THE ROLE OF STEROIDOGENIC FACTOR-1 (SF-1) IN GONADOTROPIN- RELEASING HORMONE (GnRH) RECEPTOR GENE REGULATION

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January 1998

DECLARATION

I, Carmen Poltio Pfeiffer, hereby declare that the work on which this thesis is based is my original work (except where aknowledgements indicate otherwise), and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABBREVIATIONS

α	alpha
β	Beta
β-gal	β-galactosidase
bp (s)	base pair (s)
CIP	calf intestinal phosphatase
CsCl	caesium chloride
°C	degree celsius
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphospahate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
FSH	follicle stimulating hormone
g	gram
g	gravitational acceleration
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr (s)	hour (s)
GnRH	gonadotropin-releasing hormone
GnRHR	gonadotropin-releasing hormone receptor
GSE	gonadotrope-specific element
kb	kilobases
kDa	kilodalton
λ	lamda
LB	Luria-Bertani
LH	luteinizing hormone
μg	microgram

µl	microlitre
min (s)	minute (s)
mg	milligram
mGnRHR	mouse GnRHR
ml	millilitre
mut	mutant
ng	nanogram
OD	optical density
%	percentage
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PNK	polynucleotide kinase
RNase	ribonuclease
SDS	sodium dodecyl sulphate
sec (s)	second (s)
SF-1	steroidogenic factor-1
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
TBS	Tris-buffered saline
TE buffer	Tris-EDTA buffer
TEMED	N, N, N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
v/v	volume by volume
w/v	weight by volume
wt	wild type
YT	yeast tryptone

ABSTRACT

Gonadotropin releasing hormone (GnRH) is a key reproductive hormone in vertebrates and exerts its effects via the GnRH receptor (GnRHR) to result in the synthesis and release of the gonadotropin hormones in the pituitary gonadotrope cells. GnRHR expression is likely to be regulated in a tissue- and cell- specific manner. A variety of hormones, including GnRH itself, estrogen, progesterone, inhibin, and testosterone have been shown to regulate GnRHR expression. Steroidogenic Factor-1 (SF-1), a member of the orphan nuclear receptor transcription factor family, regulates the expression of both the gonadotropin hormones in the pituitary and the steroidogenic enzymes in the gonads and adrenal gland, and provides a potential molecular mechanism for coordinate control of reproductive function. SF-1 binds to a gonadotrope-specific element (GSE) in the promoters of the gonadotropin hormones.

Our studies involved investigating whether SF-1 plays a role in tissue-specific regulation of GnRHR gene expression. A genomic clone of the mouse GnRHR gene contains a putative SF-1 site at about -15 relative to the translation start site. We demonstrate the presence of a factor with SF-1-like DNA-binding activity in the gonadotrope cell lines, αT3-1 and αT4, by gel retardation assays. DNaseI footprinting reveals that the major DNA-binding activity in αT3-1 cells on the GnRHR promoter occurs at the SF-1-like site. The SF-1-like sequence specificity of the interaction is demonstrated by gel redardation and DNaseI footprinting assays using specific and mutated oligonucleotides as competitors. Northern blot analysis suggests that GnRHR expression is not solely dependent on the presence of SF-1, as αT4 cells do not express GnRHR but a SF-1 transcript is seen in these cells. Promoter function was analysed by constructing plasmids containing 563 bp of the GnRHR gene 5' to the ATG translation start site linked to a

luciferase reporter gene, followed by transfection of these constructs into different cell lines. In addition, a mutant construct containing a mutated SF-1 site was tested. We demonstrate that this 563 bp of the GnRHR gene contains strong promoter activity in both pituitary gonadotrope (αT3-1) and somatotrope (GH_3) cells, but not in non-pituitary (COS-1) cells. Thus promoter activity appears to be tissue specific but not gonadotrope specific. The presence of a mutated SF-1 site in the 563 bp GnRHR gene fragment did not significantly effect the promoter activity, showing that binding of SF-1 protein to this site is not necessary for high levels of GnRHR expression in the pituitary gonadotropes.

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CHAPTER 1

LITERATURE REVIEW

1. Structure and function of GnRH and GnRHR

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide hormone, which controls reproductive function by stimulating the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. GnRH is secreted in a pulsatile manner, from the medial basal hypothalamus at intervals of 1-2 hours according to the species. From there it is delivered to the gonadotrophs, via the hypophysial portal circulation, where it binds to its receptor, the GnRH receptor (Ben-Menahem *et al.* 1995; Hazum and Conn, 1988). LH and FSH are secreted into the systemic circulation from the gonadotrophs in a pulsatile manner parallel to GnRH secretion. The gonadotropins in turn exert regulatory effects on the gonads to promote reproductive homeostasis (Hazum and Conn, 1988, Fig. 1.1). In addition to indirectly effecting gonadal function, GnRH has also been shown to have direct actions on gonads in some species by inhibiting steroidogenesis in males and ovulation in females (Hsueh and Jones, 1981). The ability of GnRH to stimulate reproductive function at low pulsatile doses, while suppressing them at high doses has been clinically exploited for various purposes including induction of ovulation and spermatogenesis, contraception, and for the treatment of precocious puberty, endometriosis, and steroid-dependent tumours (i.e. prostate and breast cancer) (Ziporyn, 1985).

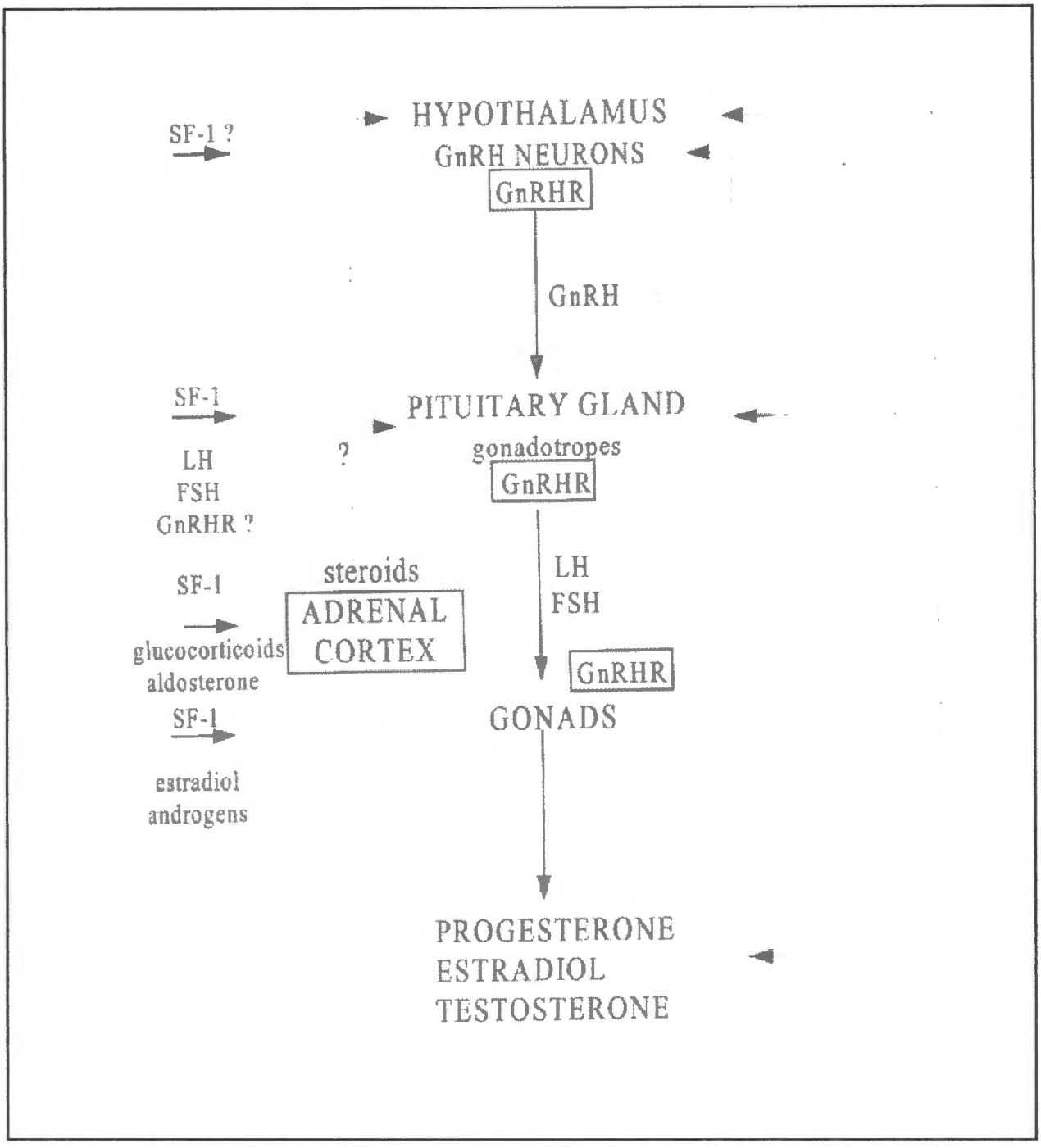

Figure 1.1. Hypothalamic-pituitary-gonadal axis. The hypothalamic peptide GnRH, is released in a pulsatile fashion into the hypophysial portal circulation and interacts with receptors (GnRH receptors) on gonadotrope cells in the pituitary (Stojilkovic *et al.* 1994). GnRH has been shown to regulate its own production in the hypothalamus. The gonadotropes secrete LH and

FSH, which effect steroidogenesis in the gonads. Sex steroid hormones, estrogens, progesterones, and testosterone have effects on LH and FSH secretion that may be mediated at the hypothalamus, the pituitary, or both (Gharib *et al.* 1990). Steroidogenic factor-1 (SF-1) is a transcription factor which has been shown to regulate estradiol and androgens in the gonads, glucocorticoids and aldosterone in the adrenal gland, and both LH and FSH synthesis in the pituitary gland (Ingraham *et al.* 1994; Lala *et al.* 1992; Lynch *et al.* 1993). SF-1 may also regulate GnRH receptor gene expression in the pituitary gland. It is also speculated that SF-1 may regulate the reproductive system at the level of the hypothalamus (Barnhart and Mellon, 1994).

1.1. Molecular structure of GnRH

Mammalian GnRH (mGnRH) was originally isolated from pig (Matsuo *et al.* 1971) and sheep (Amoss *et al.* 1971) hypothalami. Mammalian GnRH is conserved in rat (Adelman *et al.* 1986), mouse (Seeburg *et al.* 1987), human (Adelman *et al.* 1986) and frog hypothalami. Two forms of GnRH were isolated from chicken hypothalami, namely chicken GnRH I (cGnRH I) (King and Millar, 1982a and b; Miyamoto *et al.* 1983) and chicken GnRH II (cGnRH II) (Miyamoto *et al.* 1984). Four GnRHs were isolated from fish hypothalami: salmon GnRH (sGnRH) (Sherwood *et al.* 1983), lamprey GnRH (lGnRH) (Sherwood *et al.* 1986), catfish GnRH (cf GnRH) (Ngamvongchon *et al.* 1992) and dogfish GnRH (df GnRH) (Lovejoy *et al.* 1992). Seven forms of GnRH which are highly conserved, have been identified (King and Millar, 1992, Fig. 1.2).

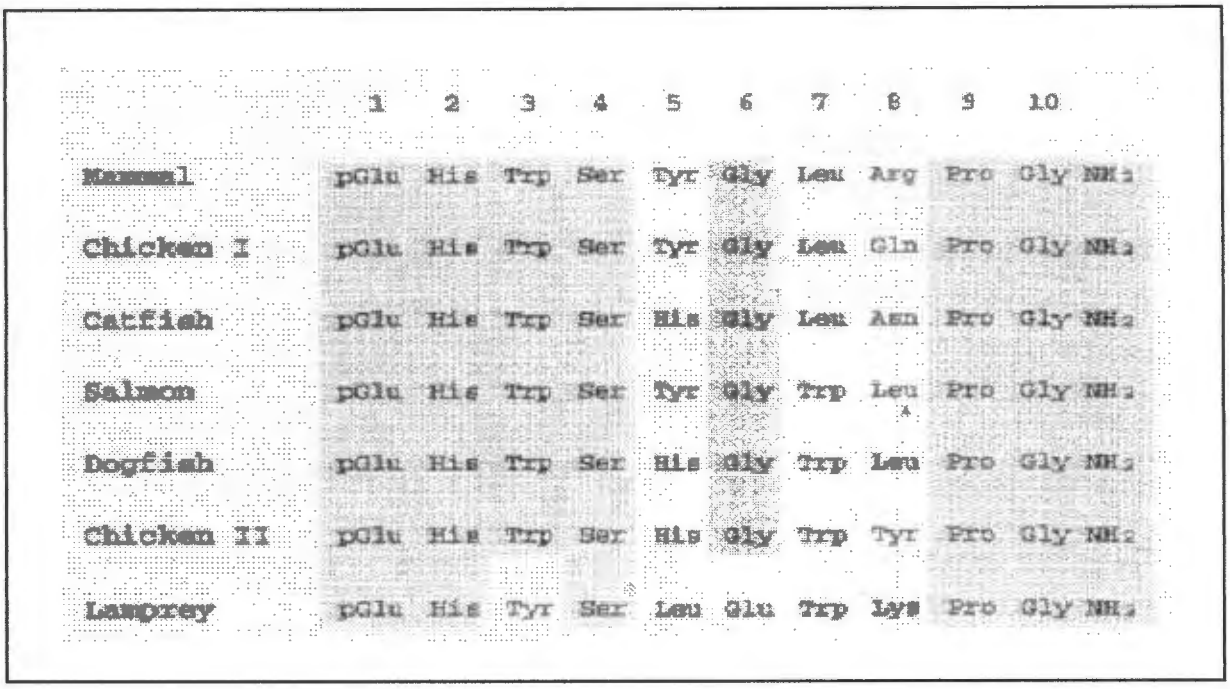

Figure 1.2. Primary structures of GnRHs isolated from vertebrate brain. The boxed areas show conserved regions (King and Millar, 1992).

Two forms of GnRH occur within a single species, namely cGnRH I and cGnRH II (King and Millar, 1992). It is thought that the main role of cGnRH I, which is variable in structure, is to regulate pituitary gonadotropin secretion while cGnRH II which is highly conserved and is more prevalent in extrahypothalamic brain areas may function as a neurotransmitter or neuromodulator. The notion that cGnRH II may function as a neurotransmitter or neuromodulator is supported by several findings which are reviewed in more detail by King and Millar (1992). GnRH exerts its effect on reproductive function by binding to GnRH receptors (GnRHRs) on gonadotrope cells, to stimulate the synthesis and release of the gonadotropin hormones from pituitary cells. Internalisation of GnRH agonists in gonadotrophs occurs via receptor-mediated endocytosis (Suarez-Quian *et al.* 1986). After internalisation, the receptor-ligand complex undergoes dissociation, followed by degradation of the ligand and partial recycling of the receptors (Schvartz and Hazum, 1987). In contrast to the rapid internalisation

of the GnRH agonists, GnRH antagonists remain bound to the cell surface for much longer (Loumaye and Catt, 1982).

1.2. The Gonadotropin-releasing hormone receptor

The GnRH receptor (GnRHR) is a 327 amino acid plasma membrane-associated protein and a member of the G-protein-coupled receptor family (Tsutsumi *et al.* 1992). The GnRH receptor has an extracellular amino terminus and seven hydrophobic membrane spanning α-helices. However, the GnRH receptor distinguishes itself from other G-protein coupled receptors in that it does not possess a cytoplasmic carboxy-terminal domain believed to be functionally important in other G-protein receptors (Fig. 1.3).

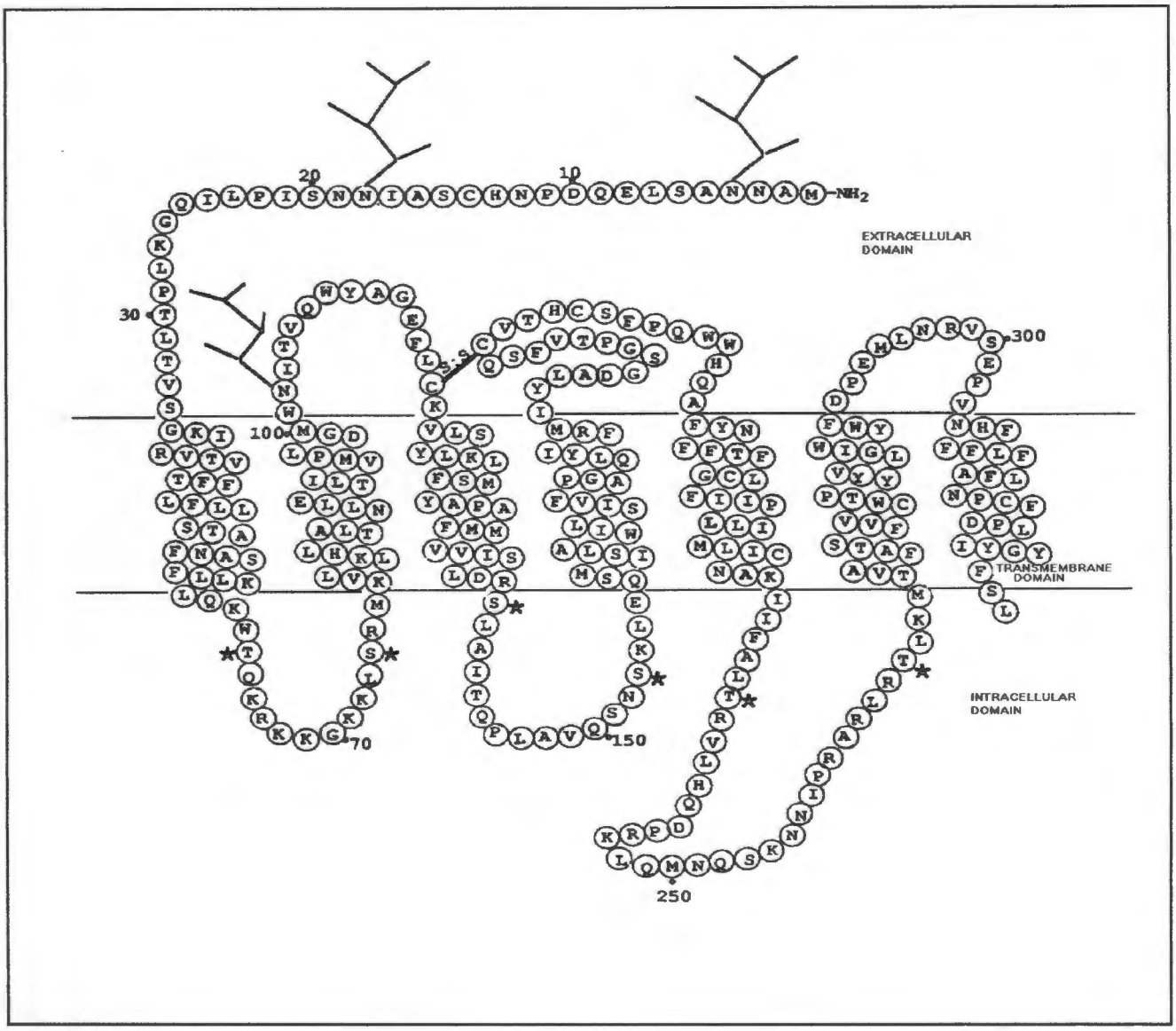

Figure 1.3. Structure of the mouse pituitary GnRH receptor. The seven putative transmembrane domains, and N-glycosylation and phosphorylation (*) sites are shown. A putative disulfide bond between the first and second extracellular loops is indicated by -S-S-. Figure taken from Stojilkovic *et al.* 1994.

The GnRH receptor is a glycoprotein and binds its ligand, GnRH with a high affinity. The N-glycosylation sites in the GnRH receptor are believed to be important for receptor expression and stability but not for ligand binding (Davidson *et al.* 1995). Six mammalian GnRH receptors have been cloned and characterised, including the mouse (Tsutsumi *et al.* 1992; Reinhart *et al.* 1992), rat (Kaiser *et al.* 1992; Eidne *et al.* 1992; Perrin *et al.* 1993), 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). The GnRH receptor is highly conserved amongst mammalian species and show more than 85% amino acid homology. The GnRH receptor is located primarily in the anterior pituitary gonadotrophs, where binding of the GnRH receptor to its ligand occurs. In addition to the gonadotrophs, GnRH receptors have been widely reported in the gonads, placenta, adrenal glands, the central nervous system and some neoplasmic tissues (Stojilkovic *et al.* 1994).

1.3. Activation of GnRH receptors

The GnRH receptor is activated when its ligand, GnRH, binds to it. A ligand receptor/G-protein coupled complex is formed which activates phospholipase C (Bruder *et al.* 1992). Phospholipase C cleaves phosphotidyl inositol 4,5,-biphosphate to diacylglycerol (DAG) and 1,4,5,-triphosphate. DAG then activates protein kinase C (PKC) which then undergoes a translocation from the cytosol to the plasma membrane. PKC is a family of enzymes, which,

when activated phosphorylates many proteins and results in the induction or activation of many nuclear transcription factors. These transcription factors may positively or negatively regulate gene expression (Fig. 1.4).

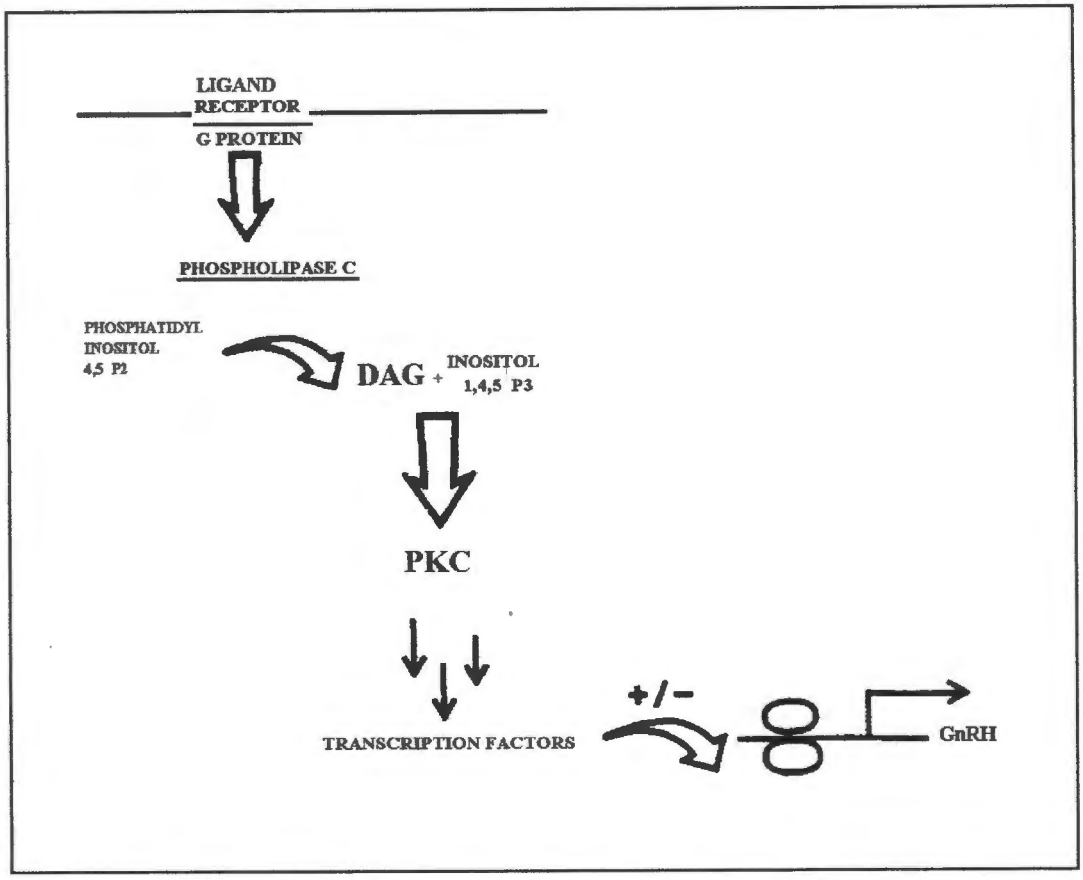

Figure 1.4. Diagram of the second messenger system in which DAG stimulates PKC to activate transcriptional factors that positively or negatively regulate gene expression (Bruder *et al.* 1992).

2. Regulation of GnRH receptor gene expression in gonadotropes

The GnRH receptor gene is likely to be regulated at several levels, for example, transcriptional, post transcriptional (RNA processing, RNA transport, RNA stability), translational and post translational modification (protein folding, transport, degradation). A major part of this

regulation is likely to be at the transcriptional level, as several studies have shown that changes in GnRH receptor number are paralleled by changes in steady state mRNA levels for the receptor (Albarracin *et al.* 1994). Kaiser *et al.* has shown that pulsatile application of GnRH to cultured rat primary pituitary cells increases receptor mRNA several-fold (1993). Multiple transcription start sites have been detected in the human, mouse and rat GnRH receptor genes, indicating that regulation of transcription may occur via start-site selection (Albarracin *et al.* 1994; Fan *et al.* 1995; Reinhart *et al.* 1997). Another level of GnRH receptor regulation may be via RNA processing as multiple and different RNA transcripts have been detected in expressing tissues (Stojilkovic *et al.* 1994). Since the receptor is only found in selected tissues, it is likely to be subjected to strict tissue-specific regulation. The GnRH receptor gene is likely to be regulated in an extremely interesting and complex manner, by both positive and negative cis-regulatory transcription elements, as well as by a variety of hormones including GnRH itself, estrogen, progesterone, inhibin, activin A and testosterone (Clayton and Catt, 1981; Fernàndez-Vàzquez *et al.* 1996;áLaws *et al.* 1990; Kaiser *et al.* 1993; Quinones-Jenab *et al.* 1996; Savoy-Moore *et al.* 1980). It is thought that regulation of the GnRH receptor gene is an important control point in the reproductive cycle, since GnRH, gonadal steroids and peptides have been shown to effect receptor mRNA levels (Eidne, 1994; Fernàndez-Vàzquez *et al.* 1996; Hamernik *et al.* 1995; Quinones-Jenab *et al.* 1996). Alarid and Mellon (1995) have shown that increased levels of cAMP inhibit GnRH receptor gene expression posttranscriptionally by destabilising mRNA. GnRH receptor gene regulation is reviewed in more detail by Leung and Peng (1996) and Stojilkovic *et al.* (1994). It is imperative to determine GnRH receptor gene structure, as well as identifying transcription start sites and DNA control regions in the promoter that may regulate GnRH receptor gene expression in order to understand the possible implications in control of the reproductive system.

