared to Dunnet's post-test.

Comparison of the regulation of bLH β - and oFSH β promoter-reporter activity in L β T2 vs. COS-1 cells

In COS-1 cells, a similar lack of response in *bLH β* and *oFSH β* promoter-reporter activity was observed in the absence of exogenous GnRHR cDNA as seen in L β T2 cells, independent of hormone concentration or duration of treatment (figure 50, A & B). Results of this study of the induction of *bLH β* and *oFSH β* promoter-reporter activity in COS-1 cells (albeit at low levels) when overexpressing GnRHR-I or GnRHR-II (figure 50, A & B) indicate that these cells are able to regulate *bLH β* and *oFSH β* transcriptional activity and that this activity, as for L β T2 cells, depends upon a

critical minimum number of receptors on the cell surface. Pulsatile administration of GnRH-1 or GnRH-2 was not required for *bLH β Luc* stimulation in COS-1 cells overexpressing any GnRHR subtype, similar to results obtained in L β T2 cells. Furthermore, these results supply evidence that transcriptional regulation of the gonadotropin β -subunit genes is not limited to pituitary gonadotrope cells, which would be consistent with another report showing induction of *oFSH β* promoter-reporter activity (~3-fold) by GnRH-1 in COS-7 cells co-transfected with the mouse GnRHR-I after 12 h continuous stimulation using 100 nM [Strahl BD *et al.*, 1998]. Moreover, more than one study has even indicated the secretion of bioactive LH and FSH from COS-7 cells [Schmidt A *et al.*, 1999; Chopineau M *et al.*, 1999], suggesting that COS-7 (and possibly COS-1) cells also contain the cellular machinery to perform the necessary post-transcriptional and translational events for the expression of the gonadotropins.

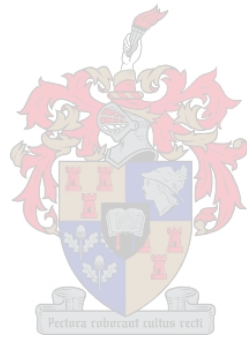
Whereas in general in L β T2 cells *bLH β* promoter-reporter activity was higher with GnRH-1 as compared to GnRH-2 via overexpressed GnRHR-I and higher with GnRH-2 as compared to GnRH-1 via overexpressed GnRHR-II, respectively, after 6 h, a similar discriminatory effect between GnRH-1 and GnRH-2 was observed in COS-1 cells overexpressing the GnRHR-II, but not the GnRHR-I (figure 50, A). Furthermore, similar to results of this study in L β T2 cells it was found that GnRH-1 and GnRH-2 possess equal abilities to stimulate *oFSH β* promoter-reporter activity in the presence of overexpressed GnRHR-I and overexpressed GnRHR-II in COS-1 cells, independent of hormone concentration or duration of treatment (figure 50, B).

Concerning time-dependent regulation of *bLH β* promoter-reporter activity it was found that a similar down-regulation of *bLH β* promoter-reporter activity as observed in L β T2 cells after prolonged (18 h) treatment was observed in COS-1 cells only when overexpressing the GnRHR-II, but not when overexpressing GnRHR-I in this study (not shown). This difference observed between L β T2 vs. COS-1 cells in the regulation of *bLH β* promoter-reporter activity is most likely due to the fact that distinct sets of G proteins, kinases, transcription factors and other classes of molecules are expressed in the two different cell-lines and highlights the danger of extrapolating results obtained in different cellular systems.

CHAPTER 5

**CONCLUDING DISCUSSION
&
FUTURE PROSPECTS**





Recent evidence for the presence of non-mammalian GnRHR-II cDNAs, together with the finding that these receptors are selective for GnRH-2 [Troskie B *et al.*, 1997; Illing N *et al.*, 1999], have triggered the interest in the existence of a human GnRHR-II. The presence of a GnRH-2-selective receptor in humans might explain earlier observations that GnRH-1 antagonists behave like agonists in reproductive tissue tumors [Eidne KA *et al.*, 1987; Emons G *et al.*, 1997] since GnRH-1 antagonists were found to have agonistic effects on the GnRHR-II [Millar R *et al.*, 2001]. It is possible that GnRH-1 antagonists interact with the putative GnRHR-II if it is present and functional in humans [Gründker C *et al.*, 2002]. Using the available sequence information for part of exons 2 and 3 [Millar R *et al.*, 1999] as well as some exon 1 sequence information of a putative human *GnRHR-II* gene, RNA from numerous available human tissue and cell types was screened for the presence of a GnRHR-II transcript containing all three exons, using dot blot analysis, RT-PCR and *in situ* hybridisation (Chapter 2 and Appendix 5). The present study indicated the widespread expression of a human GnRHR-II transcript containing exons 2 and 3 but lacking exon 1, similar to the findings of a previous report [Millar R *et al.*, 1999]. Human ejaculate was the only source where a potential full-length (containing all three exons), sense, intronless transcript was detected. From human ejaculate RNA a GnRHR-II cDNA sequence stretching from -392 relative to the translation start within exon 1 until almost the end of TM6 in exon 3 was assembled, using results of 5' RACE and RT-PCR (Chapter 2 and Appendix 5). *In situ* hybridisation further revealed that these transcripts are present in mature sperm within the ejaculate (Chapter 2 and Appendix 5). Whereas other reports demonstrated a functional role for GnRH-1 in human sperm during spermatogenesis and in the process of fertilisation [Morales P, 1998; Morales P *et al.*, 2000], which would suggest the presence of the GnRHR-I on the sperm cell surface, this is the only report of the finding of GnRHR-II transcripts in the male reproductive tract (Chapter 2 and Appendix 5).

Subsequent to this study a full-length GnRHR-II cDNA was cloned from other primates and demonstrated to be functional [Neill JD *et al.*, 2001; Millar R *et al.*, 2001]. Comparison of the human sperm GnRHR-II sequence obtained in this study with the protein coding sequences of the published primates GnRHR-II sequences revealed that the human sequence contains a frameshift mutation within the coding region in exon 1 and a premature TGA translation stop signal in exon 2, within the region encoding ECL2. The requirement for a nt insertion in exon 1 and the premature translation stop signal in exon 2 were also present in the *GnRHR-II* gene

on chromosome 1 (accession AL160282). While the functionality of a protein product resulting from translation of this human GnRHR-II transcript is questionable, evidence exists for the presence of a specific GnRH-2-responsive system in humans [Leung PCK *et al.*, 2003; Enomoto M *et al.*, 2004b]. For example, cell proliferation studies show that GnRH-2 inhibits the growth of human ovarian cancer cells that express transcripts for GnRHR-II but not for GnRHR-I, indicating that the GnRHR-II binding sites are functional in these cells [Leung PCK *et al.*, 2003]. Furthermore, in a recent study by Enomoto M *et al.* [2004b] it was indicated that GnRH-1 as well as GnRH-2 have both stimulatory and inhibitory effects on cell proliferation. Growth of a human endometrial carcinoma- and a human prostatic carcinoma cell-line is decreased by GnRH-1 and GnRH-2, whereas that of a human prostatic carcinoma- and a human mature leukemic cell-line is increased [Enomoto M *et al.*, 2004b]. However, the sensitivities of cells to the stimulatory and inhibitory effects differ with GnRH-1 as compared to GnRH-2, such that GnRH-2 is a stronger inhibitor while GnRH-1 is a stronger stimulator of cell growth, which would strongly suggest that the inhibitory and stimulatory effects of GnRH occur via different GnRHRs i.e. that the cognate GnRHR-II is also functional in humans [Enomoto M *et al.*, 2004b]. Interestingly, in a subsequent study performed by this same group it was indicated that both GnRH-1 and GnRH-2 affect actin cytoskeleton remodeling and cell migration in human prostatic carcinoma cell-lines and that these effects are mediated via the GnRHR-I [Enomoto M *et al.*, 2006]. It remains unknown whether the human GnRHR-II is involved in the effects of GnRH on cell proliferation and sperm function. Clearly further work is required to ascertain the precise nature of potentially functional protein products of the disrupted yet transcriptionally active human *GnRHR-II* gene [Pawson AJ *et al.*, 2005], possibly with the use of GnRHR-II-specific antibodies.

To further investigate the possibility that a local GnRH/GnRHR network is present in mature sperm, the distribution of transcripts for GnRHR-I, GnRH-1 and GnRH-2 in human and monkey ejaculate was also determined (Chapter 3). The results presented here indicate that transcripts for GnRH-1, GnRH-2 and the GnRHR-I are expressed in human ejaculate in addition to transcripts for the GnRHR-II (Chapter 3). These findings would be consistent with and support the view that locally produced GnRH may affect spermatogenesis and spermiogenesis and may increase the probability of conception in an autocrine/paracrine manner *in vivo*. Based on these findings, it would be interesting to compare the ability of GnRH-2 to increase sperm-ZP binding, and thereby affect fertility and reproduction, with that of GnRH-1, in the

presence of GnRHR-I- and/or GnRHR-II-specific antagonists, to further examine the functionality of a human GnRHR-II in sperm. This could be done using a well-described hemizona assay *in vitro* [Morales P, 1998].

In addition to the finding of transcripts of the GnRHR-II in human sperm, data is presented here for the first time to demonstrate that baboon, a non-human primate species, also expresses GnRHR-II transcripts in pituitary and extrapituitary tissues (Chapter 2). An extensive report is given of the various sense GnRHR-II transcripts found in human and non-human primates, which suggests that alternative splicing is an important mechanism whereby expression of the *GnRHR-II* is regulated across tissues and species (Chapter 2). Furthermore, evidence is presented that in non-human primates, similar to humans, an antisense GnRHR-II transcript with intron 2 retained is widely expressed. The finding of such a transcript across species and in numerous tissues would suggest that it has an important function, possibly by modulating the expression of the GnRHR-I as proposed by Pawson AJ *et al.* [2005].

Taken together, results of this study (Chapter 2 & 3), together with reports in the literature, would suggest that, if the human GnRHR-II protein is expressed as a functional receptor, such a receptor would be involved in cell proliferation, to stimulate or inhibit the growth of GnRH-2-responsive cancerous cells and possibly to stimulate spermatogenesis and sperm maturation and increase sperm-egg binding.



Towards further understanding of the function of GnRH-1 and GnRH-2 in the pituitary and the respective roles of the GnRHR-I relative to the GnRHR-II, transcriptional regulation of the gonadotropin subunit genes by GnRH was investigated (Chapter 4). There are numerous reports in the literature as to the effects of GnRH-1 on *LHβ* and/or *FSHβ* promoter activity and/or mRNA turnover via endogenous or expressed GnRHRs in various systems *in vivo* and *in vitro* (see Chapter 4), none of which determined the role of GnRH-2 in this regard. The present study is the first to show that GnRH-2 affects gonadotropin subunit gene expression in the LβT2 mouse pituitary gonadotrope cell-line via GnRHR-I and GnRHR-II, albeit a small effect under certain conditions, and supplies evidence that its ability to do so depends on a number of factors. These factors include the relative ratio of GnRHR-I and GnRHR-II, concentration of hormone used, method of administration and treatment duration, as well as varying concentrations of PACAP (Chapter 4). In addition, it is

demonstrated for the first time that GnRH-1 may affect gonadotropin subunit gene expression via GnRHR-II in addition to GnRHR-I.

Results of this study using GnRH-1 and GnRH-2 demonstrated a non-significant increase in *LH β* and *FSH β* mRNA levels as well as in *bLH β* and *oFSH β* transcriptional activity via endogenous GnRHR-I in L β T2 cells after 6 h continuous stimulation (Chapter 4). These results showing a small (not significant) transcriptional effect on *bLH β* by GnRH-1 and GnRH-2 via endogenous GnRHR-I (≤ 1.5 -fold) would be consistent with findings by others collectively showing that acute GnRH-1-induced LH β protein synthesis and LH secretion are more dependent upon new protein synthesis than new mRNA synthesis, with continuous stimulation via endogenous GnRHR-I in L β T2 cells [Wurmbach E *et al.*, 2001; Pernasetti F *et al.*, 2001; Nguyen KA *et al.*, 2004; Kakar SS *et al.*, 2003; Haisenleder DJ *et al.*, 1991]. However, a non-GnRH-associated increase in FSH β mRNA was observed in L β T2 cells after 24 h in culture in this study (Chapter 4). Furthermore, promoter-reporter studies revealed that basal (non-GnRH-associated) transcriptional activity of *oFSH β* is relatively high as compared to *bLH β* (Chapter 4). The finding in the present study of stimulation of *FSH β* transcription in the absence of GnRH is a first, and suggest that transcriptional effects via a non-GnRH-associated pathway may contribute to an observed non-GnRH regulated increase in FSH protein levels [Padmanabhan V & McNeilly AS, 2001].



Central to the reproductive field is the question of whether a specific FSH-releasing factor exists that would preferentially stimulate FSH β mRNA and/or FSH protein synthesis and release. Results in the literature concerning this issue are contradictory [reviewed in Millar RP, 2003]. Whereas other factors such as activin seem to be potent stimulators of FSH β mRNA [Padmanabhan V & McNeilly AS, 2001], results of this study are the first to establish that GnRH-2 may play such a discriminatory role on *FSH β* gene transcription via expressed GnRHR-I, but not GnRHR-II (Chapter 4). This is evident by comparison of the ratio of expression of transfected *oFSH β* and *bLH β* promoter-reporters via GnRH-1 with that of GnRH-2, but not when observing actual fold inductions of the respective promoters.

GnRH-2 results in a greater *oFSH β Luc:bLH β Luc* ratio as compared to GnRH-1, when overexpressing the GnRHR-I in L β T2 cells independent of the concentration of hormone used or whether hormones were administered continuously or in a pulsatile

fashion (Chapter 4). This preferential effect on *FSH β* promoter activity for GnRH-2 is not seen for overexpressed GnRHR-II, where the opposite preference is observed for most conditions tested. Thus GnRH-1 results in a greater *oFSH β Luc:bLH β Luc* ratio as compared to GnRH-2, when overexpressing the GnRHR-II in L β T2 cells with continuous stimulation or pulsatile administration at a high pulse frequency (1 pulse/0.5 h) for 6 h. These opposite selectivities for GnRHR-I and GnRHR-II on the ratios of *oFSH β Luc:bLH β Luc* promoter activity for GnRH-1 vs. GnRH-2 are intriguing and suggest another mechanism for fine control of gonadotropin regulation, which could occur by variation of relative GnRHR-I vs. GnRHR-II levels. Physiologically, it may be that the ratio of *FSH β* to *LH β* mRNA is more relevant than the absolute mRNA levels of the transcripts. These results correspond to effects seen on protein level as demonstrated by others [Millar R *et al.*, 2001; Millar RP, 2003].

When comparing the effects of GnRH-1 and GnRH-2 on transcriptional regulation of the *bLH β* and *oFSH β* -subunit genes individually, differences are observed (Chapter 4). In general, at lower concentrations (10 nM), GnRH-1 is a more potent stimulator of *bLH β* promoter activity as compared to GnRH-2 via overexpressed GnRHR-I for short-term (6 h) continuous exposure as well as for pulsatile stimulation at a frequency of one pulse every 2 h, most likely due to differences in the affinity for GnRH-1 as compared to GnRH-2 for the GnRHR-I. However, GnRH-1 and GnRH-2 result in a similar maximum fold stimulation of *bLH β* promoter activity after 6 h using saturating doses (100 nM) of hormone, showing that they have the same efficacy for regulation of *bLH β* promoter activity via GnRHR-I in this system (Chapter 4). In contrast, both saturating and sub-saturating concentrations of GnRH-2 result in a greater maximum response in *bLH β* promoter activity as compared to GnRH-1 via overexpressed GnRHR-II for 6 h, independent of whether hormone is administered continuously or in a pulsatile fashion. GnRH-2 thus seems to be a more efficacious stimulator of *bLH β* transcription via GnRHR-II, than GnRH-1 (Chapter 4). Results of this study further demonstrate that, in contrast to *bLH β* , GnRH-1 and GnRH-2 possess equal abilities at saturating and at non-saturating concentrations to stimulate *oFSH β* promoter activity via GnRHR-I and GnRHR-II, independent of the way in which hormone is presented, except at a slow pulse frequency treatment in the presence of GnRHR-II during which GnRH-2 results in a marked greater induction of *oFSH β* promoter-reporter activity as compared to GnRH-1.

In addition to the effects of GnRH-1 and GnRH-2, the effect of PACAP alone or PACAP together with GnRH-1 or GnRH-2 on gonadotropin subunit gene expression was investigated in the present study (Chapter 4). Results show that PACAP alone has no effect on endogenous α -, $LH\beta$ - and $FSH\beta$ -subunit mRNA levels or on transfected $bLH\beta$ - and $oFSH\beta$ -promoter activities after 6 h continuous treatment (Chapter 4). However, PACAP did modulate the response of the $bLH\beta$ promoter to GnRH via both GnRHR-I and GnRHR-II, and of the $oFSH\beta$ promoter via GnRHR-I but not via GnRHR-II (Chapter 4). Some of these effects were PACAP dose-independent, while others were PACAP dose-dependent and suggested differential regulation by lower concentrations of PACAP (3 nM) possibly via a PACAP-induced PKA pathway, vs. effects at 20 nM possibly via both PACAP-induced PKA and PKC pathways. Some modulatory effects of PACAP were different for GnRH-1 vs. GnRH-2, while others appeared to be equally effective for both hormones. The modulatory effects of PACAP were also different for $bLH\beta$ - vs. $oFSH\beta$, and for GnRHR-I vs. GnRHR-II, under some conditions. This study showing differences in the modulatory effect of PACAP on the $LH\beta$ - and $FSH\beta$ response to GnRH-1 as compared to GnRH-2 on transcriptional level is a first of its kind. Furthermore, the investigation into the role of the GnRHR-II in the PACAP response is also novel. Since it is evident from the results of this study that some of the effects of PACAP are concentration-dependent, future studies could include a repeat of the Northern blot analysis of endogenous α -, $LH\beta$ - and $FSH\beta$ -subunit mRNA levels but with the inclusion of 3 nM PACAP to also determine the effect of PACAP concentration on mRNA levels. It would be interesting to further investigate the kinase pathways and other downstream pathways involved in the PACAP modulatory effects.

Besides the role of peptide hormones on gonadotropin subunit gene expression, changes in GnRHR number have previously been proposed as a mechanism involved in the differential regulation of gonadotropin subunit gene expression [Kaiser UB *et al.*, 1995; Kaiser UB *et al.*, 1997a; Bédécarrats GY & Kaiser UB, 2003]. Although the current study is not a thorough investigation into the effect of varying GnRHR numbers on $LH\beta$ - and $FSH\beta$ transcriptional regulation via GnRH-1, GnRH-2 and PACAP, by comparison of results obtained in the presence of endogenous GnRHR levels with that obtained with expressed mammalian receptors, it was demonstrated that gonadotropin β -subunit gene expression depends on a minimal number of receptors on the cell surface (Chapter 4). It would appear that the cells used in this study express endogenous GnRHR at too low levels to be able to

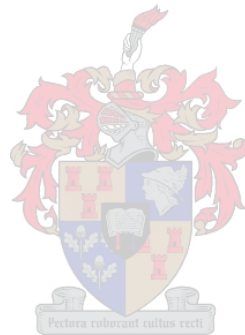
respond to a 6 h stimulation with GnRH-1 or GnRH-2 alone, independent of the concentration of hormone or the method of treatment used (Chapter 4). Although the physiological significance of the results with overexpressed GnRHRs is unclear, it is clear that GnRHR-I levels vary *in vivo* during the reproductive cycle [Kaiser UB *et al.*, 1993; Bauer-Dantoin AC & Jameson JL, 1995; Yasin M *et al.*, 1995], and thus it is possible that they reach similar levels to those obtained in cells expressing exogenous GnRHR in the present study. Ideally, these studies should be repeated in a pituitary cell-line or primary pituitary cells that express the GnRHR-I and the GnRHR-II endogenously to be able to compare the effects of GnRH-1 vs. GnRH-2 in a system where the two receptor subtypes are expressed at physiological levels. Alternatively, further studies could include experiments where the GnRHR-I and the GnRHR-II are co-transfected, to have more similar receptor levels of both, to investigate this interaction further. Other interesting variations of the promoter reporter studies and/or Northern blot analysis would include the addition of treatments with activin, inhibin and follistatin to further examine the regulation of *FSH β* (and possibly *LH β*) promoter activity and mRNA levels.

Since relatively little is known about the cell signalling pathways that mediate GnRH action at the level of gonadotropin-subunit gene transcription, it would be useful to design a study to compare the different signal transduction pathways as well as the second messengers involved in GnRH-1- vs. GnRH-2 signalling. Preferential sensitivity to distinct second messenger pathways and/or activation of different transcription factors is another possible mechanism whereby different GnRH pulse frequencies can regulate gonadotropin gene regulation [Vasilyev VV *et al.*, 2002b].

Collectively, results of this study clearly point to the possibility that the differential regulation of gonadotropin subunit gene expression by GnRH observed *in vivo* may be mediated by the presence of GnRH-1 and GnRH-2, at varying concentrations and released at varying pulse frequencies from the hypothalamus, acting via any or both GnRHR subtype(s). Furthermore, an added level of control is mediated via changes in GnRHR number or the relative expression of GnRHR-I vs. GnRHR-II on the cell surface, as well as by changing levels of PACAP released from the hypothalamus. These parameters all vary during the reproductive cycle, thereby suggesting a physiological mechanism for fine control of the pituitary response.

The findings presented in this thesis are significant since a thorough understanding of the factors that regulate *LH β* and *FSH β* gene expression is critical for the

development of efficient methods for fertility regulation and the treatment of a variety of reproductive disorders. Furthermore, they contribute to advancing the basic knowledge of regulation of gene expression.



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Table 4. Summary of distribution of human GnRHR-II transcripts detected by RT-PCR¹, Northern blot analysis² or dot blot analysis³.

Human tissue or cell type	Human GnRHR-II transcripts detected						
	Antisense exon 2-3 amplicon containing intron 2			Sense amplicon, possibly containing exon 1			
	[Millar R <i>et al.</i> , 1999] ¹	[Hutchinson E, 1997] ¹	This study ¹	[Millar R <i>et al.</i> , 2001] ²	[Neill JD <i>et al.</i> , 2001] ³	[Hutchinson E, 1997] ¹	This study ¹
Adult brain	Cerebellum, Hypothalamus, Whole brain.	Cerebellum, Whole brain.	Cerebellum, Cortex, Hypothalamus, Medulla, Midbrain, Pons.	Various brain parts.	Whole brain.		
Foetal brain	Whole brain.	Whole brain.	Cerebellum, Frontal lobe, Hypothalamus, Medulla, Midbrain, Olfactory bulb, Pons.				
Adult peripheral	Heart, Insulinoma, Kidney, Liver, Lymph node, Pancreas, Retina, Skeletal muscle, Spinal cord, Spleen, Thyroid.	Adrenals, Bone marrow, Cortex, Daudi, Insulinoma, Kidney, Liver, Lung, Lymph node, Retina, Spleen, Thyroid.	Kidney, Pituitary, Thyroid.	Heart, Pancreas.	Adrenal, Breast, Heart, Kidney, Large intestine, Liver, Lung, Pancreas, Pituitary, Skeletal muscle, Small intestine, Spleen, Stomach, Thymus, Thyroid.		
Foetal peripheral			Adrenals, Lumbar sympathetic chain, Pituitary, Retina.				

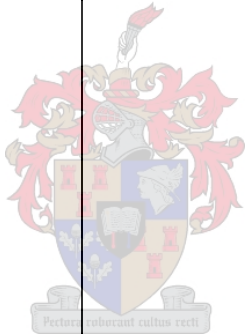
Reproductive	Ovary, Placenta, Testis, Uterus.	Placenta, Prostate, Testis, Uterus.	Ejaculate, Testis, Uterus.		Ovary, Placenta, Prostate, Testis, Uterus.		Ejaculate ¹ .
Carcinoma cell-lines or cells	MCF-7 breast.	CaCO2, MCF-7 breast, T47D.	HepG2 liver.		Colorectal, HeLa, Leukemia, Lung.		
Tissues and cells in which attempts were unsuccessful		hvec2, HUH-7, Liver, Small intestine.				Daudi, Foetal brain, Lymph node, MCF-7 breast, Placenta, T47D, Thyroid.	Adult tissues: Cerebellum, Cortex, Hypothalamus, Kidney, Medulla, Mid-brain, Pituitary, Pons, Testis, Thyroid, Uterus. Foetal tissues: Adrenals, Cerebellum, Frontal lobe, Hypothalamus, Lumbar sympathetic chain, Medulla, Mid-brain, Olfactory bulb, Pons, Pituitary, Retina. HepG2 cell-line.

Table 6: Summary of GnRHR-II cloning results from human and baboon tissues and cells.

Species	Tissue/cell type	Features of the cloned GnRHR-II cDNA transcript		
		PCR primer pair	Length (bp)**	Sequence
Human	Ejaculate	S5 & AS6	319 ¹	Part of exons 1 & 2. Fully processed. Contains a TGA translation stop signal in exon 2. Is contained within the S5 & AS10 sequence.
		S5 & AS6	419 ^Δ	Same sequence as the above S5 & AS6 319 bp sequence, except that this sequence contains a 100 bp insert between exons 1 and 2, resulting in a shift in the reading frame. Possibly the result of incomplete processing of the mRNA. Also contains the TGA translation stop in exon 2.
		S5 & AS10	542 ¹	Same sequence as the S5 & AS6 319 bp sequence except for 1 nt difference in exon 1, possibly due to a sequence error in the S5 & AS10 sequence. Continues further 3' up to the end of primer AS10, encoding part of TM6.
		AP2 & AS3 (5' RACE)	706 ¹	Part of exon 1, including 391 nt of the 5' UTR. A nt deletion at position +26 relative to the ATG translation start results in a frame shift (compared to sequences from other cloned mammalian GnRHR-II cDNAs), raising the question whether this transcript is functional in humans.
Baboon	Cerebellum	S5 & AS10	331 ⁴	Part of exons 1 & 3, but lacks exon 2, with a resultant shift in reading frame.
	Pituitary	S1 & AS11	1084 ⁷	Most of exons 1 & 3, including the translation start codon and translation stop signal and 5' UTR and 3' UTR sequences, but lacks exon 2, resulting in a shift in the reading frame.
		S8 & NUP (3' RACE)	779 ⁸	Most of exon 2 and part of exon 3. Has a 448 bp insertion between exons 2 & 3. Does not contain novel 3' sequence.
	Temporal lobe	NUP & AS7 (5' RACE)	807 ^Δ	Part of exon 2, but does not include exon 1. Does not contain novel 5' sequence.

** Number in superscript represents the sequence number. Refer to Appendix 4 for sequence information.

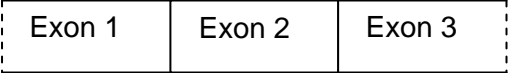

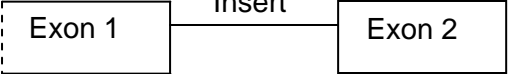

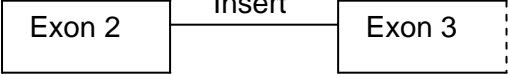
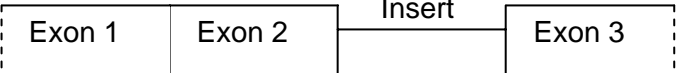
Δ Sequence data not shown.

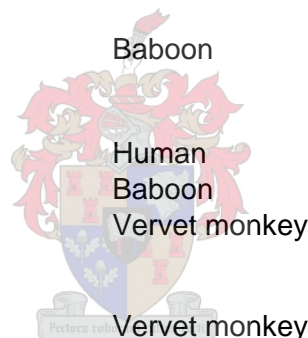
Table 7: Summary of GnRHR-II cloning results from vervet monkey.

Species	Tissue/cell type	Features of the cloned GnRHR-II cDNA transcript		
		PCR primer pair	Length (bp)**	Sequence
Vervet monkey	Ejaculate	S3 & AS13	1729 ^{6,8}	Most of exon 1, full exon 2 & most of exon 3. Starts at -37 within 5' UTR, includes the translation start codon and all seven TMs and ends 86 nt 3' to translation stop signal within 3' UTR. Has a 447 bp insertion between exons 2 & 3 in frame.
	COS-1 cells	S5 & AS10	542 ²	Part of exon 1, full exon 2 & part of exon 3. Starts within ECL1 and ends within TM6. Contains the sequences of S5 & AS6 and S10 & AS10 in a single amplicon. Consensus sequence derived from two clones is identical to published vervet monkey GnRHR-II cDNA sequence.
		NUP & AS1 (5' RACE)	244 ²	5' RACE sequence, starting within TM1 in exon 1 and continues 5' to contain 83 nt of the 5' UTR, of which 34 nt are novel compared to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988). Consensus sequence derived from four clones is identical to published vervet monkey GnRHR-II cDNA sequence except for 2 nt differences within the 5' UTR.
		S1 & AS12/ S2 & AS12	1295 ^Δ	Full coding region, from -48 relative to translation start, within 5' UTR, until 82 nt downstream of translation stop, within 3' UTR. Obtained the expected size band on an agarose gel, but not positive on Southern blot using exon 1-specific oligo AS3. Cloning attempts unsuccessful.
	Occipital lobe	S5 & AS10	542 ³	Part of exon 1, full exon 2 & part of exon 3. Starts within ECL1 and ends within TM6. Identical to published vervet monkey GnRHR-II cDNA sequence except for two nt differences, one within TM3 and one within ECL2, which would result in the incorporation of two different amino acids.
		NUP & AS7 (5' RACE)	171 ^Δ 958 ⁵	Two transcripts: i. Most of exon 2 but does not include exon 1 sequence. ii. Part of exon 1 including the translation start and 140 nt of the 5' UTR, of which 92 nt are novel compared to the published vervet monkey GnRHR-II cDNA, plus most of exon 2. Sequence derived from a single clone, contains 2 nt differences compared to published vervet monkey GnRHR-II cDNA sequence (Accession AF353988). In addition, has a 116 bp insertion between exons 1 & 2 with a resultant shift in the reading frame.
		S8 & NUP (3' RACE)	727 ^Δ	Most of exon 2 plus part of exon 3, stretching from ECL2 to TM6. Contains a 447 bp insertion between the two exons. Does not include the translation stop signal or novel 3' sequence. ^Δ

** Number in superscript represents the sequence number. Refer to Appendix 4 for sequence information.

Δ Sequence data not shown.

