DISTRIBUTION AND BIOSYNTHESIS OF LH-RH AND STRUCTURALLY RELATED PEPTIDES.

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ABSTRACT

Biologically active peptides are a class of molecules which can, in general, be grouped into families of structurally related moieties which have a wide somatic distribution and which subserve multiple physiological roles. Thus, the releasing factors of the hypothalamus or their structural analogues may carry out multiple endocrine, paracrine, or neurotransmitter functions outside the hypothalamus. This thesis describes the distribution and biosynthesis of Luteinizing Hormone-Releasing Hormone (LH-RH) and structurally related peptides in mammalian tissues and tumours.

Two strategies were employed in this investigation:

(a) Immunological screening of tissues for suitable sources of LH-RH and the partial characterization of the LH-RH immunoreactive peptides of selected tissues;

(b) Complemetary oligonucleotide screening of mRNA from certain tissues, cDNA and genomic libraries as well as genomic DNA restriction endonuclease fragments to establish the nature of the LH-RH precursor and related molecules.

Acetic acid extracts of nineteen rat tissues were assayed in serial dilution for LH-RH immunoreactivity (LH-RH-IR). An antibody directed to the middle of the LH-RH sequence was used in these screening radioimmunoassays. LH-RH-IR was found in extrahypothalamic brain areas, in gastroenteropancreatic tissues, in the retina, the submandibular gland, the thyroid and the testes.

In view of a putative role of endogeonous LH-RH-like immunoreactive peptides in intra-testicular regulation, the

molecular nature of the LH-RH-like material detected in rat testes was investigated. Acetic acid extracts of adult rat testes were partially purified by LH-RH-immunoaffinity chromatography. On Sephadex G-100 this material separated into four major peaks of >100K, ~32K, ~5K and <4K daltons. The <4K peak of LH-RH-IR eluted differently on Sephadex G-25 and analytical reverse phase HPLC than did synthetic hypothalamic LH-RH decapeptide. Antibodies directed at the C-terminus of LH-RH gave higher estimates of LH-RH-IR for all the chromatographically separated testicular polypeptides than did N-terminally and middle directed antisera. The small testicular LH-RH-like peptides displaced radiolabelled LH-RH agonist from rat pituitary membranes more effectively than did the larger immunoreactive molecules.

Monkey, pig and dog testes as well as dog Sertoli cell tumours were extracted with acetic acid and shown to contain LH-RH immunoreactive material similar to that of the rat. Human seminal plasma was also investigated as a source of LH-RH-IR. When pooled seminal plasma from azoospermic males was fractionated by gel filtration some fractions had significant amounts of the rat testicular type of LH-RH.

In order to investigate the biosynthesis of LH-RH and its structural variants, mRNA studies were performed, cDNA libraries constructed and screened together with genomic libraries and DNA restriction endonuclease fragments. LH-RH immunoreactive material was found to be associataed with membrane bound polysomes and not free polysomes in ovine hypothalami. This has been demonstrated for other secreted proteins. The Guanidinium Hydrochloride, Urea-Lithium Chloride, and Phenol-mCresol methods

of mRNA purification were used to prepare RNA for *in vitro* translation, Northern blot analysis and cDNA library construction. Rabbit reticulocyte lysate cell free synthesis as well as Xenopus oocyte translation techniques were used to determine the translatability of the prepared mRNA. Radiolabelled cell free synthesised products of hypothalamic mRNA translation were precipitated by antisera against somatostatin, but not by antisera against LH-RH. In the Xenopus oocyte translation system, immunoreactive LH-RH was detected in the incubation medium of some oocytes injected with hypothalamic mRNA. The small number of neurons synthesizing LH-RH relative to somatostatin in the hypothalamus could explain these results.

A series of oligonucleotides complemetary to the N-terminal and C-terminal coding sequences of LH-RH were synthesised by the phosphoramidite intermediate technique. The most suitable oligonucleotide probes for the LH-RH mRNA sequence were deduced from evolutionary considerations of the known vertebrate variants of LH-RH. This was achieved by accommodating the changes in the vertebrate LH-RH sequences during evolution by presuming minimal base changes in the mRNA coding for the variant peptides. The observation that there is a significantly higher incidence of specific codons for any one amino acid in the neuropeptide hormones sequenced to date, was also used to predict the most likely LH-RH mRNA sequence.

In order to investigate the nature of the LH-RH precursor, poly A mRNA was prepared from rat and sheep hypothalami, and two human mammary tumour cell lines. Rat hypothalamic mRNA species of ~500 bases which hybridized to certain probes directed to both N- and C-terminal coding sequences of LH-RH. Sheep hypothalamic

mRNA preparations had five distinct mRNA northern blot positive hybridization bands of ~1.8Kb, 1.45Kb, 1.1Kb, 0.65Kb and 0.50Kb. The variation in the size of mRNA species positive for LH-RH coding sequences in the different tissues screened could be unprocessed LH-RH hnRNA while the smaller species may result from degradation. Differential splicing during mRNA maturation could also explain the different sizes of LH-RH coding mRNAs. Alternatively, each mRNA could be an authentic mRNA arising from different genes coding for LH-RH itself or a LH-RH-like peptide. Differential splicing and 2 transcription start sites in a single gene have been determined to be the most likely explanation for these multiple RNA species.

Primer extension studies of rat and human hypothalamic poly A RNA as well as human breast tumour cell line poly A RNA, demonstrated that the most abundant LH-RH encoding mRNA in all these tissues has a 5′ end ~108 bases upstream from the 1st base of the LH-RH encoding sequence.

Chain termination DNA sequencing of one of these clones established 5′ end sequencing consistent with it being a full length clone. Examination of the human placental LH-RH cDNA sequence immediately upstream of the potential RNA polymerase II cap site indicated by this clone, revealed TATA box and CAAT box sequences as well as CAAT box associated Y box sequences and a possible enhancer core element immediately upstream from the CAAT box. The presence of another transcription initiation start site further upstream (as predicted by the human placental cDNA sequence) was supported by longer primer extended cDNAs in every tissue tested. The longer primer extended products in the hypothalamus indicated that the 1st intron in the gene is always

cleaved out during mRNA maturation in this tissue while this is not always the case in the human breast tumour cells.

GnRH cDNA enriched libraries of the preoptic area of rat hypothalamus were constructed in the plasmid pGem-3 by specific primer extension and restriction endonuclease digestion. LH-RH coding sequence positive inserts of ~330b, were obtained from clones from the latter two libraries.

The Margaret Dayhoff Protein Sequence Library was scanned for mammalian LH-RH-like amino acid sequences. Of the protein and peptide sequences reported to date, one peptide has 9 amino acids in common with mammalian LH-RH, one has 8 in common, one has 7 in common, nineteen have 5 in common, while two hundred and seventy eight have 4 in common. These sequences include variant vertebrate LH-RHs, viral, bacterial, fungal and animal proteins. It is conceivable that a large number of these similar amino acid sequences have arisen independently of the LH-RH sequence. However, the high scores achieved by some of these sequences in the Mutation Data Matrix Matching of the Dayhoff protein sequence search for LH-RH-like sequences, suggest very strongly that a considerable number have arisen from an ancestral gene common to that of LH-RH. The fact that some of these sequences occur in the simplest life forms suggests that a LH-RH-structure-related ancestral gene arose very early in evolution. It has also been noted that some of the structurally related peptides have gonadotropin releasing activity and/or LH-RH receptor binding activity in <u>in vitro</u> bioassay systems.

In summary, this thesis provides evidence for a LH-RH family of peptides in various mammalian tissues and in a wide range of species, similar to the other known families of neuropeptides.

Evidence is also provided for the existence of two transcriptional initiation sites in the only LH-RH gene characterized to date. *In situ* hybridization and S1 nuclease protection assays for LH-RH mRNA quantitation have indicated that LH-RH gene transcription may be upregulated in certain scenarios where serum LH levels are decreasing.

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CONTENTS

ABBREVIATIONS AND SYMBOLS

LH-RH	Luteinizing Hormone-Releasing Hormone (synonymous with Gonadotropin-Releasing Hormone)
LH-RH-IR	Luteinizing Hormone-Releasing Hormone Immunoreactivity
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol
NaCL	Sodium Chloride
$MgCl_2$	Magnesium Chloride
W/V	Weight per unit Volume
NaOAc	Sodium Acetate
GuHCl	Guanidinium Hydrochloride
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetate
KCl	Potassium Chloride
OD_{260}	Optical Density at 260 nanometers
SDS	Sodium Dodecyl Sulphate
MOPS	Morpholinopropanesulfonic Acid
BSA	Bovine Serum Albumin (Pentax Fraction V)
PVP	Poly Vinyl Pyrrolidone
NaPPi	Sodium Pyrophosphate
^oC	Degrees Centigrade
dNTP	Deoxynucleotide Triphosphate
~	Approximately
$CaCl_2$	Calcium Chloride
ATP	Adenosine Triphosphate
L-broth	Luria-Bertani broth
NaOH	Sodium Hydroxide
$MgSO_4$	Magnesium Sulphate
TMACl	Tetra Methyl Ammonium Chloride
DMSO	Dimethyl Sulphoxide

PBS	Phosphate Buffered Saline
TFA	Trifluoro Acetic Acid
HFBA	Heptafluoro Butyric acid
HPLC	High Performance Liquid Chromotography
Na_2HPO_4	Disodium Hydrogen Phosphate
TE	10mM Tris/HCl (pH 8.0), 1mM EDTA
TBE	10mM Tris/Borate (pH 8.3), 1mM EDTA

CHAPTER 1

INTRODUCTION: NEUROPEPTIDE DISTRIBUTION AND HETEROGENEITY

1.1 Peptide Diversity in Mammalian Brain

In the last decade a large number of peptides originally characterized in non-neuronal tissues have also been found in the mammalian central nervous system. The converse of this statement is also true. Thus, for example, some of the so-called hypothalamic regulatory factors have been reported to occur in other organs and body fluids outside the brain, while some of the so-called gastrointestinal peptides (e.g. Vasoactive Intestinal Polypeptide, VIP) have been found in the brain. Three major concepts have resulted from these findings, viz:

(i) The brain contains a wide variety of peptide-containing cell bodies or peptidergic neurones which are distributed in distinct fibre pathways. In these distinct neuronal networks, these locally synthesised peptides are seen to function as neurotransmitters in their own right or as neuromodulators which block or alter the turnover of other neurotransmitters.

(ii) Peptides either identical to or similar to the neuropeptides are distributed in a wide variety of tissues where they usually function as hormones. These non-neuronal peptides function in systems which are, in the greater part, independent of and functionally unrelated to the involvement of their counterparts in the central nervous system.

(iii) Some peptides have been co-opted for many regulatory functions in a wide variety of tissues during evolution.

Table I lists the various categories of peptides which have now been detected in the mammalian brain.

1.2 Families of Structurally Related Peptides

It has been observed that among the peptides there exist definite groups having distinct structural similarity. Thus the VIP "family" of peptides includes CCK, Secretin, Gastrin, Glucagon, GIP (Gastric Inhibitory Peptide), PHI (Peptide having N-terminal histidine and C-terminal iso-leucine amide) and GH-RF (Growth Hormone-Releasing Hormone) (Said, 1980; Tatemoto and Mutt, 1980; Guillemin et al., 1982; Rivier et al., 1982). All the members of the VIP family have considerable amino acid sequence homology.

Other peptide families include: the Opioid family of Endorphins, Neo-endorphins, Dynorphins and Enkephalins (Gubler et al., 1980; Kakideni et al., 1982); the MSH family (Liotta et al., 1982); the Oxytocin-Vasopressin and Neurophysin family (Land et al., 1982, 1983); the Insulin-Like Growth Factors (Dull et al., 1984); the CRF, Sauvagine, Urotensin family (Shibahara et al., 1983); the Gonadotropin, Thyrotropin family (Pierce et al., 1981); and the Growth Hormone, Prolactin, Placental Lactogen family (Wallis, 1975). Within each of these groups the peptides have striking sequence similarity.

TABLE 1: CATEGORIES OF PEPTIDES IN THE MAMMALIAN BRAIN

A: Hypothalamic Releasing Factors
- Thyrotropin-Releasing Hormone (TRH)
- Gonadotropin-Releasing Hormone (Gn-RH or LH-RH)
- Corticotropin-Releasing Hormone (C-RF)
- Growth Hormone-Releasing Hormone (GH-RF)
- Somatostatin

B: Pituitary Hormones
- Adrenocorticotropic Hormone (ACTH)
- Luteinizing Hormone (LH)
- Follicle Stimulating Hormone (FSH)
- Growth Hormone
- Thyrotropic Hormone
- Prolactin
- Beta Endorphin
- Alpha Melanocyte Stimulating Hormone (alpha MSH)

C: Neurohypophyseal Hormones
- Neurophysin I and II
- Vasopressin
- Oxytocin

D: Gastrointestinal Peptides
- Vasoactive Intestinal Polypeptide (VIP)
- Cholecystokinin (CCK)
- Gastrin
- Substance P
- Insulin
- Glucagon
- Secretin
- Motilin
- Neurotensin
- Methionine - Enkephalin
- Leucine - Enkephalin

E: Inverterbrate Peptides
- Hydra Head Activator
- FMRF Amide

F: Others
- Angiotensin II
- Bradykinin
- Calcitonin
- Carnosine
- Sleep peptide
- Neuropeptide Y
- Calcitonin Gene Related Product (CGRP)
- Bombesin

1.3 Mechanisms for Generating Molecular Heterogeneity

1.3.1 Differential Proteolytic Processing

Besides the peptides having structurally similar analogues in different organs, the same protein precursor molecule can give rise to different peptide products in different cell types. Thus although the pro-opiomelanocortin (POMC) precursor protein is identical in the pituitary and hypothalamus, the anterior pituitary produces ACTH, while the intermediate lobe processes all the ACTH to alpha-MSH and CLIP, and the arcuate region of the hypothalamus produces a mixture of ACTH, alpha-MSH and CLIP (Roberts et al., 1982).

The same precursor may also be processed differentially in the same tissue. Thus, somatostatin-28 (SS-28), an N-terminally extended form of the hormone somatostatin-14 (SS-14), is a biologically active hormone in its own right and derived from the same precursor in pancreatic islet cells (Lechan et al., 1983).

Bioactive peptides of different molecular size which are derived from the same precursor have also been described in the case of the endorphins (Rehfeld, 1978). In this instance alpha endorphin and gamma endorphin are respectively beta endorphin 1 to 16 and 1 to 17. Different length bioactive forms of CCK have also been described (Rehfeld, 1978). The different molecular forms of the same peptide have been implicated in various physiological states and psychiatric conditions, a review of which was given by Krieger (1983).

1.3.2 Other Post Translation Modifications

Other post-translational modifications that can give rise to molecular heterogeneity include amidation, acetylation, sulphation, phosphorylation, glycosylation and assembly of sub-units. As an example of the latter case, the gonadotropins share a common alpha subunit but have different beta subunits.

1.3.3 Differential hnRNA Splicing

Peptide heterogeneity may potentially also arise through differential splicing of nuclear RNA during mRNA maturation. Thus different mRNAs could be obtained from the same transcribed gene by the inclusion or exclusion of certain exons of the gene. When these mRNAs are translated they would give rise to different protein products which may have tracts of sequence homology. The calcitonin gene with four coding exons in the primary transcript appears to undergo differential splicing in different tissues. Thus, the CGRP (calcitonin gene related peptide) encoding sequence occurs in the calcitonin mRNA of some tissues but not others (Amara et al., 1982).

1.3.4 Gene Duplication

Gene duplication and subsequent mutation within daughter genes is the main mechanism by which families of structurally-related peptides arise. The Brockman body in the pancreas of the anglerfish has two closely related mRNAs both giving rise to SS-14 (Hobart et al., 1980). This is probably a case of recent gene duplication since there is considerable sequence homology between the two genes. The glycoprotein hormones LH, FSH, TSH and hCG share a common alpha subunit but have different beta subunits

which have arisen due to multiple gene duplications over a more extensive period of evolution. (Pierce et al., 1981).

1.4 The LH-RH Family of Peptides

There has been no evidence for a LH-RH family of peptides until very recently. Indeed, only ten years ago, the idea of non-ribosomal synthesis of LH-RH was still favoured by some investigators (McKelvy and Snyder, 1978). This method of peptide synthesis does not, however, appear to be involved in the generation of peptide diversity in mammals. In support of ribosomal synthesis, however, there was a considerable body of evidence that higher molecular weight forms of LH-RH do occur (Kerdelhue, et al., 1973; Barnea and Porter, 1975; Fawcett, et al., 1975; Millar et al., 1977, 1978, 1980; Gautron et al., 1981). None the less, no irrefutable evidence had been provided of the exact size of the precursor(s) that give(s) rise to the hypothalamic decapeptide LH-RH. Cell free synthesis followed by immunoprecipitation of the products with an LH-RH antiserum had suggested that the primary LH-RH precursor in rat, mouse and human hypothalami is a 28,000 molecular weight protein (Curtis and Fink, 1983a, 1983b). Recently the cDNA sequence of a placental LH-RH precursor has revealed that it is a 92 amino acid protein (~10,000 molecular weight) (Seeburg et al., 1984). It has since been reported that the LH-RH mRNA may undergo differential splicing in the human hypothalamus and the placenta (Adelman et al., 1986). This does not however effect the LH-RH protein precursor since the difference in splicing occurs in the 5′ untranslated region of the mRNA.

Besides the identification of a wide variety of higher molecular weight LH-RH immunoreactive proteins, four peptides with sequences dissimilar from the mammalian decapeptide have been detected in vertebrates. Thus Gln^8-LHRH has been identified in chicken hypothalami (King and Millar, 1982), while His^5, Trp^7, Tyr^8-LH-RH has been identified as another form in chicken brain (Miyamoto et al., 1984); Trp^7, Leu^8-LH-RH was isolated from salmon brain (Sherwood et al., 1983), and in the lamprey Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8-LH-RH has been described (Sherwood et al., 1985). Using antisera specific for Gln^8 LH-RH, Millar has detected Gln^8-LH-RH in mammalian extra-hypothalamic brain (Millar et al., 1987). Ovine pineals have been shown to contain a peptide similar to Gln^8 LH-RH as distinguished by chromatographic systems (Millar et al., 1986). Mammalian gonadal LH-RH has been shown to be structurally distinct from the hypothalamic decapeptide (Dutlow and Millar, 1981). Other non-neuronal sources of LH-RH immunoreactivity include the placenta (Khodr et al., 1980), pancreas (Seppala and Wahlstrom, 1979) and milk (Amarant et al., 1982). LH-RH receptors have been demonstrated in a wide variety of tissues and tumours suggesting that extrahypothalamic sources of the peptide or its related molecules have physiological roles outside the pituitary. The low circulating levels of the hypothalamic LH-RH argue against the hypothalamus being the source of the peptide involved at the non- pituitary target tissues. Tissues having LH-RH receptors include the pituitary (Clayton et al., 1979), the placenta (Currie et al., 1981), the testes (Sharpe and Fraser, 1980; Millar et al., 1982), the ovary (Popkin et al., 1983), the adrenals (Bernardo et al., 1978; Eidne et al., 1985), the

prostate (Hierowski et al., 1983) and breast tumours (Eidne et al., 1985). The different affinities of the receptors of different tissues for LH-RH agonists and antagonists could be seen as suggestive of different endogenous ligands, i.e. peptides similar to but distinct from the mammalian hypothalamic LH-RH decapeptide.

A number of lines of evidence are thus indicative of a wide distribution of LH-RH and structurally related peptides in mammalian tissues and tumours. There is also a wide distribution of so-called LH-RH receptors which display different ligand binding characteristics and there exists therefore, structural diversity of receptors. A working hypothesis that can be developed from these observations is that there may be receptor specificity for the variant forms of LH-RH-like peptides produced at different sites. The privacy of communication which is well documented in the hypothalamic hypophyseal portal system could thus be expanded to include other scenarios for specific regulatory roles of the different, but related peptides at other tissue sites. The gonadal peptide could thus be thought to function in paracrine regulation of reproductive function. There is also a developing body of evidence supportive of an autocrine growth regulatory role for a number of peptides (Stiles, 1984; Cuttitta et al., 1985). The success of LH-RH antagonists in treating certain tumours having LH-RH receptors could be an indication of a growth regulatory role of endogenous LH-RH variants in certain tissues (Tolis et al., 1982; Harvey et al., 1984; Eidne et al., 1985).

