CHARACTERIZATION OF THE HUMAN GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE AT THE MOLECULAR LEVEL

by

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Date July 31, 1995
ABSTRACT

The gonadotropin-releasing hormone (GnRH) receptor is a plasma membrane associated receptor and a member of the GTP-binding protein coupled receptor family. The interaction of the ligand, GnRH, and the GnRH receptor is a critical event in the endocrine control of reproduction. This coupling stimulates the synthesis and release of both luteinizing hormone and follicle stimulating hormone from the anterior pituitary. In addition, GnRH-GnRH receptor binding acts locally to regulate human chorionic gonadotropin secretion in the placenta and steroidogenesis in the ovary. The objective of this thesis was to isolate and characterize the gene for the GnRH receptor in human. The human GnRH receptor (GnRH-R) gene was isolated from a human genomic library derived from placental tissue. The genomic clones obtained encompassed the entire gene including its coding region (987 bp) as well as substantial 5' and 3' sequences. Sequence analysis revealed a structural organization consisting of three exons and two introns distributed over 18.9 Kb. Exon II contains only 219 bp and the remainder of the approximately 4.7 Kb transcript is distributed between exon I (1915 bp) and III (3321 bp). Sequence analysis and restriction endonuclease mapping revealed the sizes of intron A and B to be approximately 4.2 and 5.0 Kb, respectively. Sequencing of the 5' end of the gene revealed the presence of five consensus TATA
sequences clustered within a 700 nucleotide region. Primer extension analysis detected multiple transcription initiation sites associated with this group of TATA sequences. Transcription of this region up to the most 5' initiation site was demonstrated by the reverse transcription - polymerase chain reaction (RT-PCR) method. The 5' nontranslated region has a length between 703 and 1393 bp, depending on which initiation site is used. Several consensus cis - acting regulatory sequences were identified within the 5' end. These include sites for GATA-1, WAP, PEA-3, AP-1, and Pit-1. In addition, cAMP response element (CRE) - like and glucocorticoid / progesterone response element (GRE / PRE) - like sequences were found. The ability of these response elements to bind to their respective regulatory proteins (CRE binding protein for CRE; progesterone receptor for GRE/ PRE) was investigated by mobility shift assays. No DNA - protein complexes were observed for these response - like elements suggesting that the mismatches incurred could not be recognized by the respective regulatory proteins. The 3' end of the gene was also sequenced and five classical polyadenylation signals were found scattered over a region of 800 nucleotides. RT-PCR conducted on the 3' nontranslated region confirmed transcription up to the most 3' located polyadenylation signal. Factoring in the location of the most 5' initiation site and the most 3' polyadenylation signal, the total transcript covers a region of 5455 bp. The finding of multiple transcription initiation sites and polyadenylation signals raises
the possibility of tissue-specific regulation and the existence of variable transcripts for the human GnRH receptor. Genomic Southern blot analysis indicated the presence of a single copy of the gene encoding for the GnRH-R gene within the human genome. Using somatic hybrid analysis, the GnRH-R gene was also assigned to human chromosome 4. This study represents the first report on the isolation and characterization of the GnRH-R gene in any species. The characterization of the human GnRH-R gene should facilitate future investigative efforts on the delineation of possible genetic disorders for this gene, the mechanisms involved in its regulation, and on generation of improved GnRH analogues currently in use for several reproductive disorders and diseases.
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<th>Full Form</th>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3', 5'-cyclic-monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3', 5'-cyclic-monophosphate</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DEP</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphates</td>
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<tr>
<td>dATP</td>
<td>deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>dl.dC</td>
<td>polydeoxyinosinic acid and polydeoxycytidyllic acid</td>
</tr>
<tr>
<td>(dl.dC)-(dl.dC)</td>
<td>homopolymer of dl.dC</td>
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<tr>
<td>dTTP</td>
<td>deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
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<tr>
<td>ddCTP</td>
<td>dideoxycytidine 5'-triphosphate</td>
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<td>ddGTP</td>
<td>dideoxyguanosine 5'-triphosphate</td>
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<tr>
<td>ddTTP</td>
<td>dideoxythymidine 5'-triphosphate</td>
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<td>deoxyribonuclease</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>acceleration of gravity</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-1-thio-β-D-galactoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>mCi</td>
<td>millicurie</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propane sulfonic acid</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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ng  nanogram
nM  nanomolar
nt  nucleotide
NZY NZ amine/Yeast/NaCl/MgSO₄ media
OD  optical density
PAGE polyacrylamide gel electrophoresis
pcv packed cellular volume
PEG polyethylene glycol
PIPS piperazine-N, N’-bis(2-ethanesulfonic acid)
PMSF phenylmethylsulfonyl fluoride
pnv packed nuclear volume
RNA ribonucleic acid
rpm revolutions per minute
RT-PCR reverse transcription-polymerase chain reaction
SDS sodium dodecyl sulphate
s second(s)
Taq Thermus aquaticus, source of a DNA polymerase
TEMED N, N, N’, N’-tetramethylethlenediamine
Tris tris(hydroxy methyl) aminomethane
tRNA transfer RNA
UV ultraviolet
U unit(s)
vol volume
X-Gal 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
μg microgram
α alpha
β beta
γ gamma
λ lambda
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This thesis is dedicated to my mother and father, two people who have always encouraged me to follow my dreams and who were my source of strength and inspiration throughout the compilation of this work. They, above all, understood the trials and tribulations associated with research and they prepared me well for the challenge. Mom and Dad, this is for you.
I. INTRODUCTION

The gonadotropin - releasing hormone (GnRH) receptor is a member of the GTP - binding (G) protein coupled receptor superfamily. The binding of this receptor to its ligand, GnRH, plays a pivotal role in reproduction. Among its physiological functions, GnRH - receptor coupling stimulates the synthesis and release of gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. In addition, GnRH and GnRH receptors are of importance locally for gonadal steroidogenesis and for the maintenance of pregnancy. Signal transduction pathways for the GnRH receptor are generally of the phosphoinositol - phospholipase C nature (Leung and Steele, 1992).

In terms of the receptor ligand, GnRH, the existence of this neuropeptide was predicted in the 1950s (Harris, 1950), but its structure was not reported until the 1970s (Matsuo et al 1971; Burgus et al., 1972). Since then, concerted efforts have focused on its structural analysis (Sherwood et al., 1993). Comparatively fewer studies were focused on the equally important other half, the receptor. It is therefore not surprising that for the past 20 years, relatively limited information was known about the GnRH receptor. And until the early part of this decade, no
information was available on its molecular biology. However, despite the initially slow start, information on the GnRH receptor, particularly its molecular biology, is growing steadily.

Binding sites for the GnRH receptor have been identified in pituitary for a number of vertebrate species (Naor et al., 1980; Limonta et al., 1986; Peter et al., 1992; Weil et al., 1992; Pal et al., 1992; Schulz et al., 1993) including human (Wormald et al., 1985). Several extrapituitary GnRH receptor binding sites including the brain (Millan et al., 1985), gonads (Clayton et al., 1979; Clayton et al., 1980a; Clayton et al., 1980b; Bourne et al., 1980; Hazum et al., 1982), placenta (Currie et al., 1981; Iwashita et al., 1986), and neoplastic tissues (Lamberts et al., 1982; Eidne et al., 1985; Miller et al., 1985; Pahwa et al., 1989; Fekete et al., 1989; Qayum et al., 1990; Emons et al., 1992) have also been identified.
1.1 Physiological role

The GnRH receptor is an integral plasma membrane protein involved in intercellular and intracellular communication. In essence, the GnRH receptor acts as a link connecting the extracellular world to the internal control centers. The receptor itself accepts specific external cues called the first messenger (ligand; GnRH; agonists; antagonists) and transfers them into uniformly comprehensible signals, known as second messengers. These messages can then be passed onto the nucleus eliciting protein phosphorylation or dephosphorylation and eventually transcriptional activation or inactivation.

The primary physiological response of pituitary GnRH receptor binding to GnRH, a hypothalamic decapeptide, is the release of gonadotropins such as luteinizing hormone and follicle stimulating hormone into the peripheral circulation (Fig. 1). These gonadotropins in turn affect gonadal function. As well, other cellular responses such as biosynthesis of gonadotropins and GnRH receptors, up- and down-regulation of GnRH receptors, and desensitization of gonadotropes are also prominent effects of GnRH receptor activation.

In addition to the pituitary, GnRH via its receptor has been suggested to act
Fig. 1 The hypothalamic-pituitary-gonadal axis.

Schematic representation of the physiological role of GnRH. GnRH = gonadotropin releasing hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone.
hypothalamus

GnRH

pituitary

LH  FSH

gonads

steroids
directly on the ovary in an autocrine and/or paracrine fashion (Peng et al., 1994; Dong et al., 1993). In the ovary, GnRH has both stimulatory and inhibitory actions depending mostly upon experimental conditions and on the maturational stage of the ovarian follicles. Inhibitory effects of GnRH on ovarian function include reductions in gonadotropin receptor biosynthesis, gonadotropin-mediated steroidogenesis, and follicular development. Stimulatory effects of GnRH in the ovary include augmentation of steroidogenic and ovulatory processes.

Further to extrapituitary GnRH receptors, the importance of the GnRH receptor in the placenta cannot be overlooked. GnRH, present in both cyto- and syncytiotrophoblast, is the major candidate for stimulation of human chorionic gonadotropin (hCG) release (Belisle et al., 1984) from the placenta via possible autocrine and/or paracrine mechanisms. Levels of hCG peak at 11 to 14 weeks of gestation and thereafter decline steadily. Placental GnRH mRNA levels do not vary significantly during pregnancy (Lin et al., 1995), suggesting that other factors, including GnRH receptor down-regulation and desensitization, may be involved in the decline of hCG after the first trimester. Functions of hCG include the maintenance of the early corpus luteum to ensure continued progesterone until this function is taken over by the trophoblast. As well, hCG plays a role in relaxin secretion by the ovary, early development of the fetal adrenal gland, and the
regulation of male sexual differentiation (Seron-Ferre et al., 1978; Siler-Khodr, 1983).

1.2 Distribution of the GnRH receptor

1.2.a. Pituitary

Specific high affinity binding sites for GnRH, GnRH agonists, and antagonists have been characterized in the pituitaries of several species (Naor et al., 1980; Limonta et al., 1986; Peter et al., 1992; Weil et al., 1992; Pal et al., 1992; Schulz et al., 1993) including human (Wormald et al., 1985). Among the anterior pituitary cell types, sublocalization of the GnRH receptor to the gonadotrope is prevalent for all species studied. For some species, the gonadotrope is the exclusive pituitary cell locale for the receptor (Hyde et al., 1982). However this is not necessarily the case for all species. In species, such as the goldfish, GnRH has also been shown through electron microscopy studies to bind directly to somatotropes (Cook et al., 1991) and elicit GH release (Chang et al., 1990). However in general, pituitary GnRH receptors for varying species were found to share widely similar properties. As demonstrated by photoaffinity labelling, all possessed a common theme of two
subunit components for the GnRH receptor.

1.2.b. Brain

GnRH receptor binding sites have been shown to exist in several areas of the brain. Localization of GnRH receptors to the lateral septal nucleus, anterior cingulate cortex, subiculum, entorhinal cortex, and hippocampus are among the identified regions (Millan et al., 1985).

1.2.c. Gonads

Outside of the central nervous system, several reproductive tissues have demonstrated expression of the GnRH receptor. In the ovary, the GnRH receptor has been identified in granulosa and luteal cells using mainly photoaffinity labelling studies (Clayton et al., 1979; Hazum et al., 1982). Recently, using RT-PCR analysis the GnRH receptor has also been identified in the rat ovary (Moumni et al., 1994) and in human preovulatory granulosa cells (Peng et al., 1994). In testicular tissue, receptors have been localized to the Leydig cells, but not to the Sertoli cells
Photolabelled GnRH receptors in the rat gonads were found to be similar to those of the rat pituitary gland, involving two distinct subunits of 43 and 53 kilodaltons (kDa) (Iwashita and Catt, 1985). Additionally, sequencing of the rat (Olofsson et al., 1994) and human (Peng et al., 1994) ovarian GnRH-R cDNAs indicated that they were identical to the pituitary GnRH-R cDNA sequences. With identical primary structures revealed, these data further support that the rat and human ovarian GnRH receptors are identical to their pituitary counterparts.

1.2.d. Placenta

The placenta contains low-affinity GnRH binding sites distinct from the pituitary GnRH receptors (Currie et al., 1981; Iwashita et al., 1986). There remain some similarities however, in the binding subunit itself which suggests that the placenta receptor may likely be a variant of the pituitary GnRH receptor.
1.2.e. Neoplastic tissues and cell lines

GnRH receptors have been identified in pituitary adenomas (Snyder, 1985), human breast cancer tissue (Eidne et al., 1985; Miller et al., 1985), human epithelial ovarian carcinomas (Lamberts et al., 1982; Pahwa et al., 1989; Emons et al., 1992), and prostate tumours (Fekete et al., 1989; Qayum et al., 1990).

Additionally, GnRH binding sites have been identified in immortalized α-T3 gonadotropes (Horn et al., 1991), human breast cancer cell lines (Eidne et al., 1987; Kakar et al., 1994), human endometrial cancer cell lines (Emons et al., 1993), and in human ovarian cancer cell lines (Kakar et al., 1994). Ovarian cancer GnRH receptors are very similar to other extrapituitary GnRH binding sites of the low affinity and high capacity type (Emons et al., 1992). Antiproliferative effects of GnRH analogues on ovarian tumour cells (Emons et al., 1993), prostate cancers, and on non-neoplastic prostatic tissue (Fekete et al., 1989; Qayum et al., 1990; Janaky et al., 1992) have been observed.
1.3 Regulation of the GnRH receptor

Regulation of GnRH receptor levels in the pituitary gland has been well characterized during various physiological conditions. Ontogenic experiments have demonstrated changes in GnRH receptor level with developmental stage (Chan et al., 1981). Estrous cycle experiments demonstrated maximum numbers of GnRH receptors during the proestrous period prior to the onset of the preovulatory surge of luteinizing hormone (LH) (Clayton et al., 1980b; Crowder et al., 1984; Nett et al., 1987). These levels were sustained for several hours after the surge, followed by a marked decrease in receptor levels at metestrus, and a subsequent slight increase in receptor numbers during the day of estrus (Bauer-Dantoin et al., 1993). In contrast, during pregnancy and lactation, lower levels of GnRH receptors have been observed than during the estrous cycle. Further physiological studies involving castration and hypothalamic lesions have demonstrated GnRH receptor number up-regulation (Marian et al., 1981). Specifically, pituitary GnRH-R mRNA levels increased several fold in ovariectomized female and orchidectomized male rats (Kaiser et al., 1993) as well as in castrated sheep (Illing et al., 1993). These studies clearly indicate the regulation of GnRH receptors in vivo and point towards potential endocrine factors such as ovarian steroid hormones and protein hormones, including GnRH, as potential regulators of the GnRH receptor.
The ability of GnRH to regulate the expression of its own receptor in the pituitary has been well documented. In *in vitro* studies, treatment of pituitary cell cultures with physiological concentrations of GnRH have resulted in a biphasic change in GnRH receptor numbers (Conn et al., 1984). Initially, receptor activation leads to a down-regulation of receptors for GnRH (< 4 h post-treatment) which is subsequently followed by an increase in the number of GnRH receptors (9 h post-treatment). The initial down-regulation of receptors is reflective of desensitization of gonadotropes to GnRH resulting from uncoupling of receptors to second messenger systems. Mechanisms underlying homologous up- and down-regulation appear to be independently regulated.

Down-regulation of GnRH receptors occurs within a time frame of 3 to 4 h after GnRH treatment and appears to be independent of extracellular calcium. Supporting studies which raised intracellular calcium or chelated extracellular calcium (Conn et al., 1984) have and have not resulted, respectively, in receptor down-regulation. Up-regulation of GnRH receptors, on the other hand, occurs several hours following down-regulation. Increasing the numbers of GnRH receptor is dependent upon extracellular calcium and can be stimulated by compounds that raise intracellular calcium (Young et al., 1985). As well, receptor up-regulation requires that intact protein synthesis and microtubular function takes
place (Conn et al., 1984; Young et al., 1984). The mechanisms of up- and down-regulation of GnRH receptors are complex and data indicate that these processes occur through different pathways. However, for both cases, occupancy of the GnRH receptor alone is not responsible for induction of receptor up- or down-regulation.

GnRH mediated GnRH receptor up-regulation can be mimicked by treatment with adenosine 3', 5'-monophosphate analogues as well as by depolarization of pituitary cells with KCl (Young et al., 1984) or calcium ionophore, A23187 (Young et al., 1985). More recent studies on the homologous regulation of the GnRH receptor mRNA in rat pituitary cells parallel previous findings on the ability of GnRH to regulate its own receptor (Kaiser et al., 1993). Continuous stimulation by GnRH resulted in no observable change in receptor mRNA, while pulsatile administration of GnRH increased levels of receptor mRNA.

In addition to the regulation of the GnRH receptor by its own ligand, GnRH, several other factors may be involved in receptor regulation as well. In particular, gonadal steroids assume a likely role. Studies involving estradiol and testosterone replacement in ovariectomized female and orchidectomized male rats, respectively, have resulted in decreased receptor mRNA levels for both cases (Kaiser et al., 1993).
Similarly, in the absence of intact hypothalamic-pituitary connections, treatment with 17β-estradiol has been shown to increase GnRH receptor levels (Gregg and Nett, 1989). Further to gonadal steroids, treatment of sheep pituitary cell cultures with estradiol (Laws et al., 1990b; Sealfon et al., 1990) and progesterone (Laws et al., 1990a; Sealfon et al., 1990) could increase and decrease, respectively, the numbers of GnRH receptors. Treatment with inhibin has also resulted in a significant increase in GnRH receptor levels (Laws et al., 1990a; Sealfon et al., 1990). Estradiol and inhibin treatments performed in combination additively increased GnRH-R mRNAs to a higher extent than with each treatment alone. Similar results were obtained when RNA levels were measured by Northern blots (Miller et al., 1993).