Sequence make-up:	Species:	Tissue/cell type*
	Human Vervet monkey	Ejaculate ¹ COS-1 kidney cells ² , occipital lobe ³
	Human Vervet monkey	Ejaculate ¹ Occipital lobe ⁵
	Human Vervet monkey	Ejaculate ^Δ Occipital lobe ⁵
	Baboon	Cerebellum ⁴ , pituitary ⁷
	Human Baboon Vervet monkey	Widely distributed ^Δ Pituitary ⁸ , temporal lobe ^Δ Occipital lobe ^Δ
	Vervet monkey	Ejaculate ⁶



Notes:

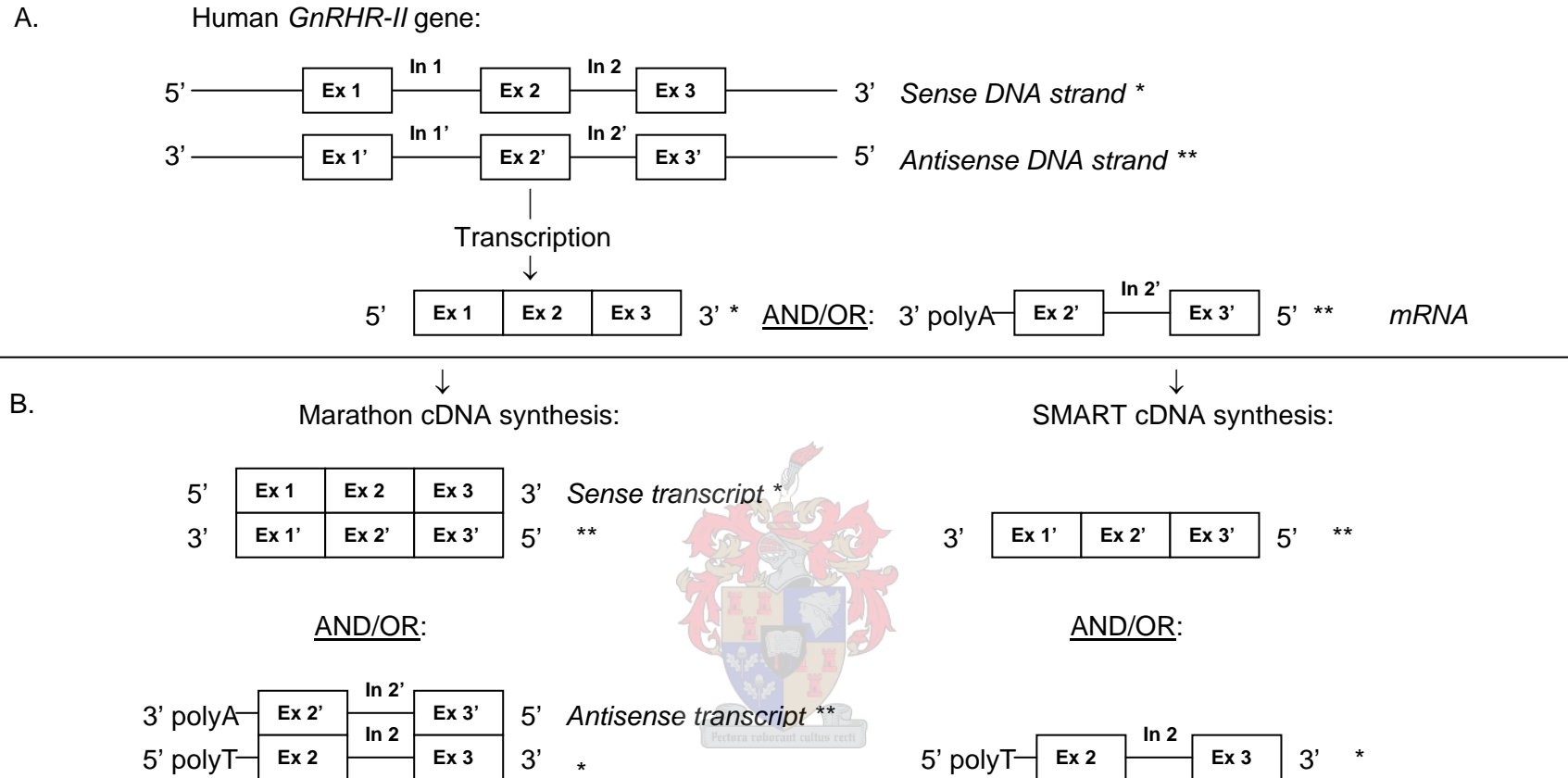
“Exon 1” may or may not include the 5’ UTR (refer to tables 1 & 2 and actual sequences).

“Exon 3” may or may not include the translation stop (refer to tables 1 & 2 and actual sequences), and never includes the polyA tail.

* Number in superscript represents the sequence number. Refer to Appendix 4 for sequence information.

Δ Sequence data not shown.

Fig 23. Summary of all sequences cloned: schematic representation.



Key

Ex Exon number, from sequence of human *GnRHR-I* gene (Kakar SS, 1997)

In Intron number, from sequence of human *GnRHR-I* gene (Kakar SS, 1997)

** Sense exon 2-specific primer will be able to anneal to this cDNA strand during PCR

* Antisense exon 3-specific primer will be able to anneal to this cDNA strand during PCR

Fig 5. Schematic view of (A) putative sense and antisense transcripts from the human *GnRHR-II* gene and (B) the putative resulting human GnRHR-II cDNAs produced with the Marathon™ and SMART™ RACE kits, for use in RACE.

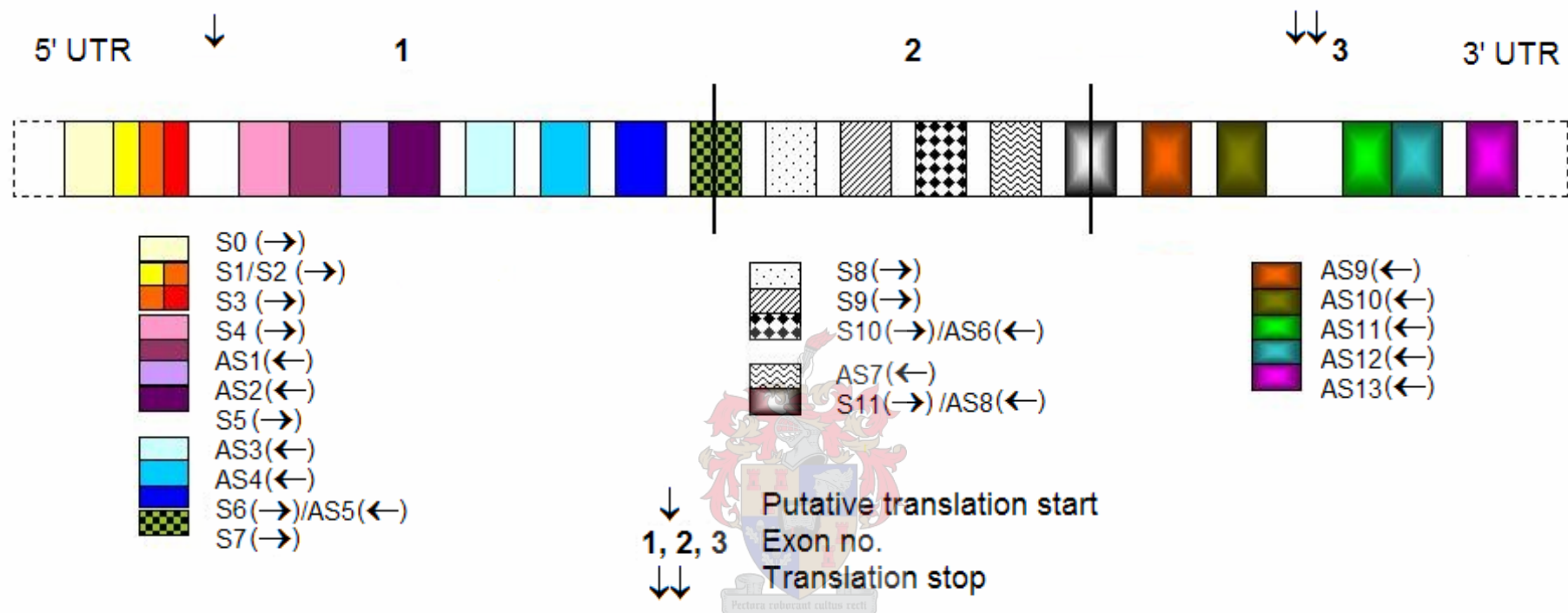


Fig 6. Schematic representation of the human GnRHR-II cDNA and relative positions of the primers used in RT-PCR, RACE, Southern blot analysis, exontrapping, and for probe synthesis for dot blot and in situ hybridisation analyses. Direction of primers is indicated as sense (→) or antisense (←).

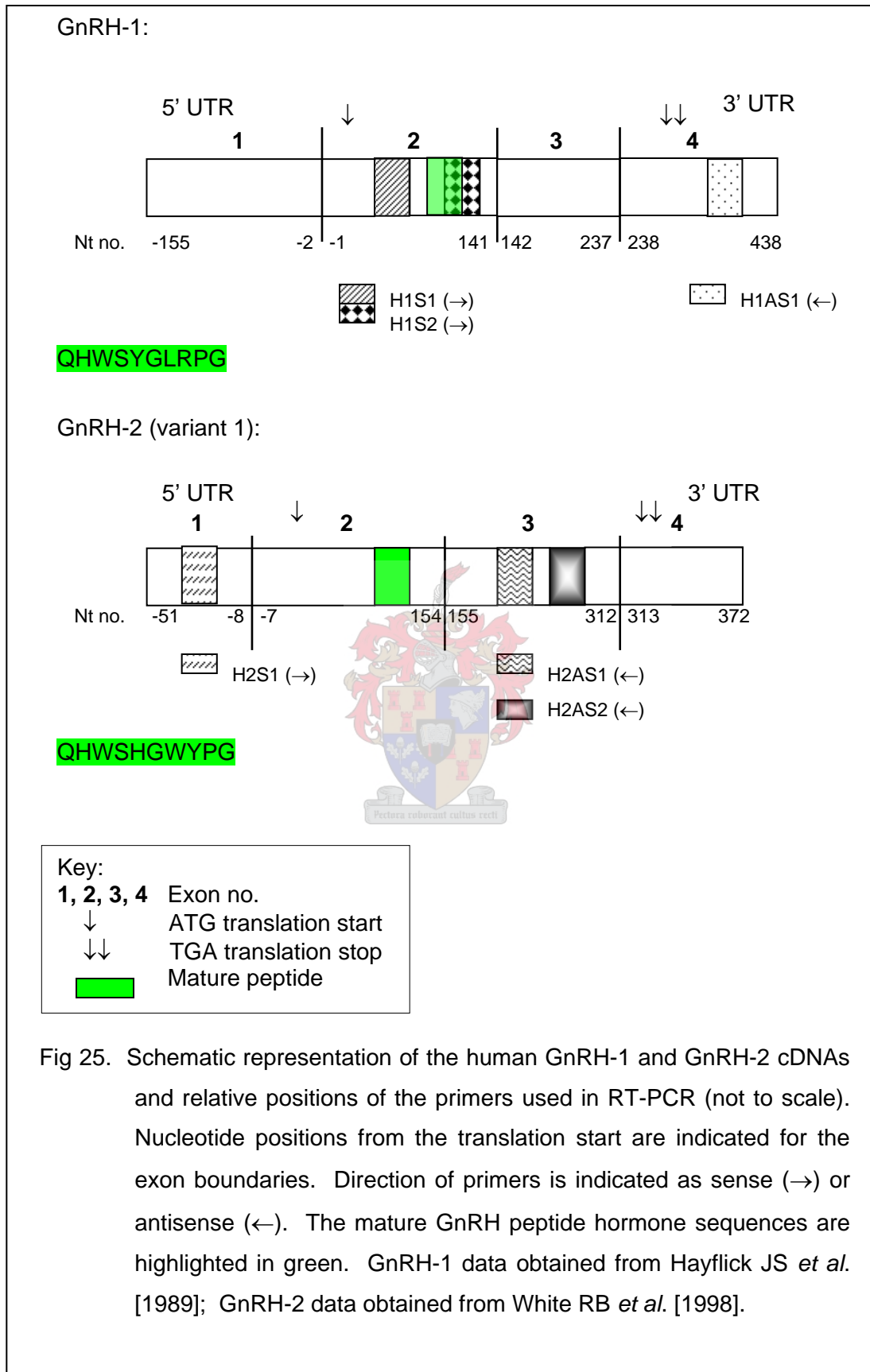


Fig 25. Schematic representation of the human GnRH-1 and GnRH-2 cDNAs and relative positions of the primers used in RT-PCR (not to scale). Nucleotide positions from the translation start are indicated for the exon boundaries. Direction of primers is indicated as sense (→) or antisense (←). The mature GnRH peptide hormone sequences are highlighted in green. GnRH-1 data obtained from Hayflick JS *et al.* [1989]; GnRH-2 data obtained from White RB *et al.* [1998].

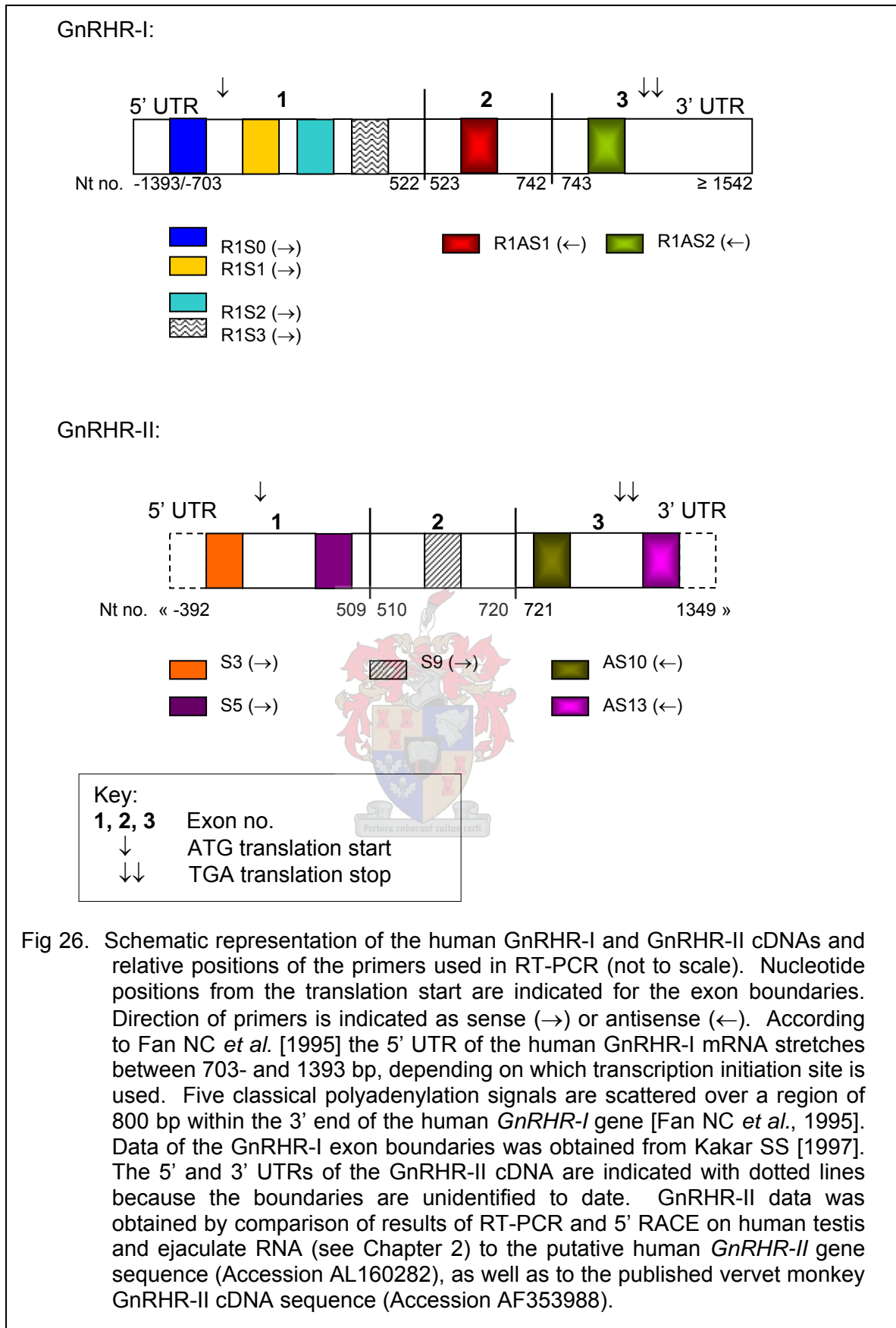


Fig 26. Schematic representation of the human GnRHR-I and GnRHR-II cDNAs and relative positions of the primers used in RT-PCR (not to scale). Nucleotide positions from the translation start are indicated for the exon boundaries. Direction of primers is indicated as sense (→) or antisense (←). According to Fan NC *et al.* [1995] the 5' UTR of the human GnRHR-I mRNA stretches between 703- and 1393 bp, depending on which transcription initiation site is used. Five classical polyadenylation signals are scattered over a region of 800 bp within the 3' end of the human *GnRHR-I* gene [Fan NC *et al.*, 1995]. Data of the GnRHR-I exon boundaries was obtained from Kakar SS [1997]. The 5' and 3' UTRs of the GnRHR-II cDNA are indicated with dotted lines because the boundaries are unidentified to date. GnRHR-II data was obtained by comparison of results of RT-PCR and 5' RACE on human testis and ejaculate RNA (see Chapter 2) to the putative human *GnRHR-II* gene sequence (Accession AL160282), as well as to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988).

Appendix 1

List of primers used in RT-PCR, RACE, Southern blot analysis, exontrapping, and for probe synthesis for dot blot and *in situ* hybridisation experiments, indicating relative positions in the relevant genes

Notes:

1. All primer combinations span exon-intron boundaries.
2. Alternative names are given for each primer. "Lab name" refers to the name of the primer as it is known in the laboratory. "Thesis name" refers to a new name given to each primer based on its position in the gene.

GnRH-1:

GnRH-1 sense primers			GnRH-1 antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
H1S1 ¹	129	Exon 2, +18 relative to translation start, within the signal peptide.	H1AS1 ¹	131	Exon 4, +382 relative to translation start, within the 3' UTR.
H1S2 ^{1,2,*}	122	Exon 2, +95 relative to translation start, within the last 4 nucleotides encoding the mature peptide.			

1. 100% homologous to human; % homology to vervet monkey not known.
2. 47.8% homology to rhesus monkey.

* Used in Southern blot analysis.

GnRH-2:

GnRH-2 sense primers			GnRH-2 antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
H2S1 ^{1,2}	132	Exon 1, -49 relative to translation start, within the 5' UTR.	H2AS1 ^{3,*}	124	Exon 3, +228 relative to translation start, within GAP.
			H2AS2 ⁴	133	Exon 3, +298 relative to translation start, within GAP.

1. 100% homologous to human; % homology to vervet monkey not known.
2. 100% homologous to rhesus monkey.
3. 86.4% homology to rhesus monkey.
4. 95.5% homology to rhesus monkey.

* Used in Southern blot analysis.

GnRHR-I:

GnRHR-I sense primers			GnRHR-I antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
R1S0 ^{1,2}	HR1S2	Exon 1, -31 relative to translation start.	R1AS1 ⁴	106	Exon 2, +675 relative to translation start.
R1S1 ³	HR1S3	Exon 1, +9 relative to translation start.	R1AS2 ⁴	HR1AS1	Exon 3, +894 relative to translation start.
R1S2 ⁴	101	Exon 1, +301 relative to translation start.			
R1S3 ^{5,*}	117	Exon 1, +458 relative to translation start.			

1. 100% homologous to human; % homology to vervet monkey not known.
 2. 58.3% homology to bonnet monkey.
 3. 87.0% homology to bonnet monkey.
 4. 100% homologous to bonnet monkey.
 5. 90.5% homology to bonnet monkey.
- * Used in Southern blot analysis.



GnRHR-II:

Percentage (%) homology to human and vervet monkey is indicated as follows:

- h: 100% homologous to human but <100% homology to vervet monkey.
- m: 100% homologous to vervet monkey but <100% homology to human.
- h,m: 100% homologous to both human and vervet monkey.
- #: <100% homology to both human and vervet monkey; % homology given.

GnRHR-II exon 1					
Sense primers			Antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
S0 ^{h,*}	S1	-77 relative to translation start, within 5' UTR.	AS1 ^h	NAS3	+163 relative to translation start.
S1 ^h	NS2	-48 relative to translation start, within 5' UTR ^s .	AS2 ^h	NAS2	+237 relative to translation start.
S2 ^m	NS2mod1	-48 relative to translation start, within 5' UTR ^s .	AS3 ^{h,*}	AS252	+317 relative to translation start.
S3 ^m	GW2S	-36 relative to translation start, within 5' UTR.	AS4 ^{h,m}	AS302	+367 relative to translation start.
S4 ^h	S4	+90 relative to translation start.	AS5 ^{h,m}	AS405	+472 relative to translation start.
S5 ^{h,m}	S210	+295 relative to translation start.			
S6 ^{h,m}	S387	+473 relative to translation start.			
S7 ^m	We1e2S	+497 relative to translation start; spans the exon 1-2 barrier, with 14 nt in exon 1 and 12 nt in exon 2.			

* Used in Southern blot analysis.

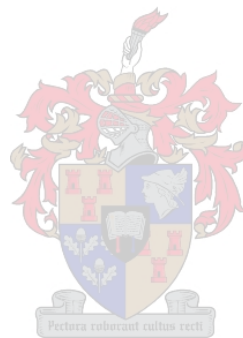
GnRHR-II exon 2					
Sense primers			Antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
S7 ^m	We1e2S	(See above).	AS6 ^{h,m}	10242	+594 relative to translation start.
S8 ^m	We2S	+524 relative to translation start.	AS7 ^m	We2AS	+654 relative to translation start.
S9 ^{h,m,*}	10377S	+554 relative to translation start.	AS8 ^m	We2e3AS	+698 relative to translation start; spans the exon 2-3 barrier, with 24 nt in exon 2 and 2 nt in exon 3.
S10 ^{h,m}	10417	+594 relative to translation start.			
S11 ^{h,m}	JHe2e3S	+704 relative to translation start; spans the exon 2-3 barrier, with 18 nt in exon 2 and 9 nt in exon 3.			

* Used in Southern blot analysis.

GnRHR-II exon 3					
Sense primers			Antisense primers		
Thesis name	Lab name	Position in gene (5' end of primer)	Thesis name	Lab name	Position in gene (3' end of primer)
S11 ^{h,m}	JHe2e3S	(See above).	AS9 [#]	10350	+763 relative to translation start.
			AS10 ^{h,m}	10070	+811 relative to translation start.
			AS11 ^h	10071	+1199 relative to translation start, 60 nt downstream of translation stop, within 3' UTR.
			AS12 ^h	NAS4	+1221 (human) relative to translation start, 82 nt downstream of translation stop, within 3' UTR.
			AS13 ^{h,m}	AS6	+1200 (monkey) or +1321 (human) relative to translation start, 60 nt or 182 nt downstream of translation stop, respectively.
			# 95.7% homologous to human and 87.0% homologous to vervet monkey.		

Other:

Name:	Description:
AP1	Marathon RACE adaptor primer 1.
AP2	Marathon RACE nested adaptor primer.
UPM	Smart RACE universal primer mix (mixture of a 45-mer and 22-mer).
NUP	Smart RACE nested universal primer.
β A1	β -actin exon 2 sense primer; 100% identity to human and mouse.
β A2	β -actin exon 3 antisense primer; mouse-specific, 96% identity to human.
T7	T7 promoter primer.
U19	U-19mer primer.



Appendix 2

Expected sizes of RT-PCR products & PCR annealing temperatures used.

Non-GnRHR-II:

Primer pair	Gene of interest	Expected size (bp) (Including primers)	Annealing temperature (°C)
H1S1 & H1AS1	GnRH-1	387 ¹	55
H2S1 & H2AS2	GnRH-2	389/368/365 (human) ² / 350 (Rhesus monkey) ³	60
R1S2 & R1AS1	GnRHR-I	396 ⁴	50
R1S1 & R1AS2	GnRHR-I	909 ⁴	53
R1S0 & R1AS2	GnRHR-1	948 ⁴	53
βA1 & βA2	β-actin	317 ⁵	50-55

Expected sizes are based on the following sequences:

- 1 Human GnRH-1 cDNA (Accession X15215).
- 2 Human GnRH-2 variant 1 cDNA (Accession NM_001501).
- 3 Rhesus monkey GnRH-2 mRNA (Accession AF097356).
- 4 Human GnRHR-I cDNA (Accession L07949).
- 5 Mouse β-actin cDNA (Accession X03672).

GnRH-RII:

Primer pair	Expected size (bp) (Including primers)	Annealing temperature (°C)
S0 & AS13	1303 ⁶ /1424 ⁸	50
S1/S2 & AS12	1295 ⁷	58
S3 & AS13	1383 (human) ⁸ /1262 (vervet monkey) ⁷	57
S4 & AS5	402 ⁹	55
S5 & AS6	319 ¹⁰	55
S5 & AS10	542 ^{10,11}	52
S7 & UPM	615 (long UP) ⁷ /592 (short UP) ⁷	60
S8 & NUP	640 ⁷	60
S10 & AS10	250 ¹¹	60
AP1 & AS5	567 ⁷	Touchdown 72→70→68
AP2 & AS3	409 ⁷	Touchdown 72→70→68
NUP & AS1	256 ⁷	55
UPM & AS2	358 (long UP) ⁷ /336 (short UP) ⁷	55
NUP & AS7	750 ⁷	60
UPM & AS8	818 (long UP) ⁷ /793 (short UP) ⁷	60

Expected sizes are based on the following sequences:

- 6 5' SMART RACE results on COS-1 and RT-PCR results on vervet monkey ejaculate RNA using primer pair S3 & AS13.
- 7 Published vervet monkey GnRHR-II [Neill JD *et al*, 2001].
- 8 Human chromosome 1 genomic DNA (Accession AL160282).
- 9 PCR amplification of P1 human genomic DNA clones.
- 10 Exontrapping results.
- 11 RT-PCR results with S10 & AS11 on testis RNA [Hutchinson E, 1997].

Appendix 3

Primer sequences (5' → 3')

GnRH-1-specific:

Sense:

H1S1: ACTCCTAGCTGGCCTTATTCTAC

H1S2: TGGAGGAAAGAGAGATGCCG

Antisense:

H1AS1: GCAACTTGGTGTAAGGATTTCTG

GnRH-2-specific:

Sense:

H2S1: GCAGCTGCCTGAAGGAGCCATC

Antisense:

H2AS1: CCAGGGCATGCTGTCGTCCAGG

H2AS2: CTGCGGTCAGCAGTGTCCGTGC

GnRHR-I-specific:

Sense:

R1S: CACAAGGCTTGAAGCTCTGTCCTG

R1S1: CAGTGCCTCTCCTGAACAGAATC

R1S2: TGGAACATTACAGTCCAATGG

R1S3: GCAAAGTCGGACAGTCCATGG

Antisense:

R1AS1: TGCATTGCAGATCAGCATGATG

R1AS2: TGATTTACTGGGTCTGACAACCTG

GnRHR-II-specific:

Sense:

S0: GAGGGCGAAGAATCAGTGGCCAAAGC

S1: CCGCTTCATACCCACACTTCATCCTCC

S2: CCGCTTCATACCCACACTGCCTCCTCC

S3: CACACTGCCTCCTCCTCAGTTTCTCTC

S4: CCCACCTTCTCGGCAGCAGCC

S5: ACCTGGAATATCACTGTTCAATGG

S6: CAGCCTGGGGACTTAGTTTCCTG

S7: TTGCCTTGCCCCAGCTGTTCTGTTT

S8: ATACCGTCCACCGAGCTGGCCAGTC

S9: TCACTCAGTGTGTCACCAAAGGCAGC

S10: GCAAGAGACCACCTATAACCT

S11: CAAGGAAGGGGAGCCATGCCCTGCTG

Antisense:

AS1: ACTGACCACAGGACTGCCAGGTT
AS2: ACTAGTAAGTCGGCGGCTGCTAAATGGATG
AS3: CATGCGATGTCCACAGCCAGCC
AS4: GGAAAGCTGCAGAATACATGGC
AS5: GAAACTAAGTCCCCAGGCTGC
AS6: GGTTATAGGTGGTCTCTTGC
AS7: CAATGCGGCTATAGCAGATGGCCATG
AS8: GGCATGGCTCCCCTTCCTTGTCTGAG
AS9: CCGGAGACGAACACGGGGACAAT
AS10: GGTGTCCAGCAGAGGATGAAGGTCAG
AS11: GGAGAGCAGGAGTAGAAGTGAG
AS12: CAGTATTTCTTTTTTGGGGGGA ACTA
AS13: GTAGAGATGGGGTCTTGCTGTGTTACC

Other:

AP1: CCATCCTAATACGACTCACTATAGGGC
AP2: ACTCACTATAGGGCTCGAGCGGC
UPM: Long: TAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
Short: CTAATACGACTCACTATAGGGC
NUP: AAGCAGTGGTAACAACGCAGAGT
 β A1: CACCACACCTTCTACAATGAGCTG
 β A2: GATCTTCATGAGGTAGTCTGTCAGG
T7: TAATACGACTCACTATAGGG
U19: GTTTTCCCAGTCACGACGT



Appendix 4: Sequence data of cloned amplicons

Sequence no

1. Cloned GnRHR-II cDNA sequence from human ejaculate containing part of exon 1, the full exon 2 and part of exon 3, stretching from TM1 to TM6.
2. Cloned GnRHR-II cDNA sequences from COS-1 vervet monkey kidney cells containing parts of exon 1, the full exon 2 and part of exon 3, obtained by 5' SMARTTM RACE and RT-PCR.
3. Cloned GnRHR-II cDNA sequence from monkey occipital lobe containing part of exon 1, the full exon 2 and part of exon 3, stretching from ECL1 in exon 1 to TM6 in exon 3.
4. Cloned GnRHR-II cDNA sequence from baboon cerebellum containing part of exon 1 and part of exon 3 but lacking exon 2, stretching from ECL1 to TM6 minus ECL2.
5. Vervet monkey occipital lobe GnRHR-II 5' SMARTTM RACE sequence, containing part of exon 1 and most of exon 2, with an insert between the two exons.
6. Cloned GnRHR-II cDNA sequence from vervet monkey ejaculate, containing most of exon 1, the full exon 2 and most of exon 3, with an insert between the exons 2 and 3.
7. Cloned GnRHR-II cDNA sequence from baboon pituitary, containing most of exon 1 and most of exon 3 but lacking exon 2, stretching from the 5' UTR to the intracellular C-terminal tail minus ECL2.
8. Additional insert sequence between exons 2 and 3 of the GnRHR-II cDNA: comparison between baboon pituitary and vervet monkey ejaculate and occipital lobe.
9. Cloned GnRH-1 cDNA sequences from human ejaculate and hypothalamus.
10. Cloned GnRH-2 cDNA sequence from human ejaculate.
11. Cloned GnRHR-I cDNA sequences from human ejaculate and pituitary.