2.1. GnRH receptor gene structure

The intron-exon structure of the human (Fan *et al.* 1994), the mouse (Albarracin *et al.* 1994; Zhou and Sealfon, 1994) and rat (Reinhart *et al.* 1997) GnRH receptor genes are similar. Exon 1 carries the 5'-untranslated region and codes for transmembrane domains (TM) I-III and a portion of TM IV. Exon 1 is 1272 bp, 584 bp, and 625 bp long in the human (Fan *et al.* 1995), mouse (Albarracin *et al.* 1994; Zhou and Sealfon, 1994) and rat (Reinhart *et al.* 1997) GnRH receptor genes, respectively. Exon 2 which is 219 bp long in the human and 217 bp long in both the mouse and rat GnRH receptor genes codes for the remainder of TM IV as well as TM V. Exon 3 codes for TMs VI and VII and the COOH-terminal tail part of receptor together with the 3'-untranslated region and is 3300 bp, 803 bp and 1476 bp long in the human, mouse and rat GnRH receptor genes, respectively (Albarracin *et al.* 1994; Fan *et al.* 1995; Reinhart *et al.* 1997; Zhou and Sealfon, 1994). The entire gene is over 20 kb long with the first intron being at least 4 kb, 15 kb and 12 kb in length in the human, mouse and rat, respectively. The second intron is 5 kb long in both the human and mouse, but only 2.5 kb long in the rat. The intron-exon structure of the mouse GnRH receptor gene is depicted in Fig. 1.5.

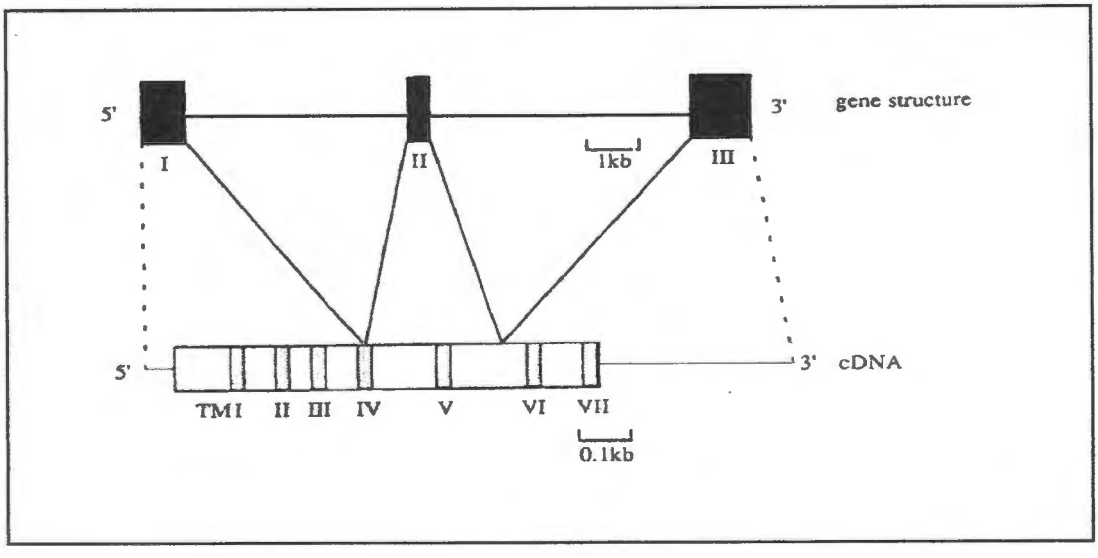

Figure 1.5. Intron-exon structure of the mouse GnRH receptor gene and structure of mouse GnRH receptor cDNA. Open boxes indicates the protein coding regions and hatched boxes are the putative transmembrane domains (Zhou and Sealfon, 1994).

2.2. Characterisation of the 5' end of the GnRH receptor cDNA

Primer extension analysis detected multiple transcription initiation start sites at positions -742, -733, -167, -144 and -43 relative to the translational start site in the human GnRH receptor gene (Fan *et al.* 1995). Major transcription start sites at -63 and -103 nucleotides relative to the start codon were detected in the mouse (Albarracin *et al.* 1994; Hapgood, unpublished data) and rat (Reinhart *et al.* 1997) GnRH receptor genes, respectively. The existence of multiple, as well as far upstream transcription start sites in the human GnRH receptor gene suggests the potential for more numerous and diverse transcription elements in the human promoter than in those of rodents.

Several consensus sites for putative transcription factors have been identified, by sequence analysis, in the region upstream of the transcription start sites of the human, mouse and rat GnRH receptor genes. Whether these binding sites have functional significance needs to be determined. Not much has been published on establishing functional activity of promoter regions, but for the mouse, Albarracin *et al.* (1994) has shown that 1.2 kb of the 5'-flanking region of the GnRH receptor gene is essential for promoter function and tissue specificity.

The TATA box, important for determining the accurate start of transcription, appears to be absent in the mouse 5'-flanking region although sequence analysis of the 5' end of the human and rat GnRH receptor genes revealed the presence of five (-57, -154, -193-, -761, -774

upstream of the start codon) and one putative TATA box at position -126 relative to the translational start site in the human and rat genes, respectively. Several CAAT sequences are interspersed among the TATA boxes (Fan *et al.* 1995; Reinhart *et al.* 1997). It is unusual for G-protein-coupled receptors to have consensus TATA and CAAT sequences. In the absence of consensus TATA and CAAT sequences G-protein-coupled receptors may be regulated by mechanisms such as GATA motifs (Aird *et al.* 1994), AT-rich elements (Wobbe and Stahl, 1990), CpG islands and transcriptional initiator elements in conjunction with Sp1 binding sites (Javahery *et al.* 1994). The human and rat GnRH receptor genes fall into the category of TATA-containing G-protein-coupled receptors, but whether the absence or presence of TATA boxes is characteristic of this receptor family needs to be further investigated, as relatively few G-protein coupled receptor promoter regions have been sequenced. A putative cAMP response element (CRE), 5'-tgaagtct-3', was detected in the human GnRH receptor gene at position -1533 to -1526 relative to the start codon and is 75% homologous to the published sequence. Sequence analysis further revealed the presence of a putative glucocorticoid/progesterone response element (GRE/PRE 5'-gttacacagtattct-3') at position -121 to -135 relative to the translation start site. The presence of these sequences suggests that cAMP and glucocorticoids or progesterone may play a role in the regulation of the human GnRH receptor gene. Consensus binding sites for eukaryotic transcription factors, such as GATA-1, Oct-1 and engrailed protein was also identified. Several PEA-3 binding sites, 5'-aggaag-3', which act as a phorbol ester responsive element in some genes, have also been detected. The human, mouse and rat GnRHR genes all have a putative AP-1 site, which has been shown to confer responsiveness to protein kinase C, in other promoters. A Pit-1 site has been detected in the human and rat GnRH receptor 5'-flank (Fan *et al.* 1995; Reinhart *et al.* 1997). Pit-1 is an anterior pituitary specific transcription factor that has been reported to be involved in the regulation of anterior pituitary

hormones. A possible GnRH-responsive element was found in the mouse GnRHR 5'-flanking region (Albarracin *et al.* 1994). A 30-bp DNA sequence (gonadotrope-specific element (GSE)) which is proposed to dictate gonadotrope-specific expression of the α-subunit of the glycoprotein hormones has been detected in the 5'-flanking region of the α-subunit gene. Barnhart and Mellon (1994) has shown that the transcription factor, steroidogenic factor-1, binds to an 8-bp stretch of nucleotides within the GSE. An identical 8-bp putative GSE or SF-1-like sequence was found in the 5'-flanking region of the mouse (Albarracin *et al.* 1994) and rat (Reinhart *et al.* 1997) GnRH receptor genes. Whether the SF-1-like sequence identified in the 5'-flanking region of the mouse GnRH receptor gene plays a role in GnRHR gene regulation remains to be investigated. The finding that SF-1 regulates gonadotropin expression and that a putative SF-1 site was detected in the putative promoter region of the GnRHR gene suggests that SF-1 may form part of a conserved global regulatory pathway co-ordinating reproductive function.

2.3. The gonadotropins

LH and FSH are glycoprotein hormones which possess N-linked carbohydrate chains attached to specific asparagine residues. The type and quantity of oligosaccharide present on the gonadotropin effects the clearance of the hormone, their bioactivity, and how they are sorted intracellularly. The gonadotropins are heterodimers, composed of a common α-subunit and a unique β-subunit that is responsible for the specificity of the hormone (Gharib *et al.* 1990).

2.4. Gonadotropin regulation

The amplitude and frequency of GnRH release determines the amount of gonadotropins secreted. Pulsatile GnRH release stimulates gonadotropin secretion, while continuous

administration leads to suppression of gonadotropin secretion (Kay *et al.* 1994). GnRH acts at the pituitary level via the GnRHR, which is activated when its ligand GnRH binds to it. This results in the activation of phospholipase C which leads to elevated levels of DAG and intracellular Ca^{2+} and results in activation of PCK (Bruder *et al.* 1992). Ben-Menahem *et al.* (1995) have shown that protein kinase C (PKC) and Ca^{2+} are involved in GnRH mediated stimulation of gonadotropin secretion and that cross talk exists between PKC and Ca^{2+}, which are involved in mediating the early and late phases of GnRH action. Sex steroids also effect gonadotropin secretion, however it is not sure whether regulation occurs because of alterations in GnRH secretion induced by the steroids at the hypothalamus or whether regulation occurs directly at the level of the pituitary gland, or both (Karsch, 1987; Knobil, 1974; Schally *et al.* 1972). Gonadal peptides, for example inhibins, activins and follistatin, have been identified that can regulate gonadotropin secretion by direct effects on the pituitary (Attardi *et al.* 1995; Ling *et al.* 1985; Padmanabhan *et al.* 1996). In addition, SF-1 has been shown to regulate the human and rat glycoprotein α and β-subunit genes in pituitary gonadotrophs by binding to a gonadotrope-specific element (GSE) in the promoter region of these genes (Barnhart and Mellon, 1994; Halvorsen *et al.* 1996a and b; Horn *et al.* 1992; Keri and Nilson, 1996).

2.5. The gonadotropins and SF-1

Tissue specific expression of the α-subunit gene is believed to be mediated by a gonadotrope-specific element (GSE) which is conserved in all mammalian α-subunit genes thus far known (Barnhart and Mellon, 1994). The GSE contains the sequence 5'-tg(a/t)cc-3', which comprises a nuclear receptor binding half-site (Laudet *et al.* 1992). Variations of this sequence are also found in the promoter regions of many different genes which play a role in steroidogenesis, sexual differentiation and adult reproductive function (Barnhart and Mellon, 1994) (Fig. 1.6).

Using the immortalised mouse gonadotroph precursor cell line αT3-1, which expresses the α-subunit and GnRHR but not LHβ or FSHβ mRNA, Barnhart and Mellon showed that SF-1 binds to the human GSE (Windle *et al.* 1990; Barnhart and Mellon, 1994). They also showed that reporter constructs containing the α-subunit GSE are expressed at higher levels in cell lines with endogenous SF-1 than in cells lacking SF-1, demonstrating the potential of the GSE as an SF-1-dependent regulatory element. These findings supports a role for SF-1 in tissue specific transcriptional activation of the α-subunit gene.

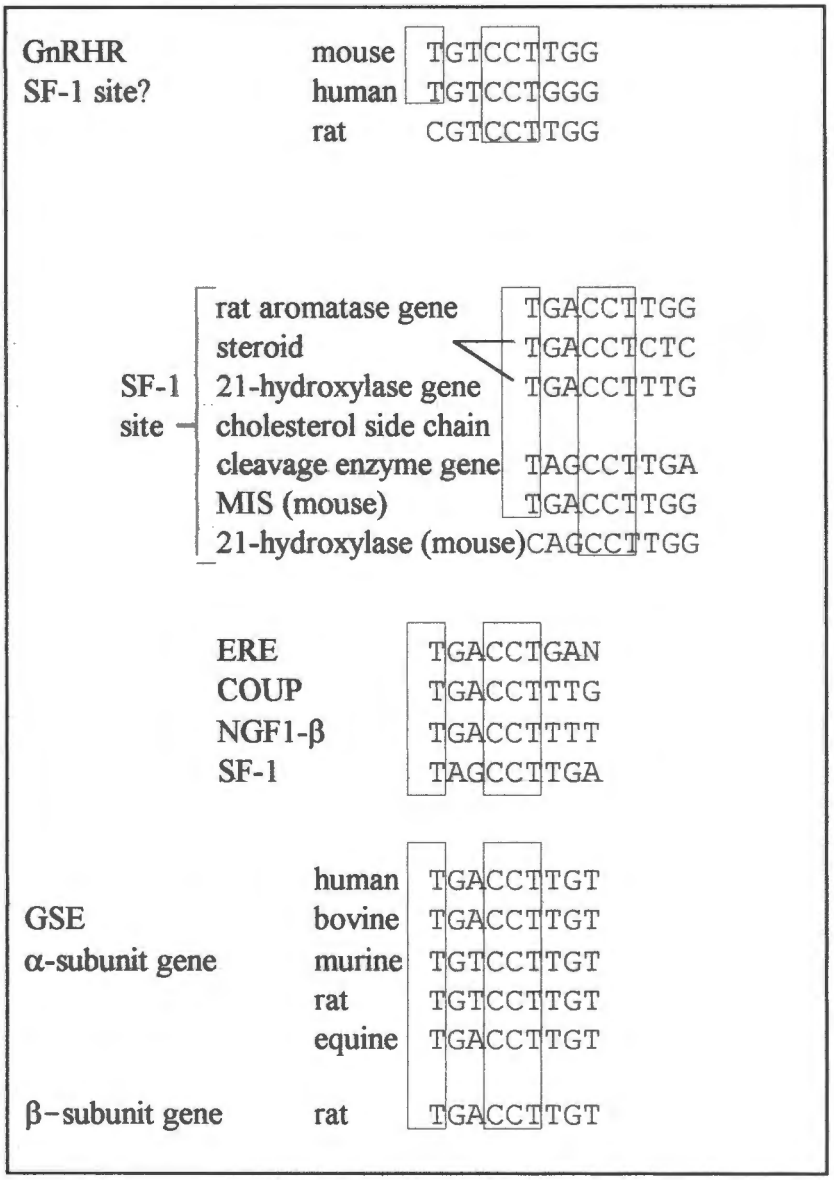

Figure 1.6. The GSE is homologous to the binding sites for members of the steroid receptor superfamily.

In addition to regulating α-subunit gene expression, SF-1 also regulates β-subunit gene expression. The rat LHβ gene promoter contains a consensus GSE (TGACCTTGT) at -127 to -119 relative to the transcription start site, while the bovine LHβ gene promoter contains a consensus GSE at -125 to -117 relative to the transcription start site. Keri and Nilson have shown that transfection of αT3-1 cells, who endogenously express SF-1, with an SF-1 expression vector, increases transcription of the LHβ promoter. Furthermore they have used transgenic mice with the wild type or mutant bovine LHβ promoter to prove the physiological significance of the LHβ SF-1 consensus sequence (1996). Co-transfection of an SF-1 containing expression vector and an LHβ luciferase reporter construct into CV-1 cells, which lack endogenous SF-1, caused a 56-fold increase in luciferase activity (Halvorson *et al.* 1996b). Taken together these results suggest that SF-1 may participate in expression of the LHβ gene by gonadotropes. Halvorson *et al.* has further shown that GnRH and the PKC system interact with SF-1 to increase LHβ activity in the rat (Halvorson *et al.*, 1996a). SF-1 and the estradiol receptor act in synergism to regulate the expression of the salmon gonadotropin IIβ subunit gene (Drean *et al.*, 1996).

2.6. Steroidogenic factor-1 (SF-1)

Steroidogenic factor-1, a 54 kDa protein, is a member of the orphan nuclear receptor transcription factor superfamily (Barnhart and Mellon, 1994). The human SF-1 protein consists of 461 amino acids and is highly homologous to those of mammalian counterparts. The gene is 30 kb long and contains seven exons, including a non-coding exon 1 (Oba *et al.* 1996).

Although SF-1, or sometimes called adrenal 4-binding protein (Ad4bp), was first described in the adrenal gland where it is a key regulator of steroidogenic enzymes in adrenocortical cells, it is also found in the testis, ovary, pituitary gonadotrope cells and embryonic ventral diencephalon which gives rise to part of the hypothalamus (Ikeda *et al.* 1993; Ikeda *et al.* 1994; Ingraham *et al.* 1994).

SF-1 was first identified through its ability to bind to and co-ordinately regulate the expression of genes in the corticosteroid biosynthetic pathway (Lala *et al.* 1992). Subsequently, SF-1 was shown to bind to and regulate the rat cytochrome P450 aromatase gene, which catalyses the conversion of androgens to estrogens (Lynch *et al.* 1993). Although SF-1 is predominantly expressed in the adrenal gland it is selectively expressed in the pituitary gonadotrope cells, where it is proposed to regulate the expression of many different gonadotrope specific genes (Barnhart and Mellon, 1994; Halvorson *et al.* 1996b; Keri and Nilson, 1996). The occurrence of SF-1 in the pituitary and hypothalamus suggests that the function of SF-1 extents well beyond regulating steroidogenic enzymes and that SF-1 is essential for reproductive behaviour (Barnhart and Mellon, 1994). This is a field of active research and knock out experiments have been done to determine the role of SF-1 (Ikeda *et al.* 1995; Ingraham *et al.* 1994; Luo *et al.* 1994)

2.7. Knock out experiments

Sequence analysis revealed that the SF-1 transcript is homologous to FTZ-F1, a gene product that regulates the expression of the *Drosophila fushi tarazu* (*ftz*) homeobox gene (Lavorgna *et al.* 1991; Ueda *et al.* 1990). In knock out experiments with mice, Luo *et al.* (1994), showed that mice in which the *Ftz-F1* gene (encoding SF-1) was disrupted, lacked adrenal glands and

gonads and had female internal genitalia. This is consistent with the fact that SF-1 regulates the biosynthesis of androgens (Lynch *et al.* 1993) and Mullerian-inhibiting substance (Shen *et al.* 1994), which are important mediators of male sexual differentiation. Studies showed that *Ftz-F1* disrupted mice lacked transcripts for the gonadotrope-specific markers LHβ, FSHβ and the GnRHR and exhibited decreased expression of the α-subunit of the glycoprotein hormones (Ingraham *et al.* 1994). These findings imply that SF-1 is required for development of cells of the gonadotrope lineage or for expression of multiple gonadotrope-specific genes. In knock out experiments, Ikeda *et al.* showed that mice in which the SF-1 gene was disrupted exhibited grossly impaired ventromedial hypothalamic nucleus structure (Ikeda *et al.* 1995). Despite their apparently normal GnRH neurons, pituitary gonadotropins and GnRH receptor were not expressed. GnRH treatment restored gonadotropin and GnRH receptor expression in SF-1 knock out mice. Curing with GnRH implies that impaired delivery of GnRH to the gonadotropes causes defects in gonadotropin and GnRH receptor expression. SF-1 knock out mice have defects in all three levels of the hypothalamus-pituitary-gonadal axis and this suggest that SF-1 plays a key role in reproductive function. Further studies on the link between SF-1, the hypothalamus and reproductive function will provide insight into the complex mechanisms of neuro-endocrine control. Recently the human gene encoding SF-1 has been cloned, which will provide new insights on SF-1 structure, thereby facilitating efforts to study the role of SF-1 in neuro-endocrine control (Oba *et al.* 1996; Wong *et al.* 1996).

2.8. DAX-1

DAX-1 encodes a 470 amino acid nuclear hormone receptor with a novel DNA-binding domain (Burris *et al.* 1996; Zanaria *et al.* 1994). Mutations in the *DAX-1* gene results in X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism (Muscatelli *et al.* 1994).

Disruption of both the *SF-1* and *DAX-1* genes results in a phenotype of adrenal insufficiency and deficiency of pituitary gonadotrophs (Ingraham *et al.* 1994; Luo *et al.* 1994; Yu *et al.* 1996), but whereas Ftz-F1-disrupted mice completely lack gonads, DAX-1 null patients have poorly developed but detectable testes. Ito *et al.* (1997) showed that DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain and suggests that the loss of this inhibitory property of DAX-1 may account in part for the phenotype of adrenal hypoplasia congenita. DAX-1 shows a similar tissue distribution to SF-1 and is present in the pituitary gland, hypothalamus, adrenal gland, gonadotropes and gonads (Burris *et al.* 1995; Guo *et al*, 1995). Burris *et al.* (1995) first described the presence of a putative SF-1 site in the 5'-flanking region of the human *DAX-1* gene. They further showed that SF-1 is able to bind the putative SF-1 response element found in the *DAX-1* promoter, *in vitro*. Yu *et al.* (1996) showed that *DAX-1* transcription is regulated by SF-1 and in 1997 Vilain *et al.* showed that the SF-1-like-site is an enhancer element within the *DAX-1* promoter. Taken together, these results suggest that SF-1 may be transcription factor that acts, at least in part, through DAX-1 in the cascade leading to normal pituitary, adrenal, and gonadal development.

In summary, sequence analysis of the regulatory region of the GnRH receptor gene has revealed the presence of a putative GSE or SF-1-like site. The aim of this study is to determine if SF-1 interacts with the putative GnRHR GSE, and whether this interaction is involved in GnRH receptor gene regulation. Understanding the mechanisms by which the GnRHR is regulated has important therapeutic implications, as it is thought that regulation of the GnRHR gene is one of the major levels of control of reproductive function.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

All materials (powders and organic solvents) used were analytical grade, unless stated otherwise. Solutions were made with distilled-deionised H_2O and sterilised by autoclaving.

2. Cells

Mouse pituitary gonadotrope precursor cell lines which expresses (αT3-1) (Windle *et al.* 1990) and which do not express (αT4) the GnRHR, as well as a mouse GnRH neuronal cell line (GT1-7) (Mellon *et al.* 1990) which may express the GnRHR under certain tissue culture conditions were obtained from Pamela Mellon (University of California, San Diego, CA). The mouse pituitary somatotrope cell line, GH_3, as well as a monkey kidney cell line, COS-1, was used in this study. GH_3 and COS-1 cells were provided by the Department of Chemical Pathology, University of Cape Town.

3. Plasmids

A genomic clone, clone III, of the mouse GnRH receptor gene was obtained from Stuart Sealfon at Mt. Sinai in New York. This clone contains 1.1 kb of the GnRH receptor gene cloned into the XbaI site of the pBluescript®SK phagemid. The 1.1 kb sequence is made up as follows: 500 bp of 5'-flanking sequence, 569 bp of coding sequence (accession number, M93108), and 38 bp of intergenic sequence (accession number, L33778) (Fig. 2.1).

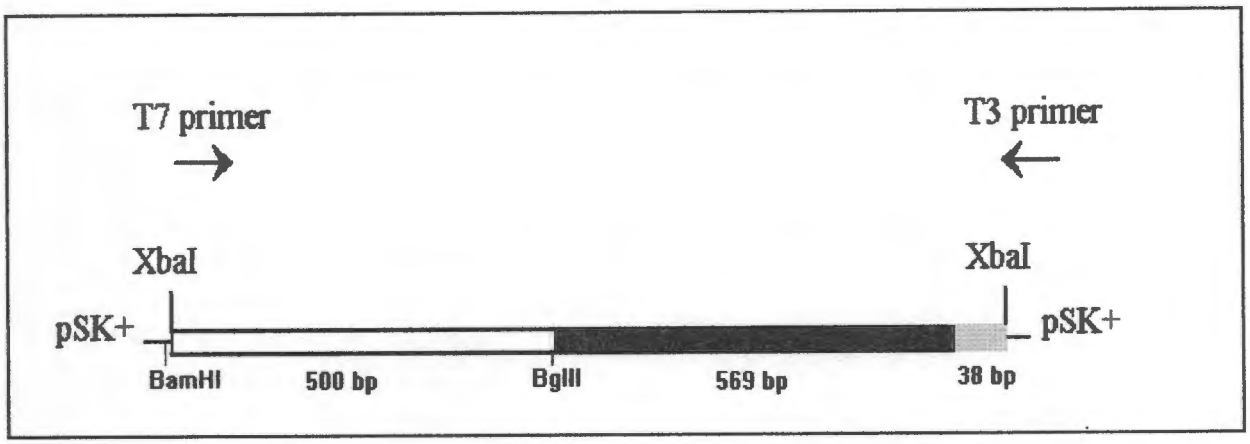

Fig. 2.1. Plasmid map of clone III. Clone III contains 1.1 kb of GnRH receptor genomic sequence cloned into the XbaI site of pBluescript SK+. The white, black and grey boxes represent the 5'-flanking region, exon 1 and intron 1, respectively.