However, in direct contrast to the above studies, Wang et al. (1988) have demonstrated a decreased GnRH receptor number in rat pituitary cell cultures treated with inhibin. This decrease in receptor number was in part due to the ability of inhibin to antagonize GnRH-stimulated synthesis of GnRH receptors (Braden et al., 1990). It is clear that further detailed studies will be necessary in order to clarify the role of inhibin in the regulation of GnRH receptor numbers.
1.4 Activation of the GnRH receptor and signal transduction

The GnRH receptor is a GTP-binding (G) protein coupled receptor and functions in calcium mobilization. Activation depends largely upon reversible binding of the ligand, GnRH. Signal transduction (Fig. 2) across the plasma membrane is dependent upon three proteins: the receptor, G protein, and the effector(s). In the case of GnRH, the effectors are enzymes involved in second messenger production and calcium ion channels. The first step in the mechanism of action of GnRH receptor involves ligand binding. Following binding of the agonist, GnRH receptors aggregate and become internalized. After internalization, the receptors become associated with lysosomes to undergo a degradation pathway and/or become associated with the Golgi apparatus and LH granules to undergo receptor recycling (Hazum and Conn, 1988). As well, binding of GnRH to a pocket formed by the transmembrane regions of the GnRH receptor leads to conformation changes in the receptor. These changes allow the receptor to associate with heterotrimeric (α, β, and γ subunits) G protein(s) and mediate the stimulatory actions of GnRH (Andrews et al., 1986; Perrin et al., 1989; Hawes et al., 1992; Stojilkovic et al., 1993).

All of the three subunits of heterotrimeric G proteins share structural and
Fig. 2 Signal transduction mechanism of the GnRH receptor.

DG = Diacylglycerol; IP$_3$ = Inositol 1, 4, 5-triphosphate; PIP$_2$ = Phosphoinositol 4, 5-bisphosphate; PLC = Phospholipase C; PKC = Protein Kinase C; PLA$_2$ = Phospholipase A$_2$; GTP = guanosine 5’ triphosphate; GDP = guanosine 5’ diphosphate.
functional diversity. Studies have revealed that the $\alpha$, $\beta$, and $\gamma$ subunits are encoded by at least 15, 4, and 8 genes, respectively. Although the major phospholipase C (PLC) activating component is the $\alpha$ subunit in most tissues, $\beta\gamma$ subunits have also been shown to be involved in PLC activation (Spiegel et al., 1992). In general though, the G proteins have been divided into two distinct classes: pertussis toxin sensitive and pertussis toxin insensitive. Those that are categorized under pertussis toxin sensitive include $G_{t1}$ (t=transducin), $G_{i2}$, $G_{i1}$ (i=inhibitory), $G_{i2}$, $G_{i3}$, and $G_o$ and have cGMP - phosphodiesterase, adenylate cyclase, $K^+$ and $Ca^{2+}$ channels, and $Ca^{2+}$ mobilizing receptors as their possible effectors. Pertussis toxin insensitive G proteins include $G_s$ (s=stimulatory), $G_{olf}$ (olfactory), $G_q$ (q=members of a ubiquitously distributed family of G proteins), $G_{11}$, $G_{12}$, $G_{13}$, $G_{14}$, and $G_{15/16}$ (G protein subtype) and have $\beta1$- and $\beta2$-PLC as well as adenylate cyclase as their effectors (Bourne et al., 1991). Studies have indicated that members of the pertussis toxin insensitive class of G proteins mediate PLC activity via GnRH in pituitary gonadotropes (Hsieh et al., 1992).

Once the activated receptor has contacted the G protein, this results in the release of $\alpha$ subunit bound GDP in exchange for GTP. This exchange is permitted due to the fact that GTP is present in the cell at higher concentrations than is GDP. Dissociation of the G protein into $\alpha$ and $\beta\gamma$ subunits leads to the activation of the $\beta$ -
forms of PLC (Spiegel et al., 1992). Activation of PLC leads to the cleavage of membrane phospholipids: phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4, 5-phosphate (PIP$_2$) into inositol 1, 4, 5-triphosphate (IP$_3$) and diacylglycerol (DAG). PIP$_2$ has been identified as the major substrate during GnRH stimulated activation of PLC in gonadotropes (Morgan et al., 1987).

IP$_3$ is the major second messenger molecule involved in calcium mobilization and represents both an early and sustained response to GnRH stimulation (Guillemette et al., 1987). GnRH stimulates a quick rise in intracellular [Ca$^{2+}$], followed by a smaller but sustained level that is in agreement with IP$_3$ release (Izumi et al., 1989). The initial peak of Ca$^{2+}$ is independent of extracellular Ca$^{2+}$, while the later sustained peak is dependent on entry of Ca$^{2+}$ through voltage sensitive and insensitive Ca$^{2+}$ channels (Virmani et al., 1990). Thus the early peak of Ca$^{2+}$ is reflective of Ca$^{2+}$ released from intracellular stores, as a result of IP$_3$ mediated Ca$^{2+}$ release from the endoplasmic reticulum. The sustained phase supports direct IP$_3$ mediated Ca$^{2+}$ entry into gonadotropes.

IP$_3$ is a comparatively more hydrophilic molecule than DAG and diffuses into the cytoplasm where it elicits the release of calcium from intracellular stores distinct
from the mitochondria. Further support for IP₃ as a calcium mobilizer was obtained in patch clamp studies involving single gonadotropes being injected with IP₃ (Tse and Hille, 1992). Catabolic routes for the termination of this second messenger are via the 5-phosphate pathway where IP₃ is dephosphorylated to inositol 1, 4-bisphosphate, inositol 4-phosphate, and inositol. Also catabolism via the 3-kinase route to inositol 1, 3, 4, 5-tetraphosphate and subsequent dephosphorylation to inositol 1, 3, 4-triphosphate has been demonstrated (Zheng, 1993).

Several lines of evidence support the idea that calcium functions as a second messenger in the acute release of LH in response to GnRH. First, when extracellular calcium is blocked, GnRH-induced LH release is inhibited (Stern and Conn, 1981; Conn et al., 1979). However when Ca²⁺ replacement occurred, LH release was re-established (Bates and Conn, 1984). Second, agents that increase intracellular Ca²⁺, specifically, also stimulate the release of LH. Third, GnRH-induced LH release, caused a measurable increase in intracellular Ca²⁺ levels. Finally, treatment of pituitary cells with Ca²⁺ channel blockers such as verapamil or methoxyverapamil blocked stimulated LH release (Conn et al., 1983), whereas utilization of a calcium channel agonist, maitotoxin, resulted in LH release (Conn et al., 1987).
Once intracellular Ca$^{2+}$ concentrations are elevated through mobilization of intracellular stores and extracellular sources, several responses take place. Calcium binds to Ca$^{2+}$ binding proteins such as calmodulin and protein kinase C (PKC) resulting in protein phosphorylation or dephosphorylation. In addition, several reports have eluded to phospholipase D (PLD) as a Ca$^{2+}$ sensitive enzyme, suggesting that Ca$^{2+}$ may have a direct role in activating PLD as well.

In addition to IP$_3$ and Ca$^{2+}$, DAG is the third potential second messenger and the other product of the PLC reaction. This second messenger functions to activate protein kinase C directly. Characteristically, DAG is a hydrophobic molecule that tends to remain within the phospholipid bilayer of the plasma membrane. It is from this locale that DAG elicits many of its GnRH stimulated responses. Initially, DAG production temporally parallels the formation of IP$_3$. The early rise in DAG is followed by a slow sustained phase of DAG production. However, the sustained phase is several magnitudes higher than IP$_3$ levels. These data suggest that other sources of DAG may be involved, including phosphatidylcholine (PC). PC is hydrolyzed readily by PLC and PLD (Billah and Anthes, 1990) into choline and phosphatidic acid (PA; interconverts with DAG). Also, receptor - coupled activation of PLD gives rise to DAG formation and may play a role in sustaining the activation of PKC.
In addition to being metabolized by PLC and PLD, PC is also hydrolyzed by phospholipase A$_2$ (PLA$_2$). A PA-specific PLA$_2$ that utilizes PA in the arachidonic acid (AA) cascade has also been associated with receptor-mediated cell activation (Axelrod et al., 1988). Turnover of DAG is rapidly achieved by conversion to PA and subsequently to PI (Nishizuka, 1992).

All three of these second messengers converge to activate PKC. The PKC family of proteins are single polypeptides consisting of a regulatory and a kinase domain. There exists at least 10 closely related isozymes of PKC (Huang and Huang, 1993). Compounds such as Ca$^{2+}$, DAG, AA, phosphatidylinositol phosphates, and phosphatidylserine for example can unmask the catalytic domain of these enzymes and cause activation. Activation of PKC results in the production of cAMP from ATP which can then proceed to influence several well documented cellular responses, including phosphorylation. In addition, a role of PKC in the control of exocytosis has also been documented (Stojilkovic and Catt, 1992). Generally speaking, PKCs are proteins that are involved in the control of cellular growth and differentiation. De-regulation of these proteins can lead to detrimental effects and eventual cell death.

In terms of cyclic nucleotide signalling in GnRH-mediated action, early
studies (Naor et al., 1975; Sundberg et al., 1976) have shown that under certain conditions, GnRH can induce a slight increase in both cAMP and cGMP. More recent studies have also suggested a role for cGMP on the regulation of GnRH-stimulated phosphoinositide turnover (Naor, 1990). However, in direct contrast, studies by Conn et al. (Conn et al., 1979) have shown that cyclic nucleotides are not involved in GnRH-stimulated LH release and it is generally accepted that cAMP is not a second messenger for GnRH. More detailed studies involving cyclic nucleotides may be necessary to end this debate.
1.5 Molecular biology of the GnRH receptor

1.5.a. General

Until the early part of this decade, there was very limited information concerning the molecular biology of the GnRH receptor. Isolation of the cDNA or gene had not been accomplished as yet in any species. Chromosomal assignment of the gene for the GnRH receptor as well as basic structural data on the receptor at any level was lacking. However, data on the isolation and sequencing of the GnRH-R cDNA in several species were to follow. The cloning of the GnRH-R cDNA was to be preceded by expressional studies using *Xenopus laevis* oocytes injected with pituitary RNA (Yoshida et al., 1989; Sealfon et al., 1990). These studies served two purposes. The first was the necessary confirmation of the presence of RNA encoding for the GnRH receptor and the second was the identification of a readily available source of mRNA for construction of cDNA libraries. The isolation of the first GnRH-R cDNA clone was derived from screening of an α-T3 (mouse) derived cDNA library. RNA produced from the mouse cDNA clone was demonstrated to encode for a functional receptor when expressed in *Xenopus* oocytes (Tsutsumi et al., 1992). Isolation of the mouse GnRH receptor cDNA (Reinhart et al., 1992; Tsutsumi et al., 1992; Perrin et al., 1993) has lead to the cloning of the cDNA for
the rat (Eidne et al., 1992; Kaiser et al., 1993; Kakar et al., 1994) GnRH receptor. More recently, cDNAs for sheep (Brooks et al., 1993; Illing et al., 1993), bovine (Kakar et al., 1993), pig, and human (Kakar et al., 1992; Chi et al., 1993) GnRH receptors have been isolated.

1.5.b. Structure

Data indicate that GnRH receptors are members of the G protein coupled receptor superfamily. These receptors consist of a single polypeptide chain containing seven hydrophobic transmembrane domains (TM) as indicated through hydropathy plots (Fig. 3). These hydrophobic regions are joined to hydrophilic extracellular and intracellular loops (E1, E2, E3; I1, I2, I3). Analysis of three dimensional conformations for G protein coupled receptors indicate that the TM domains are predominantly α-helical in nature and arranged so as to form a central hydrophilic ligand binding pocket (Baldwin, 1993). Potential N-linked glycosylation sites are present in the extracellular domain. Potential sites for phosphorylation by cAMP-dependent protein kinases and PKC are located within the first and third cytoplasmic domains.
Fig. 3 Structure of the GnRH receptor.

Amino acid sequence of the GnRH receptor (mouse; Perrin et al., 1993; Reinhart et al., 1992; Tsutsumi et al., 1992) is represented in the single letter amino acid code. Amino acids are numbered as shown and the transmembrane domains are boxed.
A highly conserved tripeptide sequence located at the junction between the end of the third transmembrane domain and the beginning of the second cytoplasmic domain is implicated in G protein coupling. This conserved sequence consists of an Asp-Arg-Tyr triplet which is found in many G protein coupled receptors to date. Cytoplasmic carboxyterminal tails have been described for all G protein receptors characterized to date. This region has been linked to the coupling of G proteins, desensitization, and internalization of G protein coupled receptors (Dohlman et al., 1991). Preliminary data on mouse and rat GnRH receptors indicate complete absence of this carboxyterminal region.

1.5.c. Interspecies relationship at the mRNA level

Utilization of molecular biology techniques has lead to the examination of mRNA levels encoding for the GnRH receptor across various species. Northern analyses of α-T3-1 and mouse pituitary mRNA have identified GnRH-R transcripts of sizes 3.5 and 1.6 Kb (Tsutsumi et al., 1992; Reinhart et al., 1992; Kaiser et al., 1992). Similarly for the rat, studies using Northern analyses and reverse transcription - polymerase chain reaction (RT-PCR) (Perrin et al., 1993) have identified a major transcript ranging in size from 4.5 (Reinhart et al., 1992) to 5-
5.5 Kb (Kaiser et al., 1992) as well as a smaller 1.8 Kb transcript. Multiple transcripts for the sheep have also been identified. Sizes of 5.4 - 6, 3.6 - 4, 2.3, and 1.3 Kb were reported (Brooks et al., 1993; Illing et al., 1993). Northern analysis of bovine pituitary RNA revealed the presence of four different transcripts (5.0, 3.5, 2.5, and 1.5 Kb), of which the 5.0 Kb was the most abundant (Kakar et al., 1993). Northern analyses conducted on human pituitary RNA have detected a single mRNA of 4.7 - 5.0 Kb (Chi et al., 1993; Zhou et al., 1994) encoding for the GnRH receptor.

Northern analyses performed on rat extrapituitary tissues such as ovary, Leydig cell, and testis have identified the GnRH receptor transcripts as well (Reinhart et al., 1992; Kaiser et al., 1992). No transcripts were observed using Northernns for rat placenta however (Reinhart et al., 1992; Kaiser et al., 1992). In situ hybridization studies have also identified receptor mRNA in the rat hippocampus and hypothalamus (Jennes and Wright, 1993). Northern analyses performed on human testis, prostate, placenta, breast, and ovary did not detect any GnRH-R mRNA (Reinhart et al., 1992; Kakar et al., 1992; Chi et al., 1993). More sensitive analysis through RT-PCR was necessary in order to detect mRNAs from human placenta, testis, prostate, and breast tissue (Kakar et al., 1994).
1.6 Binding characteristics

The cloned mouse GnRH receptor (receptor expressed from cloned cDNA) has dissociation constants which are similar to those of its native receptor (Kd) values of 0.5 nM and 0.5 to 2.9 nM in pituitary and α-T3 cells, respectively. Rat, sheep, and human cloned GnRH receptors possess Kd values in the order of 0.6 nM, 0.13 nM, and 0.9 nM when measured with agonist, des-Gly10-[D-Ala6]N-GnRH-N-ethylamide. Additionally, for sheep and human cloned GnRH receptors, Kd values of 4.9 nM and 2.8 nM were measured with GnRH. These Kd values are similar to those of the native receptor for rat (0.2 nM), sheep (0.3 nM, agonist; 4.1 nM, GnRH), and human (0.32 nM, agonist; 4.8 nM, GnRH) as measured in pituitary cells.
1.7 Clinical applications

Several important clinical applications involving the GnRH receptor have been described mainly through pharmacological studies. This involvement of the GnRH receptor in treatment is based on the knowledge that the administration of both GnRH agonists and antagonists results in the effective suppression of gonadotropin output. This desired effect is of importance in various therapeutic applications such as in the treatment of precocious puberty, endometriosis, hormone-dependent neoplasia (breast and prostate), hirsutism, and uterine leiomyomata. As well, potential use of GnRH analogues as contraceptive agents are being currently researched. On the other hand, stimulation of GnRH mediated actions has also led to the successful treatment of hypogonadotropic hypogonadism and infertility (Millar et al., 1987). These studies indicate that detailed molecular information regarding GnRH-GnRH receptor coupling is of particular value in the improvement of GnRH analogue based treatment.
1.8 Objective

Understanding the molecular biology of the GnRH receptor is currently of major interest to many reproductive endocrinologists, molecular biologists, and to all those who remain interested in understanding G protein mediated signal transduction. The importance of GnRH - GnRH receptor coupling has been well characterized as the neuroendocrine control of reproduction and is required for normal reproductive function. Reproductive roles in addition to LH and FSH synthesis and secretion include proper maintenance of pregnancy and gonadal function as well as use in clinical treatment of certain reproductive diseases and neoplasia.

The recent increase in GnRH receptor related studies is evidenced by the body of work that has surrounded the GnRH receptor of late. Molecular cloning of the rat and mouse GnRH-R cDNAs were reported by various groups and coincided with the initiation of this project. However, no reports had ever been published on the gene encoding for these receptors in any species. By the time this thesis was completed, additional GnRH-R cDNAs had been isolated in sheep, cow, pig, and human. As well the gene encoding for the mouse GnRH receptor was subsequently reported in addition to various regulatory studies on the GnRH receptor at the
mRNA level.

This investigation was primarily concerned with the isolation and characterization of the gene encoding for the human GnRH receptor. As mentioned this is as yet unchartered territory for the GnRH receptor. The results of this present investigation delineate the organization of the human GnRH-R gene in terms of structure, copy number, and location in the genome. Additionally, the findings of this thesis contribute to the better understanding of the mechanistic action and the regulatory control of the GnRH receptor.
II. MATERIALS AND METHODS

2.1 Genomic DNA library screening

2.1.a. Preparation of filters

A human genomic placental library (Stratagene, La Jolla, CA), constructed in λFIX II vector - Xho I site, was plated at a density of 20,000 to 30,000 plaque forming units (pfu) per 150 mm petri dish plate on host strain E. coli SRB(P2). Approximately 2 x 10^6 plaques were screened in the isolation of the hGnRH-R gene.

The procedure for screening was conducted according to standard protocols (Ausubel et al., 1992). Briefly, NZY broth supplemented with 0.02 % maltose was inoculated with a single colony of SRB(P2) and grown to saturation at 37 °C under conditions of good aeration (shaker, 275 rpm). A mixture of 600 μl of host cells and approximately 25,000 to 30,000 phages (100 μl of a 10^4 dilution of a 2.2 x 10^10 pfu / ml library) was incubated for 20 min at 37 °C. Ten ml of 0.7 % NZY top agar were added and the mixture was poured onto NZY agar plates. Incubation periods of 7 to 8 h at 37 °C followed. Plates were collected and placed at 4 °C for
at least 1 h before applying filters.

Plaque lifting was conducted with nylon filters which were labelled and placed face down on plates for 1 min. Orientations of the filters were marked using an 18-gauge needle and subsequently lifted from the plates and placed face up in denaturing solution (0.2 M NaOH / 1.5 M NaCl) for 2 min. Filters were then neutralized in 1.5 M NaCl, 0.4 M Tris-Cl, pH 7.6 for 5 min, washed in 2X SSC (20X SSC stock: 3M NaCl and 0.3 M Na₃citrate·2H₂O, pH 7.0) for 30 s, and placed on filter paper to dry. After drying, filters were wrapped in clear plastic and placed under UV light for 2 min on each side for DNA cross-linking. At this point filters were subjected to hybridization with a partial human GnRH-R cDNA probe (760 bp; +904 to +1663; Peng et al., 1994) and autoradiography with intensifying screens (overnight exposure).