CHAPTER 2

DISTRIBUTION OF LH-RH IMMUNOREACTIVE PEPTIDES

2.1 INTRODUCTION

Based mainly on his earlier studies of the pituitary portal circulation (Green and Harris, 1949), Harris proposed in 1955 that parvocellular neurons of the hypothalamus receive inputs from other neurons in the central nervous system via synaptic contacts and secrete factors into the portal vessels to regulate hormone release from the anterior pituitary (Harris, 1955). This basic premise has subsequently been confirmed by the isolation and elucidation of the structure of the following hypothalamic factors: thyrotropin-releasing factor (Boler et al., 1969); gonadotropin-releasing factor (Matsuo et al., 1971); somatostatin (growth hormone release inhibiting factor) (Burgus et al., 1973); corticotropin-releasing factor (Vale et al., 1981); growth hormone-releasing factor (Rivier et al., 1982; Esch et al., 1983). The chemical synthesis of these hypothalamic peptides and the development of radioimmunoassays for them, greatly extended the information about their control of anterior pituitary function. However, since the releasing factors are present in minute quantities (in the order femtomoles/mg of the hypothalamus) (Guillemin et al., 1981), biosynthesis studies have proven particularly difficult. The investigation of other possible sources of larger amounts of the peptide releasing factors became a necessary avenue of study in attempting to delineate their biosynthetic pathways. Indeed, this approach has recently rewarded investigators with the determination of the

complete structure of the somatostatin precursor in the angler fish endocrine pancreas (Hobart et al., 1980), the frog skin TRH precursor (Richter et al., 1984), and a human pancreatic islet tumour GH-RF precursor (Mayo et al., 1983). It has, in fact been known for a considerable period of time that a wide variety of tumours are a source of ectopic hormones (Bloom et al., 1979). Also, in 1966 Pearse postulated a common embryological origin for cells of the so-called diffuse neuroendocrine system (Pearse, 1966). He later suggested that the common origin was the neuroectoderm and proposed that the endocrine system was an "outpouch" of the brain (Pearse, 1969). Working on this premise numerous investigations have now shown that the nervous and endocrine systems synthesize a large number of identical or related peptides.

It was with the above facts in mind that the distribution of LH-RH-IR in extrahypothalamic brain, non-neuronal tissues and tumours was investigated.

2.2 SURVEY OF RAT TISSUE FOR LH-RH-IR

Nineteen rat organs and brain regions were dissected from 50 adult male Wistar rats. Acetic acid extracts of these tissues were assayed in serial dilution for LH-RH-IR. The LH-RH antiserum (antiserum 1076) used in the radioimmunoassays of tissue extracts has been shown to be highly specific for the middle portion of the mammalian LH-RH decapeptide (see Fig. 3.4) (Millar et al., 1984). However, since cross-reacting related peptides could conceivably be detected by this radioimmunoassay, the immuno-reactive peptides detected in certain tissues were

subjected to further characterization by radioimmunoassay with other antibodies and by high performance liquid chromatography.

2.2.1 Methods

2.2.1.1 Acetic Acid Extraction

The dissected tissues were collected on solid CO_2 and stored at -20°C. The tissues were homogenized in two volumes of 2N acetic acid at 4°C using an Ultra-Turrax homogenizer. When a uniform homogenate was obtained, extraction was further facilitated by incubation at 4°C for 2h. The homogenates were centrifuged at 18,000g for 30 minutes at 4°C and the clear supernatants were lyophilized. The lyophilized extracts were reconstituted in 0.02N acetic acid, recentrifuged at 18,000g for 30 minutes and the clear supernatant serially diluted in 0.02N acetic acid and subjected to radioimmunoassay.

2.2.1.2 Radioimmunoassay

LH-RH was iodinated by the chloramine T protocol (Hendricks et al., 1975) and purified by chromatography on Dowex 1X8 and CM-32 cellulose ion exchange resins (Millar et al., 1984). In a typical assay each assay tube contained 10,000 cpm of the peptide radiolabelled to a specific activity of approximately 1000 uCi/ug. The antiserum was used at a final dilution in the assay of 1:100,000. The assay was performed in a final volume of 500 ul of phosphate buffered saline (PBS) (pH 7.0) containing 0.1% gelatin and the protease inhibitor bacitracin at a final concentration of 10^{-4}M. All samples and standards were added to the assay tubes in 0.02N acetic acid. The assay tubes were incubated at 4°C for 18h. 750 ul of 0.5% charcoal (Norit A)

coated with 0.05% dextran T70 in PBS was added to the assay tubes and incubated at 4°C for 15 minutes to separate antibody-bound-from free-^{125}I-LH-RH. The tubes were centrifuged at 1000g for 15 minutes at 4°C and supernatants containing the antibody-bound peptides were counted in a multi-well gamma counter. The displacement from antibody of radiolabelled peptide by immunoreactive material in the standards and samples was expressed relative to the amount of radiolabelled peptide bound by antibody in the absence of unlabelled LH-RH standard ($\%B_o$). The LH-RH-IR in each tissue extract was expressed relative to the total peptide extracted as determined by the Lowry protein assay (Lowry et al., 1951).

2.2.2 Results and Discussion

The acid extracts of a number of tissues were found to contain "LH-RH binding proteins." These were evidenced in the radioimmunoassay by the higher levels of the radiolabelled peptide in the antibody bound fraction, relative to the amount bound when only radiolabelled peptide and antibody are present. In order to correct for these factors in the assay their contribution to the binding of radiolabelled peptide was measured in the absence of antibody (called the non-specifically bound-fraction). This was done for each dilution of the tissue extract in question. The amount of non-specifically bound radiolabelled peptide (evaluated in cpm) was subtracted from the amount bound in the presence of antibody, thus giving the number of counts specifically bound by antibody.

Protease contamination of samples in a radioimmunoassay is usually evidenced by less radiolabelled peptide in the antibody

bound fraction of the assay due to degradation of either the antibody or the radiolabelled peptide, or both. This would be reflected in the assay as an apparently high level of immunoreactive peptide in the sample. The presence of immunoreactivity in tissue known to contain high levels of proteases, e.g. the pancreatic LH-RH-IR, should therefore be interpreted with caution.

Table 2.1 and figure 2.1 show that LH-RH-IR was detectable in a number of tissue extracts. The specific activity of these immunoreactive peptides is in every case much less than in the hypothalamus. Besides being an accurate reflection of the LH-RH content of certain tissues, the results in certain cases could be due to the underestimation of an LH-RH-related peptide which has low cross-reactivity in this assay. The more detailed characterization of the LH-RH-IR in testes described in chapter 3 of this thesis supports the latter explanation. The pineal gland of sheep has recently been shown to contain a peptide similar to Gln^8-LH-RH (Millar et al., 1987). This peptide is known to show low cross-reactivity in the radioimmunoassay using antiserum 1076.

The presence of LH-RH-IR in <u>other areas of the brain</u> has also been documented by other workers (Silverman et al., 1976 and Ibata et al., 1983). The possibility that the LH-RH found in extra-hypothalamic brain performs a neurotransmitter or neuromodulator role deserves investigation.

LH-RH was found in a number of <u>tissues involved in reproductive functions</u>. Immunocytochemical evidence for a local, "private", i.e. paracrine, pituitary LH-RH system has been presented (Yuan Li et al., 1984). Being a target organ for

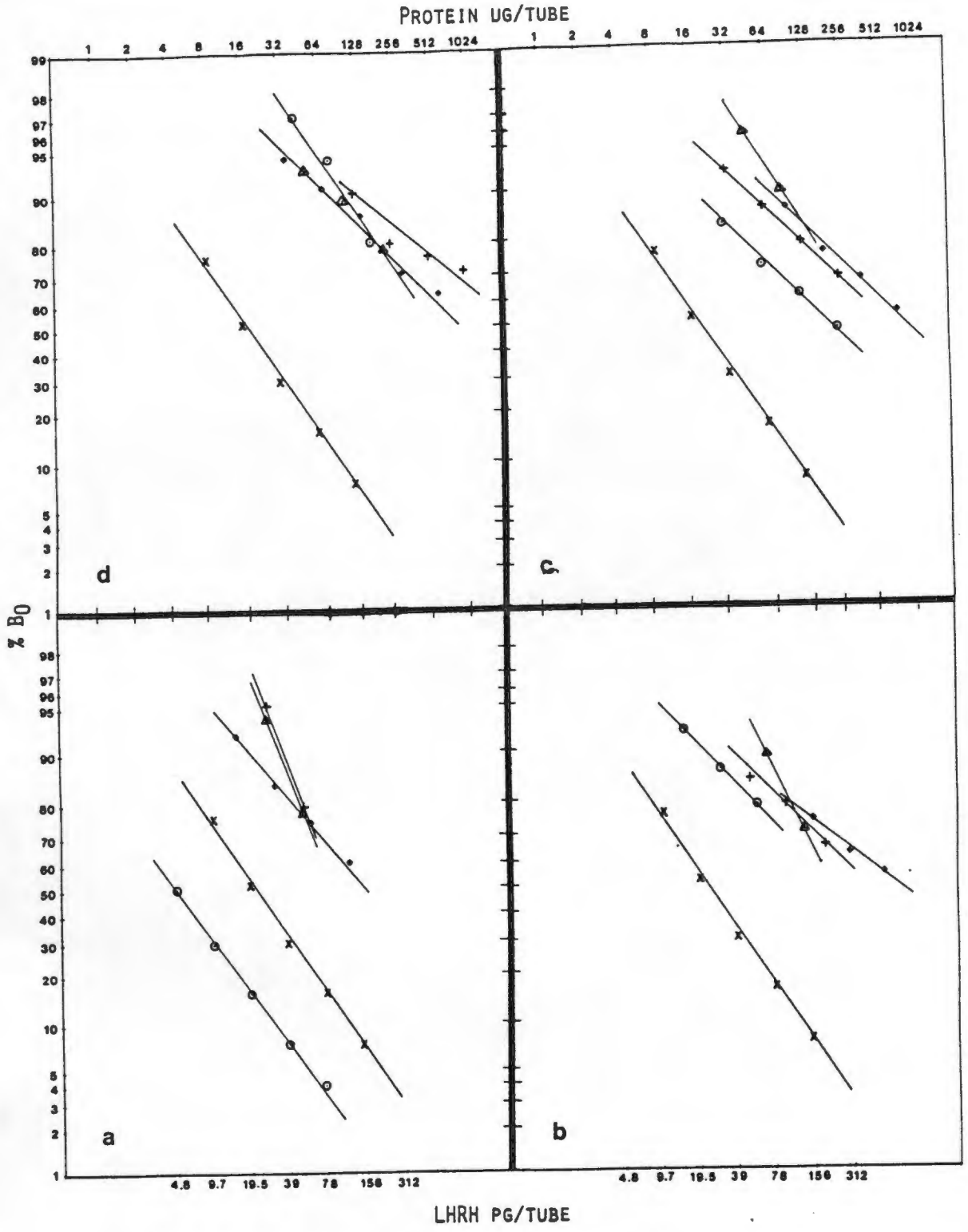

Fig. 2.1 **DISPLACEMENT OF ^{125}I LH-RH FROM ANTISERUM 1076 BY SERIAL DILUTIONS OF ACETIC ACID EXTRACTS OF RAT TISSUES** The synthetic peptide used was quantified by weight and the protein concentration of the extracts by the method of Lowry (Lowry et al, 1951).

SYMBOL	PANEL a	b	c	d
○	Hypothalamus	Pituitary	Pancreas	Thyroid
◆	Pineal	Cerebrum	Cerebellum	Skin
△	Brain Stem	Stomach	Subman. Gl.	Testis
+	Retina	Spleen	Small Int.	Kidney
×	LH-RH Standards			

TISSUE	PROTEIN (ug/tube)	^{125}I LH-RH (%Ab bound)	LH-RH (pg/tube)	LH-RH-IR (pg/mg prot)
Hypothalamus	3.9	51	20.5	5256
Pineal	44	77	8.6	195
Brain Stem	90	61	15.3	170
Retina	42	85	5.5	131
Pituitary	52	83	6.5	125
Cerebrum	101	70	11.5	114
Stomach	121	70	11.5	93
Spleen	140	65	13.0	93
Pancreas	246	48	21.5	87
Cerebellum	79	88	6.5	83
Subman. Gl.	198	72	11.0	56
Small Int.	220	73	10.8	49
Thyroid	150	81	7.3	49
Skin	200	80	7.5	38
Testis	530	64	13.5	25
Kidney	113	90	1.5	13
Adrenals	144	100	-	-
Liver	240	100	-	-
Muscle	128	100	-	-

Table 2.1 **LH-RH-IR IN 19 RAT TISSUES**
Concentrations were determined from the displacement curves shown in figure 2.1.

- At what %.
- some not parallel.
- perhaps you should comment that quant. for non parallel ones will vary depending on the level at which measured

hypothalamic LH-RH, it is also possible that the pituitary immunoreactive peptides described in this and other studies are measurable because of concentration at this site due to the peptide binding to the pituitary receptors, internalization, etc.

The testicular LH-RH-IR described here, was further characterized (chapter 3). Another source of LH-RH is the placenta (Khodr et al., 1980). It has been proposed that the LH-RH synthesized in placental cytotrophoblasts regulates the placental production of hCG in syncytiotrophoblasts (Hsueh et al., 1981). Human and bovine milk has also been shown to contain LH- RH-IR (Amarant et al., 1982). Because other hormones including TRH, prolactin, the opioid casomorphin and ACTH have been found in milk, it has been proposed that they have physiological roles in the suckling mammalian neonate (Hazum, 1983).

LH-RH-IR was found in a number of gut regions and gut associated organs. The fact that LH-RH-IR is found in different parts of the gut is an extension of the group of so-called brain and gut peptides (Powell et al., 1979). LH-RH-IR in the pancreas has also been reported by others (Seppala et al., 1980). It is, furthermore, reported that LH-RH inhibits insulin release from isolated rat pancreatic islets (Klier et al., 1980) suggesting that the immunoreactive material in the pancreas may have a regulatory role in glucose metabolism. Additional support for a role of LH-RH in glucose metabolism is the fact that glucose oxidation and incorporation into fatty acids in rabbit adipose tissue is increased by LH-RH (Murthy et al., 1974).

The presence of LH-RH-IR in the submandibular gland is also of interest since it has been demonstrated that the submandibular

glands of cats contain hypolemmal axons containing large dense core granular vesicles of the peptidergic type (Kidd et al., 1979). Another neuropeptide found in submandibular glands is vasoactive intestinal polypeptide (Wharton et al., 1979).

LH-RH-IR was also found in a number of other tissues. The presence of LH-RH in the retina is reminiscent of reports on the presence of other neuropeptides in the retina (Eskay et al., 1980). It is tempting to speculate that the LH-RH in retina has a role in vision since certain patients suffering from the LH-RH deficiency disorder, Kallmann's syndrome, present with colour blindness (Kallmann et al., 1944). LH-RH may also be implicated in the sensory pathways of smell since some Kallmann's patients suffer from anosmia and LH-RH has been reported in the olfactory system (Philips et al., 1980).

The fact that LH-RH-IR is detectable in the skin could be an indication of innervation of the skin by LH-RH neurons. The role of the peptide at the periphery could be related to its effect on glucose metabolism, should it be located in the subcutaneous adipose tissue. It is also feasible that peptide location in mammalian skin is a vestige of the large amounts of the so-called neuropeptides (e.g. TRH) found in amphibian skin (Jackson et al., 1977).

The rat thyroid contains significant amounts of LH-RH. The thyroid gland has recently also been found to contain the neuropeptides substance P, CCK, ACTH, beta endorphin, and beta lipotropin (Ahren et al., 1983; Clements et al., 1982).

Since the kidney may be a site of catabolism and clearance of LH-RH, as for proteins and peptides in general, the presence

of LH-RH-IR in kidney extracts could arise from a concentrating effect in this organ of LH-RH and/or its degradation products.

This study thus confirms the location of LH-RH-IR at a number of extrahypothalamic sites. However, due to the relatively small amounts of the peptide(s) in these tissues investigation of the mechanism of biosynthesis of LH-RH was seen to be more feasible in the hypothalamus. Further confirmation of the actual sites of synthesis of the peptide found in these extrahypothalamic tissues is required. The physiological role of the peptide(s) at these sites is certain to engender investigation in the future.

2.3 SURVEY OF TUMOURS AND CELL LINES FOR LH-RH-IR

2.3.1 Introduction

Since ectopic production of many hormones occurs in a number of tumours, acetic acid extracts of human tumours and tumour cell lines were assayed for LH-RH-IR. The cell lines included a number of mouse and human neuroblastoma cell lines, human melanoma cell lines as well as other lines of epithelial origin. Solid tumours included dog Sertoli cell carcinomas, human pancreatic tumours (including one which was shown to be producing large amounts of somatostatin) and a human buccal tumour cell line grown in nude mice.

Of all the cell lines and tumours tested only the human buccal tumour cell line was shown to be producing significant quantities of LH-RH-IR. The buccal tumour line HEp-3 was first described in 1954 and believed to be neuro-ectodermal in origin (Toolan, 1954). It has been successfully passaged in tissue culture and in nude mice (Miskin et al., 1981). This cell line

has recently been shown to synthesize high levels of VIP (Gozes et al., 1983). The nature of the LH-RH- IR in this cell line was analysed by radioimmunoassay with several antisera directed to different parts of the mammalian LH-RH, as well as by HPLC. One of the antisera (F86) used in this study had not been previously characterized. Thus, in order to define the region of the mammalian decapeptide to which this antiserum was directed, the cross reactivity of a series of LH-RH analogues and fragments in a radioimmunoassay with this antiserum were tested.

2.3.2 Methods

2.3.2.1 Characterization of Antiserum F86

This antiserum was kindly supplied by Dr. S. Lynch. The cross-reactivities of LH-RH analogues in the LH-RH radioimmunoassay using this antiserum were calculated from the concentrations (by weight) of the analogues which decreased the binding of ^{125}I-LH-RH to the antiserum by 50% (i.e. the ED_{50}). The percent cross reactivity for a particular analogue or fragment was thus estimated by dividing the ED_{50} of that peptide by the ED_{50} of standard LH-RH and multiplying by 100. From the degree of cross-reactivity of a number of analogues in the assay, deductions were made as to the specificity of the antiserum i.e. which amino acids in LH-RH are essential for binding by this antiserum.

2.3.2.2 Peptide Extraction

The protocol was essentially as outlined in 2.2.2.1 except that two extraction solutions were used, viz:

a) 2N acetic acid in methanol;

b) 2N acetic acid containing 10^{-4}M bacitracin.

These protocols were used in order to assist in the first instance with delipidation of some tumour samples, and in the second instance to inhibit proteases known to be synthesised by the tumour.

2.3.2.3 Radioimmunoassays

Six different LH-RH antisera were used to quantitate the LH-RH-IR in tumour extracts. Antibodies 1076 and 743 are directed at the middle of the LH-RH sequence (Millar et al., 1984). Antiserum 80/1 requires both the N- and C-termini for binding (King et al., 1983), while antiserum CRR11B73 is nominally C-terminally directed. Antiserum F86 was shown in this study to require the N-terminus of LH-RH for binding, while being sensitive to the conformation of the molecule as well. Antiserum YK1 has not been fully characterized.

2.3.2.4 HPLC

Three different reverse phase HPLC systems were used:

a) In the first system a micro Bondapak C18 column was used. Samples of tumour extract were injected in 1 ml 0.1% TFA. Solution A was 1% acetonitrile in 0.1% TFA and solution B, 80% acetonitrile in 0.1% TFA. A linear gradient was run from 0% to 100% B over 60 minutes at 1 ml per minute and 1 ml fractions were collected.

b) In the second system a Vydac C18 column was used. Samples were injected in 1 ml of 0.1% heptafluoro butyric acid (HFBA). Solution A was 0% acetonitrile, 0.1% HFBA and solution B, 60% acetonitrile, 0.1% HFBA. The sample was

injected onto the column which had been pre-equilibrated in 30% B and run at this % B for 10 minutes. The gradient was altered to 50% B over 20 minutes, kept at this % B for 10 minutes, altered to 100% B over 5 minutes and kept at 100% B for 5 minutes. The flow rate was 1.5 ml per minute and 1.5 ml fractions were collected.

c) In the third system a Spherisorb C18 column was used. Samples were injected in 1 ml of 0.01M ammonium acetate (pH 4.0). Buffer A was 0% acetonitrile in 0.01M ammonium acetate, while buffer B was 60% acetonitrile in 0.004M ammonium acetate. The column was equilibrated to 40% B before the sample was injected. After injection the buffer was maintained at 40% B for 20 minutes. It was then altered to 60% B over 5 minutes, kept at 60% B for 25 minutes, taken to 99% B over 5 minutes and kept at 99% B for 5 minutes. The flow rate was 1.5 ml per minute and 1.5 ml fractions were collected.

In each system, duplicate 100 ul aliquots of each fraction were lyophilized and assayed for LH-RH immunoreactivity.