A 580 bp BglII-BamHI fragment was excised from clone III after mutagenesis of the ATG to a BglII site. The constructs, wt GnRHR-LUC and mut GnRHR-LUC contain 563 bp of the GnRH receptor gene containing sequences from -500 to +63 relative to the transcription start with the wild type (TGTCCTTGG) or mutated (TGTTTTTGG) GSE respectively, cloned into the BglII site of the promoterless LUC reporter plasmid, pGL2-basic (Promega). The β-galactosidase expression vector, pCISLACZ, used in this study was provided by Dr. Arieh Katz, Department of Chemical Pathology, University of Cape Town, and has the lacZ gene (3.8 kb) cloned into the XhoI/SmaI site of the 6.27 kb pCIS vector. The plasmid SF-1 was obtained from Keith Parker (Duke University Medical Centre, Durrham, North Carolina) and contains about 2 kb of human SF-1 cDNA cloned into the EcoRI site of the 4.7 kb plasmid, pCMV5 (Andersson *et al.* 1989). Human β-actin cDNA (2.1 kb) cloned into the BamHI site of the 2.9 kb ampicillin resistant expression vector Okayama-Berg (Okayama and Berg, 1983) was provided by Sihaam Boolay (Department of Biochemistry, University of Cape Town).

4. Oligonucleotides

The sequences of oligonucleotides used are listed below:

Oligonucleotides used for sequencing

race 1	5′ GAGGTGCCTGTCCGAAGTTCTCACC 3′
m13 -20	5′ GTAAAACGACGGCCAGT 3′
T7	5′ AATACGACTCACTATAG 3′
T3	5′ ATTAACCCTCACTAAAG 3′
reverse primer	5′ AACAGCTATGACCATG 3′
GL1	5′ TGTATCTTATGGTACTGTAACTG 3′
GL2	5′ CTTTATGTTTTTGGCGTCTTCCA 3′

Oligonucleotides used for mutagenesis

1S	5′ CTTGGAGAAA GATCTCTAAC AATGCATC 3′
2S	5′ GAAGCCTGTT TTTGGAGAAA GATCTCTAAC AATGC 3′

Double-stranded oligonucleotides used for mobility shift assays

mGnRHR 'GSE'	5′ GATCCCTGTCCTTGGAGAAGATC 3′	
	3′ CTAGGGACAGGAACCTCTTCTAG 5′	GSE
mGnRHR	5′ GATCCCTGTTTTTGGAGAAGATC 3′	
mutated 'GSE'	3′ CTAGGGACAAAAACCTCTTCTAG 5′	mGSE
SF-1 aromatase	5′ GATCGATGACCTTGGGAGAGATC 3′	
	3′ CTAGCTACTGGAACCCTCTCTAG 5	SF-1

Oligonucleotides were annealed for gel mobility shift assays using the method described by Ausubel *et al.* (1987). Complementary strands were annealed at a molar ratio of 1:1, by incubating at 88°C for 2 min, followed by 65°C for 10 min, 37°C for 10 min, 25°C for 5 min and then finally placing the sample on ice.

5. Plasmid isolation

5.1. Small -scale (mini-prep) method

The Ish-Horowicz and Burke method (1981) and the Wizard Minipreps DNA Purification kit were used to extract plasmid DNA. Both methods employ the alkali-lysis method to isolate plasmid DNA.

5.1.1. Preparation of plasmid DNA using the Ish-Horowicz and Burke method

Plasmid DNA was extracted from *E. coli* cells using a modified version of the miniprep method described by Ish-Horowicz and Burke (1981). The cells were grown overnight in 5 ml of 2 X yeast tryptone (2 X YT) (1.6% tryptone (w/v), 1% yeast extract (w/v), 85.6 mM NaCl, pH 7.0) broth (ampicillin 100 µg/ml) and incubated at 37°C, with shaking. Cells were harvested by spinning in a microfuge for 2 min and the cell pellet was resuspended in 200 µl of solution 1 (25 mM Tris-Cl (pH 8.0), 50 mM EDTA, 1% glucose (w/v)). EDTA chelates Mg^{+} ions and therefore inactivates DNases, while glucose allows the formation of spheroplasts. Cells were lysed by adding 300 µl of freshly prepared solution 2 (0.2 M NaOH, 1% SDS (w/v)) and mixed by inverting the tube until the solution cleared. NaOH denatures chromosomal DNA, while SDS, a detergent, disrupts the cell membrane. After 5 min on ice 300 µl of solution 3 (3 M potassium acetate pH 4.8) was added and left on ice for a further 5 min. The decrease in pH allows selective renaturation of plasmid DNA that might have denatured after the addition of solution 2, while potassium acetate precipitates chromosomal DNA and proteins. Cellular debris was removed by spinning for 10 min at 15 000 *g* and the supernatant was transferred to a new tube. RNase A was added to a final concentration of 20 µg/ml and incubated at 37°C for 2 hr. After RNase treatment the supernatant was extracted twice with chloroform by adding an equal volume of chloroform, mixing by hand for 30 sec and then spinning for 1 min to allow phase

separation. The DNA was precipitated by adding an equal volume of 100% isopropanol to the aqueous phase and spinning for 10 min at 15 000 *g*. The DNA pellet was washed with 500 µl of cold 70% ethanol and centrifuged for 10 min. The pellet was vacuum dried and resuspended in 32 µl of H_2O. DNA was further purified by polyethylene glycol precipitation (PEG) as discussed in 5.3.2.

5.1.2. Preparation of plasmid DNA using the Wizard DNA Purification kit.

The protocol used was according to that described by the manufacturers (Promega). Cells were harvested from *E. coli* cultures as described in 5.1.1. The cell pellet was resuspended in 200 µl of Cell Resuspension Solution (50 mM Tris-Cl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A) and lysed by adding 200 µl of Cell Lysis Solution (0.2 M NaOH, 1% SDS (w/v)). Chromosomal DNA and proteins were precipitated by adding 200 µl of Neutralisation Solution (1.32 M potassium acetate pH 4.8) and spinning in a microfuge for 5 min. Wizard Minipreps DNA Purification Resin was added to the supernatant and plasmid DNA was isolated by passing the mixture through a Wizard minicolumn. The minicolumn traps plasmid DNA which is eluted with 50 µl of ultra-pure H_2O. The plasmid DNA was analysed spectrophotometrically, then by restriction enzyme digestions and agarose gel electrophoresis. The concentration of the DNA was calculated according to the formula in Sambrook *et al.* (1989), where 1 absorbance unit at 280 nm is equivalent to 50 µg DNA/ml. The purity of the DNA was estimated by the ratio between the readings at 260 nm and 280 nm. The DNA was stored at -20°C.

5.2. Large-scale (maxiprep) method

Plasmid DNA was extracted using a scaled-up version of the miniprep method of Ish-Horowicz and Burke, employing either the Nucleobond (Separations) or the Wizard Maxiprep DNA Purification kits.

5.2.1. Extraction of plasmid DNA using the Nucleobond kit

E. coli cells were grown in 10 ml of 2 X YT broth (ampicillin 100 µg/ml) at 37°C overnight, with shaking. A main culture (500 ml) was set up by diluting the starter culture 100-fold in 2 X YT broth (ampicillin 100 µg/ml) and incubating at 37°C overnight, with shaking. Cells were harvested by centrifugation at 5 000 rpm in a Beckman J2.21 centrifuge, JA-10 rotor. The cell pellet was carefully resuspended in 12 ml of solution S1 (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A). Cells were lysed by adding 12 ml of solution S2 (0.2 M NaOH, 1% SDS (w/v)), mixed gently and incubated at room temperature for 5 min. Thereafter 12 ml of solution S3 (2.8 M potassium acetate pH 5.1) was added, the suspension mixed by inverting the tube 6-8 times and incubated on ice for at least 5 min. The cell suspension was centrifuged at 12 000 rpm for 45 min at 4°C (JA-20 rotor). After the high speed centrifugation the supernatant was carefully removed from the white precipitate and loaded onto a NUCLEOBOND AX cartridge equilibrated with 5 ml of buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl, pH 6.3). After adsorption of the nucleic acid to the cartridge, it was washed twice with 12 ml of buffer N3 (100 mM Tris, 15% ethanol, 1159 mM KCl, pH 6.3). The plasmid DNA was eluted with 6 ml of buffer N5 (100 mM Tris, 15% ethanol, 100 mM KCl, pH 8.5). The purified plasmid DNA was precipitated by adding 0.7 volumes of isopropanol followed by centrifugation at 15 000 rpm for 20 min at 4° (JA-20 rotor). The DNA was washed with 70% ethanol, vacuum dried for 5 min and redissolved in TE buffer (10 mM Tris-Cl (pH 7.5), 1 mM EDTA). The

DNA was analysed as described earlier and then purified by caesium chloride (CsCl) centrifugation as discussed in 5.3.1.

5.2.2. Extraction of plasmid DNA using the Wizard Maxiprep Purification kit

Bacteria were grown up as for the Nucleobond kit. All steps were performed at room temperature. Cells were harvested as described in 5.2.1. The cell pellet was resuspended in 15 ml of Cell Resuspension Solution (50 mM Tris-Cl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A). Cells were lysed by adding 15 ml of Cell Lysis Solution (0.2 M NaOH, 1% SDS (w/v)), mixed by inverting the tubes and left at room temperature until the solution cleared (approximately 15 min). Thereafter 15 ml of Neutralisation Solution (1.32 M potassium acetate pH 4.8) was added, mixed by inversion and centrifuged at 14 000 *g* for 30 min. The supernatant was removed and centrifuged for 15 min. The DNA was precipitated by adding one half volume of isopropanol and centrifuging at 14 000 *g* for 20 min. The DNA pellet was resuspended in 2 ml of TE buffer. Ten ml of WIZARD Maxipreps DNA Purification Resin was added to the DNA solution and mixed. The Resin/DNA mix was transferred to a maxicolumn, with the tip inserted into a vacuum source. A vacuum was applied to pull the Resin/DNA mix into the maxicolumn. The maxicolumn was washed with 25 ml of column wash solution (0.2 M NaCl, 20 mM Tris-Cl (pH 7.5), 5 mM EDTA, 55% ethanol). The resin was rinsed by adding 5 ml of 80% ethanol to the maxicolumn. After the ethanol was drawn into the maxicolumn, the vacuum was allowed to draw for a further minute. The maxicolumn was inserted into a 50 ml tube and centrifuged at 1 500 *g* for 5 min in a swinging bucket rotor to dry the resin. The maxicolumn was placed back onto the vacuum source and the resin dried to completion by drawing a vacuum for another 5 min. Preheated (70°C) TE buffer (1.5 ml) was added to the maxicolumn

and left for 1 min. The DNA was eluted by centrifuging at 1 500 *g* for 5 min. The plasmid DNA was analysed as described before and thereafter purified by CsCl centrifugation.

5.3. Purification of plasmid DNA

5.3.1. CsCl centrifugation

Plasmid DNA was purified by isopycnic gradient centrifugation (CsCl centrifugation) as described in Sambrook *et al.* (1989). Ethidium bromide is a DNA intercalating agent that inserts between adjacent base pairs in the DNA double helix and reduces the buoyant density by pulling the strands apart. Covalently closed circular plasmid DNA cannot intercalate as much Ethidium Bromide as linear DNA can, and therefore a differential gradient is produced, which allows the separation of DNA in a solution of CsCl (Fig. 2.2).

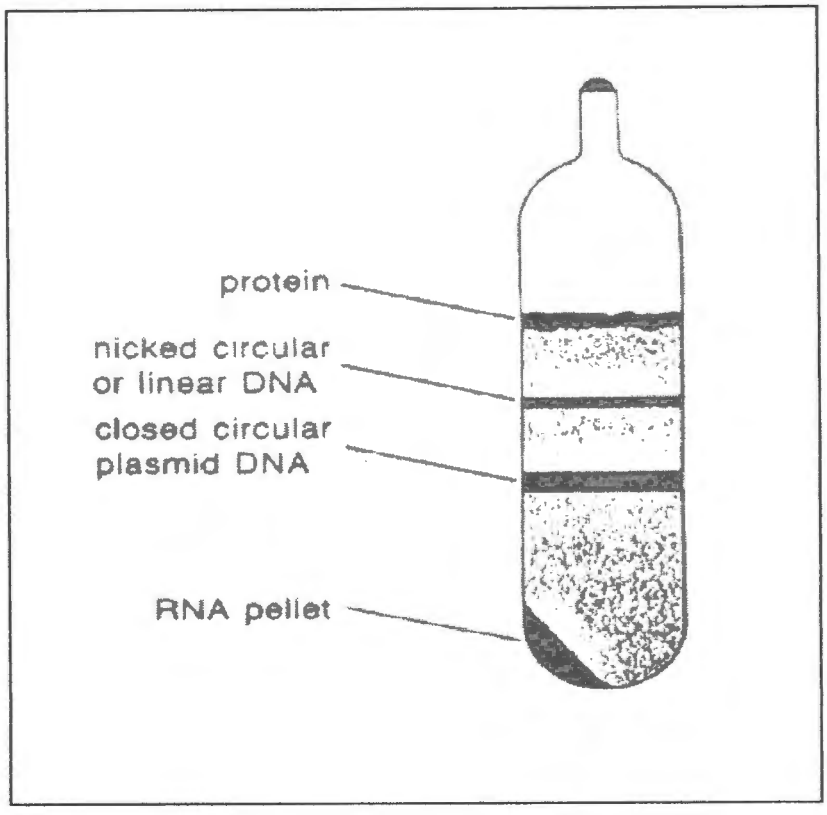

Fig. 2.2. Separation of DNA in a CsCl-Ethidium Bromide gradient (Sambrook *et al.* 1989).

The DNA to be purified (approximately 1 mg) was added to a solution consisting of 12.6 g of CsCl, 12.3 ml of TE buffer and 300 µl of 10 mg/ml ethidium bromide in a centrifuge tube. The weight of the DNA solution was adjusted so that 1 ml equalled 1.56 g. Beckman Quickseal ultracentrifuge tubes (Vti65) were filled with the plasmid DNA solution and centrifuged at 45 000 rpm overnight (20°C) in a Beckman L-70 ultracentrifuge, Vti65.2 rotor. The plasmid band was visible in normal light. The Vti65 tube was punctured and the closed circular plasmid DNA removed with a 1 ml syringe and placed into a 10 ml tube. Ethidium bromide was removed by extracting four times with two volumes of isoamyl alcohol. After the fourth extraction 6 ml of TE buffer was added to the aqueous phase and dialysed for 3 hr against three changes of 500 ml TE buffer. After dialysis the DNA was precipitated by adding one tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold 100% ethanol, and placed at -20°C overnight. The DNA was collected by centrifugation at 14 000 *g* for 30 min. The pellet was washed with cold 70% ethanol and centrifuged for 15 min. After the pellet was vacuum dried it was resuspended in 200-500 µl of TE buffer. The DNA was analysed as described before and then stored at -20°C.

5.3.2. PEG precipitations

Differential precipitation with PEG yields plasmid DNA of extremely high purity but unlike CsCl purification, does not efficiently separate nicked circular molecules from the closed circular form of the plasmid DNA (Sambrook *et al.* 1989). Miniprep DNA prepared by the alkali-lysis method as described in section 5.1.1. was purified by PEG precipitation and then used for DNA sequencing .

One quarter volume (8 µl) of 4 M NaCl and one and a quarter volume (40 µl) of 13% PEG_{8000} was added to the 32 µl of plasmid DNA isolated in 5.1.1. After thorough mixing the samples were incubated on ice for 40 min and the DNA pelleted by centrifugation at 15 000 *g* for 20 min at 4°C. After aspirating off the supernatant, the pellet was rinsed with 500 µl of cold 70% ethanol and centrifuged for 10 min. The supernatant was decanted, the DNA pellet vacuum dried for 10 min and then resuspended in 20 µl of H_2O. The DNA was analysed spectrophotometrically, then by agarose gel electrophoresis and stored at -20°C.

6. Restriction enzyme digestion

Restriction enzyme digestions were carried out using restriction buffers and enzymes as recommended by the manufacturers (Boehringer Mannheim and Amersham). The volume of enzyme used never exceeded one tenth of the total reaction volume as the enzyme contains glycerol, which may inhibit the reaction. Two restriction enzymes could be combined in a single digest provided their buffer requirements were compatible. If their buffer requirements were incompatible, the DNA was first digested with one enzyme, ethanol precipitated and then digested with the second enzyme. Digestion volumes varied according to the amount of DNA being digested, but normally 0.5 µg of plasmid DNA was digested in a total volume of 10 µl using 5 units of enzyme. Digestions were incubated at 37°C for 2-16 hr. The reactions were terminated by adding one sixth volume of 6 X stop/loading buffer (50% glycerol (v/v), 100 mM EDTA (pH 8.0), 1% SDS (w/v), 0.1% bromophenol blue (w/v), 0.1% xylene cyanol (w/v)) and analysed by agarose gel electrophoresis.

7. Agarose gel electrophoresis

To separate, identify and purify DNA fragments agarose gel electrophoresis was used. When an electric field is applied across an agarose gel, DNA, which is negatively charged, migrates toward the anode at a rate proportional to its size (Sambrook *et al.* 1989).

Agarose gel electrophoresis was performed using 1-4% agarose gels, depending on the size of the DNA fragments to be separated. Agarose (Sigma) was dissolved in 1 X TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer by heating in a microwave oven until the solution became clear. Ethidium bromide was added to the gel to a final concentration of 0.5 μg/ml to enable visualisation of the DNA by transillumination. It was cooled to approximately 50°C before being poured into a horizontal mold and allowed to solidify for 30-60 min. Thereafter DNA samples (containing stop/loading buffer to a final concentration of 1 X) were loaded onto the gel submerged in a buffer tank containing 1 X TAE. An electric field of approximately 10 V/cm was applied to the various gels. Bacteriophage λ DNA digested with PstI was used as a molecular size marker, or when very small fragments were analysed the 123 bp ladder (Boehringer Mannheim) was used. Preparative agarose gels (DNA fragments for cloning or radioactive labelling) were performed with 1-2% low melting agarose gels in 1 X TAE buffer (SeaPlague (FMC BioProducts) for large fragments or NuSieve (FMC BioProducts) for small fragments less than 1 kb). Preparative gels were run in 1 X TAE buffer at 5 V/cm. The bands were detected as above, but with a low energy hand-held UV lamp (λ=342nm) to ensure that the DNA was not damaged.

8. Purification of DNA fragments from low melting agarose

DNA was recovered from low melting agarose gels using the USBioclean MP Kit (United State Biochemical), which is based on the method of Vogelstein and Gillepsie (1979). After separation by agarose gel electrophoresis, the relevant bands were cut out with sterile blades after visualisation by a hand-held UV lamp (λ=342nm). The gel slices were placed in petri dishes, cut up, and transferred to 1.5 ml microfuge tubes. Three volumes of 6 M NaI solution were added to the agarose gel block and placed at 55°C for 5 min to dissolve the agarose. Thereafter 10 µl of glass powder suspension was added, mixed well, and allowed to sit on ice for 5 min to allow the DNA to adhere. The mixture was then microfuged for 10 sec and the supernatant removed. The glass powder pellet was washed twice with a 50-fold excess of rinse buffer and microfuged for 10 sec. The DNA was eluted by dissolving the glass powder pellet in 20 µl of TE buffer and incubating at 55°C for 5 min. After incubation the sample was microfuged for 10 sec and the DNA was recovered in the supernatant. The DNA concentration was estimated by ethidium bromide spotting (Sambrook *et al.* 1989), which entails spotting DNA samples and standards onto the surface of a 1% agarose slab gel containing 0.5 µg/ml ethidium bromide and then visualising by UV light. Solutions of λ DNA, with concentrations varying between 2.5 and 25 ng/µl, were used as standards.

9. DNA cloning

9.1. Preparation of competent cells

Competent cells were prepared using a variation of the method described by Perbal (1988). Cells were streaked onto LB (1% tryptone (w/v), 0.5% yeast extract (w/v), 171 mM NaCl, pH 7.0) agar plates and grown at 37°C overnight. Thereafter a single colony was inoculated into 40 ml of ψ broth (2% tryptone (w/v), 0.5% yeast extract (w/v), 20 mM $MgSO_4.7H_2O$, 10 mM

NaCl, 5 mM KCl) and grown at 37°C overnight, with shaking. 4 ml of the overnight culture was inoculated into 200 ml of ψ broth and shook at 37°C until the OD_{600} reached 0.46-0.60. All further steps were carried out at 4°C. Cells were collected by spinning at 5 000 rpm for 10 min (Beckman J2.21 centrifuge, JA-10 rotor). The cell pellet was resuspended into 40 ml of pre-cooled TFBI buffer (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2.2H_2O$, 50 mM $MnCl_2.4H_2O$, 15% glycerol (v/v), pH 5.8). The solution was spun at 5 000 rpm for 10 min (JA-20 rotor) and the cell pellet was resuspended into 8 ml of TFBII buffer (10 mM MOPS, 75 mM $CaCl_2.2H_2O$, 10 mM $RbCl_2$, 15% glycerol, pH 6.8). The solution was left on ice for 15 min, then frozed down and stored in 400 µl aliquots at -70°C.

9.2. Linearisation and dephosphorylation of vector

Recircularisation of vectors was minimised by removing the 5' phosphates with calf intestinal phosphatase (CIP) (Boehringer Mannheim). The method used is described in Sambrook *et al.* (1989) and is as follows: vectors (20 µg) were linearised by digesting overnight with 60 units of a suitable enzyme. After digestion the sample was extracted with an equal volume of phenol-chloroform (1:1 v/v) and the DNA precipitated by adding 2 volumes of 100% ethanol and placing at 0°C (an ice-water slurry) for 15 min. The DNA was recovered by spinning at 15 000 *g* for 10 min at 4°C and the DNA pellet was redissolved in 90 µl of 10 mM Tris-Cl (pH 8.0). The concentration of the DNA was determined spectrophotometrically and the DNA was dephosphorylated in a reaction containing 1 X dephosphorylation buffer (Boehringer Mannheim) and 0.2 units of CIP. The reaction was placed at 56°C and stopped after 30 min by adding 5 µl of 10% SDS (w/v) and 1 µl of 0.5 M EDTA (pH8.0). After mixing well the CIP was inactivated by placing at 75°C for 10 min. The reaction was cooled to room temperature then extracted once with an equal volume phenol and then with an equal volume of phenol-

chloroform (1:1 v/v). The DNA was precipitated by adding one tenth volume of 3 M sodium acetate (pH 7.0), 2 volumes of 100% ethanol and placed at 0°C for 15 min. The DNA was recovered by centrifugation at 15 000 *g* for 10 min, the pellet washed with 70% ethanol and centrifuged for a further 10 min. The DNA was resuspended in 60 µl of TE buffer and the concentration determined.

9.3. Ligation

Ligation reactions were performed using the in-gel-ligation method described in the NuSieve GTG agarose booklet, with slight modifications. The molar ratio of insert: vector used was 5:1. 25 ng of insert DNA was ligated to 48 ng of vector DNA in a reaction consisting of 1 X T4 ligase buffer (Boehringer Mannheim) and 4 units T4 DNA ligase (Boehringer Mannheim) in a final volume of 42 µl. The ligation reaction was incubated at room temperature for 16 hr. Reactions were incubated at 68°C for 5 min to melt the agarose and ligations stopped by adding EDTA to a final concentration of 12.5 mM. The samples were placed at 37°C until transformation to prevent the agarose from solidifying.

9.4. Transformation

The transformation method used was a variation of the method described in the Sambrook *et al.* (1989). *E.coli* XL-1 blue competent cells were thawed on ice and 10 µl of the ligation mix, prepared in section 9.3 (approximately 10 ng of vector) was added to 100 µl of the competent cells prepared in 9.1. Two controls were set up in parallel; a transformation control, i.e., competent cells transformed with 1.25 ng of uncut pBR322 and a contamination control, i.e., cells without DNA. After 30 min on ice cells were heat-shocked (37°C for 5 min) to induce DNA uptake and then placed on ice for 2 min. 0.9 ml of LB medium was added and the

reactions incubated at 37°C for 60 min, with shaking, to allow expression of the antibiotic resistance markers. Different aliquots of the transformation mixes were plated onto LB agar plates (ampicillin 50 μg/ml; tetracycline 15 μg/ml) and incubated at 37°C overnight. Recombinants were screened by miniprep plasmid isolation, restriction enzyme digestion, agarose gel electrophoresis and DNA sequencing.

10. DNA sequencing.

Sequencing was performed using the Sequenase 2.0 kit (United States Biochemical) or an automated DNA sequencer (373 DNA Sequencer, Applied Biosystems).

10.1. Sequencing using the Sequenase 2.0 (USB) kit

The Sequenase 2.0 kit is based on the enzymatic method described by Sanger *et al.* (1977). This method involves the use of dideoxynucleoside triphosphates (ddNTP) that, once incorporated into a growing chain prevent further extension because they lack the necessary 3'-hydroxyl group. Four reactions, each containing all four dNTPs (one radioactively labelled), are carried out. DNA polymerase may incorporate either a dNTP or a ddNTP into the growing chain. If a ddNTP is incorporated, chain elongation is terminated and this results in a population of DNA strands of various lengths, each ending with the same ddNTP. The DNA sequence can be deduced, after these fragments have been resolved, according to size, by electrophoresis on a denaturing polyacrylamide gel followed by autoradiography (Fig. 2.3).