2.1.b. Preparation of radioactive cDNA probes

Recombinant plasmids were constructed and digested with appropriate restriction endonucleases and the inserts were subsequently isolated by 5 % polyacrylamide gel electrophoresis (PAGE). Bands containing target DNAs were
Preparation of DNA (25 to 50 ng) probes was performed using a Random Primers DNA Labelling Kit (Gibco - BRL, Burlington, Ontario, Canada). Labelling of target DNAs was done in accordance to instructions supplied by the manufacturer. DNA was denatured at 100 °C for 5 min and placed on ice. Two µl each of the following deoxyribonucleoside triphosphates: dATP, dGTP, and dTTP were added. Also 15 µl of random priming buffer, 50 µCi of α[32P]dCTP (3000 Ci / mmol; Amersham, Oakville, ON, Canada), and 3 U of Klenow fragment were added. After an incubation period of 2 h at 25°C, the reaction mixture was denatured at 100 °C for 5 min prior to hybridization. The specific activity of probes obtained was 10^8 cpm / µg.

2.1.c. Hybridization with radioactive probes / Washing

Filters were incubated in prehybridization solution (50 % formamide, 5X Denhardtts [1X Denhardtts solution: 0.02 % ficoll, 0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolidone], and 5X SSPE [1X SSPE: 3 M NaCl, 200 mM Na₃PO₄, 20 mM EDTA, pH 7.4, 0.1 % SDS, and 0.1 mg / ml denatured salmon
sperm DNA) for 1 to 2 h at 42 °C. Hybridization with radioactive probes was performed overnight at 42 °C in a hybridization oven.

Filters were washed once with 1X SSC (20X SSC stock: 3M NaCl and 0.3 M Na₃citrate·2H₂O, pH 7.0) / 0.1 % SDS for 20 min at 42 °C and at least twice with 0.1X SSC / 0.1 % SDS at 52° to 65 °C for 15 min. After washing, filters were mounted wet in plastic wrap for autoradiography with Kodak XAR5 film and intensifying screens at -70 °C for overnight exposure.

2.1.d. Screening

Positive plaques were identified by aligning primary library filters and plates with their respective autoradiograms using needle punctures as reference points. A circular agar plug containing the positive plaque was removed and placed in phage dilution buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM MgSO₄) for at least 5 h. Positive plaques were replated at a density of 500 pfu per 90 mm plate and subsequent 2° and 3° screening followed. Third round positive plaques were purified and single isolates for each positive were replated and kept as stock for future analysis.
2.1.e. Preparation of liquid lysate

A concentrated phage lysate was made for each positive plaque and processed using the following protocol. NZY broth supplemented with 0.02% maltose was inoculated with E. coli SRB(P2) and grown overnight at 37 °C. A single plaque was added to 10 ml of NZY broth containing 10 mM MgSO₄ and 0.1 ml of overnight SRB(P2) cells. This mixture was shaken vigorously at 37 °C until lysis occurred (6 to 8 h). After lysis, 0.1 ml of chloroform was added to lyse any remaining cells and incubated for 1 min. Centrifugation at 3000 x g at room temperature for 10 min followed. The supernatant was retained and 0.1 ml of 1 M MgSO₄ was added to 10 ml of lysate. Lysates were processed immediately to isolate lambda DNA. Ample quantities (several micrograms) of lambda DNA were prepared from an aliquot of the lysate for future restriction analysis.

2.1.f. Preparation of lambda DNA from phage lysates

Lysate was transferred to a corex tube and the following were added: 10 ml of TM buffer (50 mM Tris-HCl, pH 7.4 / 10 mM MgSO₄), 10 μl of 5 mg / ml DNase, and 20 μl of 10 mg / ml RNase. Lysate was incubated for 1 h at 37 °C.
Thereafter 2 ml of 5 M NaCl and 2.2 g of solid PEG-6000 were added and the mixture was further incubated for 1 h on ice. Recovery of the phage DNA pellet was obtained after centrifugation at 10,000 x g for 10 min at 4 °C. The pellet was resuspended in 1 ml of TM buffer, subjected to two phenol / chloroform extractions, one chloroform / isoamyl alcohol (24:1) extraction, and an isopropanol precipitation.

2.2 Cloning of DNA

Four prominent genomic DNA segments, encompassing the entire span of the human GnRH-R gene, were cloned into pUC19 vector. Three EcoRI genomic fragments of sizes 7.7, 7.4, and 3.8 Kb were cloned in addition to a 2.7 Kb HindIII fragment.

2.2.a. Competent cells

Cells were made competent using the calcium chloride / rubidium procedure (Ausubel et al., 1991). An overnight culture of E. coli DH5α was prepared and a
portion (1 ml) added to 250 ml of LB broth. The culture was incubated at 37 °C, while shaking at 275 rpm. Cell density was monitored using a spectrophotometer and allowed to reach an O.D.₆₀₀ of 0.5 (2 to 3 h). Cells were harvested by centrifugation at 4,000 x g for 10 min at 4 °C and resuspended in 125 ml of solution A (10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0 / 10 mM RbCl). Cells were centrifuged again, resuspended in 125 ml of solution B (100 mM MOPS, pH 6.5, 50 mM CaCl₂, and 10 mM RbCl), and incubated on ice for 15 min. Cells were resuspended in solution B to a final volume of 25 ml with 15 % (v / v) glycerol. Competent cells were frozen in 0.1 ml aliquots using an ethanol / dry ice bath and stored at -70 °C.

2.2.b. Ligation

Target DNAs were cleaved to completion with the appropriate restriction endonuclease(s). Isolation of the target DNA by agarose gel electrophoresis or PAGE and subsequent purification of the DNA followed. Vectors were prepared and digested with the same restriction endonuclease(s) as the target DNA. Ligation reactions consisted of a 1 : 10 ratio of vector to insert and 20 U of T4 DNA ligase in a final volume of 40 µl. The ligation mixture was incubated overnight at 15 °C.
prior to *E. coli* DH5α transformation.

### 2.2.c. Transformation

A mixture of 0.1 ml of competent cells with 1 - 200 ng of the ligation reaction were combined and incubated on ice for 30 min. Several controls were also implemented as well. Positive and negative controls consisted of competent cells plus recombinant plasmid and competent cells without DNA or with non-recombinant plasmid, respectively. Following the ice bath incubation, the reaction was heat shocked for 1 min at 42 °C, 1 ml of LB broth was added, and the mixture was incubated for an additional 1 h at 37 °C. The ligation mixes were then plated on LB - ampicillin plates supplemented with X - Gal (40 µl of 20 mg / ml stock solution) and IPTG (40 µl of a 100 mM stock solution). Plates were incubated overnight at 37 °C and positive colonies were identified by the blue (negative) - white (positive) selection pattern. Confirmation of positive colonies was achieved by minipreparations of plasmid DNA using the alkaline lysis procedure, restriction digests, and Southern analysis. Subsequent large scale preparations of recombinant plasmid DNA were conducted for each positive clone of interest.
2.3 DNA sequencing

2.3.a. General

DNA fragments were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using the T7 Sequencing Kit (Pharmacia, Vancouver, B.C., Canada) and conducted as per manufacturer's instructions with slight modifications. All sequencing was performed using both M13 universal forward and reverse primers as well as sequence specific oligonucleotides. Analysis of the nucleotide sequences was conducted with the assistance of a program from the Genetics Computer Group Inc. (Madison, WI).

2.3.b. Preparation of template

Double-stranded DNA templates were denatured by alkali treatment prior to sequencing. Approximately 2 μg of each template DNA were combined with 2 μl of 2 M NaOH / 2 mM EDTA in a reaction volume of 10 μl. Following an incubation period of 10 min at room temperature, a sodium acetate / ethanol precipitation (7 μl of H₂O, 3 μl of 3 M sodium acetate, pH 4.8, and 60 μl of 95 %
ethanol) was performed. Precipitated DNA was collected by centrifugation at 14,000 rpm for 10 min, washed briefly with 70% ethanol, and dried in a speedvac evaporator.

2.3.c. Annealing of primer

Each denatured DNA template was resuspended in 10 μl of H₂O, combined with 2 μl of primer (50 ng/μl), and 2 μl of annealing buffer (1 M Tris-HCl pH 7.6, 100 mM MgCl₂, and 160 mM DTT). The reaction was incubated for 20 min at 37 °C and allowed to cool to room temperature (10 min) in order to promote annealing.

2.3.d. Labelling reaction

After the annealing reaction, 3 μl of Labelling Mix-dATP, 10 μCi of [α-³⁵S]dATP, and 2 μl of diluted T7 DNA Polymerase (3 U) were added and incubated for a further 5 min at room temperature.
2.3.e. Termination reaction

Four sequencing mixes, one for each nucleotide, were prepared. Into each "A" tube, 2.5 μl of A-Mix (840 μM each dCTP, dGTP, and dTTP; 93.5 μM dATP; 14 μM ddATP; 40 mM Tris-HCl pH 7.6, and 50 mM NaCl) were dispensed. Consequently for the other nucleotides, 2.5 μl of C-Mix, G-Mix, and T-Mix (identical components as A-mix, except 14 μM of ddATP is replaced by 14 μM of the respective nucleotide as identifiably labelled) were dispensed into their respective tubes. The four sequencing mixes were pre-warmed for at least 1 min before addition of 4.5 μl of the labelling reaction to each mix. An incubation period for 5 min at 37 °C followed. Reactions were stopped with 5 μl of Stop Solution (0.3 % each Bromophenol Blue and Xylene Cyanol FF; 10 mM EDTA pH 7.5, and 97.5 % deionized formamide). Each stopped reaction was heated for 5 min at 100 °C prior to loading (3 μl per well) onto a sequencing gel.

2.3.f. Denaturing gel electrophoresis

Sequencing gel apparatuses were obtained from Gibco - BRL, Burlington, Ont., Canada. Polyacrylamide 6 % / 7 M urea sequencing gels of dimensions 38
x 50 cm and 0.4 mm thickness were prepared and prerun at 45 W constant power for 30 min prior to loading. Multiple staggered loadings (3) for each reaction were conducted and gels were electrophoresed at 70 W constant power for 6 to 7 h. Subsequently, gels were processed, dried at 80°C for 1 h 20 min using a gel dryer, and subjected to autoradiography at room temperature.

2.4 Characterization of the 5' terminus of the human GnRH-R mRNA

2.4.a. Isolation of total RNA

Total RNA was prepared using the guanidinium isothiocyanate - cesium chloride procedure (Ausubel et al., 1992). Approximately 2 g of each tissue sample were homogenized using a polytron homogenizer (two or three 10 s bursts) in 15 ml of 4 M guanidinium isothiocyanate buffer containing 5 mM sodium citrate pH 7.0, 0.5 % sarcosyl, and 1 % β-mercaptoethanol. The mixture was then centrifuged for 10 min at 15,000 rpm, 4°C and the supernatant was recovered. Cesium chloride was added to the supernatant at a concentration of 1 g / 2.5 ml of supernatant and then layered onto 3 ml of 5.7 M CsCl containing 0.1 M EDTA, pH 7.5. Samples were centrifuged at 28,000 rpm in an ultracentrifuge using an SW40 rotor for 18 h
at 18 °C. RNA pellets were washed with 1 ml of diethylpyrocarbonate water and then resuspended in 10 mM Tris-HCl pH 7.4, 5 mM EDTA, and 1 % SDS. The resuspended RNA was subjected to a chloroform : n-butanol (4:1) extraction, followed by a 1 / 10 vol 3M sodium acetate pH 5.2 and 2.2 vol 95 % ethanol precipitation at -20 °C overnight. RNA pellets were resuspended in diethylpyrocarbonate water and stored at -70 °C. RNA concentration and purity was assessed by optical density readings at 260 nm and 280 nm. RNA integrity was assessed by the identification of the 28S and 18S ribosomal bands through denaturing agarose gel electrophoresis in the presence of ethidium bromide. The use of human tissue (intestine; placenta) was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. Human brain total RNA and human pituitary poly-A RNA were obtained from Clonetech, Palo Alto, CA.

2.4.b. Primer extension analysis

Primer extension analysis was conducted using three oligonucleotides, PE-1, PE-2, and PE-3 (Table 1). These primers were designed such that they would encompass the span of TATA boxes found in the DNA sequence of the genomic
Table 1. Oligonucleotides used in this investigation.

The location and orientation of primers used in this study are listed accordingly. The corresponding sequence is also shown. Numbering is sequential with the most 3’ transcription initiation site designated as +1.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-1</td>
<td>5'AGCTTCTCTGTGTACTGGCTG3'</td>
<td>-583 to -603</td>
</tr>
<tr>
<td>PE-2</td>
<td>5'CTGTTATTACACATTAATGCA3'</td>
<td>-36 to -56</td>
</tr>
<tr>
<td>PE-3</td>
<td>5'TTTATTAATCAATCTTACTGAT3'</td>
<td>+74 to +53</td>
</tr>
<tr>
<td>A</td>
<td>5'GCTTGAAGCTCTGTCCTGGGA3'</td>
<td>+679 to +699</td>
</tr>
<tr>
<td>B</td>
<td>5'TTTATGGTCACAATACTCA3'</td>
<td>-782 to -764</td>
</tr>
<tr>
<td>C</td>
<td>5'AGAGAAGCTGGTAATTCTCG3'</td>
<td>-690 to -672</td>
</tr>
<tr>
<td>D</td>
<td>5'GATGCTGGTTGTGATGGC3'</td>
<td>+766 to +749</td>
</tr>
<tr>
<td>E</td>
<td>5'ATGAATCTCTCCATCTTGGA3'</td>
<td>+1733 to +1753</td>
</tr>
<tr>
<td>F</td>
<td>5'CAACACTTGACAGATACAA3'</td>
<td>+3591 to +3609</td>
</tr>
<tr>
<td>G</td>
<td>5'TGTATCTGTACAAGTG3'</td>
<td>+3608 to +3592</td>
</tr>
<tr>
<td>H</td>
<td>5'CATATGAGCAACATGTAT3'</td>
<td>+4766 to +4749</td>
</tr>
<tr>
<td>I</td>
<td>5'AACCTATAGGAGGGAAGTG3'</td>
<td>+2189 to +2170</td>
</tr>
<tr>
<td>J</td>
<td>5'TCTTAAGGTCCAATATGT3'</td>
<td>+4062 to +4079</td>
</tr>
<tr>
<td>K</td>
<td>5'AAGTAGGATTTACACTTAAAT3'</td>
<td>+1793 to +1813</td>
</tr>
</tbody>
</table>
clones obtained in this study. Each oligonucleotide was end-labelled in a reaction containing 100 μCi [γ-32P]ATP (3000 Ci / mmol; Amersham, Oakville, ON, Canada), 100 ng of oligonucleotide, 2 μl 10X polynucleotide kinase buffer, and 4 U of T4 polynucleotide kinase. The mixture was incubated for 1 h at 37 °C and the enzyme was subsequently inactivated at 65 °C for 5 min. Approximately 2 x 10^5 cpm of each oligonucleotide primer as determined by Cerenkov counting was hybridized with 40 μg of total RNA from human brain and intestine in a reaction mixture containing 0.5 M NaCl, 40 mM PIPES pH 6.8, and 1 mM EDTA for 1 h at 42 °C. Annealed primer-RNA reaction mixtures were diluted 1:10 into reverse transcription mix containing 1 mM of each dNTP, 50 mM Tris-HCl pH 7.6, 60 mM KCl, 10 mM MgCl₂, 1 U RNAsin (Promega, Madison, Wis., USA), 1 mM DTT, and 10 U of AMV reverse transcriptase per 30 μl reaction. The reaction mixture was incubated for 2 h at 37 °C and the primer extended products were subsequently analyzed on a 6% polyacrylamide / 7.0 M urea gel. DNA sequencing reactions of M13 were used as a size standard.
2.5 Reverse-transcription polymerase chain reaction (RT-PCR)

2.5.a. Preparation of cDNA

Ten μg of total RNA per tissue sample used were reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Pharmacia, Vancouver, B.C., Canada). RNA was resuspended in a reaction volume of 8 μl, heated at 70 °C for 10 min and then chilled on ice. Thereafter, the following components were added to the heat - denatured RNA mix: 5 μl of Bulk First-Strand Reaction Mix (containing Murine Reverse Transcriptase, RNAsin, BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer), 200 mM DTT solution, and 1 μg of oligo d(T)12-18. The reaction mixture was incubated at 37 °C for 1 h and followed by a 10 min heating at 95 °C. Reverse transcribed cDNAs were stored at -20 °C. One μl aliquots were used as template for subsequent polymerase chain reaction (PCR) amplification.

In addition to total RNA being reversed transcribed, 2 μg of human pituitary poly-A RNA were also subjected to reverse transcription for 1 h at 37 °C using the First Strand cDNA Synthesis Kit under the same conditions mentioned above.
2.5.b. Polymerase chain reaction (PCR)

Primers used in PCR amplification are indicated in Table 1 and were designed based on the human GnRH-R gene sequence determined in the preceding experiments. PCR reactions were conducted in a final reaction volume of 25 μl in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.0 mM MgCl₂, 50 mM dNTPs, 0.01 % gelatin, 50 pmol / μl of sense and antisense oligonucleotides, 1 U of Taq DNA polymerase (Gibco - BRL, Burlington, ON, Canada), and overlayed with 50 μl of mineral oil. The PCR cycle profile consisted of a denaturation step at 96 °C for 30 s, an annealing step at 50 °C for 30 s, and an extension step for 90 s at 72 °C. PCR was conducted for 35 cycles in a DNA thermal cycler (Perkin Elmer) with a final extension for 15 min at 72 °C after the last cycle of amplification. Ten μl of the PCR reaction mixture were fractionated on a 1 % agarose gel and stained with ethidium bromide. Several controls were implemented during the PCR reactions. Negative controls consisted of PCR reaction mixture without cDNA in order to examine the possibility of cross-contamination between samples. Also, the possibility of genomic DNA contamination was excluded with the incorporation of nonreverse transcribed RNA as a template. PCR conducted with human β-Actin derived primers (antisense 5'-ggacctcagtacatcaagtaa-3; sense 5’-gtggaggtggtagcatgac-3'; Peng et al., 1993) was used as a positive internal
control in order to eliminate the possibility of RNA degradation and any technical problems associated with reverse transcription.