2.3.3 Results and Discussion

Antiserum F86 was shown by the cross-reactivity studies to be directed to the N-terminus of LH-RH. Thus, it does not tolerate changes in this part of the molecule and therefore cross-reacts very poorly with LH-RH analogues altered or lacking in amino acids at the N-terminus. The antiserum displays conformational constraints as well since it reacts relatively

PEPTIDE	% CROSS REACTIVITY
LH-RH	100.0
2-10 LH-RH	2.0
[Pyr^{3} Ala^{2}] LH-RH	2.2
[pNO_2 Phe^{3}] LH-RH	0.3
[Thr ^{4}] LH-RH	52.9
[His^{5}] LH-RH	178.1
[D Trp^{6}] LH-RH	26.2
[Ile^{6}] LH-RH	36.9
[Trp^{7}] LH-RH	53.8
[Trp^{7},Leu^{8}] LH-RH	3.8
[Gln^{8}] LH-RH	222.8
[Ala^{9}] LH-RH	127.2
LH-RH Ethylamide	129.1
[His^{5},Trp^{7},Tyr^{8}] LH-RH	0.5
1-2 LH-RH	< 0.002
1-3 LH-RH	< 0.002
1-5 LH-RH	0.2
3-10 LH-RH	< 0.002

Table 2.2 **CROSS REACTIVITIES OF LH-RH ANTISERUM F86 WITH ANALOGUES OF LH-RH**
The cross reactivities were calculated from the concentrations (by weight) of the analogues which decreased the binding of ^{125}I LH-RH to the antiserum by 50%. The data indicates that the antiserum requires the N-terminus of LH-RH for binding. The antiserum is conformation-sensitive as well, since alterations in the middle of the LH-RH molecule decrease the % cross-reactivity. Gly^{6} is important for binding, since the beta-II type bend induced at this position brings the N-and C-termini into close apposition.

ANTISERUM	LH-RH-IR (ng/g tissue)
1076	1.8
80.1	2.1
CRR11B73	3.0
F86	3.2
743	4.2
YK1	4.7

Table 2.3 **RADIOIMMUNOASSAY OF HEp-3 TUMOUR EXTRACTS WITH SIX LH-RH ANTISERA.**
The discrepancies in quantitation by the different antisera is most probably due to the presence in the extracts of the two forms of LH-RH-IR shown in figure 2.2.

poorly with analogues that have amino acid substitutions in the middle of the molecule. LH-RH is believed to contain a beta-II type bend in the middle of the molecule. Substitutions in this region of the molecule either stabilize or relax this conformation (Friedinger et al., 1980; Spatola, 1983).

Radioimmunoassay of the crude extract of the HEp3 tumour by the six antisera gave a range of LH-RH-IR estimates in the tumour from 1.8 ng/g tissue with antiserum 1076 to 4.7 ng/g tissue with antiserum YK1 (Table 2.3). When the extracted material was applied to the first reverse phase system (Fig. 2.2a) a major peak of immunoreactivity eluted earlier that mammalian LH-RH.

Another extract of the tumour when run on the second reverse phase system resolved into two immunoreactive peaks detected by antiserum 1076, one coincident with the elution position of the mammalian decapeptide and the other an earlier eluting peak (Fig. 2.2b).

A LH-RH-IR profile similar to that of the second HPLC system was obtained in the third system (Fig. 2.2c).

The immunoreactive material eluting earlier than the mammalian hypothalamic decapeptide was re-assayed with antisera F86, CRR11B73 and 80/1. The N-terminally directed antiserum gave an estimate of the immunoreactivity similar to that of antiserum 1076, while both the nominally C-terminally directed antiserum CRR11B73 and the N- and C-terminus requiring antiserum 80/1 did not detect the first peak. One interpretation of these data is that the earlier eluting peptide is another variant form of the LH-RH decapeptide and is modified at the C-terminus. Alternatively, it could be a C-terminally extended form, i.e. a precursor and/or an incompletely processed form of the mammalian

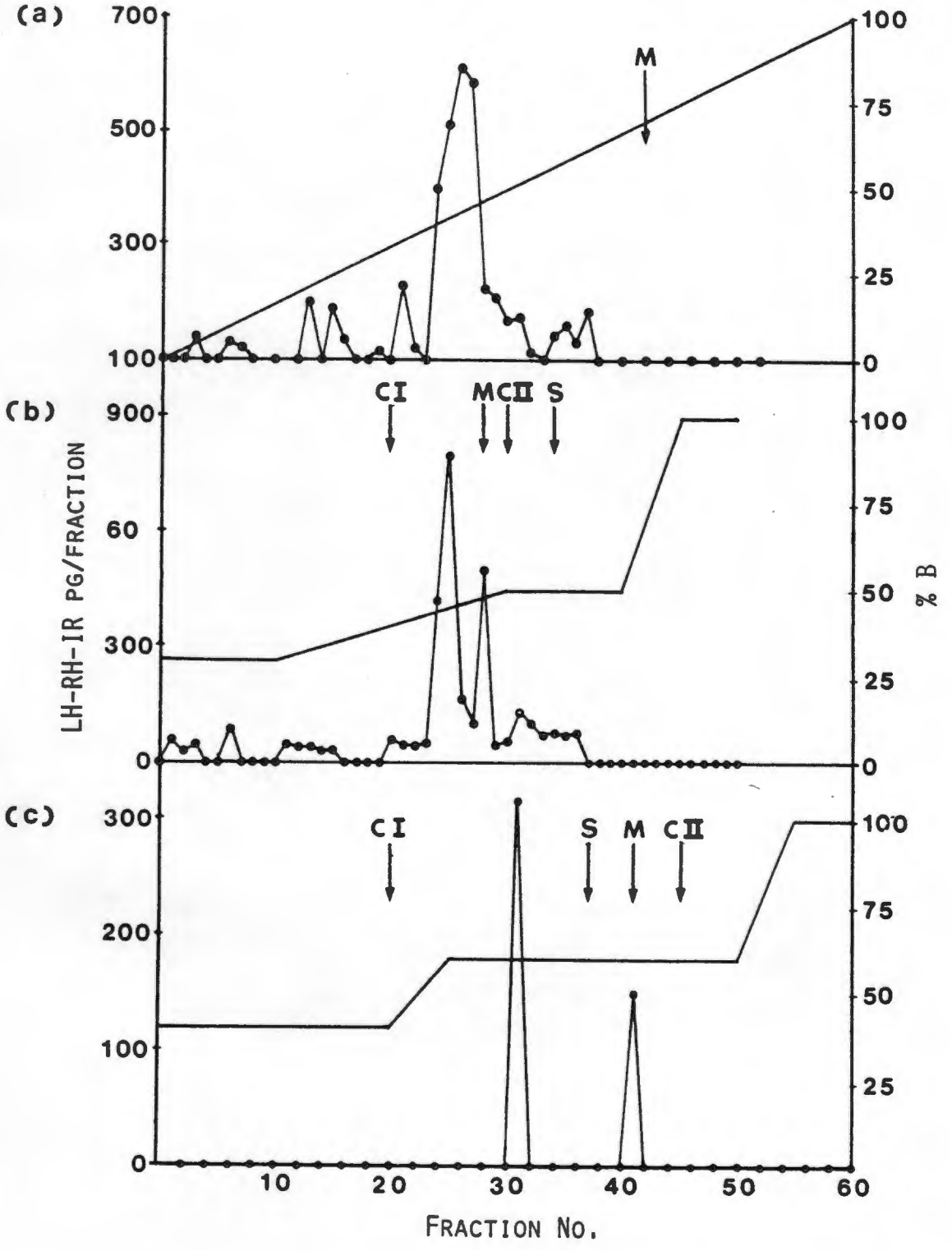

Fig. 2.2 **REVERSE PHASE HPLC OF HEp-3 TUMOUR EXTRACTS**
Three reverse phase HPLC systems known to separate Chicken I-, Chicken II-, Mammalian- and Salmon-LH-RH (see text) were used to determine the nature of the LH-RH-IR extracted from the HEp-3 buccal cell tumour. In all three systems a predominant early peak (less hydrophobic than mammalian LH-RH) was seen. This peak did not coincide with that of any of the vertebrate LH-RH's. Whether this peak is a fragment or precursor form of the mammalian peptide or a novel peptide has not been determined.

decapeptide (e.g. lacking an amidated C terminus). The fact that the mammalian decapeptide is not present in some extracts, as evidenced in the first HPLC system, could be due to processing or degrading proteolytic enzymes being present at variable concentrations from one preparation of the tumour to the next (e.g. a reflection of the stage of the tumour when harvested from the nude mice).

Since the HEp3 tumour produced large amounts of poly A mRNA (5-10% of total RNA - see chapter 5) the nature of the LH-RH precursor was further investigated by northern blot analysis and cDNA cloning as outlined in Chapter 5.

2.4 CONCLUSIONS

This survey of extra-hypothalamic tissues and tumours has demonstrated a wide distribution of LH-RH-IR. This pattern was previously described for a number of peptides initially detected either in the brain or the gastro-intestinal tract. Based on the effects of LH-RH at different sites, e.g. in the pancreas or in the gonad, it is highly likely that LH-RH-like peptides synthesized outside the hypothalamus and identical to or related to LH-RH, may serve functions different from the gonadotropin-releasing activity observed at the pituitary. The potential for a peptide to function as a neurotransmitter or releasing factor or paracrine hormone depending on its site of synthesis becomes a valid concept considering the above data and deserves future verification in the case of LH-RH and structurally related peptides.

CHAPTER 3

GONADAL LH-RH-LIKE PEPTIDES

3.1 INTRODUCTION

Porcine hypothalamic LH-RH was originally isolated by virtue of its ability to stimulate release of the anterior pituitary hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Matsuo et al., 1971). The positive regulatory role that hypothalamic LH-RH has via the pituitary gonadotropins on gonadal steroidogenesis, ovulation in the female and spermatogenesis in the male has been demonstrated in a number of vertebrates (Schally, 1978). However, repeated or prolonged administration of high doses of LH-RH or its agonists to a number of species results in inhibitory, "paradoxical" effects on reproductive function. Thus, in females, chronic administration of LH-RH agonists results in a decrease in ovarian steroidogenesis (Yoshinaga, 1979), a decrease in ovarian gonadotropin receptors (Kledzik et al., 1978), inhibition of follicular maturation and ovulation (Berquist et al., 1979), delay of puberty (Johnson et al., 1976), delay of implantation and pregnancy termination (Corbin et al., 1975), delay of parturition (Bereu et al., 1980), and decrease in uterine and oviductal growth (Rippel et al., 1976). In males, chronic LH-RH agonist treatment decreases testicular androgen production (Haug et al., 1974), decreases testicular gonadotropin receptor content (Auclair et al., 1977), decreases testicular weight (Sandow et al., 1978), inhibits spermatogenesis (Pelletier et al., 1978), and decreases the growth of the ventral prostate and seminal

vesicles (Oshima, et al., 1975). It was initially proposed that the high doses of LH-RH used in these situations cause desensitization of the pituitary to respond to the normal positive stimulation by the decapeptide on LH and FSH release. However, it has been demonstrated that high doses of LH-RH can decrease testicular function without concomitant decreases in serum gonadotropin levels (Rivier et al., 1979). These anti-fertility effects are partly due to "down regulation" of gonadal gonadotropic hormone receptors resulting from LH-RH induced elevation of pituitary FSH and LH levels (Auclair et al., 1977), but direct effects of LH-RH on the gonads have also been described (Sharpe et al., 1983). High affinity receptors for LH-RH have been demonstrated on the cell membranes of ovarian granulosa (Clayton et al., 1979) and luteal cells (Marwood et al., 1980) and on testicular interstitial cells (Millar et al., 1982; Sharpe et al., 1981). The occurence of these LH-RH receptors in the gonads and the demonstration that gonadal function is altered by LH-RH administration in hypophysectomized animals argues for a direct involvement of LH-RH-like peptides in the control of ovarian and testicular function <u>in vivo</u>. Since hypothalamic LH-RH is released into the portal veins at low concentrations (Eskay et al., 1977); is rapidly degraded by the pituitary (Griffiths et al., 1981) and is found in infinitesimally small amounts in the peripheral circulation (Eskay et al., 1977), there may be a local source of LH-RH or a related peptide within the gonad which is involved in the regulation of reproductive function via a local receptor mediated mechanism. Since a local source of LH-RH or a related peptide in the gonads has been proposed by the rationale outlined above and

since LH-RH immunoreactivity (LH-RH-IR) was found in rat testes as described in the distribution studies of the previous chapter, chromatographic and immunological cross reactivity approaches were used to further characterize the rat testicular LH-RH-IR.

3.2 RAT TESTICULAR LH-RH-IR

3.2.1 Methods

3.2.1.1 Acetic Acid Extraction

390 rat testes were collected on solid CO_2 and stored at -20°C. The testes were homogenised in two volumes of 2N acetic acid at 4°C by three 5-10 sec. bursts with an Ultra-Turrax homogenizer. Homogenates were centrifuged at 18,000g for 30 minutes at 4°C and the clear supernatants were lyophilized. The peptide concentrations in the extracts and subsequent column fractions, etc. were determined according to the method of Lowry (Lowry et al., 1951).

3.2.1.2 Immunoaffinity Chromatography

The lyophilized extract was reconstituted in 0.5M ammonium acetate (pH 7.0) and centrifuged at 18,000g for 30 minutes at 4°C. The clear supernatant was subjected to batch-wise affinity chromatography using anti-LH-RH antiserum 1076 (Millar et al., 1984) conjugated to Sepharose 4B (Axen et al., 1967). The testicular extract was mixed with the resin in roller bottles for 3 hours at room temperature. Unbound non-immunoreactive material was removed by several washes with 0.5M ammonium acetate (pH 7.0). LH-RH-IR bound to the affinity gel was eluted with five washes of 80 ml of 1.5M acetic acid (until pH 2.8 was reached) and lyophilized. To neutralize the affinity resin, the acid was

replaced by 0.5M ammonium acetate and washing continued until the pH of the eluant reached pH 7.0.

3.2.1.3 Sephadex G-100 Chromatography

The material partially purified by immunoaffinity chromatography was reconstituted in a small volume of 2N acetic acid containing 8M guanidinium chloride, incubated at 50°C for 3 hours and subjected to Sephadex G-100 chromatography. The protein content of the eluant was monitored by ultraviolet absorption at 280 nm. Immunoreactive LH-RH was detected by radioimmunoassay using antiserum 1076.

3.2.1.5 Sephadex G-25 Chromatography

The lower molecular weight immunoreactive peptides (~5000 daltons and less) eluted off the G-100 column were chromatographed on Sephadex G-25.

3.2.1.5 Reverse Phase HPLC

Lyophilized acetic acid extracts of rat testes were reconstituted in 1% TFA and slowly passed through three Waters Sep-pak C-18 cartridges in tandem. The cartridges were washed thoroughly with 1% TFA and the bound material eluted with 60% acetonitrile, 1% TFA. The eluted peptides were lyophilized and subjected to reverse phase HPLC on a Vydac C-18 column as outlined in 2.3.2.4(b). Under the conditions used in this protocol four synthetic vertebrate-LH-RH-decapeptides were shown to be separated from each other.

3.2.1.6 Antibody Binding Studies

The different molecular forms of the LH-RH-IR separated by Sephadex G-100 and Sephadex G-25 chromatography were subjected to radioimmunoassay by antisera directed against different regions of the mammalian hypothalamic LH-RH decapeptide (Millar et al., 1984). The relative quantitation of LH-RH-IR peaks by these antisera yielded information on possible differences in structure between the testicular peptides and the hypothalamic LH-RH.

3.2.1.7 Pituitary Receptor Binding

The various species of LH-RH-IR separated on Sephadex G-100 were used to displace ^{125}I labelled LH-RH agonist from pituitary membranes according to the method of Millar (Millar et al., 1982).

3.2.2 Results and Discussion

The crude acetic acid extract of 390 rat testes contained 54.5 ng of LH-RH-IR with a specific activity of 2.93 ng/g peptide using antiserum 1076. After affinity chromatography of 17.4 ng of LH-RH-IR, the specific activity increased 50-fold to 165 ng/g peptide. Sephadex G-100 chromatography of a portion of the affinity purified material revealed four major LH-RH-like peptides (I, II, IV and VI) with apparent molecular weights of >100,000, ~32,000, ~5,000 and $\leq$4,000 (Fig. 3.1). When the low molecular weight peptides from the G-100 column were subjected to chromatography on Sephadex G-25, the major peak of immuno-reactivity emerged later than the mammalian hypothalamic decapeptide (Fig. 3.2). The testicular peptides having LH-RH immunoreactivity were shown by reverse phase HPLC to include a

species that elutes in a position similar to that of chicken II LH-RH, i.e. His^5, Trp^7, Tyr^8-LH-RH (Fig. 3.3). Three other elution peaks, not coincident with any of the vertebrate LH-RH peptides were also observed on HPLC.

When all six peaks of LH-RH-IR of the G-100 column were quantitated with antisera specific for different regions of the mammalian hypothalamic decapeptide (Fig. 3.4), antiserum 744 which is directed against the -COOH terminus of the mammalian peptide (Millar et al., 1977) consistently gave higher estimations of LH-RH-IR than did antisera 1076 and R42 which recognize the middle of mammalian LH-RH and both termini respectively (Millar et al., 1977; Nett et al., 1973).

When pituitary membranes were used to assay for LH-RH receptor-binding ligands in the Sephadex G-100 peaks of testicular LH-RH-IR, it was observed that there was increased receptor-binding activity as the size of the material tested decreased (Fig. 3.5). The quantitation by this assay of testicular LH-RH receptor-binding-peptide in peak VI off the G-100 column was approximately 80 times more than the LH-RH-IR determined in this peak by antiserum 744.

The presence of a number of molecular size forms of LH-RH-IR in rat testis is an indication that a peptide similar to the hypothalamic decapeptide may be synthesized by processing from a precursor protein in the testis. The higher molecular weight LH-RH-IR species demonstrated by molecular sieve chromatography could be precursors of a peptide which when fully processed may be similar in size to the hypothalamic decapeptide. It must be noted, however, that even though strong denaturing conditions (8M guanidinium hydrochloride, 2N acetic acid) were used for the

Sephadex G-100 chromatography, the possibility of aggregation of the lower molecular weight peptides and/or their binding to other proteins under these conditions, cannot be entirely excluded. The observation that the testicular LH-RH-like peptides elute later on Sephadex G-25 than does the hypothalamic peptide is not necessarily an indication that the peptide is smaller since adsorptive behavior has been observed for a number of peptides chromatographed on molecular sieve columns (Gautron et al., 1981). Although the testicular peptide that is more hydrophobic than the mammalian hypothalamic decapeptide elutes in a position similar to that of chicken II LH-RH on HPLC, more detailed studies are required to determine whether it is in fact chicken II LH-RH.

The antibody binding studies could be viewed to be supportive of the testicular peptide being different from the chicken II LH-RH. Antiserum 744, which binds to amino acids Arg^8, Pro^9, $GlyNH_2$ of the mammalian hypothalamic peptide, recognizes the testicular peptide better than do the other antisera tested. The implication of the antibody-binding studies is that the testicular peptide shows C-terminal homology with the mammalian decapeptide. It is, however, in this region that the chicken II LH-RH (His^5, Trp^7, Leu^8-LH-RH) differs from the mammalian hypothalamic peptide. A similar study on rat testis extracts has described testicular LH-RH immunoreactive material which may share C-terminal sequences with mammalian hypothalamic LH-RH (Bhasin et al., 1983). In contrast, another study described rat testicular peptides which reacted more poorly with C-terminally-directed LH-RH antisera than antisera which recognize either both termini or the middle region of the

molecule (Turkelson et al., 1983). The conflicting reports regarding the implied structural differences between the testicular and hypothalamic LH-RH-immunoreactive peptides may be due, in part, to the presence of multiple precursor forms of the peptide in crude extracts. Proteolytic degradation and/or aggregate formation and/or co-purification of peptide binding proteins could also explain the discrepancies. Two groups have recently demonstrated gonadal LH-RH-like peptide which are more hydrophobic on reverse phase HPLC than the mammalian decapeptide (Aten et al., 1985; Iwasaki et al., 1985). The observation that pituitary membrane LH-RH receptors bind the testicular peptide better than any of the antibodies used in this study could be exploited in the further purification and characterization of this peptide.

Since large numbers of rat testes would have to be extracted in order to purify gonadal LH-RH-IR in sufficient quantities for structural analysis, other sources of the peptide were investigated.

3.3 LH-RH-IR IN TESTES OF OTHER MAMMALS

In order to investigate whether other mammalian species have testicular LH-RH-IR, acetic acid extracts were made of rabbit, monkey and pig testes. Since there are some indications that Sertoli cells are the site of synthesis of the LH-RH variant peptide (Sharpe et al., 1981) pig testis tubules were prepared as a potential source of enriched levels of the peptide. Since Sertoli cell carcinomas are known to be common in certain breeds of dog (Lipowitz et al., 1973) extracts of such tumours were also tested for LH-RH-IR.