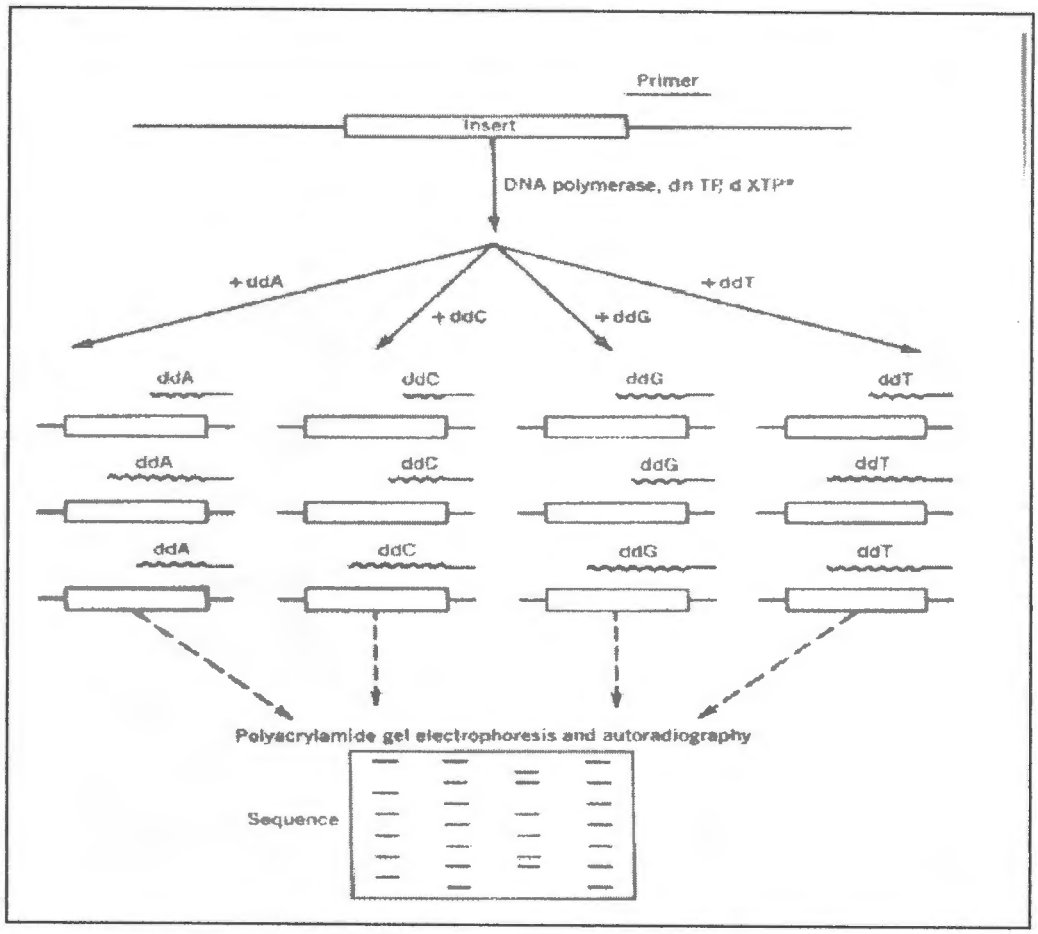

Fig. 2.3. Dideoxy reaction for sequencing. Copying of the insert DNA by DNA polymerase is inhibited at specific sites in the presence of dideoxynucleotides (Sambrook *et al.* 1989).

The protocol supplied with the Sequenase 2.0 kit was followed, with slight modifications. Double-stranded plasmid DNA was denatured using 2 different methods.

10.1.1. Alkaline denaturation

The method used is described in the Isotherm DNA sequencing kit. Approximately 5 μg of DNA, in a total volume of 20 μl, was denatured by adding an equal volume of 0.4 M NaOH, 0.4 mM EDTA. The reaction was mixed and incubated at 65°C for 15 min. The denatured DNA was precipitated by adding one fifth volume of 2 M ammonium acetate and 5 volumes of 95% ethanol, followed by incubation at room temperature for 5 min. The DNA was collected by

spinning at 15 000 *g* for 5 min and the DNA pellet washed by centrifuging with 70% ethanol. The pellet was dried in a speedy vac and resuspended in 7 µl of H_2O.

10.1.2. Denaturation by boiling

The template DNA was denatured by boiling 5 µg of DNA, in a total volume of 7 µl, for 3 min. The DNA was then immediately placed on dry ice for 30 sec and pelleted in a microfuge (Yuh-man Sun, personal communication).

The denatured DNA was annealed to different primers, with concentrations ranging between 10-20 ng, in a reaction mixture consisting of 7 µl of DNA, 2 µl of 5 X reaction buffer (20 mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl) and 1µl of primer. The annealing reaction was allowed to proceed at 37°C for 15 min then placed on ice (not longer than four hours) until the labelling reaction. The labelling reaction was carried out by adding 2 µl of 5 X dGTP labelling mix (all dNTPs except dATP), 1 µl of 100 mM DTT, 2 µl of Sequenase diluted 8-fold with enzyme dilution buffer and 0.5 µl of [α-^{35}S] dATP (10 µCi/µl, Amersham). The components were mixed and incubated at room temperature for 2 min. During incubation tubes containing 2.5 µl of the four termination mixes, ddATP, ddCTP, ddGTP and ddTTP were prewarmed at 37°C for at least 1 min. After labelling 3.5 µl of the labelling reaction was transferred into each of the microfuge tubes containing the four termination mixes. The components were mixed by gentle agitation and incubated at 37°C for 5 min before the addition of 5 µl of stop buffer (95% formamide (v/v), 20 mM EDTA, 0.05% bromophenol blue (w/v), 0.05% xylene cyanol FF (w/v)). The samples were stored at -20°C until polyacrylamide gel electrophoresis.

11. Denaturing polyacrylamide gel electrophoresis

DNA fragments were separated by polyacrylamide gel electrophoresis (Sambrook *et al.* 1989). A 6% polyacrylamide gel was prepared by mixing 5.7 g acrylamide (Merck), 0.3 g bis-acrylamide (Sigma), 42 g urea, 20 ml 5 X TBE (0.45 M Tris-borate, 0.01 M EDTA), 40 ml H_2O, and stirrring with a magnetic stirrer for 1 hour. The solution was filtered through a scintered glass funnel under vacuum. Polymerisation was initiated by adding 800 µl of 10% ammonium persulfate (w/v) and 80 µl of N,N,N',N' Tetramethlyethylenediamine (TEMED) before pouring the gel. After polymerisation (approximately 45 min) the gel was covered with Saran wrap and kept overnight, or used immediately. The gel was placed in an electrophoresis apparatus, containing 1 X TBE (0.09 M Tris-borate, 0.002 M EDTA), allowing the gel to be bathed in buffer. The gel was preheated at 90 watts for 30 min. Samples were denatured by placing at 80°C for 2 min prior to being loaded and electrophoresed at 90 watts for 1.5 to 4 hrs depending on where the sequence of interest was situated from the primer. Sequences were determined by autoradiography.

12. Autoradiography

Both denaturing gels (DNA footprinting and sequencing gels) and non-denaturing PAGE gels (gel mobility shift assays) were vacuum dried onto Whatman 3MM blotting paper before exposing to X-ray films. For gels containing ^{32}P isotope, the films (Hyperfilm, Amersham) were exposed to the gel in the presence of an intensifying screen at -70°C. Intensifying screens were not necessary if ^{35}S was used and exposure was performed at room temperature. Exposure times ranged from overnight to 1 week. After exposure, X-ray films, having been exposed to the gel in a light-excluding cassette, were removed and placed in developing solution. After 2

min the film was transferred to water for 1 min then to a fixing solution for a further 2 min. The X-ray film was rinsed with water and hung up to air dry.

13. Mutagenesis

Site-directed mutagenesis of the putative SF-1 site in the 5'-flank of the mouse GnRH receptor gene was performed using a variation of the method described by Kunkel (1985).

13.1. Preparation of single-stranded target DNA

Uracil containing single-stranded DNA was prepared by transforming clone III into competent CJ236 *E. coli* cells (dut^-ung^-), and the transformations were plated onto LB antibiotic plates (ampicillin 100 μg/ml, chloramphenicol 30 μg/ml). After overnight incubation at 37°C a single colony was inoculated into 2 ml of 2 X YT broth (ampicillin 100 μg/ml, chloramphenicol 30 μg/ml) and shaken at 37°C overnight. Thereafter 20 μl of the overnight culture was added to 4 ml of 2 X YT broth (ampicillin 100 μg/ml, chloramphenicol 30 μg/ml, uridine 0.25 μg/ml), incubated at 37°C with shaking until the OD_{600} equalled 0.5-0.8 and then 1 ml was added to 1 ml of 2 X YT broth (ampicillin 100 μg/ml, chloramphenicol 30 μg/ml, uridine 0.25 μg/ml), infected with 5 μl of VCS M13 helper phage (Strategene, $>10^{11}$ pfu/ml) and shaken at 37°C for 1 hr. This was inoculated into 25 ml of 2 X YT broth (ampicillin 100 μg/ml, uridine 0.25 μg/ml, 70 μg/ml kanamycin), and grown at 37°C overnight, with shaking. Phage-infected cells were harvested by spinning at 8 000 rpm for 30 min at 4°C (Beckman JA 2.1, JA-20 rotor). The supernatant, containing the phage, was removed and centrifuged as before. The phage was precipitated with one fifth volume of 5 X PEG/NaCl (15% PEG_{8000} (w/v), 2.5 M NaCl) and incubated on ice for 1 hr. Thereafter, it was centrifuged at 8 000 rpm for 15 min at 4°C. The pellet was drained well and resuspended in 3 ml of pre-cooled (4°) TE buffer by vigorous

vortexing. The sample was divided into 750 µl aliquots and placed on ice for 1 hr. Thereafter it was centrifuged as before and the supernatant was extracted once with an equal volume of phenol, followed by extraction with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v)) and then twice with an equal volume of chloroform-isoamylalcohol (24:1, v/v). Samples were pooled and uracil containing single-stranded DNA was precipitated by adding one volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol and placed at -20°C overnight. DNA was collected by spinning at 15 000 *g* for 30 min, the pellet washed with 70% ethanol and centrifuged for 10 min. The pellet was air dried for 10 min and resuspended in 100 µl of TE buffer. The uracil containing single-stranded DNA was analysed spectrophotometrically and by agarose gel electrophoresis.

13.2. Hybridisation of oligonucleotides to the uracil containing single-stranded DNA (USS) and primer extension

Polynucleotide kinase (PNK) was used to phosphorylate oligonucleotides 1S and 2S (section 2.4). Oligonucleotides (50 pmol) were kinased in a reaction containing 50 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.1 mM spermidine HCl, 0.5 mM ATP and 10 units of PNK in a final volume of 20 µl. Reactions were incubated at 37°C for 60 min. PNK was inactivated by placing at 68°C for 10 min. Oligonucleotides were annealed to the uracil containing single-stranded DNA by mixing 1.5 pmol of template DNA, 15 pmol of either 1S or 2S and 1 µl of 10 X PE1 buffer (100 mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$, 500 mM NaCl, 100 mM DTT) in a final reaction volume of 10 µl and placed at 70°C for 5 min. The reaction was cooled down to 30°C, placed on ice, and the uracil containing single-stranded DNA was converted to a full length, circular DNA strand by polymerisation and ligation. Polymerisation was initiated by adding the following to the annealing reaction; 1 µl of 10 X PE 2 buffer (200

mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT), 2 µl of 5 mM dNTPs, 1 µl of 10 mM ATP, 0.5 units of T7 DNA polymerase (Boehringer Mannheim) and 1 unit of T4 DNA ligase (Boehringer Mannheim). The components were mixed carefully and the reaction was placed on ice for 5 min, followed by 5 min at room temperature and 2 hr at 37°C. The reaction was stopped with 20 µl of 10 mM Tris-Cl (pH 7.6) and 20 µl of 10 mM EDTA. A polymerisation reaction with no oligonucleotide was used as a control. Polymerisation reactions were analysed by agarose gel electrophoresis and transformed into *E. coli* XL1 blue cells. Colonies were screened by miniprep plasmid isolation and restriction enzyme digestion. Mutations were confirmed by DNA sequencing.

14. Radiolabelling of DNA

Two methods were employed to label DNA.

14.1. Labelling with Klenow

DNA fragments for DNase I footprinting and double-stranded oligonucleotides for mobility shift assays were end-labelled using the Klenow subunit of DNA polymerase I (United States Biochemical). Reactions were performed as described by Sambrook *et al.* (1989). Generally 100 ng of DNA was labelled in a reaction consisting of 1 X Klenow reaction buffer (United States Biochemical), 20 µCi of α-^{32}P dCTP (10 µCi/µl, Amersham) and 1 unit of Klenow in a final reaction volume of 20 µl. Reactions were incubated at 30°C for 15 min then stopped with 1 µl of 0.5 M EDTA. Labelled DNA was purified by spinning through a Sephadex G-50 spin columm.

14.2. Megaprime labelling

Radioactive probes for Northern blot analysis were labelled using the protocol supplied with the Megaprime ™DNA labelling kit (Amersham), which is based on the method of Feinberg and Vogelstein (1984). This method uses random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. By substituting a radiolabelled nucleotide for a non-radioactive equivalent in the reaction mixture the newly synthesised DNA is made radioactive.

DNA fragments to be labelled were electrophoresed on low melting agarose gels as described in section 2.7. The desired band was excised from the gel and 3 ml of H_2O was added for every gram of agarose. The sample was placed in a boiling H_2O bath for 5 min to melt the agarose and denature the DNA. Primer solution (5 µl) was added to 25 ng of denatured DNA in a total volume of 26 µl and boiled for 5 min. The reaction was spun down and the following added: 4 µl of dATP, 4 µl dGTP, 4 µl of dTTP, 5 µl of 10 X reaction buffer (Tris-Cl (pH 7.5), $MgCl_2$, 2-mercaptoethanol), 2 µl of Klenow and 5 µl of α-^{32}P dCTP (10 µCi/µl, Amersham) in a total volume of 50 µl. The components were mixed gently and placed at 37°C for 30 min. The reaction was stopped with 5 µl of 0.2 M EDTA and purified by spinning through a Sephadex G-50 spin column.

14.3. Purification of labelled DNA

The labelled DNA was separated from unincorporated nucleotides using a Sephadex G-50 spin-column, which was prepared as follows:

A 1 ml disposable syringe was plugged with a small amount of sterile glass wool and filled with Sephadex G-50 (equilibrated with STE buffer (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA)) to 0.9 ml. The column was washed thrice with 100 µl of STE buffer by centrifuging for 1 min at 4 000 *g* in a low speed benchtop centrifuge (Lasec). The labelled DNA was made up to a volume of 100 µl with STE buffer and then applied to the column. Labelled DNA was eluted from the column by centrifugation at 4 000 *g* for 1 min. Specific activity of the fragments were typically 40 000-100 000 cpm/ng, as determined by counting 1 µl in a scintillation counter.

15. Preparation of nuclear extracts

Nuclear extracts were prepared according to the method described by Schreiber *et al.* (1989). All steps were carried out at 4°C. 1.0-5.0 $x10^6$ tissue culture cells were harvested by first washing cells with 10 ml of cold phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.75 mM KH_2PO4, 137 mM NaCl, 2.7 mM KCl), and then scraping the cells off the flasks with a rubber policeman. Cells were pelleted by centrifugation (800 *g* for 5 min) and then and stored at -70°C until nuclear extract preparation. To make nuclear extracts the cell pellets were allowed to thaw on ice and then washed with 10 ml of TRIS-buffered saline (TBS) (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) by centrifuging at 800 *g* for 5 min. The cell pellet was resuspended in 1 ml of TBS, transferred to an eppendorf tube and pelleted by spinning for 15 secs in a microfuge. The TBS was removed and the cell pellet was resuspended in 400 µl of cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF)

by gentle pipetting with a yellow tip. The cells were allowed to swell by placing on ice for 15 min, after which 25 µl of a 10% (v/v) solution of Nonidet NP-40 was added and the tube vigorously vortexed for 10 sec. The reaction was spun for 30 sec in a microfuge and the nuclear pellet was resuspended in 50 µl of cold buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). Thereafter the sample was vigorously rocked for 15 min. Nuclear extracts were centrifuged for 5 min at 12 000 *g* in a microfuge and the supernatant stored in aliquots at -70°C.

16. Mobility Shift Assays

Mobility gel shift assays were performed according to the method described by Horn *et al.* (1992). Variable amounts of nuclear extracts were incubated in standard incubation buffer. The final reaction conditions were 37.5 mM KCl, 100 mM NaCl, 7.5 mM HEPES, 10 mM Tris-Cl (pH 8.0), 8.125% (v/v) glycerol, 1.25 mM $MgCl_2$, 3.5 mM DTT, 0.675 mM PMSF, 1 mM EDTA, 0.25 mM EGTA, 2 µg pdIdC (Boehringer Mannhein) as non-specific competitor, 20 µg Bovine Serum Albumin (Molecular Biology grade, Boehringer Mannheim) and 1 ng of end-labelled DNA. Reactions were started by adding nuclear extracts and were carried out in a total volume of 20 µl for 10 min at room temperature. To resolve the complexes, the reaction mixtures were applied to 4% polyacrylamide gels (29:1 acrylamide:bisacrylamide) in 0.25 X TRIS-acetate-EDTA (pH 8.0) (TAE) buffer. Gels were pre-electrophoresed at 150 V for 2 hr at room temperature. Before the samples were loaded onto the gel, the pre-electrophoresis buffer was changed and electrophoresis was continued at 150 V for 2-3 hr at room temperature. Gels were dried and the DNA-protein complexes were visualised by autoradiography. The binding specificities of the GSE/SF-1 oligonucleotides were determined by using double-stranded DNA oligonucleotides as unlabelled competitors in the mobility shift assay. The

mobility shift incubations were carried out as above, however, the DNA competitors were included in the reaction cocktail. The molar ratio of competitor to labelled DNA used was 100:1.

17. DNase I footprinting

The DNase I footprint was performed as described by Hapgood and Patterton (1994) with slight modifications. Radiolabelled DNA fragments were incubated with variable amounts of nuclear extract. Final reaction conditions were 37.5 mM KCl, 60 mM NaCl, 6.5 mM HEPES, 10 mM Tris-Cl, 8.5% (v/v) glycerol, 1.75 mM $MgCl_2$, 4.7 mM DTT, 0.605 mM PMSF, 1 mM EDTA, 0.15 mM EGTA, 2 µg pdIdC, 40 µg BSA and 1 ng of end-labelled DNA in a total volume of 40 µl. Reactions were started by adding nuclear extracts and the DNA-protein mixture was incubated at room temperature for 10 min then placed at 4°C for at least 5 min. The sample was adjusted to 19 mM $MgCl_2$ and 19 mM $CaCl_2$, by adding 1.5 µl of 0.5 M $MgCl_2$; 0.5 M $CaCl_2$, before adding 3 µl of 16 µg/ml DNase I (grade 1, Boehringer Mannheim). The reaction was allowed to proceed at 4°C for exactly 1 min and then stopped by adding 8.4 µl of stop solution (0.7 mg/ml of proteinase K, 0.12 M EDTA, 1% (w/v) SDS) and placed at 37°C for 30 min. Thereafter one tenth volume of 3 M sodium acetate pH 5.2 was added and proteins were extracted by adding an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v) at room temperature. Reactions were vortexed for 30 sec and microfuged for 2 min. The phenol-chloroform-isoamylalcohol extraction was repeated. The DNA was precipitated by adding 150 µl of 100% ethanol, vortexing and placing at -70°C for 30 min. The DNA was collected by spinning at 15 000 rpm in a microfuge for 30 min at 4°C. The pellet was washed with 200 µl of 70% ethanol and centrifuged for 10 min. Samples were vacuum dried and resuspended in 3 µl of sequencing loading buffer (Sequenase 2.0 kit, Amersham). Samples were

stored at -20°C or immediately electrophoresed. They were denatured by placing at 90°C for 3 min, then subjected to electrophoresis on a 6% denaturing polyacrylamide gel for 1.5 hr. Gels were dried and DNA footprints were detected by autoradiography.

18. RNA isolation

RNA was extracted under RNase free conditions. All solutions were treated with Diethyl pyrocarbonate (DEPC) and latex gloves were worn at all times. RNA was extracted from tissue culture cells using the acid-guanidinium-thiocyanate-phenol-chloroform method (Sambrook *et al.* 1989). All steps were carried out at 4°C. Eukaryotic cells growing in 75 cm^2 flasks were harvested with a rubber policeman, washed with cold PBS and transferred to 15 ml centrifuge tubes. Cells were pelleted by spinning at 800 *g* for 3 min. The pellet (approximately 5 $x10^6$ cells) was resuspended in 1 ml of PBS and divided into 250 µl aliquots in microfuge tubes. An equal volume of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sarcosyl, 0.72% mercaptoethanol) was added, homogenised with a 1ml syringe and stored at -70°C until RNA extraction. RNA extraction was continued at 4°C by adding 25 µl of 2 M sodium acetate (pH 4.0), 250 µl of phenol and 50 µl of chloroform-isoamylalcohol (24:1, v/v) to each sample. After mixing, the samples were left on ice for 1 hr, then centrifuged at 10 000 *g* for 20 min. RNA was precipitated by adding an equal volume of isopropanol, placing on dry ice for 10 min, then spinning as before. The pellet obtained was resuspended in 150 µl of solution D and the RNA precipitated as before. The RNA pellet was washed with 75% ethanol by spinning at 10 000 *g* for 10 min at 4°C. It was briefly dried under vacuum, then resuspended in 25 µl of DEPC-treated H_2O by placing at 65°C for 10 min. The RNA concentration was determined spectrophotometrically where 1 absorbance unit at 260 nm equals 40 µg/ml. The

purity of the RNA was determined by the 260/280 ratio. The integrity of the RNA was analysed on a 1% formaldehyde containing agarose gel.

18.1. RNA gel electrophoresis

For RNA gels the electrophoresis apparatus was washed with DEPC-treated H_2O. RNA samples were analysed on 1% formaldehyde containing agarose gels based on a modification of the method described in Ausubel *et al.* (1987). For a minigel, gels were prepared as follows: 0.5 g agarose, 5 ml 10 X MOPS (0.4 M morpholinopropanolsulfonic acid, 100 mM sodium acetate, 10 mM EDTA, pH 6.0) and 36 ml DEPC-H_2O. The agarose was heated and allowed to cool to approximately 50°C before adding 8.5 ml 37% (v/v) formaldehyde. The gel was poured in a fume cupboard. After the gel had solidified it was covered with 1 X MOPS buffer and the RNA samples were loaded. RNA samples were dried in a speedy vac and resuspended in 10 µl of loading buffer. Loading buffer was always made fresh and included 72 µl of formamide, 16 µl of 10 X MOPS, 26 µl of 37% (v/v) formaldehyde, 8 µl of DEPC-H_2O, 10 µl of 80% (v/v) glycerol, 8 µl of saturated bromophenol blue and 10 µl of 10 mg/ml ethidium bromide. The RNA samples were electrophoresed at 80-100 V for 2 hr in 1 X MOPS as running buffer and detected by UV light. For Northern blotting 20 µg of total RNA was denatured and subjected to electrophoresis on denaturing 1% formaldehyde containing agarose gels. No ethidium bromide was added to the loading dye for preparative gels. RNA samples were electrophoresed at 80 V for 2 hr.

18.2. Mono-directional transfer

A glass dish was filled with approximately 1 litre of 20 X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), covered with a glass plate followed by 3 sheets of Whatman 3 MM paper

soaked in 20 X SSC to form a wick. The gel was placed on the Whatman paper and Hybond-N^+ membrane (Amersham) was layered on top followed by 3 sheets of Whatman 3 MM paper soaked in 20 X SSC. The area around the gel was covered with cling wrap. A stack of paper towels (approximately 5 cm thick) was placed on top of the membrane, followed by another glass plate and a mass of 0.5 kg. The RNA was allowed to transfer overnight and then fixed to the membrane by UV-crosslinking (Hoefer). To check the efficiency of transfer of the RNA from the gel to the membrane, membranes were stained with methylene blue after mono-directional transfer as follows; soaked the membrane in 5% acetic acid for 15 min at room temperature. Thereafter the membrane was transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 10 min at room temperature. The membrane was rinsed in H_2O for 10 min.

18.3. Hybridisation

Northern blot analysis was performed under conditions of high stringency. After UV-crosslinking the membrane was prehybridised in 2 X PIPES, 0.5% (w/v) SDS, 50% (v/v) formamide and 20 µg/ml of denatured herring sperm DNA for 2-4 hr at 42°C. The volume of prehybridisation solution needed was calculated according to the formula in Sambrook *et al.* (1989) where 0.2 ml of solution is required per cm^2 of membrane. Thereafter the labelled probe (section 14.2 and 14.3) was denatured by boiling for 5 min and added to the prehybridisation solution. Hybridisation was allowed to proceed overnight, at 42°C, with shaking. After hybridisation, the membrane was washed twice in 200 ml of 2 X SSPE (0.3 M NaCl, 18 mM NaH_2PO_4, 2 mM EDTA, pH 7.4), 0.1% (w/v) SDS at room temperature for 10 min, followed by 1 X SSPE, 0.1% (w/v) SDS for 15 min at 65°C, and finally in 0.1 X SSPE, 0.1% (w/v) SDS for 10 min at 65°C. The final wash was repeated 1-3 times depending on the amount of

radioactivity on the membrane. All washes were performed with shaking. The membrane was dried between two pieces of Whatman 3MM paper, wrapped in Saran wrap and signals detected by autoradiography. Membranes were stripped by placing in boiling 0.5% (w/v) SDS. The solution was allowed to cool to room temperature. The success of stripping was checked by autoradiography.