Sequence no 1: Cloned GnRHR-II cDNA sequence from human ejaculate containing part of exon 1, the full exon 2 and part of exon 3, stretching from TM1 to TM6, and aligned to published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)

Exon 1	Exon 2	Exon 3
--------	--------	--------

-392-390 -380 -370 -360 -350 -340 -330 -320 -310
 Human ejacul A R A G D L H V L E A A G I G V R L A E P H K Y T C T Q
 c gcc cgg gca ggt gat ctc cac gtc ttg gag gcc gcc ggc ata ggt gtg cgc ctg gca gaa cct cac aaa tac aca tgc acg cag

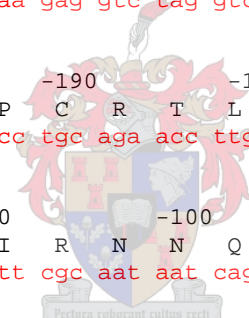
-300 -290 -280 -270 -260 -250 -240 -230
 Human ejacul A P T E S R A V G A K E V * V S R G L K P R L V C P R K
 gcc cgg act gaa tcc agg gct gta ggg gct aaa gag gtc tag gtc agt aga ggc ctg aag ccc agg ctg gtc tgt cca agg aaa

-220 -210 -200 -190 -180 -170 -160 -150 -140
 Human ejacul K E R D W Y Q I F V P C R T L T V E Q V T S S R T D G E
 aag gag cgt gat tgg tac cag atc ttc gtt ccc tgc aga acc ttg aca gtt gaa caa gtg acc tcc tcc aga aca gat gga gag

-130 -120 -110 -100 -90 -80 -70 -60
 Human ejacul S P E A E A L V N E I R N N Q L Q I L K R R A K N Q W P
 tct cca gaa gca gag gct tta gtg aac gaa att cgc aat aat cag ctc cag atc ctg aaa agg agg gcg aag aat cag tgg cca

-50 -40 -30 -20 -10 1 10 20
 Human ejacul K L T A S Y P H F I L L S F S P G H H V C R Q R H P L G
 aag cta acc gct tca tac cca cac ttc atc ctc ctc agt ttc tct cca ggc cac cat gtc tgc agg caa cgg cac ccc ttg ggg
 V/vet monkey cc gct tca tac cca cac ttc atc ctc ctc agt ttc tct cca ggc cac cac gtc tgc agg caa cgg cac ccc ttgg ggg

30 40 50 60 70 80 90 100 110
 Human ejacul S A A G E E V W A G S G V E V E G S E L P T F S A A A K
 tca gca cgc ggg gag gag gtc tgg gct gga tca gga gtg gag gtg gag ggc tca gag ctg ccc acc ttc tcg gca gca gcc aag
 V/vet monkey tca gca gtg ggg gag gag gcc tgg gct gga tca gga gtg ggc gtg gag ggc tca gag ctg ccc acc ttc tcg aca gca gcc aag



120 130 140 150 160 170 180 190
 V R V G V T I V L F V S S A G G N L A V L W S V T R R E
 Human ejacul gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gca gtc ctg tgg tca gtg aca cgg cgg gaa
 V/vet monkey gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gca gtc ctg tgg tca gtg aca cgg cgg caa
 _____ TM1 _____ ||

200 210 220 230 240 250 260 270 280
 P S Q L R P S P V R R L F I H L A A A D L L V T F V V M
 Human ejacul ccc agc cag ctg cgc ccc tct ccg gtc agg aga ctg ttc atc cat tta gca gcc gcc gac tta cta gtc act ttt gtg gtt atg
 V/vet monkey ccc agc cag ctg cgc ccc tct ccg gtc agg aca ctg ttc gcc cat tta gca gct gcc gac tta cta gtc act ttt gtg gtt atg
 ICL1 | _____ TM2 _____

290 300 310 320 330 340 350 360
 P L D A T W N I T V Q W L A V D I A C R T L M F L K L M
 Human ejacul ccc cta gat gcc acc tgg aat atc act gtt caa tgg ctg gct gtg gac atc gca tgt cgg aca ctg atg ttc ctg aaa cta atg
 V/vet monkey ccc cta gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctg atg ttc ctg aaa cta atg
 _____ ECL1 | _____ TM3 _____

370 380 390 400 410 420 430 440
 A T Y S A A F L P V V I G L D R Q A A V L N P L E S R S
 Human ejacul gcc acg tat tct gca gct ttc ctg cct gtg gtc att gga ttg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt tca
 V/vet monkey gcc atg tat tct gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt tca
 _____ ICL2 _____

450 460 470 480 490 500 510 520 530
 G V R K L L G A A W G L S F L L A F P Q | L F L F H T V H
 Human ejacul ggt gta agg aaa ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttc ccc cag | ctg ttc ctg ttc cac acg gtc cac
 V/vet monkey ggt gta agg aaa ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag | ctg ttc ctg ttc cat acc gtc cac
 _____ TM4 _____ | 2

540 550 560 570 580 590 600 610
 * A G P V P F T Q C V T K G S F K A Q W Q E T T Y N L F
 Human ejacul tga gct ggc cca gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc aag gct caa tgg caa gag acc acc tat aac ctc ttc
 V/vet monkey cga gct ggc cca gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc aag gct cga tgg caa gag acc acc tat aac ctc ttc
 ECL2

620 630 640 650 660 670 680 690 700
 T F C C L F L L P L T A M A I C Y S R I V L S V S R P Q
 Human ejacul acc ttc tgc tgc ctc ttt ctg ctg cca ctg act gcc atg gcc atc tgc tat agc cgc att gtc ctc agt gtg tcc agg ccg cag
 V/vet monkey acc ttc tgc tgc ctc ttt ctg ctg cca ctg att gcc atg gcc atc tgc tat agc cgc att gtc ctc agt gtg tcc agc cct cag
 |-----TM5-----|

710 720 730 740 750 760 770 780
 T R K G S H A P A G E F A L P R S F D N C P R V R L R A
 Human ejacul aca agg aag ggg agc cat g cc ccg gct ggt gaa ttt gcc ctc ccg cgc tcc ttt gac aac tgg ccc cgt gtt ccg ctc cgg gcc
 V/vet monkey aca agg aag ggg agc cat g cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac aat cgt ccc cgt gtt tgt ctc cgg gcc
 |-----ICL3-----|

790 800 810 820 830 835
 L R L A L L I L L T F I L C W T
 Human ejacul ctg aga ctg gcg ctg ctt atc ttg ctg acc ttc atc ctc tgc tgg aca cc
 V/vet monkey ctg aga ctg gct ctg ctt atc ttg ctg acc ttc atc ctc tgc tgg aca cct tat tac cta ctg ggt ctg tgg tac tgg ttc tct
 |-----TM6-----|

840 850 860 870
 P Y Y L L G L W Y W F S
 V/vet monkey ccc acc atg cta act gaa gtc cct ccc agc ctg agc cac atc ctt ttc ctt ttt ggc ctc ctc aat gct cct ctg gat cct ctc
 |-----ECL3-----|-----TM7-----|

880 890 900 910 920 930 940 950
 L Y G A F T F G C R R G H Q E L S I D S S K E G S G R M
 V/vet monkey ctc tat ggg gcc ttc acc ttt ggc tgc cga aga ggg cac caa gaa ctt agt ata gac tct tct aaa gaa ggg tct ggg aga atg
 |-----Intracellular C-terminal tail-----|

1040 1050 1060 1070 1080 1090 1100 1110 1120
 L Q Q E I H A L R Q Q E V Q K T V T S R S A G E T K G I
 V/vet monkey ctc caa cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca aga agt gca gga gaa aca aaa ggc att

V/vet monkey tct ata aca tct atc tga
 S I T S I *
 1130 1140

Notes:

1. The above is the combined sequence of secondary 5' RACE, using an exon 1-specific primer (AS3) in combination with the Marathon adaptor primer AP2, and RT-PCR using an exon 1-3 primer pair (S5 & AS10) in human ejaculate RNA. The sequence obtained using an exon 1-2 primer pair (S5 & AS6) is contained within this sequence, stretching from nt number 294 to 611. The 5' RACE sequence stretches from nt number -391 to 337 whereas the exon 1-3 amplicon stretches from nt number 294 to 835. A total of sixteen overlapping clones were sequenced to obtain the above consensus sequence (five clones containing 5' RACE sequence, nine clones containing exon 1-2 sequence and two clones containing exon 1-3 sequence). The human ejaculate sequence is aligned to the recently published vervet monkey GnRHR-II cDNA (accession AF353988).
2. Nucleotides are numbered from the translation start, according to the human ejaculate sequence, therefore exon 1 ends at nt position 509 whereas exon 1 ends at nt position 510 of Sequence numbers 2 to 7. The translated amino acid sequence is indicated above the nt sequences. The amino acid sequence shown is that which would be predicted from the human ejaculate nt sequence until the end of the human sequence in TM6. Thereafter the vervet monkey amino acid sequence is shown. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences are included, except the Marathon RACE adaptor primer AP2. Primers used in RT-PCR are printed in italics. Primers used in primary and secondary 5' RACE are underlined.
3. Nucleotides typed in red represent novel (unpublished) human GnRHR-II 5' sequence obtained in the 5' RACE analysis, from nt number -89 until -392, which is a total of 481 bp.
4. The position of the vervet monkey translation start codon (AGC) is bolded and highlighted in green (xxx). However, the human sequence has a nt deletion compared to the monkey sequence (the position of which is indicated by ↓) and this results in a frame shift so that the translation start codon is in a different reading frame. Interestingly, had there not been a nt deletion, the human sequence would have an AUG methionine translation start codon compared to the ACG threonine of the vervet monkey sequence. A second putative translation start, situated at nt position 279 and highlighted in yellow (xxx) would cancel the need for a nt insertion in the human sequence, but would result in a truncated protein that lacks TM1 and most of TM2.
5. There are a number of nt differences between the human ejaculate and vervet monkey sequences (highlighted in turquoise, x) due to species differences. The nt differences often result in the incorporation of a different amino acid (x) in the human translated protein sequence, compared to the vervet monkey sequence.
6. The human ejaculate sequence contain an in-frame TGA translation stop signal within exon 2, starting at nt position 534 and shown in underlined bold print. It was subsequently revealed that, upon completion of the human genome project, the TGA is present in the GnRHR-II gene on chromosome 1 (accession AL160282). At the same position, the monkey sequence contains a CGA arginine codon, creating an extended open reading frame.

Sequence no 2: Cloned GnRHR-II cDNA sequences from COS-1 vervet monkey kidney cells containing parts of exon 1, the full exon 2 and part of exon 3, obtained by 5' SMART™ RACE and RT-PCR. The 5' RACE product stretches from -82 in the 5' UTR to the end of TM1 in exon 1. The RT-PCR product stretches from ECL1 in exon 1 to TM6 in exon 3. Sequences are aligned to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)

Exon 1	Exon 2	Exon 3
--------	--------	--------

```

-82-80      -70      -60      -50      -40      -30      -20      -10      1
  R  G  G  R  R  I  S  G  Q  S  *  P  L  H  T  H  T  A  S  S  S  V  S  L  Q  A  T  T
Monkey COS-1 a cgc ggg ggg cga aga atc agt ggc caa agc taa ccg ctt cat acc cac act gcc tcc tcc tca gtt tct ctc cag gcc acc acg
V/vet monkey                                ccg ctt cat acc cac act tca tcc tcc tca gtt tct ctc cag gcc acc acg

          10          20          30          40          50          60          70          80
  S  A  G  N  G  T  P  W  G  S  A  V  G  E  E  A  W  A  G  S  G  V  A  V  E  G  S  E
Monkey COS-1 tct gca ggc aac ggc acc cct tgg ggg tca gca gtg ggg gag gag gcc tgg gct gga tca gga gtg gcg gtg gag ggc tca gag
V/vet monkey tct gca ggc aac ggc acc cct tgg ggg tca gca gtg ggg gag gag gcc tgg gct gga tca gga gtg gcg gtg gag ggc tca gag

          90          100          110          120          130          140          150          160          170
  L  P  T  F  S  T  A  A  K  V  R  V  G  V  T  I  V  L  F  V  S  S  A  G  G  N  L  A
Monkey COS-1 ctg ccc acc ttc tcg aca gca gcc aag gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gcc
V/vet monkey ctg ccc acc ttc tcg aca gca gcc aag gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gcc
                                     |-----TM1-----|

          180          190          200          210          220          230          240          250
  V  L  W  S  V  T  R  P  Q  P  S  Q  L  R  P  S  P  V  R  T  L  F  A  H  L  A  A  A
Monkey COS-1 gtc ctg tgg tca gtg
V/vet monkey gtc ctg tgg tca gtg aca cgg ccg caa ccc agc cag ctc cgc ccc tct ccg gtc agg aca ctc ttc gcc cat tta gca gct gcc
                                     |-----ICL1-----|-----TM2-----|

          260          270          280          290          300          310          320          330
  D  L  L  V  T  F  V  V  M  P  L  D  A  T  W  N  I  T  V  Q  W  L  A  G  D  I  A  C
Monkey COS-1 gac tta cta gtc act ttt gtg gtt atg ccc cta gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt
V/vet monkey gac tta cta gtc act ttt gtg gtt atg ccc cta gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt
                                     |-----ECL1-----|-----

          340          350          360          370          380          390          400          410          420
  R  T  L  M  F  L  K  L  M  A  M  Y  S  A  A  F  L  P  V  V  I  G  L  D  R  Q  A  A
Monkey COS-1 cgg aca ctc atg ttc ctg aaa cta atg gcc atg tat tct gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca
V/vet monkey cgg aca ctc atg ttc ctg aaa cta atg gcc atg tat tct gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca
                                     |-----TM3-----|

```

Monkey COS-1 V L N P L G S R S G V R K L L G A A W G L S F L L A L P
 V/vet monkey gta ctc aac ccg ctt gga tcc cgt tca ggt gta agg aaa ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc
 ICL2 TM4

Monkey COS-1 Q L F L F H T V H R A G P V P F T Q C V T K G S F K A R
 V/vet monkey cag ctg ttc ctg ttc cat acc gtc cac cga gct ggc cca gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc aag gct cga
 1|2 ECL2

Monkey COS-1 W Q E T T Y N L F T F C C L F L L P L I A M A I C Y S R
 V/vet monkey tgg caa gag acc acc tat aac ctc ttc acc ttc tgc tgc ctc ttt ctg ctg cca ctg att gcc atg gcc atc tgc tat agc cgc
 TM5

Monkey COS-1 I V L S V S S P Q T R K G S H A P A G E F A L R R S F D
 V/vet monkey att gtc ctc agt gtg tcc agc cct cag aca agg aag ggg agc cat g cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac
 ICL3 2|3

Monkey COS-1 N R P R V C L R A L R L A P L I L L T F I L C W T P
 V/vet monkey aat cgt ccc cgt gtt tgt ctc cgg gcc ctg aga ctg gct ctg ctt atc ttg ctg acc ttc atc ctc tgc tgg aca cc
 TM6
 Y Y

V/vet monkey cta ctg ggt ctg tgg tac tgg ttc tct ccc acc atg cta act gaa gtc cct ccc agc ctg agc cac atc ctt ttc ctt ttt ggc
 L L G L W Y W F S P T M L T E V P P S L S H I L F L F G
 ECL3

V/vet monkey ctc ctc aat gct cct ctg gat cct ctc ctc tat ggg gcc ttc acc ttt ggc tgc cga aga ggg cac caa gaa ctt agt ata gac
 L L N A P L D P L L Y G A F T F G C R R G H Q E L S I D
 TM7

```

          1020          1030          1040          1050          1060          1070          1080          1090
V/vet monkey tct tct aaa gaa ggg tct ggg aga atg ctc caa cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca
          S S K E G S G R M L Q Q E I H A L R Q Q E V Q K T V T S
                               Intracellular C-terminal tail

          1100          1110          1120          1130          1140
V/vet monkey aga agt gca gga gaa aca aaa ggc att tct ata aca tct atc tga
          R S A G E T K D I S I T S I *

```

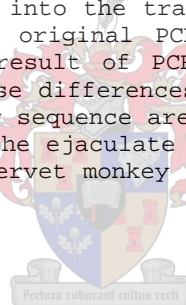
Notes:

1. The above is the combined sequence of secondary 5' SMART RACE, using an exon 1-specific primer (AS1) in combination with the SMART RACE primer NUP, and RT-PCR using an exon 1-3 primer pair (S5 & AS10) in COS-1 vervet monkey RNA. The sequence obtained using an exon 1-2 primer pair (S5 & AS6) is contained within this sequence, stretching from nt number 295 to 613 (including primers). Similarly, the sequence obtained using an exon 2-3 primer pair (S10 & AS10) is contained within this sequence, stretching from nt number 594 to 836(including primers). The 5' RACE sequence stretches from nt number -83 to 162 (excluding primers) whereas the exon 1-3 amplicon stretches from nt number 295 to 836 (including primers). A total of thirteen overlapping clones were sequenced to obtain the above consensus sequence (four clones containing 5' SMART RACE sequence, five clones containing exon 1-2 sequence, two clones containing exon 2-3 sequence and two clones containing exon 1-3 sequence). The COS-1 vervet monkey sequence is aligned to the recently published vervet monkey GnRHR-II cDNA (accession AF353988).
2. Nucleotides are numbered from the translation start. The translated amino acid sequence is indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences are included, except the SMART RACE nested universal primer NUP. Primers used in RT-PCR are printed in italics. Primers used in primary and secondary 5' RACE are underlined.
3. Novel (unpublished) COS-1 vervet monkey GnRHR-II 5' sequence was obtained in the 5' RACE analysis, from nt number -50 to -83.
4. The position of the vervet monkey translation start codon (AGC) is bolded and highlighted in green (xxx).
5. There are two nt differences within the 5' UTR between the COS-1 vervet monkey 5' RACE sequence and the pulished vervet monkey sequence (highlighted in turquoise, x). These nt differences result in the incorporation of an alanine instead of a serine in the COS-1 translated protein sequence, compared to the vervet monkey sequence.

	800		810		820		830	836					
	L	L	I	L	L	T	F	I	L	C	W	T	
M occ lobe	ctg	ctt	atc	ttg	<i>ctg</i>	<i>acc</i>	<i>ttc</i>	<i>atc</i>	<i>ctc</i>	<i>tgc</i>	<i>tgg</i>	<i>aca</i>	<i>cc</i>
V/vet monkey	ctg	ctt	atc	ttg	<i>ctg</i>	<i>acc</i>	<i>ttc</i>	<i>atc</i>	<i>ctc</i>	<i>tgc</i>	<i>tgg</i>	<i>aca</i>	<i>cc</i>
	-----TM6-----												

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated underneath. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences are included and printed in italics.
2. A total of two clones were sequenced to obtain the above consensus sequence.
3. There are two nt differences between the vervet monkey occipital lobe sequence and the published vervet monkey sequence (accession AF353988) (highlighted in turquoise, **x**), even though they both are from vervet monkey. Both nt differences would result in the incorporation of different amino acids into the translated occipital lobe sequence, compared to the published vervet monkey translated amino acid sequence. The original PCR was performed using ordinary *Taq* polymerase and it is therefore possible that these differences are the result of PCR error, but, since the above sequence is the result of sequencing of two different clones and both have these differences, it is likely that the nt differences between the vervet monkey occipital lobe and the published vervet monkey sequence are real. Interestingly, these two differences are not found in the vervet monkey ejaculate sequence, rather, in the ejaculate sequence, they are the same as the published vervet monkey sequence. In addition, the nt differences between vervet monkey ejaculate and the published vervet monkey sequence are not found in the occipital lobe sequence.



Sequence no 4: Cloned GnRHR-II cDNA sequence from baboon cerebellum containing part of exon 1 and part of exon 3 but lacking exon 2, stretching from ECL1 to TM6 minus ECL2, and aligned to published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)

Exon 1	Exon 3
--------	--------

```

295   300           310           320           330           340           350           360           370
T   W   N   I   T   V   Q   W   L   A   G   D   I   A   C   R   T   L   M   F   L   K   L   M   A   M   Y   S
B cerebellum acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctc atg ttc ctg aaa cta atg gcc atg tat tct
V/vet monkey acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctc atg ttc ctg aaa cta atg gcc atg tat tct
                                     ECL1                                     TM3

```

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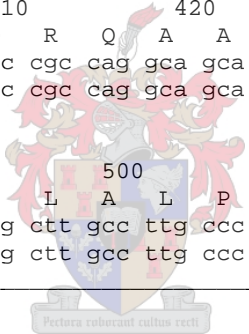
380           390           400           410           420           430           440           450           460
A   A   F   L   P   V   V   I   G   L   D   R   Q   A   A   V   L   N   P   L   G   S   R   S   G   V   R   K
B cerebellum gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt tca ggt gta agg aaa
V/vet monkey gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt tca ggt gta agg aaa
                                     ICL2

```

```

470           480           490           500           510
L   L   G   A   A   W   G   L   S   F   L   L   A   L   P   Q
B cerebellum ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag | ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~
V/vet monkey ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag | ctg ttc ctg ttc cat acc gtc cac cga gct ggc cca
                                     TM4

```



```

B cerebellum ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~
V/vet monkey gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc aag gct cga tgg caa gag acc acc tat aac ctc ttc acc ttc tgc tgc
                                     ECL2

```

```

B cerebellum ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~
V/vet monkey ctc ttt ctg ctg cca ctg att gcc atg gcc atc tgc tat agc cgc att gtc ctc agt gtg tcc agc cct cag aca agg aag ggg
                                     TM5

```