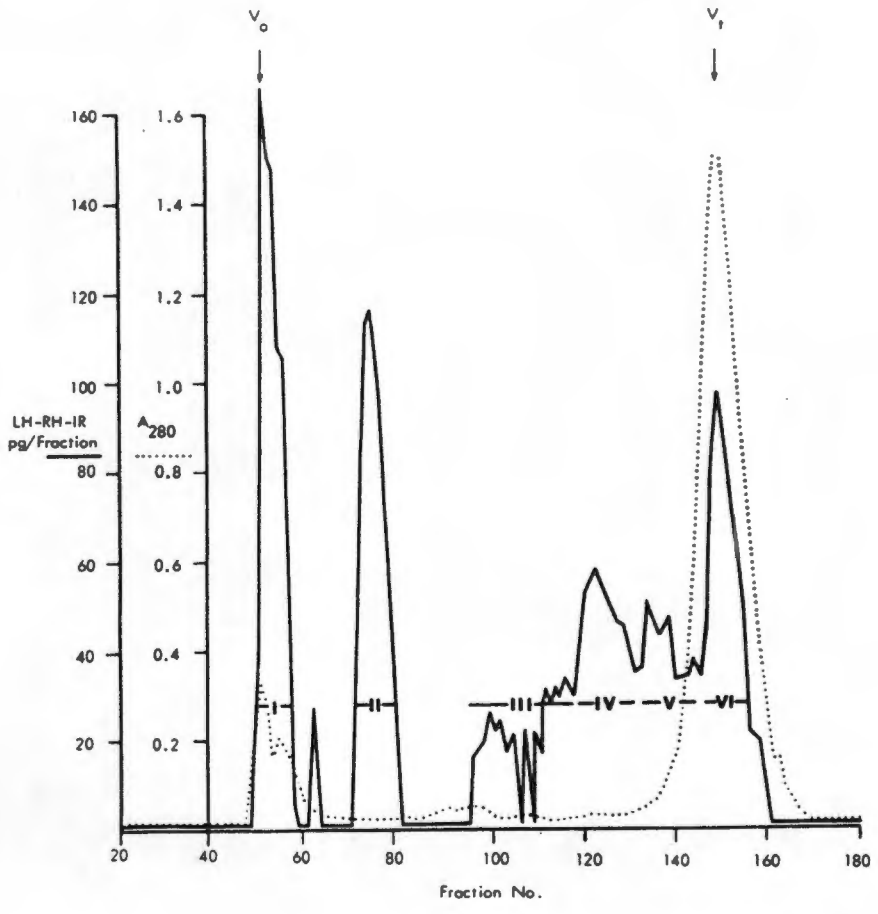

Fig. 3.1 **SEPHADEX G-100 CHROMATOGRAPHY OF AFFINITY PURIFIED RAT TESTICULAR LH-RH-IR.**
3.5ng of LH-RH-IR, partially purified by affinity chromatography was reconstituted in 2N AcOH, 8M GuHCl, incubated at 50°C for 3h, placed on a Sephadex G-100 column (86.5cm x 5cm) and eluted with 2N AcOH at a flow rate of 30ml/h at 4°C. 30min fractions were collected, lyophilized and assayed with antiserum 1076.

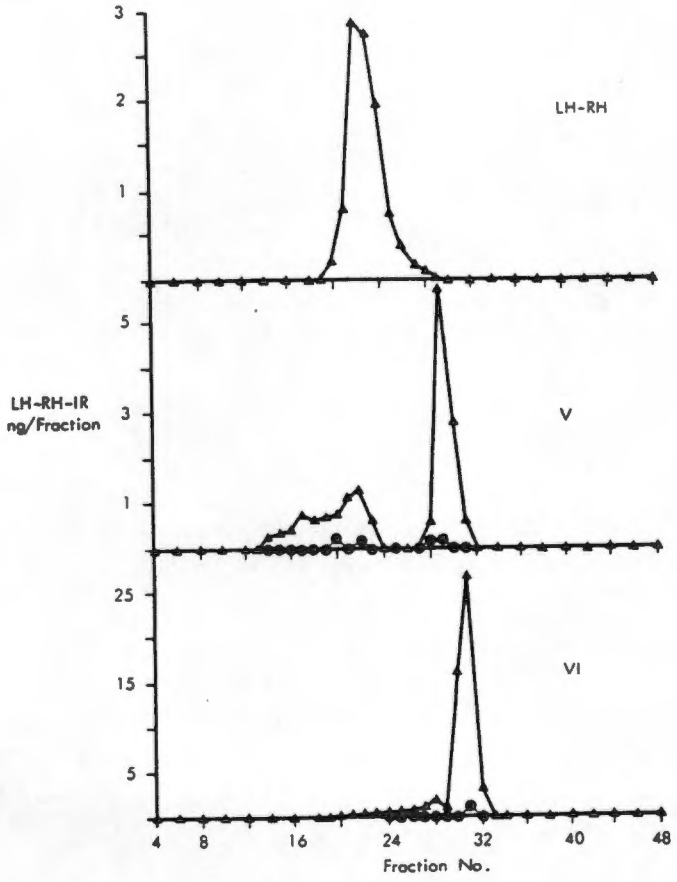

Fig. 3.2 **SEPHADEX G-25 CHROMATOGRAPHY OF LH-RH AND POOLED TESTICULAR LH-RH-IR PEAKS V AND VI OFF SEPHADEX G-100 Fig 3.1).**
The G-25 column (87.5cm x 1.7cm) was run at 4°C at a flow rate of 12ml/h and 30min fractions were collected. Material placed on the column was in 2N AcOH, 8M GuHCl and eluted with 2N AcOH. Lyophilized column fractions were assayed with antiserum 1076 and antiserum 744.

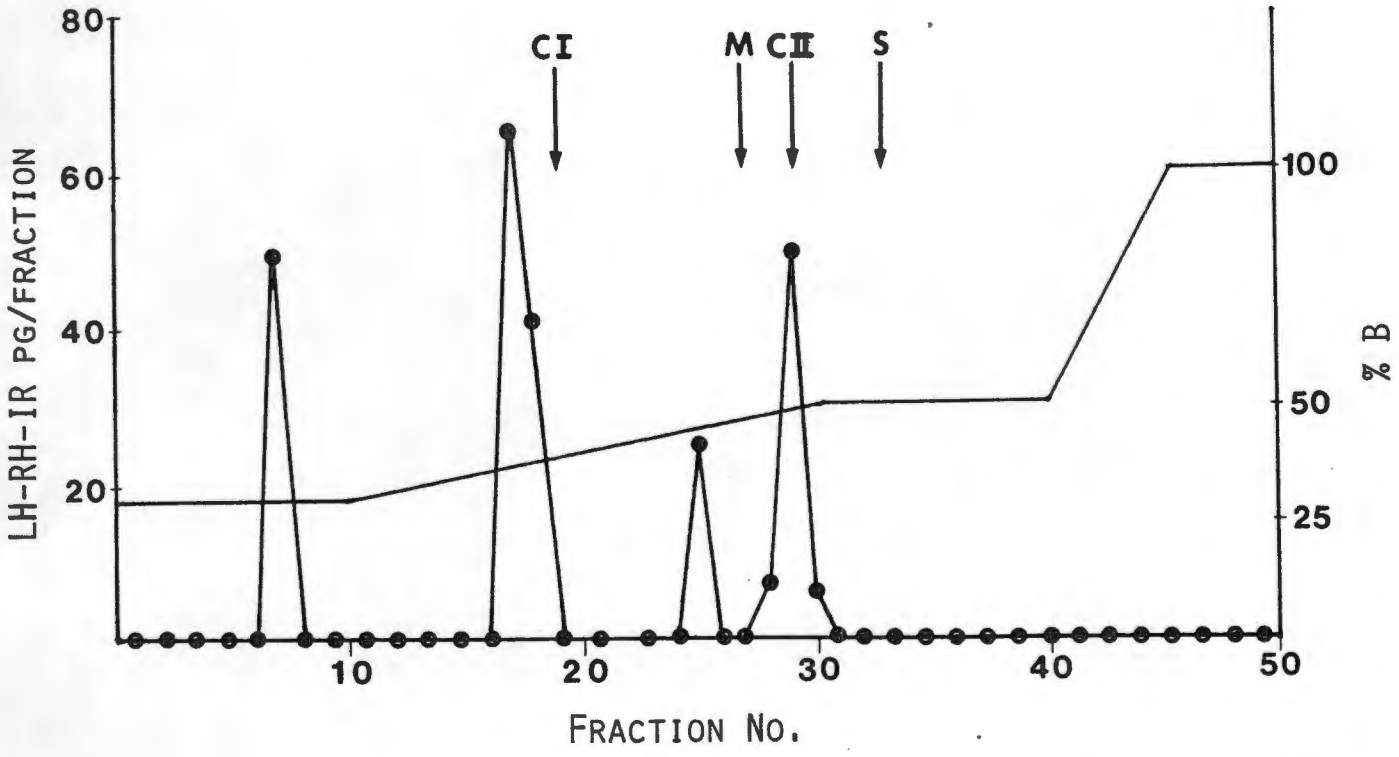

Fig. 3.3 **VYDAC C18 REVERSE PHASE HPLC OF RAT TESIS ACETIC ACID EXTRACTS.**
20 rat testes were extracted in 2N AcOH. The lyophilized extract was passed through 3 Sep-pak C-18 cartridges in 1% TFA and the retained peptides eluted in 60% acetonitrile, 1% TFA. The lyophilized eluant was applied to the column in 1 ml of the starting buffer and chromatographed as outlined in 2.3.2.4(b). Prior to this column run, fractions from a blank run, performed under identical conditions, were assayed and shown not to contain LH-RH-IR. The four vertebrate forms of LH-RH were shown to elute in the positions indicated in subsequent column runs under identical conditions.

(CI=Chicken-I LH-RH; CII=Chicken-II LH-RH; M=Mammalian LH-RH; S=Salmon LH-RH)

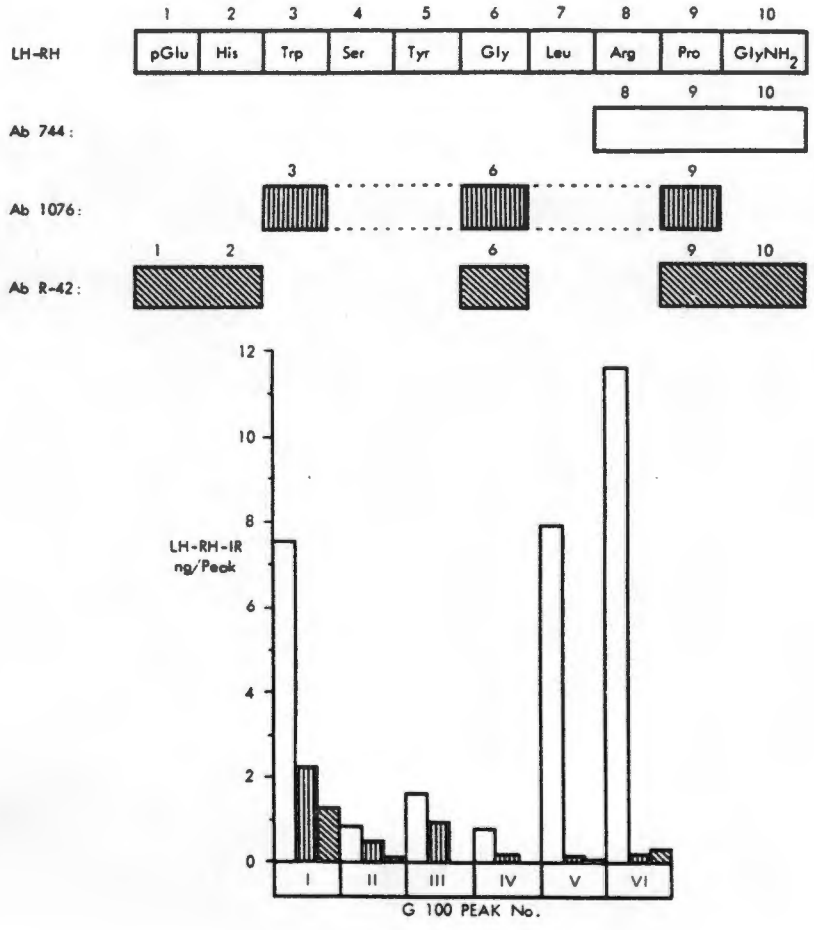

Fig. 3.4 **ESTIMATION BY THREE ANTISERA OF LH-RH-IR IN THE POOLED SEPHADEX G-100 PEAK FRACTIONS OF RAT TESTIS EXTRACT (Fig.3.1).**
The parts of the LH-RH molecule to which the three antisera are directed are indicated. This information was obtained from cross reactivity studies using LH-RH analogues and fragments in the radioimmunoassay employing these antisera (see Table 2.2). Antiserum 744 (C-terminally directed) estimated the amount of LH-RH-IR in each of the pooled peaks to a greater extent than did antisera 1076 (middle directed) and R-42 (N- and C-terminally directed).

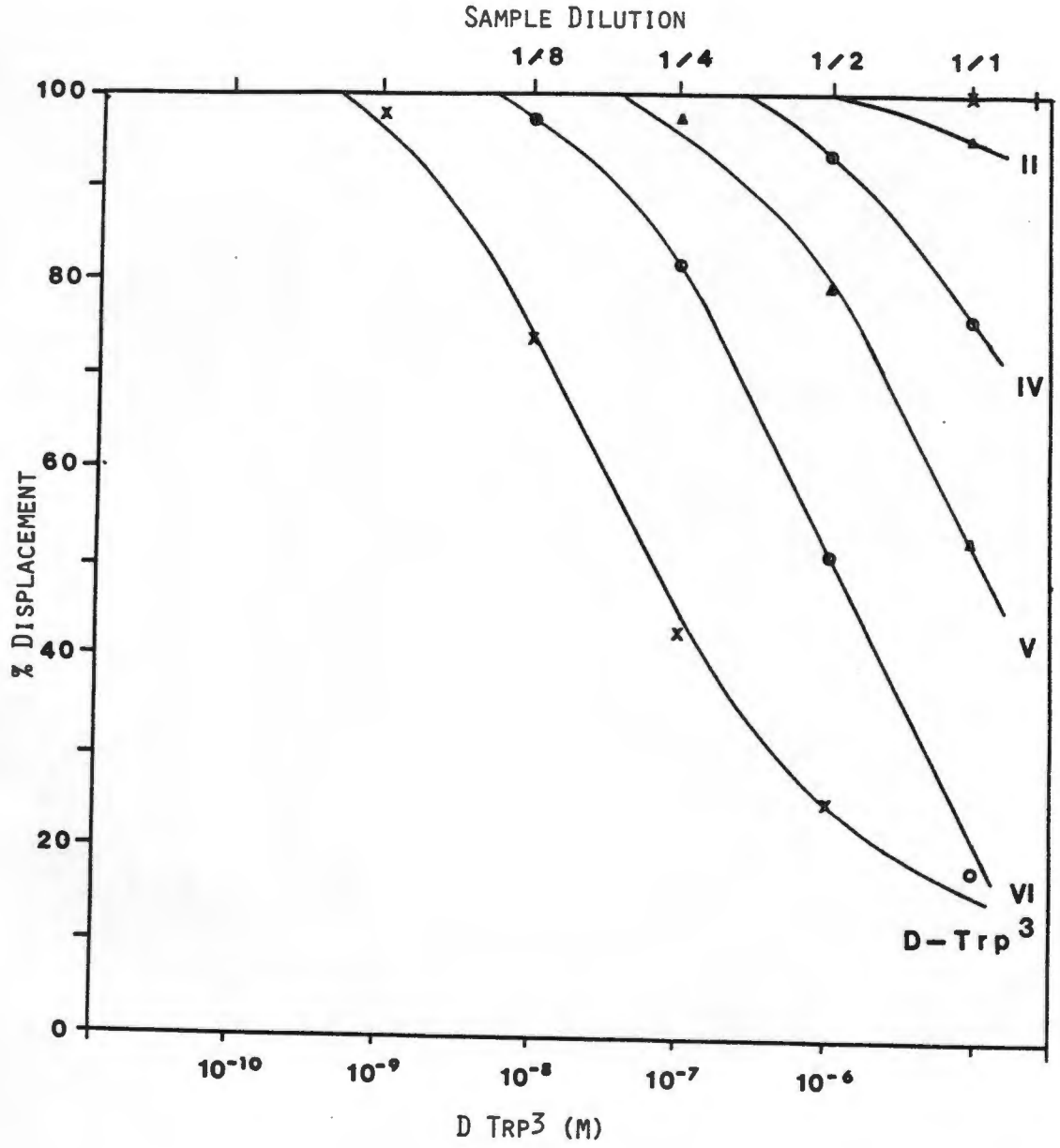

Fig. 3.5 **DISPLACEMENT OF ^{125}I LABELLED LH-RH AGONIST FROM RAT PITUITARY MEMBRANES BY TESTICULAR LH-RH-IR SIZE FRACTIONATED ON SEPHADEX G-100**
Five of the six peaks of LH-RH-IR separated on Sephadex G-100 (Fig. 3.1) were serially diluted and assayed for LH-RH receptor binding activity using rat pituitary membranes. The smaller LH-RH-IR species consistently displayed a higher affinity for the pituitary receptor of LH-RH.

3.3.1 Methods

3.3.1.1 Seminiferous Tubule Preparation

A decapsulated pig testis weighing 470 g was rinsed in a buffered solution of 2.5mM (pH 7.2), 137mM NaCl 5mM KCl, 0.7mM Na_2HPO_4. The testis was then incubated at 35°C for 15 minutes in rinse solution containing 0.4 mg% collagenase (140 IU/mg), 10 ug/ml DNase and 1% BSA. The tubules were then centrifuged at 1000 g for 10 minutes at 4°C and washed thrice with 0.9% saline at 4°C.

3.3.2. Results

Assay of testis and seminiferous tubules of different mammalian species with the three LH-RH-site-specific antisera shown in figure 3.6 gave similar relative quantitation as for the rat (see Fig. 3.4). No significant enrichment of the peptide was achieved either by removing the interstitial cells from pig testes (Fig. 3.6) or by extracting histologically confirmed Sertoli cell carcinomas of dogs (Fig. 3.7).

3.4 LH-RH-IR IN HUMAN SEMINAL PLASMA

Since high doses of exogenously administered LH-RH agonists have antifertility effects at the level of the testis it could be envisaged that certain classes of infertility in man may be due to pathologically high levels of the testicular LH-RH variant. Thus, a number of semen specimens were obtained from a fertility clinic, and assayed for LH-RH-IR.

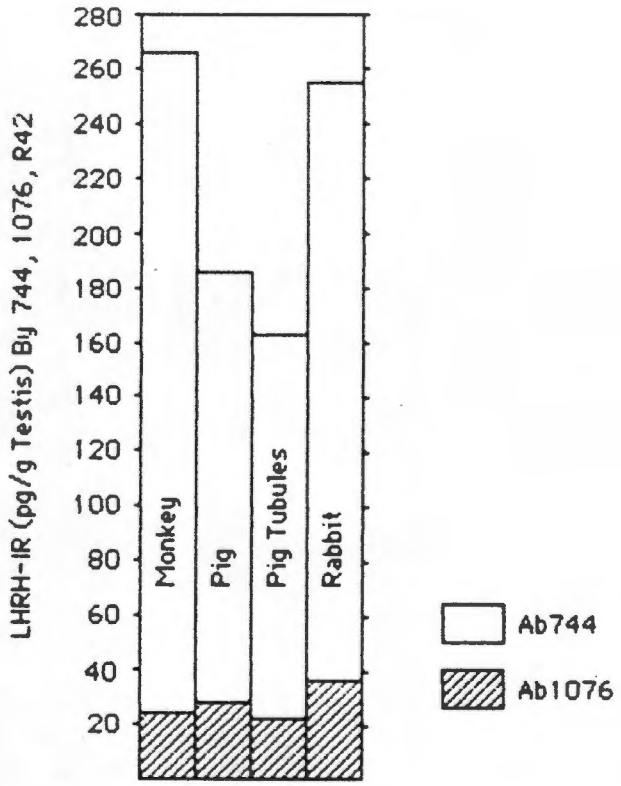

Fig. 3.6 **LH-RH-IR IN THE TESTES OF THREE VERTEBRATE SPECIES** Acetic acid extracts of monkey, pig, and rabbit testes were examined as potential sources of testicular LH-RH-IR for purification. The material in all cases was estimated to the same degree by Ab 744 and Ab 1076 as for the rat testis extract, indicating that the same peptide is present in these species. No enrichment was obtained by preparing pig tubules, i.e. enriching for Sertoli cells.