19. Cell culture

αT3-1, αT4, GH_3 and GT1-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 4500 mg glucose/litre, while COS-1 cells were grown in DMEM, supplemented with 1000 mg glucose/litre. Antibiotics (streptomycin (100 μg/ml) and penicillin (100 U/ml)) and fetal calf serum, to a final concentration of 10%, (FCS, Highveld laboratories)) were added to the media. Cells were grown in 75 cm^2 or 150 cm^2 flasks and incubated at 37°C in 5% CO_2. GT1-7 cells were grown in 75 cm^2 tissue culture flasks coated with 15 μg/ml polyornithine. Media was changed every 48 hrs. αT3-1 cells were passaged by removing media, adding 3 ml of Buffer I (140 mM NaCl, 4 mM KCl, 20 mM HEPES, 1 mg/ml BSA, 8.3 mM glucose, 1 mM EDTA, pH 7.4) to the cell monolayer and shaking the flask slightly. Cells were then sucked up with a 5 ml pipette and transferred to a 15 ml centrifuge tube. Cells were spun down at 100 *g* for 5 min and the cell pellet resuspended in 5 ml of fresh media. 50 μl of cells was mixed with 50 μl of trypan blue and the mixture was transferred to a hemicytometer and counted under a microscope. Cells were seeded into 75 cm^2 tissue culture flasks at a density of 1.0×10^6 and 10 ml of fresh media added. COS-1, αT4, GH_3 and GT1-7 cells were split with trypsin (5 mM EDTA, 1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 11.1 mM glucose and 1.25% trypsin (BDH Biochemical) for COS-1, αT4 and GH_3 cells, while 2.5% trypsin was used for GT1-7 cells, pH 7.2). Media were removed from

cells, 3 ml of trypsin added, and cells incubated at 37°C for 5 min. After cells had dislodged from the flasks, they were sucked up with a 5 ml pipette and transferred to 15 ml centrifuge tubes. Cells were counted as before and seeded into 75 cm^2 or 150 cm^2 tissue culture flasks.

19.1. Transfections

19.1.1. Transfection of αT3-1 cells

19.1.1.1. Transfection using the calcium phosphate method

Cells were seeded at a density of 2 X 10^6 into 60 mm plastic petri dishes, containing 5ml of DMEM-10% FCS. After 24 hr, subconfluent cells (approximately 80%) were transfected with the various constructs by the calcium phosphate method as described previously (Ausubel *et al.* 1987; Christine Ferguson, personal communication). Variable amounts of luciferase constructs, β-galactosidase expression vector and SF-1 cDNA were transfected. DNA, dissolved in TE buffer, was added to 31 µl of 2 M $CaCl_2$ to make up a total volume of 250 µl and then slowly added (with agitation) to 250 µl of HEPES buffer (274 mM NaCl, 42 mM HEPES, pH 7.1) and 5 µl of 100 X PO_4 solution (70 mM Na_2HPO_4, 70 mM $Na_2H_2PO_4$, pH 7.0). A precipitate was allowed to form by leaving at room temperature for 30 min. During this incubation media was removed from the cells to be transfected and the cells washed with 5 ml of HEPES-buffered Dulbecco's modified Eagle's medium (HEPES-buffered DMEM). The precipitate formed (0.5 ml) was resuspended well and then added to the subconfluent cell monolayers in the petri dishes. Cells were incubated at 37°C in 5% CO_2 with the calcium phosphate-DNA precipitates for 24 hr. After 24 hr, the media was replaced with fresh media and tranfectants were harvested after 24 hr.

19.1.1.2. Transfection using lipofectin and lipofectamine

αT3-1 cells were transfected with lipofectin® reagent (Life Technologies). The method described by the manufacturers was followed. Cells were seeded into 60mm petri dishes at a density of 2.5-5.0 X 10^6. DNA (2 μg) was mixed with 250 μl of OPTIMEM (Gibco). The DNA to be transfected was made up of 1.56 μg of pGL2-control and 0.44 μg of β-galactosidase vector. In a separate tube 250 μl of OPTIMEM was mixed with 10 μl of 1 mg/ml lipofectin. The two solutions were mixed and left at room temperature for 15 min. During this incubation media were removed from cells and they were washed with 5 ml of HEPES-buffered DMEM. Mixtures were diluted by adding 2 ml of OPTIMEM and then added to the cells (40-60% confluent, approximately 24 hr after seeding) plated in 60 mm petri dishes. Cells were incubated with the lipofectin-DNA complexes for 5.5 to 6 hr at 37°C in 5% CO_2. After the 5.5 to 6 hr incubation, 2.5 ml of DMEM-20% FCS was added to the cells and incubated overnight at 37°C in 5% CO_2. After 24 hr the media was replaced with 5 ml of DMEM-10% FCS and incubated for a further 24 hr. Cells were harvested 48 hr after transfection.

19.1.1.3. Transfection using electroporation

αT3-1 cells were transfected by electroporation using the method described by the manufacturers (Invitrogen, Electroporater II). Cells (2.6 X 10^6 per 500 μl) were added to 0.4 cm cuvettes containing 12.58 μg of pGL2-control vector and 4 μg of β-galactosidase vector. The cells were mixed with the DNA by gently tapping the cuvettes and then incubated on ice for 10 min. During this incubation the electroporator was charged, after which the cells were electroporated. The settings used were 330 V, 500 μF capacitance and ~ resistance. After electroporation samples were placed on ice for 10 min, 1 ml of pre-warmed DMEM-high

glucose media added, and then transferred to 100 mm petri dishes containing 9 ml of DMEM-high glucose media. Cells were harvested 48 hr after transfection.

19.1.2. Transfection of COS-1 and GH_3 cells

19.1.2.1. Transfection using lipofectin

COS-1 and GH_3 cells were transfected using lipofectin® reagent (Life Technologies), as described for αT3-1 cells. Cells were seeded into 60 mm petri dishes at a density of 2.5-5 X 10^5. For COS-1 cells, petri dishes were coated with poly-D-lysine. Briefly, DNA to be transfected was mixed with 250 µl of OPTIMEM. In a separate tube 250 µl of OPTIMEM was mixed with 10 µl of 1 mg/ml lipofectin. The same procedure was followed as for αT3-1 cells and transfectants were harvested 48 hr after transfection.

19.1.2.2. Transfection of COS-1 cells using DEAE-Dextran

COS-1 cells were seeded into 100 mm petri dishes, coated with poly-D-lysine, at a density of 3.0 X 10^6. Cells were grown until 80% confluent, then washed with 10 ml of HEPES-buffered DMEM. 4 ml of DNA/HBS/DEAE-Dextran mixture was added to the cells. The DNA/HBS/DEAE-Dextran mixture was made up as follows: 13.5 µg of pGL-2 control and 5.25 µg of β-galactosidase vector, and 4 ml of 3 mg/ml HBS/DEAE-Dextran diluted 10-fold in DMEM media. Cells were incubated with this mixture for 4 hr at 37°C and 5% CO_2. After 4 hr, media was aspirated off and 10 ml of 200 µM chloroquin/DMEM/2% FCS was added to the cells. The cells were incubated for a further 50 min and then washed with 10 ml of serum-free DMEM. 10 ml of DMEM/10% FCS media was added to the cells and the cells returned to the incubator. Cell were harvested for assays 48 hr after transfection.

19.1.2.3 Transfection of COS-1 cells using electroporation

COS-1 cells were transfected by electroporation using the protocol described for αT3-1 cells (19.1.1.3).

19.2. Harvesting cell extracts

Cells were washed twice with 5 ml of PBS, after which 400 µl of 1 X Reporter Lysis Buffer (RLB, Luciferase Assay kit, Promega) was added per 60 mm petri dish and the solution left at room temperature for 15 min. Cells were scraped from the dish, with a rubber policeman, and the cell lysate was transferred to a microfuge tube on ice. The lysate was vortexed for 15 sec, then centrifuged for 2 min at 4°C in a microfuge. The supernatant was stored in aliquots at -70°C.

19.3. Protein Determination

Protein concentrations were determined using the BIORAD assay reagent from Bio-Rad Laboratories, which is based on the method of Bradford (1976). Bovine serum albumin (BSA), concentrations ranging from 0-25 µg were used as standards. Briefly, 1 ml of 1 X BIORAD was added to standards and samples made up to 50 µl with H_2O or RLB, and left at room temperature for 5 min before taking the OD_{595}. The volume of cell extract used per assay varied from 2-10 µl, depending on the cell type transfected but generally 5 µl of cell extract was used for αT3-1 and GH_3 cells while 2 µl was used for COS-1 cells.

19.4. β-galactosidase (β-gal) assays

β-gal activity was measured by adding buffer Z (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4.2H_2O$, 10 mM KCl, 1 mM $MgCl_2.6H_2O$, 50 mM β-mercaptoethanol, pH 7.0) to the cell extract, in RLB, to a final volume of 200 µl. The amounts of cell extract and buffer Z used varied for different assays, but generally 40 µl of cell extract was added to 160 µl of buffer Z for αT3-1 and GH_3 cells, while only 5 µl of cell extract was used for COS-1 cells. The reaction was started by adding 40 µl of 4 mg/ml ONPG (O-nitrophenyl-β-D-galactopyranoside dissolved in solution 2 (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0) and incubated at 30°C until a yellow colour developed. The reaction was stopped by adding 100 µl of 1 M Na_2CO_3 and the OD_{420} measured. β-galactosidase activity was expressed as:

$$\beta\text{-gal activity} = {}^{OD420/hr}/_{mg\ protein} \quad \text{(b)}$$

(Herbomel *et al.* 1984).

19.5. Luciferase assays

Luciferase assays were performed as described in the Luciferase assay kit manual (Promega). Generally 20 µl of cell extract, in RLB, was added to 100 µl of luciferase assay substrate and the amount of luciferase produced was measured in a luminometer (1253-003 Luminometer, BioOrbit). 350 µl of Tris-acetate buffer (0.1 M Tris, 2 mM EDTA, pH 7.75 with acetic acid) and 100 µl of substrate were added to the cuvette, vortexed, and a background reading taken. Luciferase units was determined by adding 20 µl of cell extract to the cuvette, vortexing, and measuring light production 30 sec after adding the cell extract to the substrate. Luciferase units was expressed as:

$$\text{luciferase units} = {}^{\text{luciferase produced}}/_{\text{mg protein}} \qquad \text{(a)}$$

Normalised luciferase activity was determined by dividing luciferase units produced by β-gal activity ($^{a}/_{b}$) to correct for transfection efficiency.

20. Computer analysis

Sequencing analysis was performed using the GCG program on the University of Cape Town VAX. Sequences were compared using Bestfit, while restriction enzyme maps were determined using Map or Mapsort. β-gal and luciferase results were graphically analysed using GraphPad PRISM™ version 1.03 computer software by GraphPad Software Incorporation. Standard errors were calculated by the GraphPad PRISM program.

