Gene structure, transcripts and transcriptional regulation of primate type II gonadotropin-releasing hormone receptors

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Abstract

The type II gonadotropin-releasing hormone (GnRH) peptide is structurally conserved in species across vertebrate classes from fish to man. Cognate type II GnRH receptors have been cloned from frogs and from non-human primates and recently a partial clone of a putative human type II GnRH receptor has been identified. The objectives of this study were: to clone a full-length human type II GnRH receptor cDNA, to determine the gene structure of the human and marmoset monkey type II GnRH receptors, and to investigate the transcriptional regulation of the marmoset type II GnRH receptor.

In this study, two genomic loci in human and one locus in marmoset monkey encoding the type II GnRH receptor and RNA-binding motif protein-8 (RBM8A) have been cloned and characterised. The type II GnRH receptor and RBM8A genes overlap and are orientated in a tail-to-tail manner in the marmoset locus and in both the human loci. The locus on human chromosome 1 and the marmoset locus contain the full-length type II GnRH receptor genes, which are composed of 3 exons and two introns.

The type II GnRH receptor genes have the same structure as the human type I GnRH receptor gene and encode proteins with 90% amino acid identity to each other and 41% amino acid identity to the human type I GnRH receptor. Transcripts of the human type II GnRH receptor gene are found in a wide range of reproductive and non-reproductive tissues as well as in neural and non-neural tissues. The human transcripts apparently lack a methionine initiation codon and have a premature stop codon in exon 2. The marmoset gene does have a methionine start codon and does not have a stop codon in exon 2 and transcribes a full-length functional transcript.

The type II GnRH receptor genes at the human chromosome 1 locus and the marmoset locus are flanked by and overlap the RBM8A and peroxisome biogenesis factor 11B (Pex 11B) genes in the antisense orientation. The RBM8A gene consists of 6 exons and five introns in humans and directs the synthesis of a RBM8A protein

of 173 or 174 amino acids by alternative splicing. A second human locus, located on chromosome 14, contains pseudogenes of the RBM8A (*RBM8B*) and the type II GnRH receptor and probably originates from the locus on chromosome 1 by retrotransposition. This locus is not present in the marmoset genome.

The nucleotide identities of the 5' flanking region of the marmoset type II GnRH receptor are 90% and 39% to the human type II GnRH receptor and human type I GnRH receptor genes respectively. The marmoset type II GnRH receptor promoter contains response elements for oestrogen and progesterone and binding sites for SF-1 and CREB. 2.3 kb of the 5' flanking region of marmoset type II GnRH receptor was cloned in front of a luciferase reporter in order to study the transcriptional regulation of the type II GnRH receptor. The full-length 5' flanking region increases luciferase activity 10.8-fold when expressed in HeLa-S3 and 3.6-fold in COS-1 cells. Progressive 5' and 3' deletions indicate the presence of negative regulatory regions at –2342/-1995, -1679/-1346 and -1346/-1084 and positive regulatory regions at –1995/-1679, -766/-665 and –458/-1.

List of abbreviations

AA	Arachidonic acid
AP-1	Activator protein 1
cAMP	Adenosine-3',5'- cyclic phosphate
CRE	cAMP response element
CREB	CRE binding protein
cDNA	Complementary DNA
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Extracellular loop
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
FSH	Follicle stimulating hormone
GAP	GnRH associated peptide
GnRH	Gonadotropin-releasing hormone
GnRH-R	GnRH receptor
GPCR	G protein-coupled receptor
GRAS	GnRH receptor activating sequence
GRK	G protein-coupled receptor kinase
GnRHir	GnRH immuno reactive
GTP	guanosine triphosphate
hCG	Human chorionic gonadotropin
HPLC	High performance liquid chromatography
ICL	Intracellular loop
ICV	Intracerebroventricular
IP_3	Inositol 1,4,5-triphosphate
JNK	Jun N-terminal kinase
LH	Luteinising hormone
MAPK	Mitogen activated protein kinase

NMR	Nuclear Magnetic Resonance.
PCR	Polymerase chain reaction
PIP_2	Phosphatidyl inositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
mRNA	Messenger RNA
PACAP	Pituitary adenylyl cyclase activating polypeptide
PLD	Phospholipase D
PLA_2	Phospholipase A_2
RACE	Rapid amplification of cDNA ends
RBM8	RNA-binding motif protein-8
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SBE	SMAD binding element
SC	Subcutaneous injection
SF-1	Steroidogenic factor 1
SeCys	Selenocysteine
SECIS	Selenocysteine insertion sequence
SURG-1	Sequence underlying responsiveness to GnRH
TM	Transmembrane domain
TPA	12-O-tetradecanoylphorbol-13-acetate
TSS	Transcriptional start site
UTR	Untranslated region

Chapter 1: Introduction

The decapeptide gonadotropin-releasing hormone (GnRH) has originally been isolated from pig hypothalami. GnRH is released in a pulsatile manner from the hypothalamus and the primary role of GnRH is to stimulate the release of lutenizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary (Matsuo et al., 1971; Schally et al., 1971a; Schally et al., 1971b). LH and FSH regulate gametogenesis and steroidogenesis in the gonads (King and Millar, 1997). Later studies have shown that many structural variants of GnRH exist and that GnRH has multiple functions in addition to its role as the primary regulator of reproduction (King and Millar, 1997).

1.1 Primary structure of GnRHs

The primary structure of mammalian GnRH isolated from pig was determined to be pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Matsuo et al., 1971). After the first identification of the structure of GnRH in pig and sheep pituitary, it was thought that GnRH was identical in all species. However, studies on GnRH from various species have shown that GnRH in birds, reptiles and fish is immunologically and chromatographically different from GnRH in amphibians and mammals (King and Millar, 1979; King and Millar, 1980). This has led to the determination of the sequence of the first structural variant of GnRH, namely chicken I GnRH (King and Millar, 1982a; King and Millar, 1982b; Miyamoto et al., 1982).

Since then, 23 different variants of GnRH have been identified in species ranging from protochordates to mammals and they are generally named after the species in which they were first identified (Table 1.1). Many vertebrates have two forms of GnRH and several species of fish have three forms of GnRH. A phylogenetic analysis based on the cDNA encoding various GnRH forms has suggesed that the majority of GnRH peptides can be divided into three different types of GnRH: GnRH

I, GnRH II and GnRH III (White et al., 1998; Fernald and White, 1999; Okubo et al., 2000; Sealfon et al., 1997).

GnRH I is the primary regulator of gonadotropin release from the pituitary and has many structural variants. The variants of GnRH I include mammalian GnRH (mGnRH), guinea pig GnRH (gpGnRH), chicken GnRH I (cGnRH I), seabream GnRH (sbGnRH), catfish GnRH (cfGnRH) and medaka GnRH (mdGnRH), and may also include frog GnRH (fgGnRH), herring GnRH (hrGnRH) and dogfish GnRH (dfGnRH) (Matsuo et al., 1971; King and Millar, 1982a; King and Millar, 1982b; Bogerd et al., 1992; Lovejoy et al., 1992; Powell et al., 1994; Jimenez-Linan et al., 1997; Carolsfeld et al., 2000; Okubo et al., 2000; Yoo et al., 2000; Montaner et al., 2001). GnRH II comprises only one GnRH form, GnRH II, which is conserved in species from several vertebrate classes (Miyamoto et al., 1984; King and Millar, 1997; Sealfon et al., 1997). GnRH III comprises only salmon GnRH, and has only been identified in teleost fish (King and Millar, 1997; Fernald and White, 1999).

Additional GnRH forms have been identified and these GnRH peptides have not been classified as GnRH I, GnRH II or GnRH III. Nine GnRH peptides have been identified in three tunicate species (Powell et al., 1996; Adams et al., 2003) and two GnRH peptides in the jawless fish lamprey (Sherwood et al., 1986; Sower et al., 1993). Recently a GnRH-like peptide has also been cloned from octopus. This peptide has 12 amino acids of which 6 are identical to mGnRH. Functional studies have shown that this octopus GnRH-like peptide is able to stimulate LH release from quail pituitary cells, but only at a high concentration (Iwakoshi et al., 2002). Post-translational modification can lead to further structural variants of GnRH as exemplified by the hydroxylation of Pro^9 of mammalian GnRH, identified in rat, hamster and frog brain (Gautron et al., 1992).

Table 1.1: Primary structure of GnRH peptides.

	1	2	3	4	5	6	7	8	9	10
Mammalian #	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$Gly.NH_2$
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	$Gly.NH_2$
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	$Gly.NH_2$
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	$Gly.NH_2$
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	$Gly.NH_2$
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	$Gly.NH_2$
Frog	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	$Gly.NH_2$
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	$Gly.NH_2$
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	$Gly.NH_2$
GnRH II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	$Gly.NH_2$
Guinea pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	$Gly.NH_2$
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	$Gly.NH_2$
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	$Gly.NH_2$
Tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	$Gly.NH_2$
Tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	$Gly.NH_2$
Tunicate III	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	$Gly.NH_2$
Tunicate IV	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	$Gly.NH_2$
Tunicate V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	$Gly.NH_2$
Tunicate VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	$Gly.NH_2$
Tunicate VII	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	$Gly.NH_2$
Tunicate VIII	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	$Gly.NH_2$
Tunicate IX	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	$Gly.NH_2$

Shaded boxes indicate highly conserved regions.

The 10 amino acid length of GnRH has been conserved in species spanning 500 million years of evolution. The N- and C-termini of GnRH are highly conserved with the exception of position 2 in the guinea pig GnRH and position 3 in lamprey I GnRH (Table 1.1). Both the N- and C-termini are important for binding to the GnRH receptor and the N-terminal domain plays a major role in receptor activation (Sealfon et al., 1997). The central residues of GnRH vary, with position 8 being the most variable and with Gly^6 being conserved in higher vertebrates.

The conformation of mGnRH has been shown to include a β-II turn involving residues 5 to 8, (for review see Sealfon et al., 1997). Nuclear Magnetic Resonance (NMR) studies have shown that this conformation is stabilised by hydrogen bonds between Ser^4–Arg^8 and between $pGlu^1$–Gly^{10}-NH_2 of mGnRH (Maliekal et al., 1997). Evidence for the role of the β-II turn conformation in the affinity of mGnRH for its receptor comes from studies with GnRH analogues that are constrained in a β-II turn conformation. Substitution of Gly^6 for a D-amino acid or the incorporation of a γ-

lactam ring between residues 6 and 7 constrains GnRH in a conformation with a β-II turn, leading to analogues with high affinity (Monahan et al., 1973; Freidinger et al., 1980; Pfleger et al., 2002).

Substitution of Gly^6 of GnRH II with a D-amino acid leads to only a small increase in the affinity of this peptide for GnRH receptors compared to the increase in affinity for the same substitutions in mGnRH. It has been suggested that the conserved GnRH II is preconfigured in a β-II turn and that this accounts for its high affinity to GnRH receptors and for its conservation of primary structure (Pfleger et al., 2002).

1.2 The evolution of GnRH

GnRH plays a central role in regulating reproduction in species that evolved over 500 million years from a common ancestor. The amino acid sequence of GnRH varies and several species contain two or three forms of GnRH. Two hypotheses describe the possible evolution of various GnRH forms. The first hypothesis is based on the amino acid sequence of the decapeptides and the occurrence of specific GnRH forms across vertebrate classes (King and Millar, 1995; King and Millar, 1997). GnRH is synthesised as a preprohormone which is enzymatically cleaved to release the decapeptide. Thus, the second hypothesis on GnRH evolution is based on the amino acid sequence of the preprohormone instead of only the decapeptide (White et al., 1998; Fernald and White, 1999).

The first hypothesis proposes that a duplication of an ancestral GnRH gene occurred during, or prior to, the evolution of protochordates. The suggestion of an early duplication is based on the observation that tunicates have at least two forms of GnRH. This gene duplication has resulted in two evolutionary lineages of the GnRH peptide. The first lineage consists of GnRH II and its progenitors; the second lineage consists of the other GnRH peptides which have been identified. GnRH II, as the most conserved GnRH peptide, has been identified in cartilaginous fish, bony fish, amphibians, reptiles, birds and mammals. Its progenitors are suggested to be

lamprey GnRH I and one of the tunicate GnRHs. The second evolutionary GnRH lineage consists of mammalian GnRH with dogfish GnRH and lamprey GnRH III as its progenitors. The catfish, salmon, seabream and chicken GnRH peptides are proposed to have arisen through duplication of the mammalian GnRH gene in teleost fish and in reptiles (King and Millar, 1995; King and Millar, 1997).

The second hypothesis, based on the preprohormone sequence, has been enabled by the cloning of the gene or cDNA of the different GnRH forms from various species. Three evolutionary types of GnRH, GnRH I, GnRH II and GnRH III, were identified based on phylogenetic analysis (Fig 1.1) (White et al., 1998; Fernald and White, 1999; Okubo et al., 2000; Okubo and Aida, 2001, Millar et al, in press). The first type, GnRH I, comprises the medaka-, seabream-, mammalian-, guinea pig- and chicken I GnRH peptides. In the brain, this group of GnRH is predominantly expressed in the hypothalamus and is stimulating the release of gonadotropins from the pituitary. The second type of GnRH consists of only the GnRH II peptide and this peptide is conserved in species from fish to man. The third type of GnRH, GnRH III, is salmon GnRH. GnRH III has been identified in several species of fish, but the gene for GnRH III has not been identified in other vertebrate classes.

The origin of GnRH III (salmon GnRH) has been hypothesised to be either prior to the emergence of teleost (White et al., 1998; Fernald and White, 1999; Okubo et al., 2000) or within the teleost lineage (King and Millar, 1995; King and Millar, 1997; Carolsfeld et al., 2000). The latter hypothesis is based primarily on the decapeptide sequence and the occurrence of GnRH in different taxa. Recently the GnRH III cDNA of arowana has been cloned. Arowana is one of the most primitive teleosts and the presence of GnRH III in this species indicates that this GnRH arose either very early in teleost evolution or prior to the emergence of teleosts (Okubo and Aida, 2001).

It appears that two gene duplications took place in the evolution of GnRH. One duplication occurred prior to the emergence of vertebrates and another gene duplication took place prior to the emergence of teleosts, resulting in three paralogous GnRH forms (King and Millar, 1995; King and Millar, 1997; White et al., 1998; Fernald and White, 1999; Okubo et al., 2000; Okubo and Aida, 2001; Dubois

et al., 2002). Accordingly, one would expect three GnRH forms to occur in all higher vertebrates. However, GnRH III (salmon GnRH) has only been found in fish so far. Perhaps it will still be found in higher vertebrates including humans. However, a gene for GnRH III has not been identified in the sequenced genomes of human and mice indicating that GnRH III has been lost in the evolution of higher vertebrates (White et al., 1998; Fernald and White, 1999).

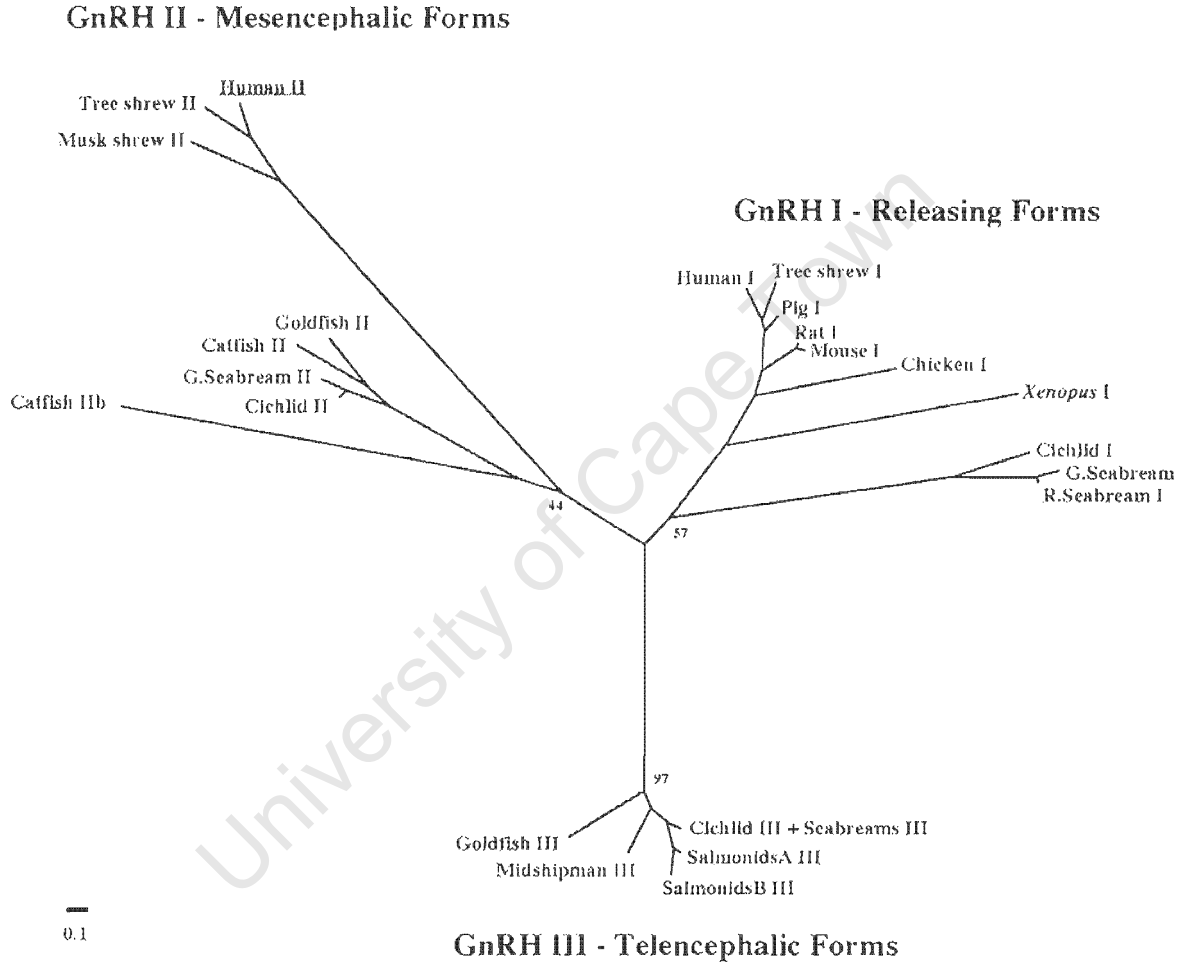

Figure 1.1. Phylogenetic tree of cDNAs encoding GnRH preprohormones. Evolutionary distances are represented by the branch length and not by the branch angles. Bootstrap values, indicating the number of times a set of sequences group together, are indicated for three important nodes of the tree (reproduced from White et al., 1998).

1.3 Tissue distribution of GnRH

1.3.1 GnRH expression in the brain

The three types of GnRH peptides have distinct expression patterns in the brain. GnRH I (medaka GnRH, catfish GnRH or seabream GnRH in species of fish) is localised mainly in the pre-optic area of the hypothalamus of fish, supporting the notion that this peptide group is responsible for the release of gonadotropins from the pituitary (Bogerd et al., 1994; White et al., 1995; Gothilf et al., 1996; reviewed in Fernald and White, 1999; Okubo et al., 2000).

The presence of GnRH I in the pre-optic area of the hypothalamus is conserved in fish, amphibians, reptiles, birds and mammals. Microdissection and radio-immuno assays (RIA), *in situ* hybridisation and immunohistochemical staining have been use to localise GnRH I (fgGnRH in *Rana dybowski* and mGnRH in *Xenopus laevis* and *Rana catesbeiana*) to predominantly the pre-optical area of the hypothalamus of newts and frogs (Hayes et al., 1994; Muske et al., 1994; Yoo et al., 2000). The area with the highest concentration of cGnRH I in reptiles and birds is the median eminence of the hypothalamus (Katz et al., 1990; Tsai and Licht, 1993). *In situ* hybridisation and immunohistochemistry has also been used to localise GnRH I (mammalian- or guinea pig GnRH) to the pre-optic area of the hypothalamus with projection to the median eminence in mammals (Silverman et al., 1987; reviewed in Muske, 1993; Kasten et al., 1996; Jimenez-Linan et al., 1997; Latimer et al., 2000; Grove-Strawser et al., 2002). In the primate, mGnRH is expressed in a scattered population of neurons, in particularly the ventral areas of the hypothalamus (Latimer et al., 2000).

The distribution of GnRH II in the brain differs from that of GnRH I. Cell bodies of neurons expressing GnRH II have been found by *in situ* hybridisation in the midbrain of all fish species investigated (Bogerd et al., 1994; White et al., 1995; Gothilf et al., 1996; reviewed in Fernald and White, 1999; Okubo et al., 2000). Studies on species from different vertebrate classes have identified GnRH II in several extra-hypothalamic and hypothalamic brain regions in addition to the expression in

midbrain. Micro dissection of reptilian (turtle) and avian (chicken) brain followed by RIA has shown that the highest concentrations of GnRH II are in the hindbrain areas of medulla and cerebellum in turtles and in the hypothalamus, medulla and midbrain of the chicken (Katz et al., 1990; Tsai and Licht, 1993).

GnRH II in the brains of mammals was first identified in the musk shrew (Dellovade et al., 1993). A study using immunohistochemical methods has located GnRH II-containing neurons to the midbrain of the musk shrew. GnRH II immuno reactive fibers are found widely scattered throughout the forebrain including the hypothalamus. The largest concentration of GnRH II fibers is found in the habenula. Electron microscopical analysis of the habenula, hybridised with a GnRH II antibody, has identified synapses with GnRH II in synaptic vesicles, suggesting a role as a neurotransmitter (Rissman et al., 1995). Cloning of the cDNA of GnRH II from the tree shrew has enabled *in situ* hybridisation analysis of GnRH II expression. Consistent with the immunological data, the mRNA encoding GnRH II is expressed in the midbrain (Kasten et al., 1996). There is some immunological evidence to the existence of GnRH II in rodents, though a gene encoding GnRH II has not been identified in the sequenced mouse genome (Chen et al., 1998; Gestrin et al., 1999; Chen et al., 2001).

The presence of GnRH II in the primate brain was first reported by Lescheid *et al* (Lescheid et al., 1997). GnRH II-producing neurons are found in the primate midbrain, as in musk shrew and tree shrew. GnRH II neurons are also found in the basal hypothalamus and the pituitary stalk of primates, which is in contrast to the expression in shrews (Lescheid et al., 1997). Cloning of the GnRH II gene in humans has enabled northern blot analysis of the GnRH II expression. GnRH II expression in the human brain is most notable in caudate nucleus, amygdala and hippocampus. The expression in midbrain has not been investigated (White et al., 1998). However, *in situ* hybridisation has identified GnRH II-expressing neurons in the midbrain, hippocampus and in the hypothalamus of the rhesus macaque (Urbanski et al., 1999). The expression patterns of GnRH I and GnRH II in the hypothalamus of rhesus macaques differ in that GnRH II is expressed mainly in discrete areas of the hypothalamus including the supraoptic, paraventricular and arcuate nuclei, whereas cells expressing GnRH I are more scattered and present

mainly in the ventro-lateral and mediobasal hypothalamus (Urbanski et al., 1999; Latimer et al., 2000). The difference in expression pattern suggests that GnRH I and GnRH II are produced by different populations of hypothalamic neurons, and this has been confirmed by a double-labelling technique combining immuno histochemistry (IHC) with a GnRH I antibody and *in situ* hybridisation with a riboprobe for GnRH II (Latimer et al., 2000).

GnRH III has been localised to the terminal nerve area and the olfactory bulb using *in situ* hybridisation on brains from the medaka, gilthead seabream and the African cichlid (White et al., 1994; White et al., 1995; Gothilf et al., 1996; Okubo et al., 2000).

In summary, the three evolutionary types of GnRH peptides have conserved patterns of expression in the brain. GnRH I is expressed mainly in the terminal nerve and the preoptic area of the hypothalamus, GnRH II is expressed mainly in the midbrain and GnRH III is expressed in fish terminal nerve and olfactory bulb.

1.3.2 GnRH expression in peripheral tissues

GnRH I has been identified in several tissues outside the brain. Seabream GnRH has been identified in spleen and in lower amounts in liver, heart, kidney and testis in the African cichlid (White and Fernald, 1998). In mammals, Tan and Rousseau identified mGnRH by extracting the peptide from human placenta followed by peptide sequencing (Tan and Rousseau, 1982). Confirmation of placental expression of mGnRH has come from cloning the cDNA and from RT-PCR done by four different groups (Seeburg and Adelman, 1984; Radovick et al., 1990; Harris et al., 1991; Kakar and Jennes, 1995). An early study found that mGnRH is expressed in neoplastic cells in breast cancer but not in normal breast tissue (Seppala and Wahlstrom, 1980). However, a recent study has shown that mGnRH is expressed in normal breast but is over-expressed in cancerous breast tissue (Chen et al., 2002b). In addition, mGnRH is expressed in three different breast cancer cell lines (Harris et al., 1991; Chen et al., 2002b). These studies indicate that mGnRH is expressed in breast cancer and may be expressed in normal breast tissue. mGnRH is also expressed in whole ovary and in granulosa cells of rats and humans (Oikawa et al.,

1990; Peng et al., 1994; Kang et al., 2001). The synthesis of mGnRH in rat and human testis has been shown, by *in situ* hybridisation, to be localised to the seminiferous tubules (Bahk et al., 1995). mGnRH has also been identified in rat spleen lymphocytes and in the human T-cell leukaemia Jurkat cell line and in a rat T-cell line (Azad et al., 1991; Wilson et al., 1995; Azad et al., 1997). RT-PCR has also shown expression of GnRH in the liver, heart, skeletal muscle, pituitary and kidney (Kakar and Jennes, 1995).

The conserved GnRH II peptide is expressed in testis of the African Cichlid (White and Fernald, 1998). GnRH-like material has been identified in synaptic boutons in frog sympathetic ganglia by immunohistochemical staining, and a later study using a GnRH II-specific antibody in RIA has identified it to be GnRH II (Jan et al., 1980; Troskie et al., 1997). Cloning of the gene for GnRH II from human has enabled RNA dot blot and RT-PCR analysis. The expression of GnRH II is 30-fold higher in kidney and 4-fold higher in prostate and bone marrow compared to any region of the brain (White et al., 1998). GnRH II has been identified by RT-PCR in surface epithelial cells of the human ovary and in granulosa-luteal cells (Choi et al., 2001; Kang et al., 2001). GnRH II has also been identified in human breast tissue and in the breast cancer cell line MCF-7 (Chen et al., 2002b). Furthermore, GnRH II is over-expressed in breast cancer tissue compared to normal tissue (Chen et al., 2002b).

Little is known about the expression of GnRH III outside the brain. A study by White and Fernald has identified transcripts of GnRH III in testis of African cichlid but not in heart, liver, spleen or kidney (White and Fernald, 1998). Northern blot analysis has identified transcripts of GnRH III in ovaries and testis of rainbow trout (Ferriere et al., 2001).

1.4 GnRH biosynthesis

GnRH I, GnRH II and GnRH III are synthesised as preprohormones. GnRH I cDNA has been identified in species of fish (Bogerd et al., 1994; White et al., 1995; Gothilf

et al., 1996; Lin and Peter, 1997) and mammalian GnRH cDNA has been isolated from *Xenopus laevis*, rat and from humans (Seeburg and Adelman, 1984; Adelman et al., 1986; Hayes et al., 1994). GnRH II cDNAs have been characterised in fish (Sommer et al., 1991; White et al., 1994; Lin and Peter, 1997), in tree shrew, macaque monkey and in humans (Kasten et al., 1996; White et al., 1998; Urbanski et al., 1999). Interestingly, two genes encoding GnRH II have been identified in goldfish (Lin and Peter, 1997). cDNAs of GnRH III have been identified in several species of fish and two genes encoding GnRH III have been identified in salmon (Bond et al., 1991; Klungland et al., 1992; Bogerd et al., 1994; Okuzawa et al., 1994; White et al., 1995; Gothilf et al., 1996; Ferriere et al., 2001).

Sequence analysis of all GnRH cDNAs characterised in species of fish, reptile and mammals show that the GnRH preprohormone consists of a signal peptide, the GnRH decapeptide, immediately followed by a conserved Gly-Lys-Arg processing site and a C-terminal protein called GnRH associated peptide (GAP). Cleavage of the signal peptide exposes a glutamine residue, which undergoes cyclisation to form the N-terminal pyro-Glu of GnRH (Seeburg and Adelman, 1984). The GnRH decapeptide is released by enzymatic cleavage at the Gly-Lys-Arg site and the glycine serves as donor of the amino group to form the C-terminal Gly-NH_2 of GnRH (Seeburg and Adelman, 1984; Adelman et al., 1986; Seeburg et al., 1987). This confirms an earlier proposal from immunological studies of Gln cyclisation, cleavage at basic pairs in a Gly-Gly-basic-basic sequence and amidation of the C-terminal Gly (Millar et al., 1977). The remainder of the GnRH precursor constitutes GAP. The length of GAP is a conserved 56 amino acids for mGnRH in tree shrew, mouse, rat and human. There is a 70% amino acid identity between human and rodent GAP, whereas GAP is less conserved between fish and mammals (Adelman et al., 1986; Seeburg et al., 1987). The GAP from the GnRH II precursor is 50% longer than the mGnRH GAP in humans, monkey and tree shrew (Kasten et al., 1996; White et al., 1998; Urbanski et al., 1999), which is in contrast to fish where GAP from GnRH II is shorter than GAP from GnRH I (White et al., 1995).

GAP has been implicated in gonadotropin release and inhibition of prolactin release from pituitaries but the data from different studies do not concur. Both the full-length

GAP and a peptide comprising the first 13 amino acids of GAP have LH and FSH releasing activity on human, baboon and rat pituitary cells *in vitro* (Nikolics et al., 1985; Millar et al., 1986b; Yu et al., 1988). In contrast, GAP and peptides of various regions of GAP do not stimulate gonadotropin release in ewes (Thomas et al., 1988). GAP inhibits prolactin release from rat pituitary cells *in vitro* and *in vivo,* and immunisation of rabbits against GAP peptides results in increased plasma levels of prolactin (Nikolics et al., 1985; Yu et al., 1988). The hypo-gonadal *hpg* mouse, in which a large part of GAP has been deleted, has a high plasma level of prolactin, which can be lowered by injection with GAP (Mason et al., 1986; Seeburg et al., 1987). In contrast, no prolactin-release inhibiting effect of GAP is seen in sheep (Thomas et al., 1988).

Another possible role for GAP is involvement in the correct processing of GnRH (Sherwood et al., 1993). In the *hpg* mouse, only part of the GnRH gene is deleted. The signal peptide, the GnRH peptide and 11 N-terminal amino acids of GAP are present in the gene, whereas the C-terminal part of GAP is deleted. This gene is transcriptionally active in hypothalamic neurons but plasma LH and FSH levels are undetectable and immunohistochemistry indicates that the mGnRH peptide is not present in the hypothalamus of *hpg* mice (Mason et al., 1986). The absence of GnRH in the *hpg* mouse may lead to a further decrease in prolactin levels due to the stimulatory role of GnRH on prolactin release observed from rat pup pituitaries (Andries and Denef, 1995).

1.5 GnRH gene structure

The GnRH gene structure has been determined in fish, rodents and humans. The GnRH gene for all GnRH forms in all species consists of four exons and three introns, and the positions of intron-exon junctions are conserved in all species investigated (Adelman et al., 1986; Mason et al., 1986; Klungland et al., 1992; Dong et al., 1996; White et al., 1998; White and Fernald, 1998). Exon 1 contains the 5' untranslated region (UTR), while exon 2 encodes the signal peptide, the GnRH peptide and the N-terminal part of GAP. Exon 3 encodes the central part of GAP

and exon 4 encodes the C-terminus of GAP and the 3' UTR. Differential splicing of intron 1 of the mGnRH gene, which is observed in human hypothalamus and placenta, affects the length of the 5' UTR and may affect expression through altered RNA stability or translation efficiency (Radovick et al., 1990). Part of the coding region of the mGnRH gene in the rat overlaps with a gene encoded by the opposite DNA strand. This gene, called SH, is transcribed in the heart, hypothalamus, placenta and T-cells in the opposite orientation to GnRH and there is speculation that it plays a role in regulating GnRH expression (Adelman et al., 1987; Sherwood et al., 1993; Wilson et al., 1995).

1.6 Regulation of mGnRH release

mGnRH is synthesised in a scattered population of neurons in the medio-basal hypothalamus. GnRH is released in a pulsatile manner from the median eminence into the specialized portal vasculature by which it is transported to the gonadotrope cells of the anterior pituitary. The pulsatility of GnRH release is implicated in the differential regulation of expression and release of LH and FSH (reviewed in Conn and Crowley, 1991; Conn and Crowley, 1994; Mercer and Chin, 1995). Pulsatile delivery of GnRH to the pituitary is necessary to maintain LH and FSH secretion, and the pulse frequency and amplitude increases at the time of the LH surge prior to ovulation (Reame et al., 1984).

A large number of neurotransmitters and growth factors have been shown to regulate GnRH release from GnRH neurons and from the immortalised GnRH-expressing mouse hypothalamic cell line GT1-7. It has been shown that norepinephrine, glutamate, nitric oxide, neuropeptide Y, TGFα, TGFβ, endothelin, galanin, activin-A, histamine and angiotensin II stimulate GnRH release from hypothalamic GnRH neurons and/or from GT1-7 cells. Corticopin-releasing hormone (CRH), prolactin, interleukin 1β and opiates inhibit GnRH release. GABA, dopamine and IGF-2 have shown both inhibitory and stimulatory effects on GnRH release (reviewed in Brann and Mahesh, 1997; Gore and Roberts, 1997).

1.7 Transcriptional regulation of mGnRH expression

The release of mGnRH from secretory granules of GnRH neurons is under complex control by various neurotransmitters. In addition, several factors have been shown to influence the synthesis of GnRH by regulating the transcription of the GnRH gene. Most work has concentrated on the regulation of the mGnRH gene from rats or humans. The study of GnRH promoters was facilitated by the creation of murine hypothalamic cell lines expressing GnRH endogenously. The SV40 T antigen oncogene was targeted to GnRH-producing neurons by either the rat or human GnRH promoters. The resulting hypothalamic tumours were removed and the cells were selected and propagated resulting in the cell lines GT1-3, GT1-7, GN11 and NLT (Mellon et al., 1990; Radovick et al., 1991). These cell lines have a neuronal morphology; they secrete GnRH, and GnRH synthesis and release from these cell lines are regulated in a manner consistent with GnRH neurons *in vivo* (Gore and Roberts, 1997).

1.7.1 The enhancer of the mGnRH promoter

The fact that GnRH is expressed in a subset of hypothalamic neurons prompted research in the requirements for neuron-specific expression. Mellon and co-workers have cloned 3 kb of the 5' flanking region of the rat GnRH gene in front of a reporter gene and transfected this construct into various cell lines (Whyte et al., 1995). Deletion analysis has identified a 300 bp enhancer 1.5 kb upstream of the transcriptional start site which, together with a 285 bp minimal proximal GnRH promoter, confers cell-specific expression. mGnRH expression levels in the GnRH neuronal cell line GT1-3 are high, whereas other cell lines tested, both neuronal and non-neuronal, show little expression. The GnRH promoter in the absence of the enhancer shows little expression in GT1-3 cells (Whyte et al., 1995). The same enhancer is shown to be sufficient in combination with the promoter to direct appropriate tissue expression in hypothalamic GnRH neurons as well as in the ovaries of transgenic animals. The enhancer alone is not able to confer GnRH-neuron-specific expression to a heterologous promoter *in vivo* or *in vitro,* indicating

that the enhancer functions together with the GnRH promoter in directing tissue-specific expression (Lawson et al., 2002).