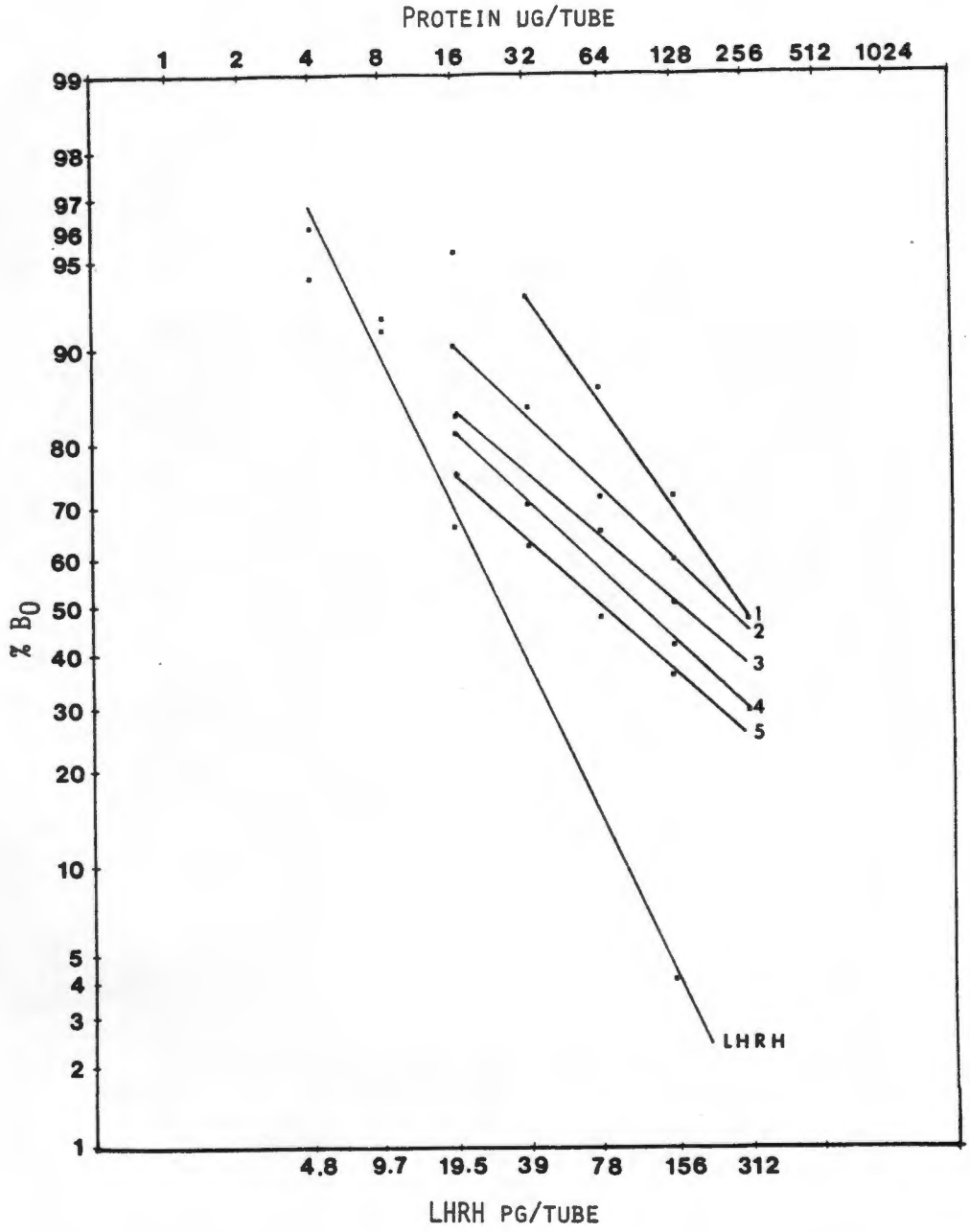

Fig. 3.7 **RADIOIMMUNOASSAY OF DOG TESTIS AND DOG SERTOLI CELL CARCINOMA EXTRACTS FOR LH-RH-IR**
Acetic acid extracts of normal dog testes (samples 1, 2 and 5) and Sertoli cell carcinomas (samples 3 and 4) were assayed for LH-RH-IR using Ab 1076. Non-parallel displacement indicating the presence of a peptide disimilar from the mammalian decapeptide was obtained in all cases. The Sertoli cell carcinomas did not prove to be an enriched source for the peptide.

3.4.1 Methods

Because of the high levels of proteases in seminal plasma (Lundquist et al., 1955) the following peptide extraction protocol was followed. Semen samples were placed directly in liquid nitrogen when collected. The snap frozen samples were placed directly into a boiling water bath and incubated at 100°C for 30 minutes. The samples were placed on ice and made 0.2N with respect to acetic acid which had been pre-cooled to 4°C. The protease inhibitors Bacitracin and p-Chloro Mercuric Benzoate were added to concentrations of 10^{-4}M and 10^{-5}M respectively (Horsthemke et al., 1980). The samples were incubated at 4°C for 30 minutes and centrifuged for 30 minutes at 18,000 g. The supernatants were lyophilized and reconstituted in 0.02N acetic acid containing the two inhibitors listed above and assayed for LH-RH-IR.

3.4.2 Results

Enhanced binding of radiolabelled LH-RH was observed when assaying all human semen extracts for LH-RH-IR. The binding of radiolabelled LH-RH by semen extracts in the absence of antibody made detection of LH-RH-IR extremely difficult. However, the presence of low levels of LH-RH-IR could be extrapolated by subtracting the binding factor component of the assay from the antibody plus binding factor components (i.e. the enhanced or total binding figure) (Fig. 3.8).

Sephadex G-25 fractionated semen samples were obtained from the laboratory of Dr. Peter Gray of the University of the Orange Free State (Gray et al., 1984). In the case of normozoospermic and oligospermic seminal plasma chromatographed on Sephadex G-25,

no LH-RH-IR was detectable in any of the column fractions. However, the chromatographed azoospermic seminal plasma had two fractions with a significant level of LH-RH-IR. The inability to detect any LH-RH-IR at all in the column fractions of the chromatographed normozoospermic and of oligozoospermic semen samples, obtained from Dr. P. Gray, could be due to the fact that stringent steps were not taken to eliminate proteolytic degradation of peptides prior to the column runs. As for the LH-RH-IR extracted from a number of species, the azoospermic seminal plasma column fractions were recognized to a greater extent by the C-terminally directed antiserum (antiserum 774) than by the antisera requiring either both termini or the middle region of the mammalian hypothalamic decapeptide for binding (Fig.3.9).

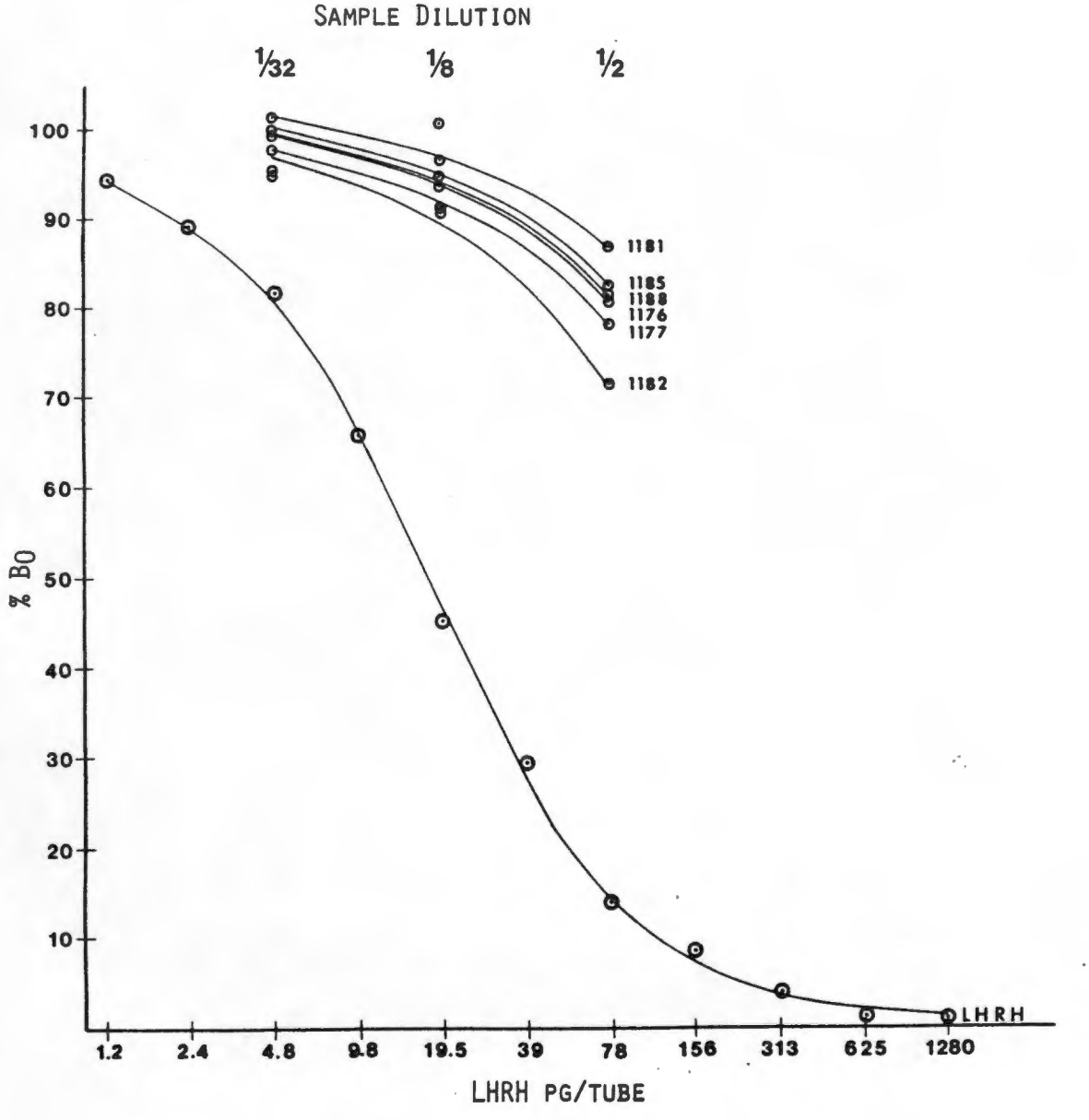

Fig. 3.8 **RADIOIMMUNOASSAY OF SERIALLY DILUTED HUMAN SEMEN SAMPLES FOR LH-RH-IR**
Assay of human semen extracts for LH-RH-IR initially showed the presence of LH-RH binding factors in all cases (evidenced by enhanced binding of ^{125}I-LH-RH in the assay). When no antibody was added to the assay tubes, the binding component due to these factors was noted. Subtraction of this "non-specific-binding" component in the assay enabled the estimation of low amounts of LH-RH-IR in all semen samples.

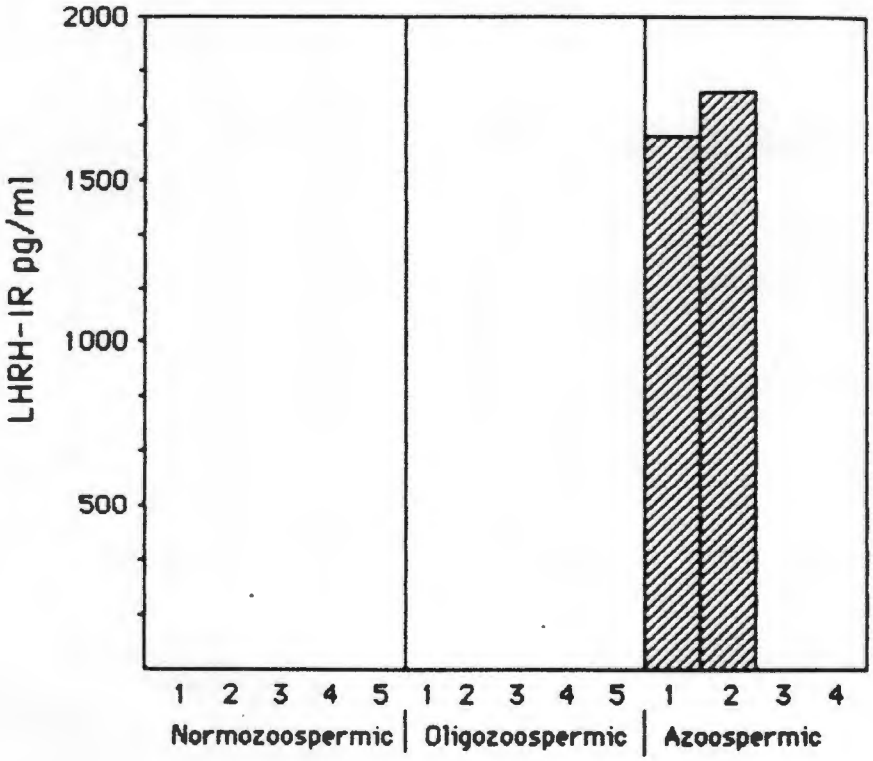

Fig. 3.9 **RADIOIMMUNOASSAY OF POOLED FRACTIONS OF SEPHADEX G-25 CHROMATOGRAPHED SEMEN EXTRACTS**
Pooled semen samples from either normo-, oligo-, or azoospermic individuals were extracted for peptides and size fractionated on Sephadex G-25 columns. Pooled fractions were assayed for LH-RH-IR using antisera 1076 and 744. LH-RH-IR was only observed in fractions of the azoospermic semen sample. Estimation by Ab 744 was higher than by Ab 1076 indicating that the same peptide found in rat, pig, dog and rabbit testes is present in human semen. Whether the relatively high levels of the peptide have a pathological role in azoospermia deserves further investigation.

3.5 CONCLUSIONS

There exist in mammalian gonads, peptides which are recognized by some antisera raised to hypothalamic LH-RH. The peptides can bind to the LH-RH receptors of pituitary gonadotrophs and may serve as the endogenous ligand for the previously described Leydig cell "LH-RH receptors". The peptide probably results from the processing by proteolytic cleavage of larger precursor forms. The physiological role of the gonadal peptide is not yet known. The effect of exogenous LH-RH on the enzymes of the steroidogenic pathway is, however, strongly suggestive of a paracrine role for the testicular peptides in regulating steroidogenesis (Jones et al., 1981, 1982; Hsueh et al., 1980). High levels of the endogenously synthesized peptide may also play a role in the pathophysiology of azoospermia, as suggested by this study. The relatively high levels detected in the seminal plasma from patients with this disorder and the fact that such a condition can be induced in laboratory animals by high levels of LH-RH agonists is supportive of such a pathological role for the peptide (Labrie et al., 1980). Evidence has recently been provided for a LH-RH-like glycoprotein of between 10,000 and 30,000 daltons in pooled human semen (Sokol et al., 1985). The peptide does not seem to be involved in the feminization observed in dogs with Sertoli cell carcinomas.

CHAPTER 4

DESIGN AND SYNTHESIS OF LH-RH OLIGONUCLEOTIDES

Synthetic oligonucleotides have proven effective in detecting complementary polynucleotides. DNA detection by oligomers employed in Southern Blot hybridization (Montgomery et al., 1978); cDNA synthesis using oligonucleotides as primers (Smith, 1980); and RNA detection by Northern Blot analysis with small synthetic probes (Thomas, 1980) have become established techniques in characterizing the mammalian genome, specific mRNAs and, by inference, specific polypeptides. The rapid chemical synthesis of oligonucleotides by the phosphoramidite intermediate technique (Caruthers, 1982) and the advances made in recombinant DNA technology have made it possible to investigate the nature of peptide hormone precursors (Eiden, 1982). The extremely low concentrations of LH-RH in hypothalamic tissue (~300 ng/g wet tissue) (Palkovits et al., 1974) has made the application of cDNA oligonucleotides and recombinant DNA technology a more feasible alternative than classical protein isolation and sequencing to establish the nature of the LH-RH precursor. As in the cases of somatostatin (Hobart et al., 1980) and the opioid family of peptides (Gubler et al., 1982), recombinant DNA techniques could potentially verify the existence of genes related to the LH-RH gene. Oligonucleotide primer extension studies could also be employed to characterize the 5′ end of LH-RH encoding mRNAs. Transcription start sites and potential promoter and enhancer sequences could then be deduced from gene sequencing studies. Solution phase hybridization and 5′ nuclease protection assays

using oligonucleotides could also be used to quantitate low levels of LH-RH mRNA.

4.1 DESIGN OF cDNA PROBES

There is a high degree of codon degeneracy for certain of the amino acids in the LH-RH decapeptide. There are in fact 110,592 unique 30-mer mRNA sequences that could code for the peptide (Table 4.1). In order to simplify the task of determining the most likely probes for mammalian LH-RH mRNA the following strategies were employed.

4.1.1 Origin of the C-and-N-Terminal Amino Acids

LH-RH is "blocked" at both termini by unusual amino acids, viz. pyroglutamic acid at the N-terminus and glycine-amide at the C-terminus. The pyroglutamic acid (pGlu) of peptides is derived from glutamine (Gln) in precursor polypeptides. In all the cases investigated to date Gln is the amino acid which becomes cyclised during the processing of the precursor polypeptide (Millar, et al., 1978, 1981; Folkers, et al., 1981; Abraham et al., 1981). Glutamic acid (Glu) can also be chemically converted to pGlu but there is no evidence that this is the origin of pGlu in ribosomally biosynthesised polypeptides.

Structural analysis of a number of C-terminally amidated peptides has revealed that they are all derived from precursors having a glycine (Gly) residue C-terminally adjacent to the amidated amino acid (Millar et al., 1978, 1981). Furthermore, a pituitary amidating enzyme has been demonstrated to have an absolute requirement for Gly on the C-terminal side of the amino

acid which undergoes oxidative transamidation (Bradbury et al., 1982).

On the basis of these studies, Gln was assumed to be the precursor of $pGlu^1$, and Gly^{11} the donor of the amino group to the C-terminal $Gly^{10}NH_2$ of LH-RH.

4.1.2 Heptadecamer Probes for N-Terminal Coding Sequences

Statistically, an oligonucleotide of "n" nucleotides occurs only once at random in a polynucleotide target when 4^n is the number of nucleotides in the polynucleotide (Thomas, 1966; Smith, 1984). For the mammalian genome as the polynucleotide ($\sim 10^{10}$ bases), n=17.

In order to synthesize fewer oligonucleotide probes for the LH-RH mRNA sequence, the mRNA sequence was divided into a N-terminal coding region and a C-terminal coding region. Heptadecamer probes for both mRNA stretches could then be used in parallel experiments to identify mRNA species having sequence homology to both probes. From the statistical argument presented above, a mRNA species which hybridizes to both groups of oligonucleotides has a high likelihood of being LH-RH encoding. The N-terminal section of LH-RH from $pGlu^1$ to Gly^6, was chosen for the synthesis of the first group of cDNA oligonucleotide probes. The third nucleotide of the Gly^6 codon was eliminated resulting in a heptadecamer sequence having only 48 possibilities. It was decided to synthesize all the likely cDNA heptadecamer probes for the LH-RH N-terminal coding sequence in three groups of 16 oligonucleotides viz. groups L1, L2, L3 (Table 4.2)

Codons		Gln[1]	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly[10]	
	5'	CAA G	CAU C	UGG	UCN AGU C	UAU C	GGN	CUN UUA G	CGN AGA A	CCN	GGN	3'
Codons per amino acid		2	2	1	6	2	4	6	6	4	4	
No of unique 30-mers		110	592									

Table 4.1 **CODON DEGENERACY FOR LH-RH AMINO ACIDS**
The high degree of redundancy in the coding for the amino acids that constitute the LH-RH decapeptide indicates that the authentic 30-mer sequence coding for LH-RH is one of 110 592 possibilities.

LH-RH N-Terminus Codons			Gln[1]	His	Trp	Ser	Tyr	Gly[4]	
		5'	CAA G	CAU C	UGG	UCN AGU C	UAU C	GGN	3'
Oligomer Mixture	L 1	3'	GTT C	GTA G	ACC	AGA G	ATA G	CC	5'
	L 2	3'	GTT C	GTA G	ACC	AGC T	ATA G	CC	5'
	L 3	3'	GTT C	GTA G	ACC	TCA G	ATA G	CC	5'

Table 4.2 **cDNA OLIGONUCLEOTIDES FOR LH-RH GLN[1] - GLY[4]**
The 48 possible unique heptadecamers complimentary to the RNA encoding the N-terminus of LH-RH were synthesized as three groups of 16 oligonucleotides each, viz. L1, L2 and L3.

4.1.3 Probes for C-Terminal Coding Sequence

The diversity in mRNA sequence for the C-terminal section of LH-RH from Gly^6 to Gly^{11} was more extensive. The heptadecamer sequence which excluded the last nucleotide of the Gly^{11} triplet included 1,536 possibilities.