CHAPTER 3

RESULTS

1. Partial sequence analysis of the mouse GnRH receptor gene

A mouse GnRH receptor genomic clone, clone III, containing 500 bp of 5'-flank, 569 bp of coding region and 38 bp of intergenic region, was obtained from Stuart Sealfon at Mt. Sinai in New York. Clone III was partially sequenced to ascertain if the correct clone was obtained, and also to determine its percentage homology with the sequence of the 5'-flanking region of the mouse GnRH receptor gene, published by Albarracin *et al.* (1994) (Fig. 3.1). Partial sequence analysis of clone III was obtained by sequencing it with various primers. The primers used are listed in section 2.4. DNA sequence analysis was performed using the GCG sequencing program. Partial sequence analysis of clone III revealed 100% DNA homology with the sequence published by Albarracin *et al.* (1994).

```
-1164  CCCACATCTT GCTGTAATGG GCTTTCTGCT GTACCCAACA GTGTATTTTG
-1114  AACTTATCTT GATTTCTTCA TATAGTAAAA CTATAAAACT TCTCAAAATT
-1064  GCTTTATTAC CAGCAAAGAC TAGGAGAGTG TGTGTAGTAG AGACTCAGTA
-1014  ACTCACTACT AAATAAATTA ATAGCATACA GTATAAAAAG AATAAACCAA
-964   CAAAAATACA CCATCCAAAC CAAAATAGCT GCCTTTACCC TAATAGTGTC
-914   CCTGCTTTGG GTGGGTGTGA TGAAAGCAAA GTAATTGGTA ATCTGATAAC
-864   AACAAACTGT GACTTACTAA ATTCCAATGG AAGTCATACC AATATTTGTA
-814   TGGCTAAATA CTATCATCAC ATAACACAGA GAAGGATTCT AAGTAGCCTC
-764   AATGTGGGGT GGTTATATAA CACAATAGGA TTATAAATTA TAAATTAGCA
-714   TATTATATCA TCAATTCAGT TCCAGGATTT ATGCAAGTAT GTAGTTTTGT
-664   TCCAGTATCT AGTTTTCCTT TCAATTTGGT ATGATAATAA CAAGTAAAAT
-614   ATTTACCCAA TTATAAAATC AAGGCAATAA CAAAAATAAT TTTACTATAA
-564   TTTCAGCATG GATAGTCTGA TCATTAAAAC CAATTGATAA ACTTAAATCT
-514   AGAATAATTG GTATTAGAAC AGGCTGCTTA AAACAGTTAA AGTACTAGCT
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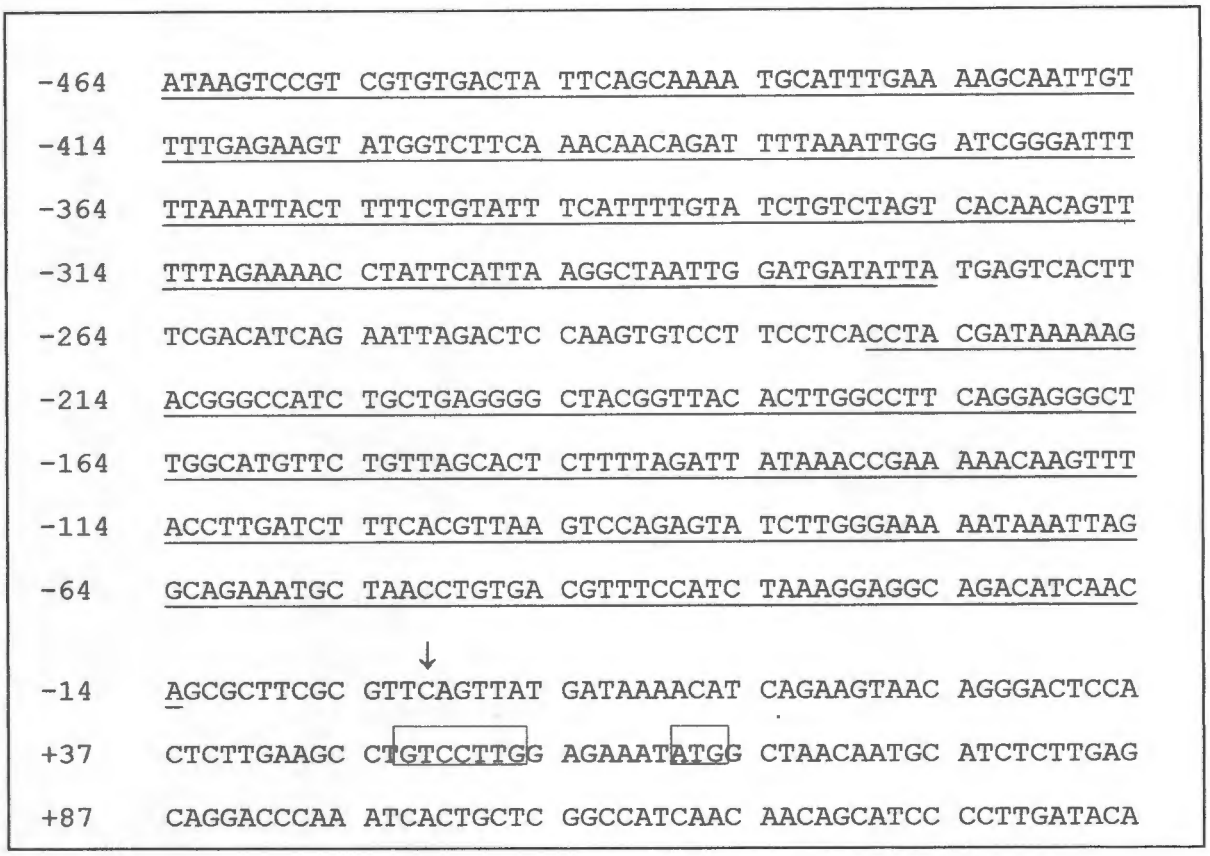

Figure 3.1. Nucleotide sequence of the 5'-flanking region of the mouse GnRH receptor gene (Albarracin *et al.* 1994). The position of the major transcription start site which is located 63 bp upstream of the translational start site is indicated by an arrowhead. The putative GSE element and the translation start site (ATG) are boxed. Nucleotide sequences obtained by sequencing clone III are underlined.

2. αT3-1 and αT4 cells contain a GSE/SF-1 binding nuclear protein(s)

Protein-DNA binding studies were performed using nuclear extracts prepared from αT3-1, αT4 and GT1-7 cells. Oligonucleotides corresponding to the mouse GnRH receptor 'GSE' (oligonucleotide GSE in the materials and methods section) and to a strong SF-1 binding site (oligonucleotide SF-1) found in the promoter of the aromatase gene were end-labelled and used as probes in gel mobility shift assays. A nuclear factor-DNA complex is seen in both αT3-1 and αT4 cells (Fig. 3.2). A second shifted complex is seen in αT3-1 cells. This is consistent with

findings of others who have identified a protein-DNA complex with similar mobility to the upper complex as SF-1 binding to its DNA-binding element, whereas a protein-DNA complex with similar mobility to the lower complex correlates to the binding of an SF-1 degradation product (Barnhart and Mellon, 1994; Lala *et al.* 1992). No similar binding complex is seen in GT1-7 cells (Fig. 3.2). The finding that a complex with indistinguishable mobility was observed when both the GSE and SF-1 oligonucleotides were used as radiolabelled probes, suggested that SF-1 is the protein in αT3-1 and αT4 cells that binds the putative mouse GnRH receptor GSE. Since it is known that αT4 cells do not express GnRHR (section 4), the presence and binding of SF-1 to the GSE in αT4 cells is not sufficient for GnRHR expression.

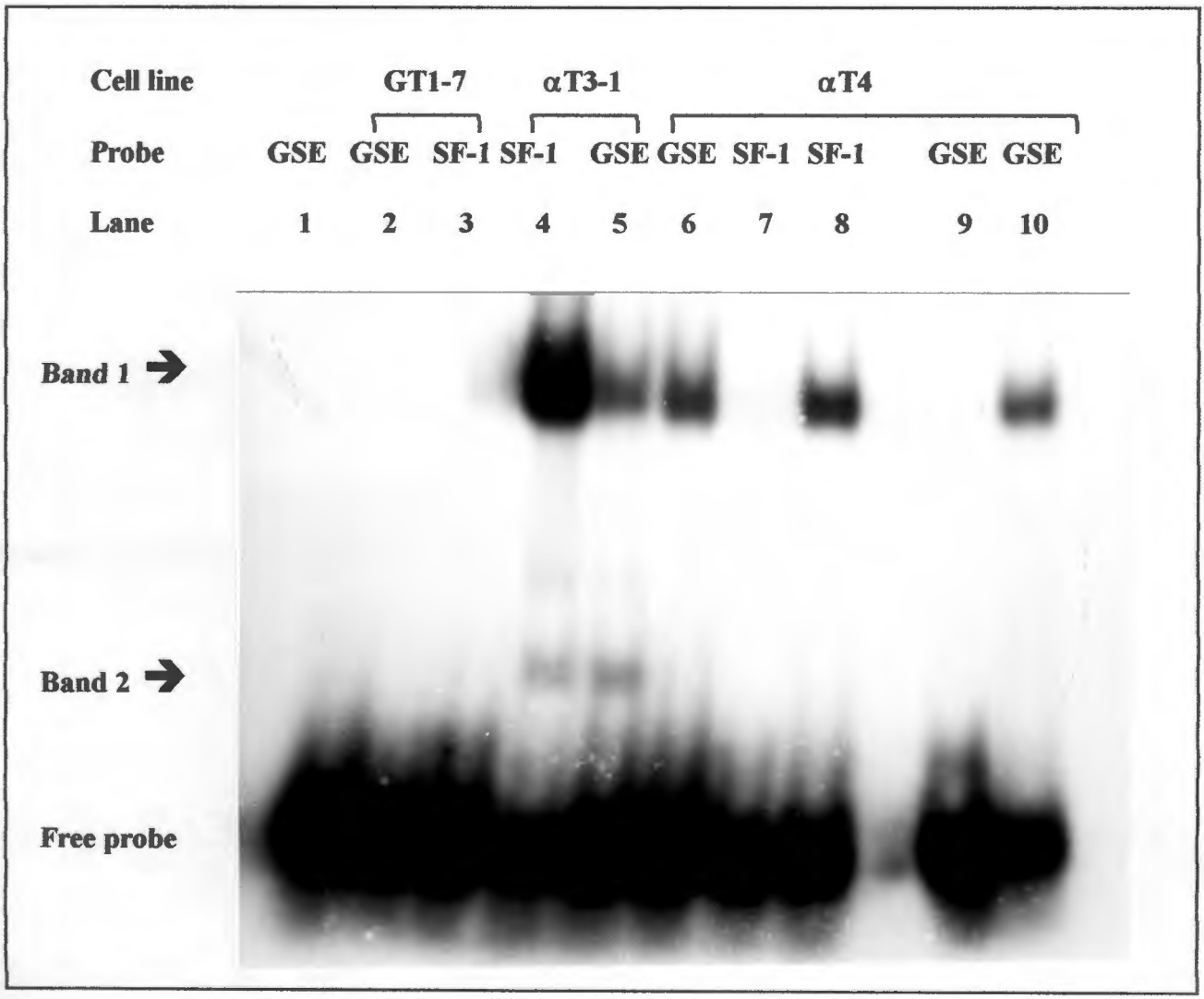

Figure 3.2. αT3-1 and αT4 cells contain a GSE/SF-1 binding nuclear protein(s). Oligonucleotides corresponding to the 'GSE' of the mouse GnRH receptor gene (lanes 1, 2, 5, 6, 9, 10) or an SF-1 binding site (lanes 3, 4, 7, 8) were used as radiolabelled probes. These probes were incubated with 5 μl of nuclear extracts prepared from αT3-1 (lanes 4, 5), αT4 (lanes 6, 8, 10) or GT1-7 cells (lanes 2, 3) and subjected to electrophoresis in a gel mobility shift assay. Band 1 represents the major shifted band obseved in both αT3-1 and αT4 cells. Band 2 (observed with αT3-1 nuclear extracts) is most likely the binding of a SF-1 degradation product to the GSE and SF-1 radiolabelled probes. Lanes 1, 7 and 9 represent mobility shift assays without adding nuclear extracts to the reactions. These are the results of several experiments.

Results in Fig. 3.2 have suggested that SF-1 is the protein in αT3-1 and αT4 cells that bind the GSE and SF-1 oligonucleotides. To further verify the sequence specificity of the DNA-protein interactions, gel mobility shift assays were performed with nuclear extracts prepared from αT3-1 cells, using the GSE, SF-1 and mutated GSE (mGSE) oligonucleotides as unlabelled competitors together with the GSE and SF-1 oligonucleotides as radiolabelled probes. Investigators have found that a 2 bp nucleotide mutation (CC⇒TT) in the GSE completely abolishes binding of SF-1 (Horn *et al.* 1992) or diminishes but does not abolish binding of SF-1 (Wilson *et al.*1993). This 2 bp nucleotide change (CC⇒TT) was incorporated into the putative mouse GnRH receptor GSE used in this study to generate a binding site with a theoretically diminished affinity for SF-1 (mGSE oligonucleotide in materials and methods). Figs. 3.3 and 3.4 illustrates gel shift experiments using the mutated GSE, the GSE and SF-1 oligonucleotides as unlabelled competitors together with either the GSE or SF-1 oligonucleotides as radiolabelled probes. The major shifted band seen when using the wild type GSE (Fig. 3.3A) and SF-1 (Fig. 3.3B) oligonucleotides as probes is completely abolished when challenged with a 100-fold

excess of cold GSE or SF-1 oligonucleotide competitors. There is substantial but incomplete competition of the mGSE oligonucleotide with the GSE and SF-1 radiolabelled probes, implying that the 2 bp change which has been incorporated into the 'GSE' only diminishes binding of SF-1, but is not sufficient to completely abolish binding (Fig. 3.3A, lane 1). These results show that the interaction between SF-1 and the 'GSE' sequence from the GnRH receptor gene is not highly sequence specific as SF-1 is still able to recognise the GSE with a 2 bp mutation. In contrast, when SF-1 is used as the probe (Fig. 3.3B), the mutated GSE shows virtually no competition (Fig. 3.3B, lane 5). The results show that while the protein(s) in the nuclear extract binds to both the GSE and SF-1 sites, it has higher affinity for the SF-1 site than the GSE and appears to have some affinity for the mutated GSE.

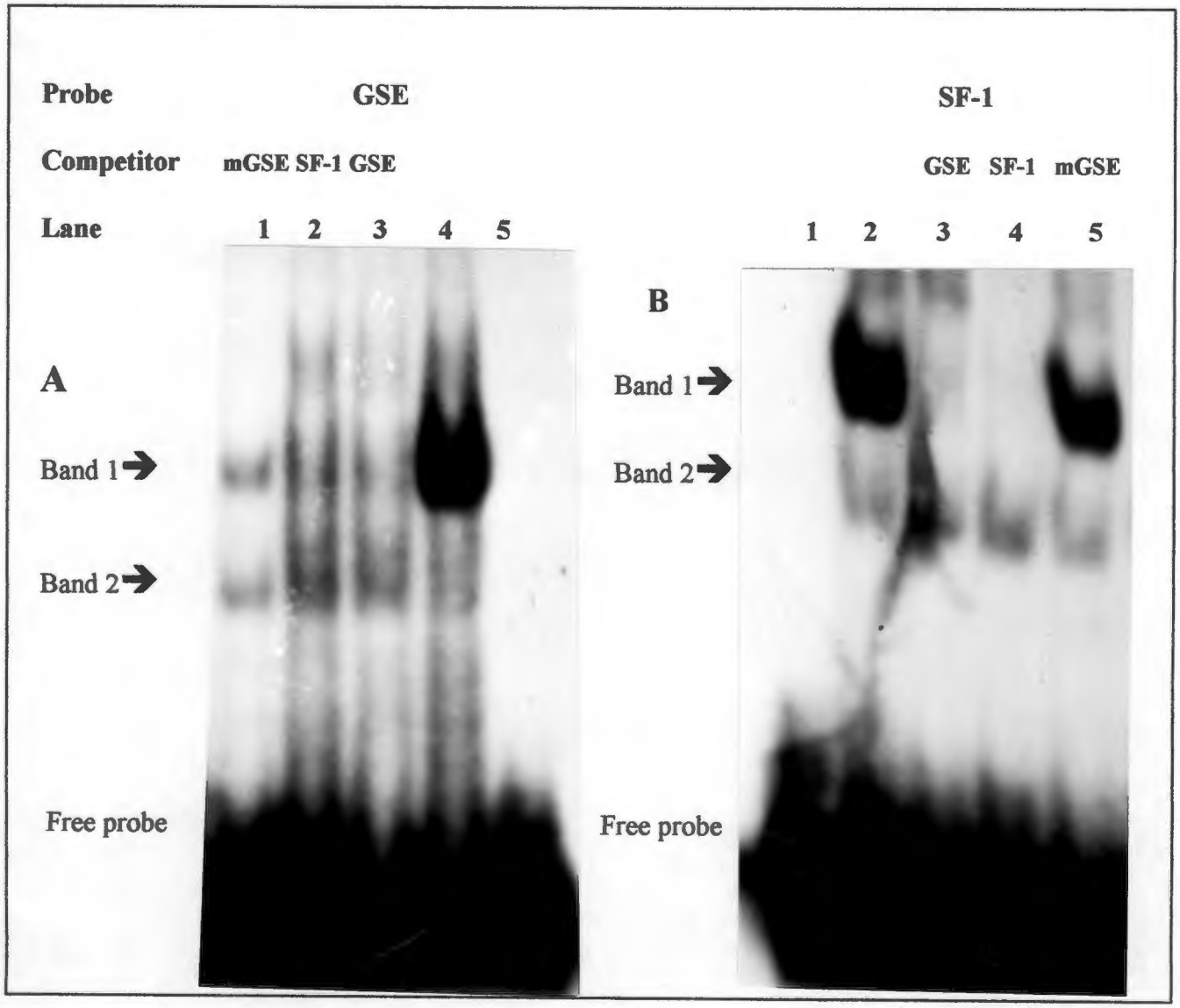

Figure 3.3. Mutations in the GSE and SF-1 sites affect SF-1 binding. Mobility shift assays using nuclear extracts prepared from αT3-1 cells (5 μl) were performed using the GSE and SF-1 oligonucleotides as radiolabelled probes and the GSE, SF-1 and mutated GSE oligonucleotides as unlabelled competitors. Competition assays were performed using unlabelled oligonucleotides at a 100-fold molar excess to radiolabelled probes. Figure 3.3A is the mobility shift assay using 1 ng of the GSE oligonucleotide as probe and contain the following oligonucleotides as competitors: lane 4, no competitor; lane 3, GSE; lane 2, SF-1; lane 1, mGSE. Lane 5 is a mobility shift reaction using the GSE oligonucleotide as probe without adding nuclear extract. Figure 3.3B is the mobility shift assay using 1 ng of the SF-1 oligonucleotide as probe and contain the following oligonucleotides as competitors: lane 2, no competitor; lane 3, GSE; lane 4, SF-1; lane 5, mGSE. Lane 1 is a mobility shift reaction using the SF-1 oligonucleotide as probe without adding nuclear extract. Band 1 represents the major DNA-protein complex formed. Band 2 most likely correlates to the binding of a SF-1 degradation product to the GSE and SF-1 oligonucleotides.

3. A SF-1-like protein binds to the mouse GnRH receptor 'GSE'

A 378 bp FokI fragment, encompassing the region -270 bp to +108 bp relative to the transcription start site (Fig. 3.1), containing the putative mouse GnRH receptor 'GSE' was excised from clone III, by restriction enzyme digestion, and used in DNase I footprinting experiments with nuclear extracts prepared from αT3-1 cells. Two different concentrations of nuclear extracts were used in the footprinting experiment. To test the sequence specificity of the DNA-protein interaction unlabelled GSE and mGSE oligonucleotides were used as competitors in the footprinting experiment. A footprint is seen over the 'GSE' which is competed away with the cold wild type GSE oligonucleotide but not with the mutated GSE oligonucleotide (Fig.

3.4). These results show that a nuclear protein(s), present in αT3-1 cells, binds over the mouse GnRHR 'GSE' and that this protein-DNA interaction is sequence specific, as the mGSE oligonucleotide fails to compete for binding. Mobility shift assays have provided strong evidence that the nuclear protein(s) in αT3-1 cells that bind the mouse GnRH receptor 'GSE' is SF-1. No other footprints could be detected in this region (Fig. 3.4). Our studies indicate that the major protein-DNA interaction over this region occur over the 'GSE'.

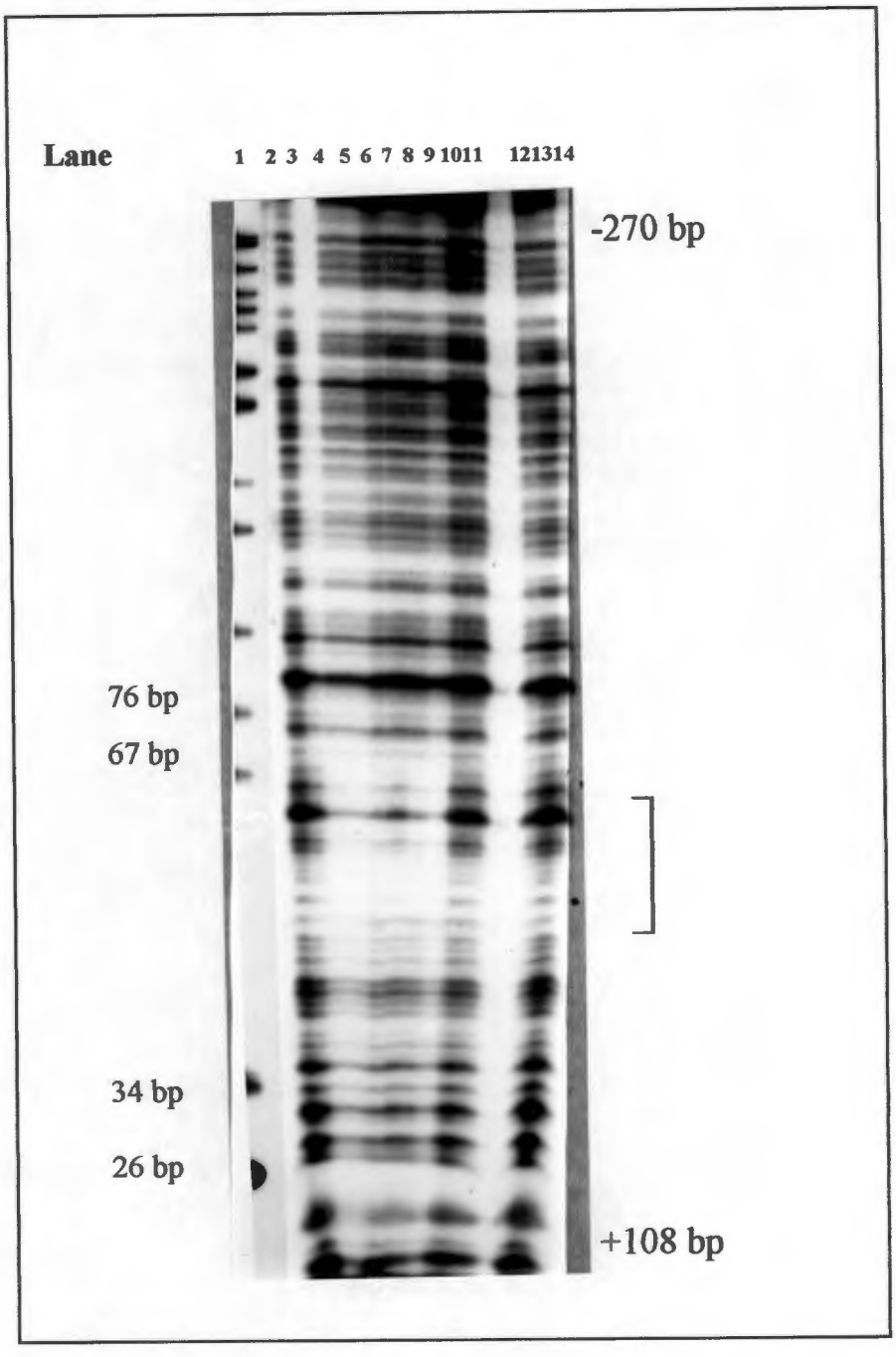

Figure 3.4. SF-1 binds over the mouse GnRH receptor 'GSE'. DNase I footprint analysis of αT3-1 nuclear proteins binding to the mouse GnRH receptor promoter. The ^{32}P-end-labelled 378 bp fragment of the mouse GnRH receptor gene (1 ng), containing the putative GSE site, was incubated with 6 µl (lanes 4, 5, 7, 8, 10, 11) and 10 µl (lanes 6, 9, 12) of αT3-1 nuclear extract partially digested with DNase I, and separated on a 6% polyacrylamide gel as described in the materials and methods. A 100-fold molar excess of the unlabelled GSE (lanes 10, 11, 12) and the mutated GSE oliginucleotides (lanes 7, 8, 9) were used as competitors. The protected region is indicated by the bracket. The fact that the faint footprint occurs over the GnRHR GSE is verified by competition experiments with the mutated and wt GSE oligonucleotides. No footprint is seen when competed with the wt GSE oligonucleotide (lanes 10, 11, 12). A footprint is seen when competed with the mutant GSE oligonucleotide (lanes 7, 8, 9). pBR322 digested with HpaII (radiolabelled by the Klenow method as discussed in the materials and methods section 14.1) is used as a molecular size marker (lane 1). DNaseI footprinting reactions using the labelled FokI fragment as probe without adding nuclear extract is shown in lanes 2, 3, 13, 14.

4. GnRH receptor expression is not solely dependent on SF-1 RNA expression

Using three different cell lines, the possible correlation between SF-1 RNA expression and GnRH receptor RNA expression was investigated. αT3-1 cells are transformed precursor gonadotrope cell lines which have been previously shown to express GnRHR RNA and SF-1 RNA (Windle *et al.* 1990, Barnhart and Mellon, 1994). αT4 cells are also transformed gonadotrope cells, but they have been shown not to express GnRHR RNA. It is not known whether they express SF-1 RNA. GT1-7 cells are transformed neuronal cells and it is not clear if they express GnRHR or SF-1 RNA (Mellon *et al.*1990, Barnhart and Mellon, 1994). Northern

blot analysis was performed on RNA extracted from αT3-1, αT4 and GT1-7 cells, using SF-1 or mouse GnRH receptor cDNA as probes. As seen in Fig. 3.5A, SF-1 message was detected in αT3-1 and αT4 cells, but was absent from GT1-7 hypothalamic neurons. When the intensity of the SF-1 signals seen in αT3-1 and αT4 were quantified with an IMAGER (Instant Imager, Packard) and standardised against β-actin values (Fig. 3.5C) we found the signal in αT4 cells 0.5 times less than that seen in αT3-1 cells. The same blot was stripped and probed with the mouse GnRH receptor cDNA. The GnRH receptor is expressed in αT3-1 cells but not in αT4 and GT1-7 cells (Fig. 3.5B). These results show that GnRH receptor gene expression is not solely dependent on SF-1 RNA expresssion.

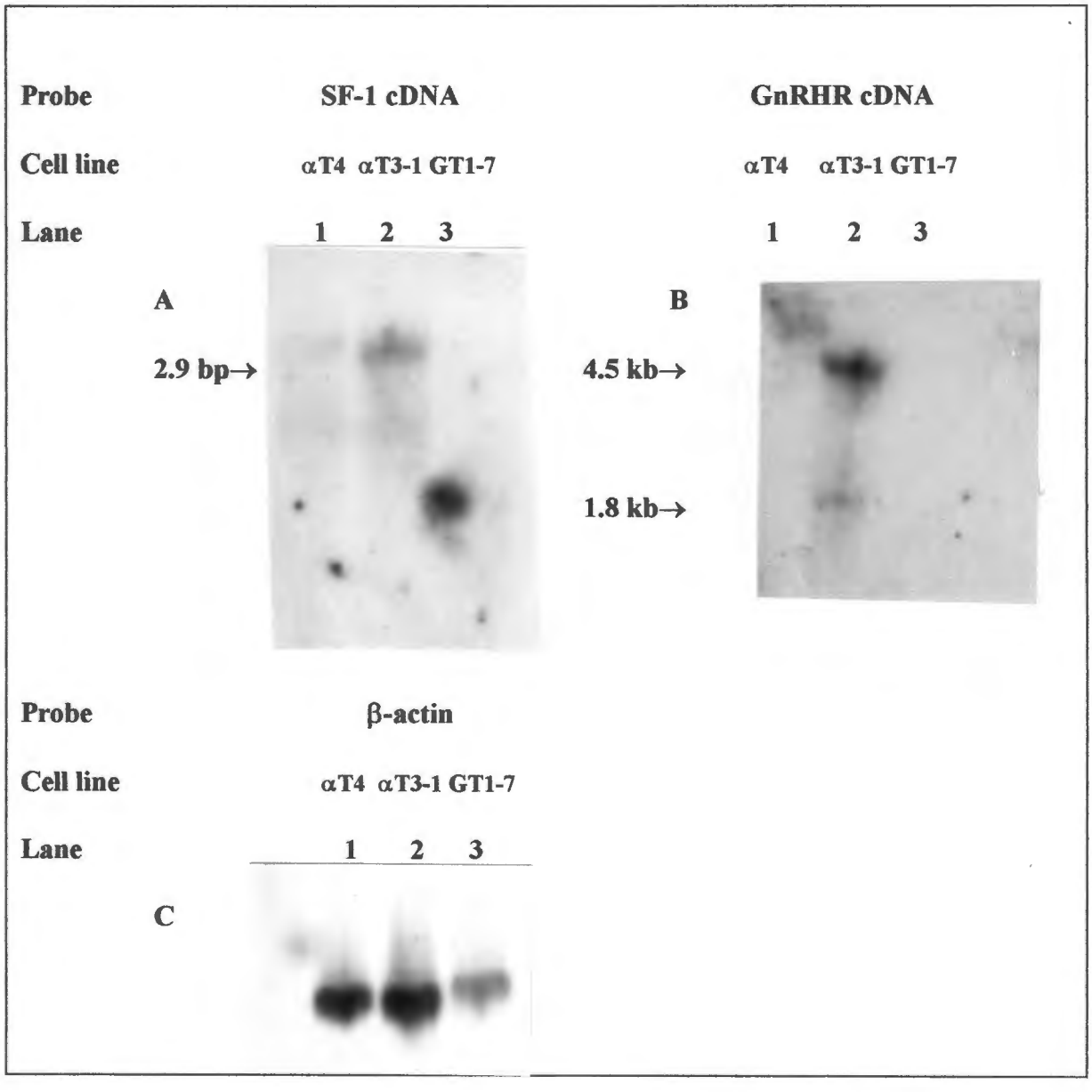

Figure 3.5. SF-1 mRNA is expressed in both αT3-1 and αT4 cells, while GnRH receptor mRNA is only expressed in αT3-1 cells. RNA samples from αT3-1 (lane 2), αT4 (lane 1)and GT1-7 (lane 3) were subjected to Northern blot analysis using either SF-1 cDNA (A), GnRH receptor cDNA (B) or β-actin (C) as probes. A single band of 2.9 kb is seen in αT4 and αT3-1 cells when probed with SF-1 cDNA (A; lanes 1 and 2, respectively). Two transcripts (a major one of 4.5 kb and a minor one of 1.8 kb) are seen in αT3-1 cells when this blot is probed with GnRHR cDNA (B; lane 2). αT4 cells do not express GnRH receptor RNA (B; lane 1). GT1-7 cells do not express SF-1 or GnRH receptor RNA (A and B; lane 3). The blob in Fig. 3.5A is an artifact which might erroneously suggest that a band representing SF-1 RNA is present in GT1-7 cells.

5. Preparation of constructs containing 563 bp of the GnRH receptor gene linked to a luciferase reporter gene for functional assays.

To evaluate the functionality of the 563 bp fragment of the mouse GnRH receptor gene (-500 to +63 relative to the transcription start site), luciferase assays were performed. Before cloning the GnRHR fragment into the pGL2-basic vector, the ATG, at +63 bp relative to the transcription start site, of the mouse GnRH receptor gene was mutated. The translation start site was mutated for two reasons, firstly to ensure that translation starts at the reporter gene and that it does not start at the ATG of the GnRH receptor gene as this may result in out of frame translation of the luciferase reporter gene and therefore in the luciferase protein not being expressed. The second reason for mutating the ATG was to generate a BglII site to facilitate cloning into the pGL2-basic vector. The restriction site was generated using oligonucleotide 1S (section 2.4) and employing Kunkel's method, as described earlier (Fig. 3.6). A 580 bp BamHI-BglII fragment (563 bp of GnRHR gene sequence and 17 bp of pBluescript®SK phagemid sequence) was

excised from clone III and cloned into the BglII site of the promoterless luciferase vector, pGL2-basic, to form wt GnRHR-LUC (Fig. 3.7). The mut GnRHR-LUC construct was formed by fusing the same 563 bp fragment, but with a CC⇒TT mutation in the 'GSE' at position +48 bp relative to the transcription start site (site-directed mutagenesis using oligonucleotide 2S) to the pGL2-basic vector (Figs. 3.6 and 3.7). Recombinants were screened by miniprep plasmid DNA isolation, restriction enzyme digestion and DNA sequencing with the luciferase primers, GL1 and GL2. Recombinants with the GnRH receptor fragment cloned in the sense direction, i.e. driving expression of the luciferase gene, were selected. Gene expression studies were performed by transfection of these constructs into mouse gonadotrope (αT3-1), mouse somatotrope (GH_3) and monkey kidney (COS-1) cells, followed by measurement of luciferase activity in cell lysates. The promoterless vector, pGL2-basic, was transfected serving as a control for basal levels of luciferase expression. The pGL2-control vector, which contains a strong SV40 promoter was used as an internal standard for measuring luciferase activity. Transfection efficiency was determined by transfection with the β-galactosidase vector, pCISLACZ.

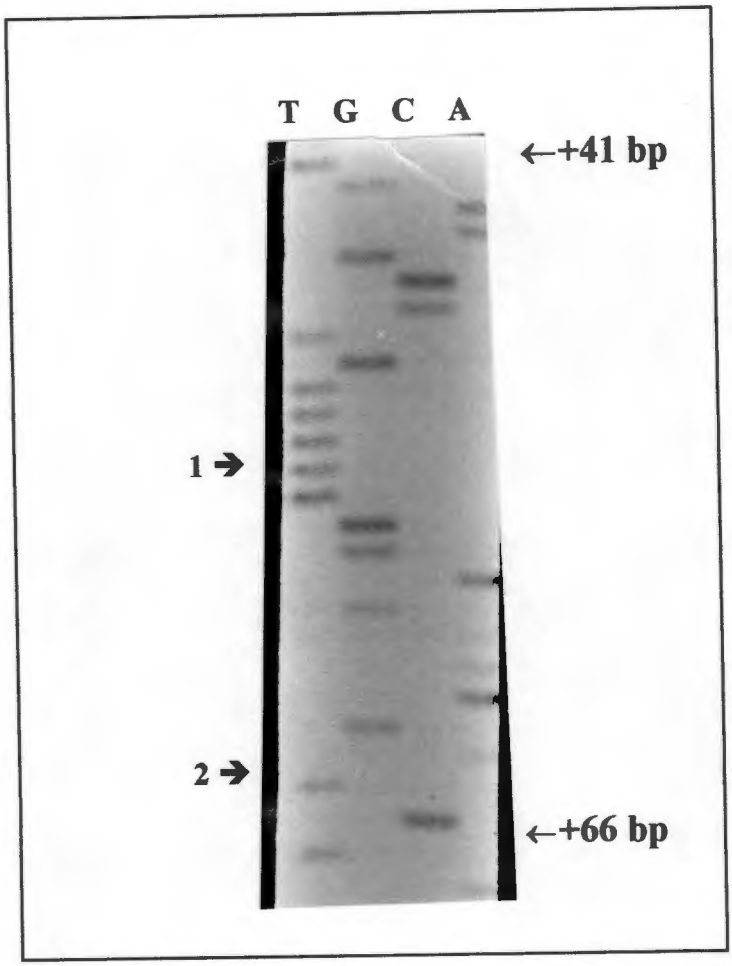

Figure 3.6. The ATG of the mouse GnRH receptor gene was mutated to a BglII site to facilitate cloning into the pGL2-basic vector. The 'GSE' element was mutated to test the functionality of this element. The first arrow shows the 2 bp nucleotide change (CC→TT) in the 'GSE', while the second arrow shows the mutation of the ATG to a BglII site. The sequencing gel autorad of the region covering +41 to +66 bp relative to the transcription start site (Fig. 3.1) is shown. The mutant sequence is shown i.e. TGAAGCCTGT**TTTT**GGAGAAA**GATCT**CTAA, where the mutated sequences are shown in bold. The wild type sequence of the GnRHR 563 bp fragment is TGAAGCCTGT<u>CC</u>TTGGAGAAA<u>T</u>AT<u>GG</u>CTAA, where the bases mutated in the mutant sequence are underlined.

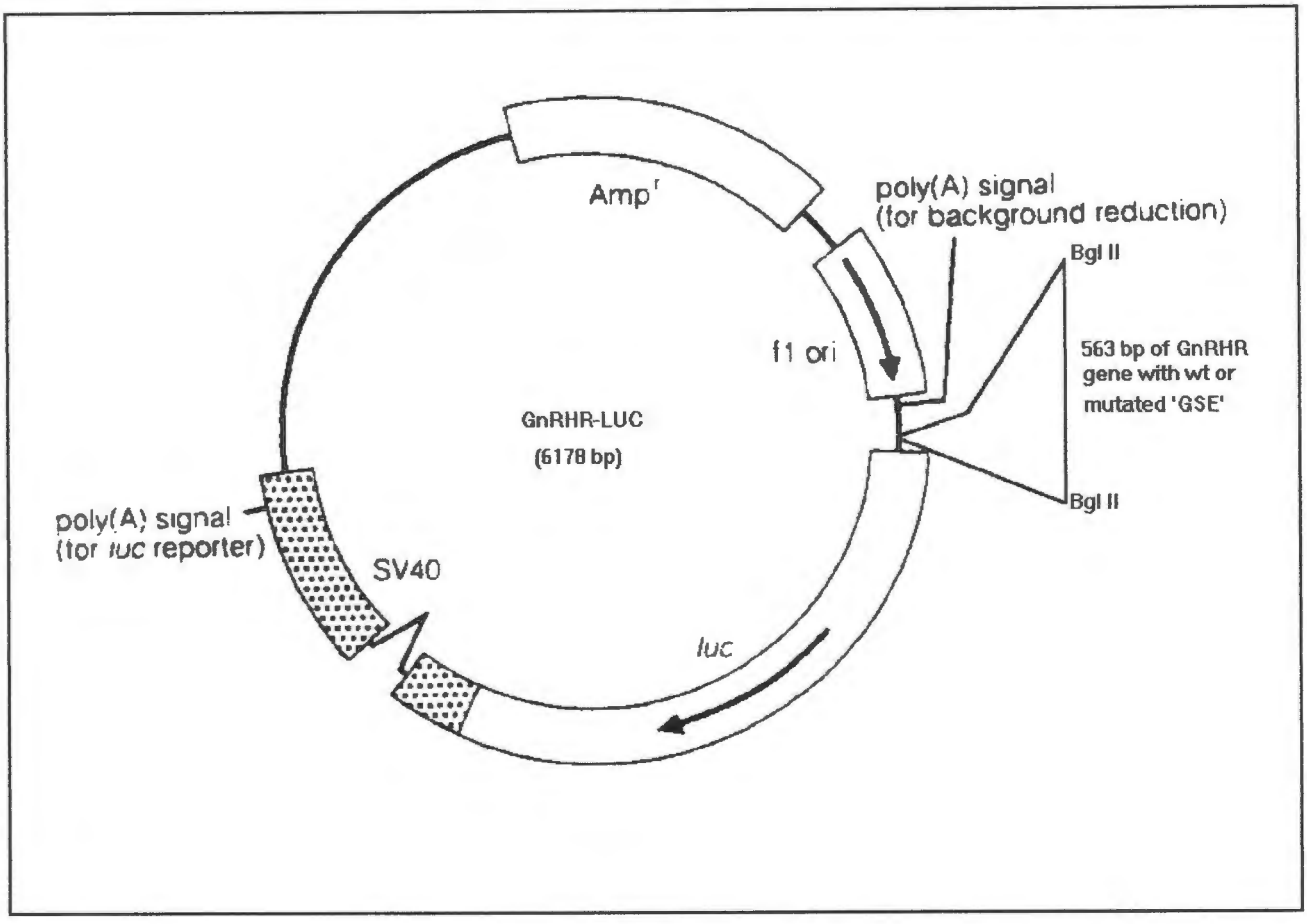

Figure 3.7. wt GnRHR-LUC and mut GnRHR-LUC contain 563 bp of wild type mouse GnRHR gene sequence and 563 bp of mutated GnRHR gene sequence, respectively, linked to the luciferase gene in the reporter plasmid pGL2-basic. After site-directed mutagenesis, as described in text, the 580 bp fragment (563 bp of GnRHR gene sequence and 17 bp of pBluescript®SK phagemid sequence) was excised from clone III and cloned into the BglII site of the pGL2-basic vector. Recombinants with the GnRH receptor gene sequence cloned in the sense direction were selected for and used in gene expression studies.

5.1. Optimising the luciferase assay

5.1.1. Determination of the linear range of light detection

The linear range of luciferase detection was determined by making serial dilutions of purified luciferase enzyme (Boehringer Mannheim) and measuring luciferase activity in a luminometer. Table 3.1 illustrates luciferase units obtained for different concentrations of purified luciferase enzyme. The linear range of light detection was obtained by using between 1 pg/µl and 10 µg/µl of purified luciferase enzyme.

Table 3.1. Determining the concentration of luciferase which produces linear light production.

Concentration of luciferase	Luciferase units
10 µg/µl	1294
1.0 µg/µl	99.00
0.1 µg/µl	12.00
0.01 µg/µl	4.000
1.0 ng/µl	0.450
0.1 ng/µl	0.042
0.01 ng/µl	0.005
1.0 pg/µl	0.001
0.1 pg/µl	0.000
0.01 pg/µl	-0.001

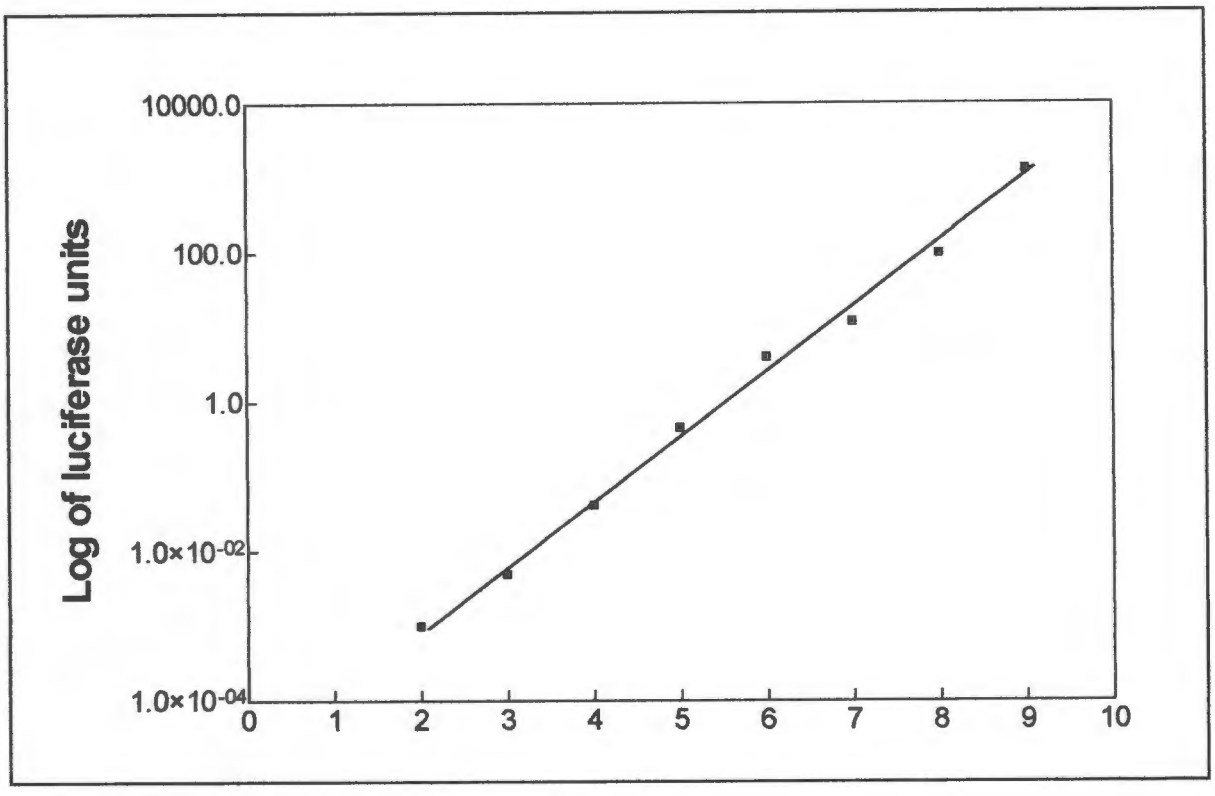