The cell specificity of the mGnRH promoter *in vivo* has been confirmed for the mouse GnRH gene. The proximal 1 kb of the mouse GnRH promoter is sufficient for expression in the hypothalamus and ovary. However, including an additional 1 kb or 2 kb of the promoter increases hypothalamic expression 3.5-fold or 17-fold respectively, suggesting the presence of an enhancer active in the hypothalamus. Ovarian GnRH expression is at its highest when constructs only contain the proximal 1 kb of the GnRH promoter. In comparison, ovarian expression of the 3 kb construct is only 0.27% of the levels achieved from the 1 kb construct, indicating the presence of a strong repressor element active in the ovary (Kim et al., 2002). A GnRH neuron-specific enhancer, similar to the one identified in rat and mouse, has been identified in the human GnRH gene. As with the rat and mouse GnRH genes, this enhancer, together with the promoter, is necessary and sufficient for targeting expression of GnRH to hypothalamic neurons in transgenic animals (Wolfe et al., 1996; Wolfe et al., 2002). In a different study however, deletion studies of the human GnRH 5' flanking region have shown no activity of this enhancer when expressed in GT1-7 cells (Kepa et al., 1996).

DNAse I protection analysis has identified several protected areas of both the mouse and the human enhancer, indicating binding of nuclear proteins to this region (Whyte et al., 1995; Wolfe et al., 2002). It has been shown, by electrophoretic mobility shift assays (EMSAs), that the homeodomain transcription factor Oct-1 binds to the enhancer of the murine GnRH promoter. Mutation of a particular binding site eliminates protein binding to this site and reduces promoter activity to 5% of wild-type activity (Clark and Mellon, 1995). In the human GnRH enhancer, Wolfe et al. have identified an octamer consensus binding site for Brn-2. A nuclear protein from hypothalamic GnRH-producing neuronal cell lines binds to this site but binding is not inhibited or supershifted by Brn-2 antibody. Over-expression of Brn-2 does increase activity of the human GnRH promoter in a GnRH-neuronal cell line (Wolfe et al., 2002). Brn-2 and Oct-1 are members of the POU family of homeodomain transcription factors and the involvement of these transcription

factors in both GnRH enhancers indicates the conservation of function of these transcription factors in the enhancer of the mouse and human GnRH promoters.

The cell specificity of the GnRH enhancer involves several factors. In the rat GnRH enhancer, the transcription factor GATA-4 is shown to bind to a particular GATA binding consensus site. Expression of a dominant negative GATA-4 decreases activity in GT1-7 cells of the GnRH promoter containing the wild-type enhancer, but not if the GATA binding site of the enhancer has been mutated. Furthermore, over-expression of GATA-4 in a fibroblast cell line increases activity of the promoter containing the wild-type enhancer but not when the GATA binding site is mutated (Lawson et al., 1996). GATA-4, which is expressed in the developing mouse embryo, is thought to be involved in the differentiation of GnRH neurons in the developing embryo. Further cell specificity of the enhancer is conferred by multiple CAAT elements. These elements are bound by a protein that appears to be only expressed in the GT1-7 cell line and may, therefore, contribute both to activity and cell specificity of the enhancer (Kelley et al., 2002).

Nitric oxide inhibits transcription of the GnRH gene but stimulates the release of GnRH from GT1-7 cells. This repression of transcription is mediated by two adjacent cis-elements of the enhancer. Mutation of either of these two elements eliminates NO inhibition of transcription. The two sites bind Oct-1 and the CCAAT/enhancer binding protein β (C/EBP)β respectively. These two sites are located at different positions in the enhancer than the Oct-1 and CAAT sites which are involved in the positive regulation of the GnRH promoter. Nitric oxide does not induce expression of Oct-1 or C/EBPβ but increases the affinity of Oct-1 for the enhancer due to increased phosphorylation (Belsham and Mellon, 2000).

GnRH expression is regulated by the pineal hormone, melatonin, in a cyclical manner. The expression of GnRH mRNA is suppressed only at 12 hours and 36 hours after the addition of melatonin to GT1-7 cells and no change in mRNA levels are seen at other time points. This suppression of GnRH expression can be inhibited by the melatonin antagonist, luzindole. The inhibitory action of melatonin has been located to five different areas of the GnRH promoter. Two of these areas

bind Oct-1 and GATA-4 respectively, as previously reported, and a third area contains the consensus binding site for transcription factors of the ROR/RZR family of nuclear receptors (Roy et al., 2001).

1.7.2 Regulation of the mGnRH proximal promoter

The proximal promoter of the rat mGnRH 5' flanking region is necessary in combination with the enhancer in order to confer cell specificity of the enhancer (Lawson et al., 2002). Several areas of the promoter are bound by GT1-7 nuclear proteins (Eraly and Mellon, 1995). One of these areas contains the consensus binding site for the transcription factor Otx2, and Otx2 has been shown to bind this site. Over-expression of Otx2 in GT1-7 cells increases activity of wild-type rat GnRH promoter, but not of the promoter in which the Otx2 binding site has been mutated. Mutation of the Otx2 site leads to decreased expression in GT1-7 cells but not in NIH3T3 cells, suggesting the involvement of Otx2 in cell specificity of the GnRH promoter (Kelley et al., 2000). Oct-1, which was shown to be important for enhancer activity, is also regulating promoter activity. DNAse I analysis has identified several protected areas of the promoter using GT1-7 nuclear extract. Two of these protected areas contain consensus binding sites for Oct-1, and Oct-1 binds to these regions of the promoter. A mutation in either of these regions results in a 60-70% decrease in expression (Eraly et al., 1998).

The mGnRH promoter has been shown to be stimulated by protein kinase C (PKC). The phosphorylation of target proteins by activated PKC leads to increased expression of proteins of the Jun and Fos families. These proteins form dimers that bind to AP-1 binding sites (or TRE, – TPA response elements) and stimulate expression of phorbol ester-activated genes. 12-O-tetradecanoylphorbol-13-acetate (TPA) is a short term activator of PKC whereas long term treatment with TPA leads to the proteolytic degradation of PKC. Prolonged treatment with TPA leads to a decrease in GnRH promoter acitivity suggesting that GnRH mRNA transcription is stimulated by PKC and the degradation of PKC decreases the expression of GnRH (Gore and Roberts, 1997). This has been confirmed by treatment with a specific PKC inhibitor, NPC 15437, which also leads to a decrease in GnRH gene

expression. The responsiveness of the GnRH promoter to TPA has been located to two areas of the promoter and the most proximal of these areas forms TPA-inducible complexes with GT1-7 nuclear proteins (Eraly and Mellon, 1995).

The mGnRH promoter activity is under hormonal regulation by estradiol, prolactin and cortisol. Estradiol decreases the expression of GnRH I in human granulosa luteal cells, an effect that is inhibited by the estradiol antagonist Tamoxifen. The regulatory cis-elements of the promoter responsible for regulation of expression by estradiol have not yet been identified (Khosravi and Leung, 2003).

Prolactin has been shown to inhibit mGnRH release from GT1-7 cells and from pituitary cells *in vitro* (Gore and Roberts, 1997). However, Wilson *et al* found that prolactin stimulates GnRH promoter activity in a rat T-cell line in a cyclical manner (Wilson et al., 1995). This difference may indicate that GnRH mRNA expression is differentially regulated in different tissues (hypothalamic GnRH neurons vs. T-cells) or that prolactin stimulates the GnRH promoter but inhibits GnRH exocytosis.

Cortisol inhibits mGnRH transcription and release from primate and rodent hypothalamus, and glucocorticoid receptors are present in the murine GT1-7 cell line. The glucocorticoid responsiveness has been located to the 459 bp proximal part of the GnRH promoter by using cortisol, an agonist and an antagonist on rat GnRH promoter deletions linked to a reporter gene. Transcription of the endogenous mouse GnRH gene is also inhibited by a cortisol agonist, confirming the cortisol responsiveness of the GnRH promoter (Chandran et al., 1994).

The transcription factor SCIP/Oct-6/Tst-1, may be involved in repression of GnRH expression. Over-expression of SCIP in GT1-7 cells decreases expression from the rat GnRH I promoter and SCIP is able to bind to the promoter; however, the physiological role of SCIP needs to be confirmed (Wierman et al., 1997).

There are some indications that the mGnRH gene might utilize two different promoters. In addition to the well characterised proximal promoter, a second transcriptional start site has been identified 500 or 560 bp further upstream in the monkey and human GnRH genes. Transcripts initiated at the upstream promoter

have been identified in testis, placenta, ovary and breast. Transcripts initiated from the proximal promoter only have been identified in hypothalamus, pituitary and cerebral cortex (Dong et al., 1996). The upstream promoter is more active in cell lines of reproductive non-neuronal origin than in GT1-7 cells. Conversely, the downstream promoter is more active in GT1-7 cells than in the placental JEG cell line. DNAse I analysis and EMSA have identified four regions of the upstream promoter which bind to nuclear proteins from JEG cells. These regions do not form protein complexes with nuclear extract from GT1-7 cells (Dong et al., 1997).

In summary, maximal cell-specific transcriptional expression of mGnRH *in vitro* and *in vivo* requires both the proximal GnRH promoter and a GnRH neuron-specific enhancer located further upstream. Several transcription factors including Oct-1, SCIP/Oct-6/Tst-1, Brn-2, GATA-4, Otx2 and C/EBPβ have been shown to be important for the transcriptional regulation of the GnRH gene.

1.7.3 The GnRH II promoter

The transcriptional regulation of the GnRH II gene is not well understood. A cDNA or gene for GnRH II has not been identified in rodents and only recently have human neuronal cell lines expressing GnRH II been identified. The GnRH II gene in one of these cell lines (TE-671) is up-regulated by cAMP, and GnRH II release into the media is also increased after stimulation with a cAMP analogue. Mutation of a cAMP response element (CRE) consensus sequence in the GnRH II promoter decreased basal expression to 0.06% of wild-type levels (Chen et al., 2001). Gonadal steroids regulate GnRH II transcription in human granulosa luteal cells. Estradiol increases expression of GnRH II in human granulosa luteal cells, an effect that is inhibited by the estradiol antagonist Tamoxifen. GnRH II expression is stimulated by a progesterone antagonist suggesting down-regulation of GnRH II by endogenous progesterone in human granulosa luteal cells. The regulatory cis-elements of the GnRH II promoter responsible for the steroidal regulation of expression have not yet been identified (Khosravi and Leung, 2003).

1.8 The functions of GnRH

1.8.1 GnRH I as a gonadotropin-releasing factor

GnRH I was first identified as a gonadotropin-releasing factor and is as such the primary regulator of reproduction in vertebrates. GnRH is released from GnRH neuron projections in the median eminence of the hypothalamus into the portal vasculature by which it is transported to the anterior pituitary. GnRH binds to receptors on gonadotrope cells in the anterior pituitary, initiating a signalling cascade resulting in the synthesis and release of LH and FSH. LH and FSH are transported via the general circulation to the gonads where they stimulate gametogenesis and production of sex steroids in both male and female vertebrates. The role of GnRH as a gonadotropin-releasing factor has been reviewed by several authours (Millar et al., 1987; Conn and Crowley, 1991; Conn and Crowley, 1994; Stojilkovic et al., 1994; King and Millar, 1997). GnRH is released from the median eminence in a pulsatile manner. The pulse frequency varies through the oestrous cycle with increased pulse frequency during the follicular phase, increased pulse amplitude during the LH surge and decreased pulse frequency during the luteal phase (Reame et al., 1984; reviewed in Mercer and Chin, 1995). The pulse frequency affects the synthesis of LHβ and FSHβ subunits differentially. High pulse frequencies preferentially stimulate LHβ synthesis and LH release, whereas low GnRH pulse frequencies preferentially stimulate FSHβ synthesis and FSH release (Dalkin et al., 1989).

The function of GnRH I and the GnRH receptor in gonadotropin-release has been well characterised in vertebrates. The co-localisation of GnRH I and GnRH II in the hypothalamus of an amphibian (Muske et al., 1994), a reptile (Tsai and Licht, 1993), a bird (Katz et al., 1990) and primates (Lescheid et al., 1997; Urbanski et al., 1999), suggests the involvement of GnRH II in stimulating the pituitary release of gonadotropins. In the chicken, GnRH II is 5-13 fold more potent than cGnRH I in stimulating LH and FSH release from pituitaries, suggesting a possible involvement of GnRH II in the regulation of gonadotropin release (Millar et al., 1986a). However, the concentration of GnRH II in the hypothalamus and in particular the median eminence of the chicken is much lower than that of cGnRH I (Katz et al., 1990;

Sharp et al., 1990). The amount of cGnRH I increases at onset of puberty in cockerels with concomitant increase in plasma LH levels, whereas no change in GnRH II levels is seen. The amount of hypothalamic cGnRH I in laying hens is higher than in out-of-lay hens, but no change is seen for GnRH II. Furthermore, immunisation against cGnRH I but not against GnRH II has resulted in lower plasma LH levels, regression of ovaries and oviducts and complete cessation of egg laying (Sharp et al., 1990). These results indicate that cGnRH (GnRH I) is the primary regulator of gonadotropin secretion in chickens.

A study performed on sheep showed that both mGnRH and GnRH II can stimulate gonadotropin-release from pituitaries *in vivo*. mGnRH is more potent than GnRH II in stimulating both LH and FSH release, but GnRH II increases FSH release more than it increases LH release (Millar et al., 2001). The three GnRH forms identified in fish have distinct areas of expression (White et al., 1995; Gothilf et al., 1996). The occurrence of GnRH I in the pre-optic area of the hypothalamus and the observation that the sizes of only the GnRH I neurons increase in territorial vs. non-territorial male African cichlids supports the notion that GnRH I is the regulator of reproduction.

1.8.2 Direct actions of mGnRH on gonads and placenta

Several diverse functions of GnRH have been identified in addition to its main function as a stimulator of LH and FSH release from the pituitary. Direct mGnRH action on both testis and ovary has been established in hypophysectomised rodents (Hsueh and Schaeffer, 1985). Hypophysectomy leads to a decrease in testicular weight and this can be countered by FSH administration. GnRH agonists are able to inhibit the maintenance of testis weight by FSH (Hsueh and Erickson, 1979b). *In vitro* treatment of leydig cells with human chorionic gonadotropin (hCG) leads to an increase in androgen production. GnRH and GnRH agonists inhibit hCG-stimulated androgen production. This inhibition is blocked by GnRH antagonists, suggesting involvement of the GnRH receptor in Leydig cells (Hsueh and Schaeffer, 1985).

Hypophysectomy leads to a decrease in plasma levels of oestrogen and progesterone in female rats. Ovarian granulosa cells obtained from hypophysectomised rats increase oestrogen and progesterone secretion to the media when treated with FSH *in vitro*. mGnRH and GnRH agonists are able to inhibit the FSH induced increase (Hsueh and Erickson, 1979a). Hypophysectomy leads to a decrease in ovary weight, which can be countered by FSH administration. The *in vivo* treatment with mGnRH or GnRH agonists inhibits this FSH stimulated increase in ovary size (Hsueh and Erickson, 1979a; Birnbaumer et al., 1985). Administration of a GnRH antagonist together with FSH has led to a further increase in ovary weight compared to FSH alone (Birnbaumer et al., 1985).

The direct ovarian role of mGnRH in follicle maturation has been evaluated by further *in vivo* studies. Hypophysectomised rats were treated with FSH alone, with FSH and mGnRH, or with FSH and GnRH antagonists and ovulation was induced with hCG after two days. Some ova were found in the fallopian tubes, and some corpus lutea (CL) were found on the ovaries of animals treated with FSH alone. No ova or CL were found in animals treated with both FSH and GnRH. An increased number of ova and CL were found in animals treated with FSH and GnRH antagonist, suggesting a direct ovarian role of GnRH in inhibiting follicle development (Birnbaumer et al., 1985). It appears that mGnRH directly regulates gonadal steroid production and follicle maturation in addition to its pituitary role.

Early studies have shown that mGnRH has a possible function of stimulating the release of hCG in placenta. The addition of mGnRH to placentas stimulates hCG release in a dose dependent manner (Khodr and Siler-Khodr, 1978; Belisle et al., 1984). The concentrations of mGnRH used were high (1.5×10^{-6} M) and the mechanism of GnRH action in the placenta still needs to be elucidated.

1.8.3 GnRH and reproductive behaviour

Many studies over the last three decades have implicated GnRH in reproductive behaviour. These studies have generally involved mGnRH or mGnRH agonists. Recently, a few studies of the role of GnRH II in reproductive behaviour have also

been undertaken. Two main aspects of the role of GnRH in reproductive behaviour have been investigated. One aspect is the association of reproductive behaviour with changes in the number of GnRH containing cells or the sizes of these cells in the brain in fish. The other important aspect is the effect exogenous GnRH or the inhibition of endogenous GnRH has on reproductive behaviour.

The studies on the effect of reproductive behaviour on GnRH generally involve exposing female or male animals to the other sex for courtship or copulation. The animals are then sacrificed and GnRH content is measured by peptide extraction, high performance liquid chromatography (HPLC) and radio-immuno assay (RIA) or by immunocytochemistry (reviewed in Rissman et al., 1997). Courtship or copulation is shown to increase the GnRH content or GnRH cell number in the hypothalamus of goldfish, newts, ringdoves and musk shrews. However, this effect may be due to pheromone exposure. Indeed, in goldfish, prairie voles and musk shrews, exposure to pheromone alone has produced the same effect (Rissman et al., 1997). Furthermore, cutting the olfactory tract in goldfish blocks the pheromone-induced increase in GnRH.

In the ferret, either copulation or vaginocervical stimulation with a glass rod induces LH release within minutes in oestrous females. Animals sacrificed during the LH surge had a decreased number of GnRH immuno reactive (GnRHir) cells in the forebrain compared to animals in which the LH-level had returned to baseline levels two days after stimulation. Thus reproductive behaviour in the form of vaginocervical stimulation, either by copulation or by using a glass rod, appears to influence the number of GnRHir cells in the forebrain of ferrets (Rissman et al., 1997). Reproductive behaviour in ringdoves is also associated with an increase in GnRHir cells in the brain. Male doves were sacrificed after 2 hours of courtship with a female and cells immunoreactive for GnRH were counted. Courting males have a significantly increased number of GnRHir cells in the habenula compared to males in isolation or castrated males (Zhuang et al., 1993). A subsequent study has identified these cells as being mast cells and the authours suggest that mast cells may serve as a means of delivery of GnRH to the habenula (Silverman et al., 1994; Zhuang et al., 1993).

The influence of GnRH on reproductive behaviour has mostly been studied using mGnRH or mGnRH agonists, but a few of the later studies use cGnRH I in birds or GnRH II in various species. In reptiles, receptivity is increased in female lizards after subcutaneous (sc) administration of mGnRH (Alderete et al., 1980). mGnRH also increases reproductive behaviour in male newts, whereas a GnRH antagonist suppresses reproductive behaviour (Moore et al., 1982). In amphibians, receptivity is increased in both intact female *Xenopus laevis* frogs and ovariectomized frogs upon mGnRH injection (Kelley, 1982). GnRH also stimulates reproductive behaviour in birds. An early study has shown that intramuscular injection of mGnRH stimulates several aspects of female ringdove reproductive behaviour (Cheng, 1977). Repeated exposure of female song sparrows to recorded male song decreases the behavioural response by females, a process called habituation. Intra-cerebroventricular administration of cGnRH I prevents habituation in these birds (Maney et al., 1997). These studies show that GnRH I is involved in stimulating courtship behaviour.

Behavioural effects of mGnRH in mammals have first been observed in female rats. Female rats in heat display lordosis, a reflex receptive posture, upon mounting by a male. This response is abolished in ovariectomized rats, but can be reinstated with injection of the combination of oestrogen and progesterone. Oestrogen alone shows no effect on lordosis. Administration of mGnRH facilitates lordosis response in oestrogen-primed ovariectomized rats to a similar extent as the oestrogen-progesterone treatment. Injection of LH or FSH shows no effect on lordosis. It appears that GnRH influences reproductive behaviour in female rats in a manner independent of the gonads and independent of LH and FSH (Moss and McCann, 1973). These results are confirmed by a study on hypophysectomised, ovariectomized oestrogen-primed female rats, showing a dose-dependent increase in lordosis after subcutaneous (sc) mGnRH injection (Pfaff, 1973). In addition, a mGnRH antagonist can inhibit reproductive behaviour in rats. Female ovariectomized rats have been treated with oestrogen and progesterone to induce lordotic response to mounting. Intracerebroventricular infusion of a potent mGnRH antagonist inhibits the lordotic response, suggesting that reproductive behaviour in the female rat is facilitated by mGnRH, possibly through the GnRH receptor (Dudley et al., 1981).

Removal of the vomeronasal organ impairs lordosis in female hamsters. This effect is reversed by mGnRH, suggesting a role for GnRH in mediating pheromonal input and reproductive behaviour (Mackay-Sim and Rose, 1986). In the female musk shrew, intracerebroventricular (icv) administration of mGnRH in intact animals reduces the latency to present the rump and wag the tail, behaviours indicative of receptivity (Schiml and Rissman, 2000). The female marmoset monkey is receptive to the male throughout the ovarian cycle. However, the female's display of proceptive behaviour in which she invites the male to copulate, occurs mainly around the time of ovulation. Treatment of ovariectomized marmosets with oestrogen stimulates low frequencies of proceptive behaviour. Proceptive behaviour is significantly increased two hours after intra-muscular administration of mGnRH (Kendrick and Dixson, 1985).

There is also some reported effect of GnRH on reproductive behaviour in male animals. In a study by Dorsa *et al.*, castrated rats have been given testosterone replacement to facilitate reproductive behaviour. Continuous exposure to GnRH results in an increase in the latency to mount after 6-7 weeks, thus showing a down-regulation of reproductive behaviour after prolonged GnRH treatment (Dorsa et al., 1981). Conversely, short-term exposure (2 hr) of male voles to mGnRH increases mount frequency and decreases intromission latency and latency to ejaculation. These results are seen both in intact animals and in castrated and testosterone-primed animals (Boyd and Moore, 1985).

An effect of GnRH II on reproductive behaviour has also been reported. An osmotic pump has been implanted in female green iguanas and a GnRH II agonist has been administered in a pulsatile manner over several weeks. An increase in male reproductive behaviour is observed 3 days after implantation of the pump in females, suggesting receptiveness of the female. The study has been conducted in whole animals and gonadal steroids may mediate the behaviour observed. Indeed, the oestrogen level reaches nadir at the same time as males exhibit increased reproductive behaviour (Philips et al., 1985). A study using female sparrows shows increased courtship behaviour 30 min after infusion of GnRH II but not cGnRH I into the third ventricle in oestrogen-primed intact animals. Plasma LH levels before

infusion and at the time of increased reproductive behaviour have were non-detectable, suggesting GnRH II does not mediate its effect through LH released from the pituitary and thereby stimulating gonadal steroid production (Maney et al., 1997).

GnRH II stimulates reproductive behaviour in female musk shrews. An initial study has shown that icv administration of mGnRH but not GnRH II stimulates reproductive behaviour in intact female musk shrews (Schiml and Rissman, 2000). Interestingly, a follow-up study has shown that GnRH II stimulates only reproductive behaviour in food-restricted animals (Temple et al., 2003). This study has also shown that mGnRH has no effect in food-restricted animals. Restricted feeding of female musk shrews is associated with a lower number of GnRH II immunoreactive cell bodies in the midbrain, and the area of GnRH II immunoreactive fibres in the median eminence is smaller compared to *ad libitum*-fed animals. The authors suggest that the role of GnRH II in mammals is to coordinate the energetic condition and reproductive behaviour and that the effect of GnRH II only becomes apparent in a nutritionally challenged situation (Temple et al., 2003; Terasawa, 2003).

Little is known about the influence of GnRH III on reproductive behaviour. In one study, terminal nerve lesion in male dwarf gourami fish has resulted in decreased nest building. The terminal nerve is the main area of expression of GnRH III and this points to a possible role of GnRH III in reproductive behaviour (Yamamoto et al., 1997).

1.8.4 GnRH in neurotransmission

GnRH immunopositive cell bodies have been identified in the olfactory nerve of the terminal nerve in gold fish. Fibres from these cell bodies project to the retina and it has been suggested that salmon GnRH acts as a neurotransmitter in the terminal nerve and retina. The localisation of GnRH in the olfactory nerve, with projections to the retina, suggests that salmon GnRH may be involved in coordination of sensory input with reproductive behaviour (Stell et al., 1984). The best-characterised neurotransmitter role of GnRH is probably in the frog sympathetic ganglion. GnRH

has been found in synaptic boutons of sympathetic ganglia (Jan et al., 1980; Troskie et al., 1997). Applying GnRH or GnRH agonists to sympathetic neurons elicits a slow depolarisation associated with the late slow excitatory postsynaptic potential. GnRH antagonists block both GnRH-induced and nerve-impulse-induced depolarisation. The depolarisation is due to the inhibition of a slow potassium current called M-current, named for its inhibition by muscarinic acetylcholine receptor agonists. Salmon GnRH, cGnRH I, mGnRH and lamprey GnRH all inhibit the M-current at high concentrations, but GnRH II is a 100-fold more potent than salmon GnRH and more than a 1000-fold more potent than mGnRH in inhibiting the M-current (Jones, 1987). This inhibition desensitises to GnRH II and involves G-protein activation and stimulation of phospholipase C (PLC) (Bosma et al., 1990). More recently, the GnRH in sympathetic ganglia has been identified as GnRH II, and a receptor with high affinity for this peptide has been identified in frog sympathetic ganglia (Troskie et al., 1997). Thus it seems likely that GnRH II is an endogenous neurotransmitter in amphibian sympathetic ganglia.

Further support for the notion of GnRH II as a neurotransmitter comes from immunohistochemistry on brains of musk shrews. GnRH II positive cell bodies in the midbrain showed projections to the medial habenula, where electron micrographs localised GnRH II to synaptic vesicles in axon terminals (Rissman et al., 1995). In addition, Moss et al. reported that neurons in the hypothalamus and the cerebral cortex respond to GnRH by a change in firing rate of the neurons (Moss et al., 1978). Taken together, the observations above suggest that GnRH functions as a neurotransmitter in frogs and possibly in fish and mammals. This leads to a suggestion that GnRH might exercise its effect on reproductive behaviour through its action as a neurotransmitter.

1.8.5 Stimulation of growth hormone release by GnRH

GnRH III is primarily expressed in the terminal nerve and the olfactory bulb. GnRH III stimulates *in vivo* and *in vitro* gonadotropin release and growth hormone (GH) release in three species of fish, gold fish, common carp and tilapia. The GH and gonadotropin release from pituitaries *in vitro* seem to occur independently of each

other since GH but not gonadotropin release is inhibited by somatostatin. Conversely, gonadotropin release but not GH release is inhibited by a dopamine agonist (Marchant et al., 1989; Lin et al., 1993; Melamed et al., 1995).

GnRH stimulation of GH release has also been observed in mammals. The GH release is increased approximately two-fold in perifused pituitaries from rat pups upon stimulation with 10 nM mGnRH. The increase in GH release is highest in 2-9 day old rats and virtually no stimulation is seen for 14-day old rats (Andries and Denef, 1995). These results indicate a role of GnRH in stimulating somatic growth through stimulating GH release from pituitary somatotroph cells.

1.8.6 GnRH in cells of the immune system

GnRH has been identified in mast cells in the medial habenula of the ringdove. The number of GnRH-positive cells increase during courtship, and mast cells may serve to deliver GnRH to medial habenula (Silverman et al., 1994; King and Millar, 1997).

Several groups have demonstrated the presence of GnRH in lymphocytes and a recent study by Chen *et al.* identified both GnRH I and GnRH II in normal T-cells and in Jurkat cells (a human T cell leukkemia cell line). These results suggest that the addition of exogenous GnRH I or II may stimulate chemotaxis and the entry of T-lymphoma cells into normal tissue (Chen et al., 2002a).

1.8.7 GnRH and cell proliferation

GnRH has been implicated in regulating the proliferation of cancer cell lines. For example, the proliferation of Jurkat cells is stimulated by mGnRH (Azad et al., 1997). On the other hand, the proliferation of several ovarian, endometrial and breast cancer cell lines is inhibited by mGnRH and GnRH II (Emons et al., 2000; Choi et al., 2001; Grundker et al., 2001; Grundker et al., 2002). The antiproliferative effect of GnRH is thought to be mediated through coupling of the GnRH receptor to the G-protein $G\alpha_i$, as opposed to the coupling to $G\alpha_{q/11}$ in pituitary gonadotrope cells. In the case of inhibition of proliferation, the mechanism is by inhibiting the

mitogenic effect of epithelial growth factor (EGF) through the induction of a phosphatase that dephosphorylates the EGF receptor (Grundker et al., 2001).

In conclusion, GnRH plays an essential role in vertebrate reproduction as the primary regulator of gonadotropin release from the pituitary. In addition, several other roles of GnRH have been established, as discussed above. GnRH in the primitive protochordate is found in neurons close to the gonads and may stimulate the gonads through GnRH released directly into adjacent blood vessels (Powell et al., 1996). This function may represent an early-evolved function of GnRH, predating evolution of the pituitary. The occurrence of GnRH in gonads of various vertebrates including humans and its function in regulating hormone production may reflect this early-evolved function of GnRH (Millar, 2001). The neuronal expression of GnRH is conserved in its role as the regulator of pituitary release of gonadotropins and also as a neurotransmitter in sympathetic ganglia, and possibly as a regulator of reproductive behaviour. In addition, GnRH has been co-opted for a role in the immune system and as a regulator of hormone secretion in the placenta.

1.9 General characteristics of G protein-coupled receptors

After release from the hypothalamus, GnRH travels via specialised portal vessels to the anterior pituitary where it binds to a cognate receptor on the cell surface of gonadotrope cells. This GnRH receptor belongs to the class of receptors containing seven transmembrane domains, ie, the G protein-coupled receptors (GPCR).

GPCRs comprise a large superfamily of receptors of about 800 members. The GPCRs can be divided into three major families based on sequence homology. Family A, the rhodopsin-like receptors, is the largest GPCR group and it contains the rhodopsin receptor, the β adrenergic receptor and related receptors including the GnRH receptors. Family B consists of the glucagon-like receptors and family C consists of the metabotropic glutamate receptors. A possible new family of GPCRs, membrane progesterone receptors, has recently been identified (Zhu et al., 2003).

GPCRs and their heterotrimeric G proteins are an important means for cells to respond to extracellular stimuli including light, neurotransmitters, odorants and hormones (Strader et al., 1995; Gether and Kobilka, 1998; Ji et al., 1998; reviewed in Gether, 2000; Shenker, 2001). Although GPCRs respond to ligands with very diverse chemical properties, several common features of GPCRs have been noted. GPCRs have seven α-helical transmembrane domains (TM) connected by alternating extracellular loops (ECL 1-3) and intracellular loops (ICL 1-3), an extracellular N-terminal domain and an intracellular C-terminal domain. Two conserved cysteine residues in ECL 2 and 3 that are involved in disulfide bridge formation which keeps ECL 2 and ECL 3 in proximity to each other, are conserved in GPCRs (Gether, 2000). The N-terminal domain is glycosylated to varying degrees in GPCRs and may be necessary for maintaining normal receptor expression levels. The intracellular loops and the cytoplasmic tail are important for interaction with G-proteins, and phosphorylation of serine and threonine residues is involved in the desensitisation of GPCRs (Shenker, 2001).

The TMs are more conserved than the extracellular and intracellular domains, but the amino acid homology is low between receptors from different GPCR families. A conserved motif in family A receptors is an aspartate in TM 2 and asparagine in TM 7. These two residues are in proximity to each other, and are necessary for the stabilisation of receptor conformation. Recent data from the crystal structure of rhodopsin indicates that the aspartate residue in TM 2 and asparagines residue in TM 7 might interact through a water molecule, which also mediates interaction of the aspartate in TM 2 with a glycine in TM 3 (Palczewski et al., 2000). A highly conserved motif in family A GPCRs is the (D/E)RY motif at the cytoplasmic end of TM 3 (Gether, 2000). This motif is involved in receptor activation. It has been proposed that ligand binding changes the conformation of the receptor so that the acidic residue (D/E) becomes available for protonation when the receptor is in the active conformation. The aspartate residue may form an ionic bond with the neighbouring arginine residue when the receptor is in the inactive conformation. This ionic bond is disrupted upon protonation of the acidic residue and the arginine residue becomes available to form other interactions. This interaction may be an ionic bond with the conserved aspartate residue in either TM 2 for most family A GPCRs or in TM 7 for the GnRH receptor (Ballesteros et al., 1998).

The seven hydrophobic α-helical TMs form a bundle in the plasma membrane (Palczewski et al., 2000). The current models for ligand binding to GPCRs suggest that small ligands like the biogenic amines bind to a pocket formed by the seven TMs, whereas peptide ligands bind to the extracellular domains and to the TMs. Large glycoprotein hormones and the neurotransmitters bind to the large extracellular domains of their cognate receptors (Ji et al., 1998; Gether, 2000; Shenker, 2001). Binding of the ligand leads to activation of the receptor. A model called the extended ternary complex model has been proposed to describe activation of the receptor. According to this model, the receptor is in equilibrium between two conformational states: the inactive state R and the active state R*. The receptor conformation spontaneously isomerises and the proportion of receptor in R conformation would account for the basal activity of the receptor in the absence of ligand. The activities of ligands reflect their ability to shift this equilibrium. The proportion of receptors in the R* conformation can be increased either by agonists,

which bind preferentially to the active R* conformation, or by mutations stabilising the R* conformation. Antagonists would inhibit agonists from shifting the equilibrium towards the R* conformation and inverse agonists bind preferentially to the R conformation, thereby lowering basal activity of the receptor (De Lean et al., 1980; Samama et al., 1993; Gether and Kobilka, 1998).

Activation of the receptor leads to the binding of G proteins to the receptor. The heterotrimeric G proteins (Gα, Gβ and Gγ) are classified into four major families according to the amino acid homology of their α subunits: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$. The Gα subunits have different effectors, for example: $G\alpha_s$ stimulates adenylyl cyclase, $G\alpha_i$ inhibits adenylyl cyclase, $G\alpha_q$ stimulates phospholipase Cβ and $G\alpha_{12}$ stimulates RhoGEF (Shenker, 2001; Neves et al., 2002). G proteins in their inactive state are in their trimeric form and bind GDP to the α subunit. Interaction with the activated receptor promotes the exchange of GDP for GTP, followed by dissociation of the Gα from the Gβγ-dimer and activation of effectors by Gα, and in some cases Gβγ. Activation of effectors by Gα is terminated upon hydrolysis of GTP to GDP by the intrinsic GTPase activity of Gα, followed by reassociation of the three G protein subunits.

Prolonged exposure to agonist leads to desensitisation of GPCRs. The desensitisation involves phosphorylation of one or several serine or threonine residues in the intracellular loops or in the C-terminal tails by either protein kinase A (PKA) or by PKC or by G protein-coupled receptor kinases (GRKs) (Shenker, 2001). Subsequently, as shown for many GPCRs, binding of adaptor proteins of the arrestin family to the phosphorylated receptor sterically inhibits binding of G proteins to the receptor, thereby attenuating the signalling. Binding of arrestin also stimulates binding of proteins involved in internalisation of the receptor. Internalised receptor vesicles either fuse with lysosomes and the receptor is degraded or the receptor is dephosphorylated and recycled to the plasma membrane (Shenker, 2001).