4.1.3.1 Natural Codon Frequency Considerations

In order to determine the most likely LH-RH C-terminus encoding sequences, the codon incidence for each amino acid represented at the C-terminus was examined in a number of neuropeptide hormone and brain protein messengers. It has been observed that there exists in eukaryotic mRNA a natural frequency of codon selection for each of the amino acids (Estratiades et al., 1977; Shine et al., 1977). In order to see whether such a preference for certain codons exists in the mRNAs of neuropeptide hormones and brain proteins, seven such mRNAs were examined. Table 4.3 indicates that such a frequency of codon selection does exist in these polypeptide messengers. This information was then used to choose the most likely codons to screen for in the mRNA for the LH-RH C-terminus (Table 4.5).

4.1.3.2 Arg^8 Codons: Evolutionary Considerations

In the case of codons for Arg, the third nucleotide is represented to a similar extent by both C and G bases. Since mammalian LH-RH may have evolved from an ancestral molecule which also gave rise to avian Gln^8-LH-RH (King et al., 1982) the codon representing single point mutations from Gln to Arg were examined. Two possibilities exist as demonstrated in Table 4.4. Since CGG (as opposed to CGA) is the codon occuring most

LH-RH C-Terminus amino acids	Gly^{6}	Leu	Arg	Pro^{7}
Codon Incidence in 7 polypeptides	GGC_{58}	$C_{133}UG_{94}$	$C_{54}GG_{28}$	CCC_{41}
	G_{26}	C_{32}	C_{30}	G_{14}
	U_{13}	A_{14}	A_{14}	U_{9}
	A_{8}	U_{17} U_{14}	A_{27} U_{9}	A_{7}

Table 4.3 **CODON FREQUENCY FOR THE LH-RH C-TERMINUS AMINO ACIDS**
The incidence of the various codons for each amino acid of the LH-RH C-terminus was determined in mRNAs encoding 7 polypeptides viz. the ACTH-precursor (Nakanishi et al, 1979), the Vasopressin precursor (Land et al,1982), the Calcitonin precursor (Jacobs et al, 1981), the Enkephalin precursor (Noda et al, 1982), the Tubulin precursor (Ginzburg et al, 1981), the Human Growth Hormone precursor (Martial et al, 1979) and the Somatostatin precursor (Hobart et al, 1980). The codon incidence in all the mRNAs for each individual amino acid followed the same general trend for all 7 polypeptides.

Amino Acid Change		Gln^{8}	->	Arg^{8}
Codon Changes	1 Mutation :	CAG	->	CGG
		CAA	->	CGA
	2 Mutations:		->	CGC
			->	CGU
			->	AGG
			->	AGA

Table 4.4 **Gln^{8} -> Arg^{8} MUTATION - POSSIBLE CODON CHANGES IF MAMMALIAN LH-RH AROSE FROM CHICKEN-I LH-RH**
A single point mutation of the second nucleotide of the Gln^{8} triplet of chicken-I LH-RH could give rise to the mammalian peptide. If this assumption is correct, it would indicate that the Arg^{8} codon is either CGG or CGA. Since the CGG codon occurs at a higher frequency in mammalian mRNA, it was chosen as the most likely codon for Arg^{8} in the mammalian peptide.

frequently in the mRNAs listed in Table 4.3, it was chosen as the most likely triplet to represent Arg^8 in the mammalian LH-RH message.

4.1.3.3 LH-RH C-Terminus: Most likely cDNA Probes

On the basis of the case presented above four oligonucleotides for the C-terminal coding region of LH-RH, were synthesized. These are shown in Table 4.5 as oligonucleotide group L4. It has been demonstrated that dG-rU mismatches do not effect duplex stability during hybridization (Lomart et al., 1975). Thus in the case of Gly^6 and Gly^{10}, three codon possibilities, viz. GGC, GGG and GGU could hybridize to the 4 probes synthesised. The cDNA triplet for Leu (GGG) would also hybridize to the CCU codon. The initial group of four probes (L4) would thus effectively hybridize to 18 unique sequences. In the event of other mismatches, the high G-C content of these four probes (16 out of 17 bases) would counteract the duplex instability to some degree (Lipsett et al., 1964; Smith, 1984).

4.1.3.4 Probes for statistically less probable LH-RH C-terminus coding sequences

Since the above reasoning is only useful in deriving the most likely codons and can not guarantee the actual sequences, other cDNA oligonucleotides representing the LH-RH C-terminus were also synthesised viz. L5-L8 (Table 4.5). In order to reduce the number of C-terminal oligomers synthesized while increasing the number of sequences screened, 14-mers were chosen. The 64 oligomers synthesized on this basis would screen for two-thirds of all the possible LH-RH C-terminal coding sequences. Since

LH-RH C-Terminus			Gly[6]	Leu	Arg	Pro	Gly[10]NH_2		
LH-RH Precursor			Gly[6]	Leu	Arg	Pro	Gly	Gly[11]	
"Preferred" Codons		5'	GGC G	CUG	CGG	CCC	GGC G	GG	3'
Oligonucleotide Mixture	L4	3'	CCG C	GAC	GCC	GGG	CCG C	CC	5'
	L5	3'	CCA G	GAA G	GCC T	GGG T	CC	5'	
	L6	3'	CCA G	GAC T	GCC T	GGG T	CC	5'	
	L7	3'	CCC T	GAA G	GCC T	GGG T	CC	5'	
	L8	3'	CCC T	GAC T	GCC T	GGG T	CC	5'	

Table 4.5 **OLIGONUCLEOTIDE PROBES FOR LH-RH C-TERMINAL CODING SEQUENCES**

L4 was synthesized as a mixture of heptadecamers. The decision to have both G and C as the third base in the case of the Gly codons in L4 was taken since 82% of the Gly residues of 7 polypeptides were encoded by these two codons (Table 4.3). CGG was the codon of choice in the case of Arg[8] for the reasons indicated in Table 4.4. L5, L6, L7 and L8 are each mixtures of 16 oligonucleotides. Thus, 64 of the 144 possible unique sequences for this section of the LH-RH encoding message would be screened by these four mixtures. Having G as the third base in the probe for the Pro[9] codon would facilitate dG:rU matching as well, enabling each of the 16 probes to hybridize to 24 unique sequences. Thus, 96 of the possible 144 coding sequences for this part of the LH-RH would hybridize to these probes.

these C-terminal probes would only serve as secondary confirmatory probes for LH-RH mRNAs detected unequivocally by the N-terminal probes, their size was not seen to be in conflict with the statistical limits deduced by Thomas and Smith (Thomas et al., 1966; Smith et al., 1984). Small probes of this length and even shorter have been demonstrated to be very effective in identifying unique polynucleotide sequences (Wallace et al., 1981; Furutani et al., 1983; Richter et al., 1984).

4.2 SYNTHESIS OF LH-RH OLIGONUCLEOTIDES

The use of solid support polymeric carriers has greatly simplified the multistep synthesis of oligonucleotides of defined sequence. The macromolecular support serves to protect one of the nucleotide chain termini and provides an efficient and easy method of separating the attached growing biopolymer from soluble reactants. The method of Caruthers employing silica as the solid support system and deoxynucleoside phosphoramidites as the building blocks was slightly modified and used to synthesize the LH-RH cDNA oligomers (Caruthers, 1982; Beaucage et al., 1981).

4.2.1 Synthesis of the Support

Synthesis of the oligonucleotide takes place in the 3′ to 5′ direction. The initial step thus involves attachment of the activated first nucleotide at the 3′ end of the oligomer to an activated silica gel support.

4.2.1.1 Activation of Silica Gel

HPLC grade silica gel was exposed to a saturated LiCl solution for 24 hours. The silica was then treated with 3-

triethoxysilylpropylamine in dry toluene for 12 hours at 20°C. After low speed certrifugation, the gel was washed three times with each of the following solvents: toluene; methanol; and 50% methanol in water. The silica was shaken in 3-triethoxysilylpropylamine in toluene at 20°C for 18 hours. It was then washed in methanol and ether and dried in vacuo. The dry silica was then suspended in anhydrous pyridine, treated with trimethylsilyl chloride for 12 hours at 20°C, washed with methanol and ether and dried in vacuo.

4.2.1.2 Attachment of Nucleoside to Activated Silica.

The dry silica was suspended in dimethyl formamide (DMF) and a solution of the 5'-dimethoxytrityl nucleoside-3'-p-nitrophenyl succinate in dioxane and triethylamine was added. The suspension was shaken at 20°C for 4 hours. Free amino groups on the silica were measured with ninhydrin. To acylate or "cap" these groups, acetic acid was added to the resin and shaken for 30 minutes, after which a negative ninhydrin test resulted. The silica was then washed with DMF followed by ether and dried in vacuo. In order to analyse the extent of nucleoside attachment to the solid support, an accurately weighed sample of silica (10-15 mg) was treated with 5 ml of 72% perchloric acid: ethanol (3:2 v/v) and the optical density of the supernatant after centrifugation was measured at 497 nm (an acid solution of dimethoxytritanol has an extinction coefficient of 70,000 cm $mole^{-1}$). A typical preparation yielded 60-80 moles of bound nucleoside per g of silica.

4.2.2 Synthesis Procedure

The derivatized nucleosides were added in molar excess to an appropriate quantity of resin by the following general scheme.

4.2.2.1 Deblocking the Attached Nucleoside

The appropriately derivatized deoxynucleoside attached covalently to the silica was treated with 2% trichloro acetic acid (TCA) in dichloromethane for 0.5 min and then washed with fresh TCA solution.

Three thorough washes each with dichloromethane, acetonitrile and dry acetonitrile, under a nitrogen atmosphere, prepared the activated nucleoside for condensation.

4.2.2.2 Condensation

Stock solutions of protected 2'-deoxynucleoside-3-N,N dimethylaminomethoxyphosphines in dry acetonitrile and sublimed tetrazole in dry acetonitrile were stored under nitrogen. These were added to the silica-attached deoxynucleoside in excess (10 molar excess for the phosphine and 50 molar excess for the tetrazole). Condensation occured under the inert gas atmosphere for 5 minutes. The silica was then washed with tetrahydrofuran (THF).

4.2.2.3 Capping

Unreacted hydroxyl groups were acylated for 2 minutes with 0.6N dimethyamino pyridine in dry THF mixed with 6N acetic anhydride in 2,6 Lutidine (4:1 v/v). The blocked nucleoside attached to the resin was then washed with THF.

4.2.2.4 Oxidation

The trivalent phosphorus was oxidised to pentavalent phosphate with 0.1M iodine in THF: 2,6 Lutidine: H_2O (2:1:1 v/v) for 3 minutes.

The solid support-bound dinucleotide was then washed with THF, methanol and dichloromethane. This reaction cycle was repeated for each nucleotide that was attached.

4.2.3 Release of Oligonucleotide from Solid Support

The methyl blocking groups were removed from the oligomers prior to their release from the support by ammoniolysis. Demethylation of the oligonucleotide on the solid support was achieved by incubating the silica with thiophenol: triethylamine: dioxane (1:1:2 v/v) for 75 minutes. After washing with methanol and dichloromethane, the silica was suspended in 25% aqueous ammonia and kept sealed at room temperature for 18 hours. Fresh ammonia was added and the reaction continued at 55°C for 12 hours. The liquid phase was then evaporated to dryness.

4.2.4 Purification of the Oligonucleotides

The dry oligonucleotide was resuspended in sterile water and an aliquot was loaded onto a 20% Acrylamide 8M Urea preparative gel and electrophoresed in TBE at 20 mA constant current for 3 hours. The oligonucleotides were visualized over a fluorescent silica plate under UV irradiation. The relevant oligomer bands, as determined from standards of different oligonucleotide length were cut from the gel with a scalpel blade. The gel slice was crushed and extracted overnight in 0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA and 0.1% SDS (Maxam et al., 1977).

The oligonucleotide was then desalted on a Sephadex G-25 column (1 cm x 10 cm) and eluted with sterile water. The pooled void volume peak of UV absorbing (OD_{260nm}) oligonucleotide was lyophilized and stored dry or as aliquots in TE at -20°C.

4.2.5 Confirmation of Oligonucleotide Sequence

The chemically synthesised oligomers were radioactively labelled at the 5' terminus with ^{32}P by the T4 polynucleotide kinase catalysed reaction (Maxam et al., 1977). The sequences were confirmed by Dr. M. Bodner employing a modified Maxam-Gilbert chemical degradation sequencing technique.

4.3 OTHER PROBES

4.3.1 A Deoxyinosine Containing Oligonucleotide

Deoxyinosine insertion in oligonucleotide probes at ambiguous codon positions has recently been described as an alternative to synthesizing probes with mixtures of nucleotides in the wobble position (Ohtsuka et al., 1985). A 17-mer probe having inosine in positions of coding degeneracy for the N-terminus codons of LH-RH was synthesized for this study by Bill Huse of Yale University. This probe (L9) should hybridize to the N-terminal coding sequences for all the vertebrate LH-RHs except the recently described lamprey sequence (Table 4.6).

4.3.2 Unique Probe Deduced from the Human LH-RH Precursor cDNA

During the course of this study, the sequence of the human placental LH-RH precursor was established (Seeburg et al., 1984). A unique 18-mer probe for the precursor mRNA was synthesized for this study by Dr. Srivastava of Chemgenes Inc., Boston. This

1 Mammalian LH-RH	Gln^{1}	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro Gly^{10}
2 Chicken-I LH-RH								Gln^{8}	
3 Salmon LH-RH							Trp^{7}	Leu^{8}	
4 Chicken-II LH-RH					His^{5}		Trp^{7}	Tyr^{8}	
Possible Codons for 1,2 and 3	5'CAA G	CAU C	UGG	UCN AGU C	UAU C	GGN	3'		
Possible Codons for 4	5'.....	.....	.....	.....	CAT	....	3'		
Probe L9	3'GTI	GTI	ACC	AGI	ITI	CC	5'		

Table 4.6 **L9 - A PROBE WITH INOSINE AT AMBIGUOUS CODON POSITIONS**
Probe L9 was designed to hybridise to the mRNAs of all the known vertebrate LH-RHs except that of the recently described lamprey peptide. The N-terminal amino acids of these LH-RH variants is conserved to a greater degree than in the lamprey peptide (Tyr^{3},Leu^{5},Glu^{6},Trp^{7},Lys^{8}-LH-RH). Having inosine in positions of coding redundancy permits hybridisation of L9 to any of the 64 possible sequences encoding these LH-RHs (Ohtsuka et al, 1985).

H.P1. LH-RH Precursor 11-16		Gly^{11}	Lys	Arg	Asp	Ala	Glu^{16}
H.P1.LH-RH mRNA	5'	GGA	AAG	AGA	GAT	GCC	GAA 3'
Oligonucleotide L10	3'	CCT	TTC	TCT	CTA	CGG	CTT 5'

Table 4.7 **OLIGONUCLEOTIDE PROBE L10 - A UNIQUE PROBE FOR THE HUMAN PLACENTAL LH-RH mRNA**
The mRNA sequence encoding the first 6 amino acids immediately C-terminal to LH-RH in the precursor was chosen to derive a unique cDNA probe for the human LH-RH mRNA and gene.

oligonucleotide (L10) is directed to a region of the precursor mRNA immediately 3′ of the LH-RH coding sequence (Table 4.7).

4.3.3 A 34-mer Common to the Human, Rat and Mouse LH-RH Precursor cDNAs

The LH-RH precursor encoding mRNA in the human placenta (Seeburg et al., 1984), human hypothalamus (Adelman et al., 1986) rat hypothalamus (Adelman et al., 1986) and as deduced from the mouse gene (Mason et al., 1986a) were compared for sequence homology. The longest stretch of sequence homology in these mRNAs was found to be a 34 base sequence encoding amino acids -6 to +6 of the precursor protein (Table 4.8). A 34-mer oligonucleotide complementary to this region of the LH-RH mRNA was synthesized for this study by the Oswel DNA service of Edinburgh (Table 4.8).

aa		-6				-1	+1				-6	
H		Val	Glu	Gly	Cys	Ser	Ser	Gln	His	Trp	Ser	Tyr	Gly	
	5'	GTG	GAA	GCC	TGC	TCC	AGC	CAG	CAC	TGG	TCC	TAT	GGA	3'
R/M		Leu	.	.	.	.	.	.	.	.	.	.	.	
	5'	T	.	.	.	.	.	.	.	.	.	.	G	3'
L11	3'	AC	CTT	CCG	ACG	AGG	TCG	GTC	GTG	ACC	AGG	ATA	CC	5'

Table 4.8 **OLIGONUCLEOTIDE PROBE L11 - A UNIQUE PROBE FOR ALL LH-RH GENES SEQUENCED TO DATE.**
Oligonucleotide L11 is directed at the longest stretch of sequence homology shared by the human (H), rat (R) and mouse (M) LH-RH genes viz. the part encoding the first 6 amino acids of LH-RH and the adjacent 6 amino acids of the leader peptide. The dots indicate stretches of sequence homology.

Chapter 5

LH-RH PRECURSOR MRNA, DNA AND GENE REGULATION STUDIES

5.1 INTRODUCTION

In order to extend the study of the nature of the LH-RH precursor and related peptides, the oligonucleotide probes synthesized as outlined in the previous chapter were used to analyse mRNA from various sources, cDNA libraries, a genomic library and restriction endonuclease digested DNA.

Thus, a number of recombinant DNA techniques were employed primarily to obtain cDNA or genomic clones which contain mammalian LH-RH decapeptide encoding sequences. A second objective was to determine whether genes related to the mammalian LH-RH encoding gene occur in man. A final objective was to investigate LH-RH gene expression in the ovariectomized - estradiol replaced rat model for investigating the hypothalamo-hypophyseal-gonadal axis. This model was thought to be most likely to demonstrate changes in hypothalamic LH-RH precursor mRNA levels (previously unmeasurable) because of the exaggerated serum LH-levels observed after ovariectomy and the marked reversal to levels within the normal range for intact animals after estradiol replacement.

In order to enrich for the rat LH-RH encoding mRNA prior to cDNA cloning, several fractionation procedures were used. Membrane bound polysomes from rat and sheep hypothalami were shown to have higher amounts of LH-RH-IR associated with them than free polysomes, and were thus used as an enriched LH-RH mRNA

source. mRNA from rat and sheep hypothalami as well as from the HEp-3 human buccal cell line were size fractionated on sucrose density gradients and certain fractions were shown to be enriched for LH-RH encoding mRNA by dot-blot hybridization to oligonucleotide probes for LH-RH N- and C-terminus encoding sequences. Poly A mRNA was size fractionated by electrophoresis on methyl mercury hydroxide containing agarose gels and RNA extracted from gel slices was analysed by dot-blot analysis as well as by translation and immunoprecipitation in the Xenopus oocyte system. cDNA libraries made from these enriched sources of LH-RH mRNA were analysed by colony hybridization, plasmid isolation, cDNA insert excision and Southern blot hybridization. Sequence analysis of positively hybridising cDNAs from all these "enriched libraries" failed to reveal sequences which encode the LH-RH decapeptide. For the purpose of simplicity, none of the techniques employed or the results obtained in the course of these inconclusive investigations is presented here.

During the course of these studies the nature of the mammalian LH-RH precursor was elucidated from the sequence of cloned human placental and hypothalamic cDNA, rat hypothalamic cDNA and mouse genomic DNA. Probe L 11 (Table 4.8) which represents the longest stretch of sequence homology shared by these clones was used as a primer to construct an enriched library from rat pre-optic area poly A RNA. cDNA clones were obtained which extended beyond the 5′ end described in the published rat hypothalamic LH-RH cDNA sequence. Together with direct primer extension studies, the new sequence information obtained from these clones pin-pointed the 5′ end of rat hypothalamic LH-RH mRNA. The primer extension studies together

with an examination of the human placental LH-RH cDNA sequence provided substantial evidence for the existence of two transcription initiation sites within the LH-RH gene. Together with the fact that the 1st intron of the LH-RH gene is not removed in some instances of mRNA maturation (e.g. in the placenta), these observations have indicated the possibility of four mature mRNAs of differing length being transcribed from the LH-RH gene. Support for this possibility was supplied by northern blot hybridization results using sheep and rat hypothalamic poly A mRNA, as well as by primer extension studies using poly A RNA from the preoptic area of rat hypothalami, human hypothalami and two human breast tumour cell lines.

Southern blot hybridization studies using the unique probe L10 (Table 4.7) indicated that two other genes with sequences related to the GAP (GnRH Associated Peptide) N-terminus may be present in man.

In situ hybridization studies using a rat hypothalamic LH-RH precursor complementary-RNA probe and LH-RH-precursor mRNA quantitation by S1 nuclease assay using probe L11, support the concept of "reciprocal regulation" of LH-RH gene transcription relative to pituitary LH release in the ovariectomized-estradiol replaced rat model.