2.6 Gel Mobility shift assay

2.6.a. Cell culture

MCF-7, a human breast cancer cell line (obtained from Dr. J. Emerman, Department of Anatomy, University of British Columbia), was used as a source of progesterone receptors for DNA-protein binding studies. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with F12 (Gibco - BRL, Burlington, ON, Canada) and 10 % fetal bovine serum and maintained at 37 °C in a humidified atmosphere with 5 % CO₂. Treatment of cells with 10 nM estradiol (Sigma, St. Louis, MO, USA) for 24 h prior to harvesting was conducted in order to increase progesterone receptor levels. Confluent monolayers of MCF-7 were harvested by cell scraping for the preparation of nuclear extract. In addition to MCF-7 extract, HeLa cell nuclear extract (Promega) was obtained for preliminary studies involving CRE binding.
2.6.b. Preparation of nuclear extract

Approximately 100 x 10⁶ cells were harvested and incubated with 10 ml of media containing 10 nM R5020 for 1 h at 37 °C. Cells were then centrifuged at 1850 x g for 10 min at 4 °C and allowed to swell on ice for 10 min in 5 packed cell volumes (pcv) of hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT). Cells were pelleted again and resuspended in 2 pcv of hypotonic buffer prior to homogenization (50 strokes) with a Dounce homogenizer using pestle type B. The homogenate was centrifuged for 20 min at 3,300 x g, resuspended in ½ packed nuclear volume (pnv) ice cold low salt buffer (20 mM HEPES pH 7.9, 25 % glycerol, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). Addition of ½ pnv of high salt buffer (20 mM HEPES pH 7.9, 25 % glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) to a final concentration of approximately 300 mM KCl was followed by a 30 min incubation at 4 °C with gentle stirring. The nuclear precipitate was harvested by centrifugation at 25,000 x g for 30 min and was dialyzed for 4 to 5 h against dialysis buffer (20 mM HEPES pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). Nuclear extracts were aliquoted into tubes and stored at -70 °C. Protein concentrations were assessed using the Bradford assay (Ausubel et al., 1992).
2.6.c. Preparation of radiolabelled double-stranded oligonucleotide probes

Oligonucleotides used in mobility shift assays are indicated in Table 2. Cyclic AMP response element (CRE) and progesterone response element (PRE) consensus sequences were obtained from Promega Gel Shift Assay Systems. Target oligonucleotides containing the CRE-like and PRE-like sequences were designed according to the human GnRH-R gene sequence revealed in this study. Oligonucleotides were labelled using 1 μl of T4 polynucleotide kinase (10 U / μl), 1 μl of 10X kinase buffer, 100 ng of oligonucleotide, 50 μCi of γ-[32P]ATP, and H2O to a final volume of 10 μl. After an incubation period of 1 h at 37 °C, the enzyme was heat inactivated at 70 °C for 10 min. A 1.5 M excess of unlabelled complementary oligonucleotide and 1 μl of 20X oligonucleotide annealing buffer (200 mM Tris-HCl pH 7.9, 40 mM MgCl2, 1 M NaCl, and 20 mM EDTA) were added. The reaction mixture was heated to 65 °C for 15 min and allowed to cool to room temperature over a period of 2 h in order to promote annealing. TE buffer (pH 7.6) was added to a final volume of 100 μl and the mixture was passed through a NAP 5 column (Pharmacia, Vancouver, B.C., Canada) to separate the labelled oligonucleotides from the unincorporated nucleotides. Eluates of labelled oligonucleotides were collected and stored at -70 °C. The level of radioactivity obtained for labelled oligonucleotides ranged from 0.5 to 1 x 10⁶ cpm / μl.
Table 2. Oligonucleotides used in mobility shift assays.

Response elements are underlined within the oligonucleotide sequence. All oligonucleotides were annealed to its complement before experiments. CRE = cAMP response element; GRE/PRE = glucocorticoid/progesterone response element; m = mutant.
<table>
<thead>
<tr>
<th>CRE</th>
<th>5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3</th>
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</thead>
<tbody>
<tr>
<td>CRE-like</td>
<td>5'-CTCCAGATCTGAAGTCTGCCTAATA-3</td>
</tr>
<tr>
<td>GRE/PRE</td>
<td>5'-CGACTGGTACACAGTGTTCTGCTAC-3</td>
</tr>
<tr>
<td>GRE/PRE-like</td>
<td>5'-CAGCAGTTACACAGTATTCTCTTCA-3</td>
</tr>
<tr>
<td>mGRE/PRE</td>
<td>5'-CAGCAGGTATACAGTATTCTCTTCA-3</td>
</tr>
</tbody>
</table>
2.6.d. DNA-protein binding assay

Binding reactions were carried out in a reaction volume of 15 µl at room temperature. Binding buffer containing 10 mM HEPES pH 7.5, 10 % glycerol, 50 mM KCl, 2.5 mM MgCl₂, 200 ng of poly (dI.dC) - (dI.dC) (Pharmacia, Vancouver, B.C., Canada), 0.1 mM EDTA, and 1 mM DTT was added to 10 µg of total protein. This reaction mixture was allowed to incubate for 10 min at room temperature. Following this incubation, 30,000 cpm of labelled oligonucleotide was added and a further incubation period occurred. The reactions were stopped with the addition of 1 µl of gel loading 10X buffer and loaded onto 4 % nondenaturing PAGE.
2.6.e. Nondenaturing PAGE

Mobility shift assays were conducted using low-ionic strength PAGE. Gels were prepared with 6.8 mM Tris-HCl pH 7.9, 1 mM EDTA pH 7.9, 4% acrylamide, 0.05% bisacrylamide, and 2.5% glycerol and allowed to pre-electrophorese with low-ionic strength buffer (6.7 mM Tris-HCl pH 7.9, 3.3 mM sodium acetate pH 7.9, and 1 mM EDTA pH 8.0) for at least 30 min at a constant voltage of 150. Gels were run at a constant 200 V with recirculation of running buffer every 30 min for approximately 2 h, dried under vacuum in a gel drier, and autoradiographed overnight.
2.7 Southern analysis

One μg of plasmid DNA per sample was digested to completion with various restriction endonucleases, electrophoresed on either 1 % agarose gels or 5 % polyacrylamide gels, and stained with 0.5 μg / ml of ethidium bromide solution. Photographs of the gels were taken with a ruler under long - wavelength UV light in order to document the positions of the bands. Gels were treated with 0.2 M NaOH / 0.6 M NaCl for 30 min at room temperature with gentle rocking. After denaturation, the gels were neutralized in 1 M Tris-HCl pH 7.4 / 0.6 M NaCl for 30 min at room temperature. Gels were transferred to a Whatman 3MM paper wick resting on a support. The wick was placed in a large tray containing several hundred milliliters of 20X SSC (3 M NaCl and 0.3 M Na3citrate·2H2O, pH 7.0) and wetted with 20X SSC. The gel was then transferred on top of the Whatman wick and any remaining air bubbles were carefully removed. Nylon membrane cut to the same or slightly smaller dimensions of each gel was wetted with 20X SSC and placed onto the gel. A dry piece of Whatman 3MM paper followed by a 10 cm thick stack of paper towels was placed on top of the nylon membrane. A glass plate was placed on top of the paper towels and the entire apparatus was compressed by the addition of a 0.4 kg weight. This transfer setup was left overnight at room temperature and dismantled the next day in order to allow for capillary transfer of
the DNA from the gel onto the membrane. Nylon membranes were dried and then irradiated by UV light for 2 min each side in order to cross-link the DNA to the membrane. Hybridization was conducted overnight with various human GnRH-R cDNA probes consisting of 396 (+904 to +1300), 364 (+1300 to +1663), or 760 (+904 to +1663) bp as well as various sequence specific oligonucleotides. Blots were washed and subjected to overnight autoradiography with intensifying screens at -70 °C.

2.7.a. Genomic Southern analysis

Human placental genomic DNA was prepared for genomic Southern analysis. Human placental tissue, approximately 1 to 2 g, was obtained and quickly minced and frozen in liquid nitrogen. The tissue was ground to a fine powder with a prechilled mortar and pestle. Ground tissue was resuspended in 1.2 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5 % SDS, and 0.1 mg / ml of freshly prepared proteinase K) per 100 mg of tissue. Samples were incubated overnight at 50 °C with gentle rocking. Afterwards, samples were extracted with an equal volume of phenol : chloroform : isoamyl alcohol and centrifuged for 10 min at 3,000 rpm. Aqueous layer was transferred to a new tube
and ½ vol of 7.5 M ammonium acetate and 2.2 vol of 95 % ethanol were added. The mixture was then centrifuged for 5 min at 3,000 rpm. The DNA pellet was washed with 70 % ethanol and resuspended in TE buffer at a concentration of 1 mg / ml. Ten µg of human placental genomic DNA were used for each restriction endonuclease digestion (EcoRI, HindIII, BamHI, and PstI). Digested genomic DNA was size fractionated on 0.8 % agarose gels overnight at a constant 50 V. The gels were then denatured, neutralized, and prepare for DNA transfer and blotting according to the same conditions mentioned previously for Southern analysis. Filters were then hybridized with a [α-32P]dCTP - labelled 760 bp human GnRH-R cDNA and PstI - digested fragments, 396 bp and 364 bp in length, respectively.
2.8 Northern analysis

Human pituitary poly-A RNA was subjected to Northern analysis. Five µg of poly-A RNA was denatured in 50 % formamide / 2.2 M formaldehyde and incubated at 60 °C for 15 min. Samples were then electrophoresed on 1 % denaturing agarose gels (20 mM MOPS, 2.2 M formaldehyde, 8 mM sodium acetate, and 1 mM EDTA pH 8.0) at a constant voltage of 140 for 2.5 h. Capillary transfer of RNA to nylon membranes was conducted overnight in 20X SSC. Nylon membranes were subsequently irradiated by UV light for 2 min each side in order to cross-link the RNA to the membrane. Hybridization was conducted with a 364 bp cDNA probe overnight, washed, and exposed to Kodak XAR-5 film overnight at -70 °C with intensifying screens.
2.9 Chromosome assignment

The chromosomal localization of the human GnRH-R gene was determined by using PCR with genomic DNA from 25 human–hamster somatic hybrid cell lines (BIOS laboratories, New Haven, CT, USA). The primers K (sense; Table 1) and I (antisense; Table 1) were used. The reaction mixtures, containing 200 ng of hybrid cell line genomic DNA, were subjected to 30 cycles of PCR using similar conditions as previously described. The reaction products were then electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.
III. RESULTS

3.1 Isolation of the human GnRH-R gene

Upon screening of the human genomic library with a 760 bp (position: +904 to +1663; Peng et al., 1994) cDNA probe under high stringency hybridization conditions, 12 genomic clones were isolated from an initial screening of approximately 1.5 x 10^6 plaques. After three consecutive rounds of screening, positive clones were purified to homogeneity and λDNA was prepared for each positive clone and digested with various restriction enzymes. Genomic DNA fragments were analyzed by Southern blot analysis using the human GnRH-R cDNA as well as oligonucleotides corresponding to the 5' and 3' nontranslated regions of the gene. One λ-clone (λ-1) contained a 7.7 Kb EcoRI fragment corresponding to the 5' end of the gene (Fig. 4 and 5) and another λ-clone (λ-2) contained 3' oriented EcoRI fragments of 7.4 Kb and 3.8 Kb in size (Fig. 4 and 5). All of these hybridized with either the cDNA or oligonucleotide probes. These fragments spanned the entire gene for the human GnRH-R and were subsequently subcloned into pUC19 vector and sequenced by the dideoxy method.
Fig. 4. Southern blot analysis of genomic fragments of the human GnRH-R gene.

Restriction digests performed on λ-1 and λ-2. Positive signals identified by hybridization to a 760 bp human GnRH-R cDNA probe (+904 to +1663). X = XbaI; E = EcoRI.
Fig. 5. Southern blot analysis of 5' and 3' oriented genomic fragments of the human GnRH-R gene.

(A) Several 5' oriented fragments as identified by hybridization with oligonucleotide A. The 7.7 Kb EcoRI fragment was subcloned into pUC19 and sequenced. (B) Multiple 3' oriented restriction fragments as identified through hybridization to a 364 bp (+1300 to +1663) human GnRH-R cDNA probe. EcoRI fragments, 3' oriented, of sizes 7.4 and 3.8 Kb were subcloned into pUC19 and sequenced. X = XbaI; E = EcoRI.
3.2 The human GnRH-R gene sequence

Approximately 6.4 Kb of the human GnRH-R gene was sequenced and is shown in Fig. 6 along with its exon - intron splice junctions and deduced protein translation. This sequence contains 905 bp of 5’ flanking region (from the most 5’ transcription initiation site), the entire 5’ nontranslated sequence (1393 bp), the entire protein coding region (987 bp; Fig. 7), the exon - intron splice sites, the entire 3’ nontranslated region (3076 bp), and additional 3’ sequence. Once the sizes of the introns have been subtracted, the size of the structural gene corroborated well with the size of the major transcript of approximately 4.7 Kb and indicated full coverage of the mRNA.

Additional analyses of the human GnRH-R gene sequence are detailed further in forthcoming sections.
Fig. 6. The nucleotide sequence of the human GnRH-R gene (excluding intronic sequence).

The coding region of exons are shown in upper case letters whereas the flanking and nontranslated regions are depicted in lower case letters. TATA boxes are denoted by patched (^^^^^) underlining and CAAT boxes are underlined. Enlarged and bold nucleotides indicate the start of transcription. The translational start codon and stop codon are represented by bold lettering. Exon - intron splice junctions are indicated by the following symbol V. Classical polyadenylation signals in the 3' end of the gene sequence are double - underlined whereas ATTAAA signals are denoted with an asterisk (*). ATTTA motifs in the gene sequence are underlined. Nucleotides of the transcript are numbered sequentially and the most 3' transcription initiation site was arbitrarily assigned the +1 position.
-1595  aagctttctg aagcataaat ctggccatac ctaccgtata ttactccatt
-1545  ctttatatatg gtaagccta aactcctttt cttggaatat aggtcttcca
-1495  gatctgaagt ctgcctaatattatcacttt ttgctttccac atacccttttg
-1445  aactttctca cattgtcttct gtgtttagcat gtgtgtgctcc agctttcaag
-1395  catgcctccc ctgtcctcat accccattcc ccagccactt attacgcttat
-1345  atgtgtgtagtc ccattccagc tctgttgcaaat cctcttcccc aatgaatcag
-1295  tccatcatta aaaaaagggaggaggg agggaggagga agaggaatgga
-1245  aagggagaaa aggagaagggaggggaagggagggagga agaggggaag
-1195  ggaagggagaagggagggagggagaagggagaa gaaagaaggggac aataaatgaa
-1145  atgatagctc taatctttttt ccccctagata tagaagacaagagcaaaaaat
-1095  atacttcact aattgtatttt cttgataaat ttttttttttt ggttttttttt
-1045  gttgtgcgtgc caccttacaa caacctttctt atgtgtatgt cttttccaatg
-995  gttatatctgt ttttgttcttt tcaaggtcatc cggccctgatc agattaactt
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-895  ttaatcaaaaa ctgattttccttaatagcaatctaatagatag ctgattttttaa
-845  gcacattaattttttgtaat tctctctgttt cagtcacagtc aatagttttttga
-795  tgtagttgataa aatatttttgc cttacaattct caggtgtaaatt aatatttttt
-745  tcctttgatcc ttatatataa tagaggtat aatatattt cagtggaaggtt ^^^^^^^
-695  tagtgagaga aagttcttttata tcgtgacagtt caaaaaggag
-645  ttcaggtaca ggacttgtctt aagctgtgctca agatcagaaa cacagccagt
-595  acacagagaa gtgtgagaga taatacagatatat caacttatat caacttatct
-545  accttttcctgt gtaaacaagct ctttaaagggg gctttgaaggt gttgttttca
-495  ctttttatca cccagcaaggg ctaagataat gtatatagta aatatatttagt
-445  aaccatttat taaaatataa aatatattta agaagtttaaa caagtataat

72
aaatgaacca ataagaatgc accatctaa tcataatatc cacttttatc
cuttaaccttg tacctgtcctt ggctgctgca gaagcaaaact tgttgccatt
tagacaatca aggtgtgtggt tttaataatt ccaatgtgaag tcctaccagt
attgatgatat aactatccag cactcaccat gaaggttaaaa gaagcaacac
agaaaaaagt cctaagtgtgt cccaatattga aatgacgaga taacctataaa ^^^^^^ 
aagaacataaat ccatatatata ctaacataaa cacatatataa tgca^ttaca ^
gcagttacac agttatctct tcaataacta gtttcttttat gcattaatgt