1.10 GnRH receptor structure

The GnRH receptor belongs to the rhodopsin/β-adrenergic receptor subfamily of GPCRs. The first GnRH receptor to be cloned was the mouse receptor, cloned from a mouse gonadotrope cell line (Reinhart et al., 1992; Tsutsumi et al., 1992). The cloning of the mouse GnRH receptor was rapidly followed by cloning of GnRH receptors from the rat (Eidne et al., 1992; Kaiser et al., 1992), human (Kakar et al., 1992; Chi et al., 1993), sheep (Brooks et al., 1993; Illing et al., 1993), cow (Kakar et al., 1993) and pig (Weesner and Matteri, 1994). To date, over 30 GnRH receptors have been identified from species of fish, amphibia, birds and mammals (Millar et al. in press) (Bogerd et al., 2002). Two or three GnRH receptors have been identified in several species of fish, amphibia and mammals. The first identified GnRH receptor in mammals is referred to as the type I GnRH receptor and this receptor is expressed in pituitary and binds GnRH I. The recently identified second receptor is referred to as type II GnRH receptor. Type II GnRH receptors have been identified in amphibian and three species of monkeys, whereas rodents only have a type I GnRH receptor (Millar et al., 2001; Neill et al., 2001; Millar, 2003a). Type III GnRH receptors have been identified in species of fish and amphibia (Millar et al. in press).

The majority of studies on the structure-function of GnRH receptors have been on mouse and human type I GnRH receptors. The human and rodent type I GnRH receptors are comprised of 328 and 327 amino acids respectively. They possess the expected overall structure of seven transmembrane domain GPCRs, with the notable absence of a C-terminal tail. Type II GnRH receptors and non-mammalian type I GnRH receptors, like all other GPCRs, do have a C-terminal tail. Residues in the tail are important for desensitisation and internalistion (Ronacher et al. unpublished Pawson et al., 2003). Ovulation in mammals requires a protracted LH surge. The mammalian type I GnRH receptors may have lost their tails during evolution in order to sustain a protracted LH surge (King and Millar, 1997).

The human and rat type I GnRH receptors have one cysteine residue in the N-terminal domain, one cysteine in ECL 1 and two cysteines in ECL 2. Incubation of

the rat receptor with the cystine-reducing agent dithiothreitol (DTT) inhibits the binding of ligand, indicating the presence and functional requirement of a disulfide bridge in the receptor (Cook and Eidne, 1997). Mutating Cys^{114} or Cys^{195} abolishes binding, indicating that these two residues form a disulfide bridge between extracellular loops 1 and 2 of the rat receptor, a conserved feature in GPCRs. Single mutation of Cys^{14} or Cys^{199} results in decreased binding, but does not abolish binding. The authors concluded that these two residues do not form a disulfide bridge important for ligand binding in the rat GnRH receptor (Cook and Eidne, 1997).

The human type I GnRH receptor has four cysteine residues in similar positions to the rat receptor. Studies with photoaffinity cross-linking of a radiolabelled ligand to the receptor followed by proteinase digestion in the presence or absence of DTT indicate the presence of a disulfide bridge between residues Cys^{114} and Cys^{196}. This disulfide bridge is between ECL 1 and ECL 2 in a position similar to the rat GnRH receptor and other GPCRs. In addition, mutational analysis has indicated the presence of a disulfide bridge between Cys^{14} and Cys^{200} of the human type I GnRH receptor (Davidson et al., 1997). The monkey type II GnRH receptors only contain two cysteine residues, one in each of ECL 1 and ECL2, corresponding to the conserved disulfide bridge in GPCRs (Millar et al., 2001; Neill et al., 2001; Millar, 2003a).

The mouse GnRH receptor contains three putative N-glycosylation sites in the extracellular domains of which Asn^4 and Asn^{18} are glycosylated, but Asn^{102} is not glycosylated. Mutation of either of these two glycosylation sites does not affect ligand binding but decreases expression of the receptor (Davidson et al., 1995). The human type I GnRH receptor contains two putative N-glycosylation sites at Asn^{18} and Asn^{102}. Mutagenesis has indicated that only Asn^{18} is glycosylated. Interestingly, incorporation of an additional glycosylation site in the human receptor at the position equivalent to the Asn^4 of the mouse receptor increases expression of the human GnRH receptor (Davidson et al., 1996a). The primate type II GnRH receptors contain a putative N-glycosylation site at Asn^5 (Millar et al., 2001; Neill et al., 2001; Millar, 2003a).

Ligand binding to type I GnRH receptors involves several residues in the TMs and ECLs. The mammalian type I GnRH receptors show selectivity in binding towards GnRH peptides with arginine at position 8. It has been postulated that this arginine residue interacts with an acidic residue in the extracellular domains of the receptor. Mutational analysis has identified a glutamate at position 301 in the mouse receptor and the equivalent Asp^{302} in the human type I GnRH receptor, which confer specificity of these receptors for Arg^8 containing GnRH. Arg^8 of the peptide and the Glu^{301} or Asp^{302} in ECL 3 of the GnRH receptor may interact transiently to stabilise the peptide in the optimal conformation for binding in its final binding pocket (Flanagan et al., 1994; Fromme et al., 2001). The motif containing Glu^{301} or Asp^{302} in ECL 3 of mammalian type I GnRH receptors is replaced with Val-Pro-Pro-Ser in type II GnRH receptors. This VPPS motif is highly conserved in type II GnRH receptors from amphibia to primates and might confer selectivity of type II GnRH receptors for GnRH II (Millar, 2003a; Millar, 2003b).

Molecular modelling of the murine GnRH receptor has predicted Lys^{121} to be situated in the ligand binding pocket and mutating this residue to Gln reduces the affinity of GnRH for the receptor 100-fold. The binding of antagonists is not affected, indicating a difference in ligand binding sites of agonists and antagonists (Zhou et al., 1995). Asn^{102} at the extracellular surface of TM 2 appears to be involved in ligand binding. Mutation of this residue to alanine has led to a larger decrease in the potency of analogues with a C-terminal glycinamide compared to analogues with an ethylamide. Asn^{102} is thought to form a hydrogen bond with the amide moiety at the C-terminus of GnRH (Davidson et al., 1996b). Asp^{98} at the extracellular surface of TM 2 is also involved in ligand binding. Mutation of this residue leads to a large decrease in second messenger production. Studies with a library of GnRH analogues with substitutions at various positions indicated that Asp^{98} interacts with His^2 of GnRH. It has been proposed that the oxygen of the Asp^{98} carboxyl group forms a hydrogen bond with the –NH group of the His^2 side chain. Molecular modelling also indicates a hydrogen bond between Asp^{98} and the peptide backbone of GnRH at position 3. In addition, an ionic interaction of Asp^{98} with Lys^{121} was suggested by molecular modelling (Flanagan et al., 2000). The amino acids Asp^{98}, Asn^{102} and Lys^{121} of the type I GnRH receptors (Asp^{97}, Asn^{101} and Lys^{120} in humans) are conserved in primate and amphibian type II GnRH receptors and may serve the

same role in these receptors (Millar et al., 2001; Neill et al., 2001; Wang et al., 2001a; Millar, 2003a;).

The conserved DRY motif at the intracellular end of TM 3 is replaced by DRS in most mammalian type I GnRH receptors and DRQ in type II GnRH receptors. Mutating the Ser^{140} has no effect on expression or signalling of the mouse receptor (Arora et al., 1995; Arora et al., 1997). Mutation of the acidic Asp^{138} to Asn leads to an increase in receptor activation, whereas mutating Arg^{139} leads to a decrease in receptor activation (Arora et al., 1997; Ballesteros et al., 1998). It has been proposed that the positively-charged Arg^{139} interacts with the neighbouring negatively-charged Asp^{138} to stabilise the inactive conformation. Interrupting this interaction by mutation to the uncharged Asn or by protonation of the aspartate residue in the wild-type receptor upon activation, is thought to free the Arg^{139} side chain to interact with another acidic residue in the TMs of receptor. This residue is proposed to be Asp^{319} in TM 7. Asp^{319} in TM 7 and Asn^{87} in TM 2 are uniquely found in mammalian type I GnRH receptors.

In other GPCRs, the Asp is in TM 2 and the Asn in TM 7, indicating that the location of the Asp and Asn residues are interchanged in the mammalian type I GnRH receptors. The drosophila GnRH receptor homologue has the usual, arrangement of Asp in TM 2 and the Asn in TM 7. The non-mammalian type I GnRH receptors have Asp in both TM 2 and TM 7 and might represent an evolutionary intermediate between the drosophila receptor and the mammalian type I GnRH receptors (Zhou et al., 1994; Flanagan et al., 1997; Flanagan et al., 1999; Gether, 2000).

It has been proposed that Arg^{139} interacts with Asp^{319} and Asn^{87} when the receptor is in the active conformation. Molecular modelling indicates that a similar active state interaction could also occur in other GPCRs which have the Asp in TM 2 and the Asn in TM 7 (Ballesteros et al., 1998). Interestingly, non-mammalian type I GnRH receptors and type II GnRH receptors have aspartate residues in both the TM 2 and TM 7 positions, indicating that the Asp/Asn motif found in most GPCRs was first mutated to Asp/Asp followed by mutation to Asn/Asp in mammalian type I GnRH receptors (Millar, 2003a).

Coupling of GPCRs to G proteins involves the ICLs and the C-terminal tail of GPCRs (Gether, 2000). The DRS motif at the intracellular surface of TM 3 of mammalian type I GnRH receptors is important for receptor activation and coupling to $G\alpha_{q/11}$, leading to an increase in second messenger production (Gether, 2000). An additional motif at the C-terminal end of ICL 3 is important for G protein coupling. Mutation of an alanine residue in ICL 3 has been shown to constitutively activate some GPCRs (Gether, 2000). Mutation of the equivalent alanine in the human type I GnRH receptor (Ala^{261}) does not lead to constitutive activation of the receptor. Mutation of this residue to an amino acid with a large side chain abolishes second messenger production whilst ligand-binding is retained, indicating that an amino acid with a small side chain is necessary at this position for efficient coupling to $G\alpha_{q/11}$ (Myburgh et al., 1998).

It has recently been shown that the GnRH receptor is also able to stimulate cAMP production through coupling to $G\alpha_s$ (Arora et al., 1998; Liu et al., 2002). Several GPCRs, which couple to $G\alpha_s$, contain a specific motif with basic amino acids in the intracellular loops or the C-terminal tail. Similar motifs containing basic amino acids are present in ICL 1 of the human type I GnRH receptor. Mutating Leu^{58} at the intracellular end of TM 1 or Leu^{73} in the basic motif of ICL 1 reduces cAMP production by 80% whereas IP_3 production is unaffected. In addition, mutation of Leu^{80} at the intracellular end of TM2 or Ser^{74} in the basic motif of ICL 1 abolishes cAMP production whilst IP_3 production is increased. This shows that the coupling to $G\alpha_s$ and $G\alpha_{q/11}$ occurs through distinct domains of the receptor. The main function of the GnRH receptor in the gonadotrope cell is attributed to its coupling to $G\alpha_{q/11}$.

1.11 Intracellular signalling of GnRH receptors

Ligand binding and activation of the type I GnRH receptor stimulates several intracellular transduction pathways leading to the synthesis and release of LH and FSH (reviewed in Kiesel, 1993; Stojilkovic et al., 1994; Kraus et al., 2001; Millar, 2001). Activation of the type I GnRH receptor in the gonodotrope cell leads to binding and activation of G proteins of primarily the $G\alpha_{q/11}$ family. Binding of the

heterotrimeric G protein to the activated receptor stimulates the exchange of GDP for GTP and induces the dissociation of Gα and Gβγ subunits. $G\alpha_{q/11}$ activates PLCβ in the plasma membrane. The activated PLCβ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Fig 1.2). IP_3, binding to IP_3 receptors in the endoplasmic reticulum (ER), stimulates the release of Ca^{2+} to the cytosol. GnRH stimulation of gonadotrope cells is associated with an initial spike in Ca^{2+} concentration followed by a plateau. The spike in Ca^{2+} concentration is due to the release of Ca^{2+} from the ER. A subsequent activation of L-type voltage-gated Ca^{2+} channels results in the influx of extracellular Ca^{2+}.

Activation of the type I GnRH receptor leads to a biphasic increase in the concentration of DAG. The initial increase in DAG concentration is due to hydrolysis of PIP_2 through a direct stimulation of PLCβ by $G\alpha_{q/11}$. The later increase in DAG concentration is due to an indirect stimulation of phospholipase D (PLD) and hydrolysis of phosphatidylcholine (Millar, 2001). GnRH stimulation of the gonadotrope cell induces phospholipase A_2 (PLA_2) activity, which leads to an increase in the concentration of arachidonic acid (AA) and leukotrienes (Kiesel, 1993). Coupling of the GnRH receptor to $G\alpha_s$ stimulates cAMP production and activation of PKA. The role of PKA in the downstream signalling leading to LH and FSH synthesis remains to be elucidated (reviewed in Kiesel, 1993; Stojilkovic et al., 1994; Millar, 2001).

The increase in DAG leads to the activation of Ca^{2+}-dependent forms of PKC (Fig 1.2). Arachidonic acid and leukotrienes may also stimulate PKC. PKC is the central mediator of GnRH signalling events leading to the synthesis and release of LH and FSH. MAPK pathways are involved in the intracellular signalling following GnRH stimulation in gonadotropes. PKC and Ca^{2+} are important for activation of both the ERK and JNK pathways in gonadotrope cells. The ERK and JNK pathways are both involved in the transcriptional regulation of the GnRH receptor and of the gonadotropin subunits (Kraus et al., 2001).

The type II GnRH receptors also couple to G proteins of the $G\alpha_{q/11}$ family. Evidence for this comes from the increase in IP_3 production upon ligand stimulation. Ligand stimulation of the type II GnRH receptor has been shown to activate the ERK and p38 MAPK pathways in COS cells transfected with the receptor (Millar et al., 2001; Neill et al., 2001; Wang et al., 2001a; Neill, 2002; Millar, 2003a;).

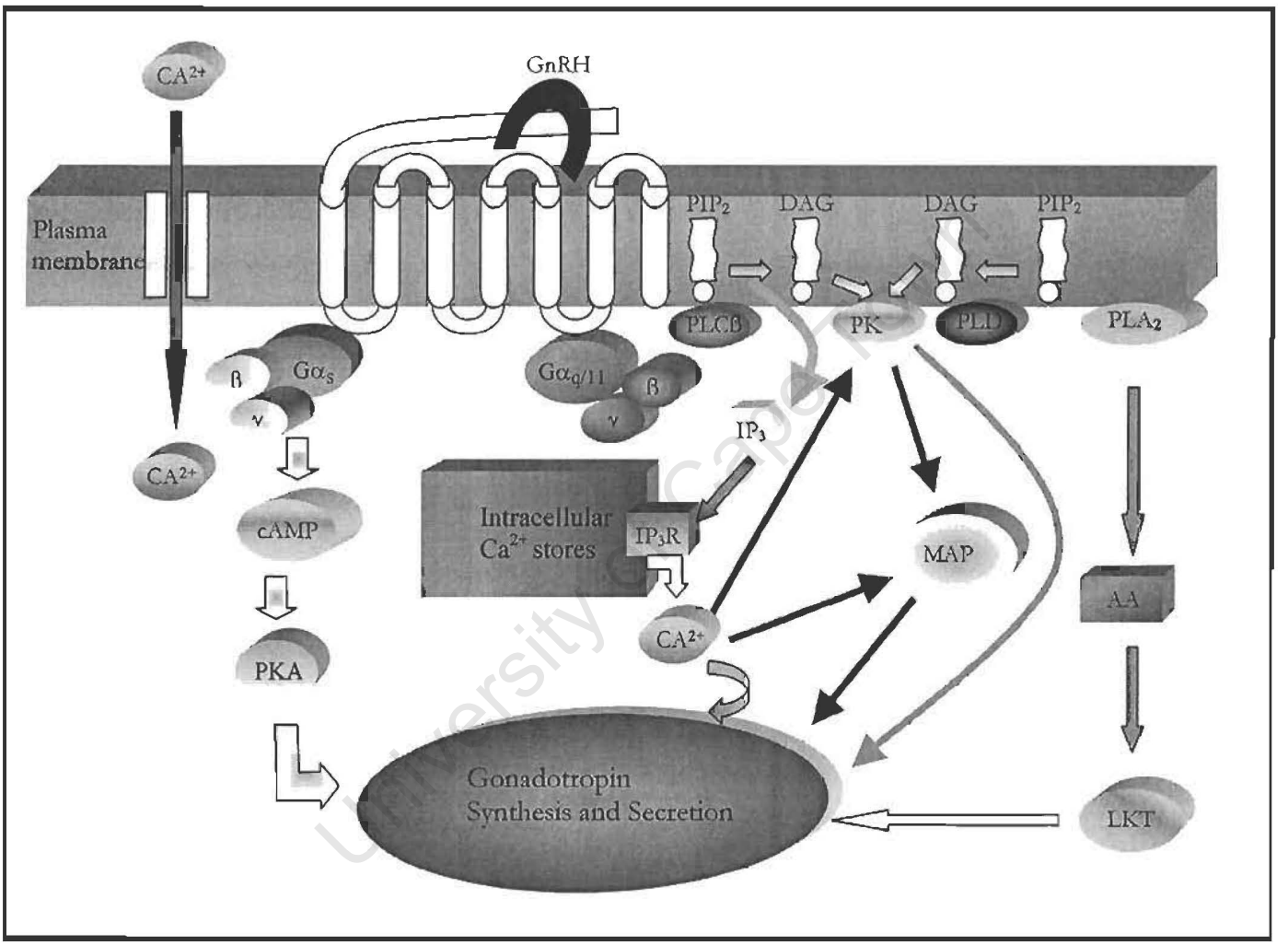

Figure 1.2. Intracellular signalling of the mammalian type I GnRH receptor. The abbreviations used are: PLCβ, phospholipase C β; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PLD, phospholipase D; PLA, phospholipase A; PKC, protein kinase C; IP_3R, IP_3 receptor; MAPK, mitogen-activated protein kinase; AA, arachidonic acid; LKT, leukotrienes; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A. (This figure is adapted from Millar et al. 2003b).

1.12 Desensitisation and internalisation of GnRH receptors

Many GPCRs desensitise rapidly upon ligand stimulation. However, the mammalian type I GnRH receptors, which uniquely lack a C-terminal tail, do not desensitise rapidly. A study by Eidne et al. has shown that the catfish GnRH receptor, which does have a tail, does desensitise rapidly. A chimaera of the rat GnRH receptor with the C-terminal tail of catfish GnRH receptor desensitises rapidly. Phosphorylation experiments have shown that the catfish GnRH receptor is phosphorylated upon ligand stimulation, whereas the rat GnRH receptor is not phosphorylated. A chimaera of the rat GnRH receptor with the C-terminal tail of the catfish GnRH receptor is phosphorylated. These results indicate that the presence or absence of a C-terminal tail determines the ability of GnRH receptors to be phosphorylated upon ligand stimulation. Furthermore, the presence of a C-terminal tail determines the ability of GnRH receptors to desensitise rapidly (Heding et al., 1998; Willars et al., 1999).

The C-terminal tail is also important for rapid internalisation. Incorporation of a stop codon in the beginning of the C-terminal tail of the chicken GnRH receptor or the catfish GnRH receptor leads to a decrease in internalisation (Pawson et al., 1998; Blomenrohr et al., 1999).

All type II GnRH receptors cloned to date have C-terminal tails and the marmoset type II GnRH receptor internalises rapidly upon ligand stimulation (Ronacher *et al.* unpublished).

1.13 Gene structure and evolution of GnRH receptors

The gene structure of mammalian type I GnRH receptors is conserved. The genes consist of three exons and two introns. Intron 1 is situated in the C-terminal end of TM 4 and intron 2 is situated in ICL 3 (Fan et al., 1994; Zhou and Sealfon, 1994; Fan et al., 1995; Kakar, 1997). The presence of introns in TM 4 and ICL 3 is conserved in species from different vertebrate classes. The GnRH receptors from

the medaka, Xenopus laevis, bullfrog and chicken have introns at the same positions as in mammalian GnRH receptors (Troskie et al., 2000; Okubo et al., 2001; Sun et al., 2001; Wang et al., 2001b). Interestingly, some of the non-mammalian GnRH receptors have an additional intron. This intron is situated in the N-terminal domain of the receptor in the medaka and chicken and is situated in the 5' UTR in Xenopus laevis GnRH receptor (Troskie et al., 2000; Okubo et al., 2001; Sun et al., 2001). The drosophila GnRH receptor homologue contains 6 introns. One of these introns, the intron in TM 4, is conserved in position compared to other GnRH receptors (Hauser et al., 1998).

The conservation of gene structure of GnRH receptors and the sequence homology of the receptors suggest a common ancestral origin of the GnRH receptor genes. The presence of a GnRH receptor homologue in an insect suggests that the ancestral GnRH receptor gene predates the separation of vertebrates and invertebrates. The occurrence of three GnRH receptors in a species of amphibia suggests that at least two duplications of an ancestral gene have occurred. An early duplication may have given rise to type I and type II GnRH receptors, and a later duplication may have given rise to the three GnRH receptors occurring in amphibians (Troskie et al., 1998; Okubo et al., 2001; Bogerd et al., 2002).

1.14 Transcriptional regulation of type I GnRH receptor expression

1.14.1 Tissue expression of the type I GnRH receptor

Type I GnRH receptors are expressed mainly in the pituitary. The receptor is expressed in gonadotrope cells in the pituitary in vertebrates and is also expressed in somatotrophs in fish. In addition, the receptor is expressed in several areas of the brain of mammals including GnRH-producing neurons of the hypothalamus. Gonadal expression of the type I GnRH receptor has been established in leydig cells of the testis, in granulosa cells and in luteal cells of the ovary. GnRH receptor mRNA has also been identified in peripheral tissues such as heart, skeletal muscle, kidney and liver and in several neoplastic tissues including breast tumours, breast

tumour cell lines, ovarian carcinomas and prostate tumours (Stojilkovic, Reinhart et al. 1994; Kakar and Jennes 1995).

The number of GnRH receptors in the pituitary changes during the estrous cycle. The concentration of GnRH receptors can be modulated by the pulse frequency of GnRH. The highest receptor concentration occurs at the time of ovulation and coincides with an increase in GnRH pulse frequency. The GnRH receptor number on gonadotrope cells is also regulated by gonadal hormones including oestrogen and activin (Stojilkovic et al., 1994; Norwitz et al., 1999b).

The change in receptor number is at least partially due to a change in the mRNA level. Cloning of the cDNAs and genes of the GnRH receptor from the mouse, rat and human has enabled characterisation of the 5' flanking regions.

The mouse GnRH receptor gene is highly expressed in the αT3-1 cell line and to a lesser extent in other cell lines (Albarracin et al., 1994). *In vivo* studies have identified the tissue-specificity of the mouse GnRH receptor promoter. Transgenic mice that harbour 1.9 kb of the mouse GnRH receptor promoter fused to a luciferase reporter gene have been created. The GnRH receptor promoter construct is expressed in pituitary, brain, testis and ovary of transgenic mice but not in kidney, spleen, pancreas, lung, heart or liver. Expression in the pituitary is 10- and 100-fold higher than expression in the brain or testis respectively (McCue et al., 1997; Ellsworth et al., 2003).

The human wild-type GnRH receptor promoter, comprising 2.2 kb of the 5' flanking region, shows only a 3-fold induction in expression in the gonadotrope cell line αT3-1 compared to promoterless reporter (Fan et al., 1995; Kakar, 1997; Ngan et al., 1999; Hoo et al., 2003). 5' and 3' deletion analysis have identified a 120 bp region, 558-677 bp upstream from the translational start codon, which gives a 13-fold induction in expression (Hoo et al., 2003).

1.14.2 Cis-elements and trans-factors involved in the regulation of transcription of the GnRH receptor

The major transcriptional start site (TSS) of the mouse GnRH receptor gene is situated 62 nucleotides upstream from the translational start codon. There is no TATA consensus sequence 30 nucleotides upstream from the TSS. Instead, there is a TATA-like element that may serve the function of a TATA box (Albarracin et al., 1994).

Eighteen transcriptional start sites and several TATA elements have been identified within the 2.2 kb human type I GnRH receptor promoter. The TSSs are situated in two regions of the promoter and the furthest upstream TSS is 1.8 kb upstream from the translational start codon (Fan et al., 1995; Kakar, 1997). There are two TSSs but no TATA elements in the 120 bp region which gives a 15-fold induction of expression in αT3-1 cells. Instead, there are two initiator elements with the consensus sequence YYAN(A/T)YY which may interact with the transcription initiation complex of transcription factors and RNA polymerase II (Hoo et al., 2003).

The transcriptional regulation of the mouse GnRH receptor gene involves several cis-elements that function as binding sites for transcription factors. A tripartite basal enhancer, situated 237-391 bp upstream from the ATG translational start site, consists of binding sites for steroidogenic factor-1 (SF-1), activator protein 1 (AP-1) and a novel element called GnRH receptor activating sequence (GRAS) (Figure 1.3) (Duval et al., 1997a; Duval et al., 1997b). The GRAS element contains a SMAD binding element (SBE) to which transcription factors of the SMAD family bind (Norwitz et al., 2002b). An additional AP-1 site is situated within the GRAS element (Norwitz et al., 2002b). Electrophorectic mobility supershift assays applying specific antibodies have identified binding of the orphan nuclear receptor SF-1 to the SF-1 binding site, binding of SMAD 4 to the SBE and binding of proteins of the Jun and Fos families of transcription factors to the AP-1 sites (Duval et al., 1997a; Norwitz et al., 1999a; White et al., 1999; Norwitz et al., 2002b; Ellsworth et al., 2003). A cAMP response element (CRE) to which CRE binding protein (CREB) binds and a cis-element called sequence underlying responsiveness to GnRH (SURG-1) have also been identified in the mouse GnRH receptor gene promoter(Figure 1.3) (Maya-Nunez and Conn, 1999; Norwitz et al., 1999a; Maya-Nunez and Conn, 2001).

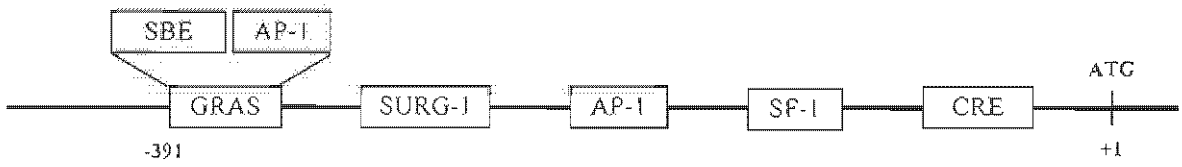

Figure 1.3. Schematic representation of the mouse GnRH receptor promoter showing cis-elements involved in the regulation of transcription. GRAS, GnRH receptor activating sequence; SBE, SMAD binding element; SURG-1, sequence underlying responsiveness to GnRH; AP-1, activating protein-1 binding site; SF-1, steroidogenic factor-1 binding site; CRE, cAMP response element. This figure is not drawn to scale.

The rat GnRH receptor promoter contains SF-1, AP-1 and GRAS cis-elements at positions equivalent to the mouse promoter and it contains an imperfect CRE element to which CREB binds *in vitro*. In addition, the rat GnRH receptor promoter contains a distal enhancer which binds transcription factors of the GATA- and LIM-families of transcription factors *in vitro* (Pincas et al., 2001a; Pincas et al., 2001b).

Most of the studies on the regulation of transcription of the GnRH receptor have been done on the mouse GnRH receptor promoter. However, a couple of studies report on cis-elements involved in the transcriptional regulation of the human type I GnRH receptor. An octamer-consensus sequence in the promoter of the human type I GnRH receptor binds Oct-1, a member of the POU family of transcription factors (Cheng et al., 2002b). A progesterone response element (PRE) to which progesterone receptors bind and an SF-1 site are also involved in regulating human type I GnRH receptor gene expression (Ngan et al., 1999; Cheng et al., 2001a).

1.14.3 Basal and cell-specific transcription of the GnRH receptor

The tripartite enhancer of the mouse GnRH receptor promoter is important for basal transcription. Mutation of either of the AP-1, SF-1 or GRAS sites leads to a 60% decrease in promoter activity. The GRAS element, when cloned in front of a heterologous promoter, confers gonadotrope-specific expression to a reporter construct. This result indicates that GRAS is important in stimulating transcription in a cell-specific manner (Duval et al., 1997a; Duval et al., 1997b).

The tripartite basal enhancer is present in the rat GnRH receptor promoter but mutation of the GRAS element in the rat promoter does not decrease expression. The basal expression of the rat promoter is increased by the presence of a distal enhancer in addition to the proximal enhancer. The effect of this distal enhancer appears to be dependent on the SF-1 site in the proximal enhancer (Pincas et al., 2001a).

A distal enhancer has not been described in the human type I GnRH receptor promoter. Three SF-1 sites are present in the proximal area of the human promoter but only one of them is important for transcription. Mutation of this SF-1 site reduces basal expression in αT3-1 cells but not in other cell lines, which suggests that cell specificity is mediated by this SF-1 site (Ngan et al., 1999). Deletion analysis of the human type I GnRH receptor promoter has identified a silencer element containg an Oct-1 binding site. Oct-1 binds to this site and mutation of this site eliminates Oct-1 binding and eliminates down-regulation of expression. Over-expression of Oct-1 also decreases expression of the promoter (Cheng et al., 2002b).

Most studies of the transcriptional regulation of the type I GnRH receptor promoter have been done in αT3-1 cells. Leung *et al.* have studied human type I GnRH receptor promoter regulation in cell lines of ovarian and placental origin. The 5' flanking region of the human GnRH receptor gene contains three active promoter regions; a proximal promoter (Cheng et al., 2000b; Cheng et al., 2001a; Cheng and Leung, 2002; Hoo et al., 2003), a medial promoter (Kang et al., 2000; Ngan et al., 2001; Cheng et al., 2002a) and a distal promoter (Cheng et al., 2000a; Ngan et al., 2000; Cheng et al., 2001b). Leung *et al.* have suggested that the proximal promoter is specific for gonadotrope cells, the medial promoter is specific for ovarian cells and the distal promoter is specific for placental cells (Cheng et al., 2002a). However, the tissue-specificity of the three promoters is uncertain. The proximal promoter is expressed in αT3-1 cells but is also expressed in the placental JEG-3 cells (Cheng et al., 2001a). The medial promoter is highly expressed in ovarian cell lines but expression in placental cell lines has not been tested (Cheng et al., 2002a). The distal promoter is highly expressed in two placental cell lines but expression in three

ovarian cell lines, SVOG-4o, SVOG-4m and IOSE-29EC, has not been tested (Cheng et al., 2001b).

1.14.4 Hormonal regulation of the GnRH receptor gene expression

The regulation of gene expression of the GnRH receptor by GnRH has been investigated *in vitro* and *in vivo* by several researchers. Treatment of intact wild-type mice with the GnRH agonist triptorelin increases GnRH receptor expression in the pituitary. Treatment with an antagonist decreases basal expression of GnRH receptor by counteracting the activity of endogenous GnRH. Antagonist treatment also inhibits GnRH agonist-stimulated GnRH receptor gene expression (Kovacs and Schally, 2001). These results are confirmed by studies on transgenic mice harbouring 1.9 kb of the mouse promoter linked to a reporter gene. Treatment with a GnRH antibody or a GnRH antagonist decreases basal GnRH receptor expression in the pituitary. No effect is seen on GnRH receptor expression in the brain. Treatment with a GnRH agonist in addition to the non-cross-reacting GnRH antibody increases GnRH receptor expression compared to treatment with antibody only (McCue et al., 1997).

In vitro studies on GnRH-regulated GnRH receptor gene expression have mainly been performed in αT3-1 cells transfected with the mouse GnRH receptor promoter. The GnRH responsiveness of the promoter involves several cis-elements. The AP-1, GRAS (containing SBE and a second AP-1 element) and SURG-1 elements are all important for the regulation of GnRH receptor transcription by GnRH. SURG-1 is less important for GnRH responsiveness than the AP-1 site and GRAS.

Mutation of the AP-1 site leads to a loss of GnRH responsiveness of the promoter (Norwitz et al., 1999a; Norwitz et al., 1999b; White et al., 1999). The importance of this AP-1 site in GnRH responsiveness has been demonstrated both *in vitro* and *in vivo*. The GnRH receptor promoter shows decreased activity in the pituitary upon administration of a GnRH antibody to transgenic mice harbouring a GnRH receptor promoter reporter construct. No effect of the antibody is seen in mice, in which the AP-1 site is mutated. These results indicate that endogenous GnRH stimulates

GnRH receptor promoter activity and that this activity is mediated by the AP-1 site (Ellsworth et al., 2003). *In vitro* analysis has confirmed the loss of GnRH responsiveness when the AP-1 site is mutated (Norwitz et al., 1999a; White et al., 1999).

Stimulation of the promoter with GnRH increases binding of AP-1 subunits to the AP-1 site. GnRH stimulates the expression of c-Fos and the phosphorylation of JunD in αT3-1 cells, and studies using eletrophorectic mobility shift assays have shown that c-Fos, FosB and JunD bind to the AP-1 site (White et al., 1999; Ellsworth et al., 2003).

Mutational analysis of the GRAS element using serial 2 bp substitutions has identified two neighbouring regions of GRAS, to which AP-1 and SMAD bind. Mutants of the AP-1 and SBE elements of GRAS do not bind *in vitro*-translated FosB or SMAD4 and they do not inhibit binding of αT3-1 nuclear proteins to GRAS. The same 2 bp mutants which do not bind FosB and SMAD 4 do not show GnRH responsiveness. Hence, the GnRH responsiveness may in part be due to the binding of AP-1 and SMAD to the GRAS element (Norwitz et al., 2002b).

Phorbol 12-myristate 13-acetate (PMA), a stimulator of PKC, increases GnRH receptor promoter activity in αT3-1 cells. Both PMA-induced and GnRH-induced stimulation of the promoter is abolished by a PKC antagonist (Norwitz et al., 1999a; White et al., 1999). GnRH stimulates both ERK and JNK pathways in αT3-1 cells. Activation of the ERK pathway by a constitutively active Raf does not stimulate GnRH receptor promoter activity. However, co-expression with a dominant negative SEK-1, an intermediate in the JNK pathway, abolishes GnRH responsiveness of the promoter (Ellsworth et al., 2003). Although GnRH stimulates both ERK and JNK, it appears that the GnRH-induced expression of the mouse GnRH receptor promoter is mediated by JNK and not ERK.

Short-term treatment (4-6 hrs) with GnRH up-regulates the GnRH receptor promoter whereas prolonged treatment with GnRH leads to down-regulation of GnRH receptors in the pituitary. Transcriptional down-regulation of the human type I GnRH

receptor by prolonged GnRH treatment has been investigated in αT3-1 cells. Stimulating the human GnRH receptor promoter for 24 hrs with a GnRH agonist reduces expression by 71%. The down-regulation is mediated by PKC and involves c-Fos and c-Jun binding to an AP-1 binding site (Cheng et al., 2000b).