```

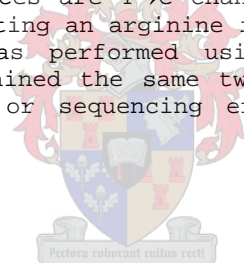
520           530           540           550           560           570           580
P   A   G   E   F   A   L   R   R   S   F   D   N   R   P   R   V   R   L   R   A   L   R   L   A
B cerebellum ~~~ ~~~ ~| cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac aat cgt ccc cgt gtt cgt ctc cgg gcc ctg aga ctg gc
V/vet monkey agc cat g| cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac aat cgt ccc cgt gtt tgt ctc cgg gcc ctg aga ctg gct
                                     ICL3                                     TM6

```

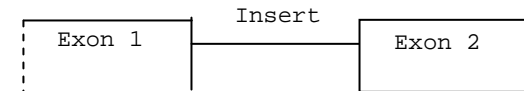
	590		600		610		620	625					
	L	L	I	L	L	T	F	I	L	C	W	T	
B cerebellum	ctg	ctt	atc	ttg	<i>ctg</i>	<i>acc</i>	<i>ttc</i>	<i>atc</i>	<i>ctc</i>	<i>tgc</i>	<i>tgg</i>	<i>aca</i>	<i>cc</i>
V/vet monkey	ctg	ctt	atc	ttg	<i>ctg</i>	<i>acc</i>	<i>ttc</i>	<i>atc</i>	<i>ctc</i>	<i>tgc</i>	<i>tgg</i>	<i>aca</i>	<i>cc</i>

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences, namely S5 and AS10, are included and printed in italics.
2. A total of two clones were sequenced to obtain the above consensus sequence.
3. Exon 1 follows directly onto exon 3. The position of the missing exon 2, at total of 211-bp, is indicated by "~".
4. The omission of exon 2 results in a shift in the reading frame, from frame 1 (exon 1) to frame 3 (exon 3).
5. There are two nt differences within exon 3 between the baboon cerebellum sequence and the published vervet monkey GnRHR-II cDNA sequence (accession AF353988). Both differences are T→C changes. Only the first nt difference would result in the incorporation of a different amino acid, incorporating an arginine instead of a cysteine into the translated baboon sequence (see highlighted (x/x) letters). The RT-PCR was performed using Expand High fidelity proofreading *Taq* polymerase. Furthermore, both clones that were sequenced contained the same two nt differences. Therefore, these nt differences are unlikely to be the result of PCR incorporation or sequencing errors. Rather, they are likely to represent species differences between baboon and vervet monkey.



Sequence no 5: Vervet monkey occipital lobe GnRHR-II 5' SMARTTM RACE sequence, containing part of exon 1 and most of exon 2, with an insert between the two exons. The sequence stretches from -83 in the 5' UTR in exon 1 to TM5 in exon 2. Sequence is aligned to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)



	-141-140	-130	-120	-110	-100	-90	-80	-70	-60	
	A G K Q R L * * T K F A V I S S R S W K G G R R I S G									
M occ lobe	ac gcg ggg aag cag agg ctt tag tga acg aaa ttc gca gta atc agc tcc aga tcc tgg aaa ggt ggg cga aga atc agt ggc									
V/vet monkey										
		-50	-40	-30	-20	-10	1	10	20	
	Q S * P L H T H T A S S S V S L Q A A T S A G N G T P W									
M occ lobe	caa agc taa ccg ctt cat acc cac act gcc tcc tcc tca gtt tct ctc cag gcc gcc acg tct gca ggc aac ggc acc cct tgg									
V/vet monkey		ccg ctt cat acc cac act tca tcc tcc tca gtt tct ctc cag gcc acc acg tct gca ggc aac ggc acc cct tgg								
		30	40	50	60	70	80	90	100	110
	R S A V G E E A W A G S G V A V E G S E L P T F S T A A									
M occ lobe	agg tca gca gtg ggg gag gag gcc tgg gct gga tca gga gtg gcg gtg gag ggc tca gag ctg ccc acc ttc tcg aca gca gcc									
V/vet monkey	ggg tca gca gtg ggg gag gag gcc tgg gct gga tca gga gtg gcg gtg gag ggc tca gag ctg ccc acc ttc tcg aca gca gcc									
		120	130	140	150	160	170	180	190	
	K V R V G V T I V L F V S S A G G N L A V L W S V T R P									
M occ lobe	aag gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gcc gtc ctg tgg tca gtg aca cgg ccg									
V/vet monkey	aag gtc cga gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gcc gtc ctg tgg tca gtg aca cgg ccg									
					TM1					
		200	210	220	230	240	250	260	270	
	Q P S Q L R P S P V R T L F A H L A A A D L L V T F V V									
M occ lobe	caa ccc agc cag ctc cgc ccc tct ccg gtc agg aca ctc ttc gcc cat tta gca gct gcc gac tta cta gtc act ttt gtg gtt									
V/vet monkey	caa ccc agc cag ctc cgc ccc tct ccg gtc agg aca ctc ttc gcc cat tta gca gct gcc gac tta cta gtc act ttt gtg gtt									
		ICL1						TM2		
		280	290	300	310	320	330	340	350	360
	M P L D A T * N I T V Q W L A G D I A C R T L M F L K L									
M occ lobe	atg ccc cta gat gcc acc tag aat atc act gtt caa tgg ctg gcc ggg gat atc gca tgt cgg aca ctc atg ttc ctg aaa cta									
V/vet monkey	atg ccc cta gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctc atg ttc ctg aaa cta									
				ECL1				TM3		

370 380 390 400 410 420 430 440
 M occ lobe M A M Y S A A F L P V V I G L D R Q A A V L N P L G S R
 atg gcc atg tat tct gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt
 V/vet monkey atg gcc atg tat tct gca gct ttc ctg cct gtg gtc att gga ctg gac cgc cag gca gca gta ctc aac ccg ctt gga tcc cgt

ICL2

450 460 470 480 490 500 510
 M occ lobe S G V R R L L G A A W G L S F L L A L P Q I C F T T L G
 tca ggt gta agg aga ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag 1 atc tgt ttt act aca ctt ggc
 V/vet monkey tca ggt gta agg aaa ctt ctg ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag

TM4

M occ lobe F C I F A S S Q S H L V M I G A G K K N R K T Y M G S M
 ttc tgc atc ttt gca tcc tcc caa tct cac ctc gtc atg att ggg gca ggg aag aag aac aga aaa aca tac atg ggg tct atg
 V/vet monkey

M occ lobe K R V S | 511 520 530 540 550 560 570 580
 aag aga gtg agc | C S C S I P S T E L A Q S L S V S P K A A S
 | tgt tcc tgt tcc ata cca tcc acc gag ctg gcc cag tcc ctt tca ctc agt gtg tca cca aag gca gct tca
 | L F L F H T T H R A G P V P F T Q C V T K G S F
 -2 | ctg ttc ctg ttc cat acc atc cac cga gct ggc cca gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc
 | ctg ttc ctg ttc cat acc gtc cac cga gct ggc cca gtc cct ttc act cag tgt gtc acc aaa ggc agc ttc
 | L F L F H T V H R A G P V P F T Q C V T K G S F

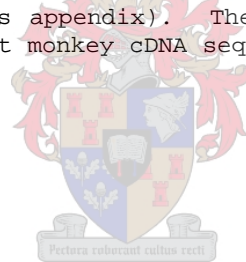
ECL2

M occ lobe R L D G K R P P I T S S P S A A S F C C H * L P C P S A
 agg ctc gat ggc aag aga cca cct ata acc tct tca cct tct gct gcc tct ttc tgc tgc cac tga ttg cca tgg cca tct gct
 K A R W Q E T T Y N L F T F C C L F L L P L I A M A I C
 aag gct cga tgg caa gag acc acc tat aac ctc ttc acc ttc tgc tgc ctc ttt ctg ctg cca ctg att gcc atg gcc atc tgc
 V/vet monkey aag gct cga tgg caa gag acc acc tat aac ctc ttc acc ttc tgc tgc ctc ttt ctg ctg cca ctg att gcc atg gcc atc tgc
 K A R W Q E T T Y N L F T F C C L F L L P L I A M A I C

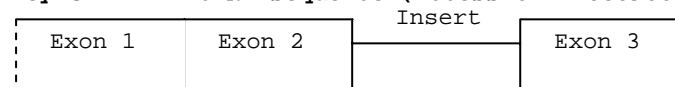
TM5

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. The sequence of the nested SMART™ RACE primer NUP is not included whereas the sequence of gene-specific primer AS7 is included and underlined.
2. The above sequence was the result of sequencing of a single clone.
3. The position of the vervet monkey translation start codon (AGC) is bolded and highlighted in green (xxx). This result confirms that the vervet monkey GnRHR-II cDNA utilises an ACG instead of ATG translation start codon.
4. Exon 1 is followed by a 116-bp insert prior to exon 2. The insert sequence is typed in italics.
5. Exon 2 follows after the insert sequence. The 116-bp insert results in a shift in the reading frame. The correct frame with the encoded amino acid sequence, had there not been a frame shift, is indicated in blue underneath the actual code.
6. There are six nt differences between the monkey occipital lobe 5' SMART™ RACE and the published vervet monkey GnRHR-II cDNA sequences, even though they both are from vervet monkey (see highlighted letters, x and x). All six nt differences would result in the incorporation of a different amino acid into the translated occipital lobe sequence, compared to the published vervet monkey translated protein sequence. The letters highlighted in pink (x) represent the nt sequences that differ from the published vervet monkey cDNA sequence, but is identical to the sequences obtained by RT-PCR using primer pair S3 & AS13 on monkey ejaculate RNA (see Sequence no 6 in this appendix). The letters highlighted in turquoise (x) represent the nt sequences that differ from both the published vervet monkey cDNA sequence as well as the monkey ejaculate RT-PCR result (see Sequence no 5 in this appendix).



Sequence no 6: Cloned GnRHR-II cDNA sequence from vervet monkey ejaculate, containing most of exon 1, the full exon 2 and most of exon 3, with an insert between the exons 2 and 3. Sequence stretches from -37 in the 5' UTR in exon 1 to the 3' UTR in exon 3, and is aligned to the published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)



	-37	-30	-20	-10	1	10	20	30	40																			
	H	T	A	S	S	S	V	S	L	Q	A	T	T	S	A	G	N	G	T	P	W	G	S	A	V	G	E	E
M ejaculate	cac	act	gcc	tcc	tcc	tca	gtt	tct	ctc	cag	gcc	acc	acg	tct	gca	ggc	aac	ggc	acc	cct	tgg	ggg	tca	gca	gtg	ggg	gag	gag
V/vet monkey	cac	act	tca	tcc	tcc	tca	gtt	tct	ctc	cag	gcc	acc	acg	tct	gca	ggc	aac	ggc	acc	cct	tgg	ggg	tca	gca	gtg	ggg	gag	gag
	50	60	70	80	90	100	110	120	130																			
	A	W	A	G	S	G	V	A	V	E	G	S	E	L	P	T	F	S	T	A	A	K	V	R	V	G	V	T
M ejaculate	gcc	tgg	gct	gga	tca	gga	gtg	gcg	gtg	gag	ggc	tca	gag	ctg	ccc	acc	ttc	tcg	aca	gca	gcc	aag	gtc	cga	gtg	gga	gtg	acc
V/vet monkey	gcc	tgg	gct	gga	tca	gga	gtg	gcg	gtg	gag	ggc	tca	gag	ctg	ccc	acc	ttc	tcg	aca	gca	gcc	aag	gtc	cga	gtg	gga	gtg	acc
	140	150	160	170	180	190	200	210																				
	I	V	L	F	V	S	S	A	G	G	N	L	A	V	L	W	S	V	T	R	P	Q	P	S	Q	L	R	P
M ejaculate	att	gtg	ctg	ttt	gtt	tct	tcg	gct	gga	ggg	aac	ctg	gcc	gtc	ctg	tgg	tca	gtg	aca	cgg	ccg	caa	ccc	agc	cag	ctc	cgc	ccc
V/vet monkey	att	gtg	ctg	ttt	gtt	tct	tcg	gct	gga	ggg	aac	ctg	gcc	gtc	ctg	tgg	tca	gtg	aca	cgg	ccg	caa	ccc	agc	cag	ctc	cgc	ccc
	220	230	240	250	260	270	280	290	300																			
	S	P	V	R	T	L	F	A	H	L	A	A	D	L	L	V	T	F	V	V	M	P	L	D	A	T	*	
M ejaculate	tct	ccg	gtc	agg	aca	ctc	ttc	gcc	cat	tta	gca	gct	gcc	gac	tta	cta	gtc	act	ttt	gtg	gtt	atg	ccc	cta	gat	gcc	acc	tag
V/vet monkey	tct	ccg	gtc	agg	aca	ctc	ttc	gcc	cat	tta	gca	gct	gcc	gac	tta	cta	gtc	act	ttt	gtg	gtt	atg	ccc	cta	gat	gcc	acc	tgg
	310	320	330	340	350	360	370	380																				
	N	I	T	V	Q	W	L	A	G	D	I	A	C	R	T	L	M	F	L	K	L	M	A	M	Y	S	A	A
M ejaculate	aat	atc	act	gtt	caa	tgg	ctg	gcc	ggg	gac	atc	gca	tgt	cgg	aca	ctc	atg	ttc	ctg	aaa	cta	atg	gcc	atg	tat	tct	gca	gct
V/vet monkey	aat	atc	act	gtt	caa	tgg	ctg	gcc	ggg	gac	atc	gca	tgt	cgg	aca	ctc	atg	ttc	ctg	aaa	cta	atg	gcc	atg	tat	tct	gca	gct
	390	400	410	420	430	440	450	460																				
	F	L	P	V	V	I	G	L	D	R	Q	A	A	V	L	N	P	L	G	S	R	S	G	V	R	K	L	L
M ejaculate	ttc	ctg	cct	gtg	gtc	att	gga	ctg	gac	cgc	cag	gca	gca	gta	ctc	aac	ccg	ctt	gga	tcc	cgt	tca	ggt	gta	agg	aaa	ctt	ctg
V/vet monkey	ttc	ctg	cct	gtg	gtc	att	gga	ctg	gac	cgc	cag	gca	gca	gta	ctc	aac	ccg	ctt	gga	tcc	cgt	tca	ggt	gta	agg	aaa	ctt	ctg

	470	480	490	500	510	520	530	540	550
	G A A W G L S F L L A L P Q				L F L F H T		I H R A G P V P		
M ejaculate	ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag				ctg ttc ctg ttc cat acc		atc cac cga gct ggc cca gtc cct		
V/vet monkey	ggg gca gcc tgg gga ctt agt ttc ctg ctt gcc ttg ccc cag				ctg ttc ctg ttc cat acc		gtc cac cga gct ggc cca gtc cct		

TM4 | 1 2

	560	570	580	590	600	610	620	630
	F T Q C V T K G S F K A R W Q E T T Y N L F T F C C L F							
M ejaculate	ttc act cag tgt gtc acc aaa ggc agc ttc aag gct cga tgg caa gag acc acc tat aac ctc						ttc acc ttc tgc tgc ctc ttt	
V/vet monkey	ttc act cag tgt gtc acc aaa ggc agc ttc aag gct cga tgg caa gag acc acc tat aac ctc						ttc acc ttc tgc tgc ctc ttt	

ECL2 |

	640	650	660	670	680	690	700	710	720
	L L P L I A M A I C Y S R I V L S V S S P Q T R K G S H								
M ejaculate	ctg ctg cca ctg att gcc atg gcc atc tgc tat agc cgc att gtc ctc agt gtg tcc agc cct cag						aca agg aag ggg agc cat		
V/vet monkey	ctg ctg cca ctg att gcc atg gcc atc tgc tat agc cgc att gtc ctc agt gtg tcc agc cct cag						aca agg aag ggg agc cat		

TM5 | ICL3

	721																			
	G E T P I P R P * S L T L D L L P L A S F I Y L P N S Y																			
M ejaculate	g	gt gag act cca att ccc agg cct taa tcc tta acc cta gac ctg ttg cct cta gca tca ttt atc tac cta cct aat agc tat																		
V/vet monkey	g21	gt gag act cca att ccc agg cct taa tcc tta acc cta gac ctg ttg cct cta gca tca ttt atc tac cta cct aat agc tat																		

	L P V T Q L W * D P N H M S S T * C * F C * I L S I I N
M ejaculate	cta cca gtc act caa ctg tgg tga gat cct aac cat atg tct agc acc tga tgc taa ttt tgt tga atc ctt tca att ata aac
V/vet monkey	cta cca gtc act caa ctg tgg tga gat cct aac cat atg tct agc acc tga tgc taa ttt tgt tga atc ctt tca att ata aac

	S * V S W T G T R E A I S I I Y S Y T P S S L K V D W V
M ejaculate	agc tga gtt agc tgg aca ggg act agg gag gca atc agt att att tat tct tat aca cca tca agt ctt aaa gta gac tgg gtg
V/vet monkey	agc tga gtt agc tgg aca ggg act agg gag gca atc agt att att tat tct tat aca cca tca agt ctt aaa gta gac tgg gtg

	A S H F Y H N P W G * E I I * S * V G K G K R V C S I L
M ejaculate	gct tca cat ttc tat cat aat ccc tgg ggg taa gag atc ata tag tcc tag gtt ggg aag ggg aaa agg gtt tgc agc att ctc
V/vet monkey	gct tca cat ttc tat cat aat ccc tgg ggg taa gag atc ata tag tcc tag gtt ggg aag ggg aaa agg gtt tgc agc att ctc

M ejaculate L L V G G K L C V T S Y A P P S V H P I L S S E A * T L
ctc ctt gta gga ggg aag ctc tgt gtc act agc tat gcc cct cca tca gtt cac cct ata ctc agt tca gaa gct tag act ctg
V/vet monkey ~~~

M ejaculate N Y S I F A K F L | 722 730 740 750 760 770
aat tac agt ata ttt gct aaa ttc cta g | A P A G E F A L R R S F D N R P R V C
V/vet monkey ~~~
| cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac aat cgt ccc cgt gtt tgt
| cc cct gct ggt gaa ttt gcc ctc cgc cgc tcc ttt gac aat cgt ccc cgt gtt tgt
ICL3
-|3

M ejaculate 780 790 800 810 820 830 840 850 860
L R A L R L A P L I L L T F I L C W T P Y Y L L G L W Y
ctc cgg gcc ctg aga ctg gct ccg ctt atc ttg ctg acc ttc atc ctc tgc tgg aca cct tat tac cta ctg ggt ctg tgg tac
V/vet monkey ctc cgg gcc ctg aga ctg gct ctg ctt atc ttg ctg acc ttc atc ctc tgc tgg aca cct tat tac cta ctg ggt ctg tgg tac
|-----TM6-----|

M ejaculate 870 880 890 900 910 920 930 940
W F S P T M L T E V P P S L S H I L F L F G L L N A P L
tgg ttc tct ccc acc atg cta act gaa gtc cct ccc agc ctg agc cac atc ctt ttc ctt ttt ggc ctc ctc aat gct cct ctg
V/vet monkey tgg ttc tct ccc acc atg cta act gaa gtc cct ccc agc ctg agc cac atc ctt ttc ctt ttt ggc ctc ctc aat gct cct ctg
ECL3 |-----TM7-----|

M ejaculate 950 960 970 980 990 1000 1010 1020
D P L L Y G A F T F G C R R G H Q E L S I D S S K E G S
gat cct ctc ctc tat ggg gcc ttc acc ttt ggc tgc cga aga ggg cac caa gaa ctt agt ata gac tct tct aaa gaa ggg tct
V/vet monkey gat cct ctc ctc tat ggg gcc ttc acc ttt ggc tgc cga aga ggg cac caa gaa ctt agt ata gac tct tct aaa gaa ggg tct
|-----|

M ejaculate 1030 1040 1050 1060 1070 1080 1090 1100 1110
G R M L Q Q E I H A L R Q Q E V Q K T V T S R S A G E T
ggg aga atg ctc caa cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca aga agt gca gga gaa aca
V/vet monkey ggg aga atg ctc caa cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca aga agt gca gga gaa aca

M ejaculate 1120 1130 1140 1150 1160 1170 1180 1190
K D I S I T S I * S * Q S I Q E Q N N I V * K F T * P T
aaa gac att tct ata aca tct atc tga tcc taa cag agt ata cag gaa caa aat aat ata gta tag aaa ttc aca taa cca acc
V/vet monkey aaa ggc att tct ata aca tct atc tga ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~ ~~~

	1200		1210		1220	1226				
	Q	V	T	Q	Q	D	P	I	S	
M ejaculate	<i>cag</i>	<i>gta</i>	<i>aca</i>	<i>cag</i>	<i>caa</i>	<i>gac</i>	<i>ccc</i>	<i>atc</i>	<i>tct</i>	<i>ac</i>
V/vet monkey	~~~	~~~	~~~	~~~	~~~	~~~	~~~	~~~	~~~	~~

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences are included and printed in italics.
2. The above consensus sequences was derived from a single clone that had been sequenced from both the T7 and the U19 sides as well as from S5 and from AS10 to create overlapping sequences.
3. The translation start codon and stop signal are bolded. This result confirmed that the vervet monkey GnRHR-II cDNA utilises an ACG instead of ATG translation start codon.
4. Exon 1 follows onto exon 2, which is followed by a 447 nt insert prior to exon 3. The position of the insert is indicated by "~" in the published COS-1 sequence.
5. The 447 nt insert does not result in a shift in the reading frame. However, it leads to the incorporation of a number of premature translation stop signals in frame, indicated by "*". This is possibly the result of unspliced intronic sequence.
6. There are a number of nt differences between the vervet monkey ejaculate and the published vervet monkey GnRHR-II cDNA sequences, even though they both are from vervet monkey. Often, the nt differences would result in the incorporation of a different amino acid into the translated ejaculate sequence compared to the published vervet monkey translated protein sequence (see highlighted (x/X) letters). There are two nt differences at positions -31 and -29, but these form part of the primer that was used in the RT-PCR amplification reaction. Therefore the translated amino acid at that position (A) is not highlighted, even though it is different from the published vervet monkey sequence. The reason for the choice of a 5' primer with a slightly different sequence compared to the published vervet monkey sequence was, because, by 5' RACE on COS-1 and vervet monkey occipital lobe RNA, these two nt differences were found. RT-PCR was performed using ordinary *Taq* polymerase and since the above sequence is the result of a single clone, the nt differences between the vervet monkey ejaculate and the published vervet monkey sequences are not necessarily real. The premature translation stop signal within exon 1, at nt position 299, is probably not real.

Sequence no 7: Cloned GnRHR-II cDNA sequence from baboon pituitary, containing most of exon 1 and most of exon 3 but lacking exon 2, stretching from the 5' UTR to the intracellular C-terminal tail minus ECL2, and aligned to published vervet monkey GnRHR-II cDNA sequence (Accession AF353988)

Exon 1	Exon 3
--------	--------

```

-49          -40          -30          -20          -10          1          10          20          30
  P   L   H   T   H   T   S   S   S   S   V   S   L   Q   A   T   M   S   A   G   N   G   T   P   W   G   S   A
B pituitary  ccg ctt cat acc cac act tca tcc tcc tca gtt tct ctc cag gcc acc atg tct gca ggc aac ggc acc cct tgg ggg tca gca
V/vet monkey ccg ctt cat acc cac act tca tcc tcc tca gtt tct ctc cag gcc acc acg tct gca ggc aac ggc acc cct tgg ggg tca gca

          40          50          60          70          80          90          100          110          120
  A   G   E   E   A   W   A   G   S   G   V   A   V   E   G   S   E   L   P   T   F   S   A   A   A   K   V   R
B pituitary  gcg ggg gag gag gcc tgg gct gga tcg gga gtg gcg gtg gag ggc tca gag ctg ccc acc ttc tcg gca gca gcc aag gtc cga
V/vet monkey gtc ggg gag gag gcc tgg gct gga tca gga gtg gcg gtg gag ggc tca gag ctg ccc acc ttc tcg aca gca gcc aag gtc cga

          130          140          150          160          170          180          190          200
  V   G   V   T   I   V   L   F   V   S   S   A   G   K   L   A   V   L   W   S   V   T   R   P   Q   P   S
B pituitary  gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aag ctg gcc gtc ctg tgg tca gtg aca cgg ccg caa ccc agc
V/vet monkey gtg gga gtg acc att gtg ctg ttt gtt tct tcg gct gga ggg aac ctg gcc gtc ctg tgg tca gtg aca cgg ccg caa ccc agc

          TM1          ICL1

          210          220          230          240          250          260          270          280
  Q   L   R   P   S   P   V   R   T   L   F   A   H   L   A   A   A   D   L   L   V   T   F   V   I   M   P   L
B pituitary  cag ctc cgc ccc tct ccg gtc agg aca ctc ttc gcc cat tta gca gct gcc gac tta cta gtc act ttt gtg att atg ccc cta
V/vet monkey cag ctc cgc ccc tct ccg gtc agg aca ctc ttc gcc cat tta gca gct gcc gac tta cta gtc act ttt gtg gtt atg ccc cta

          TM2

          290          300          310          320          330          340          350          360          370
  D   A   T   W   N   I   T   V   Q   W   L   A   G   D   I   A   C   R   T   L   M   F   L   K   L   M   A   M
B pituitary  gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctc atg ttc ctg aaa cta atg gcc atg
V/vet monkey gat gcc acc tgg aat atc act gtt caa tgg ctg gcc ggg gac atc gca tgt cgg aca ctc atg ttc ctg aaa cta atg gcc atg

          ECL1          TM3

```

		380		390		400		410		420		430		440		450												
	Y	S	A	A	F	L	P	V	V	I	G	L	D	R	Q	A	A	V	L	N	P	L	G	S	R	S	G	V
B pituitary	tat	tct	gca	gct	ttc	ctg	cct	gtg	gtc	att	gga	ctg	gac	cgc	cag	gca	gca	gta	ctc	aac	ccg	ctt	gga	tcc	cgt	tca	ggt	gta
V/vet monkey	tat	tct	gca	gct	ttc	ctg	cct	gtg	gtc	att	gga	ctg	gac	cgc	cag	gca	gca	gta	ctc	aac	ccg	ctt	gga	tcc	cgt	tca	ggt	gta
											ICL2																	

		460		470		480		490		500		510		520		530		540										
	R	K	L	L	G	A	A	W	G	L	S	F	L	L	A	L	P	Q										
B pituitary	agg	aaa	ctt	ctg	ggg	gca	gcc	tgg	gga	ctt	agt	ttc	ctg	ctt	gcc	ttg	ccc	cag	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~
V/vet monkey	agg	aaa	ctt	ctg	ggg	gca	gcc	tgg	gga	ctt	agt	ttc	ctg	ctt	gcc	ttg	ccc	cag	ctg	ttc	ctg	ttc	cat	acc	gtc	cac	cga	gct
											TM4																	
											1 2																	

		550		560		570		580		590		600		610		620												
B pituitary	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~												
V/vet monkey	ggc	cca	gtc	cct	ttc	act	cag	tgt	gtc	acc	aaa	ggc	agc	ttc	aag	gct	cga	tgg	caa	gag	acc	acc	tat	aac	ctc	ttc	acc	ttc
											ECL2																	

		630		640		650		660		670		680		690		700												
B pituitary	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~	~~~~												
V/vet monkey	tgc	tgc	ctc	ttt	ctg	ctg	cca	ctg	att	gcc	atg	gcc	atc	tgc	tat	agc	cgc	att	gtc	ctc	agt	gtg	tcc	agc	cct	cag	aca	agg
											TM5																	

		710		720		730		740		750		760		770		780		790											
						P	A	G	E	F	A	L	R	R	S	F	D	N	R	P	R	V	R	L	R	A	L	R	
B pituitary	~~~~	~~~~	~~~~	~~~~	~	cc	cct	gct	ggt	gaa	ttt	gcc	ctc	cgc	cgc	tcc	ttt	gac	aat	cgt	ccc	cgt	ggt	cgt	ctc	cgg	gcc	ctg	aga
V/vet monkey	aag	ggg	agc	cat	g	cc	cct	gct	ggt	gaa	ttt	gcc	ctc	cgc	cgc	tcc	ttt	gac	aat	cgt	ccc	cgt	ggt	tgt	ctc	cgg	gcc	ctg	aga
											ICL3																		
											2 3																		

		800		810		820		830		840		850		860		870												
	L	A	L	L	I	L	L	T	F	I	L	C	W	T	P	Y	Y	L	L	G	L	W	Y	W	F	S	P	T
B pituitary	ctg	gc	ctg	ctt	atc	ttg	ctg	acc	ttc	atc	ctc	tgc	tgg	aca	cct	tat	tac	cta	ctg	ggt	ctg	tgg	tac	tgg	ttc	tcc	ccc	acc
V/vet monkey	ctg	gct	ctg	ctt	atc	ttg	ctg	acc	ttc	atc	ctc	tgc	tgg	aca	cct	tat	tac	cta	ctg	ggt	ctg	tgg	tac	tgg	ttc	tct	ccc	acc
											TM6																	
											ECL3																	

```

      880      890      900      910      920      930      940      950      960
      M L T E V P P S L S H I L F L F G L L N A P L D P L F Y
B pituitary atg cta act gaa gtc cct ccc agt ctg agc cac atc ctt ttc ctt ttt ggc ctc ctc aat gct cct ttg gat cct ctc ttc tat
V/vet monkey atg cta act gaa gtc cct ccc agc ctg agc cac atc ctt ttc ctt ttt ggc ctc ctc aat gct cct ctg gat cct ctc ctc tat
|-----TM7-----

```

```

      970      980      990      1000      1010      1020      1030      1040
      G A F T L G C Q R G H Q E L S I D S S K E G S G R M L Q
B pituitary ggg gcc ttc acc ctt ggc tgc caa aga ggg cac caa gaa ctt agt ata gac tct tct aaa gaa ggg tct ggg aga atg ctc caa
V/vet monkey ggg gcc ttc acc ctt ggc tgc caa aga ggg cac caa gaa ctt agt ata gac tct tct aaa gaa ggg tct ggg aga atg ctc caa
|-----C-terminal tail-----

```

```

      1050      1060      1070      1080      1090      1100      1110      1120
      Q E I H A L R Q Q E V Q K T V T S R S A G E T K D I S I
B pituitary cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca aga agt gca gga gaa aca aaa gac att tct ata
V/vet monkey cag gag att cat gcc ctt aga cag cag gaa gta caa aaa act gtg aca tca aga agt gca gga gaa aca aaa ggc att tct ata

```

```

      1130      1140      1150      1160      1170      1180      1190      1200      1210
      T S I * S * Q S I * E Q N N K S L I P * D L N I S L L L
B pituitary aca tct atc tga tcc taa cag agt ata tag gaa caa aat aat aag tct tta ata cca taa gat ctt aac atc tca ctt cta ctc
V/vet monkey aca tct atc tga

```

```

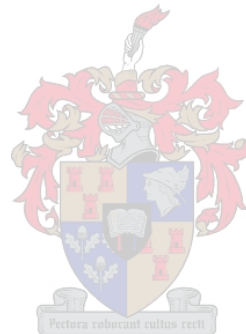
      1220      1230      1240      1247
      L L S * F P P K K K Y
B pituitary ctg ctc tcc tag ttc ccc cca aaa aag aaa tac tg

```

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nt sequences. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated. Exon boundaries are indicated with a dotted line showing exon numbers. Primer sequences, namely S1 and AS12, are included and printed in italics.
2. A total of five clones were sequenced in both directions to obtain the above consensus sequence.
3. The translation start codon and translation stop signal are bolded.
4. Exon 1 follows directly onto exon 3. The position of the missing exon 2 is indicated by "~".
5. The omission of exon 2 results in a shift in the reading frame, from frame 1 (exon 1) to frame 3 (exon 3).

6. There are a number of nt differences between the baboon pituitary sequence and the published vervet monkey GnRHR-II cDNA sequence (accession AF353988). The RT-PCR was performed using Expand High fidelity proofreading *Taq* polymerase and, furthermore, five different clones were sequenced to obtain the above consensus sequence. Therefore, these nt differences are unlikely the result of PCR- or sequencing errors. Rather, they are likely to represent species differences between baboon and vervet monkey. Often, the nt differences also result in the incorporation of a different amino acid into the translated baboon sequence compared to the published vervet monkey translated protein sequence (see highlighted (x/X) letters).



Sequence no 8: Additional insert sequence between exons 2 and 3 of the GnRHR-II cDNA: comparison between baboon pituitary and vervet monkey ejaculate and occipital lobe

	1	10	20	30	40	50	60	70	80																			
M occ lobe	gtg	aga	ctc	caa	ttc	cca	ggc	ctt	aat	cct	taa	ccc	tag	acc	tgt	tgc	ctc	tag	cat	cat	tta	tct	acc	tac	cta	ata	gct	atc
M ejaculate	gtg	aga	ctc	caa	ttc	cca	ggc	ctt	aat	cct	taa	ccc	tag	acc	tgt	tgc	ctc	tag	cat	cat	tta	tct	acc	tac	cta	ata	gct	atc
B pituitary	gtg	aga	ctc	caa	ttc	cca	ggc	ctt	aat	cct	taa	ccc	tag	acc	tgt	tgc	ctc	tag	cat	cat	tta	tct	acc	tac	cta	ata	gct	atc
		90	100	110	120	130	140	150	160																			
M occ lobe	tac	cag	tca	ctc	aac	tgt	ggt	gag	atc	cta	acc	ata	tgt	cta	gca	cct	gat	gct	aat	ttt	ggt	gaa	tcc	ttt	caa	tta	taa	aca
M ejaculate	tac	cag	tca	ctc	aac	tgt	ggt	gag	atc	cta	acc	ata	tgt	cta	gca	cct	gat	gct	aat	ttt	ggt	gaa	tcc	ttt	caa	tta	taa	aca
B pituitary	tac	cag	tca	ctc	aac	tgt	ggt	gag	atc	cta	acc	ata	tgt	cta	gca	cct	gat	gct	aat	ttt	ggt	gaa	tcc	ttt	caa	tta	taa	aca
	170	180	190	200	210	220	230	240	250																			
M occ lobe	gct	gag	tta	gct	gga	cag	gga	cta	ggg	agg	caa	tca	gta	tta	ttt	att	ctt	atg	cac	cat	caa	gtc	tta	aag	tag	act	ggg	tgg
M ejaculate	gct	gag	tta	gct	gga	cag	gga	cta	ggg	agg	caa	tca	gta	tta	ttt	att	ctt	ata	cac	cat	caa	gtc	tta	aag	tag	act	ggg	tgg
B pituitary	gct	gag	tta	gct	gga	caa	gga	cta	ggg	agg	caa	tca	gta	tta	ttt	att	ctt	gaa	cac	cat	caa	gtc	tta	aag	tag	act	ggg	tgg
	260	270	280	290	300	310	320	330																				
M occ lobe	ctt	cac	att	tct	atc	ata	atc	cct	ggg	ggt	aag	aga	tca	tac	agt	cct	agg	ttg	gga	agg	gga	aaa	ggg	ttt	gca	gca	ttc	tcc
M ejaculate	ctt	cac	att	tct	atc	ata	atc	cct	ggg	ggt	aag	aga	tca	tac	agt	cct	agg	ttg	gga	agg	gga	aaa	ggg	ttt	gca	gca	ttc	tcc
B pituitary	ctt	cac	att	tct	atc	ata	atc	cct	ggg	ggt	aag	aga	tca	tac	agt	cct	agg	ttg	gga	agg	gga	aaa	ggg	ttt	gca	gca	ttc	tcc
	340	350	360	370	380	390	400	410	420																			
M occ lobe	tcc	ttg	tag	gag	gga	agc	tct	gtg	tca	cta	gct	atg	ccc	ctc	cat	cag	ttc	acc	cta	tac	tca	ggt	cag	aag	ctt	aga	ctc	tga
M ejaculate	tcc	ttg	tag	gag	gga	agc	tct	gtg	tca	cta	gct	atg	ccc	ctc	cat	cag	ttc	acc	cta	tac	tca	ggt	cag	aag	ctt	aga	ctc	tga
B pituitary	tcc	ttg	tag	gag	gca	agc	tct	gtg	tca	cta	gct	atg	ccc	ctc	cat	cag	ttc	acc	cta	tac	tca	ggt	cag	aag	ctt	aga	ctc	tga
	430	440	447																									
M occ lobe	att	gca	gta	tat	ttg	cta	aat	tcc	tag																			
M ejaculate	att	aca	gta	tat	ttg	cta	aat	tcc	tag																			
B pituitary	att	aca	gta	tat	ttc	cta	aat	tcc	tag	c	448																	

Notes:

1. Nucleotide identities that are the same in two of the three RNAs but differ in the third are highlighted in yellow.
2. The percentage overall identities are as follows, except for the additional C at the end of the baboon pituitary sequence:
 Vervet monkey occipital lobe vs. vervet monkey ejaculate: 99.3% (3 nt differences over 447 bp)
 Vervet monkey occipital lobe vs. baboon pituitary: 98.4% (7 nt differences over 447 bp)
 Vervet monkey ejaculate vs. baboon pituitary: 99.1% (4 nt differences over 447 bp)

Sequence no 9: Cloned GnRH-1 cDNA sequences from human ejaculate and hypothalamus, aligned to the published human GnRH-1 cDNA sequence (Accession X15215)

```
18 20      30      40      50      60      70      80      90      100
      L  L  A  G  L  I  L  L  T  W  C  V  E  G  C  S  S  Q  H  W  S  Y  G  L  R  P  G  G
H sperm  a  ctc  cta  gct  ggc  ctt  att  cta  ctg  act  tgg  tgc  gtg  gaa  ggc  tgc  tcc  agc  cag  cac  tgg  tcc  tat  gga  ctg  cgc  cct  gga  gga
H hypothal  a  ctc  cta  gct  ggc  ctt  att  cta  ctg  act  tgg  tgc  gtg  gaa  ggc  tgc  tcc  agc  cag  cac  tgg  tcc  tat  gga  ctg  cgc  cct  gga  gga
X15215    a  ctc  cta  gct  ggc  ctt  att  cta  ctg  act  tgg  tgc  gtg  gaa  ggc  tgc  tcc  agc  cag  cac  tgg  tcc  tat  gga  ctg  cgc  cct  gga  gga
                                     Signal peptide                                     |_____Mature decapeptide_____|