Figure. 3.8. The log of luciferase units produced is plotted against the concentration of purified luciferase enzyme. Numbers 1, 2, 3, 4, 5, 6, 7, 8 and 9 represents 0.1 pg/µl, 1.0 pg/µl, 0.01 ng/µl, 0.1 ng/µl, 1.0 ng/µl, 0.01 µg/µl, 0.1 µg/µl, 1.0 µg/µl and 10 µg/µl, respectively. Light production is linear for the concentrations of purified luciferase enzyme ranging from 1 pg/µl to 10 µg/µl. Lyophilised luciferase enzyme (Boehringer Mannheim) was dissolved to a final concentration of 1 mg/ml in 0.5 M of ice-cold Tris-acetate buffer (pH 7.5). Serial dilutions (Table 3.1) were made using 1 X Reporter Lysis Buffer (Luciferase Assay Kit), and luciferase activity measured in a luminometer, 10 seconds after starting the reaction.

5.1.2. Determination of the optimal time for luciferase detection

Luciferase assays were optimised by taking sample readings at various time points to determine during which time frame light emittance is constant and fluctuation of luciferase activity is at a minimum. Luciferase activity decreased by between 10% and 30% after 300 seconds, but was fairly constant between 5 and 60 seconds (Table 3.2). As a consequence, luciferase readings

were always measured 30 seconds after adding our cell extracts to the substrate and thereby starting the light production.

Table 3.2. Optimising the luciferase assay by determining the decay rate of luciferase production. Readings were taken at various time points between 5 and 300 seconds after starting the reaction. Numbers 1 to 10 are the results of 10 independent assays.

	TIME IN SECONDS										
No.	5	30	60	90	120	150	180	210	240	270	300
1	4.95	4.99	4.99	4.97	4.86	4.78	4.69	4.59	4.52	4.50	4.46
2	5.69	5.63	5.59	5.54	5.55	5.50	5.43	5.36	5.30	5.24	5.13
3	0.17	0.17	0.16	0.16	0.16	0.16	0.15	0.16	0.15	0.14	0.14
4	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05
5	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.10	0.10	0.10	0.10
6	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
7	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.07	0.06
8	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
9	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.05	0.04	0.04	0.04
10	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.04

5.1.3. Optimisation of transfection conditions

5.1.3.1. Comparing lipofectin versus DEAE-Dextran transfection for COS-1 cells

A trial experiment comparing liposome-mediated transfection versus DEAE-Dextran transfection was performed to determine which method worked best, as transfection efficiencies are dependent on many variables such as cell lines and constructs to be transfected, DNA isolation methods and tissue culture conditions. Only two methods were tested for COS-1 cells as both methods were previously shown to work for transfection of COS-1 cells. After

transfection, results were analysed by doing β-galactosidase and luciferase assays as described in the materials and methods. Liposome-mediated transfection was about 1.5 X more efficient that DEAE-Dextran transfection for both β-galactosidase and luciferase activities.

Table 3.3. Lipofectin and DEAE-Dextran transfection methods were compared for COS-1 cells. After transfection, cell extracts were harvested and used in β-galactosidase and luciferase assays. Transfection efficiency was 1.5 X higher when using lipofectin as opposed to using DEAE-Dextran.

Method of transfection	β-galactosidase units	Luciferase units
Lipofectin	0.87	71
DEAE-Dextran	0.57	50

5.1.3.2. Optimising transfecting αT3-1 cells with lipofectin and varying DNA concentrations

Initially αT3-1 cells were transfected with lipofectin as lipofectin gave the best results for COS-1 cells. DNA concentrations were varied to increase the chances of getting high transfection efficiencies with these cells as others have previously reported that αT3-1 cells are finicky and difficult to transfect (Daniel Assefa, personal communication; Ian Wakefield, personal communication). After transfection cell extracts were harvested and assayed using β-galactosidase and luciferase assays, but readings obtained were too low to analyse the data and it could therefore be concluded that liposome-mediated transfection did not work for αT3-1 cells under the conditions used. These results emphasize the well established fact that the efficiency of transfection method is dependent on the cell line being transfected.

5.1.3.3. Transfecting αT3-1 cells by electroporation

To try and overcome the problems in trying to transfect αT3-1 cells, transfection of αT3-1 by electroporation was attempted. Many studies have been performed comparing electroporation to other transfection methods and in the majority of cases electroporation was always the most successful (Paulsen *et al.* 1995; Spencer, 1993). This method was not successful for transfecting αT3-1 cells as transfectants died before they could be harvested. This may indicate that transfection by electroporation is too harsh for αT3-1 cells or that maybe the voltage we used was too high, as electroporation of COS-1 cells, which served as controls, yielded positive results.

5.1.3.4. Transfecting αT3-1 cells using the calcium phosphate method.

αT3-1 cells were transfected by the calcium phosphate method as described in the materials and methods. The success of calcium phosphate transfection is dependent on the quality of the DNA to be transfected. Supercoiled DNA is more efficiently transfected than linear or nicked DNA. DNA used for calcium phosphate transfections was purified by caesium chloride gradient centrifugation as described before. The ratio of luciferase vector to β-galactosidase vector was varied in transfection experiments. The necessity to include carrier DNA in transfection experiments was also determined. After transfection cell extracts were harvested and β-galactosidase and luciferase assays performed. There was no significant difference observed when varying the ratios of DNA transfected and therefore a luciferase vector to β-galactosidase ratio of 3.5:1 was used.

Table 3.4. Transfection of αT3-1 cells by calcium phosphate precipitates. The DNA concentrations of the reporter constructs as well as the concentration of carrier DNA is shown. Normalised luciferase activity was calculated by dividing luciferase units produced by β-galactosidase units produced.

Total DNA(μg/μl)	pGL2-control (μg/μl)	β-gal (μg/μl)	carrier (μg/μl)	ratio	Normalised luciferase act.
8.8	5.87	2.93		2:1	6.76±0.31
8.8	4.1	1.17	3.52	3.5:1:3	5.28±0.44
8.8	6.84	1.95		3.5:1	4.49±0.15

5.2. Luciferase assays

The luciferase assay system was used to a) Study promoter activity of constructs containing 563 bp of the GnRHR gene, b) Determine whether promoter activity, if observed, was tissue-specific and c) Whether SF-1 played a role in promoter activity. In order to address these three issues wild type and mutant GnRHR-LUC constructs were transfected into αT3-1, GH_3 and COS-1 cells. SF-1 cDNA was also transfected into αT3-1 and COS-1 cells to determine the role of SF-1 in possible promoter activity. The pGL2-control vector, containing the SV40 promoter and the promoterless pGL2-basic vector were used as positive control and negative controls, respectively. The pCISLACZ vector served as an internal standard to monitor transfection effeciency. αT3-1 cells were transfected using the calcium phosphate method as described in the materials and methods (section 2.19.1.1.1), while GH_3 and COS-1 cells were transfected using lipofectin (2.19.1.2.1).

5.2.1. Results of transfection of αT3-1 cells

Table 3.5. DNA concentrations used for calcium phosphate transfections into αT3-1 cells. β-galactosidase and luciferase activity was measured using the formulae given at the bottom of the table. Abbreviations used in table: cont, pGL2 positive control; basic, pGL2 negative control; wt, wt GnRHR-LUC; mut, mut GnRHR-LUC; SF-1, steroidogenic factor-1; β-gal, β-galactosidase; lucif, luciferase; rel.act., relative activity

Concentration of DNA transfected (μg)							Activity measured			
Total	cont	basic	wt	mut	β-gal	SF-1	β-gal[b]	lucif[a]	a/b	rel.act.
36	28				8		7.70	31.09	4.04	1.00
36	28				8		7.80	23.24	3.00	0.74
36	28				8		0.94	3.70	3.96	0.97
36	28				8		0.87	3.45	3.96	0.97
36	28				8		0.82	3.06	3.73	0.92
36		28			8		0.00	0.00	0.00	0.00
36		28			8		0.00	0.00	0.00	0.00
36		28			8		0.00	0.00	0.00	0.00
36		28			8		0.00	0.00	0.00	0.00
36		28			8		0.00	0.00	0.00	0.00
36			28		8		18.00	37.25	2.10	0.52
36			28		8		18.10	33.42	1.85	0.46
36			28		8		1.30	2.15	1.65	0.41
36			28		8		1.17	2.50	2.14	0.53
36			28		8		1.08	1.75	1.62	0.40
36			28		8	14	4.00	20.48	5.12	1.27
36			28		8	14	4.10	18.24	4.45	1.10
36			28		8	14	0.49	1.40	2.86	0.71
36			28		8	14	0.65	1.90	2.92	0.72
36			28		8	14	0.88	1.85	2.10	0.52
36				28	8		7.20	18.45	2.57	0.63
36				28	8		4.90	12.86	2.62	0.65
36				28	8		0.96	1.25	1.30	0.32
36				28	8		1.30	2.25	1.73	0.43
36				28	8		1.10	3.35	3.05	0.75
36				28	8	14	3.80	19.50	5.13	1.27
36				28	8	14	6.30	35.40	5.62	1.40
36				28	8	14	0.57	2.35	4.12	1.02
36				28	8	14	0.48	3.20	6.70	1.65
36				28	8	14	0.50	2.55	5.10	1.26

a= luciferase activity = $^{\text{luciferase units}}/_{\text{mg protein}}$

b= β-galactosidase activity = $^{\text{OD420/}}\text{hr}/_{\text{mg protein}}$

a/b= nomalised activity, i.e. luciferase activity per β-galactosidase activity

relative luciferase activity= activity relative to the pGL2-control plasmid, i.e a/b for experiment divided by a/b for pGL2 positive control plasmid

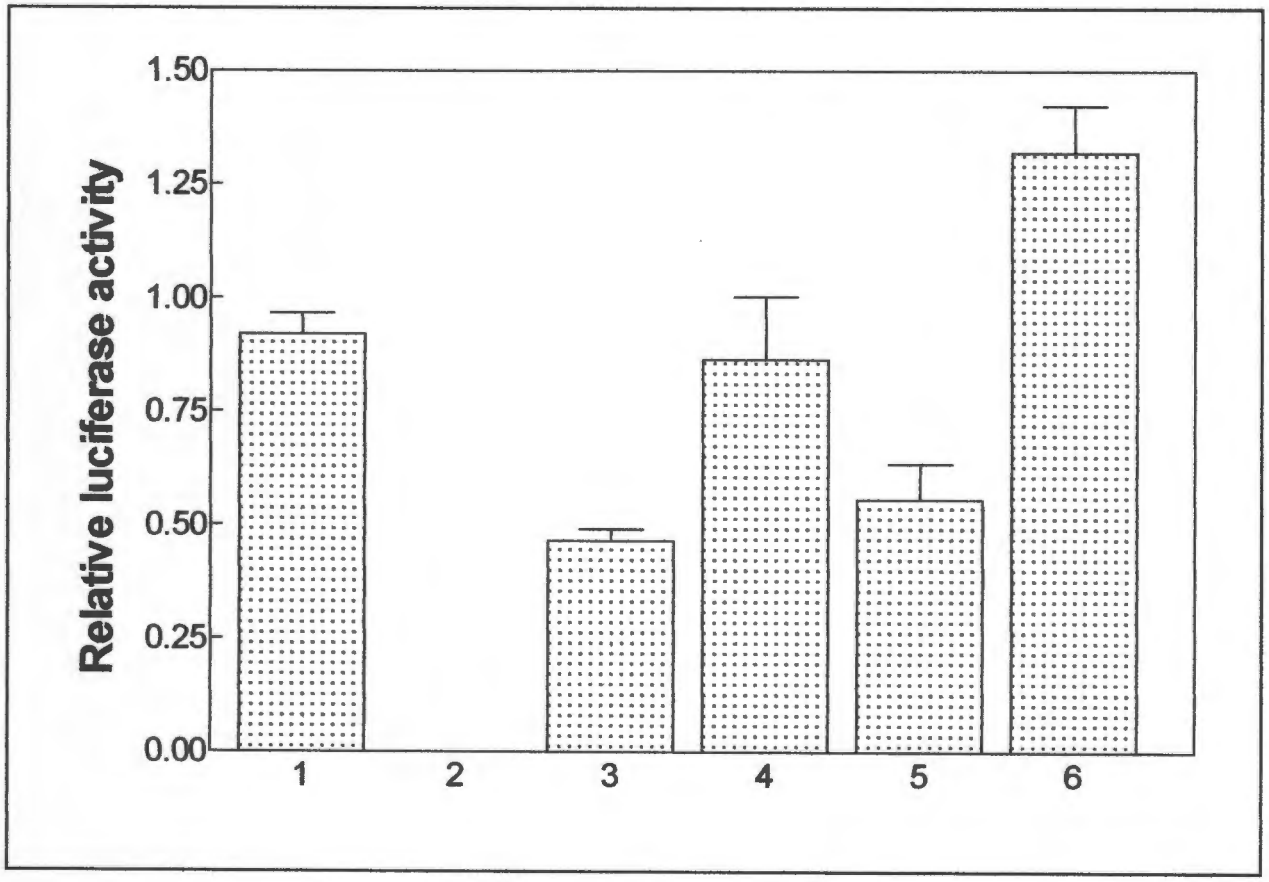

Figure 3.9. αT3-1 cells were transiently co-transfected with the following constructs, pGL2-control, pGL2-basic, wt GnRHR-LUC, mut GnRHR-LUC, β-galactosidase and SF-1 cDNA as described earlier. DNA concentrations used for transfections are showed in Table 3.5. Numbers 1, 2, 3, 4, 5 and 6 represents transfection with the pGL2-control, pGL2-basic, wt GnRHR-LUC, wt GnRHR-LUC plus SF-1 cDNA, mut GnRHR-LUC and mut GnRHR-LUC plus SF-1 cDNA, respectively. Each bar represents the mean ± standard error of five experiments.

5.2.2. Results of transfection of GH_3 cells

Table 3.4. Concentrations of DNA used for lipofectin mediated transfection and β-galactosidase and luciferase activity measured in GH_3 cells. Abbreviations used in table: cont, pGL2 positive control; basic, pGL2 negative control; wt, wt GnRHR-LUC; mut, mut GnRHR-LUC; β-gal, β-galactosidase; lucif, luciferase; rel. act., relative activity

Concentration of DNA transfected (μg)						Activity measured			
Total	cont	basic	wt	mut	β-gal	β-gal[b]	lucif[a]	a/b	rel.act.
6.25	4.86				1.39	0.46	0.64	1.39	1.00
6.25	4.86				1.39	0.77	1.04	1.34	0.96
6.25		4.86			1.39	0.00	0.00	0.00	0.00
6.25		4.86			1.39	0.00	0.00	0.00	0.00
6.25			4.86		1.39	0.82	0.95	1.15	0.72
6.25			4.86		1.39	0.27	0.48	1.80	1.30
6.25				4.86	1.39	0.90	1.28	1.42	1.02
6.25				4.86	1.39	0.94	1.26	1.30	0.94

a= luciferase activity = $^{\text{luciferase units}}/_{\text{mg protein}}$

b= β-galactosidase activity = $^{\text{OD420}}/\text{hr}/_{\text{mg protein}}$

a/b= nomalised activity, i.e. luciferase activity per β-galactosidase activity

relative luciferase activity= activity relative to the pGL2-control plasmid, i.e a/b for experiment divided by a/b for pGL2 positive control plasmid

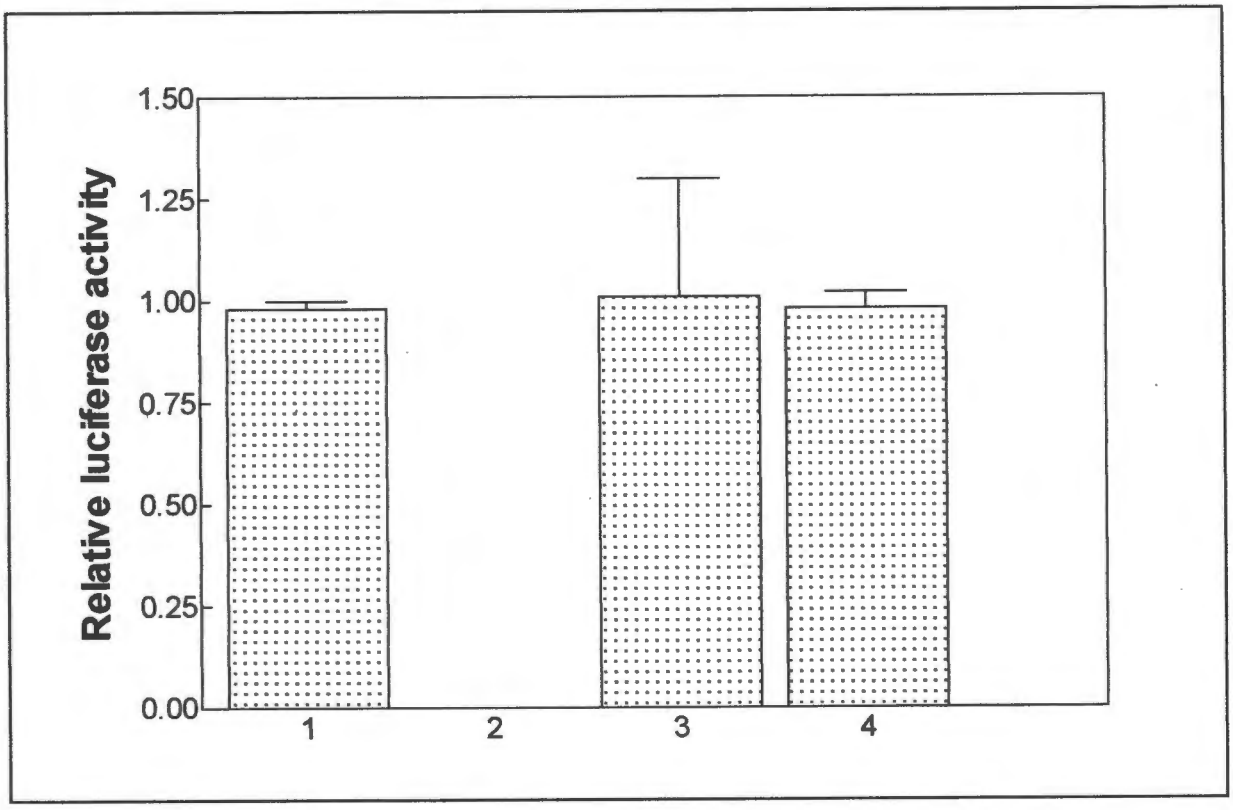

Figure. 3.10. GH_3 cells were transiently co-transfected with the following constructs, pGL2-control, pGL2-basic, wt GnRHR-LUC, mut GnRHR-LUC and β-galactosidase as described earlier. DNA concentrations used for transfections are showed in Table 3.5. Numbers 1, 2, 3 and 4 represents transfecting with pGL2-control, pGL2-basic, wt GnRHR-LUC and mut GnRHR-LUC, respectively. Each bar represents the mean ± standard error of one experiment done in duplicate.

5.2.3. Results of transfections with COS-1 cells

Table 3.7. Concentrations of DNA used for lipofectin mediated transfection and β-galactosidase and luciferase activity measured in COS-1 cells. Abbreviations used in table: cont, pGL2 positive control; basic, pGL2 negative control; wt, wt GnRHR-LUC; mut, mut GnRHR-LUC; β-gal, β-galactosidase; SF-1, steroidogenic factor-1; lucif, luciferase; rel. act., relative activity

Concentration of DNA transfected (µg)							Activity measured			
Total	cont	basic	wt	mut	SF-1	β-gal	β-gal[b]	lucif[a]	a/b	rel.act.
6.25	4.86					1.39	250	147.9	0.59	1.00
6.25	4.86					1.39	232	71.43	0.31	0.53
6.25	4.86					1.39	3154	992.8	0.32	0.54
6.25	4.86					1.39	2707	1123	0.41	0.70
6.25		4.86				1.39	0.00	0.00	0.00	0.00
6.25		4.86				1.39	0.00	0.00	0.00	0.00
6.25		4.86				1.39	0.00	0.00	0.00	0.00
6.25		4.86				1.39	0.00	0.00	0.00	0.00
6.25			4.86			1.39	258.5	1.99	0.008	0.01
6.25			4.86			1.39	371.9	0.754	0.002	0.003
6.25			4.86			1.39	2911	4.42	0.002	0.003
6.25			4.86			1.39	4247	6.41	0.002	0.003
			4.86		2.74	1.39	52.25	1.363	0.03	0.05
9.0			4.86		2.74	1.39	57.74	0.952	0.02	0.03
9.0			4.86		2.74	1.39	265.7	4.42	0.02	0.03
9.0			4.86		2.74	1.39	190.7	1.92	0.01	0.02
6.25				4.86		1.39	482	1.11	0.002	0.003
6.25				4.86		1.39	459	1.09	0.002	0.003
6.25				4.86		1.39	4369	6.79	0.002	0.003
6.25				4.86		1.39	3631	3.26	0.001	0.002
9.0				4.86	2.74	1.39	41.23	0.70	0.02	0.03
9.0				4.86	2.74	1.39	40.7	0.224	0.006	0.01
9.0				4.86	2.74	1.39	306.6	3.34	0.01	0.02
9.0				4.86	2.74	1.39	303.4	2.85	0.01	0.02

a= luciferase activity = $^{\text{luciferase units}}/_{\text{mg protein}}$

b= β-galactosidase activity = $^{\text{OD420}}/\text{hr}/_{\text{mg protein}}$

a/b= nomalised activity, i.e. luciferase activity per β-galactosidase activity

relative luciferase activity= activity relative to the pGL2-control plasmid, i.e a/b for experiment divided by a/b for pGL2 positive control plasmid

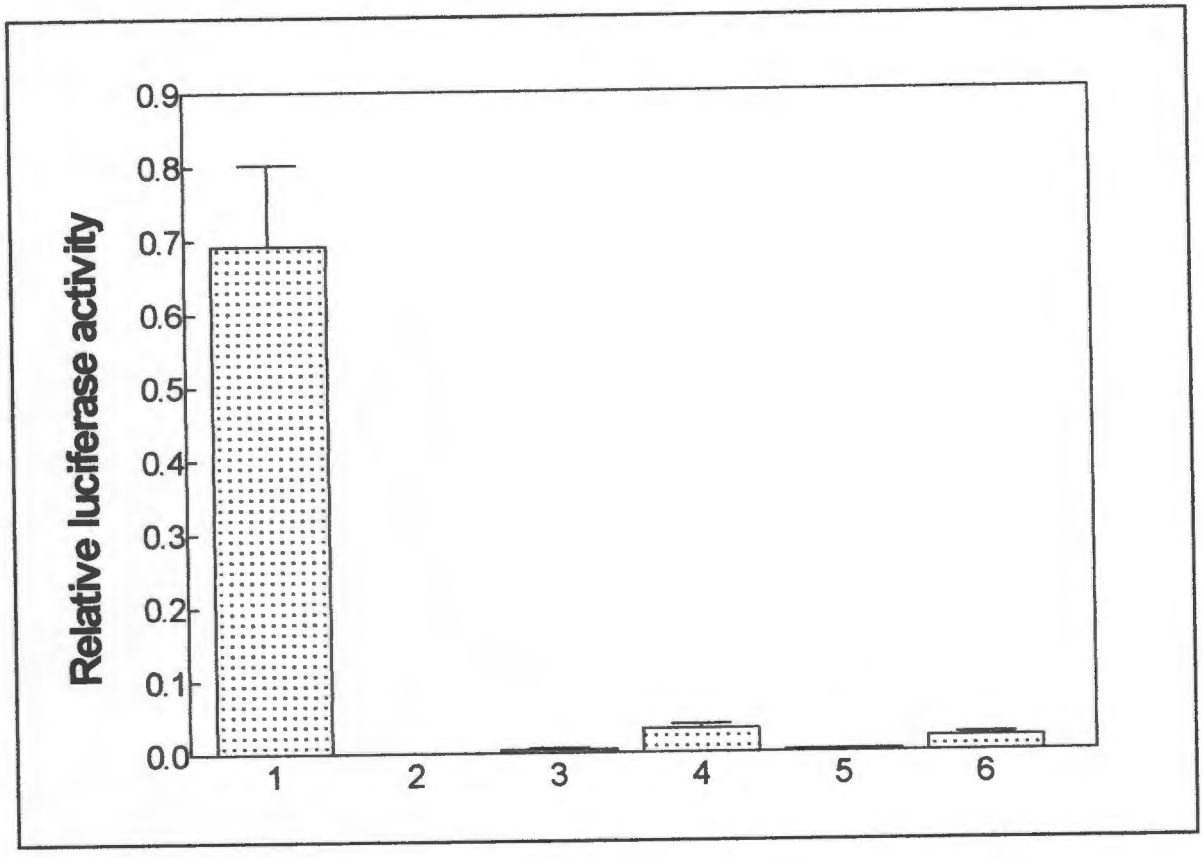

Figure. 3.11. COS-1 cells were transiently co-transfected with the following constructs, pGL2-control, pGL2-basic, wt GnRHR-LUC, mut GnRHR-LUC, SF-1 and β-galactosidase cDNA as described earlier. DNA concentrations used for transfections are showed in Table 3.5. Numbers 1, 2, 3, 4, 5 and 6 represents transfecting with pGL2-control, pGL2-basic, wt GnRHR-LUC, wt GnRHR-LUC plus SF-1 cDNA, mut GnRHR-LUC and mut GnRHR-LUC plus SF-1 cDNA, respectively. Each bar represents the mean ± standard error of four experiments.

5.3. Analysis of results of functional assays

5.3.1. 563 bp of the GnRHR gene 5' to the translation start site is sufficient for promoter activity

To test promoter activity of the 563 bp fragment of the GnRHR gene constructs were transfected into αT3-1 and GH_3 cells. No luciferase activity was produced by the pGL2-basic vector. No significant difference in luciferase activity produced by the wt GnRHR-LUC and the

mut GnRHR-LUC constructs was detected in any of the cells. Both constructs produced about half the luciferase activity produced by the pGL2-control plasmid in αT3-1 cells (wt GnRHR-LUC and mut GnRHR-LUC produced 45% and 55%, respectively) (Fig. 3.9). In GH_3 cells constructs produced a similar level of luciferase activity compared to the pGL2-control plasmid (activity of both constructs relative to the pGL2-control plasmid was 100%) (Fig. 3.10). These results indicated that the 563 bp fragment of the GnRHR gene contains strong promoter activity in both αT3-1 and GH_3 cell lines with activity in GH_3 cells being higher than that produced in αT3-1 cells (activity of GnRHR constructs relative to the pGL2-control plasmid was 100% in GH_3 cells compared to 50% in αT3-1 cells). The reason for the lower activity of both constructs in αT3-1 cells relative to GH_3 cells may be due to the presence of an inhibitor in αT3-1 cells, binding to a site other than the SF-1 site. Alternatively, an enhancer factor interacting with the promoter at a site other than the SF-1 site, present in GH_3 cells but absent from αT3-1 cells, may be necessary for the higher levels of GnRH receptor gene expression observed in GH_3 cells relative to αT3-1 cells.

5.3.2. Promoter activity of the 563 bp fragment is tissue-specific

To test for tissue specificity of the 563 bp fragment constructs were transfected into the transformed pituitary cell lines, αT3-1 and GH_3, as well as into an unrelated cell type, a transformed kidney cell line, COS-1 cells. No significant promoter activity was produced in the non-pituitary cell line, COS-1 (Fig. 3.11) whereas high levels of promoter activity was observed in both αT3-1 and GH_3 cells. Luciferase activity of the wt GnRHR-LUC and mut GnRHR-LUC constructs was between 140-233 X less than that produced by the pGL-2 control vector in COS-1 cells, whereas luciferase activity of the wt GnRHR-LUC and mut GnRHR-LUC constructs was 50% and 100% relative to the pGL2-control plasmid in αT3-1 and GH_3 cells,

respectively. These results indicated that the 563 bp fragment of the mouse GnRH receptor gene is sufficient to activate gene expression in a tissue-specific manner in both αT3-1 and GH_3 cells, but not in an unrelated cell type (COS-1 cells).

5.3.3. Tissue-specific promoter activity of the 563 bp fragment of the GnRHR gene is not dependent on SF-1

To determine the role of SF-1 and the 'GSE' on tissue-specific promoter activity of the 563 bp fragment of the GnRHR gene, the wild type and mutant luciferase constructs were transfected into αT3-1, GH_3 and COS-1. αT3-1 cells contain endogenous SF-1, whereas GH_3 and COS-1 cells do not. The results for αT3-1 cells showed that the 'GSE' on its own is not essential for promoter activity as similar promoter activity is produced by the wild type and mutant (where the SF-1 site is mutated) luciferase constructs (Fig. 3.9). Interestingly, GH_3 cells, which do not express endogenous SF-1 protein, produced even higher promoter activity than αT3-1 cells, when transfected with both the wt and mut GnRHR-LUC constructs, strongly supporting the finding that SF-1 protein is not essential for high levels of GnRHR promoter activity. In COS-1 cells, co-transfection of the wt GnRHR-LUC construct with SF-1 cDNA caused no significant difference in the low promoter activity obtained with the wt GnRHR construct alone, implying that the absence of SF-1 alone is not responsible for low basal expression in COS-1 cells.

Taken together, these results showed that the 563 bp fragment of the GnRH receptor gene used in this study is sufficient for high basal expression in the pituitary cell lines, αT3-1 and GH_3 cells, but not in the unrelated cell line, COS-1 cells. Also, it seems that the presence of the 'GSE' sequence and SF-1 protein is not crucial for promoter activity as mutations of the 'GSE' do not