GnRH responsiveness of the mouse GnRH receptor promoter has been studied in the GGH_3 cell line in addition to αT3-1 cells. The GGH_3 cell line is a murine somatotroph cell line stably expressing the mouse GnRH receptor. The GnRH responsiveness in these cells appears to be partially mediated by a $G\alpha_S$-mediated increase in cAMP. The cAMP-induced expression is mediated through a CRE in the proximal promoter to which CREB binds (Lin and Conn, 1998; Lin and Conn, 1999; Maya-Nunez and Conn, 1999; Maya-Nunez and Conn, 2001). In contrast, forskolin, an activator of PKA, inhibits GnRH-induced expression of the mouse GnRH receptor in αT3-1 cells. Forskolin also inhibits GnRH-stimulated activation of JNK and inhibits GnRH-induced binding of AP-1 to the AP-1 binding element of GRAS (Ellsworth et al., 2003). The difference in response of the GnRH receptor promoter to GnRH may be a result of using different cell lines. The GGH_3 and αT3-1 cells are of somatolactotrope and gonadotrope origins respectively.

Transcription of the GnRH receptor gene is regulated by several factors in addition to GnRH. These factors include activin A, glucocorticoids, progesterone and pituitary adenylate cyclase-activating polypeptide (PACAP). Two groups of researches have found that activin stimulates GnRH receptor promoter activity. Activin treatment of αT3-1 cells transfected with the GnRH receptor promoter results in a 2.5-fold increase in transcriptional activity. Follistatin, an activin antagonist, abolishes the activin-stimulated increase in transcription and follistatin alone decreases the basal promoter activity (Fernandez-Vazquez et al., 1996; Duval et al., 1999). A third group of researchers has found that activin alone does not stimulate GnRH receptor promoter activity; it only augments GnRH-induced activity. This activity of activin is mediated by GRAS. Mutations of the GRAS element affect GnRH-induced and activin-augmented transcription. Mutations which eliminate binding of AP-1 and SMAD to GRAS abolish the activin responsiveness of the promoter (Duval et al., 1999; Norwitz et al., 2002a; Norwitz et al., 2002b).

The mouse GnRH receptor gene is regulated by glucocorticoids. Dexamethasone, a glucocorticoid agonist, stimulates an 80% increase in GnRH receptor promoter activity in GGH3 cells. The glucocorticoid responsiveness of the promoter is eliminated when the AP-1 binding site of the basal enhancer is mutated (Maya-Nunez and Conn, 2003).

Progesterone decreases human GnRH receptor promoter activity in αT3-1 cells. Interestingly, progesterone is necessary for basal GnRH receptor promoter activity in the placental JEG-3 cell line. Inhibition of endogenous progesterone with either a progesterone antagonist or with an inhibitor of progesterone synthesis decreases promoter activity in JEG-3 cells. Progesterone responsiveness is conferred by a progesterone response element in the human GnRH receptor promoter, to which progesterone receptors bind *in vitro*. Over-expression of progesterone receptors A and B (PR-A and PR-B) decreases promoter activity in αT3-1 cells. Over-expression of PR-A decreases promoter activity and over-expression of PR-B increases promoter activity in JEG-3 cells. Progesterone and PR-B have opposite effects on GnRH receptor promoter activity in αT3-1 cells vs JEG-3 cells (Cheng et al., 2001a).

PACAP stimulates glycoprotein hormone α-subunit synthesis in the pituitary and it weakly stimulates LH release *in vivo*. Recently, Pincas *et al* have shown that PACAP also stimulates the promoter activity of the rat GnRH receptor gene transfected in αT3-1 cells (Pincas et al., 2001b). This is consistent with the stimulation of the mouse GnRH receptor promoter by cAMP in GGH_3 cells (Maya-Nunez and Conn, 1999; Maya-Nunez and Conn, 2001) and in contrast to the inhibition of GnRH-stimulated mouse GnRH receptor promoter activity by forskolin in αT3-1 cells (Ellsworth et al., 2003). Two elements of the GnRH receptor promoter are necessary in order to confer PACAP responsiveness. These elements are the SF-1 site of the basal enhancer and an imperfect CRE element in the proximal area of the promoter. In addition, a short sequence stretch, immediately upstream from the SF-1 site, is necessary for PACAP responsiveness (Pincas et al., 2001b).

1.15 Concluding remarks

GnRH was first isolated three decades ago. Since then, the role of GnRH as the primary regulator of reproduction through the hypothalamic-pituitary-gonadal axis has been well established. GnRH stimulates the release of LH and FSH from the pituitary in fish, reptiles, birds, amphibians and mammals. In addition to its role in gonadotropin release, GnRH has a diversity of functions. Some of these functions are important in reproduction, for example, the role of GnRH in reproductive behaviour and the stimulation of hormone release in placenta and in gonads. Most species contain at least two forms of GnRH. GnRH I is the primary regulator of gonadotropin release, whereas the function of GnRH II is less clear.

The role of GnRH in gonadotropin release is mediated by the type I GnRH receptor in gonadotrope cells. GnRH receptors are members of the GPCR family of transmembrane receptors. Two types of GnRH receptors have been identified in species of fish, reptiles and mammals, whereas amphibians have three types of the GnRH receptor. The type II GnRH receptor has recently been identified in monkeys and a partial type II GnRH receptor has been described in humans.

1.16 Aims

The conservation of sequence of the GnRH II peptide in species ranging from fish to mammals suggests and important function for this peptide. GnRH II appears to affect reproductive behaviour in several species (Maney et al., 1997; Temple et al., 2003) and this effect might be due to the action of GnRH II as a neurotransmitter (Jones, 1987; Bosma et al., 1990). Recently, GnRH II was identified in humans. However, the function of GnRH II in humans has not yet been determined. The occurrence of GnRH II with one or two other forms of GnRH within the same species suggested that more than one GnRH receptor might exist. Indeed, type II GnRH receptors, with high affinity to GnRH II, have now been cloned from amphibians and primates. Transcripts with homology to a putative human type II GnRH receptor have been identified (Millar et al., 2001; Neill et al., 2001; van Biljon et al., 2002; Millar, 2003a; Morgan et al., 2003). These cDNAs all have a premature stop codon in exon 2 of the receptor gene and they don't have a methionine start codon.

This thesis describes the cloning of a cDNA for the human type II GnRH receptor as well as the expression pattern in various tissues. The gene structure and genomic organisation of the human and marmoset monkey type II GnRH receptors are determined and lastly, promoter elements of the marmoset type II GnRH receptor gene are characterised.

Chapter 2: The human type II GnRH receptor: cDNA, gene structure and genomic organisation

2.1 Summary

The type II GnRH peptide is structurally conserved in species across vertebrate classes from fish to man. Cognate type II gonadotropin-releasing hormone (GnRH) receptors have been cloned from frogs and from non-human primates and recently a partial clone of putative human type II GnRH receptor has been identified. The aim of this study was to clone a full-length human type II GnRH receptor cDNA.

In this study, cDNA of the human type II GnRH receptor has been found in a wide range of reproductive and non-reproductive tissues as well as in neural and non-neural tissues. The cDNA encodes a protein of 41% amino acid identity to the human type I GnRH receptor, 90% amino acid identity to the marmoset monkey type II GnRH receptor and 93% amino acid identity to both the African green monkey and rhesus monkey type II GnRH receptors. However, the human transcripts apparently lack a methionine initiation codon and have a premature stop codon in exon 2.

Two genomic loci, each encoding both the human type II GnRH receptor and RNA-binding motif protein-8 (RBM8A), have been cloned and characterised. In both loci the genes overlap and are orientated in a tail-to-tail manner. In addition, the type II GnRH receptor gene overlaps with peroxisome biogenesis factor 11B (Pex 11B) in a head-to-head manner. The locus on chromosome 1 contains the full-length type II GnRH receptor gene (*GNRHR2*) which is composed of 3 exons and 2 introns. This gene has the same structure as the human type I GnRH receptor gene (*GNRHR*). In the antisense orientation, this locus contains the RBM8A gene, which consists of 6 exons and 5 introns and directs the synthesis of a RBM8A protein of 173 or 174 amino acids by alternative splicing.

A second locus, located on chromosome 14, contains pseudogenes of RBM8 (*RBM8B*) and the type II GnRH receptor and probably originated from the locus on chromosome 1 by retrotransposition.

2.2 Introduction

GnRH plays a central role in regulation of reproduction through the hypothalamic-pituitary-gonadal axis. The GnRH decapeptide acts on GnRH receptors on gonadotrope cells in the pituitary, resulting in the release of FSH and LH, which in turn regulate sex steroid and gamete production in the gonads (Millar et al., 1987; Conn and Crowley, 1991; Conn and Crowley, 1994). This GnRH peptide, designated GnRH I, has many structural variants (Jimenez-Linan et al., 1997; King and Millar, 1997; Sealfon et al., 1997; Yoo et al., 2000; Carolsfeld et al., 2000; Okubo et al., 2000; Montaner et al., 2001). In addition, most vertebrates have a second form of GnRH, GnRH II. GnRH II, which was first identified in the chicken, is structurally conserved in species ranging from fish to humans (Sherwood et al., 1993; King and Millar, 1997; White et al., 1998).

The GnRH II peptide is found in extra-hypothalamic regions of the brain and in peripheral tissues, whereas the GnRH I peptide is found mainly in the hypothalamus. The brain regions with the highest concentration of GnRH II are midbrain and habenula. Cell bodies immunoreactive for GnRH II have been identified in the midbrain and these cell bodies have projections to the habenula, where GnRH II is found in presynaptic vesicles (Rissman et al., 1995; Lescheid et al., 1997). GnRH II is found in ovarian cells and breast tissue in humans. GnRH II is also found in kidney and in bone marrow and expression in these tissues is higher than in the brain (White et al., 1998; Choi et al., 2001; Kang et al., 2001; Chen et al., 2002b).

The structural conservation and the wide tissue distribution of GnRH II suggest that GnRH II may have an important function. There is some evidence pointing to a possible role of GnRH II in preferential FSH release in sheep. GnRH II is able to bind and activate type I GnRH receptors and the effect of GnRH II on gonadotropin release from the pituitary might be mediated by the type I GnRH receptor (Millar et al., 2001). GnRH II stimulates reproductive behaviour in birds and in mammals. Female song sparrows show increased courtship behaviour upon GnRH II

administration whereas GnRH I has no effect (Maney et al., 1997). Reproductive behaviour is also stimulated by GnRH II in a primitive mammal, the musk shrew (Temple et al., 2003). In addition, GnRH II has a role as a neurotransmitter. GnRH and GnRH agonists elicit a slow depolarisation of bullfrog sympathetic neurons. This depolarisation is primarily due to the inhibition by GnRH II of a specific voltage-dependent potassium current called the M-current (Jan et al., 1980; Jones, 1987; Troskie et al., 1997). It might be that GnRH II regulates reproductive behaviour through its action as a neurotransmitter.

The occurrence of more than one form of the GnRH ligand within a single species and the wide tissue expression of GnRH II suggest that there may also be more than one form of the GnRH receptor and that this putative type II GnRH receptor would mediate the functions of GnRH II. The discovery of GnRH II in mammals, including humans, has prompted the search for putative type II GnRH receptors. PCR has been performed on genomic DNA from different species with primers designed to the conserved areas of TM 6 and TM 7 of type I GnRH receptors in order to clone a putative cognate type II GnRH receptor for the GnRH II peptide. The employment of this approach has enabled the identification of ECL 3 of a type II receptor in a lizard (*Agama atra*) and in the African clawed frog *(Xenopus laevis*) (Troskie et al., 1998). Only recently have full-length type II GnRH receptor cDNAs been cloned from bullfrog (Wang et al., 2001a), *Xenopus laevis* (B. Blackman, A. Katz and R. Millar, unpublished), marmoset monkey (*Callithrix jacchus*) (Millar et al., 2001), rhesus monkey (*Macaca mulatta*) and African green monkey (*Cercopithecus aethiops*) (Neill et al., 2001).

A partial clone of a putative type II GnRH receptor in humans has been identified. The ECL 3 sequence of lizard and *Xenopus laevis* was used to query human EST databases. EST sequences, encoding ECL 2 until the end of the receptor, were identified and have a 42% amino acid identity with the human type I GnRH receptor. However, the EST sequences identified were all in the antisense orientation (Troskie et al., 1998; Millar et al., 1999). RT-PCR, performed on mRNA from different tissues, has shown a wide tissue distribution of mRNA transcripts of this gene; however, the intron between exons 2 and 3 is retained in all tissues which were examined. Subsequently, it was found that exons 2 and 3 of the human type II

GnRH receptor are part of the 3' untranslated region of the ribonucleoprotein RNA-binding motif protein-8 gene (*RBM8A*), which is transcribed in the opposite orientation to the receptor gene (Millar et al., 1999; Conklin et al., 2000).

RBM8A is a ribonucleoprotein and a member of a family of proteins characterised by having a RNA binding motif of approximately 90 amino acids (Burd and Dreyfuss, 1994). These proteins bind single-stranded RNA and function in post-transcriptional maturation and processing of heteronuclear RNA. Recently, three groups of researchers employing the yeast two-hybrid screen have also cloned RBM8A. The first group demonstrated that RBM8A interacts with the human homologue of the drosophila mago nashi protein (MAGOH), a protein involved in germ plasm development in drosophila (Zhao et al., 1998; Zhao et al., 2000). The second group showed that RBM8A associates with a putative tumour suppressor, ovarian cancer 1 (OVCA1) (Salicioni et al., 2000). The third group showed that RBM8A (alternative name: Y14) is the cargo of the nuclear transport receptor RanBP5 (Kataoka et al., 2000). Further studies show that RBM8A associates with spliced mRNA in the nucleus. RBM8A forms part of a multiprotein complex called exon-exon junction complex (EJC), which binds to mRNA upstream of mRNA splice sites and is thought to communicate the position of exon-exon junctions in spliced mRNA to the cytoplasm. The position of exon-exon junctions relative to stop codons is relevant for targeting transcripts with premature stop codons for down-regulation in the cytoplasm by a mechanism called none-sense mediated decay. (Le Hir et al., 2000; Kataoka et al., 2001; Kim et al., 2001a; Kim et al., 2001b; Le Hir et al., 2001).

Northern blot analysis has revealed that RBM8A is ubiquitously expressed and that the major transcripts are 0.9 kb, 3.2 kb and 5.5 kb in length. There appears to be polymorphism in the predicted amino acid length of RBM8A. Its length is reported to be 174 (Conklin et al., 2000; Kataoka et al., 2000) or 173 (Zhao et al., 2000) amino acids, while a third group of researchers reports on both lengths (Salicioni et al., 2000). The chromosomal assignment of RBM8A is also contradictory. Two groups of researchers report that RBM8A is located on chromosome 14 (Conklin et al., 2000; Salicioni et al., 2000) and one of these groups (Salicioni et al., 2000) has also suggested that chromosome 1 may contain a RBM8A pseudogene. However, Zhao et al. reports that the RBM8A is located on chromosome 1 (Zhao et al., 2000).

The aim of this study was to clone a full-length human type II GnRH receptor cDNA and to characterise fully the type II GnRH receptor and RBM8A genes in humans.

2.3 Materials and methods

2.3.1 RNA isolation

Total RNA was prepared from the following human tissues: placenta, pituitary, testis, foetal brain, foetal lung, adult brain, cerebellum, occipital lobe, thalamus, hypothalamus, frontal cortex, pons, prostate, uterus, lymph node, small intestine, spleen, kidney, retina, bone marrow, spinal cord, insulinoma and thyroid. In addition, RNA was isolated from the following cell lines: K562 (a human chronic myeloid leukemia cell line), Jurkat (a T-cell line), MCF-7 and T47D (breast cancer cell lines). RNA was isolated using Tri-Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA was quantified spectrophotometrically and visualised on a 0,8% agarose TAE gel. Poly(A)$^+$ RNA was isolated from 1-3 mg total RNA using the PolyATtract mRNA isolation system (Promega, Madison WI, USA) according to the manufacturer's specifications.

2.3.2 cDNA preparation

cDNA was made from poly(A)$^+$ RNA using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) with an antisense oligo 10071 (Fig 2.1 and Table 2.1) specific to the 3' UTR of the human type II GnRH receptor or an anchored oligo (dT) primer according to the manufacturer's protocol. In brief, 1 μg poly A$^+$ RNA was reverse transcribed for 1 hour at 42°C using MMLV reverse transcriptase. Second strand cDNA was synthesised for 1.5 hours at 16°C with a cocktail of *E.coli* DNA polymerase I, RNase H and *E.coli* DNA ligase, followed by filling in ends with T4 DNA polymerase and ligation of a double-stranded Marathon cDNA adaptor.

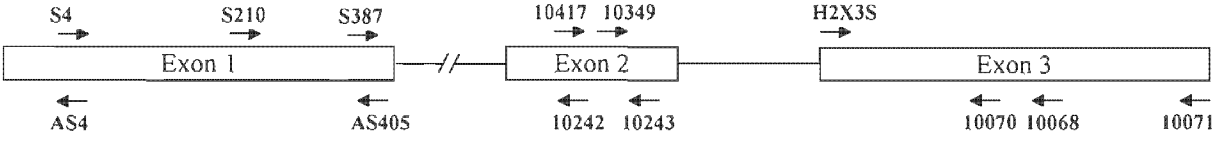

Figure 2.1. Schematic representation of the human type II GnRH receptor gene. The exons are indicate as boxes, the introns as lines and the primers used in this study are indicated by arrows.

Table 2.1. Oligonucleotides used for cloning the human type II GnRH receptor cDNA.

	Oligonucleotide sequence (5' – 3')
10071	GGAGAGCAGGAGTAGAAGTGAG
10417	GCAAGAGACCACCTATAACCT
10063	CTGACCTTCATCCTCTGCTGGACACC
10068	GAGCATTCTCCCAGACCCTTCTTTAG
10349	ATAACCTCTTCACCTTCTGCTGCCT
10070	GGTGTCCAGCAGAGGATGAAGGTCAG
T7	GTAATACGACTCACTATAGGG
U19	GTTTTCCCAGTCACGACGT
S210	ACCTGGAATATCACTGTTCAATGG
S387	CAGCCTGGGGACTTAGTTTCCTG
10243	GCAGAAGGAGGCAGCAGAAGG
10242	GGTTATAGGTGGTCTCTTGC
AP1	CCATCCTAATACGACTCACTATAGGGC
AP2	ACTCACTATAGGGCTCGAGCGGC
AS405	GAAACTAAGTCCCCAGGCTGC
AS4	GGCTGCTGCCGAGAAGGTGGG
S4	CCCACCTTCTCGGCAGCAGCC
H2X3S	GCACTGCAGGTGAATTTGCCC
ZRNP1A	TTCTGCTGCGTCTTCGGCCA
ZRNP1S	TGGCGGACGTGCTAGATCTTCA

2.3.3 PCR amplification

PCR amplification was performed between exon 2 and exon 3 on 5 µl of a 1:50 dilution of Marathon cDNA from placenta, pituitary, testis, foetal brain, adult brain, cerebellum, occipital pole, thalamus, pons, prostate, lymph node, small intestine, spleen, kidney and thyroid tissue and from Jurkat, MCF-7 and T47D cell lines, using sense primer 10417 and antisense primer 10068 (Fig 2.1). Each template was amplified with Taq polymerase (Promega, Madison WI, USA) for 35 cycles at (1 minute@ 94°C, 1 minute @ 60°C, 1 minute @ 72°C) followed by a 10 minutes extension at 72°C. 5µl of a 1:50 dilution of the PCR product from the first PCR was amplified for 30 cycles in a nested PCR using sense primer 10349 and antisense primer 10070 under the same cycling conditions as in the first PCR. PCR products were gel purified using USBioclean (Amersham Biosciences, Little Chalfont, UK), cloned in pMOS*blue* (Amersham Biosciences, Little Chalfont, UK) and sequenced with vector specific primers T7 and U19 using ABI Big Dye Terminator sequencing

kit (Applied Biosystems, Foster City, CA, USA). PCR amplification between exon 1 and exon 3 was performed on cDNA from pituitary, pons, frontal cortex, cerebellum, occipital pole, thyroid, hypothalamus, placenta, kidney, prostate and testis with sense primer S210 and antisense primer 10068. Nested PCR amplification of the exon 1/exon 3 PRC product was done between exon 1 and exon 2 using sense primer S387 and antisense primer 10243. The first PCR and the nested PCR reactions were done under the same conditions as the PCR between exons 2 and 3 and the PCR products were Southern blotted when necessary. Positive PCR products were cloned in pMOS*blue* and sequenced as described above.

2.3.4 Southern blot analysis of RT-PCR products

The PCR products were separated on a 1% agarose TAE gel. The DNA in the gel was denatured for 2 x 15 minutes (1.5M NaCl, 0.5M NaOH), neutralized 2 x 15 minutes (1.5M NaCl/0.5M Tris-HCl pH 7.0) and transferred overnight to a nylon membrane (Magnacharge, Osmonics, Westborough, MA, USA) using 20 x SSC. The membrane was rinsed in 2 x SSC and fixed by incubating for 20 minutes on Whatman paper soaked in 0.4M NaOH (Ausubel et al., 2001). The membrane was prehybridised for 45 minutes at 42°C, hybridised for 1hr 30 minutes at 42°C with an exon 2-specific oligo 10242 that had been labelled with the ECL 3'oligo labelling system according to the manufacturer's protocol (Amersham Biosciences, Little Chalfont, UK), followed by stringency washes, the final wash being 20 minutes in 1 x SSC, 0.1% SDS at 55°C.

2.3.5 5' Rapid amplification of cDNA ends (5' RACE)

5 µl of a 1:50 dilution of marathon cDNA from human pituitary, uterus, retina, bone marrow, spinal cord, foetal lung, foetal brain, insulinoma and K562, the chronic myeloid leukemia cell line, were PCR amplified. The PCR cycling parameters were: 2 minutes @ 94°C followed by 5 cycles (30 seconds @ 94°C, 30 seconds @ 65°C, 2 minutes @ 72°C) another 30 cycles (30 seconds @ 94°C, 30 seconds @ 63°C, 2 minutes @ 72°C) followed by a 5 minute extension at 72°C using the adaptor primer

AP1 and the gene-specific antisense primer AS405. 5 µl of a 1:50 dilution of the first reaction were used in PCR with a nested adaptor primer AP2 and nested gene-specific primer AS4 under the following cycling parameters: 2 minutes @ 94°C followed by 5 cycles (30 seconds @ 94°C, 30 seconds @ 65°C, 1 minute @ 72°C) and 30 cycles (30 seconds @ 94°C, 30 seconds @ 63°C, 1 minute @ 72°C) and 5 minute extension at 72°C. The PCR products were purified from an agarose gel using QiaQuick (Qiagen, Hilden, Germany), cloned into pMOS*blue* and sequenced with vector-specific primers T7 and U19 using DYEnamic ET Dye Terminator on a Megabace 500 automated sequencer (Amersham Biosciences, Little Chalfont, UK).

2.3.6 Sequencing and analysis of genomic P1 clones

Three P1 genomic clones (9792, 9793 and 9794) were previously identified by screening a human genomic P1 library (Genome Systems, St. Louis, MO, USA). The library was screened with a 415 bp probe, spanning exon 3 between primers 10063 and 10071, of the human type II GnRH receptor (Millar et al., 1999). The three genomic clones were subjected to Southern blot analysis. The clones were digested with restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII and *Spe*I, electrophoresed, transferred to a nylon membrane and fixed as described above. Probes for exon 1 and exon 3 of the human type II GnRH receptor and a probe to RBM8A were PCR amplified from genomic clone 9794 using the following pairs of primers: exon 1: S4 and AS405, exon 3: primers H2X3S and for RBM8A: primers ZRNP1A and ZRNP1S. Probes were random labelled (Prime-a-Gene, Promega, Madison WI, USA) with ^{32}P-dCTP (Amersham Biosciences, Little Chalfont, UK) and purified with NucleoTrap (Machery-Nagel, Düren, Germany) after addition of 1µg herring sperm DNA. Membranes were prehybridised for 2 hours at 42°C in 6 x SSC, 0.4% SDS, 5 x Denhardt, 20 mM NaH_2PO_4 and 0.5 mg/ml herring sperm DNA and hybridised with denatured probe overnight in the same solution at 42°C. The membranes were washed 3 times in 6 x SSC, 0.1% SDS, once in 2 x SSC, 0.1% SDS, both at 42°C, followed by a 7-hour exposure of film at -70°C.

Fragments of the genomic clones that were positive on Southern blots were subcloned into pBluescript II SK(-) (Stratagene, La Jolla, CA, USA). Three

subclones were made from genomic clone 9794, a 4.3 kb *Sac*I-*Bam*HI fragment (containing exon 1 and the upstream area of the type II GnRH receptor gene), a 5.2 kb *Bam*HI-*Bam*HI fragment (containing the area from exon 1 to exon 3) and a 5.4 kb *Bam*HI-*Eco*RI fragment (containing the area downstream of the GnRH receptor, including the RBM8A coding region and 3 kb upstream of the RBM8A start codon). Two subclones were made from clone 9792, a 4.3 kb *Sac*I-*Bam*HI fragment and a 5.2 kb *Bam*HI-*Bam*HI fragment, containing exons 1, 2 and 3 of the type II GnRH receptor gene. Two subclones were made from P1 clone 9793; a 2.0 kb *Bam*HI-*Eco*RI fragment (containing exons 2 and 3, and 1 kb upstream of exon 2 of the type II GnRH receptor gene) and a 2.9 kb *Bam*HI-*Bam*HI fragment (containing part of exon 3 of the type II GnRH receptor, the RBM8 coding region and 1.5 kb upstream of the start codon of the RBM8 gene). Successive deletions of inserts of each clone were made using Erase-a-Base (Promega, Madison WI, USA). The deletions were cloned into pBluescript II SK(-) and sequenced. Sequence analysis was performed using DNAMAN (Lynnon Biosoft, Quebec, Canada).

2.4 Results

2.4.1 Human type II GnRH receptor cDNA

A previous study identified transcripts with homology to a putative type II GnRH receptor gene in humans. These transcripts were all in the antisense orientation and were part of the RBM8A transcript, which is encoded in the antisense orientation (Millar et al., 1999). In contrast, the cDNA used in this study was synthesised using an antisense primer complementary to the 3' end of the GnRH receptor coding sequence instead of an oligo (dT) primer. This ensured that the cDNA made was from transcripts in the GnRH orientation and avoided overlapping antisense transcripts encoding RBM8A.

Nested PCR with sets of oligonucleotide primers corresponding to exons 2 and 3 was carried out on cDNA prepared from various human tissues and cell lines in order to identify transcripts of the type II GnRH receptor that have spliced out intron 2. PCR between exons 2 and 3 yielded a band of about 220 bp in pituitary, placenta, prostate, testis, occipital pole, pons, foetal brain, adult brain, lymph node, small intestine, spleen, kidney, the Jurkat and MCF-7 cell lines (Table 2.2). The size of the PCR products suggests that intron 2 is spliced out and that these transcripts are sense transcripts. A band of 670 bp, corresponding to unspliced transcripts, was present in thyroid, cerebellum, thalamus and the T47D cell line. Sequencing of the 220 bp PCR products from prostate, testis, spleen and lymph node confirmed splicing of intron 2.

Sequences corresponding to exon 1 of the human type II GnRH receptor were obtained by a BLAST search using exon 1 of the human type I GnRH receptor (hGnRH-RI) as a query sequence (Conklin et al., 2000). An EST sequence (Genbank acc no. AA954764) with 49% identity to the area between TM2 and TM4 of the hGnRH-RI was identified. Oligonucleotide primers, based on the EST sequence and on genomic sequence flanking the EST sequence, was used in PCR together with oligonucleotide primers corresponding to exons 2 and 3 in order to identify spliced transcripts of the human type II GnRH receptor containing exon 1.

PCR amplification products were screened by Southern blotting with an oligonucleotide specific for exon 2. Spliced transcripts were found in pituitary, pons, frontal cortex, cerebellum, occipital pole, thyroid, hypothalamus, placenta, kidney, prostate and testis. The cloning and sequencing of these bands identified an open reading frame with 38% homology to the human type I GnRH receptor between TM3 and TM6 (Kakar et al., 1992; Chi et al., 1993). However, a premature stop codon is present 9 codons into exon 2 of the putative type II GnRH receptor (Fig 2.3).

Table 2.2. Expression of the human type II GnRH receptor transcripts in tissues and cell lines*.

Tissues and cell lines		
Adult brain Foetal brain Hypothalamus Occipital pole Frontal cortex Spinal cord Cerebellum Pons	Neuronal tissue	Non-reproductive tissue/cells
Pituitary Lymph node Small intestine Retina Spleen Bone marrow Insulinoma K562 leukemia cells Kidney Jurkat Thyroid Foetal lung	Non-neuronal tissue/cells	
Testis Prostate Placenta Uterus MCF-7		Reproductive tissue/cells

*Transcripts have been identified by PCR (introns were spliced out) or 5' RACE on cDNA libraries.

Transcripts of variable length were identified by 5' RACE in pituitary, uterus, retina, bone marrow, spinal cord, insulinoma, foetal lung, foetal brain and K562 cells. The shortest transcript, found in retina, is initiated 28 nt upstream of the codon equivalent to the methionine start codon in the marmoset type II GnRH receptor (Fig 2.2). The majority of transcriptional start sites (TSS) are situated at positions –380 to –334 nt relative to the codon that is equivalent to the methinoine start codon in marmoset type II GnRH receptor (nucleotide number 1 in Fig 2.2 and 2.5). The longest transcripts are found in human pituitary and in foetal brain (Fig 2.2). The deduced amino acid sequence of exon 1 of the human type II receptor is 94% identical to that of the type II GnRH receptor of marmoset monkey (Millar et al., 2001). The overall amino acid identity of the human type II GnRH receptor is 90-93% to other primate GnRH receptors and 41% to the human type I GnRH receptor (Table 2.3)(Fig 2.3). The deduced amino acid sequence of the human type II GnRH receptor used for alignment and comparison with other receptor sequences is based on the sequence between the codons equivalent to the start and stop codons of the marmoset type II GnRH receptor assuming that selenocysteine is incorporated at the stop codon in exon 2 of the human type II GnRH receptor. The receptor has a structure typical of G protein-coupled receptors, including a c-terminal tail, which is uniquely absent from the mammalian type I GnRH receptors. Interestingly, translation of the 5' RACE sequence has revealed that in the human type II GnRH receptor there appears to be no methionine start codon in the frame with homology to GnRH receptors (Fig 2.2).

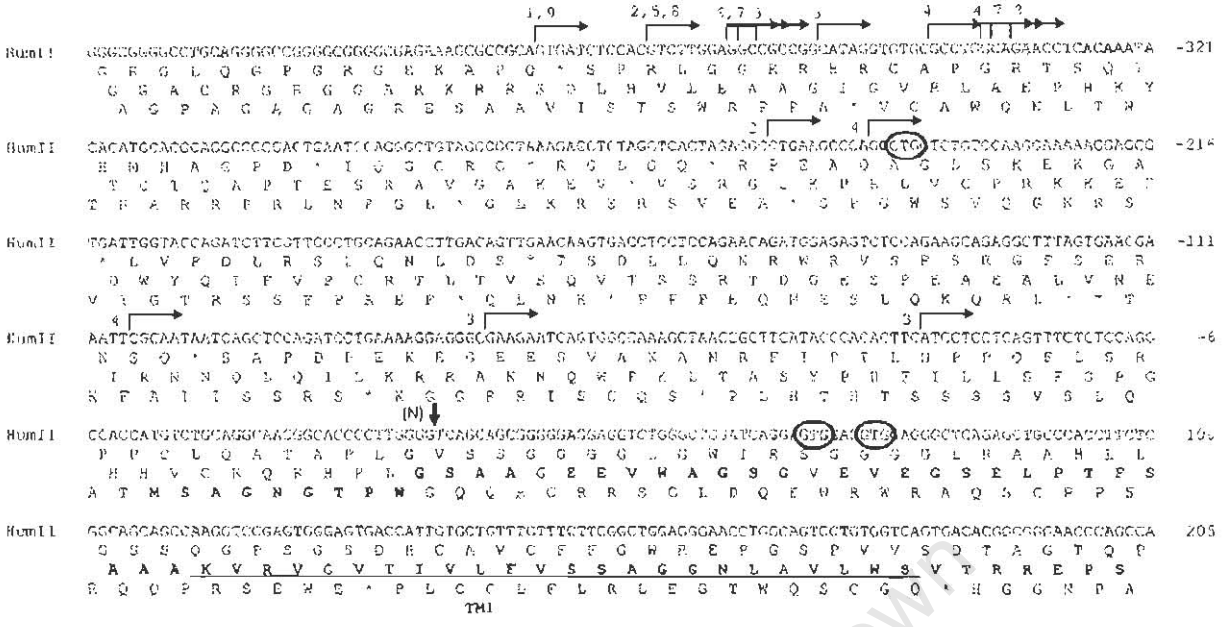

Figure 2.2. 5' Area of the human type II GnRH receptor translated in three frames. The frame with homology to GnRH receptors is in bold and the possible single nucleotide deletion is indicated by a vertical arrow. Transmembrane domain 1 is underlined. Putative non-methionine translation initiation sites in-frame are circled. Transcriptional start sites as predicted by 5' RACE are indicated by horizontal arrows. Numbers indicate the tissue; 1:pituitary, 2:uterus, 3:retina, 4:bone marrow, 5:spinal cord, 6:human insulinoma, 7:human chronic myeloid leukemia cell line K562, 8:foetal lung and 9:foetal brain.

Table 2.3. Amino acid identities of GnRH receptors.

	HGnRH-R2
HGnRH-R2*#	100 %
MtGnRH-R2	90 %
RhMGnRH-R2	93 %
GnMGnRH-R2	93 %
XlGnRH-R2	52 %
HGnRH-R1	41 %
ChGnRH-R	43 %
CfGnRH-R	46 %
BfGnRH-R1	51 %
BfGnRH-R2	43 %
BfGnRH-R3	54 %

* Abbreviations for species are: H, human (*Homo sapiens*); Mt, Common marmoset monkey (*Callithrix jacchus*); RhM, Rhesus monkey (*Macaca mulatta*); GnM, African green monkey (*Cercopithecus aethiops*); Xl, African clawed frog (*Xenopus laevis*); Ch, Chicken (*Gallus domesticus*); Cf, African catfish (*Clarias gariepinus*); Bf, Bullfrog (*Rana catesbeiana*).

GenBank accession numbers used for sequence comparison: HGnRH-R2 AF403014; MtGnRH-R2 AF368286; RhMGnRH-R2 AF353987; GnMGnRH-R2 AF353988; XlGnRH-R2 (B. Blackman *et al.* unpublished); HGnRH-R1 S60587; ChGnRH-R AJ304414; CfGnRH-R X97497; BfGnRH-R1 AF144063; BfGnRH-R2 AF153913; BfGnRH-R3 AF144062.

Figure 2.3 Sequence alignment of GnRH receptors. The transmembrane domains (TM 1-7) are underlined and conserved residues are in bold print. The stop codon in ECL 2 of the human type II GnRH receptor is indicated with an a star. Hum II, human type II GnRH receptor; Mar II, marmoset type II GnRH receptor; Xen I, *Xenopus laevis* type II GnRH receptor; Hum I, human type I GnRH receptor.