      110      120      130      140      150      160      170      180
      K  R  D  A  E  N  L  I  D  S  F  Q  E  I  V  K  E  V  G  Q  L  A  G  T  Q  R  F  E
H sperm  aag  aga  gat  gcc  gaa  aat  ttg  att  gat  tct  ttc  caa  gag  ata  gtc  aaa  gag  gtt  ggc  caa  ctg  gca  gga  acc  caa  cgc  ttc  gaa
H hypothal  aag  aga  gat  gcc  gaa  aat  ttg  att  gat  tct  ttc  caa  gag  ata  g
X15215    aag  aga  gat  gcc  gaa  aat  ttg  att  gat  tct  ttc  caa  gag  ata  gtc  aaa  gag  gtt  ggt  caa  ctg  gca  gaa  acc  caa  cgc  ttc  gaa
|___PS___|                                     GnRH-associated peptide

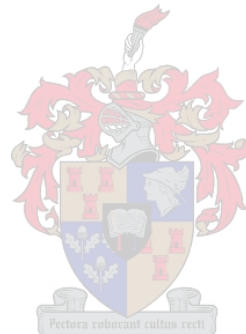
      190      200      210      220      230      240      250      260      270
      C  T  T  H  Q  P  R  S  P  L  R  D  L  K  G  A  L  E  S  L  I  E  E  E  T  G  Q  K
H sperm  tgc  acc  acg  cac  cag  cca  cgt  tct  ccc  ctc  cga  gac  ctg  aaa  gga  gct  ctg  gaa  agt  ctg  att  gaa  gag  gaa  act  ggg  cag  aag
H hypothal  tgc  acc  acg  cac  cag  cca  cgt  tct  ccc  ctc  cga  gac  ctg  aaa  gga  gct  ctg  gaa  agt  ctg  att  gaa  gag  gaa  act  ggg  cag  aag
X15215    tgc  acc  acg  cac  cag  cca  cgt  tct  ccc  ctc  cga  gac  ctg  aaa  gga  gct  ctg  gaa  agt  ctg  att  gaa  gag  gaa  act  ggg  cag  aag
                                     GnRH-associated peptide

      280      290      300      310      320      330      340      350
      K  I  *  I  H  W  A  R  R  N  D  H  Y  *  H  D  L  S  I  I  L  T  L  K  I  Y  N  P
H sperm  aag  att  taa  atc  cat  tgg  gcc  aga  agg  aat  gac  cat  tac  taa  cat  gac  tta  agt  ata  att  ctg  aca  ttg  aaa  att  tat  aac  cca
H hypothal  aag  att  taa  atc  cat  tgg  gcc  aga  agg  aat  gac  cat  tac  taa  cat  gac  tta  agt  ata  att  ctg  aca  ttg  aaa  att  tat  aac  cca
X15215    aag  att  taa  atc  cat  tgg  gcc  aga  agg  aat  gac  cat  tac  taa  cat  gac  tta  agt  ata  att  ctg  aca  ttg  aaa  att  tat  aac  cca

      360      370      380      390      400  404
      L  N  T  C  K  W  Y  E  F  Q  K  S  L  H  Q  V
H sperm  tta  aat  acc  tgt  aaa  tgg  tat  gaa  ttt  cag  aaa  tcc  tta  cac  caa  gtt  gc
H hypothal  tta  aat  acc  tgt  aaa  tgg  tat  gaa  ttt  cag  aaa  tcc  tta  cac  caa  gtt  gc
X15215    tta  aat  acc  tgt  aaa  tgg  tat  gaa  ttt  cag  aaa  tcc  tta  cac  caa  gtt  gc
```

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nucleotide sequences. The signal- and mature peptides, processing site (PS) and GnRH-associated peptide are indicated. Primer sequences are included and printed in italics.
2. There are 3 nucleotide differences between the human sperm and the published human GnRH-1 sequence. The differences are highlighted. Two of these nucleotide differences also result in the incorporation of different amino acids, which are highlighted as well.
3. The human sperm and hypothalamus sequences are the same up to where the hypothalamus sequence ends.
4. The human sperm sequence is a consensus sequence derived from 2 clones whereas the hypothalamus sequence is the result of sequencing of a single clone.



Sequence no 10: Cloned GnRH-2 cDNA sequence from human ejaculate, aligned to the published human GnRH-2 variant 1 cDNA sequence (Accession NM_001501)

```

-49      -40      -30      -20      -10      1      10      20
  Q L P E G A I S S T A L P * A A M A S S R R G L L L
H sperm  g cag ctg cct gaa gga gcc atc tca tcc aca gct ctt cct tga gca gcc atg gcc agc tcc agg cga ggc ctc ctg ctc
NM_001501 g cag ctg cct gaa gga gcc atc tca tcc aca gct ctt cct tga gca gcc atg gcc agc tcc agg cga ggc ctc ctg ctc

30      40      50      60      70      80      90      100      110
  L L L L T A H L G P S E A Q H W S H G W Y P G G K R A
H sperm  ctg ctg ctg ctg act gcc cac ctt gga ccc tca gag gct cag cac tgg tcc cat ggc tgg tac cct gga gga aag cga gcc
NM_001501 ctg ctg ctg ctg act gcc cac ctt gga ccc tca gag gct cag cac tgg tcc cat ggc tgg tac cct gga gga aag cga gcc
                                     |_____Signal peptide_____

120      130      140      150      160      170      180      190
  L S S A Q D P Q N A L R E P G R A L D T A A G S P V Q
H sperm  ctc agc tca gcc cag gat ccc cag aat gcc ctt agg ccc cca gca ggc agc cca gtc cag
NM_001501 ctc agc tca gcc cag gat ccc cag aat gcc ctt agg ccc cca gga agg gcc ctg gac act gca gca ggc agc cca gtc cag
_____ | _____Mature decapeptide_____ | | _____PS_____ | _____GnRH-associated peptide_____

200      210      220      230      240      250      260      270
  T A H G L P S D A L A P L D D S M P W E G R T T A Q W
H sperm  act gcc cat ggc ctc cca agt gat gcc ctg gct ccc ctg gac gac agc atg ccc tgg gag ggc agg acc acg gcc cag tgg
NM_001501 act gcc cat ggc ctc cca agt gat gcc ctg gct ccc ctg gac gac agc atg ccc tgg gag ggc agg acc acg gcc cag tgg
                                     GnRH-associated peptide

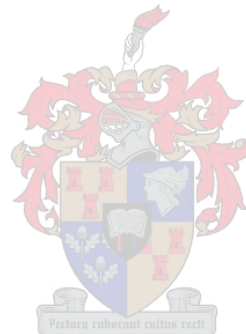
280      290      300      310      318
  S L H R K R H L A R T L L T A
H sperm  tcc ctt cac agg aag cga cac ctg gca cgg aca ctg ctg acc gca g
NM_001501 tcc ctt cac agg aag cga cac ctg gca cgg aca ctg ctg acc gca g
                                     GnRH-associated peptide

```

Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nucleotide sequences. The signal- and mature peptides, processing site (PS) and GnRH-associated peptide are indicated. Primer sequences are included and printed in italics. The translation start site is bolded.
2. The human sperm sequence and the published human GnRH-2 sequences are exactly the same for the amplified region, except that the sperm sequence has a 21 nt deletion at the 5' end of exon 3.

3. The human sperm sequence is a consensus sequence derived from sequencing of 4 clones.
4. There are two nucleotide positions at which the sperm sequence can be degenerate (highlighted in grey):
 - Nucleotide number 223: t/c (2 clones: t and 2 clones: c)
 - Nucleotide number 266: a/g (2 clones: a and 2 clones: g)Thus, two of the human sperm clones contain the same sequence as published while two clones differed in two nucleotide positions.



		430		440		450		460		470		480		490		500												
	L	A	I	T	R	P	L	A	L	K	S	N	S	K	V	G	Q	S	M	V	G	L	A	W	I	L	S	S
H sperm	ctg	gct	atc	acg	agg	ccc	cta	gct	ttg	aaa	agc	aac	agc	aaa	gtc	gga	cag	tcc	atg	gtt	ggc	ctg	gcc	tgg	atc	ctc	agt	agt
H pituitary	ctg	gct	atc	acg	agg	ccc	cta	gct	ttg	aaa	agc	aac	agc	aaa	gtc	gga	cag	tcc	atg	gtt	ggc	ctg	gcc	tgg	atc	ctc	agt	agt
L07949	ctg	gct	atc	acg	agg	ccc	cta	gct	ttg	aaa	agc	aac	agc	aaa	gtc	gga	cag	tcc	atg	gtt	ggc	ctg	gcc	tgg	atc	ctc	agt	agt

ICL2

		510		520		530		540		550		560		570		580												
	V	F	A	G	P	Q	L	Y	I	F	R	M	I	H	L	A	D	S	S	G	Q	T	K	V	F	S	Q	C
H sperm	gtc	ttt	gca	gga	cca	cag	tta	tac	atc	ttc	agg	atg	att	cat	cta	gca	gac	agc	tct	gga	cag	aca	aaa	gtt	ttc	tct	caa	tgt
H pituitary	gtc	ttt	gca	gga	cca	cag	tta	tac	atc	ttc	agg	atg	att	cat	cta	gca	gac	agc	tct	gga	cag	aca	aaa	gtt	ttc	tct	caa	tgt
L07949	gtc	ttt	gca	gga	cca	cag	tta	tac	atc	ttc	agg	atg	att	cat	cta	gca	gac	agc	tct	gga	cag	aca	aaa	gtt	ttc	tct	caa	tgt

TM4

ECL2

		590		600		610		620		630		640		650		660		670										
	V	T	H	C	S	F	S	Q	W	W	H	Q	A	F	Y	N	F	F	T	F	S	C	L	F	I	I	P	L
H sperm	gta	aca	cac	tgc	agt	ttt	tca	caa	tgg	tgg	cat	caa	gca	ttt	tat	aac	ttt	ttc	acc	ttc	agc	tgc	ctc	ttc	atc	atc	cct	ctt
H pituitary	gta	aca	cac	tgc	agt	ttt	tca	caa	tgg	tgg	cat	caa	gca	ttt	tat	aac	ttt	ttc	acc	ttc	agc	tgc	ctc	ttc	atc	atc	cct	ctt
L07949	gta	aca	cac	tgc	agt	ttt	tca	caa	tgg	tgg	cat	caa	gca	ttt	tat	aac	ttt	ttc	acc	ttc	agc	tgc	ctc	ttc	atc	atc	cct	ctt

TM5

		680		690		700		710		720		730		740		750												
	F	I	M	L	I	C	N	A	K	I	I	F	T	L	T	R	V	L	H	Q	D	P	H	E	L	Q	L	N
H sperm	ttc	atc	atg	ctg	atc	tgc	aat	gca																				
H pituitary	ttc	atc	atg	ctg	atc	tgc	aat	gca	aaa	atc	atc	ttc	acc	ctg	aca	cgg	gtc	ctt	cat	cag	gac	ccc	cac	gaa	cta	caa	ctg	aat
L07949	ttc	atc	atg	ctg	atc	tgc	aat	gca	aaa	atc	atc	ttc	acc	ctg	aca	cgg	gtc	ctt	cat	cag	gac	ccc	cac	gaa	cta	caa	ctg	aat

ICL3

		760		770		780		790		800		810		820		830		840										
	Q	S	K	N	N	I	P	R	A	R	L	K	T	L	K	M	T	V	A	F	A	T	S	F	T	V	C	W
H sperm																												
H pituitary	cag	tcc	aag	aac	aat	ata	cca	aga	gca	cgg	ctg	aag	act	cta	aaa	atg	acg	gtt	gca	ttt	gcc	act	tca	ttt	act	gtc	tgc	tgg
L07949	cag	tcc	aag	aac	aat	ata	cca	aga	gca	cgg	ctg	aag	act	cta	aaa	atg	acg	gtt	gca	ttt	gcc	act	tca	ttt	act	gtc	tgc	tgg

TM6

```

      850      860      870      880      890      900      910      917
      T   P   Y   Y   V   L   G   I   W   Y   W   F   D   P   E   M   L   N   R   L   S   D   P   V   N
H sperm
H pituitary act ccc tac tat gtc cta gga att tgg tat tgg ttt gat cct gaa atg tta aac agg ttg tca gac cca gta aat ca
L07949      act ccc tac tat gtc cta gga att tgg tat tgg ttt gat cct gaa atg tta aac agg ttg tca gac cca gta aat ca
      _____|____ECL3____|____TM7____--

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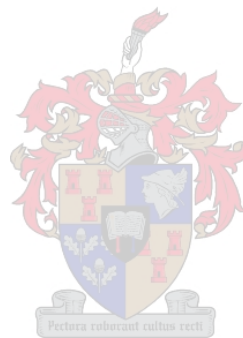
Notes:

1. Nucleotides are numbered from the translation start. Translated amino acid sequences are indicated above the nucleotide sequences. Primer sequences are included and printed in italics. The translation start site is bolded. Transmembrane domains (TM) and intracellular (ICL) and extracellular (ECL) loops are indicated underneath.
2. The human pituitary positive control as well as the human sperm sequences are identical to the published GnRHR-I cDNA sequence, which had been obtained by cloning the cDNA from whole human pituitary. This result confirms that humans acquire a single gene for the GnRHR-I, which is situated on chromosome 4 [Kakar SS, 1997].
3. The human sperm and pituitary sequences are results of sequencing of two clones.



Appendix 5: First publication

Van Biljon W, Wykes S, Scherer S, Krawetz SA, Hapgood J. Type II gonadotropin-releasing hormone receptor transcripts in human sperm. *Biology of Reproduction* 2002; **67**: 1741-1749.¹



1. In this publication it was stated that a gene is ubiquitously transcribed in the antisense orientation to the *GnRHR-II* gene on chromosome 14. This gene had been previously reported by others to encode a ribonucleoprotein [Conklin DC, Rixon MW, Kuestner RE, Maurer MF, Whitmore TE, Millar RP. Cloning and gene expression of a novel human ribonucleoprotein. *Biochimica et biophysica acta* 2000; **1492**: 465-468]. However, more recent evidence indicates that a functional transcript for the ribonucleoprotein RBM8A is transcribed from the gene on chromosome 1, and not from the gene on chromosome 14. It appears that a transcript from the gene on chromosome 14 would be non-functional for the ribonucleoprotein [Faurholm B, Millar RP, Katz AA. The genes encoding the type II gonadotropin-releasing hormone receptor and the ribonucleoprotein RBM8A in humans overlap in two genomic loci. *Genomics* 2001; **78**: 15-18.].

Appendix 6: Second publication

Hapgood JP, Sadie H, Van Biljon W, Ronacher K. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *Journal of Neuroendocrinology* 2005; **17**: 619-638.



Type II Gonadotropin-Releasing Hormone Receptor Transcripts in Human Sperm¹

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ABSTRACT

GnRH regulates reproduction via the well-characterized mammalian pituitary GnRH receptor (type I). In addition, two homologous genes for a second form of the GnRH receptor (type II) are present in the human genome, one on chromosome 14 and the second on chromosome 1. The chromosome 14 gene is ubiquitously transcribed at high levels in the antisense orientation but lacks exon 1, required to encode a full-length receptor. In comparison, the chromosome 1 gene contains all three exons. The issue of whether this gene is transcribed in any human tissue(s), and whether these transcripts encode a functional receptor protein, remains unresolved. We have directly addressed this by screening a panel of human RNAs by hybridization and RT-PCR. These analyses showed that, unlike the chromosome 14 gene, chromosome 1 gene expression is limited and of low abundance. Exon 1-containing transcripts were detected by *in situ* hybridization in mature sperm and in human post-meiotic testicular cells. Further sequence analysis revealed that although all the potential coding segments were present, the human transcripts, like the gene, contain a stop codon within the coding region and a frame-shift relative to other mammalian GnRH receptors. Although this suggests that the human gene may be a transcribed pseudogene, a functional type II GnRH receptor cDNA has recently been cloned from monkeys. Given the well-established role of GnRH in spermatogenesis and reported evidence of type II GnRH receptor immunoreactivity in human tissues, it is possible that the chromosome 1 gene is functional.

gene regulation, gonadotropin-releasing hormone receptor, sperm, spermatogenesis, testis

INTRODUCTION

GnRH is a key reproductive hormone in all vertebrates, including humans. Upon binding to its receptor on the cell membrane of pituitary gonadotropes, GnRH triggers the release of the gonadotropins, namely LH and FSH. These hormones in turn regulate most of the reproductive functions in both sexes. GnRH and GnRH analogues have extensive application in the treatment of human diseases. For example, they are utilized to treat infertility, precocious puberty, polycystic ovarian syndrome and breast cancer in

women, delayed puberty in boys, prostatic cancer in men, and in the protection of gonadal tissue during radiotherapy and chemotherapy [1].

The well-characterized pituitary GnRH receptor (or so-called type I receptor), a member of the seven transmembrane G-protein coupled family of receptors, has been cloned and its structure and function have been studied extensively in both mammalian and nonmammalian vertebrates [2–8]. Interestingly, most vertebrates, including man, have two or more forms of the GnRH peptide. In man, a second form of the GnRH hormone has been detected in the kidney, prostate, bone marrow, and brain [9]. In addition, GnRH receptors have been detected by ligand-binding studies in the pituitary, placenta, ovary, testis, adrenal glands, lymphocytes, and the central nervous system, as well as cancers of the breast and prostate [10–14]. Thus it is possible that GnRH(s) and its receptor(s) may, in addition to the established endocrine role, have paracrine and autocrine functions in several reproductive and nonreproductive tissues. In human testis, for example, it may act as a neuroendocrine hormone [15]. In amphibia, GnRH is present in the sympathetic ganglia where it acts as a neuro-modulator [16]. In fish, GnRH-producing cells interact with neurons that control sperm duct and oviduct contractility [17]. Furthermore, several differentiated lymphocytes produce GnRH or a GnRH-like peptide [18]. Taken together, these observations suggest that more than one form of the GnRH receptor exists within a single species.

Indeed, a second form of the GnRH receptor has recently been cloned from several nonmammalian vertebrate organs such as goldfish brain and pituitary [2], frog midbrain and pituitary [3], and chicken pituitary [4], as well as from mammalian vertebrate organs such as marmoset pituitary [19], COS-1 (vervet monkey kidney) cells, and rhesus monkey pituitary [20]. The mRNAs of all the mammalian GnRH receptors cloned to date are encoded by three exons, and the gene structures are conserved. Database searches have revealed that apart from the known type I pituitary receptor gene there are at least two other GnRH receptor genes in the human genome, which are located on separate chromosomes. A gene that encodes exons 2 and 3 as well as intron 2 of a putative type II GnRH receptor is located on chromosome 14. A second human type II GnRH receptor gene containing all three putative exons (exons 1, 2, and 3) is located on chromosome 1 (accession AL160282). It has been reported that the chromosome 14 gene is abundantly expressed in a wide range of human tissues, but the transcripts are in the antisense orientation with respect to the type II receptor sequence [21]. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blotting analyses showed that the gene is transcribed in most human tissues examined. However, an intronic sequence equivalent to intron 2 was retained, reflecting the absence of donor

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and acceptor splice sites for these transcripts ([21] and our unpublished results). Interestingly, the chromosome 14 transcript encodes the 3' untranslated region of a novel human ribonucleoprotein mRNA, RBM8 (accession AF127761) [21, 22]. In comparison, little is known of the expression of the chromosome 1 gene. This includes whether a functional, full-length transcript for a human type II GnRH receptor, arising from transcription of the gene on chromosome 1, is expressed in human pituitary or any other tissue. The results of our studies that directly address this issue are presented.

MATERIALS AND METHODS

Patient Samples

For RT-PCR analysis, human semen samples pooled from a number of male donors were obtained from the Andrology Department, Groote Schuur hospital (Cape Town, South Africa). Adult human postmortem tissue was obtained from the Salt River Mortuary under the supervision of pathologists from the University of Cape Town (UCT) Medical School (Cape Town, South Africa), after approval from the Medical Ethics Committee at UCT. Postabortion human fetal tissue was obtained by Nicola Illing (Department of Molecular and Cell Biology, UCT, Cape Town, South Africa) who obtained permission from the Medical Ethics Committee at UCT. For *in situ* hybridizations, formalin-fixed, paraffin-embedded testicular samples were provided by Harper hospital (Detroit, MI). Human semen samples from a normal male donor were obtained from the *in vitro* fertilization (IVF) clinic, Hutzel hospital (Detroit, MI). Semen samples had an average concentration of 15×10^7 sperm/ml and contained less than 2% immature germ cells and/or somatic contaminants.

Human Dot Blot

Dot blot analysis was performed using radiolabeled, double-stranded exon 1-specific human type II GnRH receptor DNA to probe a commercially available human RNA Master blot (Clontech, Palo Alto, CA) containing poly(A)⁺ RNA from 50 different human tissues. The DNA probe was obtained by PCR amplification of a 402-base pair (bp) fragment from human genomic DNA using primer pair S1 (5' CCC ACC TTC TCG GCA GCA GCC 3') and AS2 (5' GAA ACT AAG TCC CCA GGC TGC 3'). A 25-ng aliquot of the probe was labeled for 1 h at 37°C with the use of the Megaprime DNA labeling system (Amersham, Buckinghamshire, England) and 50 μ Ci [α -³²P]dCTP as described by the supplier. The labeled probe (10⁹ dpm/ μ g) was purified on a G-25 spin column. Hybridization, using 25 ng of probe in 5 ml of solution, was performed as described in the Master blot manual, except for the replacement of sheared salmon testis DNA with sheared herring sperm DNA. Washes were as follows: five 20-min washes with solution 1 (2 \times SSC, 1% SDS) at 65°C and two 20-min washes with solution 2 (0.1 \times SSC, 0.5% SDS) at 55°C. The Master blot membrane was exposed to Hyperfilm for four overnights prior to autoradiographic detection.

Isolation of Total RNA

Total RNA was isolated from fresh human semen by cesium chloride-guanidinium isothiocyanate ultracentrifugation as described [23]. In brief, cells were pelleted for 10 min at 2000 \times g and resuspended in 4.5 ml of guanidinium solution per 0.5-g pellet. Prior to ultracentrifugation, 1 g of cesium chloride was added per 2.5 ml of homogenate. Dithiothreitol (DTT) and RNasin ribonuclease inhibitor (Promega, Madison, WI) were added to the purified RNA to final concentrations of 1 mM and 1 U/ μ l, respectively. Human fetal RNA was obtained from Nicola Illing. Total RNA from other human tissues was isolated with the use of TRI-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol.

Reverse Transcriptase-Polymerase Chain Reaction

A 1- μ g aliquot of total RNA was reverse-transcribed in a 20- μ l reaction volume using 200 ng of a hexanucleotide mix (Roche, Randburg, South Africa) and 200 U of Superscript II RNaseH⁻ reverse transcriptase (Gibco-BRL, Paisley, Scotland). Reverse transcriptase-reactions included 1 mM dNTPs, 1 \times Gibco first strand cDNA buffer, 10 mM DTT, 20 U RNasin, and 0.1 mg/ml BSA and were performed at 42°C for 1 h. Poly-

merase chain reactions were performed with 10 μ l of first-strand cDNA and 5 U *Taq* DNA polymerase in a 50- μ l reaction volume using combinations of human type II GnRH receptor exon 1-, exon 2-, and exon 3-specific oligonucleotide primers (see Fig. 2). Twenty picomoles of each gene-specific primer were used. The sense primers were for exon 1, S2 (5' ACC TGG AAT ATC ACT GTT CAA TGG 3'); for exon 2, S3 (5' GCA AGA GAC CAC CTA TAA CCT 3'); and for mouse β -actin, β -S (5' CAC CAC ACC TTC TAC AAT GAG CTG 3'). Antisense primers were for exon 1, AS1 (5' CAT GCG ATG TCC ACA GCC AGC C 3') and AS2 (5' GAA ACT AAG TCC CCA GGC TGC 3'); for exon 2, AS3 (5' GGT TAT AGG TGG TCT CTT GC 3'); for exon 3, AS4 (5' GGT GTC CAG CAG AGG ATG AAG GTC AG 3') and AS5 (5' GGA GAG CAG GAG TAG AAG TGA G 3') for the 3' untranslated region; and for mouse β -actin, β -AS (5' GAT CTT CAT GAG GTA GTC TGT CAG G 3'). Cycling parameters were as follows: 2.5 min denaturing at 93°C, followed by 35 cycles of 1 min denaturing at 93°C, 1 min annealing at 55°C and 1 min extension at 72°C, and a final extension of 10 min at 72°C. The RT-PCR products were visualized on 1% agarose gels containing 0.5 μ g/ml ethidium bromide.

5' Rapid Amplification of cDNA Ends

Double-stranded Marathon-ready cDNA for 5' rapid amplification of cDNA ends (RACE) was prepared from 1 μ g of total RNA from human semen. Hexanucleotide primers (5 μ M) were used for first-strand cDNA synthesis using AMV reverse transcriptase and all other components of the Marathon cDNA amplification kit (Clontech). Adaptor-ligated double-stranded cDNA was diluted 1:25 in Tricine-EDTA buffer, which was supplied with the RACE kit. A 5- μ l aliquot of RACE-ready cDNA and 10 picomoles of each primer were used per 50 μ l reaction. Primary 5' RACE was performed with the adaptor-specific AP1 primer (5' CCA TCC TAA TAC GAC TCA CTA TAG GGC 3') in conjunction with exon 1-specific antisense oligo AS2. Nested 5' RACE was performed using 5 μ l of the primary reaction, adaptor-specific AP2 primer (5' ACT CAC TAT AGG GCT CGA GCG GC 3') and antisense gene-specific primer AS1, which is internally nested to AS2, in a final volume of 50 μ l. Touchdown PCR was performed according to the manufacturer's instructions using 36 cycles for the primary and 30 cycles for the secondary PCR reactions, respectively. Products of 5' RACE were purified, cloned, and sequenced as described below.

Southern Blot Confirmation of RT-PCR and 5' RACE Results

The PCR products were transferred to Hybond N⁺ nylon membranes by capillary blotting after electrophoresis. The DNA was cross-linked to the membranes with a UV crosslinker (Amersham). Membranes were subsequently probed with type II GnRH receptor gene-specific oligonucleotides that were labeled and detected using the ECL 3'-oligo labeling and detection system (Amersham). Hybridizations were performed at 42°C in 0.25 ml of hybridization solution per square centimeter of membrane. A 10-ng aliquot of fluorescein-11-dUTP-labeled oligonucleotide was added per milliliter of hybridization solution. Signals were detected by autoradiography.

Cloning and Sequencing of RT-PCR Amplification Products

The RT-PCR products were purified from low melting-point agarose using the Macherey-Nagel Nucleospin extract 2-in-1 system (Separations, Düren, Germany) according to the manufacturer's instructions. The purified DNA was ligated to the pMOSBlue vector and used for subsequent transformation of competent MOSBlue cells using the pMOSBlue blunt-ended cloning kit (Amersham). After plating, white colonies were isolated and screened by PCR for the presence of the expected size insert prior to plasmid DNA isolation and sequencing. Plasmid DNA was isolated with the use of the Wizard Plus SV miniprep DNA purification system (Promega) and sequenced at the Core Sequencing facility, University of Stellenbosch, South Africa. Several clones were sequenced in both directions to obtain a consensus sequence.

Synthesis of cRNA Probes for *In Situ* Hybridization Studies

A 402-bp subcloned fragment containing part of exon 1 of the human type II GnRH receptor gene (see above) was ligated into the multiple

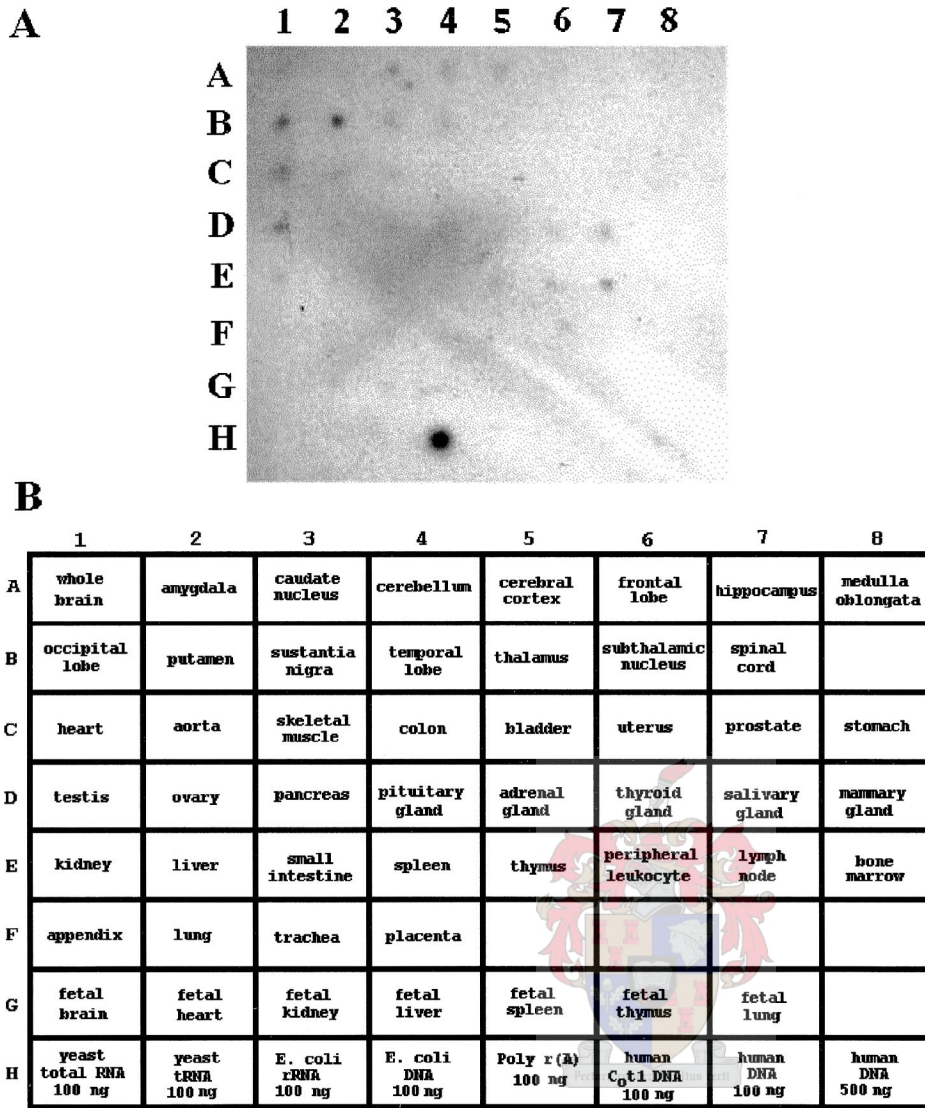


FIG. 1. A) Autoradiogram of a dot blot showing the tissue distribution of exon 1-containing poly A⁺ RNA of the human type II GnRH receptor gene. B) Diagram of the human RNA Master blot showing the type and position of poly A⁺ RNAs and controls dotted on the positively charged nylon membrane.

cloning site of the pGEM-T vector (Promega), possessing flanking T7 and SP6 bacterial phage promoters. The resulting construct was then sequenced to confirm orientation. The construct was linearized by digestion with either *Bam*HI or *Not*I restriction endonucleases in order to generate the corresponding sense or antisense RNA probes, respectively. A 1-μg aliquot of purified linearized template and 80 μCi [³⁵S]UTP (>1000 Ci/mmole, Amersham) was subsequently used to synthesize cRNA probes using the Maxiscript in vitro transcription system (Ambion, Austin, TX) according to the manufacturer's instructions.