gtaataacag caactacaat atttagataaa ttataaaaac caaggaataa ^^^^^^ 
atatataaaac tgattaacgc ttatctcta acttaagcat ggattggatc
agtaagatgg atatataaatat tctgatcag tcgattgtgtg tagattcataat
tttaaatatat catttgggttg aagataaatc ttaagtaataata tatttgtcca
gtttccgagt gctcaacagtt tttttggaac aggaaacaaa agaaattgg
 ttgagaaaatttgtagatatcc ttaagacata ggttatattaatatgagatctg
 tgttttcatt tttttcatt tacttttact actcagtgtgtg ttgagacagac
actaactata atatattttt agaaaaatttt ttaaaaagtttatatattcat
aatatcatga ctgacatttt tataaataaa ttaagcgttgtg acattcatcc
tcaacattt ggagagttgtg tgaagaggcg acatctgtgt gaggtgtctac
agttacagtt ggccctcaga atgcgttttgg cctgctctgt ttagacatctc
tgttggtatta ccaatcacaa aacaagtttaa ccttgatcctt tcacatcagat
atatcagggg acaaaaatgct acatacgctct aacacgtgtc agcttcccct
ctaaagaagg cagaaatc aaacggcttta agatccgtggtt acaataaat
atcagagata ccagagacac aaggtttgaa gctctgtccttt gggaaatAT
GGCAAAACAGT GCCTTCCTTG AACAGAATCA AAATCAGTTGT TGACGCCATCA
ACAACAGCAT CCCACTGATG CAGGGCAACC TCCCCACTCT GCCTTGTCT
GGAAAGATCC GAGTGACGGT TACTTTCTTC CTTTTTCTGC TCTCTGCGAC
CTTTAATGCT TCTTTCTTGT TGAAACCTCA GAAGTGGACA CAGAAGAAAG
AGAAAGGGAA AAAGCTCTCA AGAATGAAGC TGCTCTTAAA ACATCTGACC
TTAGCCACC TGTTGGAGAC TCTGATTGTC ATGCCACTGG ATGGGATGTG
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 ATCTTACCAC TGAACCGGGT CTTTCATCAG GACCCCCACG AACTAAAAAT
GAATCAGTCG AAGACAATATA TACAAAGAGC ACGGTGAAAG ACTCTAAAAA
TGACGGTTCG ATTTGCCACT TCATTTTCTG TCTGCTGGAC TCCCTACTAT
GTCTAGGAA TTTGATTTTG GTTTGATCCGT GAAATGTTAA ACAGGTTGTC
AGACCCAGTA AATCTACTCT CTTTTTTCTT TGCCTTTTTA AACCCTAGCT
TTGATCCACT TATCTATGGT TCTTTCTCTT TGCATAGAAG TTTTCTTTTA
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acctaaatg taattctttg aagaaacctag ttttcaagctc aatctcttttt
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gtttctttatc acttttcttt gcataataa tgtactaatata tttaaaatag
cttcagccta aggcacaagg atgccaaaaa aacaagaggtg agaaaccaca
2056  acacaggtct aaactcagca tgctttggtg agtttttctc caaaaggggc
2106  atattagcaaa tttaggttgt atgtcatata atacatagag cacagagccc
2156  ttgccccata atatcaactt tccccctcatat agtttaaag aaaaaaattg
2206  aatctatat tctctttggc ttgaaagca tttcgtacata tgtgaggagtct
2256  agtaacacac ccacaccaacc actccagcaa ccctgacaaga ctttgagttag
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2556  gatcttacta aatccatatt tcataaaata cttgagaataa tttaaaaaacaa
2606  attaagcaaa tggcacaagca aaaagatgct tggatacaca aaggaactct
2656  gggagagaaaa ctacagctggca aacagtctct gcactgatca acatcaagagacagaacat
2706  gtcaggggaa ggaagaaag atctttgatgc agggttttctt aacctgcagtt
2756  ctatgcacaa cactatatatt ccctgaaatt tttttatttt aagcgaatatt
2806  gttatatttt gtgcatttat aaaaaataac aattatctgaa tagactagac
2856  tggccacacga gcacagctggca caactaacac ctatgcctat tccacattaa
2906  tagtatggtt tccaaatatgt tgtgcacaca caagacccct tttatgtaatt
2956  caggttctgtg tctctccctt cctagaaaaa tggaaaaagga ctaaaaatat
3006  gggagtataa tacccttatt attgtaaaaa aacgcatgcc tttaaagaaa
3056  tttagccatta taacattttt taaataggta atagitcattt tattttatat
3106  gagctgatga ctgccccaa aaaaagatatgt cttgataattt ctaatactcc
3156  aggtgttgggt tttatggaag aacagtttgg cacaatcttg ccggttctttt
3206  tttcttttga tttcttttcc ctacacttaat gttttttagt gtttgtctctgt
3256  tgttttttttta atacccata ctaccttttca atctgtcttc taatgc
3306  gtttctttaa atacggttc ggtgatgcag ggtcgtgtctt tataattttt
3356  taaaagcctt ttgagcataa gattttttcc gtttcttttc aatctcctagc
3406  ttcgtatgtg ctgcgtttcttt ctacatagat ttcgtgacat ttttttttctt
3456  tgtttttgtt ccacttttgc gggtgatgct attgacgaag gtttcttggctt
3506  ttttcttttt ctttttcttt tttttttttt tttaaaagata aattcttttttt
3306  cctctgtcta ctcactccttt cattcatagc ttcttttcca ttaaactcat
3356  accttttaatt aaccaattca tggccccagt tctacagttga attggacaag
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3456  tcccaagattt tagatgtttct gtgagataaca cagggtatga gataagttga
3506  aaacaaaaac gttgctccta agtctctcttc atatacttat tcctaaactg
3556  ataatctctta cttctggatt taaaatcaa aataacacac ttgtagctgt
3606  acaatctcaag ggttttatca cacaaggttt aacgaatgtta tttcagtgttg
3656  gtctctctttg tggagtccttt atggactctct tggagtcctgg gattggccca
3706  ggcatttttt tttttttcctt attactctaa gggctatgcc aatactcgcg
3756  tcaggttctc taaccctcgt tcctgacaa aatatatatat atttcaatgt
3806  atttttattt ttagctcttt tggagacaaa aaaaacagtta
3856  cctggagggg atggacccagc tggccccacagc agctctagac aaaaaaagaag
3906  gtttaagagtt cgctgtgagcc ttggtatata taataagaaa tgtatttacc
3956  taataaatata atatcatcta ttcatacttt tataatgcaaa ctaaaatatg
4006  taataatggga aacaagattg cttcaatagt ctttttgttt tcaaaaaacac
4056  aaaccccttt aaggttcatt atgttaataaa aacaataaca caataataatt
4106  ttcttatatga atattaggtt tcataataata taatgttaaa ttctatattt
4156  ataattgatat atataaacta aatatatatgg cacaaagaat aatatggtct
4206  ttgaaattaa agatatttca ctcacagcac atatatcttc atttgatatt
4256  aacaatcatttt attttatggtc cttattataat aaaaaggtgag gactcctttgt
4306  aaaaaaggaa atgttcacacgagatcacatct atatactgag atattggaga
4356  ttcttatattg gtttcttttt ctttacttag cctataaaac tagttaaaaa
4406  ttggaatttc tttaagcaat tcaaggtaag cattactaat ctaataactaa
4456  ttagatattt ctttttaggg atctcttttg gaaggttacc
4556  caaacattat ctaccaggga acatagcata aattagtctg aaatttcctg
4606  agagtgacct tgtcttagaa cttaggtggt agtcatgaag agataatgtt
4656  tttaggcagt taaaatacttt ctagaactcc atctatattta cctgtggtcc
4706  actttcctac attgaaccaa tgccttgggc ttctctaatt actatacatt
4756  gtgctcatat gaataaaaga aattttaaaa gaaaaaaa

77
Fig. 7. Nucleotide sequence of the human GnRH-R coding region.

The nucleotide sequence of the complete protein coding region for the human GnRH-R is shown. Numbering of the nucleotides begins with +1 at the translational start (ATG) codon. The amino acid translation is represented below its respective nucleotide sequence in its three and one letter code.
3.3 Comparative analysis of the human GnRH-R gene

Software from the Genetics Computer Group, Inc. was employed in the analyses of the human GnRH-R gene. "FASTA" searches were performed comparing the human GnRH-R gene to the entire data base of GenBank and EMBL (European Molecular Biology Laboratory). "BESTFIT", "GAP", and "PILEUP" alignments were implemented in the 5' flanking and 5' nontranslated as well as in the translated, 3' flanking, and 3' nontranslated species comparisons.

FASTA searches revealed profiles of sequence similarity between the human GnRH-R gene and other genes. These genes included those for human wgle1, human STS, human β-globin, and orangutan β- and δ-globins. In particular, one prominent area of sequence similarity (-1300 bp to -1150 bp) was shared by all. This stretch of repetitive sequence, classified as an Alu repeat, was 74.6 % (wgle1), 70.8 % (STS), 68.5 % (human β-globin), and 64.7 % (orangutan β- and δ-globin) identical to the human GnRH-R Alu repeat.

Comparison of the human GnRH-R gene to that of the mouse gene as well as to the cDNAs of other species revealed notable sequence identity. Sequence comparisons for some species in the 5' and 3' ends were made on very limited
lengths of nontranslated regions. The results of the sequence comparisons are illustrated in Table 3 and Fig. 8.

As expected, the coding region for all species examined exhibited the highest degree of sequence identity. The sheep, cow, pig, mouse, and rat shared 89.5 %, 89.1 %, 88.4 %, 85.4 %, and 85.2 % sequence identity, respectively, with the human GnRH-R coding region. Notable sequence identity existed between the human and the mouse in the 5' end (5' of translational start) over a distance of 1.5 Kb. Lower sequence identity of 36.6 % in the 5' end (≈700 bp) was evidenced between the rat and human. Short stretches (all that was available) of notable sequence identity also existed for the other species, in particular, for the cow (70.5 %) and sheep (58.8 %). Sequence comparisons of the 3' end (3' of the stop codon), with lengthier sequences of up to 1.3 Kb, demonstrated relatively high sequence identity between the sheep (70.2 %), cow (68.1 %), pig (64.0 %), and human species. Lower scores were obtained for both the mouse (48.5 %) and rat (47.4 %).

Amino acid comparisons of the protein coding region of the human GnRH-R gene with the above mentioned species were also conducted (Fig. 9). High amino acid identity was observed between species. Obvious species polarity existed in terms of the number of amino acids which code for the receptor. Human, pig, cow,
Table 3. Interspecies sequence comparison of the GnRH-R gene and cDNA.

Comparisons between species of the 5’ end, 3’ end, and coding region for the GnRH-R gene (mouse) and cDNA (other species) are shown. Comparisons are represented numerically as percentage of sequence identity to the human gene. In addition, the length of sequence comparison is numerically indicated in parentheses.
<table>
<thead>
<tr>
<th>Species</th>
<th>5' End</th>
<th>Coding Region</th>
<th>3' End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>************</td>
<td>**************</td>
<td>************</td>
</tr>
<tr>
<td>Cow</td>
<td>70.5% (~50 bp)</td>
<td>89.1%</td>
<td>68.1% (~1 Kb)</td>
</tr>
<tr>
<td>Sheep</td>
<td>58.8% (~40 bp)</td>
<td>89.5%</td>
<td>70.2% (~1 Kb)</td>
</tr>
<tr>
<td>Pig</td>
<td>------</td>
<td>88.4%</td>
<td>64.0% (~1 Kb)</td>
</tr>
<tr>
<td>Mouse</td>
<td>57.3% (~1.5 Kb)</td>
<td>85.4%</td>
<td>48.5% (~1.3 Kb)</td>
</tr>
<tr>
<td>Rat</td>
<td>36.6% (~700 bp)</td>
<td>85.2%</td>
<td>47.4% (~1 Kb)</td>
</tr>
</tbody>
</table>
Fig. 8. DNA sequence alignment and comparison of GnRH-R coding regions among various species.

The nucleotide sequences of the mouse (m), rat (r), cow (b), sheep (s), human (h), and pig (p) GnRH-R coding regions as compared using the "PILEUP" alignment from the GCG (The Genetics Computer Group, Inc., Madison WI) software system. Identical nucleotides among species are indicated with an asterisk (*).
| m | CACTCTTGAA AGCTCTTCTT GAGAAA.TA TGCTACTATA TGCACTCTTT |
| r | CACTCTTGAA GGGGCTTCTT GAGAAA.TA TGCTACTATA TGCACTCTTT |
| b | GAGCTCTGAA CTGTACTAGG CATATAAGGA TGGAAACAG TGACTCTCTT |
| s | GAGCTCTGAA CTGTACTAGG CATATAAGGA TGGAAACAG TGACTCTCTT |
| h | AAGGCTTGAA GTCTGCTCCG TGGGAATATA TGCCAAACAG TGCTCTCTCT |
| p | **       ** TGGATCTAGT GGGAAAGAGA TGCCAAACAG TGCTCTCTCT |

| m | GAGCAGGACC CAATCTCAGG CGCGGCTTATG AGCAACAGCA TCCCCCTT |
| r | GAGCAGGACC CAATCTCAGG CGCGGCTTATG AGCAACAGCA TCCCCCTT |
| b | GAACAGAATG AAAACCACTG TTCAGCGATC AACAGCAGCA TCCTACTT |
| s | GAACAGAATG AAAACCACTG TTCAGCGATC AACAGCAGCA TCCTACTT |
| h | GAACAGAATG AAAACCACTG TTCAGCGATC AACAGCAGCA TCCTACTT |
| p | GAACAGAATG AAAACCACTG TTCAGCGATC AACAGCAGCA TCCTACTT |

| m | ACAGGGCAAG CTCCCGACTC TAACCGTATC TGGAAAGATC CGAGTGACCG |
| r | ACAGGGCAAG CTCCCGACTC TAACCGTATC TGGAAAGATC CGAGTGACCG |
| b | ACCAGGCAGG CTCCCGACCC TGACCCTATC TGGAAAGATC CGAGTGACCT |
| s | ACCAGGCAGG CTCCCGACCC TGACCCTATC TGGAAAGATC CGAGTGACCT |
| h | ACCAGGCAGG CTCCCGACCC TGACCCTATC TGGAAAGATC CGAGTGACCT |
| p | ACCAGGCAGG CTCCCGACCC TGACCCTATC TGGAAAGATC CGAGTGACCT |

| m | TGACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |
| r | TGACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |
| b | TTACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |
| s | TTACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |
| h | TTACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |
| p | TTACTTTCTT CCTTTTCTAT CCTCTACTCT CCTTCAATGC TTCTCTT |

| m | TGAAAGCTGC AGAAGTGGAG TCAGAAGAGG AAGAAAGGAA AAAAGCTCCT |
| r | TGAAAGCTGC AGAAGTGGAG TCAGAAGAGG AAGAAAGGAA AAAAGCTCCT |
| b | TTGAAACTTC AGAATTGGAG TCAAAGGAAA GAGAAGAGGA AAAAACTCCT |
| s | TTGAAACTTC AGAATTGGAG TCAAAGGAAA GAGAAGAGGA AAAAACTCCT |
| h | TTGAAACTTC AGAATTGGAG TCAAAGGAAA GAGAAGAGGA AAAAACTCCT |
| p | TTGAAACTTC AGAATTGGAG TCAAAGGAAA GAGAAGAGGA AAAAACTCCT |

| m | AAGGATGAG GACATGTGAG CTCAGATCGC TTCAGACAG TCTAGAGAG |
| r | AAGGATGAG GACATGTGAG CTCAGATCGC TTCAGACAG TCTAGAGAG |
| b | GAGAATGAGG TGGCTTATGAA AATCTGTTGC TGGGCACTG TCTGCTGAG |
| s | GAGAATGAGG TGGCTTATGAA AATCTGTTGC TGGGCACTG TCTGCTGAG |
| h | GAGAATGAGG TGGCTTATGAA AATCTGTTGC TGGGCACTG TCTGCTGAG |
| p | GAGAATGAGG TGGCTTATGAA AATCTGTTGC TGGGCACTG TCTGCTGAG |

| m | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
| r | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
| b | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
| s | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
| h | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
| p | CTCGATCGTC CATGCCACTG GATGGGATGT GGAATATTAC TGTTCAGTGG |
and sheep all possessed 328 amino acids, whereas, the rat and mouse receptors possessed only 327 amino acids. Identical amino acids shared between species are also indicated in Fig. 9.
Fig. 9. Alignment of the amino acid sequences of various species.

The derived amino acid sequences for the human, pig, cow, sheep, rat, and mouse GnRH receptors are aligned. Commonly conserved residues are depicted with the number symbol (#). Regions of the proposed transmembrane domains are double underlined (= = =).
<table>
<thead>
<tr>
<th>Species</th>
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<th>Length</th>
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<tr>
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</tr>
<tr>
<td>Human</td>
<td>---------</td>
<td></td>
</tr>
<tr>
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<tr>
<td>Mouse</td>
<td>89.3 %</td>
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</table>
3.4 Structural organization of the human GnRH-R gene

DNA sequencing and sequence comparison to the published human GnRH-R cDNA sequences (Kakar et al., 1992; Chi et al., 1993) revealed that structurally the GnRH-R gene consists of three exons and two introns (Fig. 10). Exon II consists of 219 bp while exon I and exon III consist of up to 1915 and 3321 bp, respectively. GT - AG consensus rules were followed for all splice junctions found (Table 4). Additionally, RT-PCR was conducted with human pituitary cDNA as a template in order to confirm transcription up to the most 5' and 3' ends of the major 4.7 Kb transcript (Fig. 13 and 18). Amplification of a 1456 bp product of the expected size resulted when PCR was conducted with oligonucleotides C and D (Table 1). However, when oligonucleotide B, a primer located upstream of the most 3' transcription initiation site, was used in combination with D no amplification product was observed (Fig. 13). Additionally, oligonucleotide combinations of E with G and F with H resulted in the amplification of products of the expected sizes of 1876 and 1176 bp, respectively (Fig. 18). For all primer combinations employed, no bands were amplified from negative controls consisting of no cDNA, intestinal cDNA, or nonreverse transcribed human pituitary poly-A RNA as a template. As well, amplification using β - Actin primers on cDNA templates resulted in the successful production of fragments of the expected size (Fig. 14).
Fig. 10. The human GnRH-R gene structure.

A schematic representation of the human GnRH-R gene. (A) Organization of the three genomic EcoRI (E) subclones. (B) Sequencing strategy employed for the human GnRH-R gene. Arrows indicate the orientation of oligonucleotides and the extent of sequence obtained. (C) Exon-intron localization. (D) Structure of the human GnRH-R cDNA. Open boxes indicate the protein coding regions and hatched boxes are the putative transmembrane domains. (E) The relative positions of the human cDNA probes used in this study. Representative scale is illustrated.
A clon1 clone2 clone3

B

E

5'

PstI

2kb

I

0.1kb

I

E

C

5'

1kb

I

II

III

IV

V

VI

VII

D

5'

1.0kb

TM I II III IV V VI VII

3'

cDNA

0.1kb

E

5' A B C

3'

cDNA probes

B PstI C
Table 4. The exon - intron organization of the human GnRH-R gene.

The splice-junctions share the consensus sequences for the donor and acceptor sites described by Breathnach and Chambon, 1981. Numbers in parentheses are relative to the translational initiation codon.
<table>
<thead>
<tr>
<th>Exon</th>
<th>Exon size (bp)</th>
<th>5'Boundary</th>
<th>Intron (kb)</th>
<th>3'Boundary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1915</td>
<td>ACCACAG (+1125)</td>
<td>gtaga...tagag [\approx 4.2]</td>
<td>TTATACCA</td>
</tr>
<tr>
<td>2</td>
<td>219</td>
<td>CCCCACG (+1445)</td>
<td>gtag...aacag [\approx 5.0]</td>
<td>AACTACCA</td>
</tr>
<tr>
<td>3</td>
<td>3319</td>
<td>GAAAAAA AA (+4766)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 Promoter region of the human GnRH-R gene

A 7.7 Kb EcoRI genomic fragment was subjected to restriction and Southern analyses resulting in the subcloning of a 2.7 Kb HindIII genomic fragment containing the 5' end of the human GnRH-R gene (Fig. 11). The 3' end of this subclone contained the segment of exon I carrying the translational start site for the human GnRH receptor. Nucleotide sequencing of the 2.7 Kb fragment was performed and analysis of the 5' end of the human GnRH-R gene revealed the presence of five consensus TATA boxes (TATAAA; -14, -111, -150, -718, and -731; Fig. 5 and 12) residing in close proximity to one another in a cluster-like arrangement. Several CAAT boxes were also found well interspersed among the TATA boxes.

In addition, inspection of the 5' nontranslated region for the human GnRH-R gene revealed the presence of several ATG (AUG; mRNA) codons upstream of the major reading frame (Fig. 6).
Fig. 11 The 2.7 Kb HindIII subclone.