2.4.2 Human type II GnRH receptor gene structure

In order to characterise the type II GnRH receptor gene (*GNRHR2*) three previously identified human P1 genomic clones (9792, 9793 and 9794) were subcloned and sequenced (Millar et al., 1999). The gene symbol *GNRHR2* has been approved by HGNC, Human Genome Nomenclature Committee, London, UK. The P1 clones were digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII or *Spe*I and separated on an agarose gel. The DNA was transferred to a nylon membrane and the blots were hybridised with probes to exon 1, exon 2-3 or RBM8A (Fig 2.4).

The use of exon 3 as a probe in Southern blots of the genomic clones demonstrates that clones 9792 and 9794 are identical, but differ from 9793 (Fig. 2.4A). Probing with exon 1 shows that clones 9792 and 9794 contain exon 1 and they display an identical banding pattern (Fig. 2.4B). In contrast, clone 9793 does not harbour exon 1. As the GnRH type II receptor and RBM8A genes overlap and are in opposite

orientations, we probed the Southern blots of the genomic clones with DNA spanning the protein coding region of RBM8A. Fig. 2.4C shows that all three genomic clones contain RBM8A; however, the bands in clone 9793 are different from the restriction bands of 9792 and 9794. The faint bands for the *Eco*RI and *Eco*RV digests of 9794 are probably due to a loading error. The results of the Southern blot were confirmed by PCR amplification with pairs of primers specific to exon 1, 3 and RBM8A (data not shown).

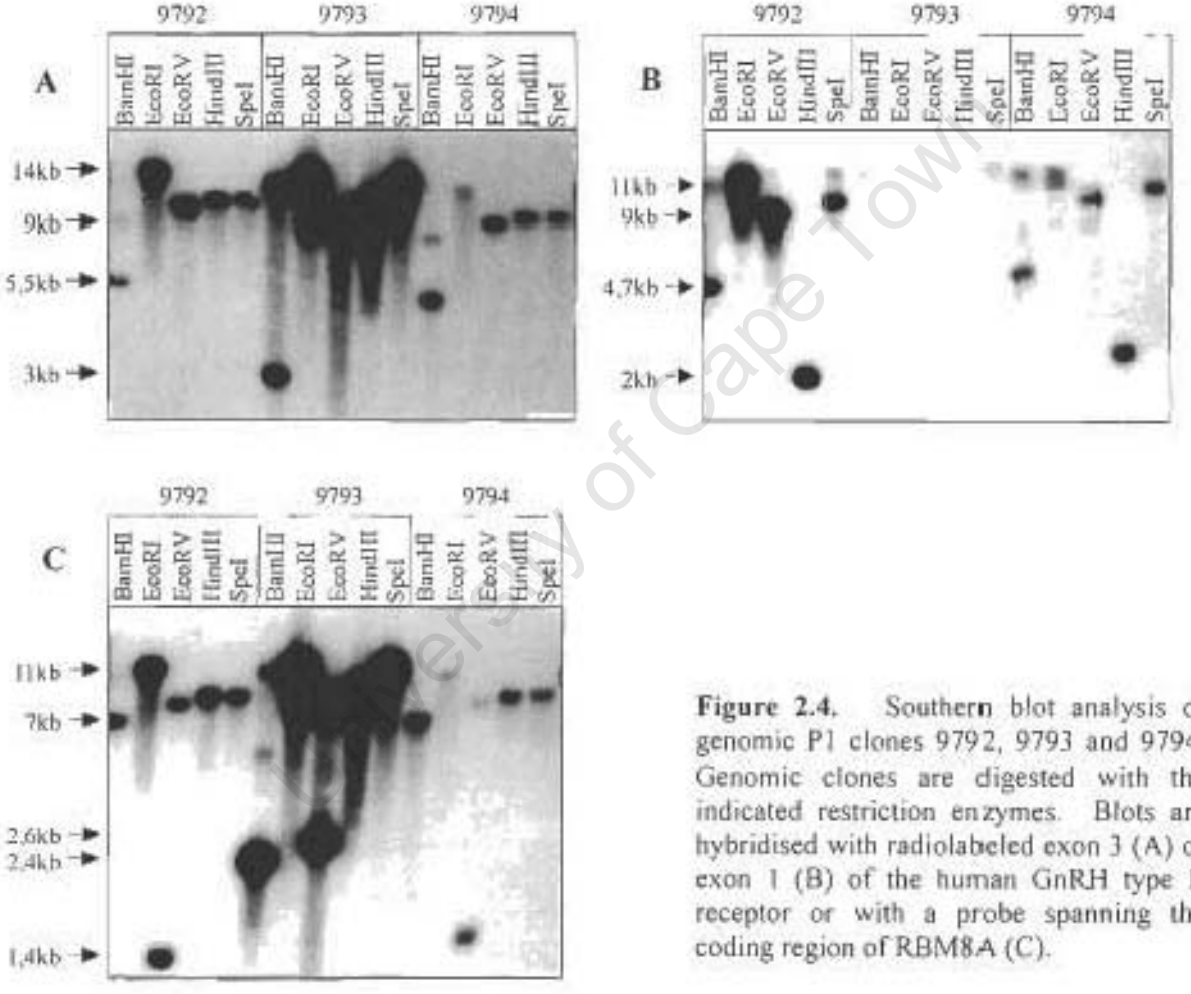

Figure 2.4. Southern blot analysis of genomic P1 clones 9792, 9793 and 9794. Genomic clones are digested with the indicated restriction enzymes. Blots are hybridised with radiolabeled exon 3 (A) or exon 1 (B) of the human GnRH type II receptor or with a probe spanning the coding region of RBM8A (C).

Further characterisation of the type II GnRH receptor gene was achieved by cloning and sequencing a 12 kb region of clone 9794. This region was found to stretch from 1.3 kb upstream from exon 1 of the type II GnRH receptor gene to 5.2 kb downstream from exon 3 (3 kb upstream from exon 1 of the RBM8A gene as the genes are in opposite orientations). A comparison of the genomic sequence with the sequence of the PCR product of the type II GnRH receptor cDNA confirmed the

presence of two introns (Fig 2.5). The splice donor gt- and acceptor –ag consensus sites of intron 1 and of intron 2 are conserved. The 4.2 kb intron 1 is situated in TM 4 and the 449 bp intron 2 is situated in ICL 3 of the receptor in postitions similar to the human type I GnRH receptor (Fig 2.5).

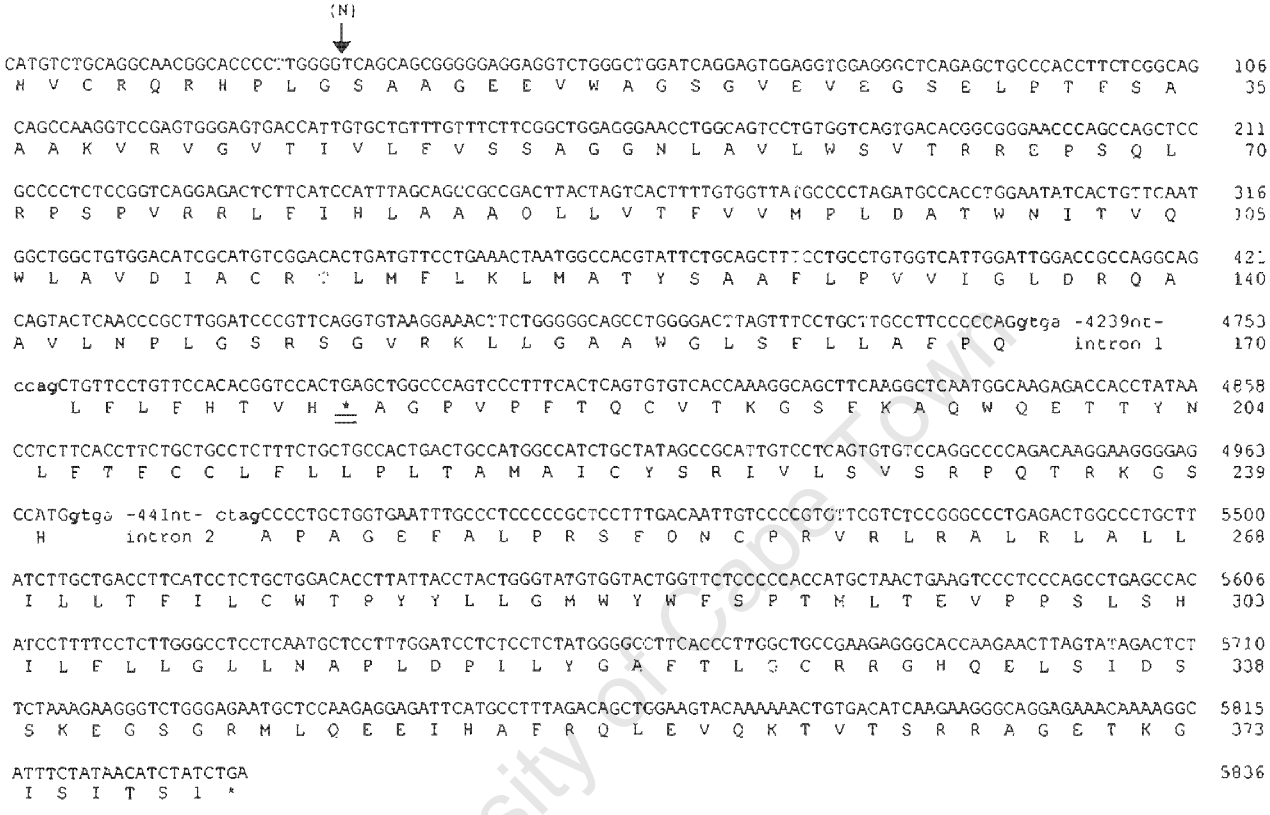

Figure 2.5. Gene structure of the human type II GnRH receptor gene. The two introns and their sizes are indicated. The intron splice sites are in bold print. The stop codon in exon 2 is double underlined. The vertical arrow indicates the position of a single nucleotide deletion in the sequence of the human type II GnRH receptor gene.

2.4.3 The human RBM8A gene

Sequencing of the 12 kb region of the P1 genomic clone 9794 in the region downstream of exon 3 of the type II GnRH receptor identified a sequence with homology to the RBM8A transcript in the antisense orientation. A comparison of this genomic sequence with the published cDNA for RBM8A (Conklin et al., 2000; Salicioni et al., 2000; Zhao et al., 2000) shows that the RBM8A gene (the gene symbol *RBM8A* has been approved by HGNC, Human Genome Nomenclature Committee, London, UK), is composed of six exons which are separated by five

introns and which stretch over 1545 bp from the translational start codon to the stop codon (Fig. 2.6). The nucleotide sequence of the coding region of the exons is 100% identical to the published RBM8A cDNA. The length of exon 1 (from the ATG) is 67 bp and exons 2, 3, 4, 5 and 6 are 60 bp, 78 bp, 138 bp, 152 bp and 2255 bp respectively. All introns begin with gt- and end with –ag splice site consensus sequences. The introns are 282 bp, 131 bp, 190 bp, 304 bp and 113 bp for introns 1, 2, 3, 4 and 5 respectively.

2.4.4 The RBM8B and type II GnRH receptor pseudogenes

Southern blot analysis of the three genomic clones 9792, 9793 and 9794 (Fig. 2.4) indicates that clone 9793 is different from clones 9792 and 9794 since it lacks exon 1 of the type II GnRH receptor gene and displays different restriction bands for exon 3 of the receptor gene and for the RBM8A gene. In order to characterise the 9793 genomic clone further, two subclones spanning a length of 4.9 kb and encompassing exons 2 and 3 of the type II GnRH receptor gene and the RBM8A gene were sequenced. A sequence comparison of clone 9793 with 9794 identified a 2.8 kb region homologous to 9794. Sequence alignment shows that the homology extends 50 bp upstream of the translational start site for RBM8A and includes an intronless RBM8A sequence, exons 2 and 3 of the type II GnRH receptor gene (in antisense orientation) and ends 506 nucleotides upstream of exon 2 of the type II GnRH receptor gene (Fig. 2.7).

Translation of the open reading frame has revealed a protein with 96% amino acid identity to RBM8A. However, there are 7 amino acid changes, one of them to a stop codon at a position equivalent to amino acid 84 of the wild-type RBM8A. These findings indicate that this RBM8A gene is a pseudogene. This pseudogene has been designated *RBM8B* (the gene symbol has been approved by HGNC, Human Genome Nomenclature Committee, London, UK). Two additional features typical of pseudogenes have been identified in *RBM8B* of clone 9793 (Maestre et al., 1995). A 16 bp dA stretch is located 20 nucleotides downstream of the second polyadenylation consensus sequence of *RBM8B* of clone 9793 (Fig. 2.6 and 2.7). The second feature is the presence of short direct repeats on either side of the

pseudogene. These short direct repeats are represented by two direct repeats of 7 nucleotides (TTTAAAT) and 11 nucleotides (GTTTTTTTTTT) found on either side of the RBM8B pseudogene of clone 9793 and are not found flanking the RBM8A gene of clone 9794 (Fig. 2.7).

The 3' untranslated region of *RBM8B* encodes (in the antisense orientation) exon 3, exon 2, intron 2 and part of intron 1 of the type II GnRH receptor. This gene has 4 amino acid changes and it has 98% amino acid identity with *GNRHR2* on genomic clone 9794. This GnRH type II receptor gene lacks exon 1 and may also be considered to be a pseudogene.

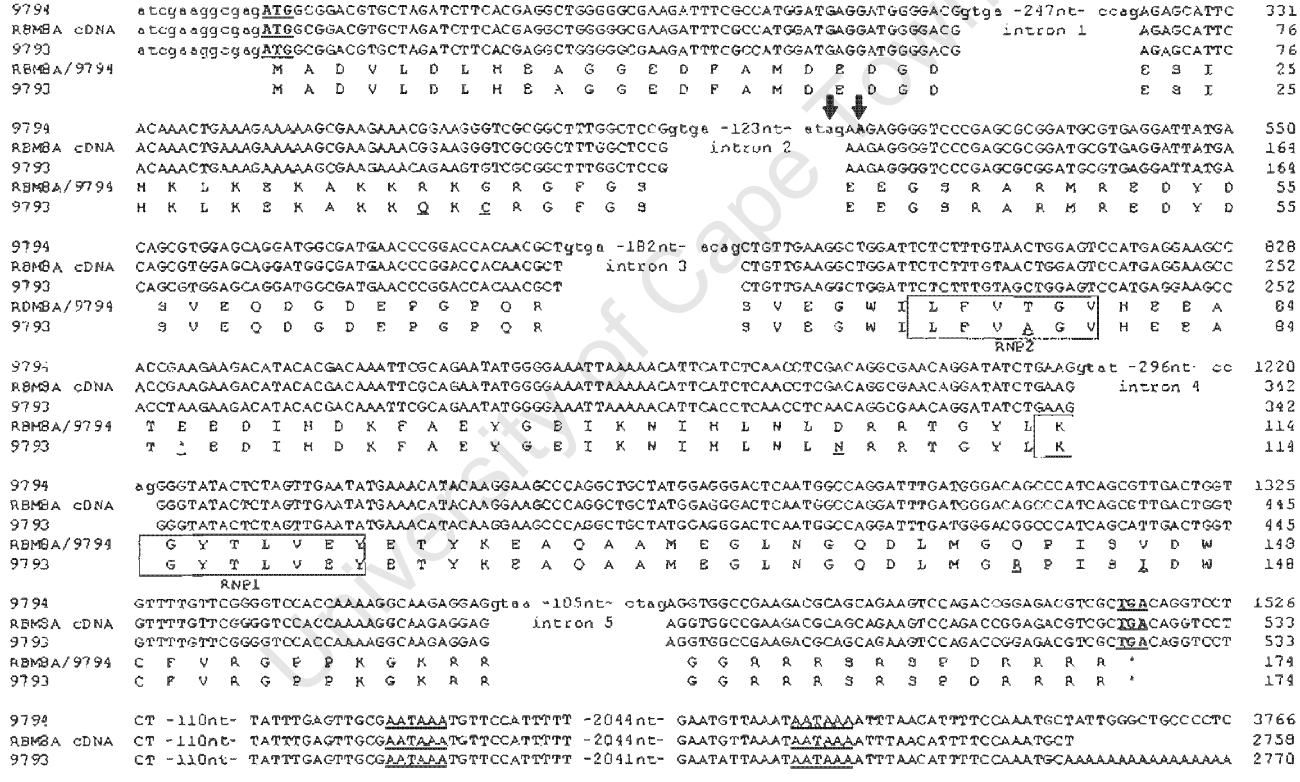

Figure 2.6. Nucleotide and amino acid sequence alignment of the RBM8A coding region of genomic clones 9793 and 9794 with the cDNA sequence of RBM8A. The amino acid sequence encoded by the RBM8A cDNA is identical to the deduced amino acid sequence of clone 9794 and is indicated as RBM8A/9794. The start and stop codons are underlined and in bold. Nucleotides of exons are written in uppercase letters and introns are written in lowercase letters. The positions of introns in the sequence of clone 9794 and their sizes are indicated, with the mRNA splice donor and acceptor sites shown in bold. The RNA binding consensus sites (RNP1 and RNP2) are boxed. The amino acids which are different in clone 9793 compared to clone 9794 are underlined. The stop codon in exon 4 of the RBM8A of clone 9793 is indicated by an asterisk. Two polyadenylation consensus sequences are underlined. The vertical arrows indicate the two mRNA splice acceptor sites at the beginning of exon 3.

2.4.5 Chromosomal assignment of *GNRHR2, RBM8A* and *RBM8B*

A chromosomal assignment using BLAST search of the GenBank unfinished high-throughput sequences (HTGS) database was carried out. The use of the *RBM8A* sequence of clone 9794 or the sequence corresponding to exon 1 of the type II GnRH receptor yielded a clone (Genbank acc no AL160282) which is located on chromosome 1 position q12.1-21.3. In contrast, a search done with the *RBM8B* sequence from clone 9793 as query identified 2 overlapping clones (Genbank acc numbers: AL122057 and AL132778) as having the highest homology. These clones are situated on chromosome 14. These findings show that both the RBM8A and the type II GnRH receptor genes are located at two genomic loci. One locus on chromosome 1q12.1-21.3 encodes RBM8A and the type II GnRH receptor, and the second locus on chromosome 14 contains *RBM8B* and type II GnRH receptor pseudogenes.

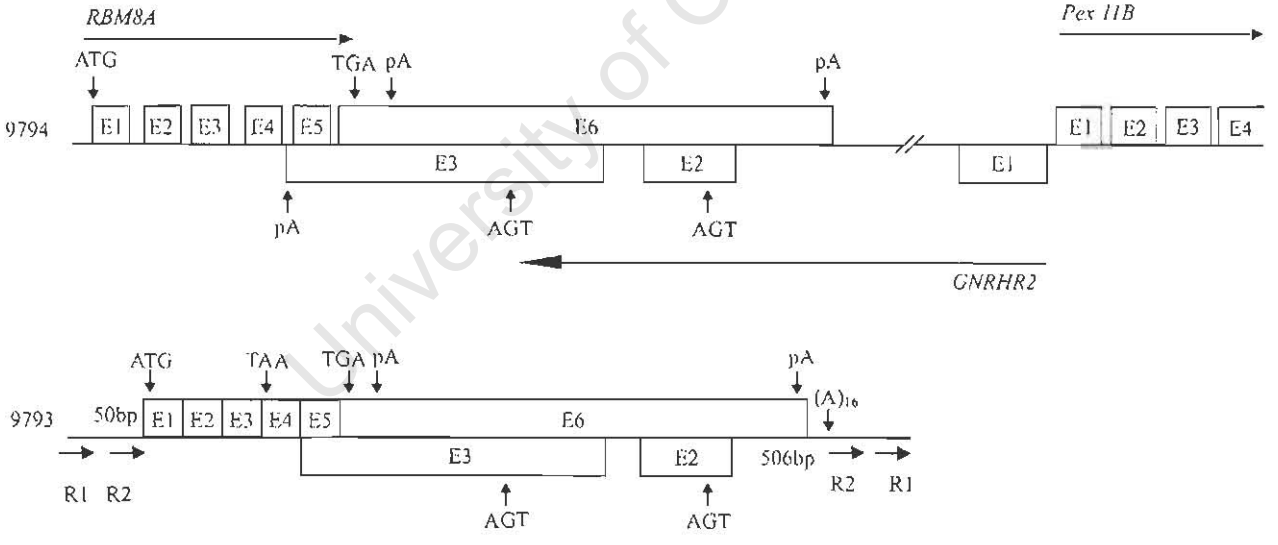

Figure 2.7. A schematic representation of the gene structure of the two loci identified containing the RBM8A gene and the type II GnRH receptor gene, shown in the orientation of RBM8A. The directions of transcription of the RBM8A gene and of the type II GnRH receptor gene (*GNRHR2*) in the antisense orientation are indicated. Exons numbered E1-6 of the RBM8A gene and E1-4 of Pex 11B (peroxisome biogenesis factor 11B) are shown above the solid line whereas exons E1-3 of the type II GnRH receptor gene are shown below the line. Start codons, stop codons and polyadenylation signals (pA) are indicated by vertical arrows. The area of homology between clones 9793 and 9794 extends 50 bp upstream of the start codon of RBM8A to 506 bp upstream of exon 2 of the type II GnRH receptor gene. The two direct repeats R1 and R2 (R1-TTTAAAT, R2-GTTTTTTTTTT) flanking the area of homology are indicated on clone 9793.

2.5 Discussion

Our results explain the previous finding of EST sequences with homology to a putative type II GnRH receptor in the antisense orientation. The EST sequences encode exons 2 and 3 of the GnRH II receptor but the homology is in the antisense orientation (Millar et al., 1999). In the present study, we have found that the human type II GnRH receptor gene overlaps and is oriented in a tail-to tail manner with the gene for a protein named RBM8A. The coding regions do not overlap but a polyadenylation consensus sequence for the RBM8A gene is situated within the type II GnRH receptor gene. Our results show that the EST sequences do not represent transcripts of the type II GnRH receptor gene but are part of the 3' UTR of the RBM8A transcript.

In this study, we identified transcripts of the human type II GnRH receptor gene. The intron between exons 2-3 of the type II GnRH receptor is retained in the EST sequences because the intron splice consensus sequences are not present when read in the antisense orientation, the orientation in which these EST sequences code for RBM8A. The intron splice consensus sequences are present in the sense orientation and the intron can only be spliced out from the sense transcript.

We identified cDNAs with spliced out intron 2, indicating they are transcripts of the human type II GnRH receptor gene. Further analysis using PCR on cDNA with primers to exon 1 and 5' RACE also identified transcripts of exon 1 of this gene, supporting the finding that the human type II GnRH receptor gene exists and is transcriptionally active. We used PCR based analysis to show that the type II GnRH receptor is widely expressed in both neural and non-neural tissues and in both reproductive and non-reproductive tissues (Table 2.2). The expression pattern of *GNRHR2* has been confirmed by RNA dot blot arrays and by Northern blots on multiple human tissues and cell lines (Millar et al., 2001; Neill et al., 2001; van Biljon et al., 2002; Millar, 2003a; Morgan et al., 2003). The identification of transcripts for this receptor in a wide variety of tissues is consistent with the wide distribution reported for the type II GnRH peptide in primates (Lescheid et al., 1997; White et al., 1998; Urbanski et al., 1999; Choi et al., 2001; Kang et al., 2001; Chen et al., 2002b).

The human type II GnRH receptor has a high amino acid identity with the monkey type II GnRH receptors (90% with marmoset and 93% with the rhesus and African Green monkeys), but the human type II GnRH receptor has lower homology to the human type I GnRH receptor (41%) (Table 2.3) (Kakar et al., 1992; Chi et al., 1993; Millar et al., 2001; Neill et al., 2001: Neill et al., 2002). The human type II GnRH receptor cDNA, as identified in this study and by other groups (Van Biljon et al., 2002; Morgan et al., 2003), contains a stop codon in exon 2. It is possible that, in some tissues, the alternative amino acid selenocysteine is incorporated in the place of the stop codon (Atkins and Gesteland, 2000). The amino acid alignments are based on this assumption.

The human type II GnRH receptor has a structure typical of seven transmembrane domain G protein-coupled receptors, including a c-terminal tail. The presence of a C-terminal tail in the type II GnRH receptor is in contrast to the mammalian type I GnRH receptors which do not have a tail (Fig 2.3). The c-terminal tail has been shown to be important for desensitisation and internalisation in GnRH receptors and in GPCRs in general (Kakar et al., 1992; Chi et al., 1993; King and Millar, 1997; Pawson et al., 1998; Heding et al., 1998; Willars et al., 1999).

Three amino acid residues of the human type I GnRH receptor, Asp^{98}, Asn^{102} and Lys^{121}, which are involved in ligand binding, are conserved in the human type II GnRH receptor cDNA. In contrast, the acidic residue in ECL 3 of the human type I GnRH receptor which confers selectivity towards mGnRH is not conserved in the type II GnRH receptors. This region of ECL 3 has the sequence VPPS in the human type II GnRH receptor and this sequence is conserved in type II GnRH receptors (Millar et al., 2001; Neill et al., 2001; Wang et al., 2001a; Millar, 2003a). A (E/D)R(Y/S) motif present at the intracellular end of TM 3 of most GPCRs is thought to be involved in receptor activation. This motif is DRQ in the human type II GnRH receptor and other type II GnRH receptors suggesting a possible conservation of function of these residues (Gether, 2000; Millar, 2003a).

The human type II GnRH receptor seems to have no methionine start codon. Translation of the 5' RACE sequence in three frames has not revealed any

methionine start codon in the frame with homology to GnRH receptors. Insertion of any nucleotide between codon 10 and 11 of the human receptor will reconstitute an open reading frame with a methionine and this frame will have 9 of the 10 N-terminal amino acids identical to that of the marmoset receptor (human: MSAGNGTPWG, marmoset: MSAVNGTPWG) (Figs 2.2 and 2.3). This finding shows that a single nucleotide deletion is present in the human type II GnRH receptor gene.

The human type II GnRH receptor has a stop codon which is eight amino acids into exon 2 (Fig. 2.5). The type II GnRH receptors from monkeys, frogs and the pig have an arginine at this position indicating that the human receptor might also have had an arginine at this position and that it was later mutated to a TGA stop codon (Millar et al., 2001; Neill et al., 2001; Wang et al., 2001a; Morgan et al., 2003) (Blackman *et al* unpublished)(Pig EST BF702918). A BLAST search has identified two EST sequences in cow and chimpanzee with homology to exon 2 of type II GnRGnRH receptorH-Rs. Interestingly, the bovine and chimpanzee type II GnRH receptors also have stop codons in exon 2. In the cow, the stop codon is shifted exactly one codon upstream in relation to the arginine or stop codon in other species, suggesting an independent evolution of the stop codon in cattle (Morgan et al., 2003)(chimpanzee EST AG122659, cow EST AV604131).

The presence of a stop codon in exon 2 and the lack of a methionine start codon indicate that the human type II GnRH receptor gene may be non-functional. However, the cloning of functional type II GnRH receptors from pig and from three different monkey species, the existence of the GnRH II peptide in humans and the immunodetection of type II GnRH receptors in human pituitary and brain all indicate that a functional type II GnRH receptor might exist in humans (White et al., 1998; Millar et al., 2001; Neill et al., 2001; Neill et al., 2002).

Studies on stimulation of cell proliferation also indicate the presence of a second GnRH receptor in humans. Both GnRH II and an mGnRH agonist have antiproliferative effect in three cell lines, Hec1A, Ishikawa and EFO-21, all of which express both the type I GnRH and type II GnRH receptor transcripts. In a fourth cell line, SKOV-3, in which only type II GnRH receptor transcripts were detected, GnRH II had antiproliferative effect whereas a mGnRH agonist had no effect on

proliferation (Grundker et al., 2002). Moreover, a knock down of type I GnRH receptor in cell lines expressing both receptor transcripts abrogated the mGnRH agonist stimulated decrease in proliferation whereas the antiproliferative effect of GnRH II was unaffected (Grundker et al., 2003). Further support for the expression of a human type II GnRH receptor comes from a study on the effect of mGnRH and GnRH II on the expression of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) in human decidual stromal cells. mGnRH stimulates expression of both uPA and PAI-1 and this stimulatory effect of mGnRH is inhibited by cetrorelix, a mGnRH antagonist. GnRH II stimulates the expression of uPA and inhibits the expression of PAI-1. In contrast to the effect of mGnRH, the effect of GnRH II on uPA and PAI-1 expression is not inhibited by cetrorelix, suggesting the effect of GnRH II is not mediated by the type I GnRH receptor (Chou et al., 2003).

The human type II GnRH receptor gene may code for a functional receptor, as there are examples of the use of non-ATG codons for the initiation of translation, such as CUG, ACG or GUG (Kozak, 1991; Kozak, 1997; Kozak, 1999; Ronsin et al., 1999; Huez et al., 2001). Indeed, the type II GnRH receptor of the African Green monkey also lacks a methionine start codon (Neill et al., 2001) (Genbank accession no. AF353988). In this species the lack of methionine start codon is due to a single T to C nucleotide substitution, resulting in an ACG codon in the position equivalent to the methionine start codon in the marmoset and rhesus monkeys. The ACG codon may encode a methionine by forming a weak two basepair interaction with the Met-tRNA anticodon (Kozak, 1999). 5' RACE analysis of human type II GnRH receptor transcripts has revealed the existence of several possible in-frame alternative translational start codons in the human type II GnRH receptor (Fig 2.2).

It is also posible that an ATG start codon could be generated by RNA editing. RNA editing can be defined as a modification of the RNA sequence from that of the genomic sequence other than RNA splicing, capping and polyadenylation (Chester et al., 2000). Two nucleotide modifications have been described in mammals, both involving deaminases. C to U editing in apolipoprotein B was the first RNA editing described in mammals (Chen et al., 1987; Chester et al., 2000). RNA editing of the serotonin 5-HT_{2C} receptor and the glutamate receptor involves deamination of

adenosine to inosine, which is translationally read as guanosine (Sommer et al., 1991; Simpson and Emeson, 1996; Burns et al., 1997; Chester et al., 2000). However, none of the 5' RACE products or RT-PCR products sequenced in this study show that RNA editing resulting in a methionine start codon has occurred.

Another possibility for generating a transcript for a functional type II GnRH receptor is removal of the frameshift mutation by RNA splicing. Indeed, a search of the Genbank human EST databases has identified a transcript (EST BG036921) from a prostate adenocarcinoma cell line, in which a 200 nucleotide sequence of exon 1 containing the frameshift is spliced out. This transcript has an in-frame methionine start codon but part of the N-terminal tail, all of TM 1 and ICL 1 and part of TM 2 has been spliced out. This transcript has been generated by utilizing an alternative splice acceptor in intron 1. The result is premature termination instead of the splicing out of the whole of intron 1. The length of this protein is predicted to be 91 amino acids, which is clearly not a full-length type II GnRH receptor (Morgan et al., 2003).

The early termination of translation due to the stop codon in exon 2 can be overcome by either alternative splicing, RNA editing or by translational events. No RNA editing or splicing that eliminates the stop codon in exon 2 has been seen in any of the transcripts sequenced in this study. Another possibility for overcoming the early termination is the incorporation of the alternative amino acid selenocysteine (SeCys) encoded by the TGA stop codon. SeCys is an amino acid with the same structure as cysteine but with a selenium moiety in place of sulphur (reviewed in Atkins and Gesteland, 2000; Hatfield and Gladyshev, 2002). A mechanism has been described in which SeCys is incorporated into proteins at the positions of TGA codons without termination of translation at this codon. This requires a specific SeCys tRNA and it requires proteins which bind to a specific hairpin loop structure in the 3' UTR of the mRNA (Fagegaltier et al., 2000a; Low et al., 2000; Tujebajeva et al., 2000). This hairpin loop structure, called selenocysteine insertion sequence (SECIS), is conserved in transcripts for proteins that incorporate SeCys (Berry et al., 1991; Lescure et al., 1999; Atkins and Gesteland, 2000; Fagegaltier et al., 2000b; Hatfield and Gladyshev, 2002). A hairpin loop structure with some similarity to SECIS is present in the 3' UTR of the human type II GnRH

receptor transcript. However, the structure lacks a conserved AAA/G motif. Furthermore, COS cells transfected with the human type II GnRH receptor cDNA show no evidence of incorporation of ^{75}SeCys into proteins of the expected size for the receptor (Morgan et al., 2003). However, the system may be too insensitive to demonstrate incorporation in view of the poor expression of GnRH receptors.

Two features of the human type II GnRH receptor gene have to be overcome in order for this gene to encode a functional full-length receptor. Mechanisms have been described which can overcome both the frameshift in exon 1 and the stop codon in exon 2. However, no evidence of RNA editing or SeCys incorporation has been forthcoming. Furthermore, no full-length cDNA of the type II GnRH receptor has been identified in the human EST dabases at NCBI. It is indeed possible that the human type II GnRH receptor gene is a pseudogene. The chimpanzee also has a stop codon in exon 2 whereas other monkey species have an arginine. The cow has a stop codon and this stop codon may have evolved independently of the stop codon in humans (Morgan et al., 2003). Interestingly, rodents appear not to have any type II GnRH receptors or GnRH II genes. Southern blot analysis of mouse genomic DNA with a marmoset type II GnRH receptor cDNA as probe did not reveal any positive bands (this study, data not shown). PCR amplification of mouse genomic DNA with degenerate primers only amplified the type I GnRH receptor from mouse (this study, data not shown). The same primers were able to amplify type II GnRH receptors from other species. In addition, BLAST searches of the mouse and rat genome databases at Genbank did not reveal any type II GnRH receptor or GnRH II peptide genes. Only mGnRH and type I GnRH receptors were identified (this study, data not shown).

It appears that a functional type II GnRH receptor may not be present in some mammals. However, the GnRH II peptide is present in humans. It may be that GnRH II acts through the type I GnRH receptor in the absence of the type II GnRH receptor. The type II GnRH receptor has high affinity for the GnRH II peptide but GnRH II can also bind to and activate the type I GnRH receptor. The affinity of type I GnRH receptors for GnRH II is approximately 10-fold lower than the affinity for GnRH I. It may be that high concentrations of GnRH II in specific tissues may have

a physiological effect by activating the type I GnRH receptor (Millar et al., 2001; Millar, 2003a).

The possibility that the type II GnRH receptor cDNAs with a stop codon (human, chimpanzee and cow) encode a short protein that is initiated downstream of the stop codon is an interesting hypothesis (Morgan er al., 2003). A Kozak consensus sequence for initiation of translation is situated 120 nucleotides downstream of the stop codon in exon 2 of the human type II GnRH receptor cDNA (Fig 2.5)(Kozak 1999). A protein translated from this start site would contain sequence equivalent to ICL 3, TM 6, ECL 3, TM 7 and the c-terminal tail of the type II GnRH receptor. It may be that this receptor variant influences the function of the type I GnRH receptor. Grosse et al. showed that a splice variant of the human type I GnRH receptor expressed in pituitary inhibits the production of second messenger upon stimulation of the full length type I GnRH receptor. They also showed that this is due to a decrease in cell surface expression of the type I GnRH receptor when it is co-expressed with the splice variant (Grosse et al., 1997). Similar findings have been reported in bull frogs. Splice variants of the bull frog type III GnRH receptors, which do not induce signal transduction when transfected alone, inhibit signal transduction of the full length bull frog type III GnRH receptor upon ligand stimulation (Wang et al., 2001b). The initiation of translation from the ATG in exon 2 of the human type II GnRH receptor gene has not been shown. Furthermore, the presence of the stop codon in exon 2 might reduce the mRNA of the human type II GnRH receptor through the mechanism of nonsense-mediated decay (NMD). The presence of an intron downstream of a stop codon in the triosephosphate isomerase gene has been shown to down-regulate mRNA levels in this way (Cheng et al., 1994).