significantly alter GnRH receptor gene expression and co-transfection with SF-1 cDNA, in cells lacking endogenous SF-1, does not significantly effect GnRH receptor gene expression.

The results on co-transfection of αT3-1 cells with SF-1 cDNA together with GnRHR-LUC constructs are difficult to explain. Since αT3-1 cells contain endogenous SF-1 protein, co-transfection with SF-1 cDNA would thus be expected to have no effect. It is possible that the slight (i.e. 2-fold) increase in promoter activity observed in αT3-1 cells on co-transfection with SF-1 cDNA may be due to effects unrelated to an increase in SF-1 protein levels.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Transient transfection studies show that the 563 bp fragment of the GnRH receptor gene, used in this study, contains strong promoter activity and that the regulatory elements for tissue-specific expression of the mouse GnRH receptor gene are present within this region. This region of the mouse GnRH receptor gene confers tissue-specific but not gonadotrope specific expression, as promoter activity is observed in both αT3-1 and GH_3 cells, but not in COS-1 cells. Further studies in other cell lines will have to be done to establish more fully the tissue specific expression. These findings are consistent with those of Albarracin *et al.* (1994) who showed that 1.2 kb of the 5'-flanking region of the mouse GnRH receptor gene contains regulatory elements for tissue-specific expression. It appears that sequences 5' to -500 bp relative to the transcription start site are not necessary for this activity, since we have shown that only 500 bp of the 5'-flanking region of the mouse GnRH receptor gene (our constructs contained the gene from -500 bp to +63 relative to the transcription start site) are necessary for promoter activity and tissue-specific expression.

The role of SF-1 in GnRH receptor gene regulation was also examined in this study. Analysis of of the mouse GnRH receptor gene from -500 bp to +63 bp relative to the transcription start site, has revealed the presence of a SF-1-like binding site at +48 bp relative to the transcription start site. Gel mobility shift and DNaseI footprinting assays, using nuclear extracts prepared from αT3-1, αT4 and GT1-7 cells, showed that a factor with SF-1-like binding activity binds over the GnRH receptor 'GSE'. A weak footprint was seen over the putative GnRH receptor GSE. No other footprints were seen on the fragment from -270 bp to +108 bp. This could

indicate that 1) no other major protein-DNA interactions occured in this region, or, 2) *in vitro* conditions of the footprint experiment may not optimise binding of other factors, or may not be able to mimic conditions *in vivo* due to, for example, the role of higher order structure (a particular chromatin structure). Although competition with unlabelled GSE and SF-1 oligonucleotides in gel mobility shift and DNaseI footprinting experiments were performed to verify the sequence specificity of the DNA-protein interactions, we cannot as yet conclude that the nuclear protein(s) in αT3-1 and αT4 cells that bind the SF-1-like site is indeed SF-1. This could be verified in future by gel mobility shift experiments with antibodies to SF-1 the protein.

SF-1 is a transcription factor that regulates steroid biosynthetic enzyme gene expression in gonadal and adrenocortical cells, as well as regulating gonadotropin gene expression (Barnhart and Mellon, 1994; Lala *et al.* 1992; Lynch *et al.* 1993). These findings imply that SF-1 may form part of a global pathway regulating reproductive function. The role of SF-1 in GnRH receptor gene expression was investigated. Transfection experiments were conducted in αT3-1, GH_3 and COS-1 cell lines with constructs containing gene sequences from -500 bp to +63 bp relative to the transcription start site of the mouse GnRH receptor gene, with wild type or mutated SF-1-like sites. αT3-1 and COS-1 cells were also co-transfected with the wt or mut GnRHR constructs plus SF-1 cDNA. The luciferase constructs we have used, containing 563 bp of the GnRH receptor gene extended until the ATG translation start site (at +63 bp relative to the transcription start site) and therefore included the SF-1-like site (at +48 bp relative to the transcription start site). The luciferase construct containing 1.2 kb of 5'-flanking region of the GnRH receptor gene prepared by Albarracin *et al.* (1994) was truncated in the middle of the putative SF-1 site, so they were not able to determine the effect of the presence of a functional SF-1 site in GnRH receptor gene expression. The results of this study revealed that luciferase

constructs with both the wild type and mutant SF-1-like sites showed similar promoter activity in αT3-1 and GH_3 cells. In COS-1 cells, transfection with the wt or mut GnRHR-LUC constructs produced no promoter activity. Co-transfection of the GnRH receptor promoter constructs, with either the wild type or mutated SF-1-like sites together with SF-1 cDNA showed no significant increase in GnRHR gene expression in COS-1 cells. However, in αT3-1 cells we observed a 2-3-fold increase in luciferase activity when the GnRHR-LUC constructs were co-transfected together with SF-1 cDNA. These results imply that an excess of SF-1 slightly increases gene expression in αT3-1 cells. It is difficult to explain these results as αT3-1 cells express endogenous SF-1 protein and co-transfection with SF-1 cDNA would be expected to have no effect on promoter activity.

The results of the wt and mut constructs in αT3-1 cells, which contain endogenous SF-1 protein, imply that SF-1 binding to the SF-1-like site is not necessary for the high level of promoter activity in these cells. If SF-1 was necessary for expression, we would have expected to see low expression with the mutated construct. However, the results of our gel shift experiments showed that SF-1 protein can bind weakly to the mutated 'GSE' in the GnRHR gene. Therefore it is possible that the high level of activity obtained with the mutated 'GSE' construct was due to some binding of SF-1 protein.

However, the results in GH_3 and COS-1 cells support the conclusion that SF-1 binding to the GSE is not necessary for high levels of GnRHR gene expression. GH_3 cells do not contain SF-1 protein but show higher levels (100% luciferase activity relative to control) of expression of the wt and mut constructs than αT3-1 cells (50% luciferase activity relative to control). Co-transfection of SF-1 cDNA with the wt or mut construct did not significantly increase GnRHR

gene expression in COS-1 cells. If SF-1 was necessary for promoter activity, we would have expected to see low activity in the absence of SF-1 cDNA and high activity for the wt construct in the presence of SF-1 cDNA, which was abolished for the mutated construct plus SF-1 cDNA. However, we do not have direct evidence that the SF-1 protein is expressed by the SF-1 cDNA in COS-1 cells. The lack of activity of the GnRHR promoter in COS-1 cells may also not be entirely due to the absence of SF-1 protein but also to the absence of other tissue-specific factors. No promoter activity was observed when these cells were transfected with the wt or mut constructs suggesting that GnRHR gene expression is tissue-specific and not expressed in COS-1 cells.

Taken together, these results show that SF-1 alone is not necessary for high levels of expression of the GnRHR gene. Similar experiments were done by Duval *et al.* (1997) who showed that a 58% reduction in promoter activity was observed in αT3-1 cells, when a 'GSE' or SF-1-like site was mutated. The reason for the discrepancy between their results and ours is difficult to explain, since the experimental errors in our assays (Fig. 3.9) is low enough to be able to detect a 60% reduction in promoter activity. Our findings suggest that the presence of an SF-1 site and SF-1 protein is not an absolute requirement for GnRH receptor gene expression. This is in agreement with the results of Duval *et al.* (1997).

αT3-1 and αT4 cells are both transformed gonadotrope cell lines developed by Windle *et al.* (1990). They showed that αT4 cells do not express GnRHR RNA, while αT3-1 cells do. We were intrigued by this finding and hoped to use αT4 cells as a vehicle to investigate the role of SF-1 in GnRHR gene expression. We showed by gel shifts and Northern blotting that αT4 cells express SF-1 RNA and most likely SF-1 protein. This has not been previously demonstrated, to

our knowledge. From these findings we can conclude that GnRHR expression is not solely dependent on SF-1 expression.

GT1-7 cells are transformed neuronal cells (Mellon *et al* 1990). Some reports show that GnRHR is expressed in these cells (Krsmanovic *et al* 1993; Li et *al.* 1996). However, contradictory or unclear report are found in the literature. Thus we thought that, as for αT4 cells, these cells would be a useful model to shed light on the role of SF-1 in GnRHR expression. Gel shifts and Northern blot analysis have revealed that neither SF-1 nor GnRHR RNA are expressed in these cells, under our conditions.

The results obtained in this study showed that a major 4.5 kb GnRH receptor mRNA and a less abundant 1.8 kb mRNA is present in αT3-1 cells. These findings are consistent with those of Albarracin *et al.* (1994), who also found two species of GnRHR mRNA in αT3-1 cells. As discussed in the introduction, the heterogeneity of the GnRHR trancripts may result from multiple transcription start sites, alternative splicing of the gene or alternative polyadenylation signals. Although the presence of multiple transcription start sites may play a role in generating multiple mRNA species, the difference in size of the transcripts derived from these start sites would not be large enough to account for the almost three kilobase difference in the two major mRNA species detected in the mouse. This suggests that other mechanisms may contribute to the generation of these multiple transcripts of the GnRH receptor gene. The GnRH receptor gene is likely to be regulated in a complex manner. We have shown that it is subject to strict tissue-specific regulation, which appears to be independent of SF-1 in the cells we have investigated. SF-1 may act in conjunction with another factor(s). Reports have suggested that SF-1 may interact with another factor, DAX-1, to effect gene regulation. It is possible that SF-1

regulates DAX-1 gene expression since SF-1 binds upstream of the DAX-1 gene (Burris *et al.* 1995; Ito *et al.* 1997; Vilain *et al.* 1997; Yu *et al.* 1996). We do not yet know the significance of this interaction but it is likely that these two proteins act in conjuction to regulate gene expression.

Our findings suggest that SF-1 is not necessary for tissue-specific expression of the GnRHR gene, but that sequences from -500 bp to +63 bp relative to the transcription start site of the GnRHR gene is necessary to confer tissue-specificity. This is supported by the observation that SF-1 gene expression is not itself restricted to gonadotropes (Ikeda *et al.* 1993; Ikeda *et al.* 1994; Ingraham *et al.* 1994). Tissue specificity may be a result of the interaction of multiple factors binding to sites in the regulatory region of the GnRH receptor gene, possibly in conjunction with SF-1. If so, some of these sites may be absent in our construct. Further studies on the regulation of SF-1 itself, and its interaction with other transcription factors will shed light on its possible involvement in GnRH receptor gene regulation.

It is possible that our results in transformed cell lines do not accurately reflect the *in vivo* situation. αT3-1 cells are transformed precursor gonadotrope cells (Windle *et al.* 1990) which express the α subunit but not the β subunit of the LH and FSH. Thus they do not exhibit some of the functions of fully differentiated gonadotrope cells. Some of the factors necessary for SF-1 dependent GnRHR expression may be absent from the cell line. In addition, SF-1 may require a ligand to exert its regulatory role on the GnRHR promoter, and this ligand may be absent in our tissue culture system. SF-1 may be involved in mediation of another factor(s) involved in intracellular signalling, such as the PKC or PKA system. These possibilities need to be investigated. Alternatively, SF-1 may have nothing to do with GnRHR expression.

Gel shift and footprint analysis have been used in this study to show that the 'GSE' site on the GnRHR binds a protein(s) *in vitro* which could be SF-1. However, no direct evidence is available to show that it is SF-1, and not another transcription factor which binds to a similar binding site. It is known that the SF-1 recognition site is similar to that of some members of the steroid receptor superfamily (Barnhart and Mellon, 1994). Also, *in vitro* binding is not a proof of functionality, so the GSE site on the GnRHR promoter may also have nothing to do with GnRHR expression.

The GnRHR 'GSE' occurs at +48 bp relative to the transcription start site, i.e. it is in the 5' untranslated region of the mRNA transcript and not 5' to the transcription start site. Some genes have transcriptional regulatory elements in the untranslated region but most to date appear in the non-transcribed regions. Maybe the real activity of the GnRHR 'GSE' *in vivo* may be translational regulation which also requires the presence of the coding region of GnRHR in the transcript. Many reports have been published that elements in the untranslated regions are involved in translational regulation (Boado *et al.* 1996; Manzella and Blackshear, 1990; Sonenberg, 1994; Timmer *et al.* 1993). It would therefore be interesting to see whether the 'GSE' plays a role in translational regulation of the GnRH receptor.

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APPENDIX A

MEDIA

Luria-Bertani (LB) agar/broth:

agar:

bacto-tryptone	10.0g
bacto-yeast extract	5.0g
NaCl	10.0g
agar	12.0g
H_2O	to one litre

pH 7.0

broth:

bacto-tryptone	10.0g
bacto-yeast extract	5.0g
NaCl	10.0g
H_2O	to one litre

pH 7.0

2 x Yeast Tryptone (2 X YT) broth:

bacto-tryptone	16.0g
bacto-yeast extract	10.0g
NaCl	5.0g
H_2O	to one litre

pH 7.0

ψ broth:

bacto-tryptone	20.0g
bacto-yeast extract	5.0g
$MgSO_4.7H_2O$	4.93g
NaCl	0.58g
KCl	0.37g
H_2O	to one litre

pH 7.0

APPENDIX B

BUFFERS AND SOLUTIONS

Buffers and solutions used for plasmid DNA isolation

Solution 1:

1M Tris-Cl (pH 8.0)	2.5 ml
0.5M EDTA (pH 8.0)	10.0 ml
20% (w/v) Glucose	5.0 ml
H_2O	to 100 ml

Solution 2:

10M NaOH	2.0 ml
25% (w/v) SDS	4.0 ml
H_2O	to 100 ml

Solution 3:

5M Potassium Acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	to 100 ml

TE buffer:

1M Tris-Cl (pH 7.5)	1.0 ml
0.5 M EDTA (pH 8.0)	0.2 ml
H_2O	to 100 ml

Buffers and solutions used for agarose gel electrophoresis

Tris-acetate buffer (50X):

Tris base	242.0 g
0.5M EDTA (pH 8.0)	100.0 ml
glacial acetic acid	57.1 ml
H_2O	to one litre

6X Stop/Loading buffer:

Glycerol	50.0 ml
0.5M EDTA (pH 8.0)	20.0 ml
10% (w/v) SDS	10.0 ml
Bromophenol Blue	0.1 g
Xylene Cyanol	0.1 g
H_2O	to 100 ml

Buffers used for preparation of competent cells

TFBI buffer:

Potassium Acetate	0.294 g
$RbCl_2$	0.121 g
$CaCl_2.2H_2O$	0.147 g
$MnCl_2.4H_2O$	0.990 g
Glycerol	15.0 ml
H_2O	to 100 ml

pH 5.8

TFBII buffer:

MOPS	0.1045 g
$RbCl_2$	0.5520 g
$CaCl_2.2H_2O$	0.0610 g
Glycerol	7.5 ml
H_2O	to 50 ml

pH 6.8

Buffers and solutions used for DNA sequencing

Tris-borate buffer (5X):

Tris base	54.0 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20.0 ml
H_2O	to one litre

6% polyacrylamide gel mix:

acrylamide	5.7 g
bis-acrylamide	0.3 g
urea	42.0 g
5X TBE	20.0 ml
H_2O	to one litre

Buffers and solutions used for mutagenesis

10X PE1 buffer:

1M Tris-Cl (pH 7.5)	5 ml
1M $MgCl_2$	5 ml
5M $NaCl_2$	5 ml
1M DTT	5 ml
H_2O	to 50 ml

10X PE2 buffer:

1M Tris-Cl (pH 7.5)	10.0 ml
1M $MgCl_2$	5.0 ml
1M DTT	5.0 ml
H_2O	to 50 ml

Solutions used for DNA labelling

STE buffer:

1M Tris-Cl (pH 8.0)	10.0 ml
0.5M EDTA (pH 8.0)	0.2 ml
0.5M NaCl	0.02 ml
H_2O	to 100 ml

Buffers and solutions used for nuclear extract preparation

PBS:

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O	to one litre

pH 7.4

TBS:

NaCl	8.0 g
KCl	0.2 g
Tris base	3.0 g
H_2O	to one litre

pH 7.4

Buffer A:

1M HEPES (pH 7.9)	0.5 ml
1M KCl	0.5 ml
0.5M EDTA (pH 8.0)	0.01 ml
0.2M EGTA	0.025 ml
1M DTT	0.05 ml
0.2M PMSF	0.125 ml
H_2O	to 50 ml

Buffer C:

1M HEPES (pH 7.9)	1.0 ml
5M NaCl	4.0 ml
0.5M EDTA (pH 8.0)	0.1 ml
0.2M EGTA	0.25 ml
1M DTT	0.05 ml
0.2M PMSF	0.25 ml
H_2O	to 50 ml

Buffers and solutions used for RNA isolation and Northern Blot Analysis

Solution D:

Guanidinium Thiocyanate	23.63 g
1M Sodium Citrate (pH 7.0)	1.25 ml
10% (w/v) Sarcosyl	2.5 ml
β-mercaptoethanol	0.36 ml
DEPC-treated H_2O	to 50 ml

10X MOPS buffer:

Morpholinopropanolsulfonic acid	41.86 g
Sodium Acetate	4.10 g
EDTA (disodium salt)	3.72 g
DEPC-treated H_2O	to one litre

pH 6.0

20X SSC:

NaCl	175.3 g
Sodium Citrate	88.2 g
H_2O	to one litre

pH 7.0

20X SSPE buffer:

NaCl	175.3 g
NaH2PO4.H2O	27.6 g
EDTA (disodium salt)	7.4 g
H_2O	to one litre

pH 7.4

Buffers and solutions used for cell culture

Buffer I:

NaCl	4.10 g
KCl	0.15 g
HEPES	2.40 g
BSA (fatty acid free)	0.50 g
Glucose	0.75 g
0.5M EDTA (pH 8.0)	2.0 ml
H_2O	to 500 ml

pH 7.4

Trypsin:

For COS-1 cells

EDTA (disodium salt)	0.20 g
NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Glucose	0.20 g
Trypsin	1.25 g
H_2O	to 100ml

pH 7.2

For GT1-7 cells

EDTA (disodium salt)	0.20 g
NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Glucose	0.20 g
Trypsin	2.50 g
H_2O	to 100 ml

pH 7.2

HEPES buffer:

NaCl	0.8 g
HEPES	0.5 g
H_2O	to 50 ml
	pH 7.10

100x PO_4:

Na_2HPO_4	0.50 g
NaH_2PO_4	0.42 g
H_2O	to 50 ml
	pH 7.0

Buffer Z:

Na_2HPO_4	1.70 g
$NaH_2PO_4.2H_2O$	1.25 g
KCl	0.15 g
1M $MgCl_2.6H_2O$	0.2 ml
β-mercaptoethanol	790 µl
H_2O	to 200 ml
	pH 7.0