5.2 cDNA Cloning and Analysis of Transcription Start Sites

5.2.1 Methods

5.2.1.1 mRNA Isolation

RNA from the preoptic areas of rat hypothalami was extracted by the single step RNA procedure (Chomczynski and Sacchi, 1987). 5 g of frozen rat hypothalamic preoptic area were homogenised

with an Ultra Turrax homogeniser in 14 ml of 4 M Guanidinium Thiocyanate, 25 mM Sodium Citrate (pH 7.0), 0.5% Sarcosyl and 0.1 M beta-Mercaptoethanol (Solution D). 1.4 ml of 2 N NaOAC (pH 4.0) was added to the homogenate and mixed thoroughly, followed by 14 ml of H_2O saturated phenol and 2.8 ml of $CHCl_3$: isoamylalcohol (49:1). The solution was mixed vigorously for 10 seconds, incubated on ice for 15 minutes and centrifuged at 10 000 g for 20 minutes at 4°C. RNA was precipitated from the aqueous phase by addition of one volume of isopropanol followed by centrifugation at 10,000 g for 20 minutes at 4°C. The RNA pellet was resuspended in 5 ml of solution D, reprecipitated with isopropanol, washed with 75% ethanol at room temperature, dried by evacuation and resuspended in binding buffer for application to an oligo dT cellulose column. An aliquot was removed for quantitation by monitoring the OD_{260}/OD_{280} ratio.

5.2.1.2 Poly A mRNA Preparation

Poly A RNA was prepared on oligo dT cellulose chromatography by a modification of the method of Pelham (Pelham and Jackson, 1976). 7 mg of total RNA at a concentration of 1 mg/ml in binding buffer (10 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.05% SDS) was heated to 65°C for 5 minutes and applied directly to 2 ml (packed bed volume) of oligo dT cellulose (pre-equilibrated in binding buffer) mixed thoroughly and allowed to cool to room temperature. The resin was packed into a 5 ml syringe and the effluent re-applied to the column three times. The column was washed with 40 ml of binding buffer at a flow rate of ~0.2 ml/min. The poly A RNA was eluted by 10 applications of 400 ul of elution buffer (10 mM Tris/HCl (pH 7.5), 0.05% SDS). Poly A

RNA was pelleted from the 10 fractions by addition of 50 ul of 3 M NaOAC (pH 7.0) and 450 ul of isopropanol, incubation on crushed dry ice for 10 minutes and centrifugation at 10 000 g at 4°C for 20 minutes. Pellets were washed with 75% ethanol and evacuated dry.

5.2.1.3 cDNA Cloning

10 ug of Rat Preoptic Area poly A RNA was heat denatured in distilled water (dH_20) at 65°C for 3 minutes and placed directly on ice. The RNA was reverse transcribed in a 100 ul reaction in a mixture containing 50 mM Tris/HCl (pH 8.2), 140 mM KCl, 8 mM $MgCl_2$, 500 uM each dNTP, 20 units of Human Placental Ribonuclease Inhibitor and 20 units of Avian Myeloblastoma Virus Reverse Transcriptase in the presence of either 100 ng of oligonucleotide L11 or 2.5 ug of Oligo d(T) 12-18. The cDNA cloning strategy employed is outlined in Fig. 5.1.

a) Specific first strand synthesis

The oligonucleotide L11 primed reaction was incubated for 30 minutes at 42°C, heated to 65°C for 3 minutes, placed on ice for 3 minutes, 20 units of AMV Reverse Transcriptase added and the reaction repeated for 30 minutes at 42°C. This procedure was repeated ten times in order to increase the amount of specific primer extended cDNA. Second strand synthesis was performed by diluting the first strand reaction mix to 200 ul and adding DTT to 5 mM, 50 units of E. coli DNA polymerase 1, and 2 units of E. coli RNase H. After a 2 h incubation at 16°C, the reaction was terminated with 12 uM EDTA (pH 8.0), phenol: chloroform extracted and ethanol precipitated from 0.5 M ammonium acetate.

b) Selected second strand synthesis

After first strand synthesis, the oligo-d(T) 12-18 primed reaction mixture was used to resuspend the double stranded DNA pellet from the oligonucleotide L11 initiated synthesis. The second strand from the latter reaction was then used as a selective primer for 2nd strand synthesis off the oligo dT primed cDNA.

The reaction mixture was boiled for 3 minutes, chilled on ice, centrifuged for 3 minutes in a microcentrifuge and the supernatant transferred to a new tube. 5 ug of DNase-free RNase was added to the tube together with dNTPs to a final concentration of 200 uM and 80 units of reverse transcriptase. After incubation at 42°C for 2 h, the reaction volume was doubled and a further 20 units of reverse transcriptase and 10 units of DNA polymerase I large fragment were added. Second strand synthesis was continued for 1 h at 37°C and after the addition of a further 10 units of DNA polymerase I large fragment, for a further 4 h at 15°C. The reaction was stopped with 12 uM EDTA (pH 8.0), phenol : chloroform extracted and ethanol precipitated from 0.3 M NaOAc.

c) Restriction endonuclease selection of cDNA inserts

A BamHI restriction endonuclease site is present in a part of the rat hypothalamic LH-RH precursor cDNA representing the 3' untranslated region of the mRNA. This information was used for further enrichment of LH-RH precursor cDNA clones in the library.

The ethanol precipitated ds cDNA was thus redissolved in dH_2O and digested with 40 units of BamHI enzyme in the appropriate buffer for 2 h at 37°C.

After organic solvent extraction and ethanol precipitation the DNA larger than 250 bases was size selected on a 4% acrylamide gel run in TBE buffer (1 x TBE = 89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) DNA was retrieved from the gel slice by electrophoresis onto a DEAE membrane also in TBE buffer. The DNA was eluted from the membrane by boiling in 500 ul of TE.

100 ng of BamHI, SalI cut pGem vector DNA was added to the dsDNA and precipitated from 0.3N NaOAc with one volume of isopropanol. The ethanol washed pellet was redissolved in 20 ul of 50 mM Tris/HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM DTT and 1 mM ATP. 5 Units of T4 DNA ligase was added and the reaction incubated at 15°C for 18h.

d) Transformation of E. coli

E. coli K-12 strain (HB 101) were grown to 0.5 OD_{600} units in 20 ml of L-broth (1% Bacto-tryptone, 1% NaCl, 0.5% Bacto-yeast extract). After centrifugation at 3 000 g for 5 minutes, the bacterial pellet was resuspended in 10 ml of 10 mM Tris/HCl (pH 7.3) containing 50 mM $CaCl_2$ and repelleted at 4°C. The bacteria were resuspended in 2 ml of the above buffer and incubated at 4°C for 5 minutes. To 200 ul of this suspension was added 20 ul of recombinant pGem in ligation buffer. The bacterial suspension was kept at 4°C for 40 minutes followed by 42°C for 2 minutes. 0.5 ml of L broth was added to the suspension and incubated at 37°C for 30 minutes. 25 ul aliquots were spread on L-agar plates

containing 40 ug/ml of ampicillin and bacterial growth allowed to proceed for 18 h at 37°C.

e) Colony hybridization

~3 X 10^4 recombinant colonies were transferred to duplicate nylon filters from agar plates. The filters were placed on Whatman 3 M paper saturated with 0.5 N NaOH and 1.5 M NaCl for 5 minutes. The filters were tranferred to 3 M paper saturated with 1.5 M NaCl, 0.5 M Tris/HCl (pH 8.0) for neutralization. The neutralization step was repeated twice and the filters were air dried. DNA was fixed to the filters by exposure to ultra-violet light for 2 minutes. The filters were prewashed at 56°C for 5 h in a solution of 0.3 M NaCl, 0.03 M sodium citrate, and 1% SDS. Prehybridization was performed at 56°C for 2 h in 6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.25% skim milk powder, 0.06% NaPPi, 1% SDS and 100 ug/ml sonicated salmon sperm DNA. Oligonucleotide L11 was labelled to a specific activity of 10^7 cpm/pmole with 10 units T4 polynucleotide kinase using 100 uCi of [gamma-^{32}P]-ATP (5 000 Ci/mmole) in 10 ul of 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM spermidine. The labelled oligonucleotide was separated from free ATP on a G-25 spin column in 100 ul of TE. 50 ul of labelled oligonucleotide was heated to 65°C for 3 minutes and added directly to the prehybridization solution. Hybridization was allowed to proceed at 55°C for 18 h. The filters were washed 3 times for 30 minutes each in 0.2 X SSC, 0.06% NaPPi, 1% SDS at 55°C, allowed to air dry and subjected to autoradiography at -70°C in the presence of Cronex amplifying screens.

How many hybridized

f) Plasmid minipreparations

Colonies which hybridized to probe L11 were picked off the original plate and grown in 40 ml of L broth overnight. Plasmid DNA was prepared by the alkaline extraction method (Birnboim and Doly, 1979). The cDNA inserts were removed from the plasmids by digestion with the enzymes EcoRI and BamHI. The cleaved DNA was electrophoresed on a 0.8% agarose gel in 1 X TBE. The DNA was visualized by fluorescence under UV irradiation after staining with 0.5 ug/ml ethidium bromide aqueous solution. The DNA was denatured in the gel by soaking in 1.5 NaCl, 0.5 M NaOH for 1 h. The gel was neutralized by soaking for 1 h in 1.5 M NaCl, 1 M Tris/HCl (pH 8.0). The DNA was transferred to nylon filters by the Southern blotting technique (Southern, 1975). The filters were UV fixed and prehybridization and hybridization performed as outlined above for colonies.

g) Supercoiled DNA sequencing

The plasmid DNA with the longest insert that hybridized to oligonucleotide L11 was purified for sequencing by dissolving the minipreparation DNA from 20 ml of liquid culture in 160 ul of H_2O, adding 40 ul of 4 M NaCl, 200 ul of 13 polyethylene glycol (MW 8 000), mixing well and incubating at 4°C for 30 minutes. Supercoiled DNA was selectively pelleted by microcentrifugation at 4°C for 20 minutes. The pellet was washed in 70% ethanol, dried and redissolved in sterile water at a concentration of 1 ug/ul.

2 ug of supercoiled DNA was denatured in the presence of 30 ng of oligonucleotide primer (pGEM-3, T7 promotor primer or SP6 promotor primer) by incubating in 20 ul of 0.2 N NaOH, 0.2 mM

disodium EDTA for 5 minutes at room temperature. NaOAc was added to 0.3N and the DNA precipitated by incubation at -20°C after the addition of 2.5 volumes of ethanol. After microcentrifugation and washing in 70% ethanol the DNA pellet was dissolved in 9 ul of sterile water. To this was added 1 ul of reverse transcriptase buffer (340 mM Tris/HCl pH 8.3, 500 mM NaCl, 60 mM $MgCl_2$, 50 mM DTT), 1 ul of reverse transcriptase (20 units) and 4 ul of [alpha-^{32}P]-dATP (10 mCi/ml, >400 Ci/mmole).

To 3 ul of each of the specific nucleotide mixes (G, A, T or C), each containing nucleotides and the appropriate dideoxynucleotide at the concentrations described by Zagurski et al. (Zagurski et al., 1985) was added 2.8 ul of the primer/ template/enzyme/label mixture and transcription performed at 42°C for 10 minutes. The reactions were stopped by adding 5 ul of 90% formamide containing 20 mM EDTA, 0.3% bromophenol blue and 0.3% xylene cyanol. 2 ul of each chain-termination sequence reaction was heated to 70°C for 3 minutes and loaded directly onto a pre-electrophoresed 6.6% sequencing gel (6.6% polyacrylamide, 50% urea, 1 x TBE) and run at 1500 - 2000 volts. A second gel loading was performed when the xylene cyanol from the first loading reached the bottom of the gel. The gel run was terminated when the bromophenol blue of the second loading reached the bottom of the gel. Runs of this nature were sufficient to deduce 250-350 bases of sequence.

5.2.1.4. Primer Extension

Oligonucleotide L11 was labelled to a specific activity of ~10^9 cpm/ug with T4 polynucleotide kinase. 40 ng of labelled primer and 10 ug of Poly A RNA were heated to 70°C in 12 ul of 50

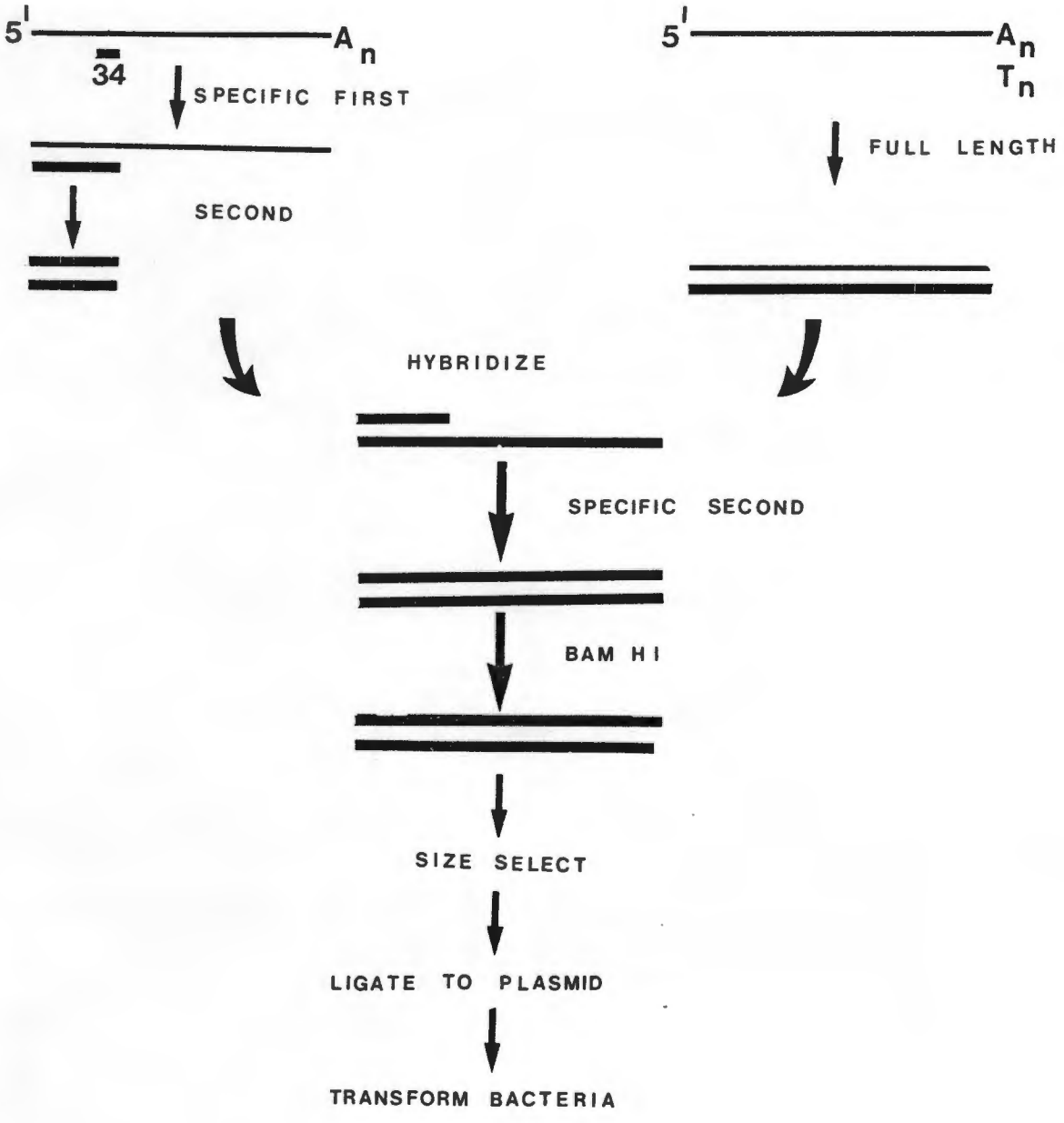

Fig. 5.1 **SPECIFIC FIRST AND SECOND STRAND SYNTHESIS PROTOCOL FOR A RAT PREOPTIC AREA cDNA LIBRARY ENRICHED FOR THE LH-RH PRECURSOR cDNA.**
Oligonucleotide L11 was used as a specific primer for LH-RH precursor cDNA synthesis. After 2nd strand synthesis, hybridization to full length 1st strands was carried out and specific full length 2nd strands were synthesized. The cDNA was size selected and directionally cloned into the pGem vector.

mM Tris/HCl (pH 8.0), 8 mM $MgCl_2$, 50 mM KCl, 4 mM DTT and slowly cooled to 55°C. The annealed primer and RNA were precipitated with one tenth volume of 3mM NaOAc and one volume of isopropanol, then resuspended in the same buffer containing dNTPs, at a final concentration of 500 uM each in a final volume of 20 ul. Ten units of reverse transcriptase were added and the reaction allowed to proceed for 30 min at 42°C. After phenol-chloroform extraction and ethanol precipitation the extension products were analysed on 0.8% agarose alkaline gels or on 8% denaturing acrylamide gels. The alkaline gels were dried down prior to autoradiography while the acrylamide gels were autoradiographed directly.

5.2.1.5 Northern Blotting and Hybridization

20 ug of rat or sheep poly A RNA was run on 1.5% Agarose gels containing 2.2 M Formaldehyde 50 mM Morpholinopropane-sulphonic acid (MOPS) (pH 7.0), 10 mM NaOAc and 1 mM EDTA. Dry RNA pellets were reconstituted in the same buffer used for casting the gel but containing 50% Formamide. The dissolved RNA was heated to 55°C for 15 min. 2 ul of 50% glycerol, lmM EDTA, 0.4% Bromophenol Blue, 0.4% Xylene Cyanol was added and the RNA loaded onto the gel and electrophoresed overnight at 30 V in gel casting buffer without formaldehyde. Prokaryotic and eukaryotic ribosomal RNAs were used as molecular weight markers and visualized on a UV box after staining with 0.5 ug/ml ethidium bromide in 0.1 M beta-mercapto ethanol. RNA was transferred out of the gel onto a nylon membrane by overnight capillary transfer as for Southern Blots, and fixed to the membrane by UV irradiation for 5 min. Prehybridization was performed at 42°C in

6 x SSC, 0.25% skim milk powder, 0.5% SDS 0.06% NaPPi, 5 mM EDTA (pH 8.0), 100 ug/ml sonicated salmon sperm DNA and 100 ug/ml yeast transfer RNA. Oligonucleotide probes L1 and L9 labelled to a specific activity of 10^8 cpm per pmole were added to the prehybridization solution after heating at 65°C for 3 min. Hybridization was allowed to proceed at 42°C for 18 hours. Three 10 min washes in 4 x SSC, 0.06% NaPPi, 0.5% SDS were performed at 42°C and the filters subjected to autoradiography at -70°C in the presence of Cronex amplifying screens.

5.2.2 Results and Discussion

The single step RNA purification protocol proved to be very effective in obtaining high yields of intact RNA free of protein and DNA. Yields of 1.4 ug total RNA/mg tissue (wet weight) with OD_{260}/OD_{280} ratios of >2.0 were achieved. Thus from 5 g of rat pre-optic area 7 mg of total RNA was obtained.

After oligo dT chromatography ~100 ug of poly A RNA was obtained from the 7 mg of total RNA applied. First strand cDNA synthesis using oligonucleotide L11 gave a predominant product of ~125 bases (Fig. 5.3) while the predominant range of oligo d(T) 12-18 primed cDNA was between 1.6 and 2.2 Kb which coincides with the average length of eukaryotic mRNA.

The second strand synthesis on the long cDNA template using the denatured specific second-strand as primer, together with the restriction enzyme selection of inserts, proved to be an effective protocol for the rare LH-RH precursor mRNA. Thus, of the 3 x 10^4 clones screened, 38 hybridized to oligonucleotide L11. Of the 20 minipreparations tested 18 had inserts of ~340 bases long, the remaining 2 being only marginally shorter.