Analysis of genomic clones and a comparison of the genomic and cDNA sequences have shown that the human type II GnRH receptor gene (*GNRHR2*) consists of 3 exons and 2 introns (Figs 2.5 and 2.7). Intron 1 is 4.2 kb in both the human type I and type II GnRH receptors and is situated at the identical positions in TM4 in both receptor genes (Fan et al., 1994; Fan et al., 1995; Kakar, 1997). The position of intron 2 is in intracellular loop 3 of the receptor, similar to the position of intron 2 in the human type I GnRH receptor gene. However, intron 2 is 449 bp in the type II receptor gene while the length of intron 2 is 5 kb in the type I GnRH receptor gene

(Fan et al., 1994; Fan et al., 1995; Kakar, 1997). The gene structure of the type I GnRH receptor has been retained in the type II GnRH receptor gene. The conserved gene structure suggests that the two genes have evolved through the duplication of an ancestral gene. The 41% identity between the receptor proteins and the presence of type II GnRH receptors in frogs (Table 2.3) indicate that this duplication was an early evolutionary event. No other full-length human type II GnRH receptor gene has been found through searches of the genome databases at GenBank.

Sequencing of clone 9794 in the 3' region of *GNRHR2* identified sequence with homology to the *RBM8A* transcript in the antisense orientation. The sequence of this RBM8A gene, which consists of 6 exons and 5 introns (Figs 2.6 and 2.7), is identical to the published RBM8A cDNA (Conklin et al., 2000; Kataoka et al., 2000; Salicioni et al., 2000; Zhao et al., 2000). The two reported polyadenylation consensus sequences of the RBM8A gene are indicated on Figs 2.6 and 2.7. The first polyadenylation consensus sequence is located 124 bp after the *RBM8A* stop codon and results in the 0.9 kb transcript, while the second polyadenylation consensus sequence situated 2 kb further downstream results in the 3.2 kb transcript seen on Northern blots (Conklin et al., 2000; Salicioni et al., 2000; Zhao et al., 2000). Exon 2, the coding region of exon 3 and 506 bp of intron 1 of the type II GnRH receptor are located between the two polyadenylation consensus sequences. Therefore, only the largest transcript (3.2 kb) should include the type II GnRH receptor sequence (in the antisense orientation) as previously reported in EST sequences (Millar et al., 1999).

Two RBM8A transcripts have been reported and they encode a protein of either 173 or 174 amino acids (Conklin et al., 2000; Kataoka et al., 2000; Salicioni et al., 2000; Zhao et al., 2000). These proteins differ by one amino acid at position 44. Transcripts that code for two glutamic acids at positions 43-44 (in the sequence SEEGS) encode a protein of 174 amino acids, whereas transcripts coding for only one glutamic acid (position 43 only, having the sequence SEGS) encode a protein of 173 amino acids. This difference can be explained by alternative splicing. There are two splice acceptor sites at**agaag**AGG at the intron 2 exon 3 boundary (Fig. 2.6). Splicing between exon 2 and exon 3 utilizing the first -ag will result in a

transcript coding for two glutamic acids at positions 43-44, while a splicing event utilizing the second -ag will generate a transcript coding for a single glutamic acid at position 43. Transcripts with one or two glutamic acids at the homologous position have also been identified in the pig and mouse RBM8 genes (Accession numbers: AW620129, BE031709, AU067487, BF228229), suggesting that a similar alternative splicing may occur in pig and mouse.

The type II GnRH receptor gene overlaps two genes in the antisense orientation. The type II GnRH receptor gene overlaps the Pex 11B gene (peroxisome biogenesis factor 11B) in a head-to-head manner in addition to the tail-to-tail overlap with RBM8A (Morgan et al., 2003). The coding region of Pex 11B (Genbank accession number NM_003846) starts 323 bp upstream of the transcriptional start site (TSS) of the type II GnRH receptor gene identified in pituitary and foetal brain (Fig 2.2). An EST sequence (Genbank accession number AL 138314) reveals the existence of a putative transcriptional start site of Pex 11B lying only 104 bp upstream of the TSS of the type II GnRH receptor, suggesting that the promoters of Pex 11B and the type II GnRH receptor overlap.

Analysis of the genomic clone 9793 identified an RBM8B gene with several amino acid changes, one of them to a stop codon, compared to the RBM8A gene on clone 9794. In addition, this gene contains features typical of processed pseudogenes. The findings indicate that *RBM8B* on clone 9793 originated from the 3.2 kb *RBM8A* transcript by reverse transcription and insertion into the genome (Weiner et al., 1986; Maestre et al., 1995; Esnault et al., 2000; Weiner, 2000). The poly A tail of the pseudogene transcripts is represented by the dA stretch (Figs 2.6 and 2.7) and the direct repeats are the result of target-site duplication upon insertion of the reverse transcribed transcript into the genome.

The 3' UTR of *RBM8B* encodes (in the antisense orientation) exon 3, exon 2, intron 2 and the distal part of intron 1 of the type II GnRH receptor. This type II GnRH receptor gene lacks exon 1 and may consequently also be considered a pseudogene. This receptor gene lacks the typical pseudogene characteristics (e.g. it has an intron) because is has apparently been retrotransposed as part of the

RBM8B transcript. To my knowledge, this is the first example of two pseudogenes which overlap each other and are encoded by opposite DNA strands.

The chromosomal assignment of *RBM8A* in literature is contradictory. Two groups report that *RBM8A* is located on chromosome 14 (Conklin et al., 2000; Salicioni et al., 2000) and one of these groups (Salicioni et al., 2000) also suggests that chromosome 1 may contain a *RBM8A* pseudogene. A third group reports that *RBM8A* is located on chromosome 1 (Zhao et al., 2000). The assignment *RBM8A* to chromosome 14 can be explained by detection methods that are biased towards the detection of the RBM8A gene without introns. cDNA has been used as a probe in fluorescence *in situ* hybridisation (Salicioni et al., 2000), and an intron-spanning primer that corresponds to the end of exon 1 and the beginning of exon 2 has been used in the GeneBridge 4 hybrid panel system (Conklin et al., 2000). The correct assignment of *RBM8A* to chromosome 1 by Zhao *et al.* is due to the use of a primer in the GeneBridge 4 panel analysis, which corresponds to an area where the pseudogene *RBM8B* is different by one nucleotide from *RBM8A* and therefore has enabled detection of the RBM8A gene only (Zhao et al., 2000).

Chapter 3: Gene structure and promoter analysis of the marmoset type II GnRH receptor

3.1 Summary

Most vertebrates have a second form of GnRH in addition to the GnRH that regulates reproduction. This second GnRH has been designated GnRH II. Receptors for the conserved type II GnRH peptide have been cloned from amphibians and from primates, including the marmoset monkey. The aim of this study was to determine the gene structure and the transcriptional regulation of the marmoset type II GnRH receptor.

A marmoset genomic library was screened and sequencing of genomic clones revealed that the marmoset type II GnRH receptor gene consists of 3 exons and 2 introns. PCR and Southern blot analysis showed that the type II GnRH receptor and RBM8A genes are adjacent and orientated in a tail-to-tail manner, like in the human. However, in contrast to the human, these genes appear to occur only at a single locus in the marmoset genome.

2.3 kb of the 5' flanking region of the marmoset type II GnRH receptor gene was cloned in front of a luciferase reporter gene in order to study the transcriptional regulation of the type II GnRH receptor. The 5' flanking region increases luciferase activity 10.8-fold when expressed in HeLa-S3 and 3.6-fold in COS-1 cells. Progressive 5' and 3' deletions identified negative regulatory elements in the regions –2342/-1995, -1679/-1346 and -1346/-1084 and positive regulatory elements at –1995/-1679, -766/-665 and –458/-1.

In addition, a minimal promoter containing the proximal 458 bp of the marmoset type II GnRH receptor 5' flanking sequence was identified. This minimal promoter was able to stimulate a 6.6-fold increase in luciferase activity in HeLa-S3 cells compared to promoterless control.

3.2 Introduction

Most vertebrate species contain at least two different GnRH peptides. GnRH I regulates reproduction through binding to 7 TM G-protein coupled type I GnRH receptors on the cell membrane of pituitary gonadotrope cells. The subsequent release of LH and FSH into the general circulation stimulates gonadal production of sex steroids and gametes in both male and female vertebrates.

In addition to the type I GnRH peptide, which varies in sequence between species, most vertebrates have at least one additional GnRH. This GnRH peptide (GnRH II) is structurally conserved in species from primitive fish to man, suggesting an important function (King and Millar, 1995; King and Millar, 1997). GnRH II has been implicated in reproductive behaviour in sparrows and in musk shrews (Maney et al., 1997; Temple et al., 2003). There are also indications of a possible role of GnRH II in preferentially stimulating FSH release in sheep (Millar et al., 2001). The best characterised role of GnRH II is possibly its function as a neurotransmitter in the sympathetic ganglia of bullfrog. GnRH II inhibits a potassium current (M-current) leading to a slow depolarisation of sympathetic neurons (Jan et al., 1980; Jones, 1987).

A receptor specific for GnRH II has been cloned from frog sympathetic ganglia (B. Blackman, unpublished result) and from bullfrog hindbrain (Wang et al., 2001a). Type II GnRH receptors have also been cloned from three species of monkey and from humans but the transcripts in humans encode a non-functional receptor (Millar et al., 2001; Neill et al., 2001; van Biljon et al., 2001) (Chapter 2 this thesis). The primate type II GnRH receptors have 90-93% amino acid identity to each other and 52-54% amino acid identity to the frog type II GnRH receptors. The amino acid identity to the human type I GnRH receptor is 41% (Chi et al., 1993; Millar et al., 2001; Neill et al., 2001; Wang et al., 2001a). As with the type I GnRH receptors, the type II GnRH receptors couple to G proteins of the $G\alpha_{q/11}$ family. The affinity of type II GnRH receptors is approximately 40-fold higher for GnRH II than for mGnRH (Millar et al., 2001). GnRH II is also more potent than mGnRH in stimulating second messenger production by all type II GnRH receptors (Millar et al., 2001; Neill et al.,

2001; Wang et al., 2001a; Neill, 2002). A striking feature of type II GnRH receptors is the presence of a C-terminal tail. This is in contrast to type I GnRH receptors which do not have a C-terminal tail. The C-terminal tail of non-mammalian type I GnRH receptors has been implicated in agonist-stimulated desensitisation and in rapid internalisation (Heding et al., 1998; Pawson et al., 1998).

The type II GnRH receptors are widely expressed. The tissue distribution of the primate type II GnRH receptors has been investigated by means of RT-PCR, cloning, Northern blot and RNA dot blot analysis. The type II GnRH receptor is expressed in many reproductive tissues including the pituitary, testis, ovary, oviduct, uterus, prostate, breast and placenta. The type II GnRH receptor is also expressed in many areas of the brain including the midbrain, pons and cerebellum, and in several areas of the forebrain including the hypothalamus. In addition, the type II GnRH receptor is present in many peripheral tissues (Millar et al., 2001; Neill et al., 2001; van B ljon et al., 2002) (Chapter 2 of this thesis).

The wide tissue distribution of the type II GnRH receptor mRNA is similar to the tissue distribution of the type I GnRH receptor transcript. The mammalian type I GnRH receptor was initially thought to be expressed only in the pituitary. Indeed, the function of the GnRH receptor in the gonadotrope cells of the pituitary has been well characterised. However, later studies identified type I GnRH receptor mRNA in ovary and testis (Kaiser et al., 1992; Reinhart et al., 1992; Tsutsumi et al., 1992; Chi et al., 1993). Expression has also been detected in the breast and prostate by RT-PCR and there are indications of expression of the type I GnRH receptor in breast tumours, placenta, T-cells, liver, heart, skeletal muscle and kidney (Belisle et al., 1984; Kakar et al., 1992; Kakar et al., 1994; Kakar and Jennes 1995; Wilson et al., 1995; King and Millar 1997).

The basal expression of the murine type I GnRH receptor in αT3-1 cells is regulated by a tri-partite enhancer. This enhancer includes binding sites for AP-1 and SF-1 and a novel element called GRAS (Duval et al., 1997a; Duval et al., 1997b). A mutation of any of the three elements reduces expression, and mutation of all three elements abrogates expression in αT3-1 cells transfected with a promoter reporter

construct. A similar enhancer has been described in the rat but an additional upstream enhancer is necessary for maximal activity (Pincas et al., 2001a). A tri-partite basal enhancer similar to the murine enhancer has not been described for the human gene. However, a SF-1 binding site has been identified in the human type I GnRH receptor promoter 134 bp upstream of the ATG. A mutation of this binding site decreases expression by 80% in αT3-1 cells but not in COS-7 or SKOV-3 cells. This implicates the SF-1 binding site in gonadotrope-specific expression of the type I GnRH receptor (Ngan et al., 1999).

Transcription of the type I GnRH receptor is up-regulated by GnRH. The GnRH responsiveness of the murine promoter is mediated by the AP-1 and GRAS sites of the basal enhancer. A mutation of either GRAS or AP-1 abolishes the GnRH responsiveness of the promoter. AP-1 binds to both the AP-1 and GRAS sites. In addition, transcription factors of the SMAD family bind to GRAS. The GnRH responsiveness is mediated by PKC and probably the JNK of the MAPK pathways (Norwitz et al., 1999a; Norwitz et al., 1999b; White et al., 1999; Norwitz et al., 2002b; Ellsworth et al., 2003).

The mouse type I GnRH receptor promoter is regulated by activin A and glucocorticoids in addition to its regulation by GnRH. Activin stimulation of the promoter is also mediated by the GRAS element of the basal enhancer (Norwitz et al., 2002a; Norwitz et al., 2002b). The glucocorticoid responsiveness of the mouse type I GnRH receptor is mediated by the AP-1 site of the basal enhancer (Maya-Nunez and Conn, 2003). PACAP stimulates the transcriptional activity of the rat GnRH receptor gene through the SF-1 element of the basal enhancer and through a CRE in the proximal promoter (Pincas et al., 2001b). Progesterone responsiveness of the human type I GnRH receptor gene is mediated by a progesterone response element in the proximal promoter (Cheng et al., 2001a; Maya-Nunez and Conn, 2003).

Two additional promoter regions have been identified in the 5' flanking region of the human type I GnRH receptor. These promoter regions are preferentially active in cell lines of placental or ovarian origin, suggesting a possible differential promoter

usage of the type I GnRH receptor in different tissues (Cheng et al., 2001b; Cheng et al., 2002a).

The mammalian type I GnRH receptor genes and the human type II GnRH receptor gene consist of 3 exons and 2 introns. Several non-mammalian GnRH receptor genes have been identified with an additional exon and intron in the 5' UTR or in the N-terminal area of the coding sequence (Troskie et al., 2000; Okubo et al., 2001; Sun et al., 2001).

The recent cloning of the marmoset type II GnRH receptor cDNA sets the stage for elucidating the gene structure and the transcriptional regulation of mammalian type II GnRH receptors.

3.3. Materials and methods

3.3.1 Genomic library screening

A genomic DNA library (in lambda vector EMBL3) of a male Common marmoset monkey (*Callithrix jacchus*) was very kindly provided by Prof. Shoji Kawamura, University of Tokyo, Japan. A probe for library screening was PCR amplified from the marmoset type II GnRH receptor cDNA (Millar et al., 2001) by using a sense primer to TM 2, MarTM2S and an antisense primer to TM 7, MarTM7AS (Table 3.1, Fig 3.1). The DNA was purified from an agarose gel using NucleoSpin Extract (Machery-Nagel, Düren, Germany) and the DNA was quantified by gel electrophoresis. 300ng DNA was random labelled with ^{32}P-dCTP in 4 reactions (75 ng in each reaction) and purified as described in Chapter 2.

Approximately $2x10^6$ plaque-forming units (PFU) were plated on a total of 20 plates (host, *E.coli* XL-1 Blue) and lifted onto Magnacharge nylon membranes (Osmonics, Westborough, MA, USA). The DNA was denatured by incubating the membranes with the DNA side up for 7 minutes on Whatman paper soaked in denaturing solution (0.5M NaOH/1.5M NaCl). The DNA on the membranes was neutralized for 3 minutes on Whatman paper soaked in neutralizing solution (1.5M NaCl/0.5M Tris-HCl pH7.2) and the membranes were rinsed in 2 x SSC. The DNA was fixed by incubating the membranes for 20 minutes on Whatman paper soaked in 0.4M NaOH. The membranes were briefly rinsed in 5 x SSC and air dried.

The membranes were prehybridised for 2 hours at 60°C in 6 x SSC, 0.4% SDS, 5 x Denhardt, 20mM NaH_2PO_4, 0.5 mg denatured herring sperm DNA (hsDNA) and hybridised with denatured probe overnight at 60°C in the same solution but without Denhardt. The membranes were washed for 20 minutes at room temperature (2 x SSC, 0.1% SDS), 20 minutes at 60°C (2 x SSC, 0.1% SDS), 20 minutes at 60°C (1 x SSC, 0.1% SDS) and were exposed to film overnight at -70°C. 20 plaques were picked, plated on separate plates and then blotted and a second screening was performed as described above. 20 plaques were picked (one plaque per plate) for a third screening, resulting in three independent positive clones. Liquid lysate was

made from two plaques of each positive clone (Ausubel et al., 2001) and DNA was isolated using Nucleobond L-50 (Machery-Nagel, Düren, Germany).

Southern blotting was performed on 6 lambda DNA preps (two DNA preps for each independent clone). The lambda clones were digested with *Bam*HI, *Sal*I, *Bam*HI-*Sal*I or left uncut. The DNA was separated on a 0.8% agarose gel and depurinated in 0.25M HCl for 30 minutes. The DNA in the gel was denatured, neutralized, transferred to a nylon membrane and fixed as described in Chapter 2.

Probes were PCR amplified from cloned type II GnRH receptor cDNA (Millar et al., 2001) with primers to exon 1, MarstartEI and MarTM2A, and for exon 2-3, MarEC2S and MarTM6AS (Table 3.1, Fig 3.1). The DNA was purified from the gel as described above, random labelled with ^{32}P-dCTP and purified as described in Chapter 2.

The blots were hybridised with either an exon 1 probe or an exon 2-3 probe as described above but with 0.1mg/ml hsDNA instead of 0.5mg/ml. Stringency washes were performed as described above, followed by overnight exposure to film at –70°C. The presence of exon 2 and exon 3 in all three lambda clones was confirmed by PCR with primers MarEC2S and MarTM6AS. The presence of exon 1 on lambda clones 1 and 2 and the absence of exon 1 on clone 3 was confirmed by PCR with primers MarstartEI and MarTM2AS2.

Table 3.1. Oligonucleotide primers used in this study.

	Oligonucleotide sequence (5' – 3')
MarTM2S	CACTGTTCAGTGGCTGGCTGG
MarTM7AS	CAAAGGAGCATTGAGGAGGCCA
MarstartEI *	TATGAATTCATCCTCCTATCTCCAGGC
MarTM2A	TTCCAGGTGGCATCTAGGG
MarEC2S	CAGTTCCCTTCACTCAGTGTGC
MarTM6AS	CGGGGAAAACCAGTACCACAG
MarTM2AS2	AAGAGTCTCCTGACCGGAGAG
MarEC2.1AS	TGGCTCCCCTTCCTTGTCCG
MarTM32S	GTATGCTGCAGCTTTCCTGCC
MarEC2.2AS	GGGGCTGGACACACCGAGCA
EMBL R	GTTCATTACTGAACACTCGTCCG
MarI2.1S	GTGCAAGCTGTCTTCACCAG
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG
PGL3adapS *	CGGGCCCGATATCCCGGGAATTCTCGAGA
PGL3adapAS *	GATCTCTCGAGAATTCCCGGGATATCGGGCCCGGTAC
MtstartEcoRI *	ATTAGAATTCGGTGGCCTGGAGATAGGAGGATG
5'SD1 *	ATATGGGCCCGCCCCGGCTCGGCGGCC
5'SD2 *	ATATGGGCCCCCTCCTGCGCGACCCAACG
5'SD3 *	ATATGGGCCCAGAAAGCGAGTGGAGCTGGC
5'SD4 *	ATATGGGCCCCCTGTCTTTGAGCCTGTCTGC
MtPro100f	ATGTAGCTAGCGGGCTTGGCTCTGAGCACTG
MtPro100r	ATGTACTCGAGCGTGACTAGGGGCGAGAAGT

* Restriction sites are underlined.

3.3.2 PCR analysis of marmoset genomic DNA and lambda genomic clones

Genomic DNA was prepared by homogenising 1 gr of marmoset monkey liver with mortar and pestle under liquid N_2. Powdered tissue was resuspended in 12 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 8, 25 mM EDTA pH8, 0.5% SDS, 0.1 mg/ml proteinase K), incubated overnight at 50°C and then followed by phenol extraction. DNA was precipitated from the aqueous phase with ½ volume 7.5 M ammonium acetate and 2 volumes ethanol, washed in 70% ethanol, dried and resuspended in TE buffer. RBM8A was PCR amplified from marmoset and human genomic DNA or from marmoset lambda DNA clone 3 with primers ZRNP1S and ZRNP1A (Table 2.1). PCR between exon 2 of the type II GnRH receptor and RBM8A was done with primers MarEC2S and ZRNP1S for marmoset RBM8A and with primers 10417 (Fig 2.1; Table 2.1) and ZRNP1S for human RBM8A.

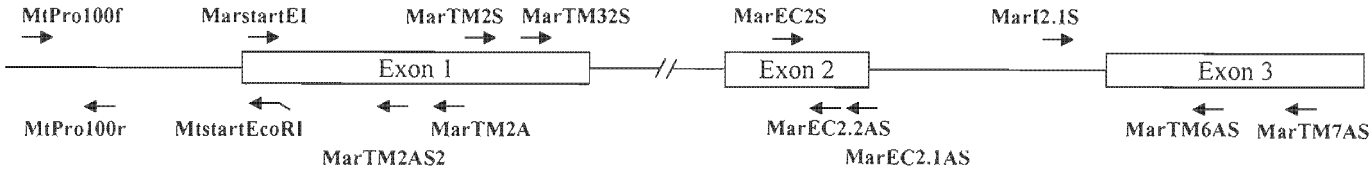

Figure 3.1. Schematic representation of the marmoset monkey type II GnRH receptor gene and the location of the indicated oligonucleotides. The exons are shown as boxes, the introns as lines and the primers used in this study are indicated by arrows.

A cDNA fragment, containing Intron 1 plus flanking exon 1 and exon 2 sequences, was PCR amplified from marmoset genomic DNA and from lambda clone 2 using primers MarTM2S and MarEC2.1AS. The PCR products were purified from an agarose gel as described above, quantified by gel electrophoresis and sequenced with primers MarTM32S and MarEC2.2AS using DYEnamic ET Dye Terminator on a Megabace 500 automated sequencer (Amersham Biosciences, Little Chalfont, UK). A cDNA fragment, containing exon 2, Intron 2 and exon 3, was PCR amplified from lambda clone 3 using primers MarEC2S and the vector primer EMBL R. The PCR products were purified from an agarose gel as described above, quantified by gel electrophoresis and sequenced with primers MarEC2S, MarI2.1S and MarTM7AS.

3.3.3 5' RACE on marmoset type II GnRH receptor cDNA

Total RNA was extracted from whole brain of a male marmoset and poly A^+ RNA was isolated, as described in Chapter 2. Marathon cDNA was made from 1 μg poly $(A)^+$RNA using the Marathon cDNA kit (Clontech, Palo Alto, CA, USA) with a random hexamer nucleotide as primer. 5' RACE was performed on 1μl of a 1:50 dilution of cDNA with adaptor primer AP1 and gene-specific primer MarTM7AS. 5μl of a 1:50 dilution of the above PCR product were amplified using adaptor primer AP2 (Table 2.1) and nested gene-specific primer MarTM6AS (Table 3.1). The PCR product was purified from a gel using QiaQuick (Qiagen, Hilden, Germany), cloned into pMOS*blue* and sequenced with vector primers T7 and U19 as described in Chapter 2.

3.3.4 Sequencing of the 5' flanking region of the marmoset type II GnRH receptor gene

Lambda clone 1 was digested with *Sal*I-*Bam*HI and a 3 kb fragment, containing part of exon 1 and the upstream area, was cloned into pBluescript II SK (-), yielding clone MtProSK. Transposon insertions were made for sequencing the insert by using the EZ::TN <KAN-2> insertion kit (Epicentre, Madison, Wi, USA) according to the manufacturer's instructions. In brief, 0.2μg of plasmid MtProSK was incubated with 0.05 pmole of the EZ::TN <KAN-2> transposon and with transposase for 2 hours at 37°C followed by transformation of competent *E.coli* DH10B. Kanamycin and ampicillin resistant colonies were picked for colony PCR screening with vector-specific primer T7 and transposon-specific primer KAN-2 FP-1 or KAN-2 RP-1. DNA was prepared using NucleoSpin Plasmid (Machery-Nagel, Düren, Germany) from colonies with the transposon inserted at appropriate places, followed by sequencing of the DNA with transposon primers KAN-2 FP-1 and KAN-2 RP-1.

3.3.5 Generation of promoter constructs of the marmoset type II GnRH receptor gene

2.3 kb of the marmoset promoter region between the *Sal*I site and the ATG start codon was cloned into the firefly luciferase reporter vector pGL3basic (Promega, Madison WI, USA) in which the multiple cloning site (MCS) had been replaced for the ease of making subsequent deletions (appendix 5.1). The MCS was replaced by incubating 20 μl (20 μM) of each of two oligonucleotides PGL3adapS and PGL3adapAS (Table 3.1, appendix 5.1) for 2 minutes at 94°C and 10 minutes at 25°C, followed by ligation into *Kpn*I-*Bgl*II digested pGL3basic yielding pGL3. MtPro (-2342/-1), containing 2.3 kb of the promoter, was constructed by ligating two fragments of the marmoset promoter into pGL3. The first fragment consisted of a 2.14 kb *Apa*I-*Kpn*I fragment of MtProSK. The second fragment consisted of a 200 bp *Kpn*I-*Eco*RI fragment containing the region immediately upstream of the ATG. This fragment was generated by PCR amplification of a 250 bp region immediately upstream of the ATG using primers MtstartEcoRI and KAN2-RP with a transposon insertion clone as template. The PCR product was digested with *Kpn*I and *Eco*RI

and the 200 bp fragment was purified from an agarose gel. The two fragments were ligated into *Apa*I-*Eco*RI digested pGL3, yielding MtPro (-2342/-1) (appendix 5.3).

Progressive deletions were made from the 5' end of MtPro. MtPro was digested with *Bst*XI, *Pvu*II, *Stu*I, *Nco*I, *Sma*I or *Dra*I and the ends of the *Bst*XI and *Nco*I digests were made blunt with T4 DNA polymerase (Amersham Biosciences, Little Chalfont, UK). The digests were cut with *Eco*RI, purified from an agarose gel and cloned in *Eco*RV-*Eco*RI digested and dephosphorylated pGL3, yielding constructs 5'BstxI (-1995/-1), 5'PvuII (-1679/-1), 5'StuI (-1346/-1), 5'NcoI (-1084/-1), 5'SmaI (-766/-1) and 5'DraI (-458/-1).

Four constructs, SD1EI (-715/-1), SD2EI (-665/-1), SD3EI (-615/-1) and SD4EI (-565/-1), were made with 50 bp progressive 5' deletions from the *Sma*I site. MtPro was digested with *Dra*I-*Eco*RI and a 460 bp fragment was purified. Four PCR products were amplified from template MtProSK with sense primers 5'SD1, 5'SD2, 5'SD3 or 5'SD4 and antisense primer MarTM2AS2, followed by digestion of the PCR products with *Apa*I and *Dra*I (appendix 5.3). The 260 bp, 210 bp, 160 bp or 110 bp fragments of the digested PCR products were ligated together with the 460 bp *Dra*I-*Eco*RI fragment of MtProSK into *Apa*I-*Eco*RI digested pGL3.

Progressive deletions were made from the 3' end of MtPro. Constructs 3'DraI (-2342/-458), 3'SmaI (-2342/-766), 3'NcoI (-2342/-1084), 3'StuI (-2342/-1346), 3'PvuII (-2342/-1679) and 3'BstxI (-2342/-1995) were made by digestion of MtPro with *Dra*I, *Sma*I, *Nco*I, *Stu*I, *Pvu*II or *Bst*XI and the ends of the *Bst*XI and *Nco*I digests were made blunt with T4 DNA polymerase. The digests were then cut with *Apa*I, purified from gel and cloned in *Apa*I-*Sma*I digested and dephosphorylated pGL3. Sequence analysis identified a *Bgl*II site 197 bp upstream from the ATG start codon of the marmoset promoter and a *Bgl*II site in the vector immediately downstream of the insert. The 3' deletion construct 3'BglII (-2342/-197) was made by digesting MtPro with *Bgl*II and purifying and religating a 7 kb fragment.

A 100 bp fragment, 665 to 766 bp upstream of the ATG, was cloned in front of the heterologous thymidine kinase promoter, upstream of the luciferase reporter gene. The 100 bp region was PCR amplified using the primers MtPro100f and MtPro100r

with the full-length promoter construct MtProSK as template. The PCR products were purified, digested with *Nhe*I and *Xho*I, gel purified and cloned into *Nhe*I-*Xho*I digested and dephosphorylated pTAL (Clontech, Palo Alto, CA, USA) (appendix 5.2), yielding construct (-766/-665) TK-Luc.

3.3.6 Cell culture and transfections

COS-1 cells and HeLa-S3 cells, a human cervical carcinoma cell line, were obtained from American Type Culture Collection (ATCC) and were maintained in 150cm^2 flasks (Corning, New York, USA) in Dulbecco's modified Eagle's medium (DMEM, Highveld Biological, Lyndhurst, RSA) containing 10% foetal bovine serum (Delta Bioproducts, Kempton Park, RSA) in a 10% CO_2 incubator at 37 °C.

DNA for transfections was prepared from 250 ml overnight culture of *E.coli* DH10B in 2 x YT broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) with 100 μg/ml ampicillin. DNA was isolated using the Nucleobond PC500 maxiprep kit (Machery-Nagel, Düren, Germany). HeLa-S3 cells were plated at a density of 100 000 cells/well of a 24-well plate (NUNC, Roskilde, Denmark) 4-5 hours prior to transfection. Transfections were performed using FuGene 6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Each marmoset promoter firefly luciferase construct (150 ng/well) was co-transfected with 50 ng/well of pRL-TK, a renilla luciferase control reporter vector (Promega, Madison WI, USA). The renilla luciferase gene is under the control of the thymidine kinase promoter, which gives a low level of constitutive activity. The renilla luciferase activity was used to normalize the experimental firefly luciferase data for transfection efficiency (see appendix 5.1 for a map of pRL-TK). For each well, the DNA for transfection and 0.75 μl FuGene 6 were each diluted in 50 μl serum free DMEM. The diluted DNA and the diluted FuGene 6 were combined and incubated for 25-45 minutes at room temperature before being added to the cells. Each transfection was performed in duplicate. The transfection medium with FuGene 6 and DNA was changed 24 hours after transfection to DMEM with 10% foetal bovine serum supplemented with PS (10 U/ml penicillin and 80 μg/ml streptomycin) and the cells were harvested 48 hours after transfection.

COS-1 cells were transfected using the DEAE-dextran method. 200 000 cells were seeded in 12-well plates 20 hours prior to transfection. The cells were incubated for 4 hours in serum-free DMEM with DEAE-dextran and with 1.5 μg promoter reporter construct and 0.5 μg pRL-TK. This was followed by a 1 hour incubation in DMEM containing 2% foetal bovine serum and 200 μM chloroquine and by a 1.5 minute incubation in 10% DMSO. The cells were grown for 48 hours in DMEM with PS before harvest.

3.3.7 Luciferase assay

Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison WI, USA) on a LuminoSkan Ascent luminometer (Thermo Labsystems, Helsinki, Finland) according to the manufacturers' protocols. In brief, cells were harvested 48 hrs after transfection by lysis in 250 μl Passive Lysis Buffer for 20 minutes at room temperature followed by one freeze-thaw cycle. Lysates were briefly centrifuged before 10 μl was loaded onto a 96 well luminometer plate. The luminometer was first set to inject 50 μl firefly luciferase substrate LARII, followed by a 5 second shake step and 5 seconds of measurement after which 50 μl of Stop & Glo was injected, followed by a 5 second shake step and 5 seconds of measurement of renilla luciferase activity.

3.3.8 Sequence and data analysis

All DNA sequence analysis was done using DNAMAN (Lynnon Biosoft, Quebec, Canada). Analysis of the 5' flanking region for possible promoters was done with PromoterInspector (Genomatix Gmbh) (Scherf et al., 2000) or Promotorscan (Prestridge, 1995). Analysis for potential transcription factor binding sites was done with MatInspector (Genomatix) (Quandt et al., 1995).

Each firefly luciferase measurement was normalized to the level of renilla activity. The results were then calculated as fold-change relative to the average level of expression of promoterless pGL3 in each experiment. The data were analysed using the method of analysis of variance. The method is used to identify the sources of variation in the data, and tests of significance, using student's t-test, are

carried out using the appropriate mean square for error (SAS statistical package, SAS Institute, Cary, NC, USA). The sources of variation are: experiments, promoter constructs, error (experimental) and error (sampling). The error (experimental) is the residual variation after accounting for variation between experiments and variation between constructs. The mean square for this error is the appropriate mean square for carrying out tests of significance involving the constructs. The error (sampling) is the sampling error between duplicate transfections.

3.4. Results

3.4.1 Marmoset genomic library screening

In order to study the gene structure of the marmoset type II GnRH receptor a genomic DNA library of the Common marmoset monkey was screened with a cDNA probe encoding the marmoset type II GnRH receptor. The library screening yielded three independent clones. Two DNA preparations were made of each of the positive lambda clones and they were subjected to Southern blot and PCR analysis. The clones were digested with *Bam*HI, *Sal*I, *Bam*HI-*Sal*I or left uncut for the Southern blot.

Probing with an exon 1 probe shows that clones 1 and 2, but not clone 3, contain exon 1 of the type II GnRH receptor (Fig 3.2A). The second DNA preparation of lambda clone 2 (DNA prep 2b) does not show any positive band for the exon 1 probe. This is probably due to low DNA yield as determined by gel electrophoresis (gel photo not shown). The *Bam*HI, *Sal*I and *Bam*HI-*Sal*I digests give bands of >12 kb, 8 kb and 3 kb respectively when hybridised with the exon 1 probe. Probing with an exon 2-3 probe shows that all three lambda clones contain exon 2 and/or exon 3 (Fig 3.2C). The banding pattern is identical for clones 1 and 2 but different for clone 3. The *Bam*HI, *Sal*I and *Bam*HI-*Sal*I digests of clones 1 and 2 give bands of 5 kb, 8 kb and 5 kb respectively, whereas the same digests give bands of >12 kb, 9-10 kb and 9-10 kb respectively for clone 3 when hybridised with the exon 2-3 probe.