In Situ Hybridization

The hybridization protocol was similar to that described previously [24]. Both paraffin sections and sperm smears were prepared using Vectabond (Vector, Burlingame, CA) silane-coated slides. In brief, the slides were pretreated by digestion with 1 μg/ml proteinase K (Invitrogen, Carlsbad, CA) at 37°C for 30 min followed immediately by acetylation with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0, at room temperature for 10 min. The pretreated slides were then hybridized at 42°C overnight in a 200-μl hybridization solution containing 10⁶ cpm of the labeled cRNA probe, 1 mg/ml tRNA, 0.1 M DTT, 50% formamide, 300 mM NaCl, 50 mM Na₂EDTA, 10% PEG-8000, and 1× Denhardt reagent. Following hybridization, the slides were washed through a series of progressively higher stringency washes at 50°C as described elsewhere [24]. Subsequent to washing, the slides were coated with a thin film of a 1:1 mixture of Kodak NBT-2 (Eastman Kodak, Newhaven, CT) emulsion in 0.3 M ammonium acetate prewarmed to 45°C. The slides were air-dried vertically at room temperature and then exposed at 4°C for 2-3 days. Following autoradiographic development, the tissue sections and sperm smears were counterstained with a hematoxylin and eosin histological stain.

RESULTS

Tissue Distribution of Exon 1-Containing Human Type II GnRH Receptor Transcripts

Our studies initially focused on determining whether exon 1-containing transcripts from the chromosome 1 gene

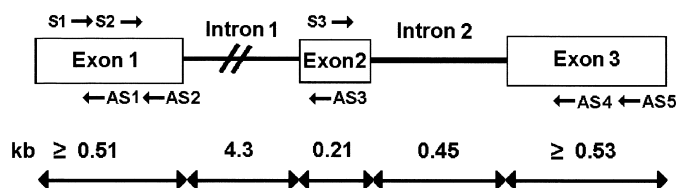


FIG. 2. Schematic diagram of the human type II GnRH receptor gene on chromosome 1, indicating the relative positions of the primers used for RT-PCR, Southern blotting, and RACE. Assignment of exon-intron boundaries was determined from the cDNA sequences. Assignment of exon and intron numbers was based on the assumption that no additional 5' and/or 3' introns and/or exons exist. Neither the 5' nor the 3' ends of the cDNA are defined. The minimum size of exon 1 is based on the position of the predicted ATG translation start codon. The minimum size of exon 3 is based on inclusion of 84 bases of the 3' untranslated region.

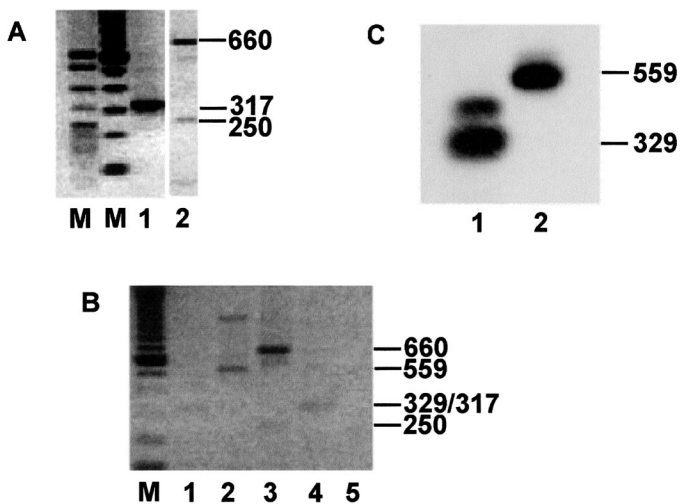


FIG. 3. Amplification of type II GnRH receptor transcripts from human testis and sperm by RT-PCR. **A**) An ethidium bromide-stained agarose gel of the results with testis total RNA is shown. The lanes are marked as follows: M, molecular weight markers; 1, 317-bp product obtained with the β -actin control primers; 2, 250-bp and 660-bp product obtained with the exon 2-exon 3 primer pair S3/AS4. **B**) An ethidium bromide-stained agarose gel of the results with sperm total RNA is shown. The lanes are marked as follows: M, molecular weight markers; 1, 329-bp product obtained with the exon 1-exon 2 primer pair S2/AS3; 2, 559-bp product obtained with the exon 1-exon 3 primer pair S2/AS4; 3, 250-bp and 660-bp product obtained with the exon 2-exon 3 primer pair S3/AS4; 4, 317-bp product obtained with the β -actin control primers; 5, negative control. **C**) An autoradiogram of a Southern blot of the gel in **B** is shown. The blot was probed with F-11-dUTP-labeled exon 1-specific oligo AS1. All six lanes, including the marker lane, were probed. Results are only shown for lanes 1 and 2 since no signals were obtained for the other lanes with the exon 1 probe.

were expressed in any human tissue(s). The rationale for this approach was that transcripts from the gene on chromosome 14, although abundant and widely distributed, would not contain exon 1. Expressed sequence tags containing exon 1 have been identified in the National Center for Biotechnology Information database from a prostate adenocarcinoma cell-line (accession BG036291) and from RNA pooled from testis, B-lymphocytes, and fetal lung cells (accession AA954764). The existence of these expressed sequence tags indicated that transcripts for the gene on chromosome 1 are expressed at least in these tissues, and possibly in others.

We examined the distribution of exon 1-containing human type II GnRH receptor transcripts in a variety of human tissues using dot blot analysis (Fig. 1). Hybridization with an exon 1-specific DNA probe under high stringency conditions yielded a convincing, albeit faint, positive signal for putamen (Fig. 1, B2). In addition, signals could also be detected for the following tissues (see Fig. 1): caudate nucleus (A3), cerebellum (A4), occipital lobe (B1), adult heart (C1), testis (D1), salivary gland (D7), peripheral leukocyte (E6), and lymph node (E7). Cross-hybridization with bacterial chromosomal DNA (Fig. 1, H4) is evident; however, the other negative controls including yeast total RNA, yeast tRNA, *E. coli* rRNA, Poly r(A), human C₀t1 DNA, and human DNA (100 and 500 ng) did not react with the probe and confirm its specificity for the chromosome 1 type II GnRH receptor. The fidelity of the positive signals was assessed by RT-PCR on total RNA from human tissues that were available.

RT-PCR was performed on total RNA from 22 different human tissues, cells, or cell lines that were available (see

Fig. 2). The adult human tissues were cerebellum, cortex, hypothalamus, kidney, medulla, midbrain, pituitary, pons, and testis. The human fetal tissues were adrenals, cerebellum, frontal lobe, hypothalamus, medulla, midbrain, lumbar sympathetic chain, olfactory bulb, pituitary, pons, and retina. The human cells were total ejaculate and HepG2 hepatocyte carcinoma cells. Several sets of PCR primers were utilized. These included the exon 1-specific primers in combination with primers to either exon 2 (that would yield a 329-bp intronless amplicon) or exon 3 (that would yield a 559-bp intronless amplicon), as well as an exon 2–3 primer pair (that would yield a 660- or 250-bp amplicon, respectively, if an intron was present or absent). These results are summarized in Figure 3. As expected, the unprocessed 660-bp chromosome 14 product was amplified from almost all human RNA samples (Fig. 3 and results not shown). This product was also present in testis and mature sperm (Fig. 3A, lane 2, and Fig. 3B, lane 3, respectively). In contrast, the processed 250-bp exon 2–3 product, arising from transcription of the chromosome 1 gene, was detected only in testis and mature sperm (Fig. 3A, lane 2, and Fig. 3B, lane 3). Furthermore, exon 1-containing transcripts were detected only in mature sperm (Fig. 3B, lanes 1 and 2). Hybridization analysis of the RT-PCR products revealed the presence of both the exon 1–2 (329 bp) and exon 1–3 (559 bp) intronless chromosome 1 transcripts in sperm (Fig. 3C).

To further examine the distribution of the human chromosome 1 type II GnRH receptor transcripts in testis and ejaculated sperm, *in situ* hybridization analyses were then performed using both sense and antisense exon 1-specific riboprobes. Bright-field photomicrographs summarizing these results are shown in Figures 4 and 5. In human testis the presence of chromosome 1 type II GnRH receptor sense transcripts is indicated by the marked deposition of silver grains within the adluminal region of the seminiferous epithelium (Fig. 4A). Although some luminal collapse resulting from the fixation process is evident, there is sufficient cellular detail to localize the signal. Silver grains were confined to the various types of differentiating haploid spermatids and not observed in association with spermatogonia, spermatocytes, Sertoli cells, or stromal cells (Fig. 4A). This is consistent with the view that the type II GnRH receptor gene on chromosome 1 is transcribed during the haploid phase of spermatogenesis. In contrast, human testis hybridized with the probe for the antisense transcript exhibited a sparse, nonspecific distribution of silver grains (Fig. 4B). *In situ* hybridization also revealed the persistence of human chromosome 1 type II GnRH receptor transcripts in mature sperm (Fig. 5). Sperm hybridized with the probe for the sense transcript displayed a concentrated, specific deposition of silver grains over the entire surface of the head, indicating the presence of human type II GnRH receptor sense transcripts (Fig. 5A). In contrast, human sperm hybridized with the corresponding control probe for the antisense transcript displayed a sparse and nonspecific background distribution of silver grains (Fig. 5B).

Cloning and Sequencing of Human Type II GnRH Receptor Transcripts from Ejaculated Sperm

A processed human chromosome 1 type II GnRH receptor transcript of the predicted size and containing all three exons was amplified from human sperm total RNA by RT-PCR (see Fig. 3, B and C). The 5' end of the cDNA was determined by 5' RACE. A consensus sequence spanning the full putative coding region for the human type II GnRH

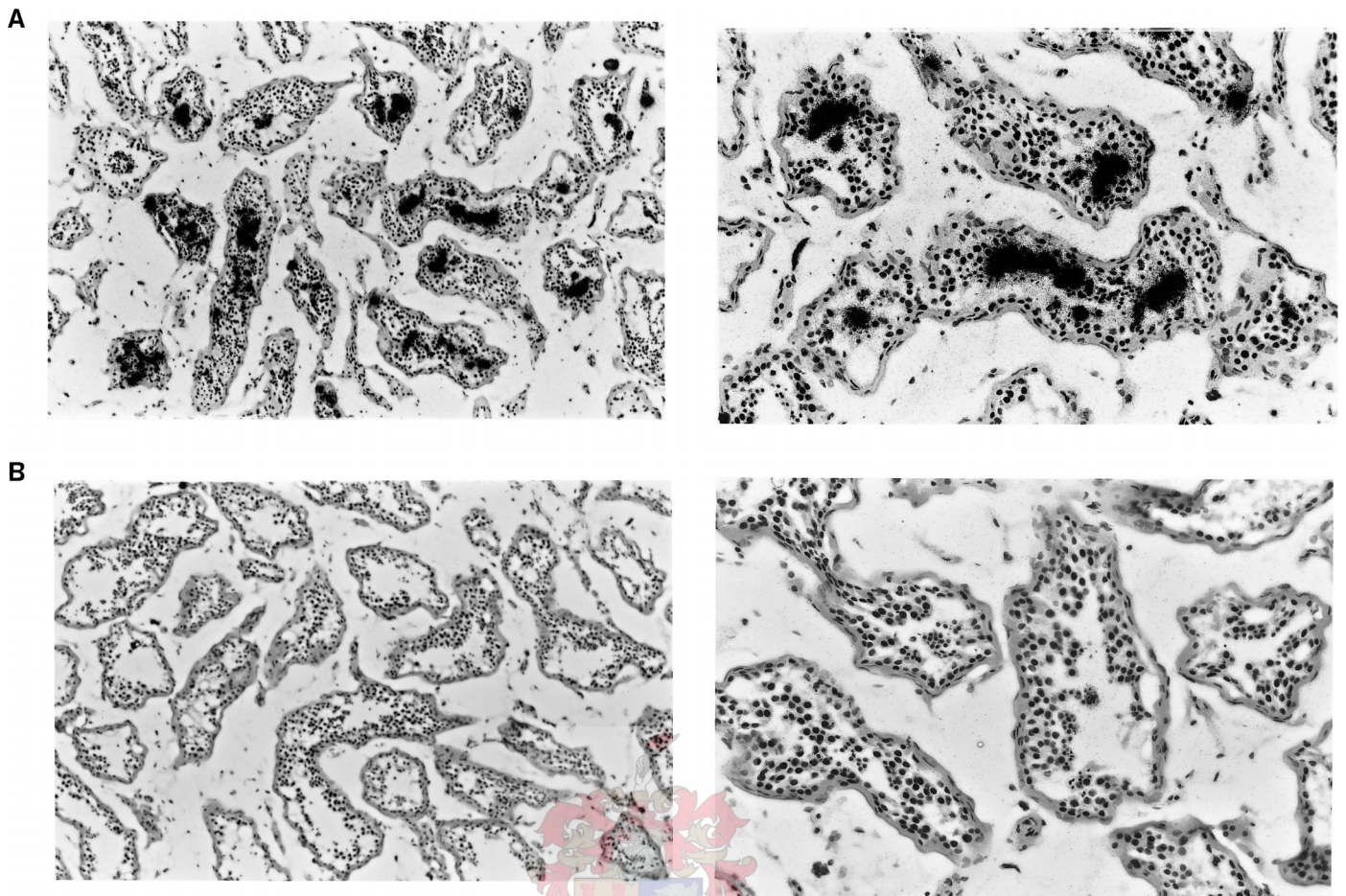


FIG. 4. In situ localization of type II GnRH receptor transcripts in human testis. Testis hybridized with a ^{35}S -labeled riboprobe for the sense transcript (A) or control riboprobe for the antisense transcript (B) are shown at $\times 200$ and $\times 400$ original magnification.

receptor cDNA was compiled by cloning and sequencing of three overlapping RT-PCR products (accession AY077708) and is shown in Figure 6. Several clones from each individual PCR reaction were sequenced. Of note, a UGA translation stop or selenocysteine codon was detected within exon 2 of the chromosome 1 transcript. In addition, alignment of the human and vervet monkey (accession AF353988) protein sequences revealed that the human cDNA would require the insertion of a single G residue in the 5' end region to encode a protein homologous to the monkey type II GnRH receptor (Fig. 6). The consensus coding sequence was then compared to the corresponding chromosome 1 gene sequence (accession AL160282) and found to be identical except for two nucleotide positions within the coding region. This would result in the substitution of a valine for an alanine residue and a serine for an arginine residue at amino acid positions 220 and 232, respectively, if the cDNA is translated.

DISCUSSION

Two genes for a type II GnRH receptor are found in the human genome. The chromosome 1 gene is comprised of exons 1, 2, and 3, whereas the chromosome 14 gene is comprised of only exons 2 and 3 of the putative receptor. Our dot blot results indicate that human transcripts containing exon 1 of the chromosome 1 type II GnRH receptor are neither abundantly nor widely expressed, in contrast to the expression of the exon 2–3 chromosome 14 transcripts.

The faint positive signals obtained for only some tissues indicate that the exon 1-containing chromosome 1 transcripts are either not expressed in most tissues or are expressed at very low levels. This is supported by our RT-PCR findings that exon 2–3 amplicons are abundant for most of the human RNAs, whereas exon 1-containing amplicons are weakly, if ever, detected. Human type II GnRH receptor transcripts have been detected by others using either dot blotting with an exon 3-specific riboprobe for the sense transcript [20] or by Northern blotting using an exon 1 specific double-stranded DNA probe [19] on selected poly(A)⁺ RNA tissue arrays. Interestingly, the reported tissue distribution patterns differ, which may reflect the use of different probes. Although our observation using an exon 1-specific probe of relatively strong signals in putamen, occipital lobe, cerebellum, caudate nucleus, and heart is consistent with that of Millar et al. [19], we did not detect a similar, relatively strong signal in other brain tissues. We did, however, detect a relatively strong signal in human testis, leading us to further examine this site of expression.

This is the first report of the cloning of a potentially full-length type II GnRH receptor transcript from the gene on chromosome 1 in any human tissue or cell type. Sequencing of the chromosome 1 sperm transcripts showed the presence of a UGA translation stop codon within exon 2, as well as a frame shift within exon 1 when compared to the recently cloned vervet monkey type II GnRH receptor (accession AF353988) [20] (see Fig. 6). Both the stop codon

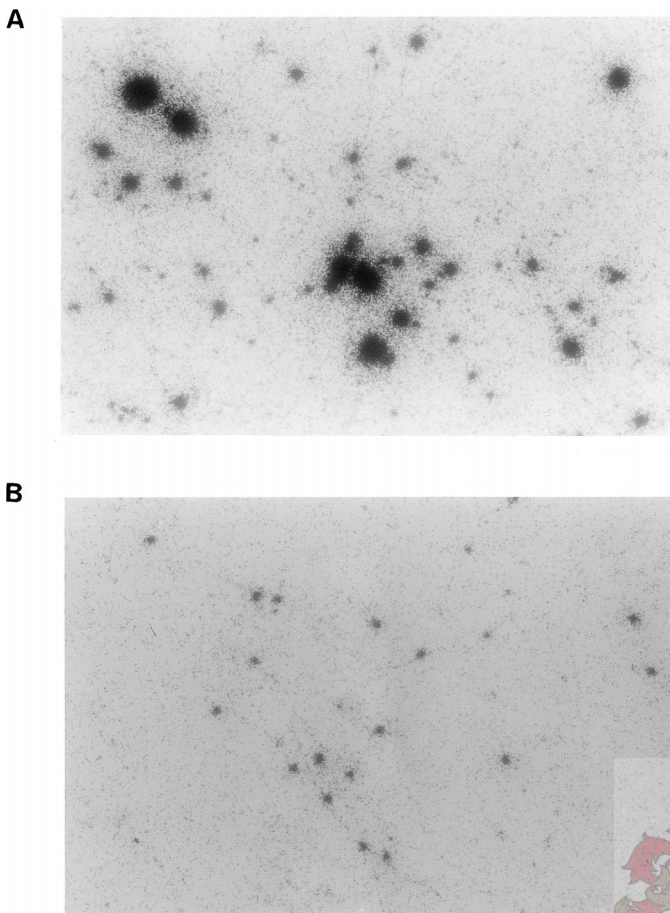


FIG. 5. In situ localization of type II GnRH receptor transcripts in human sperm. Human sperm hybridized with a ^{35}S -labeled riboprobe for the sense transcript (A) or control riboprobe for the antisense transcript (B) are shown at $\times 400$ original magnification.

and frame shift are also found in the human type II GnRH receptor gene on chromosome 1 (accession AL160282). Overall there is a 99.7% identity between the cDNA and gene sequence, with only two nucleotide differences within the coding region, most likely due to variation between individuals. The site of initiation of translation was predicted from the cDNA sequence of the monkey type II GnRH receptor. Interestingly, translation of the human cDNA would be predicted to initiate at an ATG codon like most eukaryotic cDNAs, whereas translation of the monkey cDNA starts with an unusual ACG. Overall, there is a 96.5% identity between the coding regions of the human and monkey type II GnRH receptor cDNAs and a 92.9% identity at the amino acid level (see Fig. 6).

Many human tissues were screened by RT-PCR and/or RACE for the presence of intronless transcripts containing the three exons. Ejaculated sperm was the only source where a potential full-length intronless transcript, resulting from the gene on chromosome 1, was detected. The failure to detect exon 1-containing sperm transcripts in testis total RNA by RT-PCR may have been due to their degradation postmortem and/or during the RNA isolation procedure from the testis. However, in situ hybridization revealed the presence of exon 1-containing transcripts in both testis and mature sperm. The in situ localization of type II GnRH receptor transcripts to the adluminal region of the seminiferous epithelium is consistent with the distribution of other haploid-specific mRNAs [24] and suggests that the human

chromosome 1 type II GnRH receptor gene is postmeiotically expressed in round and elongating spermatids. These transcripts are distributed throughout the entire sperm head in a similar manner as reported for other sperm transcripts [25]. A central query to be resolved is whether this transcript is functional in sperm.

Given that the mRNA contains a stop codon and would also require a nucleotide insertion to create the correct open reading frame, it seems likely that this gene is a transcribed pseudogene that, although functional in other primates, is nonfunctional in humans [20]. This may reflect the involvement of the type II GnRH receptor in the induction of mating behavior of other primates that are seasonal breeders [20], unlike humans. Pseudogenes are a consequence of gene duplication via either retrotransposition or duplication of genomic DNA [26]. The human genome contains a large number of pseudogenes, most of which are retrotransposons or processed pseudogenes, which lack introns and arise from single-stranded RNA. An intron-containing GnRH receptor pseudogene on chromosome 1 would thus have originated by DNA duplication. Pseudogenes derived from duplicated genomic DNA are most likely to be on the same chromosome as their paralogous functional partners, although they can also be inserted into a different chromosome by a duplication and translocation process [26]. Clearly, the latter process would have had to occur to result in a GnRH receptor pseudogene on chromosome 1 and a paralogous gene on chromosome 14. Most pseudogenes are promoterless and are therefore not transcribed. However, transcripts for some pseudogenes have been identified, arising most likely from DNA duplication of the promoter elements in parallel with the coding regions or from insertion of the pseudogene near the promoter of another gene [26]. Interestingly, spermatogenic cells have a high tendency to express processed retrotransposons. For example, 10 of the 14 retrotransposons that have been retained as functional genes in mammals are expressed in testis [27]. Taken together, although expression of pseudogenes does occur in mammalian spermatogenic cells, the expression in these cells of a GnRH receptor pseudogene on chromosome 1 would appear to be a rare event. Furthermore, the detection of immunoreactivity to the type II GnRH receptor protein in human pituitary and brain tissue by Millar et al. [19] would suggest that the gene on chromosome 1 is not a pseudogene.

Nevertheless, it is difficult to envisage how transcripts from a gene containing a stop codon and a frame shift within the coding region could result in a full-length, functional G-protein coupled receptor. It has, however, been shown that 5-transmembrane G-protein coupled receptors, lacking transmembrane helices 1 and 2, are functional [28]. Thus one possibility is that a functional, truncated, immunoreactive protein containing transmembrane helices 3–7 is expressed. This could occur if translation begins at the second AUG, situated at the end of transmembrane helix 2 (see Fig. 6), were it not for the stop codon within extracellular loop 2. RNA editing, with a single base transition within the UGA stop codon, could be involved in generating a functional truncated protein. Alternatively, a full-length functional protein could be generated by an additional RNA editing event involving the insertion of a G residue near the 5' end of the transcript. It has been shown that the monkey type II GnRH receptor cDNA contains a CGA arginine codon instead of a stop codon, creating an extended open reading frame [19, 20]. The presence of a UGA stop codon within the human sequence may represent a mech-

the transcript to correct the frame shift in the amino terminus, an event that appears to be extremely rare.

Although the functionality of these transcripts remains to be confirmed, the presence of GnRH and GnRH receptors has been shown to play a role during spermatogenesis, sperm maturation, and fertilization [39]. GnRH hormone has been localized to the seminal plasma [39] and has been shown to increase the binding of sperm to the ovum, an effect that is inhibited by GnRH antagonists [40, 41]. In addition, GnRH has also been shown to function as a local regulator in human placenta, where GnRH or GnRH-like peptides are synthesized by cytotrophoblasts and syncytiotrophoblasts during embryogenesis [42]. The mammalian type II GnRH receptor has been demonstrated to specifically regulate FSH secretion, a peptide hormone involved in the development of meiotic spermatocytes and postmeiotic spermatids [43]. Sperm have also been shown to express receptors for other hormones or signaling molecules such as the estrogen receptor [44] and A1 adenosine receptor [45]. Furthermore, a number of neuroendocrine hormones and growth factors, including GnRH, are produced by the testis [15]. The presence of these receptors and hormones is consistent with the presence of a network of intratesticular hormonal regulators, where hormones can function in a paracrine or autocrine manner due to their isolation from the rest of the body by a blood-testis barrier [15]. The expression of functional type II GnRH receptor transcripts in human sperm could be part of the existing network of intratesticular or neuroendocrine hormonal regulation governing spermatogenesis. Although some of the above-mentioned functions could be mediated by the type I GnRH receptor, the expression of a functional type II GnRH receptor protein in the testis and sperm would be consistent with these reports.

In summary, we have cloned a transcript of the gene on chromosome 1 for the human type II GnRH receptor from human sperm. This transcript, although containing all the exons required for a full-length receptor protein, contains a stop codon and a frame shift, which are also present in the gene. Although this would suggest that the gene is a transcribed pseudogene, several lines of evidence from the literature suggest otherwise. There is evidence for a functional role for a type II GnRH receptor protein in human sperm and testis. Furthermore, immunoreactivity data strongly suggest that a protein is expressed from the human chromosome 1 gene [19]. Thus if the gene is not a pseudogene, the transcript could possibly be translated as a truncated, immunoreactive protein or edited to result in translation of a full-length protein, possibly containing selenocysteine. However, given that RNA editing and/or incorporation of selenocysteine are rare events, the latter possibility seems unlikely. Further experiments using specific antibodies directed against domains encoded by sequences both 5' and 3' to the stop codon would be necessary to clarify whether a full-length or truncated type II GnRH receptor protein is expressed in sperm or in the developing zygote.

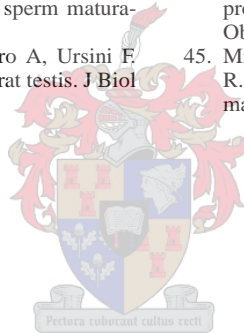
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REVIEW ARTICLE

Regulation of Expression of Mammalian Gonadotrophin-Releasing Hormone Receptor Genes

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Key words: GnRH receptor, transcriptional regulation, mammals, gene expression, tissue-specific, cell-specific.

Abstract

Gonadotrophin-releasing hormone (GnRH), acting via its cognate GnRH receptor (GnRHR), is the primary regulator of mammalian reproductive function, and hence GnRH analogues are extensively used in the treatment of hormone-dependent diseases, as well as for assisted reproductive techniques. In addition to its established endocrine role in gonadotrophin regulation in the pituitary, evidence is rapidly accumulating to support the expression and functional roles for two forms of GnRHR (GnRHR I and GnRHR II) in multiple and diverse extra-pituitary mammalian tissues and cells. These findings, together with findings indicating that mutations of the GnRHR are linked to the disease hypogonadotropic hypogonadism and that GnRHRs play a direct role in neuronal migration and reproductive cancers, have presented new therapeutic targets and intensified research into the structure, function and mechanisms of regulation of expression of GnRHR genes. The present review focuses on the current knowledge on tissue-specific and hormonal regulation of transcription of mammalian GnRH receptor genes. Emerging insights, such as the discovery of diverse regulatory mechanisms in pituitary and extra-pituitary cell types, nonclassical mechanisms of steroid regulation, the use of composite elements for cell-specific expression, the increasing profile of hormones involved in regulation, the complexity of kinase pathways that target the GnRHR I gene, as well as species-differences, are highlighted. Although further research is necessary to understand the mechanisms of regulation of expression of GnRHR I and GnRHR II genes, 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.

Gonadotrophin-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, and travels to the pituitary gland via the portal hypophyseal vasculature (1). Upon GnRH binding to its G protein-coupled receptor (GPCR) on the plasma membranes of pituitary gonadotroph cells, a range of intracellular signalling pathways are activated that ultimately regulate the synthesis and secretion of the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH). In turn, gonadotrophins stimulate sex hormone synthesis and gametogenesis in the gonads to ensure reproductive competence. 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 (2–6). GnRH II was originally isolated from chicken brain and its precise role in mammals remains to be elucidated (7). 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 (8). GnRH I released from the hypothalamus is the hormone that appears to be sufficient for gonadotrophin regulation in the mammalian pituitary (4).

Besides the well-established role for GnRH I and GnRHR I in gonadotrophin regulation in the pituitary, the detection of both forms of the hormone and receptor (Table 1) (4) in multiple mammalian nonpituitary tissues and cells suggests numerous and diverse autocrine, paracrine and endocrine extra-pituitary roles for GnRHs and GnRHRs (2–5, 9). These

TABLE 1. Summary of Mammalian Tissues and Cell Lines Where GnRHR I mRNA and/or Protein have been Detected.