Configurational alignment of the 2.7 Kb HindIII subclone (containing the promoter region of the human GnRH-R gene) within the 7.7 Kb EcoRI genomic clone. Corresponding gene structure is depicted below. Stippled and solid black boxes indicate nontranslated region and coding region of the human GnRH-R gene, respectively. Intronic regions are shown as solid lines. E=EcoRI; H=HindIII.
Fig. 12. **Organization of the human GnRH receptor promoter region.**

The positions of the TATA and CAAT sequences are emphasized. The mRNA cap sites (▼) for the human GnRH receptor transcripts are indicated by the respective nucleotides in lower case letters. Nucleotides of the transcript are numbered sequentially and the most 3’ transcription initiation site was arbitrarily assigned the +1 position.
3.6 Characterization of the 5' terminus of the human GnRH-R mRNA

Continued analyses on the 5' end of the gene proceeded. Experiments to determine the transcription initiation site(s) for the human GnRH-R gene were conducted using primer extension analysis. Moreover, since several potential promoters for the human GnRH-R gene existed, mapping of the 5' terminus (or termini) of the human GnRH-R mRNA was undertaken to examine which one(s) may be functional. Five transcription initiation sites were identified through primer extension analysis. Primer extension was performed on human brain total RNA using three synthetic oligonucleotides, PE-1, PE-2, and PE-3 (Table 1). It should be noted that in preliminary studies, human pituitary poly-A RNA was used in addition to brain total RNA. Because early results demonstrated similar findings for both the pituitary and the brain, utilization of the more available source of human brain total RNA for primer extension experiments was undertaken. PE-1 resulted in two bands at positions -669 bp and -690 bp, respectively (Fig. 13A). PE-2 and PE-3 revealed three signals (-101 and -124 bp, Fig. 13B; +1, Fig. 13C). Human intestinal total RNA, as a negative control, showed no signals when extended with PE-1, PE-2, or PE-3. All five transcription initiation sites were found to be positioned in close proximity to the TATA boxes (Fig. 12).
Fig. 13. Analysis of the human GnRH-R gene by primer extension analysis.

Oligonucleotides PE-1 (panel A), PE-2 (panel B), and PE-3 (panel C) were end-labelled and hybridized to 40 µg each of human brain (B) and intestinal (I) total RNA. Extended products were analyzed on a 6% polyacrylamide/7.0 M Urea gel. Standard lanes A, C, G, and T indicate M13 DNA sequencing reactions which were used as a size standard. Positions of the positive bands are indicated numerically and by arrows.
3.7 RT-PCR analysis of the 5’end of the human GnRH-R gene

To confirm the results of the primer extension analysis, PCR was performed using oligonucleotide B, a primer positioned upstream of transcription initiation site -690, and oligonucleotide D, which is located downstream of transcription initiation site -690 (Table 1). No cDNA amplification product was observed (Fig. 14). However, when PCR was performed using C, an oligonucleotide located immediately 3’ to transcription initiation site -690, and oligonucleotide D (Table 1), amplification of a 1456 bp product of the expected size resulted (Fig. 14). No bands were amplified from nonreverse transcribed human pituitary poly-A RNA as a template (Fig. 14).
Fig. 14. Analysis of the 5' end of the human GnRH-R gene.

A) PCR amplification of the 5' nontranslated region of the human GnRH-R gene. Two μg of human pituitary poly-A RNA, reverse-transcribed (Pit) or nonreverse transcribed (C), were amplified by PCR using primers B + D (1) and C + D (2) for 35 cycles. Molecular size standard is indicated as MW. A product consisting of 1456 bp is indicated. B) Southern blot analysis using a PE-3 oligonucleotide probe of the 1456 bp DNA fragment obtained through PCR analysis. Abbreviations and markings are the same as for Fig. 14A.
Fig. 15. β - Actin controls.

Ethidium bromide stained gels showing DNA fragments of expected size (524 bp) generated through RT-PCR with β-Actin derived primers. Molecular size standard is depicted by MW, P indicates pituitary cDNA as a template, and I represents intestinal cDNA as a template.
\( \beta\text{-Actin} \)

\[
\begin{array}{c}
\text{MW} \\
\text{P} \\
\downarrow \\
516\text{bp} \\
\end{array}
\]
3.8 Transcription factor binding sites (in addition to TATA and CAAT) and regulatory elements

The Genetics Computer Group, Inc. "TF sites" file, with a database of 2,036 recognition sites for transcription factors, was used to detect the presence of potential binding sites in the 5' end of the human GnRH-R gene. The TF sites computer file targeted only those binding sites which were perfect matches to the human GnRH-R gene. Additionally, a BESTFIT comparison was conducted to identify potential cis-acting regulatory elements.

Using BESTFIT analysis, two putative regulatory elements were located. A putative cAMP responsive element (CRE) (Roesler et al., 1988) was identified at position -1490 to -1483 (Fig. 16). Analysis of the sequence for the CRE (5’-TGAAGTCT-3’) revealed that it was identical to the published consensus sequence (5’-TGACGTCA-3’) at six out of eight base pairs. A putative glucocorticoid/progesterone response element (GRE / PRE; 5’-GTTACACAGTATTCT-3’; Fig. 16), with one base pair deviation from a functional GRE / PRE (Beato et al., 1989), was located at position -78 to -92.

Consensus binding sites for some typical transcription factors (Faisst et al.,

113
1992) were also identified. Notably, those for GATA-1, WAP, engrailed protein (Ohkuma et al., 1990), PEA-3 (Xin et al., 1992), AP-1, and Pit-1 were found (Fig. 16).
Fig. 16. Cis - acting DNA regulatory sequences of the human GnRH-R gene.

Sequences of gene regulatory protein binding sites for the human GnRH receptor. The +1 position was assigned to the most 3' located transcription initiation site.
3.9 Analysis of GRE / PRE - like and CRE-like sequences in the human GnRH-R gene

Computer analysis of the human GnRH-R gene revealed the presence of two potential regulatory elements which possess sequence similarity to a GRE / PRE and a CRE, respectively. The nucleotide sequence and position of these motifs are shown in Fig. 6. As well, all oligonucleotides used in this study are shown in Table 2. The CRE - like sequence differed in two positions from the consensus CRE whereas the GRE/PRE - like sequence differed in one position from a functional GRE / PRE (two positions from consensus sequence). Experiments involving mobility shift assays were conducted in order to examine the binding of the respective proteins to these sequences.

Nuclear extract from hormone - treated (E2; R5020) MCF-7 cells were used as a source of progesterone receptors for analysis of GRE / PRE sequence - protein binding. CRE studies were performed with nuclear extract from HeLa cells (preliminary experiments; data not shown) and MCF-7 cells. Similar results were obtained for either extract. Thereafter for ease and consistency, all gel shift assays were performed with MCF-7 extract. Negative controls consisted of binding reactions without extract. In addition, for the GRE / PRE - like motif, a double
point mutation of the consensus sequence also functioned as a negative control. Positive controls consisted of consensus sequences for both CRE and GRE / PRE. The results of gel shift assay experiments are shown in Fig. 17 and 18. Retarded DNA - protein complexes were observed for the positive controls as expected. CRE- and GRE / PRE - like sequences yielded no retardation complexes and therefore failed to bind to their respective protein factors. As well, no binding of proteins were observed for negative controls.
Fig. 17. Mobility shift assay of the CRE-like element.

All gel shift assays were performed with 30,000 cpm of labelled oligonucleotide [1, 2 (consensus CRE), and 3 (CRE-like)], 200 ng of poly (dI.dC) - (dI.dC) [1, 2, and 3], and with (2 and 3) or without (1) nuclear extract. Bound protein complexes are indicated.
DNA-protein complex

free DNA probe
Fig. 18. Mobility shift assay of the GRE / PRE - like element.

All gel shift assays were performed with 30,000 cpm of labelled oligonucleotide (1, 2 (GRE/PRE consensus), 3 (GRE/PRE - like), and 4 (GRE/PRE mutant), 200 ng of poly (dI.dC) - (dI.dC) (1, 2, 3, and 4), and with (2, 3, and 4) or without (1) nuclear extract.
1 2 3 4

- DNA-protein complex

- free DNA probe
3.10 Analysis of the 3' end of the human GnRH-R gene

The 3' end of the human GnRH-R gene was contained within a 3.8 Kb EcoRI gene fragment. Configurational alignment of the 3.8 Kb clone within the human GnRH-R gene structure is depicted in Fig. 10. A 3095 bp segment of the 3' end of the gene was sequenced (Fig. 6). Five classical AATAAA polyadenylation signals (+3954, +4080, +4283, +4460, and +4767) compatible with the reported size of the human GnRH-R mRNA were identified. They are located entirely within an 800 bp nucleotide region in a cluster-like format similar to the TATA boxes in the 5' end. Several ATTAAA sequences (+4212 and +4465), a functional variant of the classical AATAAA signal were also identified. Additionally, several ATTTA mRNA instability motifs (+3841, +3983, +4178, +4263, and +4490) (Shaw and Kamen, 1986) were found.

PCR analysis was conducted with pituitary cDNA as a template in an attempt to localize the major polyadenylation site. Various primer combinations were employed in order to amplify the 3' nontranslated region (Table 2). Oligonucleotide combinations of E with G and F with H resulted in the amplification of products of the expected sizes of 1876 and 1176 bp, respectively (Fig. 19). Using nonreverse transcribed RNA as a template for all primer combinations employed, no DNA
Fig. 19. Analysis of the 3’ end of the human GnRH-R gene.

A) PCR amplification of the 3’ nontranslated region of the human GnRH receptor. Two μg of human pituitary poly-A RNA, reverse-transcribed (Pit) or nonreverse transcribed (C), were amplified by PCR using primers E + G and F + H (2) for 35 cycles. Molecular size standard is indicated as MW. The corresponding amplification products of the expected sizes (1876 and 1176 bp) are indicated. B) and C) Southern blot analyses of the 1876 bp (B) and 1176 bp (C) products generated through PCR amplification. (B) was probed with oligonucleotide I and (C) was probed with oligonucleotide J. All abbreviations are as in Fig. 19A.
MW C1 C2 Pit1 Pit2

1876bp
MW C1 C2 Pit1 Pit2

1176bp ➤
fragments were amplified (Fig. 19A). Results obtained confirm transcription of the entire 3' nontranslated region up to the polyadenylation signal at position +4767.
3.11 Genomic Southern blot analysis

Genomic Southern blot analysis was performed to detect the GnRH-R gene directly from human genomic DNA. Genomic DNA from human placenta was digested with each of four restriction endonucleases: EcoRI, BamHI, HindIII, and PstI. Each digest generated multiple fragments that hybridized to the 760 bp human GnRH-R cDNA probe (Fig. 20A). However, when this probe was segmented with PstI digestion to generate a 5’ oriented 396 bp probe (Fig. 20B) and a 3’ oriented 364 bp probe (Fig. 20C), the number of DNA fragments decreased. As well, there was a marked polarization of results consistent with the type of probe used. For example, three fragments (7.7, 7.4, and 3.8 Kb) hybridized to the 760 bp probe (Fig. 20A), only the 7.7 Kb fragment hybridized to the 396 bp probe (Fig. 20B), and the 7.4 and 3.8 Kb moieties hybridized to the 364 bp probe (Fig. 20C). The hybridization pattern of the genomic Southern blot is in agreement with the restriction map obtained from the isolated clones and indicates that the gene for the human GnRH-R spans approximately 19 Kb and is present as a single copy in the human genome.
Fig. 20. Genomic Southern blot analysis of the human GnRH-R gene.

Ten $\mu$g of human genomic DNA was digested with each EcoRI, BamHI, HindIII, and PstI and hybridized with either 760 bp (+904 to 1663; A), 396 bp (+904 to +1300; B) or 364 bp (+1300 to 1633; C) human GnRH-R cDNA probes.
3.12 Chromosomal assignment

The human GnRH-R gene was assigned to a specific human chromosome by conducting somatic cell hybrid analysis. DNA isolated from 23 human-hamster somatic hybrid cell lines representing coverage of all human chromosomes and their parental cell lines (BIOS laboratories) were used to examine the presence of the GnRH-R gene by PCR using primers (E and I) specific for the human GnRH receptor. Segregation of the human GnRH-R gene to a unique human chromosome was based on the observable pattern of the human band in the various hybrids. As shown in Fig. 21, PCR conducted on the cell line DNAs, electrophoresed on 1% agarose, and stained with ethidium bromide, detected a major product of expected size (397 bp) from a human cell line and two hybrid cell lines, #852 (9) and #1079 (22). However, this 397 bp product was not observed in the hamster cell line or the rest of the hybrid cell lines. Analysis of the PCR results with the provided human chromosome localization tabulation sheet identified chromosome 4 as the carrier of the GnRH-R gene (Table 4).
Fig. 21. Chromosomal assignment of the human GnRH-R gene to chromosome 4.

DNA from 25 human-hamster somatic hybrid cell lines and their parental cell lines were amplified by 30 cycles of PCR using primers derived from the human GnRH receptor sequence. A 397 bp product was obtained from two hybrid cell lines (nos. 852 and 1079) and the human parental cell line, but not from the hamster parental cell line or the other hybrid cell lines (representative cell line no. 983).
Table 5. Chromosome contents of human-hamster hybrid cell lines and the assignment of the human GnRH-R gene.

The gene content of each hybrid was determined through PCR analysis. The presence [+] or absence [-] of the gene is indicated. [+] = chromosome present in at least 70 %; [(+)]= chromosome present in 40-60 %; [-]= chromosome absent; [(-)]= chromosome present in 5-30 %; d = multiple deletions in 5q; D = deleted at 5q15.1-5q15.2.
3.13 Northern blot analysis of the human GnRH-R mRNA

The human GnRH receptor possesses a mRNA which is expressed at relatively low levels. Therefore, the use of poly-A RNA was incorporated in the study of human GnRH-R mRNA expression in the pituitary. Human pituitary poly-A RNA was subjected to Northern analysis using a 364 bp human GnRH-R cDNA as a probe. An approximately 4.7 Kb transcript specific for the GnRH receptor was detected from 5 μg of poly-A RNA (Fig. 22). No signal was obtained from human intestinal poly-A RNA which served as a negative control (Fig. 22).
Fig. 22. Northern blot analysis of human pituitary and intestinal RNA.

Five μg of poly-A RNA from the pituitary (1) and intestine (2) were analyzed. Hybridization was performed with a 364 bp human GnRH-R cDNA probe (+1300 to +1663).
IV. DISCUSSION

At the outset, very little information regarding the gene for the GnRH receptor was available. The isolation of the GnRH-R gene itself had not been accomplished in any species. I had at my disposal, however, a human GnRH-R cDNA clone which was isolated through cDNA library (pituitary) screening with a rat GnRH-R cDNA probe (Peng et al., 1994). From this point, I had a basis from which to begin my work on the isolation and characterization of the human GnRH-R gene. The work presented here represents the first report on the isolation and characterization of the GnRH receptor gene and was published in 1994 (Fan et al., 1994). Additional information on the gene was reported subsequently in 1995 (Fan et al., 1995).

Prior to the isolation of the human GnRH-R gene, various analytical studies were first conducted. Among these studies, in particular, the copy number of the GnRH-R gene in the human genome was assessed. Genomic Southern blot analysis was performed on restriction digested human genomic DNA. Evidence indicated a banding pattern that is consistent with the notion of a single copy gene encoding for the GnRH receptor. Elaborating further on the digestion pattern obtained, a multiple
and complex banding of 3, 3, 4, and 4 was observed for each endonuclease, EcoRI, BamHI, HindIII, and PstI, respectively, with human GnRH-R cDNA probing (760 bp). All bands could be accounted for with additional probing with the 364 bp and 396 bp cDNA probes and are in agreement with restriction mapping of the human GnRH-R genomic clones which were later obtained. As well, the EcoRI genomic fragments as identified by genomic Southern analysis are identical to the 7.7, 7.4, and 3.8 Kb genomic clones obtained subsequently through genomic library screening. The implications of these findings on the extrapituitary GnRH receptors that have been identified are far reaching. These findings indicate that despite the tissue-specific differences of the GnRH receptor, the receptors for GnRH are encoded for by one gene. Therefore other control mechanisms at the level of the gene must be important for these tissue-related differences in the GnRH receptor.

Chromosomal assignments studies were also conducted on the GnRH receptor. Use of somatic cell hybrid analysis was successful in segregating the gene for the GnRH receptor to chromosome 4. The assignment of the human GnRH-R gene to chromosome 4 is identical with the localization of several other genes belonging to the superfamily of G protein coupled receptors. These include the genes encoding for \( \alpha 2 \)-C4 adrenergic receptor (Kobilka et al., 1987), cholecystokininin receptor A (de Weerth et al., 1993), D5 dopamine receptor (Polymeropoulos et al., 1991; Eubanks
et al., 1992), endothelin-A receptor (Hosoda et al., 1992), and neuropeptide Y Y1 receptor (Herzog et al., 1993). Recent analysis on the subchromosomal localization of the human GnRH-R gene was performed using DNA isolated from these studies (Leung et al., 1995). Using fluorescent in situ hybridization (FISH), the human GnRH-R gene was mapped to 4q21.2. Incorporating the use of a larger 15 Kb λ-2 genomic clone as a probe served to increase the precision of chromosome localization. Additionally, Kaiser et al., (Kaiser et al., 1994) using somatic cell hybrid analysis have localized the GnRH-R gene to an adjacent band on human chromosome 4q13.1-q21.1. This work was confirmed by Morrison et al., (Morrison et al., 1994) using a 1.9 Kb cDNA probe localizing the human GnRH-R gene to 4q13.2-13.3. The mapping of the GnRH-R gene to its precise chromosomal site should facilitate further studies on the identification of closely linked genes. As well, investigations of possible chromosomal rearrangements involving the GnRH-R gene and its consequent implications on fertility may be further studied.

Studies focusing on the mRNA for the GnRH receptor were also conducted. Northern analysis performed on human pituitary poly-A RNA using a 364 bp human GnRH-R cDNA probe identified a ≈ 4.7 Kb transcript. This size of mRNA for the human GnRH receptor was consistent with values of 4.7 and 5.0 Kb reported from other laboratories (Kakar et al., 1992; Chi et al., 1992). Comparison of this data
to the sequence for the human GnRH-R cDNA (Kakar et al., 1992; Chi et al., 1992) revealed that the entire coding region (987 bp) for the receptor encompassed less than half of its mRNA, leaving ample sequence for 5' and 3' nontranslated regions. More importantly from these data, it became evident that a considerable proportion of the transcript was unaccounted for in the isolated cDNA clones of the GnRH receptor. Moreover, from these data it was expected that the gene for the GnRH receptor would likely be greater than 15 Kb.

Isolation of the human GnRH-R gene through high stringency genomic library screening proved my initial hypothesis regarding gene size correct. Based on Southern analysis, restriction map analysis, and sequence data, the gene encoding for the human GnRH receptor spans approximately 18.9 Kb.