PCR amplification with primers to exon 2 and exon 3 (MarEC2S – MarTM6AS) confirmed the presence of exon 2 and exon 3 in all three lambda clones. PCR amplification with exon 1 primers (MarstartEI - MarTM2AS) confirmed the presence of exon 1 in clones 1 and 2 and the absence of exon 1 in clone 3 (results not shown). The Southern blot and the PCR anlysis indicate that clones 1 and 2 are identical and contain exons 1, 2 and 3 of the type II GnRH receptor gene but not RBM8A. Clone 3 contains RBM8A and exons 2 and 3 of the receptor gene.

3.4.2 Structure of the marmoset type II GnRH receptor gene

Sequence analysis of the cDNA of the marmoset type II GnRH receptor identified *Bam*HI sites at positions equivalent to exons 1 and 3 of the human type II GnRH receptor. The Southern blot on *Bam*HI digested lambda clones probed with an exon 2-3 probe shows a band of approximately 5 kb (Fig 3.2C). A band of similar size was obtained when probing *BamH*I digested human genomic clones with human type II GnRH receptor cDNA probe (Chapter 2). This suggests that introns (or a single intron) of sizes equivalent to the human type II GnRH receptor gene are present in the marmoset type II GnRH receptor gene (Chapter 2). PCR between exon 1 and exon 2 of lambda clone 2 and of marmoset genomic DNA gave a band of 4.6 kb (result not shown), indicating that the length of intron 1 is 4.2 kb. The exon/intron boundaries were sequenced and the alignment of the genomic sequence with the cDNA sequence confirmed that intron 1 is at the same position in TM4 as it is for the human type I and type II GnRH receptors. The sequencing of a PCR product between exon 2 and the vector primer EMBL R of lambda clone 2 revealed the presence of a 750 bp intron 2 at the same position as intron 2 in the human type II GnRH receptor gene in ICL 3 (Chapter 2). The splice-donor and splice-acceptor consensus sites -gt and -ag are conserved for both introns (Table 3.2).

5' RACE was performed in order to identify the transcriptional start site (TSS). PCR and nested PCR with adaptor-specific primers (AP1 and AP2) and primers to exon 3 of the type II GnRH receptor were performed on Marathon cDNA from marmoset whole brain. Cloning and sequencing of the 5' RACE products revealed that a transcriptional start site is located 567 bp upstream of the methionine start codon. This transcript however has exon 2 spliced out. The splice acceptor site at the intron 2 / exon 3 junction is used when splicing out intron 1, with the result that an area containing intron 1, exon 2 and intron 2 is spliced out, giving rise to a transcript with exon 1 spliced to exon 3. This transcript is not a full-length transcript of the marmoset type II GnRH receptor and may be part of an aberrant splicing of the full-length transcript. The TSS of the full-length wild-type transcript might be at the same position as the TSS identified in this study. Alternatively, the TSS will differ if the full-length wild-type transcript is the result of different promoter usage.

An alignment of the 5' RACE sequence with the genomic sequence of the marmoset type II GnRH receptor shows that a putative third intron of 293 bp (position -472/-180 relative to the ATG translational start codon) in the 5' UTR is spliced out as well. However, this putative intron is part of an aberrant transcript. A full-length 5' RACE transcript containing exons 1, 2 and 3 has not been identified in this study and the putative third intron may or may not be present in the full-length wild-type transcript.

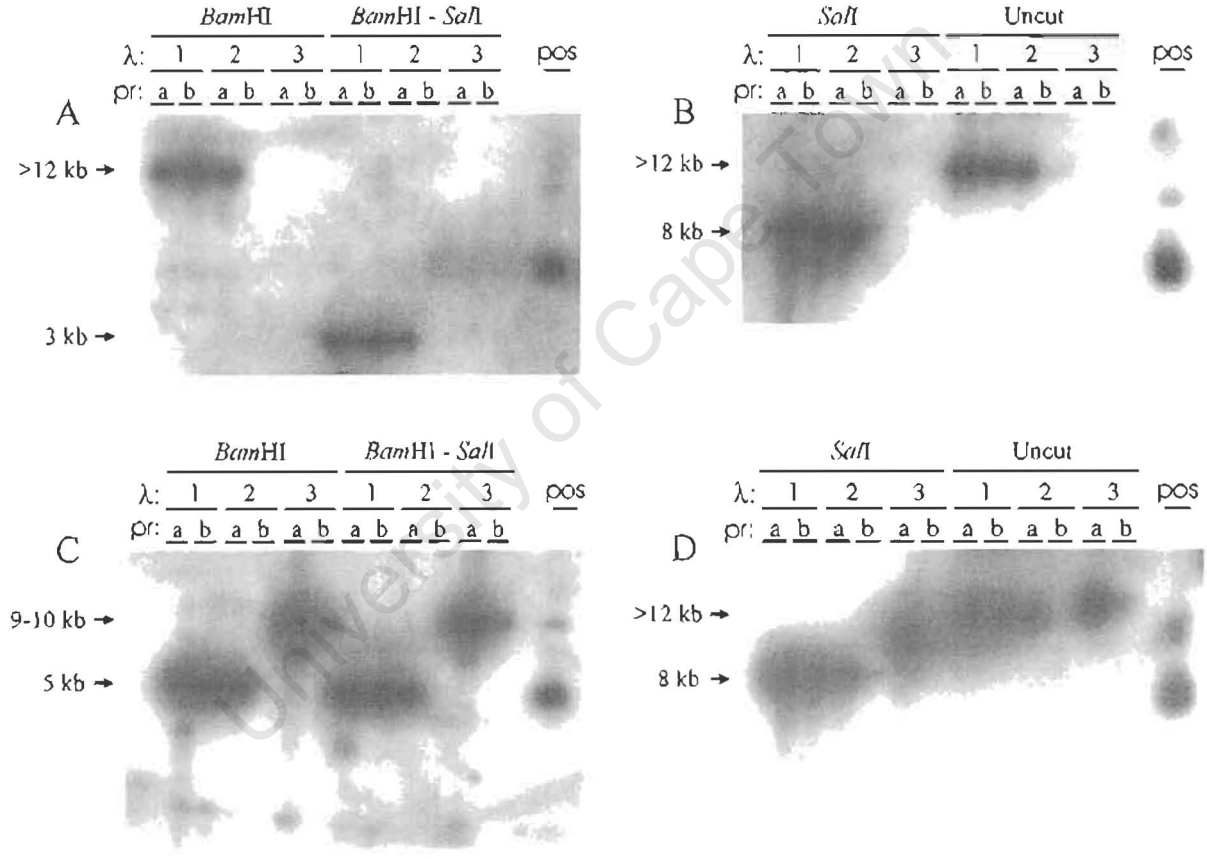

Figure 3.2. Southern blot analysis of marmoset genomic lambda clones. Two DNA preparations (a and b) of each of the lambda clones 1, 2 and 3 were digested with the indicated enzymes, separated on an agarose gel and transferred to a nylon membrane. The membranes were hybridised with a ^{32}P-dCTP labelled probe specific for exon 1 (A and B) or exon 2-3 (C and D) of the marmoset type II GnRH receptor cDNA. Pos, indicating positive control, contains 1 ng of marmoset type II GnRH receptor cloned in pCDNA3.1, uncut.

Table 3.2 Exon-intron junction sequences of the marmoset type II GnRH receptor gene[#]

	Sequence
Intron 1	CCCAG**gt**gagtgacg - 4.2 kb - tcccctcc**ag**CTGTT
Intron 2	CCATG**gt**gagaatcc - 730 bp - tgaattct**ag**CCCCT

Exon sequence is shown in uppercase letters and intron sequence in lowercase letters. The intron splice donor and acceptor consensus sequences are in bold print.

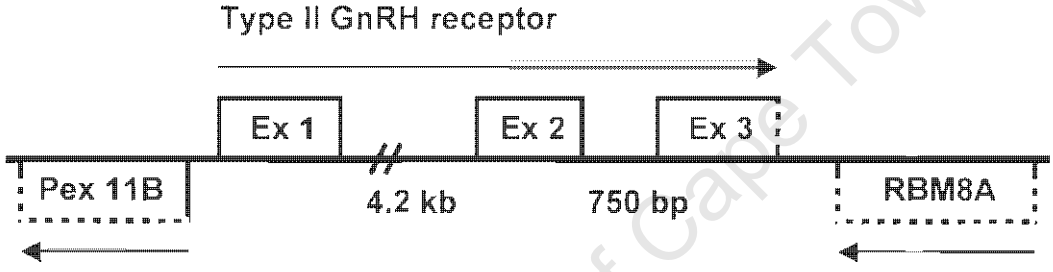

Figure 3.3. Schematic representation of the structure of the marmoset type II GnRH receptor gene. The exons are indicated by boxes. Dashed lines indicate either exons of unknown size (exons 1 and 3 of the type II GnRH receptor gene) or an unknown number of exons (RBM8A and Pex 11B, peroxisome biogenesis factor 11B).

3.4.3 Genomic organisation of the marmoset type II GnRH receptor and RBM8A genes

PCR analysis was employed to investigate the gene organisation of the RBM8A and type II GnRH receptor genes in the marmoset. PCR with primers to exon 1 and exon 6 of the human RBM8A gene gives an expected 1.5 kb band for the positive control containing a genomic clone of the human type II GnRH receptor chromosome 1 locus (Fig 3.4, lane 11). The same primers give a 1.8 kb band for marmoset genomic DNA (Fig 3.4 lane 1) and marmoset genomic lambda clone 3 but not clone 1 (Fig 3.4, lanes 5 and 3). This confirms previous PCR and Southern blot results, which indicate the presence of RBM8A on lambda clone 3 but not on clone 1. The presence of RBM8A and the type II GnRH receptor on the same genomic clone indicates that the two genes are in proximity to each other in the marmoset genome. A faint band of a different size is seen for lambda clone 1 (Fig 3.4, lane 3); however, a band of the same size is seen in the negative control (Fig 3.4, lane 7) indicating that this band is probably non-specific. The RBM8A primers are located at the translational start and termination sites of RBM8A. The larger bands for marmoset (1.8 kb for marmoset and 1.5 kb for human) indicate that the marmoset RBM8A gene contains more intron sequence than the human gene.

PCR amplification using the RBM8A primers on genomic DNA of the marmoset gives a single band of 1.8 kb, but two bands are obtained for human genomic DNA (Fig 3.4, lanes 1 and 9). The 1.5 kb band corresponds to the human RBM8A gene on chromosome 1 and the 0.5 kb band corresponds to the intron-less RBM8B pseudogene on chromosome 14. The presence of only one band (1.8 kb) for marmoset genomic DNA indicates that the marmoset genome does not contain a locus that is homologues to the retrotransposed RBM8B locus on chromosome 14 in humans.

PCR between the type II GnRH receptor and RBM8A genes gives a single 4 kb band for marmoset genomic DNA and for marmoset clone 3 (Fig 3.4, lanes 2 and 6). The same PCR gives a 3 kb band for the human genomic *Eco*RI subclone but no bands for marmoset lambda clone 1 (Fig 3.4, lanes 12 and 4). The same primers

used in PCR on human genomic DNA give, as expected, two bands of 3 kb and 2 kb (Fig 3.4, lane 10), corresponding to the human chromosome 1 and 14 (intron-less RBM8B) loci respectively. The PCR reaction with primers to type II GnRH receptor and RBM8A confirms that these two genes are adjacent and shows that they are orientated in a tail to tail manner in marmoset monkey (Fig 3.4 and 3.3).

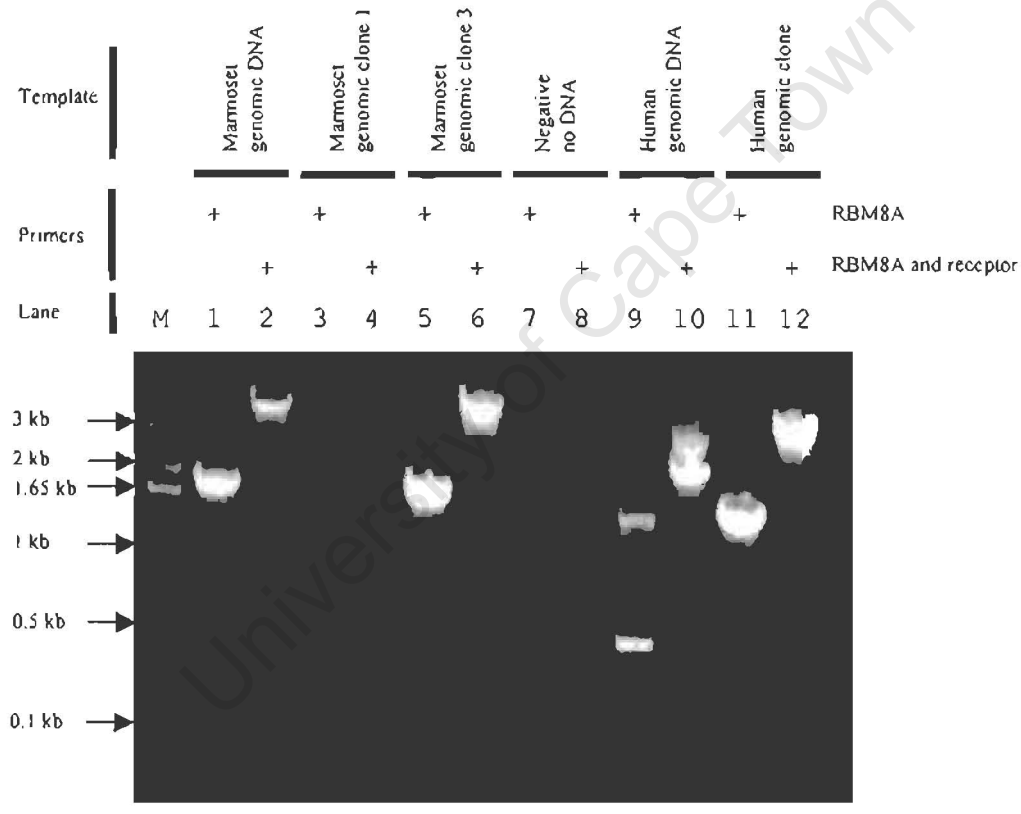

Figure 3.4. PCR analysis of the genomic organisation of the marmoset type II GnRH receptor gene in relation to the marmoset RBM8A gene. Primers ZRNP1S and ZRNP1A were used to amplify marmoset or human RBM8A, lanes 1, 3, 5, 7, 9 and 11. Primers ZRNP1S and MarEC2S were used for PCR between marmoset type II GnRH receptor and RBM8A, lanes 2, 4, 6 and 8. Primers ZRNP1S and 10417 were used in PCR between human type II GnRH receptor and RBM8A, lanes 10 and 12. Lanes 1-2, marmoset genomic DNA; 3-4, marmoset lambda clone 1; 5-6, marmoset lambda clone 3; 7-8, negative control, no template; 9-10, human genomic DNA; 11-12, positive control, genomic *Eco*RI subclone containing human *GNRHR2*.

3.4.4 Prediction of promoter regions of the marmoset type II GnRH receptor gene

The cloning of the marmoset type II GnRH receptor gene and the 5' flanking region enabled sequence analysis of the 5' flanking region for predicted promoter elements. The sequence of the 5' flanking region was analysed using two different algorithms in order to locate which area of the 5' flanking region might contain a promoter. The program Proscan predicted the presence of a single promoter region between nucleotides –666 to -416 (Prestridge, 1995) (Fig 3.5). The TSS was predicted to be at position –431, 30 bp downstream of a muscle-type TATA box. Analysing the sequence with PromoterInspector identified one possible promoter region between –509 to –298 (Scherf et al., 2000). An analysis of the 2.3 kb 5' flanking region with MatInspector identified 225 putative transcription factor binding sites (Quandt et al., 1995). The putative transcription factor binding sites in the area between the *Sma*I (-766) and *Dra*I (-458) sites, as well as putative oestrogen receptor, progesterone receptor and SF-1 binding sites, are depicted in figure 3.5.

A sequence alignment showns that the nucleotide identity between the 5' flanking regions of the human type I GnRH receptor and the marmoset type II GnRH receptor is 39%. The nucleotide identity between any two random sequences is also 39%. This indicates that there is no overall sequence conservation between the 5' flanking regions of the human type I GnRH receptor and the marmoset type II GnRH receptor. Binding sites for AP-1 (TGAGCTA), SURG-1 (GCTAATTG) and GRAS (TGCTAGTCACAACA), all of which are important in the regulation of the mouse type I GnRH receptor, are not present in the marmoset type II GnRH receptor 5' flanking region. However, a SF-1 binding site, a CREB binding site and two progesterone response elements are found in the marmoset promoter at –364/-358, -535/-515, –337/-319 and –156/–130 respectively. No androgen receptor binding sites are found in the 2.3 kb 5' flanking area but an oestrogen receptor binding site is present at 1.5 kb upstream of the ATG.

The homology between the 5' flanking regions of the human and marmoset type II GnRH receptor genes is 90% and many putative transcription factor binding sites which are present in the marmoset are also present in the 5' flanking region of the human type II GnRH receptor gene (appendix 5.4).

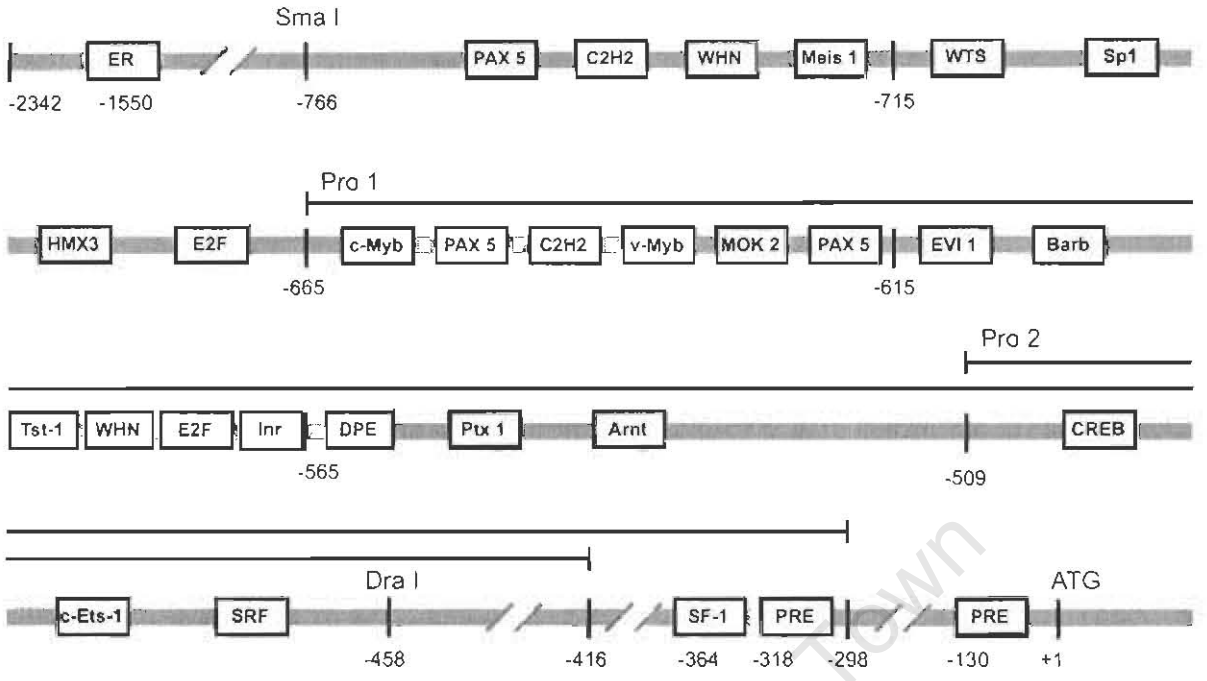

Figure 3.5 Prediction of promoter regions of the marmoset type II GnRH receptor gene. 2.3 kb of sequence upstream of the translational start codon was analysed for possible promoter sequences using either Proscan (Pro1) or PromoterInspector (Pro2). Putative transcription factor binding sites between the *Sma*I and *Dra*I sites of the 5' flanking region were identified using MatInspector. NF-1, nuclear factor 1; PAX 5, B cell-specific activating protein; C2H2, rat C2H2 Zn finger protein; WHN, winged helix protein; WTS, wilms tumor suppressor; SP-1, stimulating protein 1; HMX, H6 homeodomain HMX3/Nkx5.1; MOK-2, ribonucleoprotein associated zinc finger protein; EVI 1, ectotropic viral integration site 1 encoded factor; Barb, barbiturate-inducible element; Tst-1, POU-factor Tst-1/Oct-6; Ptx-1, pituitary homeobox 1; Arnt, aryl hydrocarbon receptor; CREB, cAMP-response elemen binding proteint; c-Ets-1, (p54); SRF, serum response factor; SF-1, steroidogenic factor-1; ER, oestrogen receptor; PRE, progesterone response element (Quandt et al., 1995); Inr, Iinitiator; DPE, downstream promoter element.

3.4.5 Transcriptional regulation of the marmoset type II GnRH receptor gene

In order to characterise the promoter elements of this gene a 2.3 kb area upstream of the ATG methionine start codon was cloned in front of a promoterless luciferase reporter gene (construct MtPro (-2342/-1)) and was transfected into HeLa-S3 and COS-1 cells. The luciferase measurements show a significant increase in promoter activity (10.8-fold, $p < 0.0005$) of the MtPro construct compared to promoterless luciferase reporter vector (pGL3) for HeLa-S3 cells (Fig 3.6A). The same 5' flanking

region of the marmoset type II GnRH receptor shows less activity (3.6-fold induction) in COS-1 cells (Fig 3.6B). An increase in reporter activity of the marmoset promoter constructs, compared to promoterless control, indicates that the 5' flanking region of the marmoset type II GnRH receptor gene contains a promoter which is active in HeLa-S3 cells and to a lesser extent in COS-1 cells.

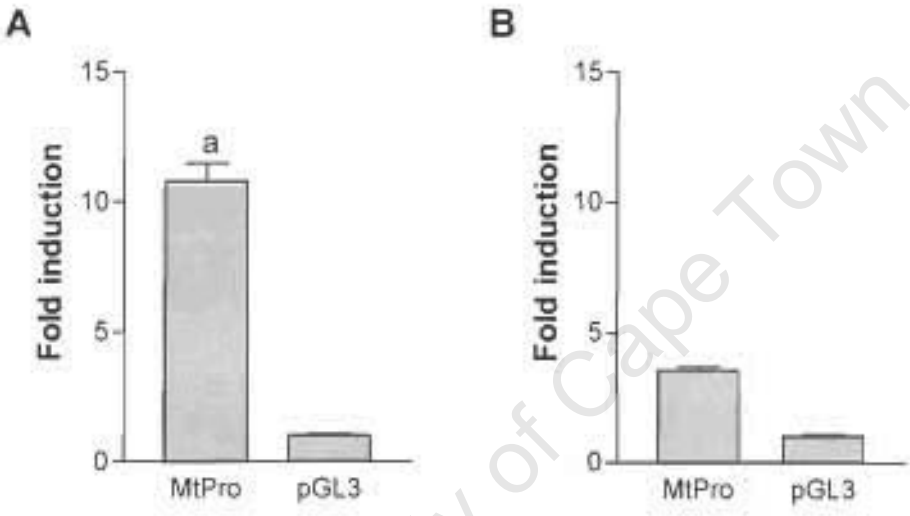

Figure 3.6. Expression of the marmoset type II GnRH receptor promoter in HeLa-S3 (A) and COS-1 cells (B). 2.3 kb of the marmoset type II GnRHR promoter, cloned in front of firefly luciferase (MtPro (-2342/-1)), or the promoterless pGL3 luciferase vector were transiently co-transfected with pRL-TK to normalize for transfection efficiency. The firefly luciferase activity was normalized for renilla luciferase activity and the relative promoter activity was calculated as fold induction compared to pGL3. The values represent the mean ± SEM of seven independent experiments, each performed in duplicate; a: $P < 0.0005$ *vs.* pGL3 for HeLaS3 cells (A) and the mean of one experiment performed in triplicate for COS-1 cells (B).

Progressive 5' and 3' deletions of MtPro were constructed and analysed in HeLa-S3 cells in order to map promoter elements in the 2.3 kb 5' flanking region of the type II GnRH receptor gene (Fig 3.7). The deletion of 347 nucleotides at the 5' end of MtPro increases expression of luciferase compared to the full-length MtPro {10.8- and 19.6-fold induction for MtPro and 5'BstxI (-1995/-1) respectively} (Fig. 3.7). This increase in expression indicates the presence of a negative regulatory element

within the region between nucleotides –2342 and –1995 relative to the translational start codon. The deletion of a further 316 nucleotides to the *Pvu*II site results in a decrease in expression (19.6- vs. 11.9-fold induction for constructs 5'BstxI and 5'PvuII). This indicates that a positive regulatory element is present in the region between nucleotides –1995 and –1679. Both the 5' deletion constructs 5'StuI and 5'NcoI show increases in expression when compared to 5'PvuII (25.8- and 34.0-fold induction for 5'StuI and 5'NcoI compared to 11.9-fold induction for 5'PvuII). This result indicates that negative regulatory elements are present in the region between the *Pvu*II and *Nco*I sites.

The highest expression of the 5' deletion constructs has been obtained for the 5'NcoI and 5'SmaI constructs, with 34.0- and 33.0-fold induction in expression, respectively. This shows that the proximal 766 nucleotides from the *Sma*I site to the ATG start codon of the marmoset type II GnRH receptor gene are sufficient for maximal basal transcription. The overall effect of the region upstream of the *Nco*I site is to reduce activity of the promoter, probably due to the presence of negative regulatory elements.

A further deletion of the region between the *Sma*I (-766) and *Dra*I (-458) sites shows an 80% decrease in expression. This result suggests that strong positive regulatory elements are present in this area. The 5'DraI construct, which contains 458 nucleotides immediately upstream of the ATG start codon, shows 6.6-fold induction over the promoterless pGL3. This region is the minimal promoter identified in this study. However, the promoter activity is much lower than for the full-length promoter or the 5'SmaI deletion construct.

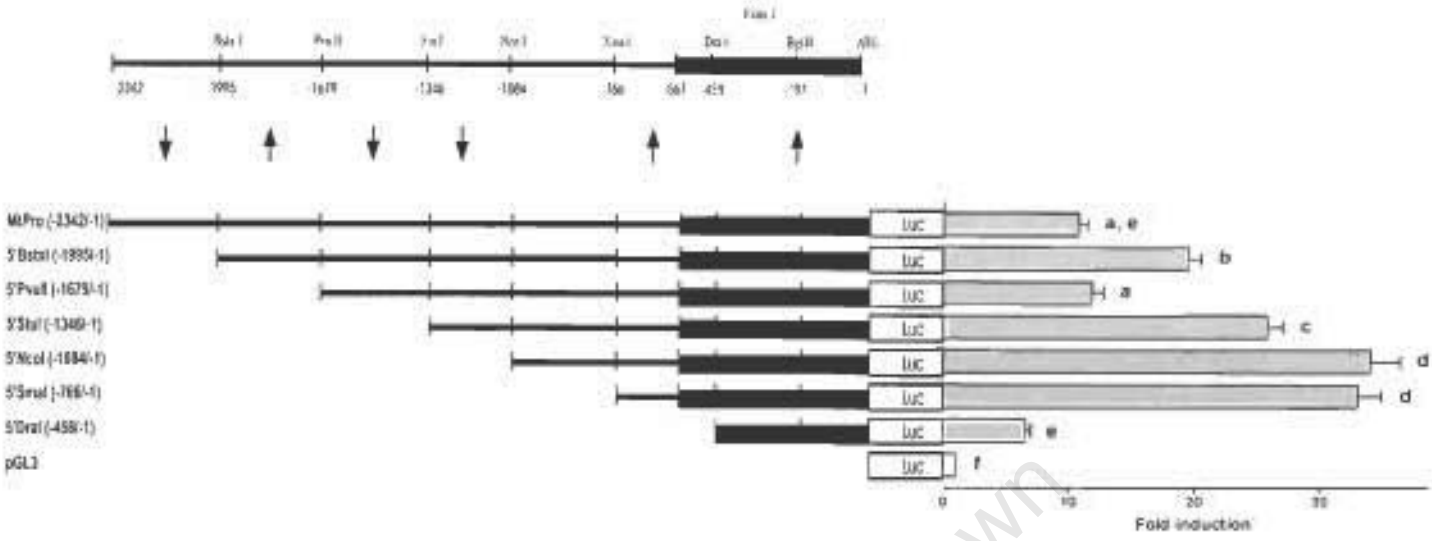

Figure 3.7. Progressive 5' deletion analysis of the marmoset type II GnRH receptor promoter in HeLa-S3 cells. HeLa cells were transfected with 5' deletion mutants and co-transfected with pRL-TK in order to normalize for transfection efficiency. Luciferase activities were measured 48 post-transfection. The firefly luciferase activity was normalized for renilla luciferase activity and the relative promoter activity was calculated as fold induction compared to promoterless pGL3. The values represent the mean ± SEM of seven independent experiments, each performed in duplicate. Significant differences between constructs are indicated by different lower case letters to the right of the bars, $P < 0.01$. The translational start site ATG and exon I are indicated. The vertical arrows indicate regions containing positive or negative regulatory elements.

Further analysis was done by testing the activity of 3' deletions of the promoter reporter construct. The 3' deletion constructs 3'BglII (-2342/-197) and 3'DraI (-2342/-458) both show increased activity compared to full-length MtPro (Fig 3.8). The luciferase activity for both the 3'BglII and 3'DraI constructs is higher than for MtPro (14.8- and 36.5-fold induction for 3'BglII and 3'DraI compared to 10.8-fold induction for MtPro). The increases in luciferase activity of 3'BglII and 3'DraI may indicate the presence of negative regulatory elements in the regions between the *Dra*I and *Bgl*II sites and between *Bgl*II and the ATG start codon. Alternatively, the increase in expression may be due to a shorther 5' UTR, leading to an increase in translation efficiency. A further 3' deletion to the *Sma*I (-766) site results in an 83% decrease in luciferase activity (comparing 6.2-fold induction for 3'SmaI with 36.5-fold induction over pGL3 for 3'DraI). This result indicates that the region between

nucleotides –766 (*Sma*I site) and –458 (*Dra*I site) contains a strong positive promoter element or promoter elements. This result confirms the finding from the 5' deletions. Results from both the 5' deletions and the 3' deletions indicate that the area between *Sma*I (-766) and *Dra*I (-458) is important for transcriptional activity of the marmoset type II GnRH receptor promoter.

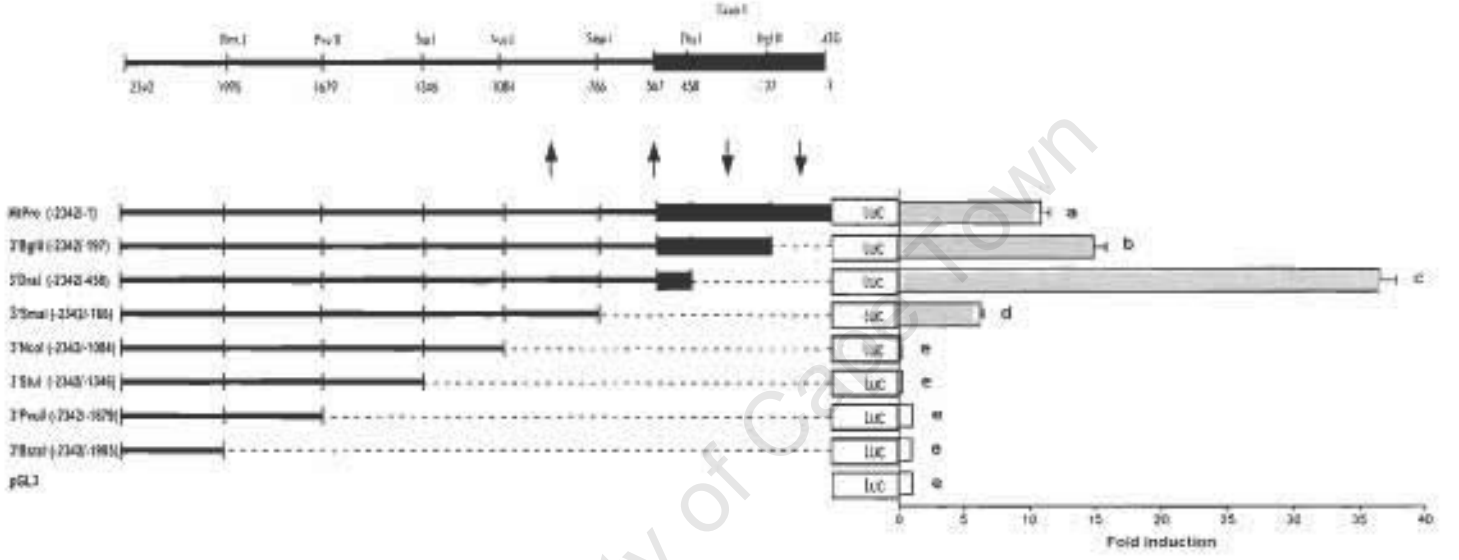

Figure 3.8. Progressive 3' deletion analysis of the marmoset type II GnRH receptor promoter in HeLa-S3 cells. HeLa cells were transfected with 3' deletion mutants and co-transfected with pRL-TK to normalize for transfection efficiency. Luciferase activities were measured 48 post-transfection. The firefly luciferase activity was normalized for renilla luciferase activity and the relative promoter activity was calculated as fold induction compared to promoterless pGL3. The values represent the mean ± SEM of seven independent experiments, each performed in duplicate. Significant differences between constructs are indicated by different lower case letters to the right of the bars, $P < 0.01$.

Both the 5' deletions (Fig 3.7) and 3' deletions (Fig 3.8) show that the area between the *Sma*I (-766) and *Dra*I (-458) sites are important for transcription. A more detailed 50 bp deletion mapping was performed in order to locate which region within the *Sma*I-*Dra*I area is important for transcription (Fig 3.9). Primers were designed with 50 bp intervals and PCR amplified fragments were ligated with the *Dra*I-*Eco*RI fragment in pGL3. The resulting four constructs SD1EI (-715/-1), SD2EI (-665/-1), SD3EI (-665/-1) and SD4EI (-615/-1) have 5' progressive 50 bp deletions

in the area between the *Sma*I site (-766) and the putative TSS (-567). The first deletion construct, SD1EI (-715/-1) and the 5'SmaI construct show higher expression compared to the full-length MtPro construct. This is consistent with the previous experiment (Fig 3.7) indicating the presence of negative regulatory elements upstream of the *Sma*I site. The first two deletions, SD1EI and SD2EI, each result in a decrease in reporter activity (30.8-fold, 20.9-fold and 11.2-fold induction for constructs 5'SmaI, SD1EI and SD2EI). The decreases in expression for these two deletions indicate the presence of positive regulatory elements in each of the 50 bp regions between (-766) and (-665). Further 5' deletions until the *Dra*I site show only a small decrease in luciferase activity (constructs SD3EI (-615/-1), SD4EI (-565/-1) and 5'DraI (-458/-1) compared with SD2EI (-665/-1)). The SD2EI and SD3EI constructs, which both contain the putative TSS at -567, show a level of activity similar to the *Dra*I and SD4EI constructs which lack the putative TSS. The results from the 50 bp deletion mapping show that the 100 bp area between -766 and -665 contains promoter elements which are important for the transcription of the marmoset type II GnRH receptor gene.