Tissue	Species	mRNA	Protein	Reference
Normal tissues, primary cells				
<i>Pituitary</i>				
	Mouse	+ (1)	+ (5)	(173)
	Rat	+ (2)	+ (6)	(156, 174)
	Guinea pig	+ (2)	+ (7)	(175)
	Bovine	+ (2)	+ (6)	(176, 177)
	Sheep	+ (2)	+ (6)	(133)
	Wallaby	+ (2)		(78)
	Possum	+ (2)	+ (6, 7)	(178)
	Human	+ (2)	+ (6)	(85)
	Bonnet monkey	+ (3)	+ (8)	(179)
	Marmoset monkey	+ (1, 3)	+ (7)	(180, 181)
	Macaque monkey	+ (1)		(180)
	Pig	+ (2)		(182)
GH, TSH cells	Human		+ (9)	(183)
<i>Hypothalamus</i>				
	Rat	+ (3)	+ (6)	(185, 186)
	Bovine	- (3)		(176)
	Wallaby	- (3)		(187)
<i>Brain</i>				
	Sheep	- (3)		(133)
	Human	+ (3)		(38)
	Wallaby	- (3)		(187)
Placode-derived	Rat	+ (3)	+ (7)	(50)
Pre-optic area	Rat	+ (3)	+ (6)	(185, 188)
Hippocampus	Bovine	- (3)		(176)
Enteric neurones	Rat	+ (3)		(189)
<i>Placenta</i>				
	Human	+ (1, 3)	+ (6)	(190, 191)
IEVT	Human	+ (3)	+ (7)	(163)
Primary trophoblasts	Human	+ (3)	+ (7)	(163)
<i>Endometrium</i>				
	Human	+ (3)		(15)
	Human	+ (3)	+ (6)	(192)
	Human	+ (3)		(193)
<i>Myometrium</i>				
	Human	+ (3)	+ (6, 7)	(194, 195)
	Bovine	- (3)		(176)
	Human	+ (3)	+ (7)	(194)
Decidua	Human		+ (7)	(196)
<i>Ovary</i>				
	Wallaby	+ (3)		(78)
	Bovine	- (3)		(176)
	Human	+ (3, 4)	+ (7)	(85, 197)
Granulosa-luteal	Human	+ (3)	+ (7)	(38, 197)
Surface epithelium	Human	+ (3)	+ (7)	(159, 198)
	Rat	+ (3)	+ (6)	(199, 200)
Interstitial	Rat	+ (1)		(79)
Granulosa	Rat	+ (1)		(79)
Corpus luteum	Rat	+ (1)	+ (6, 7)	(79, 201)
	Human		+ (6)	(202)
Theca	Rat	- (1)		(79)
Luteal cells	Bovine	- (3)		(176)
<i>Breast/mammary gland</i>				
	Rat	+ (3)	+ (6)	(203)
	Mouse	- (3)		(81)
	Human	+ (3, 4)	- (6)	(85, 204)
<i>Testis</i>				
	Wallaby	+ (3)		(78)
	Human	+ (3, 4)		(85)
	Rat	+ (3)	+ (6)	(199, 205)
	Bovine	- (3)		(176)
Interstitial	Rat		+ (6)	(205)
Leydig	Rat		+ (6)	(206)
Sperm	Human		+ (9)	(207)
<i>Prostate</i>				
	Human	+ (3, 4)		(85)
	Rat	+ (3)		(208)
<i>Immune system</i>				
Lymphocytes	Mouse	+ (3)		(87)

TABLE 1. Continued

Tissue	Species	mRNA	Protein	Reference
T cells	Human		+ (7)	(14)
Mononuclear blood cells	Human	+ (3)	+ (7)	(88)
Spleen	Human	- (3)		(85)
	Bovine	- (3)		(176)
<i>Liver</i>	Human	+ (3)		(209)
	Wallaby	- (3)		(187)
	Bovine	- (3)		(176)
	Sheep	- (4)		(133)
<i>Pancreas</i>	Hamster		- (6)	(210)
	Human		- (6)	(211)
<i>Kidney</i>	Human	+ (3)	+ (6)	(209, 212)
	Bovine	- (3)		(176)
	Sheep	- (4)		(133)
<i>Adrenal</i>	Bovine	- (3)		(176)
	Sheep	- (4)		(133)
<i>Heart</i>	Human	+ (3)		(209)
	Sheep	- (4)		(133)
<i>Skeletal muscle</i>	Human	+ (3)		(209)
	Human	- (3)		(38)
	Wallaby	- (3)		(187)
	Rat		- (8)	(213)
<i>Submaxillary glands</i>	Rat	+ (1, 3)	+ (9)	(214)
<i>Digestive tract</i>	Rat	+ (1)	+ (9)	(12)
Gastric parietal cells	Rat	+ (1)	+ (9)	(13)
<i>Spinal chord</i>	Sheep	+ (3)		(86)
<i>Retina</i>	Vole	+ (3)		(11)
Cancer tissues, cancer cell lines				
<i>Pituitary</i>				
Pituitary adenoma	Human	+ (1, 3)		(215)
α T3-1	Mouse	+ (4)	+ (6)	(184)
L β T2	Mouse	+ (4)	+ (7)	(96)
<i>Hypothalamus</i>				
GT1-7	Mouse	+ (4)	+ (6)	(39)
FNC-B4	Human	+ (3)	+ (7)	(10)
<i>Liver</i>				
HepG2 (hepatocarcinoma)	Human	+ (3)	+ (7)	(89, 216)
<i>Pancreas</i>				
	Hamster		+ (9)	(90)
	Human		+ (6)	(211)
<i>Skin</i>				
BLM, Me15392 (melanoma)	Human	+ (3)	+ (6-8)	(217)
<i>Placenta</i>				
JEG-3 (choriocarcinoma)	Human	+ (3)	- (7)	(218, 219)
JEG-3	Human	+ (3)	+ (7)	(163)
<i>Endometrium</i>				
	Human		+ (6, 7)	(220)
	Human	+ (3)	- (6)	(219)
	Human	+ (3)	+ (6)	(195)
HEC-1A	Human	+ (3)	+ (7)	(221)
	Human	+ (3)	- (7)	(219)
Ishikawa	Human	+ (3)	- (7)	(219)
<i>Uterus</i>				
Leiomyosarcoma	Human	+ (3)	+ (6)	(222)
Myoma	Human	+ (3)	+ (6)	(195)
Cervical	Human	- (3)	- (6)	(195)
<i>Ovary</i>				
	Human	+ (3)	+ (7)	(223, 224)
	Human	+ (3)	+ (6)	(225)
Epithelial	Human	+ (3)	+ (6)	(195)
Stromal	Human	+ (3)	+ (6)	(195)
Germ-cell derived	Human	- (3)	- (6)	(195)
	Rat		+ (6)	(200)
OVCAR-3	Human	+ (3)	+ (7)	(159)
Caov-3	Human	+ (3)	+ (7)	(226)
SK-OV3	Human		+ (7)	(227)
EFO-21, EFO-27	Human		+ (6, 7)	(228)
SVOG-4o, SVOG-4 m	Human	+ (3)	+ (8)	(111)
<i>Breast</i>				
	Human	+ (3)	+ (6)	(204, 223)
MCF-7	Human	+ (3)	+ (6, 7)	(229)
			(6)	(23)

TABLE 1. Continued

Tissue	Species	mRNA	Protein	Reference
MDA-MB-157, MDA-MB-231	Human		+ (6, 7)	(229)
ZR-75-1	Human		+ (6, 7)	(229)
Sk Br 3	Human		+ (6, 7)	(229)
MXT	Mouse		+ (9)	(90)
<i>Prostate</i>				
	Human	+ (3)	+ (6)	(223, 230)
	Rat	+ (3)	+ (6)	(208, 231)
Dunning R3327	Rat	+ (3)		(208)
DU145	Human	+ (1)		(232)
LNCaP	Human	+ (1)	+ (8)	(232, 233)
PC-3	Human	+ (3)	+ (6, 7)	(90, 223)
TSU-Pr1	Human	+ (3)	+ (7)	(234)

+, Expression in specific tissues. -, expression investigated and found not to occur. Numbers refer to detection method: (1) = *in situ* hybridisation ; (2) = cDNA cloned ; (3) = RT-PCR ; (4) = Northern Blot; (5) = *Xenopus* oocyte expression; (6) = ligand binding assay; (7) = hormone response; (8) = Western Blot; (9) = Immunohistochemistry or immunocytochemistry.

include neuronal migration during development (10), neuro-modulation in the brain to affect sexual behaviour (4), possible modulation of visual processing in the eye (11), digestive tract function (12), inhibition of gastric acid secretion (13), adhesion chemotaxis and homing in T cells (14), human chorionic gonadotrophin (hCG) release in the placenta (15), steroidogenesis in ovarian cells (16–19), proliferation in melanoma cells (20), sperm function and sperm–oocyte interactions (21) and growth inhibition in reproductive tumours (22–26). However, the specific roles of each form of the hormone and receptor in these various tissues and cells remain to be elucidated. Recently, there has been an upsurge in the available literature on GnRHRs. GnRH and its analogues are extensively used in the treatment of hormone-dependent diseases, as well as for assisted reproductive techniques (6). More recently, they have been proposed as novel contraceptives in men and women (6, 27). The finding that naturally occurring mutations of the GnRHR are linked to the disease hypogonadotrophic hypogonadism, which results in delayed puberty, has also recently stimulated interest in GnRHR function (28). Thus, the presence of multiple forms of GnRH and its receptor in mammals, as well as the emerging multiple roles thereof, have presented new therapeutic targets and intensified the search for novel interventive GnRH analogues.

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 gonadotroph cell surface (29). 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 gonadotrophs, including by GnRH itself (29–33). New insights have recently been obtained about other hormones [melatonin (34), adrenal and sex steroids (35–37), activin (20), hCG (38, 39)], as well as the intracellular signalling pathways (40–44) and transcription factors involved (45–49) in regulating mammalian GnRHR transcription in diverse tissues (10, 50, 51) from different species (35, 40, 41, 45, 52). 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 anticancer therapy. The present review focuses on transcription of mammalian GnRHR genes, including the hormones that are involved, the signalling pathways that are activated and the promoter elements and transcription factors that mediate responses to multiple signals in various tissues.

Mammalian GnRHR genes

The structures of the mouse (53), rat (54), human (55), pig (56) and sheep (57) GnRHR I genes have been characterised. 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 three exons separated by two 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) (Fig. 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 whereas exon 3 encodes TM 6 and 7 (58).

Candidate genes for GnRHR II can be found at two different loci in the human genome (2, 4). The first is located on chromosome 1 and overlaps in the antisense orientation with the gene encoding the RNA-binding motif protein-8A (RBM-8A) (59). 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. A premature stop codon (UAA) is located inframe within exon 2 in the human gene, suggesting that the gene products are nonfunctional. A second human locus containing a pseudogene for GnRHR II and RBM-8A is on chromosome 14. GnRHR II genes have also been detected in other mammalian genomes. The premature stop codon found in the human gene is conserved in the chimpanzee GnRHR II gene (7), whereas a fully functional GnRHR II gene is present in other primates, such as the marmoset monkey (8), rhesus monkey and African green monkey (60). GnRHR II genomic

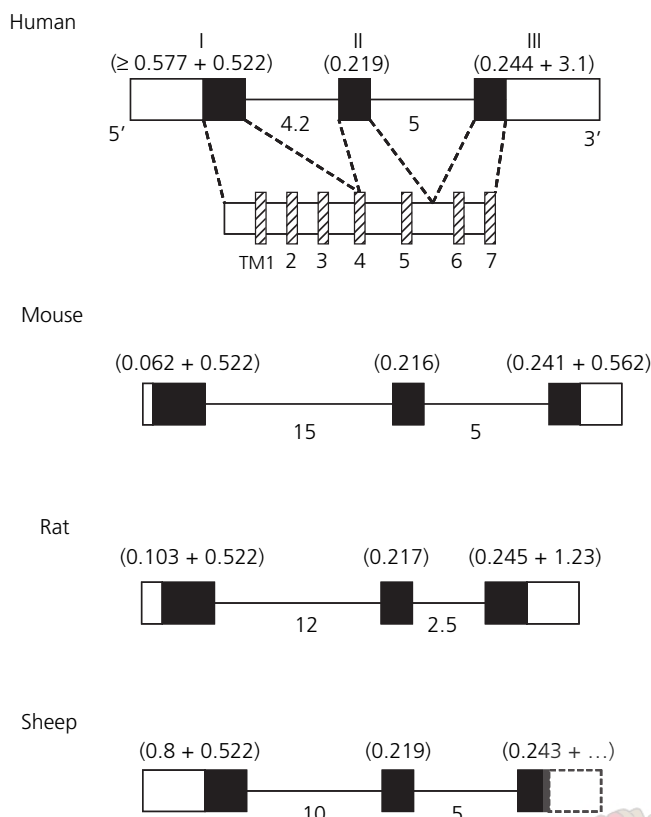


Fig. 1. Structural organisation of the the gonadotrophin-releasing hormone receptor I gene in human, mouse, rat and sheep. Exons are represented by blocks, with portions of exons containing coding sequences shown as dark areas, and untranslated regions (UTR) shown as light areas. Sizes of exons are shown as the sum of the sizes of the coding and noncoding portions of each exon. The size of the sheep 3' UTR has not been 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 the most-3' transcription start site as identified by Kakar *et al.* (98) for human pituitary tissue, and the size of the 3' UTR is as established by Fan *et al.* (55) for human brain tissue. For the mouse gene, the 5' UTR is relative to the major transcription start site as identified by Albarracin *et al.* (97) in α T3-1 cells and by Sadie *et al.* (49) in mouse pituitary tissue. For the rat gene, the size of the 5' UTR is given relative to the major transcription start site as identified by Reinhart *et al.* (54) for rat pituitary tissue. For the human gene, the correlation of coding regions with protein structure is indicated, as adapted from (58), and is identical for the other species shown. The figure was adapted from (53–55, 57, 58, 98, 99, 172). Note that the figure is not drawn to scale.

sequences for some nonprimate mammals have also been identified, and potentially encode functional proteins in pigs and dogs, but not in sheep and cows. The gene is completely deleted from the mouse genome (4), but a gene remnant is present in the rat genome (5).

Expression and physiological roles of mammalian GnRHRs

Both GnRHR I (Table 1) and GnRHR II (4) transcripts and, in some cases, GnRHR I protein (Table 1) have been detected in pituitary as well as extra-pituitary tissues and cell lines from several mammalian species. However, expression of endogenous GnRHR II protein has not been conclusively

shown in any mammalian species. One study shows immunodetection of putative GnRHR II extracellular loop 3 in mammalian pituitary tissues, however, positive signals were also detected in mouse pituitary tissue (8), raising doubts about the specificity of the antibody. Expression of a full-length human GnRHR II protein would appear unlikely because, apart from the premature stop codon within exon 2, the human transcript lacks a methionine translation initiation codon. Several as yet unsubstantiated theories have been postulated as to how such a transcript could result in the synthesis of a functional receptor protein (7, 61). However, a truncated form of the human GnRHR II may play a modulatory role in GnRHR I expression by perturbing normal processing of GnRHR I (62). Although recent indirect evidence suggests that a functional human GnRHR II protein may be expressed in cancer cells (63), no definitive functional role has been established to date for any mammalian GnRHR II (3).

Pituitary

It is well established that GnRHR I protein is expressed in mammalian pituitary gonadotrophs, where its primary role is to regulate LH and FSH synthesis and release (1). GnRHR I expression in the pituitary is regulated during foetal development, during sexual maturation (showing differential patterns between sexes) and during the reproductive cycle and pregnancy in the adult (51, 64, 65). In adult mammals, activation of the GnRHR I results in stimulation of diverse intracellular signalling pathways in the anterior pituitary, the nature of which depends on the cellular context (the relative concentrations of receptors and G-proteins vary during the oestrous cycle) (66, 67). In mouse gonadotroph cell lines, GnRHR I has been shown to couple to G_q/G_{11} in α T3-1 cells and to G_q/G_{11} and G_s in L β T2 cells (42). In rat primary pituitary cell cultures, the rat GnRHR I can couple to G_s , G_i/G_o and G_q/G_{11} , to modulate the activity of both the protein kinase A (PKA) and protein kinase C (PKC) pathways (41). Several recent reports have further unravelled the downstream kinase pathways in gonadotroph cell lines showing that GnRH can activate ERK, JNK and p38 MAPK in both α T3-1 and L β T2 cell lines, in various ways including via both PKA- and PKC-dependent and -independent pathways (41, 68). These pathways then differentially regulate synthesis and release of the gonadotrophins, via mechanisms that are not well defined. A feedback mechanism whereby PKC regulates the affinity of the GnRHR for GnRH has been reported, which suggests a novel form of 'inside-out' signalling (44). Furthermore, it has recently been shown in α T3-1 cells that the appropriate organisation of the GnRHR I into low-density membrane microdomains on the cell surface appears critical in mediating GnRH I-induced intracellular signalling (43). The important role played by the GnRHR in gonadotrophin 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 gonadotrophin and sex steroid hormone levels, in the absence of abnormalities in the hypothalamic-pituitary axis (6, 69). The majority of these mutated receptors are mislocalised proteins, exhibiting altered

membrane trafficking (70) and endoplasmic reticulum retention that can be restored to function by pharmacological chaperones (71).

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 that may code for functionally relevant truncated GnRHR I proteins (9, 72). In the gonadotrophs, ligand-mediated GnRHR I activation also leads to an increase in the expression and enzyme activity of nitric oxide synthase (NOS) I (73), the enzyme responsible for producing the signalling molecule nitric oxide (NO). In particular, this up-regulation occurs during pro-oestrus (74), and a role for NO in gonadotrophin release, fertility (75) and mating behaviour (76) has been suggested although the link between GnRHR I activation and NOS remains unclear. In some mammals (4), functional GnRHR II mRNA has been detected in pituitaries. Because stimulation with GnRH II has been shown to result in preferential FSH release in sheep (8), it is tempting to speculate that both GnRHR II and GnRH II may be involved in regulation of gonadotrophin synthesis and release in the pituitary gonadotrophs. GnRHR I immunoreactivity has also been detected in human thyrotrophs and somatotrophs, suggesting additional roles for GnRHR I in the pituitary other than gonadotrophin regulation in gonadotrophs (9).

Extra-pituitary

Reproductive tissues

In female reproductive tissues, paracrine/autocrine actions of GnRH via GnRHR I play a role in normal breast (77) and ovarian (78, 79) development, regulation of the menstrual cycle, early establishment and maintenance of pregnancy (80) and in lactation (81, 82). GnRHR I mRNA and/or protein has been detected in normal human breast tissue and several ovarian compartments, in endometrial tissue and in placental trophoblasts, cytotrophoblasts and syncytiotrophoblasts (Table 1) (9). Endometrial GnRH may play a paracrine/autocrine role in the early stages of implantation by modulating placental hCG secretion (15), which is involved in establishment and maintenance of pregnancy. The ovarian levels of GnRHR I and GnRH I mRNA vary during the oestrous cycle in the rat, where they are thought to play a local role in preparing the ovary for ovulation (83). Recent findings have also revealed additional novel functions for GnRHRs in the human ovary, including inhibition of gonadotrophin-regulated steroidogenesis and suppression of hCG-stimulated progesterone production in granulosa-luteal cells (16–19).

In male reproductive tissue, paracrine/autocrine actions of GnRH I via GnRHR I play a role in both testis and sperm development (78), as well as sperm motility and sperm–oocyte interactions (21). Although GnRHR II transcripts have been detected in mature human sperm and postmeiotic testicular cells, these appear not to be functional (61). GnRH or GnRH-like peptides produced in the human testis and prostate, and detected in human seminal plasma (84), may all be part of complex autocrine and paracrine regulatory circuits, because the GnRHR I is expressed in the human testis and prostate (85).

Non-reproductive tissues

Hypothalamic GnRH neurones have been found to express GnRHR I, which is proposed to function in an autocrine fashion to regulate GnRH release (50). Furthermore, recent findings in rat primary GnRH neuronal cells suggest that the GnRH-activated Ca^{2+} signalling and autocrine regulation of GnRH release could provide a mechanism for regulated GnRH I secretion during embryonic neuronal migration (50). Support for a role for GnRHRs in neuronal differentiation and migration also comes from work on human foetal olfactory epithelial cells (10). In addition, the detection of both GnRHR I (Table 1) and GnRHR II (4) transcripts in many mammalian brain tissues has supported a role for GnRH I and/or GnRH II as a neurotransmitter or neuromodulator. This hypothesis is supported by the expression of both GnRH I and GnRHR I in the spinal cord of sheep (86), as well as functional evidence *in vivo* for an integral role for GnRH in sexual behaviour in mammals via several different brain tissues (4). In addition, the detection of GnRH and GnRHR I transcripts and/or protein in T-cells (14), spleen (87) and gastric parietal cells (13), combined with functional evidence, suggests other autocrine/paracrine roles for GnRHRs in immunomodulation (87, 88), such as adhesion chemotaxis and homing in T cells (14), and inhibition of gastric acid secretion (13).

Cancer cells

It is widely accepted that continuous administration of GnRH analogues inhibits growth of several reproductive tissue-derived tumours and that this effect may be mediated via GnRHRs expressed on these cells (20, 22–26). However, the antiproliferative effects of GnRH analogues on human melanoma cells (20) 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 (89, 90) and/or interaction and interference with epidermal growth factor receptor mitogenic signalling (91, 92). Some investigators have recently provided indirect evidence to support a functional role for a putative human GnRHR II in mediating the antiproliferative effects of GnRH analogues in human endometrial, leukaemic and prostate cancer cells (63).

Regulation of GnRHR gene transcription in different mammalian tissues and cell lines

Regulation of expression of GnRHR numbers has been shown to occur at the transcriptional, translational and post-translational level. A well-known mechanism for ligand-mediated post-translational down-regulation of GPCR numbers on the cell surface involves desensitisation, internalisation and degradation. Whereas type I mammalian GnRHRs have been shown to internalise slowly due to the lack of a C-terminal tail (40), a recent study showed that the marmoset monkey GnRHR II, which has a C-terminal cytoplasmic domain, internalises rapidly (93). Homologous regulation of translation efficiency from GnRHR mRNA has also been shown to occur in α T3-1 cells (94). However, very little research has been reported on post-transcriptional

regulation of GnRHR I gene expression. The present review will thus focus on transcriptional mechanisms.

Work carried out in animals, in primary cells, as well as in several model cell lines, has contributed towards an emerging understanding of the complex transcriptional regulatory pathways by which mammals regulate transcription of GnRHR I genes. Much of the detailed molecular mechanisms of gene regulation of the mouse, rat and human GnRHR I genes have been investigated in mouse pituitary cell lines. The α T3-1 cell line is a precursor gonadotroph cell line that retains several differential functions of gonadotrophs, such as gonadotrophin α -subunit expression, synthesis and secretion, as well as expression of GnRHR I and receptor-dependent responsiveness to GnRH I. However, these cells differ from mature primary gonadotrophs in that they do not express or secrete the gonadotrophin beta-subunits LH β and FSH β (95). The L β T2 mouse pituitary cell line is more differentiated, exhibiting more pronounced gonadotroph-like characteristics, such as expression and secretion of the gonadotrophin α -subunit and both gonadotrophin-specific β -subunits (96). Extensive characterisation of the human GnRHR I gene promoter has been performed in human reproductive tissue-derived cell lines.

Promoter characterisation, basal and cell-specific expression (Fig. 2)

To date, the 5' flanking regions of the mouse (97), rat (54), human (55, 98) and sheep (57) GnRHR I genes have been characterised. Although 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 (99). There are several highly homologous regions within the proximal 500 basepairs of the mouse, rat, human and sheep promoters (99). A number of *cis*-elements have been conserved, in sequence as well as position, supporting their role as important functional elements. No functional characterisation of mammalian GnRHR II promoters has as yet been published, and therefore this section will focus on regulation of transcription of the mammalian GnRHR I gene.

The mouse GnRHR I proximal promoter was the first to be isolated and characterised (97). The major transcription start site in primary pituitary tissue (49) and α T3-1 cells (49, 97), is located at -62 (all numbering is relative to the translation start site) and is not associated with a consensus TATA box. In addition to this site, Clay *et al.* (100) identified other pituitary transcription start sites at -90 and -200 bp in α T3-1 cells. Gonadotroph-specific activity of the mouse promoter in α T3-1 cells is conferred by a tripartite basal enhancer, which includes binding sites for steroidogenic factor-1 (SF-1) at -244/-236, and activator protein-1 (AP-1) at -336/-330, respectively, as well as an element originally termed GnRHR-activating sequence (GRAS) at -391/-380 (102). The pan-pituitary homeobox transcription factor Pitx-1 has been shown by chromatin immunoprecipitation assay to interact with AP-1 in intact L β T2 cells, and functional evidence in other cell types indicate that this interaction might be important for GnRHR I gonadotroph-specific, basal promoter activity (103). In addition, the promoter region around

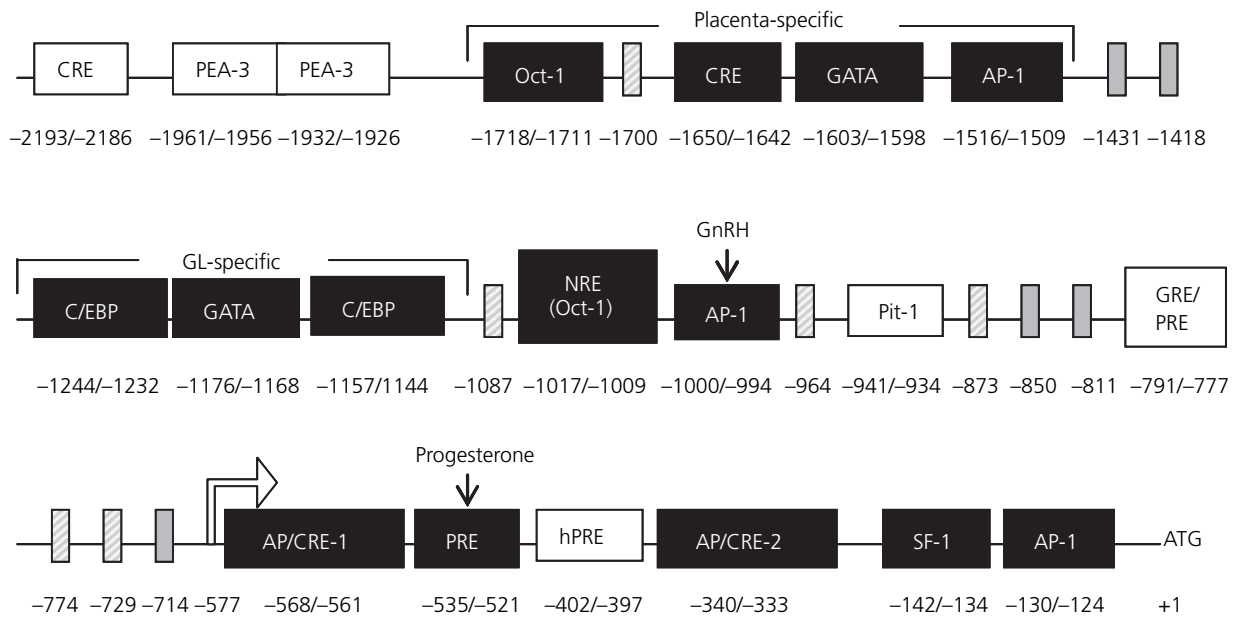
-360, shown to bind LHX3 homeodomain protein *in vitro* and in intact cells, was recently demonstrated to be important for mouse GnRHR I basal promoter activity in α T3-1 cells (104). Experiments with transgenic mice suggest tissue-specific promoter usage for the mouse GnRHR I gene, because 1900 bp of mouse GnRHR I 5' flanking sequence can drive reporter expression in pituitary, brain and testis, but not in the ovary, indicating an essential requirement for promoter elements located further upstream for ovary-specific expression *in vivo* (105).

In the rat proximal GnRHR I promoter, the transcription start site in α T3-1 cells was initially found to be 103 bp upstream from the start codon, with a putative TATA box 23 bp upstream from the transcription start site (54). A different study group later identified five major transcription start sites in α T3-1 cells, four of which are clustered around -103, and one situated at -30, along with several minor start sites (99). Maximal gonadotroph-specific expression of the rat GnRHR I is conferred by multiple regulatory domains within 1260 bp of 5' flanking region. The proximal 183 bp constitutes a self-sufficient, but fairly weak promoter which confers basal but not gonadotroph-specific activity. A distal GnRHR-specific enhancer (GnSE), located between -1135 and -753, contains binding sites for GATA-related and LIM homeodomain-related factors, and facilitates gonadotroph-specific expression through functional interaction with an SF-1 site at -245 (99, 106) (Fig. 2). The mechanisms involved in gonadotroph-specific expression of the mouse and rat GnRHR I are therefore clearly different, although both involve SF-1 sites. An AP-1 site in the rat promoter is also involved in basal promoter activity, but has no influence on the GnSE function. The function of the proximal rat promoter and the GnSE is supported by results obtained in transgenic mice, showing that the proximal 1.1-kb rat GnRHR I promoter is sufficient to drive gonadotroph-specific expression. Furthermore, 3.3 kb of the rat promoter was found to drive cell-specific expression of the transgene in gonadotrophs and certain areas of the brain (51).

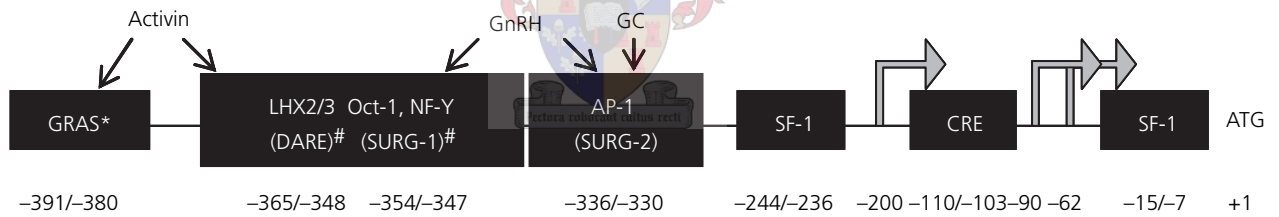
The 5' flanking regions of the human and sheep genes are much more complex than that of the mouse and rat genes, with the presence of multiple transcription start sites and CAP sites (57, 107). Although the sheep proximal 5' flanking region is structurally similar to the mouse promoter, it has greater sequence homology to the human promoter (57). No further functional characterisation of sheep promoter elements has been performed. In stark contrast to the single start site identified in mouse pituitary tissue (49), 18 transcription start sites have been identified for the human GnRHR I gene in human pituitary tissue (98). These start sites are located between -1748 and -577 and are well dispersed among several TATA and CCAAT boxes. The proximal 173 bp of the human 5' flanking region, although not a self-sufficient promoter, is critical for basal promoter activity in α T3-1 cells (108). However, characterisation of the human pituitary promoter has been hampered by unavailability of human gonadotroph cell lines, and the results in mouse cell lines may not be physiologically relevant. The mouse, rat and human promoters all contain several SF-1 sites, with at least one site in each promoter occurring in the 5' untranslated region. For the human promoter, this site is situated at -140/-134 and is

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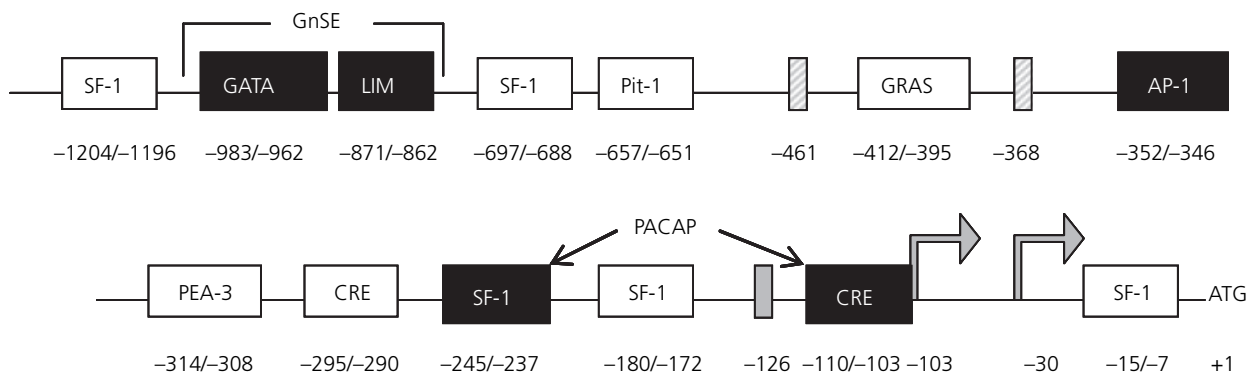
Human



Mouse



Rat



primarily responsible for mediating high cell-specific expression in α T3-1 cells (108), whereas the same function has not been assigned for similar sites in the mouse and rat promoters (situated at -15/-7 in both species) (49). An upstream Oct-1 binding at -1718 is also required for basal activity of the human promoter in α T3-1 cells (109).