Sequencing of the entire coding region for the human GnRH receptor revealed 100% sequence identity with its corresponding cDNA sequence. Analyses revealed that the gene structure for the human GnRH receptor consisted of a three exon - two intron motif. Two exon - intron splice sites were identified through sequence comparison of genomic sequence to its cDNA counterpart as well as by using GT - AG consensus splice sequences. These findings classify the GnRH receptor as belonging to the intron containing division of G protein coupled receptors. A
subsequent study on the mouse GnRH-R gene (Zhou and Sealfon, 1994) demonstrated identical findings of a three exon and two intron organization as well as identical locales for splice junctions.

Exon I of the human GnRH-R gene carries the 5’ nontranslated region and part of the reading frame encompassing transmembrane domains I through III as well as a portion of transmembrane domain IV. The second exon codes for the remainder of the IVth transmembrane domain together with the Vth transmembrane domain of the deduced amino acid sequence. The third exon for the human GnRH-R gene encodes the carboxy-terminal part of the open reading frame and the 3’ nontranslated region of the receptor. Of particular interest is the finding of two introns present within the GnRH receptor gene, since many G protein coupled receptor genes examined to date are intronless (Probst et al., 1992). The first intron was determined to localize within the IVth transmembrane domain and the second intron was assigned to the third intracellular loop of the deduced amino acid sequence. Among the genes encoding for G protein coupled receptors, introns (if present) generally tend to be positioned between the transmembrane domains. Notwithstanding, there are several known exceptions to this as exemplified by the human rhodopsin and opsin genes (Nathans et al., 1984, 1986) and the human serotonin (5-HT2) receptor gene (Chen et al., 1992). Interestingly, in terms of its
structure, the GnRH-R gene greatly resembles the human 5-HT2 receptor in several respects. Both possess a three exon - two intron gene structure and a gene size of \( \approx 20 \text{ Kb} \). Additionally, both receptors exhibit a major transcript of \( \approx 5 \text{ Kb} \).

High sequence identity among species for coding regions of the GnRH receptors existed. The coding region of human shared greater than 85 % sequence identity with mouse, rat, sheep, cow, and pig. Notably, the human GnRH receptor was encoded for by 328 amino acids which was identical to that of sheep, cow, and pig, but in contrast to the 327 amino acids described for both mouse and rat. Comparisons at the amino acid level, however, revealed similar results of greater than 85 % identity between human and other species. However, higher identity (>90 %) existed at the regions of the transmembrane domains. In particular, transmembrane domains II, III, V, VI, and VII are the most highly conserved. Regions of high conservation also existed in extracellular loop 1.

Interestingly, the deduced amino acid sequence revealed that the human GnRH receptor possessed several distinguishing features. Among these include the absence of a carboxy-terminal tail which has been previously described for the mouse and rat receptors (similar case subsequently reported for cow, sheep, and pig). This region has been linked to the coupling of G proteins, receptor desensitization, and
internalization (O’Dowd et al., 1988; Dohlman et al., 1991) in several G protein receptors. Notable exceptions include the β-adrenergic receptor which does not internalize (Hertel et al., 1990) despite the presence of a carboxy tail. As for the case of the GnRH receptor, it seems likely that throughout evolution the receptor has delegated those functions specific for the carboxy tail to other regions or that there exists a level of functional redundancy within the receptor. In support of this, a number of potential PKC phosphorylation sites, suggested to play a role in receptor desensitization, are present in transmembrane domains I and III for the human GnRH receptor.

The human GnRH receptor also did not retain the characteristic Asp-Arg-Tyr tripeptide motif of the G protein receptors. Instead, the receptor for GnRH substitutes a serine for the conserved tyrosine at position 140 thereby creating another potential phosphorylation site. In accord with these differences, the human GnRH receptor contained a reciprocal interchange of an asparagine at position 87 (other receptors; aspartate) and an aspartate (other receptors; asparagine) at position 318. This situation has also been described for all other GnRH receptors isolated to date. This amino acid switch is reportedly necessary for GnRH receptor - ligand binding (maintenance of ligand - binding conformation) as well as for signal transduction from the ligand - binding site to the regions involved in G protein
activation (Sealfon et al., 1993). It is interesting that these switches are necessary for the GnRH receptor when in other receptors the conservation of aspartate 87 and asparagine 318 are necessary for normal agonist binding and G protein coupling. In addition, two potential glycosylation sites at asparagines, position 18 and 102, have also been identified from the deduced amino acid sequence for the human GnRH receptor. These glycosylation sites have been implicated to be important for normal receptor expression and plasma membrane localization (Sealfon et al., 1993).

The GnRH receptor distinguishes itself from other G protein coupled receptors in other ways as well. In particular, the GnRH receptor possesses a long and highly basic first intracellular loop uncharacteristic of the G protein receptor family. It also possesses a seventh transmembrane domain which has an unusually high phenylalanine content.

Despite these unique differences, comparison of the human GnRH receptor amino acid sequence to other G protein coupled receptors also demonstrated evidence of similarity. The human GnRH receptor shares a number of amino acids which are highly conserved among the G protein receptors (Savarese and Fraser, 1992; Baldwin et al., 1993). Most of these are located within the transmembrane domains and are likely to be constituents of the ligand binding pocket of the
receptor. Amino acids such as proline in transmembrane domains II, IV, V, VI, and VII are highly conserved. In addition, other conserved residues include asparagine at positions 53 and 315, tryptophan at position 164, serine at position 167, and tyrosine at position 323. A highly conserved sequence of the G protein receptors, phenylalanine-X-X-cysteine-tryptophan-X-proline-tyrosine, is found in transmembrane domain VI. Additionally in the human GnRH receptor, there are two cysteine residues located in extracellular loop 1 and 2 which have also been found to be highly conserved among G protein receptors. Many of these amino acids mentioned above have suggested importance in either maintaining the structure (disulphide bridges, kinking of α-helices, etc.) or function (coupling to G protein, signal transduction) of G protein receptors suggesting why they are so highly conserved (Zhang and Weinstein, 1993).

The remarkable conservation of the transmembrane domains between the GnRH receptor and the other G protein coupled receptors suggests that these genes may have evolved from a common precursor more than 1 billion years ago (Klein et al., 1988). Mechanisms of convergent evolution that appear to have played a role in generating the multiplicity of the G protein receptor family involve gene duplication (Ohno, 1970) and retroposition (Brosius, 1991).
In accord with this, the high conservation of amino acids seen among the GnRH receptors of several species also suggests that the receptor itself performs a crucial function in reproduction of the organism.

Once the coding region for the human GnRH-R gene was sequenced and analyzed, continued sequencing and analysis of both the 5' and 3' ends followed. Points of focus included determining the localization of the human GnRH-R gene’s promoter and control region. As well, analysis of the 3’ end of the gene was undertaken to answer the question of polyadenylation and possible differential RNA processing.

4.1 Analysis of the 5’ end of the human GnRH-R gene

As previously mentioned, a lengthy 5’ nontranslated region was anticipated. Therefore, sequencing was performed for greater than 1.5 Kb upstream from the translational methionine start site. Analysis of this sequence for a specific DNA sequence(s) which contained the start site for RNA synthesis and signals where RNA synthesis should begin ensued. This sequence of interest, collectively called the promoter, has been identified in many genes by an A - T rich consensus region or
a G - C rich island. Genes possessing TATA promoters are generally associated with more regulated genes. Those that have G - C rich promoters have traditionally been linked to housekeeping genes. As for the human GnRH-R gene, five A - T rich regions described as TATAAA consensus sequences were identified within the 1.5 Kb stretch of 5’ sequence. TATAAA sequences are important in binding TF IID which in turn directs the correct initiation of RNA polymerase II for RNA synthesis. Additionally, several CAAT motifs were also located within this 5’ sequence. CAAT sequences are located in most eukaryotic promoter regions at positions -50 to -129 (from the transcriptional start) and are required for efficient transcription (Benoist et al., 1980). The CAAT motifs found within the human GnRH-R gene promoter region were located well within this range and found to cluster around the respective TATA sequences.

Many genes in the literature have been characterized as having only one promoter. However the finding of multiple TATA boxes within a single gene is not uncommon (Srikantha et al., 1990; Kawamura et al., 1992). What is unusual is the presence of consensus TATA boxes among the G protein coupled receptors sequenced to date. Instead, many of these genes have been identified as containing G - C rich promoter regions, including the genes for the mouse follicle stimulating hormone receptor (Huhtaniemi et al., 1992), human thyrotropin releasing hormone
receptor (Gross et al., 1991), and the human endothelin-A receptor (Hosoda et al., 1992). Of special note is the interesting dichotomy that exists in the rat luteinizing hormone receptor (Tsai-Morris et al., 1991). This receptor possesses a "mixed promoter"; several TATA boxes as well as G - C rich regions and, therefore, partially places itself along with the human GnRH receptor into the TATA-containing group of G protein coupled receptors.

It should be noted however that relatively few G protein coupled receptor promoter regions have been sequenced. Therefore whether or not the absence or presence of TATA boxes in the promoter region constitutes an identifying characteristic of the majority of this receptor family remains to be further clarified.

Nevertheless, the finding of multiple putative promoters for the human GnRH-R gene reveal several exciting possibilities. First, this finding suggests that the control region for the human GnRH-R gene is complex and highly regulated which is in accordance with the many detailed regulatory data that have been widely reported. More importantly, the existence of multiple promoters suggests the alternative use of promoters, possibly in a tissue-specific manner. Tissue-specific use of promoters found in the human GnRH-R gene may explain the differences exhibited between the receptors in different tissues.
The finding of consensus TATA sequences in the 5' end of the human GnRH-R gene was one step forward in establishing that the entire gene, including the 5' regulatory region for the human GnRH receptor, had been isolated. Further evidence came in the form of primer extension studies which defined the 5' termini for the human GnRH-R gene.

All five transcription initiation sites found for the human GnRH-R gene were positioned in close proximity to the TATA boxes. The compact and clustering nature of both the TATA sequences and the transcription initiation sites made assigning TATA boxes to their respective initiation sites a complex task. However, based on conforming spatial standards, TATA boxes at position -14, -111, and -150 were found to be related to transcription initiation sites +1, -101, and -124, respectively. As well, TATA boxes positioned at -718 and / or -731 are likely to be responsible for transcription initiation sites -669 and / or -690. Three out of five transcripts initiated from a cytosine base (+1, -101, and -669), while the remainder started from a thymine (-124) and an adenosine (-690). It has been observed that many but not all eukaryotic gene transcriptions initiate from an A residue (or another purine) surrounded by a pyrimidine rich region. This is true for transcription initiation site -690 which initiates from an adenosine. For the four other mRNA cap sites, they are pyrimidines and do not seem to follow this generalization. However,
it is probable that the A / G nucleotides positioned immediately next to these pyrimidines are the true sites of transcription initiation. This error in measurement (± 1 nucleotide) is likely due to using a single stranded template as a ladder rather than a double stranded one.

Major sites of transcription initiation for the human GnRH-R gene were also documented. Notable variability in the intensity of transcription initiation signals were obtained through primer extension analysis. These differences were reflective of the message level for the human GnRH receptor. Transcription initiation sites +1, -101, and -669 were accessed as major start sites of transcription for the human GnRH-R gene as evidenced by higher intensity levels. Transcription initiation -669 was deemed the major start site based on band intensity from primer extension results and on supporting RT-PCR data and mRNA size. Notably weaker signals located at -690 and -124 were classified as minor sites of transcription initiation.

The finding of multiple transcription initiation sites for the human GnRH-R mRNA brings forth the possibility that alternative transcription initiation sites are utilized in the pituitary, as well as, at extrapituitary sites and thus may in part account for some of the complexities observed regarding the regulation of expression of this gene.
Further confirmation of the primer extension results came in the form of RT-PCR analysis. Secondary in nature, these findings also served to confirm the isolation of the human GnRH-R gene in its entirety as well as the complete structure of the human GnRH-R gene.

PCR data confirmed transcription of the immediate 3' sequence from transcription initiation site -690 through to the coding region of the human GnRH-R gene. Identification of a 1393 bp 5' nontranslated region corresponding to transcription initiation site -690 is consistent with the size of the human GnRH-R mRNA of approximately 4.7 Kb as well as with reported PCR findings of Chi et al., (Chi et al., 1993) which predicted a size of at least 1.3 Kb of additional 5' nontranslated region (from the translational start) for the human GnRH receptor. Transcripts possessing extensive 5' nontranslated regions such as is the case for the human GnRH-R gene are not uncommon among vertebrates in general. Examples among others include those for human GnRH (Seeburg and Adelman, 1984) and human preproenkephalin B (Horikawa et al., 1983). Further to the RT-PCR data, the finding of no amplifiable product upstream of transcription initiation site -690, excluded the possibility of additional 5' initiation sites. Taken together, these data support the primer extension findings mentioned above.
It seems likely that the human GnRH-R gene is highly regulated at the transcriptional level as revealed through the discovery of multiple initiation sites and putative promoters for this gene. More recent reports have suggested that another control switch, at the level of translation, may play a larger role in the regulation of genes than previously ascribed. This possibility of additional regulatory control in existence for the human GnRH-R gene was supported by several lines of evidence. First, the presence of an extensive 5' nontranslated region supports the notion of additional controls. In vitro studies have shown that a longer 5' nontranslated region increases the efficiency of translation over that of a shorter one and may be necessary for the efficient initiation of translation in some genes (Kozak, 1991).

Second, the presence of several ATG (AUG) codons upstream of the major reading frame for the human GnRH-R gene also points toward additional translational controls in effect. Of the vertebrate mRNAs analyzed to date, fewer than 10 % have been found to contain upstream initiation codons (Geballe and Morris, 1994). Interestingly, many of these mRNAs were identified as those having a fundamental role in cell proliferation and development. In particular, mRNAs specific for proto - oncogenes, growth factors, and cell-surface receptors were of special note.
It is therefore of interest when several ATG codons were located upstream of the reading frame for the human GnRH receptor since it is almost always from the first ATG codon that translation initiates. Examination of the flanking sequences around the upstream ATGs indicated poor sequence identity to conserved initiation motifs. These data support the possibility of ribosomal ATG skipping, that is ribosomes scan sequence for an optimal ATG match from which translation is initiated. This somewhat rarely studied event has been observed in other genes (Geballe and Morris, 1994).

Collectively, these data support the existence of potential translational controls in operation. Detailed studies focusing on the translational control of GnRH receptor expression and on the potential regulators themselves may be necessary in order to fully understand the underlying mechanisms involved in GnRH receptor regulation.

In addition to identifying several upstream ATGs, the human GnRH-R gene was evaluated for potential cis - acting regulatory sequences in an attempt to understand the receptor’s regulation at the genomic level.

Physiological paradigms of GnRH receptor regulation abound in rat and sheep
models studied thus far. Similar detailed studies concerning the regulation of the human GnRH receptor are still lacking. Nevertheless in animal models, several parallel observations can be drawn. Various regulators such as GnRH, 17β-estradiol, progesterone, inhibin, and cAMP play a role in the regulation of the pituitary GnRH receptor (Miller et al., 1993; Sealfon et al., 1990; Laws et al., 1990b). The 5′ end of the human GnRH-R gene was therefore evaluated for binding sites which have been demonstrated to confer responsiveness.

No similar sequence to an estrogen responsive element (ERE) could be found using BESTFIT or TF SITES analysis. This was somewhat unexpected since several regulatory studies in rat and sheep have demonstrated estradiol mediated up-regulation of GnRH receptors. However, these findings do not rule out that an ERE may be found beyond the 5′ and 3′ sequence obtained or within the intronic regions of the gene. These data do suggest, more importantly, that estrogen may have a complex indirect role in the regulation of the GnRH receptor, such as, in activating other genes whose products in turn trigger the delayed secondary response in receptor expression.

Further searches for potential responsive elements were successful however. Two responsive elements of particular interest were identified after computer
analysis. These two sequences were identified using BESTFIT comparisons and consequently were not perfect matches to their respective consensus sequences. In addition, these two sequences were not identifiable using the "perfect match" TF SITES file. An element with sequence similarity to a CRE was located at -1490 to -1483. The consensus CRE sequence of 8 bp has also been identified in the regulatory region of other genes that are activated by cAMP. Further, this sequence is recognized by a specific gene regulatory protein called the CRE - binding (CREB) protein. CREB is phosphorylated by protein kinase A in response to increased cAMP levels which in turn stimulate transcription of these genes. The finding of a CRE - like sequence supports regulatory studies which have shown cAMP (or analog) involvement in mimicking GnRH mediated GnRH receptor up - regulation (Young et al., 1984) and suggests a direct stimulatory pathway for cAMP.

Additionally, a 15 bp GRE / PRE - like sequence (-78 to -92) was also identified within the 5’ end of the human GnRH-R gene using BESTFIT analysis. The consensus sequences for the GRE and PRE are identical. They are composed of two half site sequences of 6 bp separated by a variable region of 3 bp and bind to glucocorticoid and progesterone receptors (GR; PR). Regulatory studies in rat and sheep pituitary cell cultures have identified progesterone as a regulator of GnRH receptor expression. Reports have indicated progesterone action as inhibitory on the
receptor's expression (Laws et al., 1990b; Sealfon et al., 1990) while others have established a stimulatory role for progesterone (Miller et al., 1993). A search for negative response elements revealed limited information regarding these sequences. There is a total of 11 such elements published for the glucocorticoid receptor (Akerblom and Mellon, 1991). These elements were searched for throughout the human GnRH-R gene and no observable sequence similarity existed.

It should be noted as well that the human GnRH-R gene was also searched for potential GRE/PREs using the "perfect match" TF SITES computer analysis. This analysis identified 4 isolated and identically matching GRE/PRE half sites within the receptor gene sequence. However the functional importance of isolated half sites is doubtful. Genes which have been identified as having a single response element half site have never demonstrated their involvement in gene regulatory control. Further, for genes with well isolated multiple half sites, data on their involvement in gene regulation have never been reported as well.

It is evident that controversy still abounds regarding the regulatory effects of potential regulators. Whether these regulators have primary or secondary responses in gene expression remains to be seen. However, it is hoped that the findings of these regulatory elements may facilitate the clarification of some of these
discrepancies. Initial steps towards this clarification were undertaken in subsequent studies described below.

Mobility shift assays were employed in order to examine potential CRE and GRE / PRE binding activity. This DNA - protein binding assay served to answer the question of CRE and GRE / PRE function in vitro.