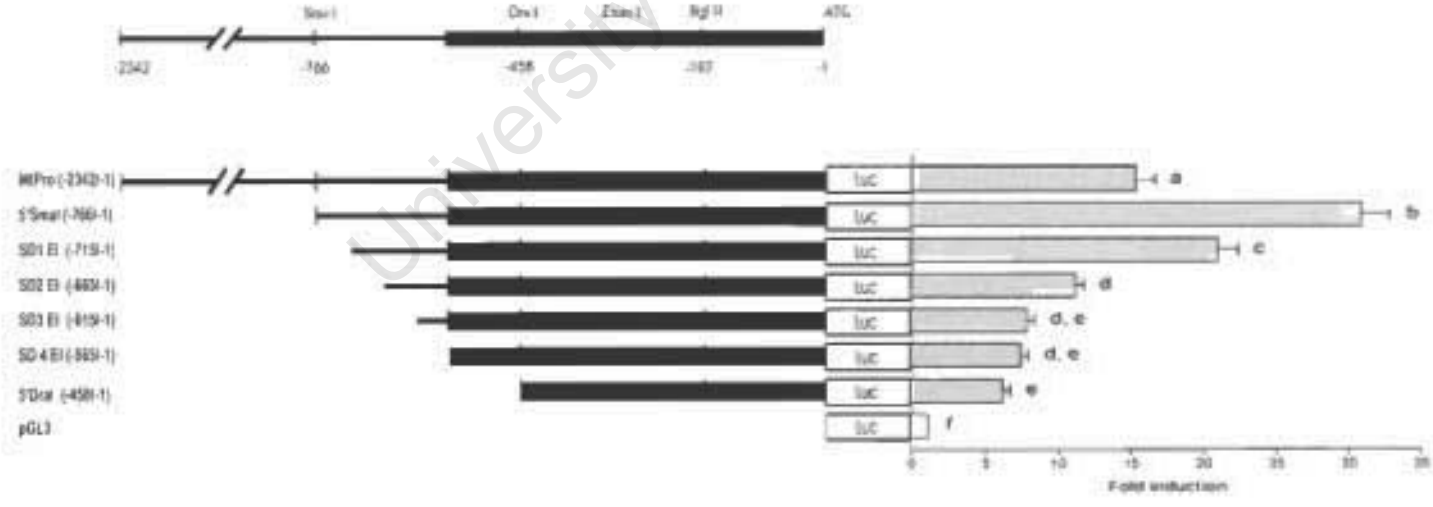

Figure 3.9. Functional fine-mapping of the -766 to -567 region of the type II GnRH receptor promoter. Progressive 5' deletions of 50 bp were generated in the area between the *Sma*I site (-766) and the transcriptional start site TSS (-567). HeLa-S3 cells were transfected with 5' deletion mutants and co-transfected with pRL-TK to normalize for transfection efficiency. The relative promoter activity was calculated as fold induction compared to promoterless pGL3. The values represent the mean ± SEM of six independent experiments, each performed in duplicate. Significant differences between constructs are indicated by different lower case letters to the right of the bars, $P < 0.01$.

The 50 bp 5' deletion analysis showed that a 100 bp region, 665 to 766 bp upstream of the ATG, is important for expression of the marmoset type II GnRH receptor in HeLa-S3 cells. We cloned this region in front of a heterologous promoter in order to confirm the importance of this region for transcription of the receptor gene. We cloned the 100 bp fragment into the multiple cloning site of pTAL (TK-Luc), which is upstream of the promoter of the Herpes simplex virus thymidine kinase and upstream of the luciferase reporter, yielding construct (-766/-665) TK-Luc. The 100 bp region resulted in a significant increase in expression compared to vector with thymidine kinase promoter only (3.8-fold induction of construct (-766/-665) TK-Luc compared to TK-Luc, $P < 0.001$)(Fig 3.10).

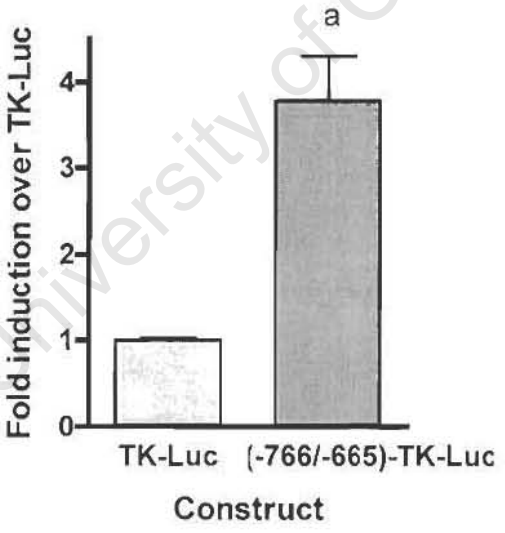

Figure 3.10. Activity of a 100 bp (-766/-665) region of the marmoset type II GnRH receptor promoter in front of a heterologous promoter in HeLa-S3 cells. A 100 bp fragment (-766/-665) of the promoter was cloned into the expression vector pTAL (TK-Luc) in front of the TK minimal promoter, upstream of luciferase, yielding construct (-766/-665) TK-Luc. The construcs TK-Luc and and (766/-665) TK-Luc were co-transfected with pRL-TK to normalize for transfection efficiency. The activity of the 100 bp region was calculated as fold induction compared to TK-Luc. The values represent the means of three independent experiments, each performed in duplicate; a: $P<0.001$ *vs.* TK-Luc.

3.5. Discussion

Recently, receptors for the conserved type II GnRH peptide have been cloned from primates (Millar et al., 2001; Neill et al., 2001) and transcripts of a human homologue have been identified (Chapter 2, this study) (Millar et al., 1999; van Biljon et al., 2002; Morgan et al., 2003). The type II GnRH receptor is widely expressed, suggesting different transcriptional control compared to the type I GnRH receptor, which is expressed predominantly in the pituitary.

A marmoset monkey genomic library was screened in this study and a comparison of the sequence of genomic clones with the published sequence of the marmoset monkey type II GnRH receptor cDNA identified three exons and two introns (Millar et al., 2001). The positions of intron 1 and intron 2 in the marmoset and human type II GnRH receptor genes are identical. The length of intron 1 is 4.2 kb in both species, whereas intron 2 is 750 bp in the marmoset compared to 450 bp in the human. The mammalian type I GnRH receptor genes contain three exons and two introns in the human, mouse and sheep (Zhou and Sealfon, 1994; Fan et al., 1995; Campion et al., 1996; Kakar, 1997). The positions of intron 1 in TM 4 and intron 2 in ICL 3 are conserved in the primate type II GnRH receptors and the mammalian type I GnRH receptors. 5' RACE identified a putative third intron of 293 bp in the marmoset type II GnRH receptor gene. However, the splicing of this third intron was seen in an aberrant transcript and the intron may or may not be spliced out in the full-length wild-type transcript.

The positions of introns 1 and 2 are also conserved in the Medaka type II GnRH receptor and in the chicken and *Xenopus laevis* type I GnRH receptor genes (Troskie et al., 2000; Okubo et al., 2001; Sun et al., 2001). However, these non-mammalian receptors have an additional exon and intron located in the N-terminal domain of the receptor or in the 5' UTR. A third intron has not been found in any of the 5' RACE reactions done on various human tissues (Chapter 2, this thesis). An alignment of the human and marmoset type II GnRH receptor sequences in the area of this novel putative intron shows that the splice acceptor site is conserved in both

species. However, a single nucleotide difference has been identified in the splice donor sequence. The marmoset has the conserved splice donor consensus site -gt, while the human sequence has a –gc at the equivalent position. It appears that a single nucleotide mutation in the human type II GnRH receptor gene has eliminated the mRNA splice donor site necessary for splicing out the sequence equivalent to this putative intron of the marmoset type II GnRH receptor.

A characterisation of the genes encoding type II GnRH receptor in the human (Chapter 2) has shown that the type II GnRH receptor gene and the ribonucleoprotein RBM8A gene overlap and are orientated in a tail to tail manner in two genomic loci. A PCR analysis of genomic clones and of genomic DNA confirms the same tail-to-tail orientation of the two genes in marmoset monkey (Fig 3.3, & 3.4).

An analysis of the 5' flanking sequence of the marmoset type II GnRH receptor gene identified a marmoset homologue of the human Pex 11B gene. The head-to-head orientation of the type II GnRH receptor and Pex 11B genes in human is conserved in the marmoset monkey.

PCR amplification of RBM8A from marmoset genomic DNA gives only one band, whereas two bands are observed for human genomic DNA (Fig 3.4). In addition, PCR between the type II GnRH receptor and RBM8A genes gives only one band for marmoset genomic DNA but two bands for human genomic DNA. These results indicate that the marmoset monkey has only one locus for RBM8A and that the retrotransposed locus found on chromosome 14 in the human is not present in the marmoset. Our results indicate that the chromosome 14 locus in the human genome arose after the divergence of the human and the marmoset monkey ancestors.

The 5' area upstream of the coding sequence of the marmoset type II GnRH receptor gene has been analysed for putative promoter regions using the computer programs Proscan and PromoterInspector (Prestridge, 1995; Scherf et al., 2000). There is an overlap between the promoter region predicted by PromoterInspector at –509 to –298 upstream of the translational start site and the promoter region

predicted by Proscan at –666 to –416 (Fig 3.5). Proscan predicts that the TSS is located at –431, which is 30 bp downstream of a muscle-type TATA box. However 5' RACE analysis performed in this study maps a putative TSS to –567. No TATA box (consensus sequence TATAAA) has been identified upstream of the TSS at –567, indicating that the type II GnRH receptor promoter is TATA-less, like the mouse type I GnRH receptor (Albarracin et al., 1994). Other GPCRs with TATA-less promoters include LH-, FSH- and TSH receptors and the proximal promoter of the human type I GnRH receptor, suggesting that other transcription initiator sequences than the TATA box are responsible for determining the TSS in these promoters (Huhtaniemi et al., 1992; Ikuyama et al., 1992; Hoo et al., 2003).

Basal transcription in eukaryotes is dependent on the binding to the promoter of RNA polymerase II (RNA pol II) and several general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) (Pedersen et al., 1999). RNA pol II and some of the general transcription factors consist of several subunits. The pre-initiation complex of RNA pol II and general transcription factors consists of approximately 30 polypeptides (Pedersen et al., 1999). A subunit of TFIID, called TATA binding protein (TBP), binds to the TATA box 30 bp upstream of the TSS and is involved in determining the location of TSS. TBP, however, can also bind to TATA-less promoters 30 bp upstream of TSS and might serve the same function in determining the location of transcription initiation in these promoters (Wiley et al., 1992).

Two additional promoter sequence elements, the Initiator and the downstream promoter element (DPE), have been identified in eukaryotes. The Initiator (consensus sequence PyPyAN(T/A)PyPy) is found at the TSS in some TATA-containing and in some TATA-less promoters, and DPE is involved in determining the location of transcription initiation (Smale, 1997). The DPE (consensus sequence (A/G)G(A/T)CGTG), located approximately 30 bp downstream of the TSS, binds TFIID and is important for transcription of TATA-less Initiator-containing promoters (Burke et al., 1998). The sequence of the marmoset type II GnRH receptor gene around the TSS contains a putative Initiator (CGAAACG) with 4 of 6 nucleotides conforming to the consensus sequence (Fig 3.5) and a putative DPE (CTGCGTG) which is found 25 bp downstream of the Initiator. The human type II GnRH receptor gene contains the putative Initiator and DPE elements at a similar position.

However, transcription of the human gene appears by 5' RACE not to be initiated in this area but further downstream (Fig 2.2).

Regulation of the type I GnRH receptor invoves binding sites for SF-1, CREB and the progesterone receptor. A comparison of the 5' flanking region of the human type I GnRH receptor gene to the marmoset type II GnRH receptor gene indicates that there is no sequence homology between the two promoters. Nevertheless, binding sites for SF-1, CREB and the progesterone receptor are present in the proximal area of the marmoset type II GnRH receptor promoter. In addition, there is an oestrogen response element 1.5 kb upstream of the ATG in the marmoset type II GnRH receptor gene. A mutational analysis is needed in order to establish whether these binding sites are functional in regulating the transcription of the marmoset type II GnRH receptor gene. The 5' flanking regions of the marmoset and human type II GnRH receptors have a high homology of 90% nucleotide identity. Many of the transcription factor binding sites in the marmoset promoter are conserved in the human type II GnRH receptor promoter (appendix 5.4) and a mutational analysis might reveal which of these are functional.

The 5' flanking region of the marmoset type II GnRH receptor has been cloned into the promoterless firefly luciferase reporter vector and expressed in HeLa-S3 and COS-1 cells. Neill et al. cloned the African green monkey type II GnRH receptor cDNA from COS-1 cells (Neill et al., 2001). This indicates that the type II GnRH receptor promoter is active in these cells. Our results show a low activity of the marmoset type II GnRH receptor promoter in COS-1 cells (3.6-fold induction). Our results show a significant increase (10.8-fold induction compared to pGL3, $p<0.0005$) in luciferase activity for the full-length 2.3 kb MtPro construct expressed in HeLa-S3 cells. The expression in HeLa-S3 cells is consistent with previous work in which a RNA dot blot of human poly (A^+) RNA has indicated expression of the human type II GnRH receptor gene in this cell line (Neill et al., 2001). Our result shows that the 2.3 kb 5' flanking region of the marmoset type II GnRH receptor gene contains a promoter. This promoter is more active in HeLa-S3 cells than in COS-1 cells.

5' deletions of the marmoset promoter and the expression of these deletions in HeLa-S3 cells revealed three regions (-2342/-1995, -1679/-1346 and -1346/-1084) with putative negative regulatory elements within each region. Three regions, –1995/-1679, -766/-458 and –458/-1, containing positive regulatory elements were also identified (Fig 3.7). This indicates that several cis-elements are involved in regulating the transcription of the type II GnRH receptor gene. The overall effect of the four regions upstream of the *Nco*I site (-1084) is a negative regulation of expression, as a deletion of the area upstream of –1084 (5'NcoI) results in an increase in expression compared to the full-length construct MtPro. These regions are further than 500 bp upstream of the area of the putative TSS and may contain repressor or activator binding sites respectively. Results obtained with the 3' deletion constructs revealed that these areas, in the absence of the promoter region between the *Nco*I site (-1084) and the ATG (Fig 3.8), have no promoter activity on their own.

Deletion of the region between –766 and –458 results a large decrease in expression. Progressive 50 bp 5' deletions identified two adjacent regions with regulatory effect between –766 and the putative TSS (Fig 3.9). The importance of these two regions was confirmed by cloning this region in front of the heterologous TK promoter and expressing this construct in HeLa-S3 cells (Fig 3.10). The –766 to –715 region shows positive regulatory activity (Fig 3.9), and contains several potential binding sites for transcription factors including that of Nuclear factor1, B-cell-specific activating protein and Meis 1 binding site (Fig 3.5). The –715/-665 region also shows positive regulatory activity (Fig 3.9) and it contains potential binding sites for Wilms tumor suppressor, Stimulating protein 1 (SP1), H6 homeo domain transcription factor and E2F (Fig 3.5).

A study, in which each of the potential binding sites is mutated, would indicate which of the potential transcription factor binding sites are active in the marmoset type II GnRH receptor promoter. Alternatively, since not all transcription factor consensus binding sites have been established, a scanning mutation in which blocks of sequence are replaced might identify regions important for expression which are not predicted by sequence analysis. This approach has been employed to identify

positive regulatory elements for both the mouse and human type I GnRH receptors (Norwitz et al., 1999a; Ngan et al., 2000; Hoo et al., 2003).

Interestingly, the construct 5'DraI with the putative TSS deleted still retains promoter activity. This construct shows 6.6-fold induction over promoterless luciferase control vector (Fig 3.7). This result shows that an alternative proximal promoter, consisting of 458 bp immediately upstream of the ATG start codon, is able to drive expression of the type II GnRH receptor gene in the absence of the promoter around the putative TSS at –567. However, the promoter activity of the 5'Dra construct is significantly lower than the activity of the full-length construct. The occurrence of two functional promoter regions in the type II GnRH receptor gene is paralleled by the occurrence of several putative promoters for the type I GnRH receptor. Three promoter regions have been identified within the proximal 1.8 kb 5' flanking region of the human type I GnRH receptor, and the differential expression of each promoter in various cell lines suggests different tissue specificities of the three promoters (Ngan et al., 2000; Ngan et al., 2001; Cheng et al., 2001b; Cheng et al., 2002a; Hoo et al., 2003).

The progressive 3' deletions showed that deleting the first 197 or 458 bp upstream of the ATG results in an increase in expression. This increase in reporter activity might be a translational effect. The transcripts of the 3' deletion constructs would have a shorter 5' UTR than transcripts of the full-length promoter. Translation initiation involves ribosomal scanning of the mRNA for ATG codons and translation is initiated when the ribosme encounters an ATG (Kozak, 1999). The shorter 5' UTR in the 3' deletion constructs has fewer competing ATG codons which may explain the increase in reporter activity.

In summary, the marmoset type II GnRH receptor gene, which consists of 3 exons and 2 introns, overlaps with and is orientated in a tail-to-tail manner with RBM8A in one genomic locus. The full-length promoter contains several putative negative and positive regulatory elements.

Chapter 5: Conclusions

The results from this thesis, and from other studies, show that a transcript of a type II GnRH receptor is expressed in humans (Millar et al., 2001; Neill et al., 2001; van Biljon et al., 2002; Millar, 2003a; Morgan et al., 2003). However, a functional receptor encoded by this transcript has not been identified. So far, no studies have shown RNA editing, RNA splicing, incorporation of selenocysteine or the use of alternative translational start sites which translates the cDNA into a full-length human type II GnRH receptor. This suggests that the type II GnRH receptor genes in humans are pseudogenes and this is apparently also the case in chimpanzee and cattle, both having a stop codon in exon 2 of the type II GnRH receptor gene. It may be that the GnRH II peptide has a functional role through the type I GnRH receptor in animals lacking a type II GnRH receptor. GnRH II binds to and signals through the human type I GnRH receptor when expressed in COS cells, although with a 10-fold decrease in affinity compared to mGnRH (Millar *et al.,* 2001). Local expression levels of GnRH II in certain tissues may be high enough for signalling through the type I GnRH receptor. However, a couple of studies indicate that specific effects of GnRH II in certain human cells and cell lines are not mediated by the type I GnRH receptor (Grundker et al., 2002; Grundker et al., 2003; Chou et al., 2003). The mechanism with which GnRH II excerts its effect on these cells is not known.

The gene structure of the human type I and primate type II GnRH receptors are similar (Zhou and Sealfon, 1994; Fan et al., 1994; Fan et al., 1995; Kakar, 1997). This, and the sequence homology, is indicative of the evolutionary relatedness of the type I and type II GnRH receptor genes. Interestingly, the human and marmoset type II GnRH receptor genes are flanked by two genes, RBM8A and Pex11B, in the antisense orientation. In addition, the human genome contains a second, retrotransposed, locus containing the RBM8B gene. This locus is not present in marmoset monkeys, suggesting it arose after the divergence of marmosets and humans. The mouse is interesting in that the Pex11B and RBM8A genes are

adjacent but there is no type II GnRH receptor gene inbetween them and no type II GnRH receptor gene has been identified elsewhere in the mouse genome.

Although there is sequence homology between the human type I and marmoset type II GnRH receptor genes, there is no overall sequence homology in their 5' flanking regions. Nevertheless, several of the cis-elements which are important for transcription in human, mouse or rat type I GnRH receptor are present in the promoter area of the marmoset type II GnRH receptor gene. It remains to be shown whether these cis-elements, SF-1, PRE and CRE, are involved in regulating the transcription of the marmoset type II GnRH receptor gene. Furthermore, an interesting aspect would be the hormonal regulation of type II GnRH receptor gene expression.

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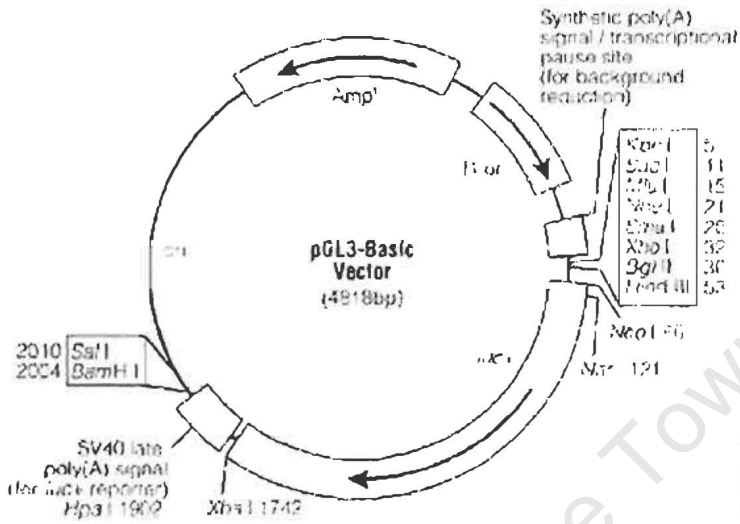
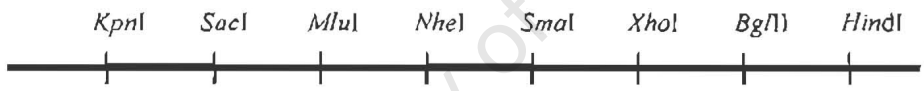
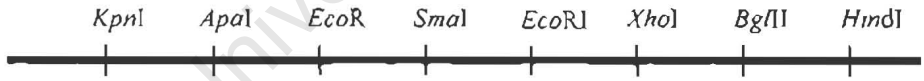
Chapter 5: Appendices

5.1. Maps of pGL3basic, pGL3 and pRL-TK



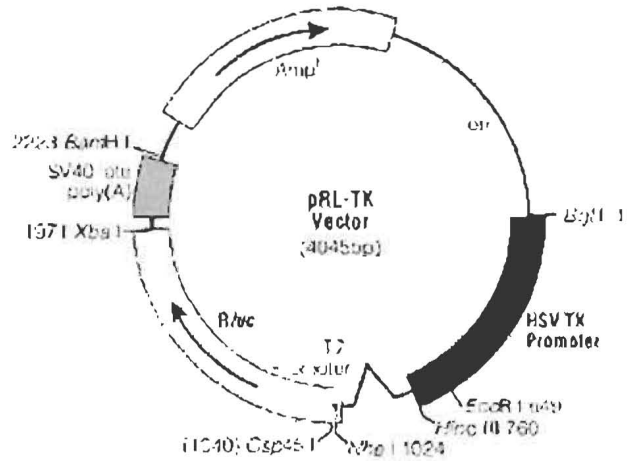


Multiple cloning site of pGL3 basic

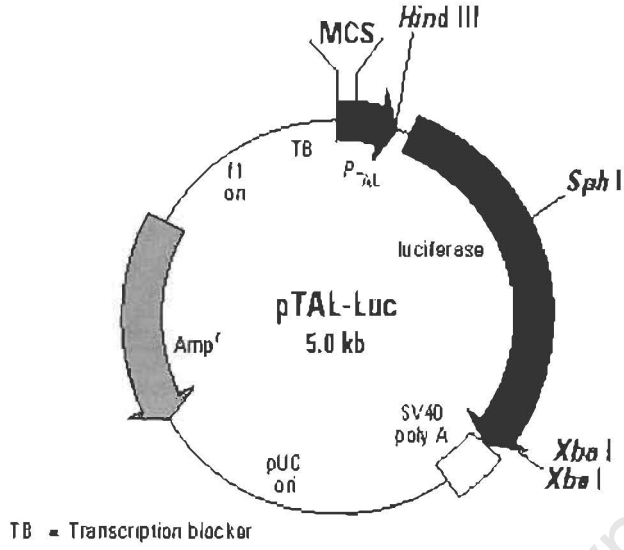


Multiple cloning site of pGL3

(This is the vetor used for the promoter-reporter constructs)

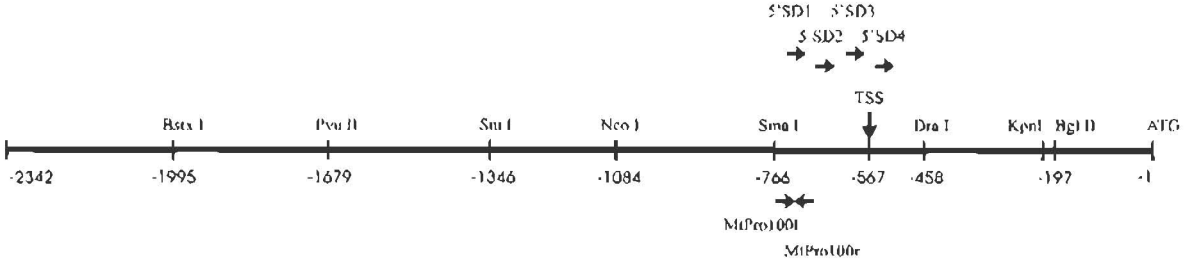


5.2. Map of pTAL



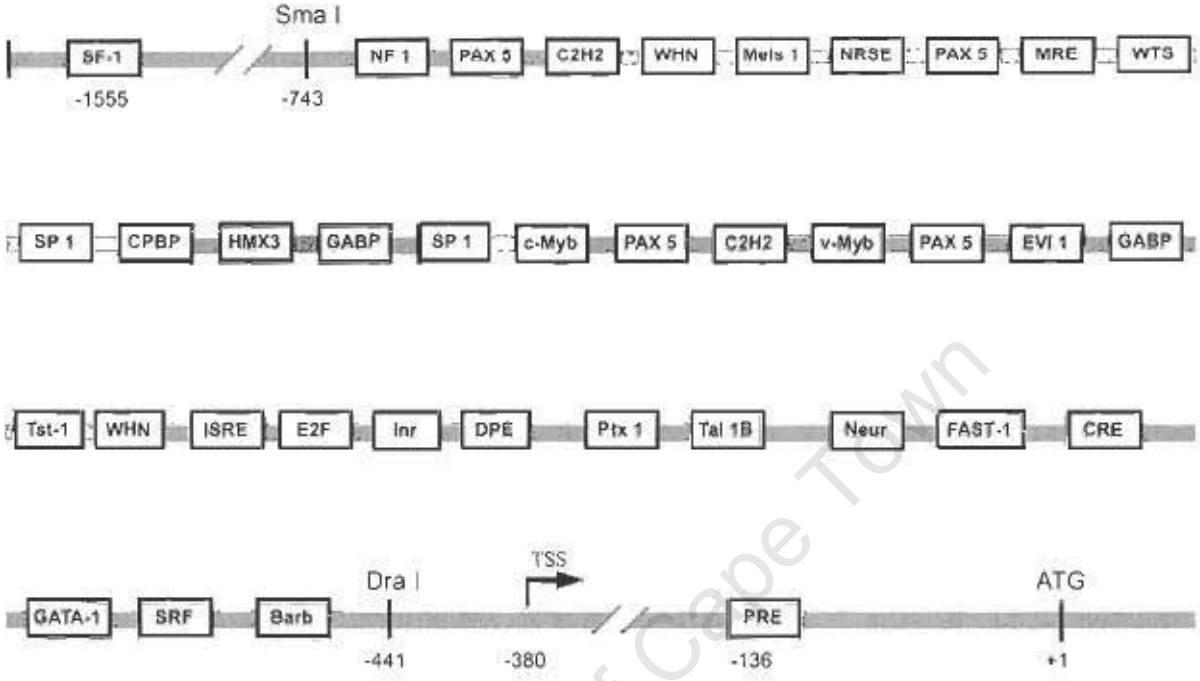
A 100 bp region of the marmoset type II GnRH receptor promoter was cloned into the *Nhe*I and *Xho*I sites generating construct (-766/-665) TK-Luc.

5.3 Schematic illustration of primers used to generate promoter deletions of the marmoset type II GnRH receptor gene



The restriction enzyme sites used for making deletions and their position relative to the translational start codon are indicated. The primers used for making the 50 bp 5' deletion constructs and the 100 bp construct in pTAL are indicated by horizontal arrows. The putative transcriptional start site is indicated by a vertical arrow.

5.4. Putative transcription factor binding sites of the human type II GnRH receptor promoter



The area of the human type II GnRH receptor 5' flanking region homologues to the *SmaI-DraI* area of the marmoset promoter in figure 3.5 was analysed for putative transcription factor binding sites with MatInspector. NF-1, nuclear factor 1; PAX 5, B-cell specific activating protein; C2H2, rat C2H2 Zn finger protein; WHN, winged helix protein; NRSE, neural restrictive silencer element; MRE, metal transcription factor 1; WTS, wilms tumor suppressor; SP-1, stimulating protein 1; CPBP, core promoter binding protein; HMX, H6 homeodomain HMX3/Nkx5.1; GABP, GA binding protein; EVI 1, ectotropic viral integration site 1 encoded factor; Barb, barbiturate-inducible element; Tst-1, POU-factor Tst-1/Oct-6; ISRE, interferon response element; Ptx-1, pituitary homeobox 1; Tal 1B, tal-1beta/E47 binding site; Neur, NEUROD1 binding site; FAST-1, FAST-1 SMAD interacting protein; GATA-1, GATA binding factor 1; CRE, cAMP-response element; SRF, serum response factor; SF-1, steroidogenic factor 1; PRE, progesterone response element (Quandt et al., 1995); Inr, initiator; DPE, downstream promoter element; TSS, putative transcriptional start site.

5.5 Publications as outcome of this thesis

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

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

Faurholm, B., Millar, RP. and Katz, A. Marmoset type II GnRH receptor, gene structure and promoter analysis. (in preparation)

5.6 Nucleotide sequence of the human type II GnRH receptor and RBM8 genes

The sequence of the two loci in human both containing type II GnRH receptor and RBM8 genes have been deposited with Genbank under the accession numbers AF403012, AF403013 and AF403014.

5.7 Nucleotide sequence of the marmoset type II GnRH receptor promoter region

This sequence is derived from a genomic clone of the marmoset type II GnRH receptor gene. 2.3 kb of of the clone upstream of the ATG start codon was sequenced. The sequence below has been deposited in GenBank under the accession number AY676461.

```
1       GTCGACATGG TGAAACCCCG TCTCTACTAA AAATACAAAA AAGTAGCTGG ATATGGTGGC
61      GCGTGCCTGT AATCCCAGCT ACTCAGGAGG CTGAGGCAGG AGAATTGCCT GAACACAGGA
121     GGCGCAGGTT GCAGTGAGCC GAGATCACGC CATTGCACTC CAGCCTGGTA ACAAGAGCAA
181     AACTCGGTCT CACAAAAATA AATAAATAAA TAAATAAATA AAAATAAAGA GCAGAAACAG
241     TTCATTTTTC TTTTTTTCTT TTTTCAAGAC AGGGTTTCAC CATATTGGTC AGGCTGCTCT
301     CAAACTCCTA ACCTCAGGTG ATCCGCCTGC CTCAGCCTCC CAAAGTGCTG GGATTATAGG
361     CGTGAGCCAC CACGCCTGGC AACAGTTCAT TTTCCAATTT CCAATTTAAT TTTTGTGCCA
421     GTTTACTTTT CTCAAACTTG CCCCATTCTC TCCAATCCTC CTAATTACTT CCATACCCCT
481     GCTCTCTCGG CTCAACTGGA CGCAAGCAGG GGGTAAGTGC AGAGCCAAAT GAGGAAGAAC
541     AGGGGCAGAA GGTACAGGAA GGAGGGACCT AGGAAGGAGA GGTGCGGCGG AGGAAGGGTT
601     GGGGAGATAA AAGAAAAGAC AGAGTTACTT ACGCTTTCTT CCAAGGCTCA GGTGGCCCTC
661     CAGCTGTCGG ATCTGTTTCT GTAACTCAGG ACTGGCTCCA TGCCTCTGCA GCGCATGGCC
721     AAGAAGAGAG CAAGCATACT GGGCGGCCCT AGAGGAGAGG GCAGGCAAGG TAAGGTGGAG
781     ACCTCCCTGA CCTTCCGCTC AAAACTATCC TCCAAAAGGC AAAGGCAGGC TTCCCCCTTT
841     TCCAGTTTGA GACCACTCTC TTTCTTCCTC CATGCCCATA TCTCACCATT TCTCCAGGCC
901     AGGGGAAGGG AACGGGATAA GGAGAAAAAA GAGCCTAGGA AGGGGTGAGG AGCACTACTG
961     AATGTTGAGG AAATAAGAAG TCTGACAATG ATGAGGCCTG AGGGGCCAGT GAAGGCCGCA
1021    GGGGTCCATC ACCAGCACAT CGGCGTGAGC ATGTACGTGA TCGATGAGGG GGAGGCAGAC
1081    AGTTGCAGGG CATTTTATTC ATCAGAATAG CTGAATAAGA GATTTCAAGG CCCTCTAGCT
1141    GAATGGAGAC CAAACGCCAG AGCGGCGGCA GAGGAGTTCC CGAATGACAG CACGGTGAGG
1201    GGACATGGAA ACTGGGAGTG CCTTCTCAGC TCTGGGGGTA CAGTCCAGGG CTACCCGCCA
1261    TGGGGCGAAT GCTCCTCATT CCATCACCGC CCAGTGAGGA CGCTGTCGGT CGTATGTGGA
1321    CACCGCAACC CTGAGCGGCC TCCCCGCTGG CCCACTCATG CCTCAGTTTC CCCATCTCTT
1381    CCGCGAGCGG AAAAGAATCA TCTCCTCAAG AGCGGCTCAA ATCTGCGCCT CACGCGGTCC
1441    GTTCCGGCCC CCGTAGCCAG TATTAACCCT TCTTGACCCT CCGTAGCCTG AACTGACCTC
1501    GCCTCACCAG TCTTCCTCTG CCCGTCCCTC CCCCCACCCC CATTCCTATT CGGGCCGCAC
1561    CTACACAGCC GCTCCCGGGC TTGGCTCTGA GCACTGAAGC GGACCCAGGC GTCCATGACA
1621    GCGGCAGCCC CGGCTCGGCG GCCCCGCTCC CGCCCCACTT CTCGCCCCTA GTCACGCCTC
1681    CTGCGCGACC CAACGGTGCC TTGCGAGGGA CAAACAACAT CAGAAAAGAA AGCGAGTGGA
1741    GCTGGCCCTT AAAGCGGAAT TTCAGGACGC GAAACGCCTG TCTTTGAGCC TGTCTGCGTG
1801    CTACTGTGCC TGTCTCCGTC AGGATGTCCC CTTATGAGAC GACCTCTACT CTCTAAGCAC
1861    GCCTCCAGCG GTACGTTCTT ATTTAAATAT TCCGCACAAA GGGCGTGGTC TGCAGGGGCG
1921    GGGCGGGGCC TGCAGGGGCG GGGCGGGGCG AGAAAGCGCC GCAGTGATCT CCTCGCCTTG
1981    GAGGCTGGGA TAGGTGTGCC CCCGGCAGAA CACCCTAAAC ACACTTGCAC GCAGGCTCTA
2041    GCAGAATCCA GGGCTATAGG GACTAAAGAG GTCTAGGGCA GTAGAGGCCC GAAACCCAGG
2101    CTGATCTGTC CAAGAGAAAA GAAGAAAAAA CGAAGTTTGG TACCAGATCT TCGTTCCCTG
2161    CAGGACCCTG ACAGTTGGAC GAGTGACCTC CTCCAGAACA TACGGAGAGT CTCCAGACGC
2221    AGAGGCTTTA AGGAAGGAAA TTCGCAATAA TCAGCTCCAG ATCCTGAAAA GGAGGGCGAA
2281    GAATCAGTGG CCAAATCTAA CCGCTTCATA CTCACACTTC ATCCTCCTAT CTCCAGGCCA
2341    CCATG
```