The regulatory elements involved in expression of the mouse, rat and sheep GnRHRs have not been characterised in cells other than pituitary cell lines. However, cell-specific *cis* and *trans* elements have recently been identified for the human promoter in ovarian, placental and neuronal medulloblastoma cell lines. Expression of the human GnRHR I gene in both α T3-1 mouse gonadotroph cells and OVCAR-3 human ovarian carcinoma cells requires two promoter regions, located between -771/-557 and between -1351/-1022 (110). However, different *trans*-acting factors appear to bind to these regions in the different cell-types, possibly providing a mechanism for cell-selective expression (110). Two additional upstream promoters are responsible for high expression levels in human placental and ovarian granulosa-luteal cells, respectively (111). The granulosa (Fig. 2) cell-specific promoter is situated between -1300 and -1018, and contains a GATA element and two putative CCAAT/enhancer binding protein (C/EBP) motifs that were shown to be crucial in regulating GnRHR I transcription in the human ovarian granulosa-luteal cell lines SVOG-4o and SVOG-4m (111). GnRHR I expression in human placental cells requires a distal promoter region, located between -1737/-1346, in combination with a proximal region, between -707 and -167 (109). At least five placental transcription start sites were identified within the distal promoter region (109). A strong negative regulatory element is located between -1018 and -771, with a strong positive regulatory region between -771 and -577 (109). The distal placenta-specific promoter also contains an Oct-1 and an AP-1 binding site, required for basal expression in placental cells and other cell-types, as well as a cAMP response element (CRE) and a GATA element, essential for placenta-specific expression (109). Taken together, these studies indicate that various reproductive tissues differentially utilise downstream and upstream promoter elements and transcription factor binding sites for tissue-specific transcription of the human GnRHR I gene (111).

The transcription factor Oct-1 appears to regulate basal GnRHR I gene expression both positively and negatively,

depending on the species and cell-type. As already mentioned, Oct-1 is required for basal expression of the human GnRHR I gene in several cell types, including placental, ovarian and gonadotroph cell lines, via an Oct-1 binding site at -1718 (109). On the other hand, in placental JEG-3 cells, ovarian OVCAR-3 cells and α T3-1 cells, Oct-1 acts as a potent repressor of the human GnRHR I promoter via a negative regulatory element (NRE) at position -1017 (112). Oct-1 is also involved in basal and GnRH-stimulated activity of the mouse GnRHR I promoter in α T3-1 cells via the SURG-1 (Sequence Underlying Responsiveness to GnRH) element (48).

The mouse CRE has been found to be essential for basal promoter activity in some pituitary cell lines, such as L β T2 gonadotroph cells (Sadie *et al.*, unpublished data) and GGH₃ somatolactotroph cells (113), but the rat CRE does not appear to be involved in basal promoter activity in α T3-1 cells (114). A CRE at position -1650 is required for placenta-specific expression of the human GnRHR I gene (109). These findings indicate a cell- and/or species-specific contribution of CREs to basal GnRHR I expression levels.

Transcriptional regulation of GnRHRs in the pituitary and in gonadotroph cell lines by physiological signals

GnRH

Homologous regulation of the GnRHR I is a physiologically relevant mechanism for increasing pituitary sensitivity to GnRH during ovulation (31). GnRH I activation of GnRHR I is thus a potent stimulus for increased expression of multiple genes including the gene encoding the GnRHR itself. GnRH I regulates the GnRHR I in a biphasic manner, with initial (short-term) exposure to hormone leading to an increase in receptor expression, whereas prolonged exposure leads to receptor down-regulation (32). It is widely accepted that pulsatile GnRH I stimulation is essential for appropriate GnRHR I expression levels, at the same time avoiding receptor down-regulation due to continuous hormonal stimulation (115). GnRH I pulse frequency and amplitude vary with physiological state, during the oestrous cycle in mammals and the menstrual cycle in humans, as well as during puberty and menopause (32). Regulation of pituitary GnRHR I mRNA levels and receptor numbers by GnRH I also differs between sexes (29). Recent experiments in transgenic mice show that mutation of the AP-1 site at

Fig. 2. Functional elements in the gonadotrophin-releasing hormone receptor I promoter regions of human, mouse and rat. Shaded boxes and striped boxes represent TATA and CCAAT elements, respectively. Black boxes represent elements that have been functionally characterised. White boxes represent putative elements that have been identified through promoter sequence analysis. 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 *et al.* (98) for human pituitary tissue, is indicated. Other transcription start sites, as identified for human brain (55), pituitary (98) and placental tissues (109), 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 characterised upstream of the GRAS element. This figure was adapted from (9) and (72), and other references quoted in the text. *GRAS contains binding sites for SMAD, AP-1 and FoxL2 proteins (45, 47). #Several functional elements overlap in this region. The positions for DARE and SURG-1 are indicated. LHX2 was specifically shown to bind DARE (154), but LHX3 was shown to bind an overlapping site (104). 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 the figure is not drawn to scale.

-336 leads to a loss of GnRH I regulation of the mouse GnRHR I promoter (105).

The effects of GnRH I on GnRHR I protein and/or mRNA levels in primary pituitary cultures and cell lines suggest a direct mechanism of GnRH on pituitary cells, with a combination of both transcriptional and post-transcriptional mechanisms regulating GnRHR I expression levels. In attempts to mimic the situation *in vivo*, rat pituitary cultures were stimulated with GnRH I in a pulsatile fashion, resulting in increased GnRHR I mRNA levels (30). The mechanism appears to involve MAPK and possibly also cAMP/PKA (116, 117). Different pulse frequencies were found to have different effects on GnRHR I mRNA, with higher pulse frequencies causing maximal stimulation (118).

In α T3-1 cells, continuous stimulation with GnRH I appears to decrease endogenous GnRHR I levels via post-transcriptional mechanisms, although transcriptional mechanisms also contribute (94, 119). By contrast, the expression levels of mouse GnRHR I promoter-reporter constructs transfected into α T3-1 cells increase in response to 100 nM GnRH I after 4–6 h of continuous stimulation (32). This GnRH I responsiveness was mapped to two regions, designated SURG-1 and SURG-2 (32). SURG-1 contains binding sites for nuclear factor Y (NF-Y) and Oct-1, and it was shown by chromatin immunoprecipitation assays that GnRH increased binding of these factors to SURG-1 in intact cells (48). SURG-2 contains the AP-1 site described earlier. GnRH I responsiveness via SURG-2 appears to be mediated by PKC-induced activation of JNK which increases expression, activity and binding of AP-1 proteins to SURG-2 (46). SURG-1 and SURG-2 can respond to GnRH I independently, but the AP-1 element is critical for conferring maximal GnRH I responsiveness (32). These findings are in agreement with the results obtained in transgenic mice (46). However, in the mouse promoter, responsiveness to GnRH I also involves binding of Smad and AP-1 factors to another composite element called GRAS, which occurs further upstream at position -391/-380 (Fig. 2) (45, 52). This is discussed in more detail below.

Down-regulation of the transcriptional activity of the transfected human GnRHR I promoter-reporter construct by 24 h of continuous GnRH agonist treatment in α T3-1 cells is also mediated via an AP-1 element in a PKC-dependent fashion (120). In L β T2 cells, endogenous GnRHR I mRNA and protein levels are up-regulated upon long-term pulsatile GnRH I stimulation (96, 121), whereas long-term continuous stimulation down-regulates receptor levels (121). By contrast, both continuous and pulsatile stimulation induced only a small increase in the activity of a transfected 1.2 kb mouse GnRHR I promoter-reporter construct in L β T2 cells (121). Conn *et al.* (122) studied the regulation of mouse GnRHR I promoter activity in the GGH₃ cell line, which was engineered by stably transfecting GH₃ rat somatotroph cells with rat GnRHR I cDNA. Several intracellular signalling pathways were found to be involved in mediating the up-regulation of the mouse GnRHR I promoter activity by GnRH I in these cells, such as PKA (123), PKC and the Ca²⁺ signalling pathway (124). However, unlike the results in α T3-1 cells (31, 32), the AP-1 site does not appear to be involved (35). Although the PKA pathway mediates homologous

regulation of the mouse GnRHR I promoter in GGH₃ cells, this is not the case for the mouse or human promoters in α T3-1 cells, most likely reflecting differences in GnRHR I G-protein coupling between the cell lines. However, functional studies do indicate a role for the PKA pathway and cAMP response elements (CREs) in regulating GnRHR I mouse, rat and human promoter activity. These promoters all contain functional CREs and are up-regulated by activators of the PKA pathway in α T3-1 cells (49, 114, 125). It is thus likely that in pituitary or extra-pituitary cells in which the GnRHR I can couple to G_s, homologous regulation will involve the PKA pathway. Other factors likely to be involved in mediating PKA responses, as shown in α T3-1 cells, are CREB (94) for the rat and SF-1 (49, 114) for the rat and mouse promoters.

Steroids

Studies in rat, sheep and cow conclude that oestradiol increases the level of GnRHR I mRNA and protein in pituitary (126, 127) consistent with a requirement for a strong, prolonged LH surge for ovulation during the preovulatory phase of the reproductive cycle. Experiments in ovariectomised transgenic mice harbouring a sheep GnRHR I promoter-reporter construct, as well as experiments in sheep primary pituitary cells (128–131), suggest that transcription is the predominant mechanism of oestradiol up-regulation of GnRHR I numbers in the pituitary. However, oestradiol stimulation of α T3-1 cells was found to down-regulate GnRHR I numbers (132), whereas oestradiol stimulation of L β T2 cells had little effect on endogenous GnRHR I gene expression (96, 132). These conflicting results highlight the apparent discrepancies that may occur when using transformed cell lines compared to primary cells that contain mixed cell populations. In addition, one group reported that the GnRHR I mRNA levels increase before an increase in circulating concentration of oestradiol (133), leading them to postulate that a decrease in progesterone, rather than an increase in oestradiol, is required for up-regulation of GnRHR I numbers.

In most mammals, high levels of progesterone correlate with reduced GnRHR I protein levels in pituitary and reduced pituitary responsiveness to GnRH I, such as that occurring during the luteal phase of the menstrual cycle and during pregnancy (126, 134, 135). In sheep pituitary cells, progesterone was found to dramatically down-regulate GnRHR I numbers within 48 h (129, 136), consistent with a direct effect of progesterone on the pituitary. Progesterone was also able to prevent oestradiol- and inhibin-induced increases in GnRHR I mRNA levels in these cells. Recent results with the human GnRHR I promoter in α T3-1 cells, showing that progesterone administration and overexpression of progesterone receptor (PR) isoforms inhibited GnRHR I promoter activity (137), suggest that, at least for the human promoter, repression by progesterone occurs via direct transcriptional effects on the GnRHR I promoter in gonadotrophs. Furthermore, this negative effect was shown to occur via a glucocorticoid response element (GRE)/progesterone response element (PRE) at -535/-521, which has 75% homology to a consensus progesterone response element (Fig. 2), and to which PR isoforms were

shown to bind *in vitro* (137). In the same study, a half-PRE binding site was shown to be located at -402/-397. However, this site did not play a role in the progesterone-mediated transcriptional effects. Interestingly, another putative GRE/PRE is located further upstream (55), but its function remains unknown.

In male rats, pituitary GnRHR I mRNA levels appear to be repressed by testosterone because a negative correlation exists between mRNA levels and testosterone concentrations in serum (30, 64). GnRHR I numbers in primary pituitary cultures from male rats decreased after treatment with α -dihydrotestosterone (138), consistent with *in vivo* results and suggesting direct actions of α -dihydrotestosterone on the pituitary. By contrast, α -dihydrotestosterone up-regulated GnRHR I mRNA levels in L β T2 cells (139).

It is well documented that chronic or prolonged stress results in inhibition of gonadotrophin secretion and inhibition of reproduction in mammals, whereas the effects of acute stress are less clear and can even stimulate reproduction (140). Although the mechanisms whereby stress regulates reproduction in mammals are not well defined, there is evidence that glucocorticoids play an important role in modulating pituitary responsiveness to GnRH I, as part of a feedback mechanism from adrenal to pituitary (140, 141). Further evidence for direct actions of glucocorticoids on pituitary is provided by findings that cortisol inhibits GnRH-induced LH release from bovine and porcine primary pituitary cells (142, 143). One mechanism whereby glucocorticoids may regulate GnRH responsiveness in pituitary may be via modulating GnRHR levels. Rosen *et al.* (144) showed that glucocorticoids augmented GnRH I-induced increase in GnRHR I numbers in castrated testosterone-replaced male rats. However, earlier studies in rats did not show a change in GnRHR I levels after treatment with corticosterone (138, 145). In sheep, administration of cortisol led to a decrease in GnRHR protein, but did not reduce GnRHR I mRNA levels (146). These experiments suggest that varying effects of glucocorticoids on GnRHR I levels may depend on species, the cellular milieu, and the dose, type and duration of glucocorticoid administration. However, a direct positive transcriptional effect of glucocorticoids on the mouse GnRHR I promoter has been established. Glucocorticoids increased endogenous GnRHR I mRNA levels in L β T2 cells, whereas pretreatment with GnRH I further augmented this increase (96). Glucocorticoids can also directly up-regulate activity of the mouse GnRHR I promoter in GGH₃ cells (35). Although the tested 1.2 kb of 5' flank of the mouse gene does not contain a classical GRE (Fig. 2), the glucocorticoid-responsive region of the mouse GnRHR I promoter was mapped to the AP-1 site at -336 in GGH₃ cells (35). The results from this study suggest that liganded glucocorticoid receptor interacts directly or indirectly with AP-1 proteins, such as c-Jun, to increase GnRHR I transcription (35).

Other physiological regulators

Activin and inhibin, both members of the transforming growth factor- β family of proteins, are produced by primary gonadotrophs (147), α T3-1 (148) and L β T2 cells (149), and exert autocrine/paracrine effects on pituitary cells. Activin-A stimulates the rate of synthesis of new GnRHRs in rat

pituitary cell cultures (150), and decreases receptor numbers in sheep pituitary cultures (151). Inhibin was found to prevent the stimulation of receptor synthesis by GnRH I in rat pituitary cultures (152), but increases GnRHR I mRNA levels (129) and receptor numbers (151) in sheep pituitary cultures. Whether these differences are species-specific or due to different experimental conditions is not known. In α T3-1 and L β T2 cells, long-term stimulation with activin-A up-regulates endogenous GnRHR I mRNA synthesis and mouse GnRHR I promoter-reporter activity (148, 149), and pretreatment of α T3-1 cells with activin enhances the response of the mouse GnRHR I promoter to GnRH I (45). Follistatin blocks the activin-mediated stimulation at both mRNA and promoter level. In addition, follistatin decreases the basal activity of the mouse GnRHR I promoter in α T3-1 and L β T2 cells, indicating that endogenous activin maintains basal GnRHR I expression levels in these cells (45, 148, 149). Activin responsiveness of the mouse GnRHR I promoter was mapped to the GRAS element (153) described earlier, together with a region immediately downstream from GRAS, termed DARE (down-stream activin regulatory element) (154) (Fig. 2). The mouse GRAS element is a composite regulatory element for which the functional activity in α T3-1 cells depends on the proper organisation and assembly of a multiprotein complex, which includes Smad, AP-1 and FoxL2 proteins (47). Basal GnRHR I promoter activity, as well as responsiveness to GnRH I and to activin require binding of Smad factors to the Smad binding element, as well as binding of AP-1 to a novel AP-1 element contained within GRAS (Fig. 2) (45, 52). The LIM-homeodomain protein LHX2 was shown to bind the DARE sequence *in vitro* (154). 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' (154). 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 (106, 154), suggesting that the rat DARE sequence is nonfunctional for activin responsiveness.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a hypothalamic peptide hormone that modulates pulsatile GnRH I release from the hypothalamus and responsiveness to GnRH I, as well as regulates gonadotrophin subunit expression (155). The mouse, rat and human GnRHR I promoters have all been shown to be regulated by PACAP in α T3-1 cells (49, 114, 125). For the rat and human promoters, this has been shown to involve PKA (114, 125). 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 the SF-1 binding site at position -245/-237, along with binding sites for additional factors, whereas PARE II contains an imperfect cAMP response element (CRE) at position -110/-103 that can bind CREB (114). Both the SF-1 site and the imperfect CRE are conserved in relative position in the mouse GnRHR I promoter (Fig. 2), raising the possibility that a similar mechanism could be responsible for the PACAP response of the mouse promoter.

Transcriptional regulation of GnRHRs in extra-pituitary tissues and cell lines by physiological signals

Ovary and placenta

By contrast to results obtained in primary rat pituitary cultures (30, 156), homologous regulation of GnRHR I by GnRH I has not been consistently observed in rat primary granulosa cells (157, 158). Treatment of human granulosa cell lines (SVOG-4o and SVOG-4m) with high and low doses of GnRH II induced a significant decrease in GnRHR I mRNA levels, whereas GnRH I induced a down-regulation at high and an up-regulation at low doses, showing that the two ligands regulate GnRHR I transcription differentially (16). Responsiveness of GnRHR I transcription to oestradiol appears to vary between ovarian cell types. In human primary ovarian surface epithelial cells, as well as in OVCAR-3 ovarian cancer cells, treatment with oestradiol caused a significant down-regulation of GnRHR I mRNA (37, 159, 160). In human granulosa-luteal cells, short-term oestradiol treatment (6 h) increased GnRHR I mRNA levels, whereas long-term treatment (48 h) decreased GnRHR I mRNA levels (161). This observation is consistent with what was found *in vivo* (30, 162). A recent study in ovarian cancer cells demonstrated that oestradiol represses GnRHR I transcription in a ER α -dependent and ER β -independent way, via an AP-1-like motif at -130/-124 (9, 37). Repression of GnRHR I promoter activity by oestradiol did not involve direct binding of the ER to the AP-1 site, suggesting that the ER interacts with other proteins bound to this motif, such as c-Jun or c-Fos. Other physiological modulators of GnRHR I expression in human primary granulosa-luteal cells are hCG (38) and melatonin (34), although the mechanisms are not well defined. hCG down-regulates GnRHR I mRNA levels in primary ovarian granulosa-luteal cells without changing GnRH I expression (38). Until recently, regulation of reproductive function by melatonin was assumed to be restricted to the level of the pituitary and the hypothalamus. However, the presence of melatonin in the follicular fluid and of melatonin binding sites in the ovary suggests a role for this hormone in the ovary. In support of this, melatonin reduces both GnRH I and GnRHR I mRNA levels in human primary granulosa-luteal cells (34), suggesting that melatonin directly regulates ovarian function.

In the human choriocarcinoma JEG-3 and the immortalised extravillous trophoblast I EVT placental cell lines, the human GnRHR I mRNA is up-regulated after 24 h of continuous stimulation with GnRH I (163). This may be a tissue-specific mechanism to help maintain GnRH I-stimulated hCG secretion throughout pregnancy. The kinase pathways and transcription factors mediating this response have not been determined, but may involve the PKC pathway, as shown in α T3-1 cells, and/or perhaps the PKA pathway, because the human gene is up-regulated by activators of the PKA pathway, via binding of CREB to two AP-1/CRE elements (9). Progesterone has a positive effect on GnRHR I promoter activity in the JEG-3 placental cell line in contrast to the repression observed in α T3-1 cells (137). The GRE/PRE at position -535/-521 was shown to mediate PR regulation in both α T3-1 gonadotroph and JEG-3 placental cells (137). Furthermore, it was shown that

both PR-A and PR-B isoforms bound to the PRE *in vitro* and that the balance between PR-A and PR-B overexpression in the different cell lines can determine the response to progesterone (137). Whereas PR-A inhibits transcription in both placental and pituitary cells, PR-B activates transcription in placental cells, and inhibits transcription in pituitary cells.

Brain

In the GT-17 hypothalamic GnRH neuronal cell line hCG was found to down-regulate the expression of the GnRHR I as well as of GnRH I (39). By down-regulating the GnRH/GnRHR system, hCG may disrupt the autocrine regulation in hypothalamic GnRH neurones. Results obtained in the TE671 neuronal medulloblastoma cell line showed that progesterone has a potent negative effect on human GnRHR I promoter activity, and up-regulates GnRH I expression (36). Overexpression of PR-A increased sensitivity towards progesterone-mediated repression of the GnRHR I gene, whereas PR-B reversed the PR-A-induced repression, suggesting that negative regulation PR occurs in the absence of overexpression via endogenous PR-A (36).

Immune cells

Consistent with the idea that immune function is differentially regulated during the reproductive cycle, expression of GnRHR I in lymphocytes was shown to vary throughout the oestrous cycle and parallels expression in pituitary (87). *In vitro* administration of GnRH and oestradiol increased GnRHR I mRNA levels in immune cells (87) but the mechanisms remain unclear.

Discussion and future perspectives

GnRHRs are expressed in multiple mammalian tissues and cell types (Table 1) and have diverse functional roles, in addition to the established endocrine role for gonadotroph GnRHRs in regulation of reproductive physiology. Furthermore, the profile of hormones that regulate expression of the GnRHR I is expanding and now includes adrenal glucocorticoids, gonadal sex steroids, as well as hCG, activin, inhibin, follistatin, PACAP, melatonin and GnRH. It is well-established that sex steroids and glucocorticoids feedback and inhibit the HPG axis at the hypothalamic, pituitary and gonadal levels, providing mechanisms for fine-tuning reproductive function (140). Studies also suggest additional connections between physiological processes, demonstrating that the neuroendocrine, immune, inflammatory and stress-response systems are functionally integrated and bidirectionally regulated (164–169). Although the target genes and intracellular mechanisms of such feedback regulation are poorly defined, the GnRHR is emerging as a potential target gene for facilitating cross-talk between these systems in multiple tissues via autocrine/paracrine and endocrine signalling. The identification of two forms of both the mammalian hormone and receptor, expressed in multiple tissues, and in many cases coexpressed, further increases the diverse signalling potential of the GnRH/GnRHR system in mammals. However, further work is needed to clearly establish the presence of GnRHR I and/or GnRHR II in the various

tissues and cells, using receptor-subtype specific functional and morphological methods.

Studies to determine the promoter elements, transcription factors and detailed molecular mechanisms of transcription regulation have focused on the mouse, rat and human GnRHR I promoters. No characterisation of the mammalian GnRHR II promoters has been published to date, most likely due to the absence of suitable model systems where the GnRHR II is known to be endogenously expressed. Most of the GnRHR I work has been performed using transient transfections in cell lines, using the human, mouse and rat GnRHR I promoters transfected into α T3-1 and L β T2 mouse pituitary gonadotroph cell lines, and, more recently, with the human GnRHR I promoter transfected into human ovarian, placental and neuronal medulloblastoma cell lines. Some work has also been performed in primary cells, which usually contain mixed cell populations. It is clear that there are many apparent discrepancies between the results obtained in the various model systems, highlighting the difficulties inherent in finding suitable model systems that are physiologically relevant. These discrepancies may be due in part to the absence of native chromatin structure when comparing responses of transfected promoter constructs versus endogenous genes, or due to cell-specific differences between cell lines. In addition, indirect effects of one cell type on other cell types in primary cultures, or the use of heterologous expression systems may result in discrepancies. Finally, variations in experimental procedures such as doses and times and method of administration (e.g. continuous versus pulsatile) of hormonal stimulation, and culture conditions may lead to different results. Some experiments in transgenic mice have been particularly helpful in confirming the results obtained *in vitro*, such as the finding that an AP-1 site in the mouse promoter is necessary for homologous regulation (46). Two elegant studies have also recently addressed the issue of protein–DNA interactions in intact cells by employing the technique of chromatin immunoprecipitation assays to identify factors binding to the endogenous mouse GnRHR I promoter, to confirm the results obtained *in vitro* (48, 103). Certainly, future studies will be helpful in determination of protein–DNA interactions in intact cells for other factors.

Despite the limitations of the present model systems, several interesting insights into the mechanisms of GnRHR I regulation have emerged. Experiments performed in transgenic mice indicate that the mouse gene does not exhibit multiple, widely spaced, tissue-specific promoter usage in the pituitary, brain and testis, although they suggest that a different promoter that is upstream of –1900 in the mouse GnRHR I gene is used in the ovary (105). Similarly, experiments with transgenic mice harbouring the rat GnRHR I promoter found that a single 3.3-kb promoter is capable of driving transcription in gonadotrophs and multiple brain tissues (51). The human promoter uses different promoters in the pituitary compared to the placenta, and even within different ovarian cell types, although these regions all occur within the first 2 kb of 5' flanking region (109–111). Evidence is emerging from work on the mouse and human GnRHR I promoters that mechanisms of regulation of transcription in the ovary appear to differ substantially as compared to other tissues investigated to date (105, 110, 111).

Mouse, rat and human promoters also appear to exploit the concept of multiple commonly expressed transcription factors binding to a composite element in a particular structural organisation, to achieve tissue-specific expression. In addition, the mouse GnRHR I promoter uses the complex SURG and GRAS composite elements to achieve homologous regulation in α T3-1 cells (32, 45, 48, 52, 102, 154). Although the components of these complex elements differ between species, they appear to often rely on some common factors. The human, mouse and rat promoters all contain several SF-1 sites, with at least one being involved in gonadotroph-specific expression (102, 106, 108), although SF-1 is expressed in several other tissues. Interestingly, the mouse and human promoters contain at least one AP-1 site shown to be involved in mediating homologous regulation via the PKC pathway in the pituitary (46, 120). However, the apparent lack of an AP-1 site in the sheep promoter (57) suggests that the PKC pathway may not be involved in homologous regulation in this species. It appears likely that both the rat and mouse promoters could employ similar mechanisms for PACAP regulation (49, 114), based on the presence of conserved promoter elements, whereas species-specific differences appear to exist for their regulation by activin (106, 154). Mechanisms of homologous regulation also appear to be cell-specific, and are likely to depend on the physiological state of the cells, as shown by different findings for GnRHR I regulation in rat primary granulosa cells (157, 158) versus primary rat pituitary cultures (30, 156).

In the light of the potential role of GnRHR genes as targets for cross-talk between various physiological systems, it is of great interest to determine whether adrenal and sex steroids directly regulate expression of GnRHR genes, and whether these mechanisms are transcriptional or post-transcriptional. Studies in some mammals show repressive effects with progesterone (126, 134, 135) and stimulatory effects with oestrogen (29, 126, 170, 171) on GnRHR I expression in the pituitary. However, experiments in primary cells and cell lines from pituitary and extra-pituitary origins reveal no consistent picture, suggesting species-, cell- and/or promoter-specific differences in response to these steroids (37, 96, 129, 132, 136, 137, 161). To date, no classical oestrogen or androgen response elements have been identified in the mouse, rat, human or sheep GnRHR I promoters. Although the same applies for glucocorticoid and progesterone response elements in the mouse, rat and sheep promoters, several PRE/GREs have been identified for the human promoter (137). Data are accumulating to suggest that steroid regulation of these promoters may occur via nonclassical pathways other than up-regulation via binding of homodimers of steroid receptors to steroid response elements. Several recent studies pinpoint a direct transcriptional effect of some steroids on mammalian GnRHR I promoters. A direct effect of oestradiol on the human GnRHR I promoter in human ovarian and breast cancer cell lines has been established, mediated via an AP-1 site (37). Interestingly, different ER isoforms exhibited different effects on the promoter, with the oestradiol response being ER α -dependent, but ER β -independent (37). An AP-1 site is also involved in glucocorticoid regulation of mouse GnRHR I promoter activity in the GGH₃ rat somatolactotroph cell line (35). However, in this case, an up-regulation is

observed in response to glucocorticoids. Because it is well established that steroid receptors can inhibit transcription of several target genes via interference with the actions of AP-1, the up-regulation of mouse GnRHR I promoter activity via AP-1 may represent a novel mechanism. Progesterone has been shown to both up-regulate [in a human placenta cell line (137)] and down-regulate [in the mouse α T3-1 gonadotroph (137) and human medulloblastoma (36) cell lines] transcription of the human GnRHR I promoter, via binding of the PR to a PRE at -535/-521. Reminiscent of the situation with the ER described above, different isoforms of the PR have differential cell-specific effects on the human promoter (36, 137). Taken together, these ER and PR results suggest that, for the human promoter, variations in the levels of receptor isoforms may be a widely used mechanism for differential tissue-specific regulation in pituitary and extra-pituitary tissues. This could allow differential expression levels of the GnRHR I by varying the relative concentrations of receptor isoforms in response to different signals and thereby integrate connections between multiple physiological processes.

Continued research on the regulation of expression of mammalian GnRHRs is important for understanding reproductive endocrinology and could lead to novel insights on receptor function.

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