Before these experiments could be performed, the first task was to obtain sufficient quantities of the CREB protein and the progesterone receptor. After a detailed review of the literature, HeLa cells and MCF-7 cells were chosen as sources for these proteins. MCF-7 cells were pre-treated with estradiol in order to increase the progesterone receptor levels by approximately five - fold and consequently increase the efficiency of nuclear extract. Nuclear extract prepared from both cell lines yielded similar results for CRE experiments indicating sufficient concentrations of CREB in MCF-7 cells as compared to HeLa cells. This was not surprising since reports have indicated cAMP involvement in the regulation of various genes in these cell lines. Thus, all remaining experiments were conducted with MCF-7 as a source for both CREB and progesterone receptor proteins. Once the initial steps were accomplished in these DNA - protein binding assays, experiments proceeded with the actual testing of these regulatory elements.
As mentioned above, progesterone and cAMP have been implicated in the regulatory control of the GnRH receptor and the findings of these regulatory elements at first seem to support these regulatory data. However, once mobility shift assays were performed on both the CRE - like and GRE / PRE - like sequences, no binding of protein to these elements was found. The absence of protein - DNA complexes indicated that the CRE and GRE / PRE elements found within the human GnRH-R gene were functionally void. These findings were not surprising however based on the nucleotide alterations which these elements possessed.

Two mismatches out of a total of 8 bp, as demonstrated through gel shift experiments, represented a significant degree of dissimilarity for the CRE - like element. This amount of variability could not be compensated due to the inability of the CREB protein to recognize this sequence as a true CRE. In addition, this suggests that the two mismatches of a "C" to an "A" and an "A" to a "T" are crucial to CREB protein binding. Also, these data imply that cAMP either acts indirectly in the control of the human GnRH-R gene or a functional CRE may be located elsewhere within the gene.

Gel-shift assays also demonstrated no protein-DNA complex for the human
GnRH-R GRE / PRE related sequence. Detailed examination, of the base pair changes for the GRE / PRE - like sequences found in the human GnRH-R gene, revealed insights as to why this sequence was shown to be nonfunctional in DNA - binding assays.

The base pairs of crucial importance in GRE / PRE elements have been defined (Beato et al., 1993). At position 1 in the half site (Table. 2), the presence of a thymine (T) base is necessary for glucocorticoid receptor (GR) and progesterone receptor (PR) binding. The 5’ methyl group of the T base has been implicated as the receptor contact site. Modification of this base has been shown to interfere with the binding of GR (Cairns et al., 1991). Further, a T base residing in position 3, has been shown to be essential for discrimination of GRE / PRE sequences. The base at the fourth position is the only site not contacted by the receptor. Nonetheless, the preferred base in this position is still a pyrimidine. The fifth position is occupied by a cytosine (C) and has been shown to be very important for binding of the GR and PR. This site has been identified as the most highly conserved position in GRE / PRE sequences isolated to date. The sixth and last position of the half site is occupied by a thymine in 60 % of all GRE/PRE sequences, although mutation to a C base does not interfere with GR or PR binding.
All positions mentioned above are occupied by the requisite base in the human GnRH-R GRE / PRE related sequence. However, the one position that is not conserved within the human GnRH-R GRE / PRE-like element is the guanine (G) base at position 2. Instead an adenosine is found in this position for the human GnRH-R gene. Contact of the GR and PR (even the estrogen receptor) to the guanine at the second position of the half-site is achieved by interaction with the N-7 position as demonstrated by methylation experiments (Scheidereit and Beato, 1984; Truss et al., 1991). This highly conserved G base is of paramount importance to a functional GRE / PRE since it is the essential component involved in the high affinity binding of receptors to their hormone response elements. This base has been suggested to be the contact site for an amino acid side chain, such as arginine 489, arginine 496 or arginine 466 in rat GR, which is conserved throughout the steroid hormone receptors. Therefore it is concluded that this G to A base switch in the human GnRH-R GRE / PRE, is the underlying reason why this steroid response-like element is nonfunctional in the human GnRH-R gene.

Although, these data have supported mainly indirect regulatory pathways for GnRH receptor expression in humans, it should be remembered that the regulatory studies at the physiological and mRNA level for the pituitary have been performed in mainly animal models only. Therefore the possibility of different regulatory
responses for cAMP, progesterone, and other hormone regulators in the human can not be excluded. In accord with this, many genes have been reported to possess very different responses in human tissues than in other animal models.

In addition to investigating potential regulatory elements, consensus binding sites for some typical transcription factors known to regulate eukaryotic promoters were also examined using TF SITES analysis. As mentioned, this computer analysis identifies only those sequences within the human GnRH-R gene which are perfect matches to the transcription factor binding sites located within the database. Therefore all sites identified are most likely active within the human GnRH-R gene.

A GATA-1 binding site was found in close proximity to TATA -150 and may likely contribute to its regulation. The engrailed binding site was found to overlap with TATA -150. The engrailed protein has been shown in in vitro studies to negatively regulate genes by competing with transcription factor IID for binding sites. This transcription factor may have a similar role in the regulatory control of the human GnRH-R gene. Additionally, several PEA-3 binding sites (Xin et al., 1992) were located upstream of TATA -731 and -718. Of special note, the binding site for PEA-3 has also been recognized to function as a phorbol ester binding site in some genes. This finding is in accordance with several regulatory studies which
have shown a role for phorbol esters in the regulation of the human GnRH receptor. A binding site for WAP (whey acidic protein; Lubon and Hennighausen, 1987), a mammary gland specific protein, was also found within the promoter region. Interestingly, an AP-1 binding site, which would confer protein kinase C responsiveness, was located in close proximity to TATA box -731 and -718. These TATA boxes are the suggested control region for the major 4.7 kb transcript of the human GnRH receptor. These data suggest that AP-1 may have a potential role in the regulation of the GnRH receptor.

Of special interest is the identification of a Pit-1 binding site (Faisst et al., 1992) within the receptor sequence. Pit-1, an anterior pituitary specific transcription factor, has been reported to be involved in the regulation of anterior pituitary hormones, such as, in the activation of the growth hormone (Bodner et al., 1988) and prolactin (Ingraham et al., 1990) genes. Similarly, Pit-1 transcription factor may have possible implications on the regulation of the anterior pituitary GnRH receptors through activation of the human GnRH-R gene.

Further analysis of the human GnRH-R gene sequence revealed the presence of a truncated (150 bp) Alu repetitive element (Fig. 5; position -1300 to -1150; Kariya et al., 1987). Alu repeats are transposable elements, generally 300 bp in
size, and consist of two alternating monomer units. Alu sequences are able to move and create target-site duplications when it inserts. Alu repeats are characteristically present in primate species and are remarkably abundant within the genome. Approximately 500,000 repeats are present per haploid human genome constituting 5% of human DNA. In other words, Alu repeats are present on average about once every 5,000 nucleotide pairs.

Functionally speaking, it is unclear as to what Alu sequences do. It has been suggested that they are derived from retroposition of an internally deleted host-cell RNA gene, 7SL, which encodes for the RNA component of the signal-recognition particle (SRP) that functions in protein synthesis (Chen et al., 1985). As well, Alu sequences have been implicated as start sites in DNA replication (Ariga, 1984), inhibitors of gene conversion (Hess et al., 1983), hot areas for recombination (Rogers, 1985), modulators of chromatin structure (Duncan et al., 1981), and as stabilizing sequences for cytoplasmic mRNA (Robertson and Dickson, 1984).

Comparisons of the Alu sequence in the human GnRH-R gene to other Alu-like sequences of other genes indicate notable sequence similarity. In addition, based on species distribution and sequence similarity, evidence suggests that these repetitive sequences have multiplied to high copy relatively recently (Deininger and
Daniels, 1986). It is tempting to speculate that these highly scattered and abundant sequences in the genome have had major effects on the expression of many nearby genes, including that for the human GnRH receptor.

4.2 Analysis of the 3' end of the human GnRH-R gene

Once the analysis of the 5’ end of the GnRH-R gene had been accomplished, efforts were focused on examining the 3’ sequence. It was hoped that the 3’ end would reveal some clues as to how the GnRH-R gene’s complex regulatory mechanisms might be achieved.

The 3’ sequence revealed a complex pattern of multiple polyadenylation signals much like the situation found for the TATA boxes and initiation sites at the 5’ end of the human GnRH-R gene. Five polyadenylation signals were of the classical AATAAA type found for eukaryotic genes. However, two additional polyadenylation signals possessed the sequence ATTAAA which has been shown to be a functional variant of the classical termination signal. The impact of these findings suggested that there were potentially seven sites for 3’ termination. Further support came in the finding of several ATTTA motifs (Shaw and Kamen, 1986),

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which have been implicated in mRNA instability and are notably present in many rapidly degraded mRNAs, suggesting that the site of transcriptional termination is contained within this 3' sequence obtained.

However, without a full length human GnRH-R cDNA being available, the major polyadenylation site remains unknown. In order to delineate the most 3' polyadenylation site used in the pituitary, PCR analysis of the 3' end with pituitary cDNA was conducted. PCR amplification of the entire 3' sequence up to the polyadenylation signal at position +4767 was successful. Several controls implemented excluded any confounding factors which may have compromised these results.

Summing up the figures of approximately 1.4 Kb of 5' nontranslated (most 5'), 3.1 Kb of 3' nontranslated, and 1 Kb of coding region brings the amount of mRNA accounted for at 5.5 Kb. These values corroborate well with the size of the human GnRH-R mRNA of 4.7 Kb including 200 to 400 bp allotted for a poly-A tail. Therefore these findings strongly indicate that the functional polyadenylation site for this transcript is located well within the 3' nontranslated region sequenced.

Additionally, the finding of multiple polyadenylation signals is of significant
value, particularly for its potential impact on the expression pattern of the human GnRH receptor. The likelihood of differential RNA processing, occurring through the use of alternative cleavage / polyadenylation signals and consequently the existence of multiple human GnRH-R transcripts, must be considered in light of these present findings. Support for the potential use of alternative termination signals (and / or alternative promoters and initiation sites) in the regulation of the human GnRH receptor came in 1994 with the finding of minor transcripts of sizes 2.5 and 1.5 Kb in the pituitary (Kakar et al., 1994). From the Northern data presented here, polyadenylation signal +4767 is likely to be responsible for the major transcript of 4.7 Kb. As for the minor transcripts, examination of the gene sequence reveals an additional termination signal located at +2595, which in light of the recent data, may likely be responsible for the smaller messages based on spatial location. It is highly doubtful that the 1.5 Kb transcript encodes for a functional receptor and it is speculated that this message is a truncated transcript.

Overall, the analysis of the human GnRH-R gene revealed that structurally, it possessed the characteristics of a highly complex and regulated gene. Discoveries of multiple putative promoters, initiation sites, and polyadenylation signals elude to intricate regulatory mechanisms in operation. Any one of which could be attributable to its multi-factorial regulation.
One further analysis of the human GnRH-R gene was performed in an attempt to elucidate any closely related sequences to the GnRH-R gene. Overall analysis of the gene for the human GnRH receptor revealed several interesting discoveries. Comparison to the other 75 G protein coupled receptors isolated to date revealed little sequence similarity. This was of particular interest considering that a common origin for this receptor family has been predicted. Relatively little sequence similarity suggests that considerable divergence has occurred throughout evolution or perhaps the single precursor theory should be expanded to include the involvement of several evolutionary precursors.

Some sequence similarity existed, however, between the human GnRH receptor and a few G protein receptors. These included the human oxytocin receptor (Kimura et al., 1992), the human V2 vasopressin receptor (Birnbaumer et al., 1992), the rat V2 vasopressin receptor (Lolait et al., 1992), and the rat V1A vasopressin receptor (Morel et al., 1992) which all shared approximately 25% identity to the human GnRH receptor.

Interestingly, the GnRH receptors in general have no significant sequence identity to the yeast STE2 receptors (Burkholder and Hartwell, 1985; Marsh and Herkowitz, 1988), except to a minor degree in the transmembrane domains. The
ligand for the STE2 receptor is α-factor, a yeast pheromone, that has close sequence similarity to GnRH in its amino-terminus and is known to bind to GnRH receptors. In addition to its structural similarities, the α-factor and GnRH also share functional commonality. In rat pituitary gonadotropes, α-factor has been demonstrated to stimulate the release of gonadotropins, an action that was blocked by GnRH antagonists (Loumaye and Catt, 1982). However, despite these structural and functional similarities between α-factor and GnRH, no significant sequence identity is observed between their respective receptors.
V. SUMMARY AND CONCLUSIONS

This investigation has been concerned with the isolation of the human GnRH-R gene and its consequent characterization at the genomic level.

The major procedures employed during this thesis are summarized as follows:
1) High stringency genomic library screening with a human GnRH-R cDNA probe was conducted. 2) Southern blot and restriction analyses of genomic clones were performed in order to confirm the authenticity of the clones as being specific for the human GnRH-R gene. 3) Subcloning of the 7.7 Kb, 7.4 Kb, 3.8 Kb, and 2.7 Kb genomic fragments into pUC19 vector was performed. Dideoxy DNA sequencing of the isolated subclones was performed using universal forward and reverse primers and sequence specific oligonucleotides. All sequence analyses were conducted using GCG Computer Group Inc. software. 4) Characterization of the 5’ terminus for the human GnRH-R gene was carried out using primer extension analysis, RT-PCR analysis, and Southern analysis. 5) MCF-7 cell lines were cultured to confluency as a source of PR and CREB proteins. 6) DNA-protein binding studies were performed using gel mobility shift assays. 7) Southern blot and RT-PCR analyses were used to confirm both the structure and the 3’ terminus of the human GnRH-R
gene. 8) Genomic Southern blot analysis was performed in order to detect the human GnRH-R gene directly from chromosomal DNA. 9) Somatic - cell hybrid analysis was employed in the segregation of the human GnRH-R gene to a unique chromosome in the genome. 10) Northern blot analysis was employed in order to investigate the number and size of mRNA transcripts encoding for the human GnRH receptor.

The literature review dealt with the general aspects of GnRH receptors, including function, distribution, and regulation. The results of this thesis were presented, illustrated, and discussed. It was found in this investigation that:

1. Genomic library experiments resulted in the isolation of twelve positive clones for the human GnRH-R gene after tertiary screening.
2. After purification of λ-clones (1 and 2) to homogeneity, a 5' or 3' oriented λ-DNA was identified and confirmed by Southern analysis.
3. Restriction endonuclease digestions revealed that λ-1 contained a 5' oriented 7.7 Kb EcoRI genomic fragment of interest. Also it was observed that λ-2 contained 3' oriented EcoRI fragments of 7.4 Kb and 3.8 Kb in size.
4. Nucleotide sequencing revealed that the entire gene for the human GnRH-
R gene was contained within these isolated genomic clones. Also through sequencing, the coding region was found to be identical to the subsequently published human cDNA as well as exhibit >85% sequence identity to other species.

5. Structurally, the human GnRH-R gene was demonstrated to possess three exon and two introns distributed over a span of 18.9 Kb. Exon I, II, and III consisted of 1915 bp, 219 bp, and 3321 bp, respectively. Intron A and B consisted of approximately 4.2 Kb and 5.0 Kb, respectively. The coding region, 5' nontranslated, and 3' nontranslated were distributed over 987 bp, ~1.4 Kb, and ~3.1 Kb, respectively.

6. Five consensus TATA sequences were revealed in the control region of the human GnRH-R gene through sequencing.

7. Five transcription initiation sites spatially related to the TATA sequences were also identified by primer extension analysis.

8. Consensus transcription factor binding sites were located within the 5' end of the gene.

9. A CRE - and GRE / PRE - like sequences were identified as potential regulatory elements for the human GnRH-R gene.

10. Mobility shift assays demonstrated the absence of protein binding to these regulatory elements.
11. Five classical polyadenylation sites in addition to several ATTTA motifs were found within the 3' end of the human GnRH-R gene.

12. Genomic Southern analysis identified that a single gene encodes for the human GnRH receptor in the genome.


Prior to this work, molecular characterization of the GnRH receptor was limited to the cDNA level. Similar studies on the gene had not been accomplished. In addition, the reported cDNA sequences represented only a small fragment of the GnRH receptor. Therefore this left several inherent and unique properties of the GnRH receptor unknown. Questions concerning its regulation, promoter, and control region as well as its 3' termination region, structural organization, and location in the genome remained unanswered. Experiments of this investigation served to answer these questions in addition to others which could not be investigated by analysis of the cDNA alone but rather required the detailed study of the gene itself.
In summary, the experiments of this thesis demonstrated for the first time, the isolation and characterization of the GnRH-R gene in any species. The implications of the human GnRH-R gene being single copy in nature suggests that the tissue-specific differences observed for some GnRH receptors are likely attributable to fine regulatory controls at the genomic level. This hypothesis was supported by the finding of an extensive 5' leader sequence, multiple promoters, initiation sites, and polyadenylation signals for the human GnRH-R gene. Structurally complex 5' and 3' control regions as demonstrated in these present studies are consistent with the multi-factorial regulation of the GnRH receptor previously described and point towards their involvement in the complex expression pattern of the GnRH receptor. Equally significant, these data support the existence of variable transcripts encoding for the receptor by the use of differential promoters and/or termination signals in the expression of the GnRH receptor.

Collectively, these data delineated the entire locus of the human GnRH receptor and provided the foundation for subsequent DNA-binding studies to determine regulatory sequences involved in the regulation of the human GnRH-R gene. DNA-binding studies performed in this study support indirect regulatory pathways for cAMP and progesterone in the control of human GnRH receptor regulation via related sequences identified in the promoter region.
In addition, the assignment of chromosome 4 as the carrier for the human
GnRH-R gene should facilitate future studies on the identification of closely linked
genes and on the examination of possible chromosomal rearrangements involving the
human GnRH receptor. Of note, use of a genomic clone isolated from this
investigation has already resulted in the subchromosomal localization of the human
GnRH-R gene to 4q21.2.

The isolation and characterization of the human GnRH-R gene as
demonstrated in this present investigation establishes a basis for which analyses
concerning the evaluation and identification of possible genetic disorders of the
GnRH receptor can be accomplished. Further, cloning and sequencing of the human
GnRH-R gene should allow for the production of improved analogues of GnRH for
use in clinical treatment of certain reproductive maladies. Moreover, cloning of the
receptor allows questions to be raised beyond the immediate world of peptide
hormone biology. The receptor sequences revealed in this study should help in the
understanding of what parts of the conserved structure are essential for proper
receptor function (ligand binding; recognition of specific signal transduction G
proteins).

Future characterizations of the GnRH-R gene in other species and tissues will
be necessary in order to fully understand both the regulation and mechanism of action of the GnRH receptor. Of primary importance will be the determination of the major promoter used in the pituitary and in other tissues. Technically, these experiments should be straightforward and would require the use of constructs containing the 5' promoter sequences and transfection assays. In addition to examining promoter activity, these transfection assays would also serve as an experimental system for which transcriptional regulation of the human GnRH-R gene at the genomic level could be directly studied. Additionally, it will be of interest to assess what potential tissue-specific controls, delineated in this investigation, are actually in operation in various tissues. Preliminary studies focusing on a possible molecular basis for the tissue-related differences of the GnRH receptors may be achieved by utilizing GnRH receptor expressing tissues along with RT-PCR analysis and DNA sequencing. It is clear that these studies in addition to others are essential to further the molecular biological understanding of the GnRH receptor.
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