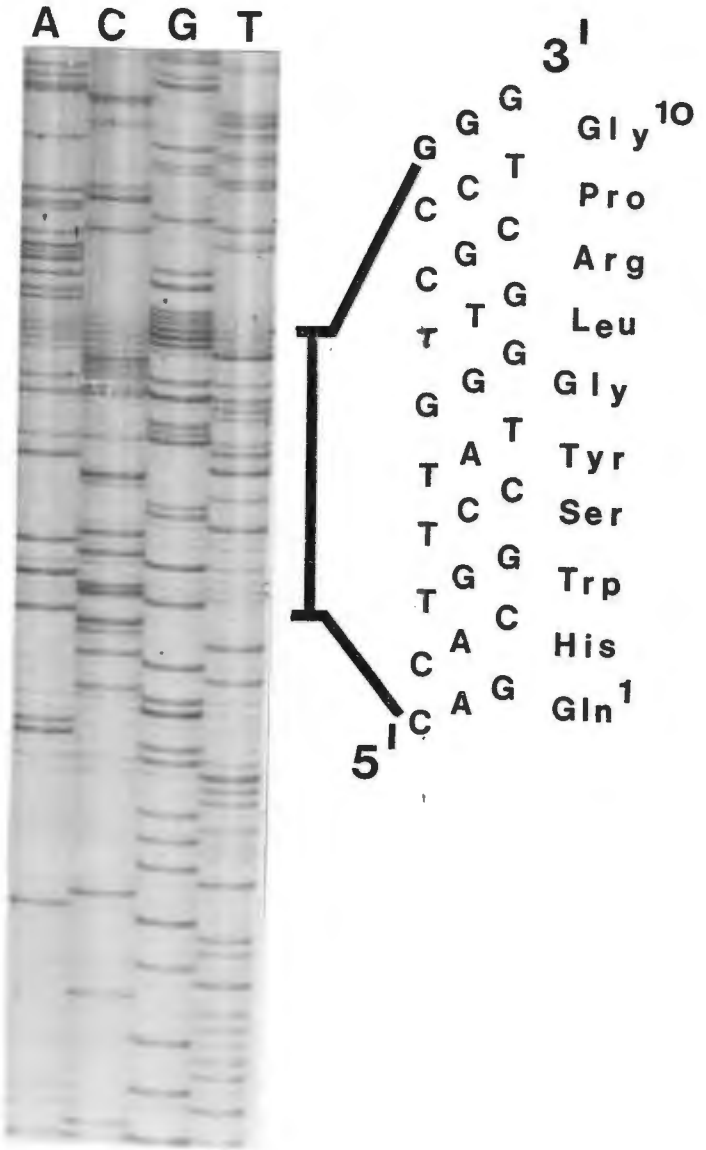

Fig. 5.2 **CHAIN TERMINATION SEQUENCING OF DENATURED SUPERCOILED DNA.**
T7 RNA polymerase primer was used to sequence a cDNA insert from the enriched rat pre-optic area library by the protocol outlined in Fig. 5.1. The insert hybridized to probe L11. The sequence indicated on the right is that of the LH-RH decapeptide. The cDNA sequence extended 6 bases beyond the published 5'-end of the message. The success of the method employed was evidenced by the fact that the insert was extended downstream of the region of the cDNA corresponding to probe L11.

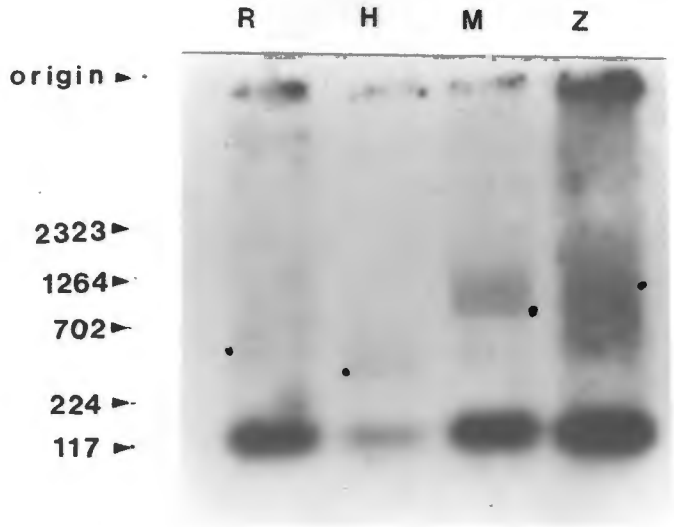

Fig. 5.3 **L11 PRIMER - EXTENSION USING POLY-A RNA FROM HYPOTHALAMI AND BREAST TUMOUR CELL LINES AS TEMPLATE**
10 μg of poly-A RNA from rat pre-optic area (R), human hypothalamus (H), or two breast tumour cell lines, MDA-MB-231 (M), and ZR-75-1 (Z) were reverse transcribed using 5'-end labelled oligonucleotide L11. A 125-base product was produced from RNA from all tissues. Longer transcripts were also obtained in each tract. Fig. 5.3(b) provides an explanation for these results.

Double stranded sequencing of one of the clones by the method of Zagurski et al., showed that it was identical to the rat hypothalamic cDNA described by Adelman et al., except for the extension of six bases at the 5' end, viz. 5'GAAGAT- (Fig. 5.2, Fig. 5.4a). The first three bases at the 5' end conform to a typical "cap" or transcription start site i.e. Pyr-A-Pyr (Fig. 5.4a). If this is indeed the transcription initiation site used by RNA polymerase II an AT-rich TATA box would be expected 25-35 bases upstream (Goldberg, 1979; Shenk, 1981). Since the upstream region of the LH-RH gene has been sequenced in the human placental LH-RH precursor cDNA (Seeburg and Adelman, 1984) and in the mouse (Mason et al., 1986), the published sequences were examined for this and other typical promotor elements. In the case of the human (as reflected in the placental mRNA sequence) the typical TATAA sequence occurs between bases -50 to -55 relative to the proposed cap site (Fig. 5.4a). In the mouse gene this sequence occurs between positions -47 to -51 relative to the proposed initiation point. Since this TATA box is relatively far upstream from the CAP site, primer extension studies were performed using poly A RNA from human and rat hypothalamus and from the human breast tumour cell lines MDA-MB-231 and ZR-75-1 (Fig. 5.3). The major primer extended product from all four tissues ran at a position of 125 bases on alkaline agarose gels and on 8% acrylamide sequencing gels. This coincides exactly with the 5' terminal sequence of the cDNA clone described above. Since the reverse transcriptase catalysed reaction was performed after heat denaturation and in the presence of high concentrations of dNTPs (500 uM each), the primer extension probably went to completion. Also, since a longer primer

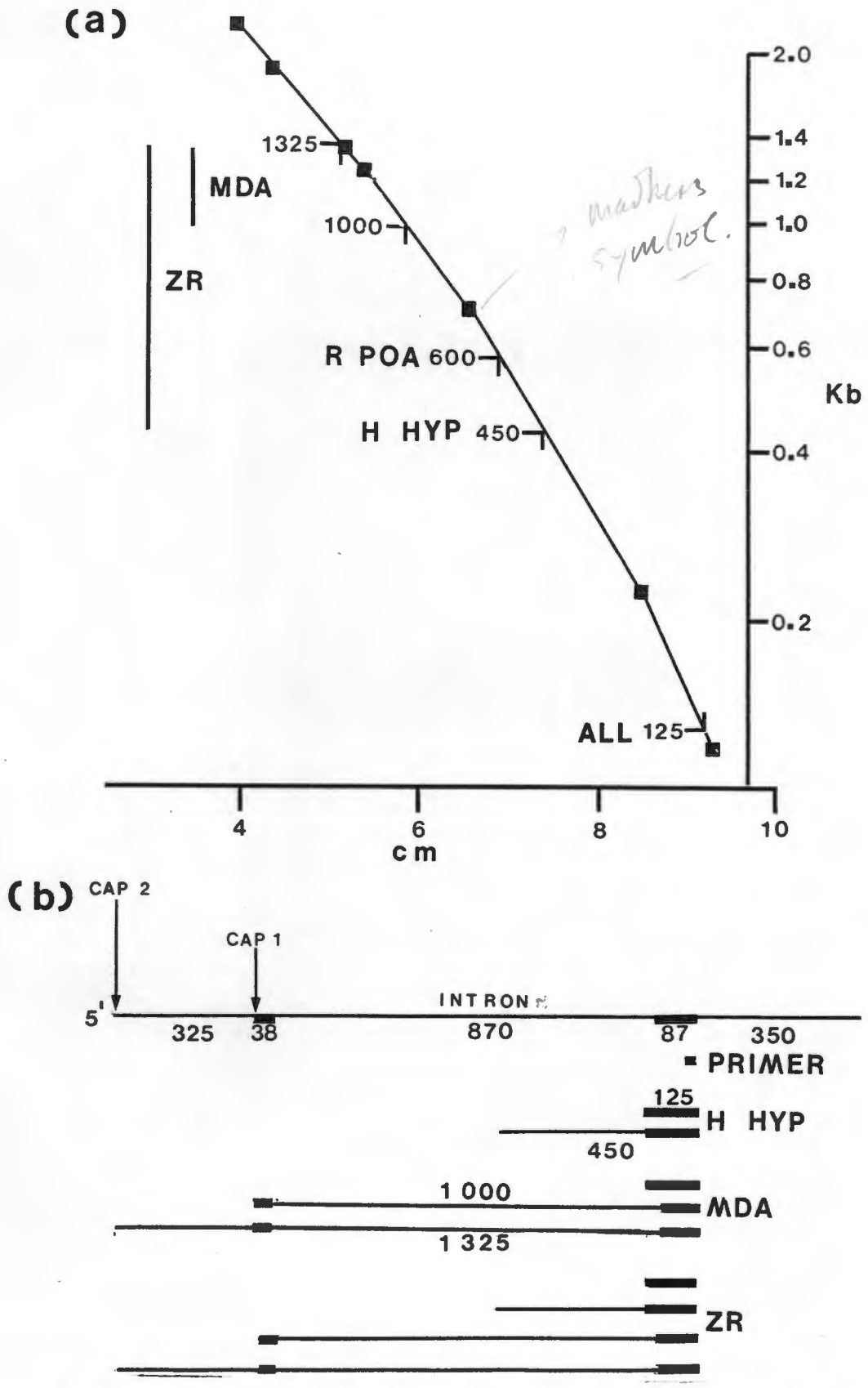

Fig. 5.3 **EVIDENCE FOR TWO TRANSCRIPTION START SITES IN THE LH-RH GENE.**
The sizes of the primer extended cDNAs are consistent with two transcription start sites in the gene. The 5' region of the untranslated human placental LH-RH cDNA includes an intron transcript as evidenced from the sequencing of rat and human hypothalamic mRNAs. The two start sites and the possible inclusion of the intron transcript makes possible the existence of 4 LH-RH mRNAs in certain tissues, e.g. in the breast tumour cell line ZR-75-1.

extension transcript is observed in all four cases, it is unlikely that this sequence represents a premature stoppage point for the enzyme.

Although the 5′ end or cap site of mRNA is generally believed to represent the transcription initiation site of a gene, there is evidence for some primary transcripts being cleaved or degraded at the 5′ terminus and the resultant newly exposed 5′ ends being capped (Shenk, 1981). Vaccinia cores have been shown to contain a 5′ phosphate-phosphoribonucleotide kinase which facilitates such a restriction by converting the 5′-terminal monophosphate resulting from degradation to a 5′-terminal triphosphate (Spencer et al., 1978). RNA degradation studies have demonstrated specific RNA endonucleases and 5′ to 3′ exonucleases in nuclei (Furuichi et al., 1977) as well as 5′ termini particularly susceptible to degradation (Piechaczyk et al., 1985; Rabbits et al., 1985). Multiple early cap sites have been shown in the SV40 genome (Reddy et al., 1979; Thompson et al., 1979; Haegeman and Fiers, 1980), other viral genes (Soeda et al., 1980; Baker and Ziff, 1981) as well as the sea urchin H2A gene (Hentschel et al., 1980). There is also a distinct hierachy of cap site preferences: A>G>>U>C (Baker and Ziff, 1981). One could thus propose that transcription beginning at a particular initiation site could give rise to a primary transcript with a poor capping site and/or a labile 5′ terminus which is rapidly degraded to expose a better and/or more stable capping site. This could be a possible reason for the longest primer extended products in this study ending ~50 bases downstream and not 25-35 bases downstream from the proposed TATA box.

Examination of the human placental LH-RH cDNA sequence around the proposed transcription initiation site in the gene reveals a number of features characteristic of DNA binding sites for transcription factors that can distinguish between certain genes (for reviews see Serfling et al., 1985; McKnight and Tjian, 1986; Maniatis et al., 1987).

Firstly, three potential enhancer core elements related to the SV40 enhancer elements (Herr and Clarke, 1986) and other viral enhancers (Gruss, 1984) (Fig. 5.4b) occur in close proximity to or incorporate:

a) The proposed CAAT box;

b) The proposed TATAA box;

c) The proposed transcription initiation site. (Fig. 5.4(a)).

Secondly, two sequences with a strong resemblance to the octameric enhancer elements of the immunoglobulins, chicken histone 2B, Xenopus U2 and SV40 genes (Herr et al., 1986) are also in close juxtaposition to the CAAT box and transcription start site proposed here for the LH-RH gene (Fig. 5.4c).

Thirdly, an inverted repeat of a pentanucleotide incorporating the proposed CAAT box, viz. 5' ATTGA 3', occurs immediately downstream from the proposed CAP site (Fig. 5.4a). The family of NF-Y proteins has been shown to bind CAAT box pentanucleotides in the reverse orientation in a number of genes. These include all class II major histocompatibility genes, the herpes simplex thymidine kinase gene, human alpha- and beta-globin genes and the Moloney sarcoma virus LTR genes (Dorn et al., 1987 (a), 1987 (b)). The existence of these well characterized enhancer and promotor consensus sequences in close apposition to the proposed transcription initiation site could

be advanced as another reason for the relatively long distance between the TATAA box and the proposed transcription initiation site. The binding of proteins to the proposed enhancer core elements, the octameric sequences and the reverse CAAT box sequence in the gene could conceivably prevent RNA polymerase II from beginning transcription at the usual positions 25-35 bases downstream from the TATAA box. Whether these previously described DNA-binding proteins bind to the LH-RH gene at these points and whether the proposed "crowding" results in transcription initiation at a position ~50 bases downstream from the TATAA box, deserves further investigation.

An inverted repeat sequence, suggestive of partial two fold symmetry occurs at the proposed start site of transcription, viz. $^{-9}$CTT^{-6}C and $^{-1}$GAA^{+3}G (ie. positions -9 to +3). Similar regions of partial or "hyphenated" two-fold symmetry have been described in the vicinity of transcription start sites of a number of genes including the adenovirus E1a gene (-11 to +9), the mouse beta-globin gene (-17 to-2) and the chicken ovalbumin gene (-7 to +7) (Ganon et al., 1979). Whether this partial two fold symmetry facilitates transcription initiation by RNA polymerase II at this particular site in the LH-RH gene also deserves further investigation.

Footprinting, <u>in vitro</u> transcription, and transfection studies using deletion and point mutations in this region of the LH-RH gene are necessary to validate the above contentions inferred from the human DNA sequence. The three enhancer elements described here and which are similar to the SV40 enhancer core elements are similar to elements in the immunoglobulin heavy chain gene. The relevant immunoglobulin

heavy chain enhancer operates much less efficiently in fibroblasts than in lymphoid cells suggesting a tissue specific role for this element. Recently, certain RNA polymerase III transcripts, believed to arise from introns in postnatal-onset neuronal-specific genes and in certain immortalized rodent cell line genes have been proposed as enhancers of RNA polymerase II gene expression (McKinnon et al., 1986). A consensus enhancer sequence GTGGTAG in the so-called A-box of the RNA polymerase III promotor is similar to the typical enhancer elements described in Fig. 5.4 . It is believed that RNA polymerase III transcription of neuronal "identifier" sequences may facilitate expression of neuron-specific RNA Polymerase II transcription units (McKinnon et al., 1986). The transcription unit described here for the LH-RH gene complies with this proposed model of synchronous positive regulation of neurone specific genes.

Since the placental LH-RH precursor mRNA extends further upstream from the transcription initiation site proposed here, another transcription initiation site must occur in the gene. The primer extension studies showed that both these initiation sites are used in the hypothalamus and in the breast tumour cell lines.

In the hypothalamus, transcripts of the 1st intron are always excised while this isn't the case in the breast tumour cell lines. In the case of the human hypothalamus, the longer primer extended product (450 bases) indicates that the first initiation site is ~325 bases upstream from the one proposed here (Fig. 5.3). Examination of the gene sequence at this point will be necessary to confirm this site. In the case of the rat gene, the upstream transcription initiation site, as interpreted from

the longer (~600 bases) primer extended product is ~475 bases from the start site described here. Northern blot hybridization studies using 20 ug of rat hypothalamic poly A RNA per tract and with probes L1 and L9 revealed two bands representing RNA molecules of ~500 bases and ~1000 bases (Fig. 5.5). This further supported the interpretation of the primer extension experiments being due to the existence of two transcription initiation sites in the LH-RH gene at the positions described above (Fig 5.3).

Since the sequence representing the first intron in the gene is sometimes not removed from the mature RNA, the existence of two transcription initiation sites in the gene provides the possibility for four possible mRNA species of differing length result from the same gene. In the case of the breast tumour cell line MDA-MD-231 the smeared band between ~1000 bases and ~1325 bases could be due to the use of both transcription initiation sites and the inclusion of the first intron sequences in the mature mRNAs. The broader smear in the case of the cell line ZR-75-1 could be due to both transcription initiation sites being used and the intron being spliced out or left in, i.e. due to all 4 messages being present. All attempts to improve the resolution of these bands were unsuccessful. Examination of the placental LH-RH precursor mRNA for inverted repeat sequences revealed the presence of 19 such stretches of 8 bases or longer while only two such repeats occur in the shorter hypothalamic LH-RH precursor mRNA sequences. The potential for secondary structure and thus multiple reverse transcriptase stoppage points in the longer mRNA molecules presents a probable explanation for the smearing observed in the alkaline agarose gel tracts of the two breast tumour cell line primer extended products.

```
-167
5' AGGGCTTTATGTGAGGATTTTTAAAAAATTACCATTAAAAAAAAAAAAGCATAGTCCATTTGCA

-103                                -74                          -51
   GTATATAATTTACCAGCAGGAAAGATTTCAATGTCCTGGAAAAATTCCCTATAAAAAGGAAGAT
                                OCTAMER.
 -41                                -10   -5   +1 +4  +8              +26
   AGGAAAACAGAAAAGTCACAGTACTCAACCTACTTCAAGGGAAGATTGATCTTTTTGGCTCTCT
                                      OCTAMER.     TAACT
 +27       +37
   GCCTCTAACAG^...3'
          INTRON 1
```

(a) Human Placental LH-RH cDNA 5' end

SV 40 Enhancer Elements	A.	5' GTGGAATGTG	3'
	B.	GCAGAAGTAT	
	C.	GTGGAAAGTC	
Potential LH-RH Enhancer Elements	A.	$^{-89}$ CAGGAAAGAT $^{-80}$	
	B.	$^{-51}$ AAAGGAAGAT $^{-42}$	
	C.	$^{-5}$ AAGGGAAGAT $^{+5}$	

(b) Comparison of SV 40 early region promotor enhancer elements with LH-RH gene sequences in the region of a postulated trancription start site.

SV 40 Octameric Control Element	1.	5' A-TGCAAAT	3'
	2.	A-TGCAAAG	
LH-RH Octameric Sequence	1.	$^{-81}$ ATTTCAA-T $^{-74}$	
	2.	$^{-10}$ ACTTCAA-G $^{-3}$	

(c) Comparison of octameric enhancer elements in the SV 40 genome with sequences in the vicinity of a proposed LH-RH gene promotor.

Fig. 5.4 **A PUTATIVE PROMOTOR UNIT FOR THE HUMAN LH-RH GENE.**

(a) Rat preoptic area LH-RH cDNA sequence and primer extension studies using rat and human RNA indicate that transcription is probably initiated at position +1 in the rat and human LH-RH genes. Potential enhancer repeat sequences are highlighted or underlined in the case of the octameric element. The CAAT and TATAA box promotor elements as well as other potential cap sites are underlined. The underlined sequence CTTC at positions -6 to -9 is an inverted repeat of GAAG at positions +1 to +4. The reverse CAAT box at position +4 is indicated by the sequence of the complementary strand beneath it.

(b) and (c) show that there is substantial sequence homology between these enhancer elements and the well characterised SV40 enhancer sequences.

The potential for secondary structure within the mRNA representing the first intron of the gene presents the intriguing possibility of the regulation of translation rates at the level of splicing. A precedent for this type of translation inhibition by "antisense" secondary structure at the 5′ end of the mRNA is in the case of the c-myc oncogene where exon I - exon II hybridisation is believed to result in a low rate of translation. Translocation or rearrangement of the gene at these positions results in high levels of the c-myc protein with resultant transformation, e.g. Non-Hodgkins lymphoma and murine plasmocytomas (Piechaczyk et al., 1985; Rabbits et al., 1985).

Added to this possibility of LH-RH mRNA translation inhibition by including the 1st intron in the mature message is the possible inhibition by antisense RNA arising from transcription of the other DNA strand of the gene, as proposed by Adelman et al. (Adelman et al., 1987). Such inhibition has been demonstrated in a number of prokaryotic systems (Green et al., 1986) and enzymes which specifically unwind RNA hybrids have been shown in Xenopus oocytes (Bass et al., 1987; Rebagliati et al., 1987).

LH-RH gene expression studies thus provide an ideal system for investigating whether regulation at the level of RNA-RNA interaction occurs in eukaryotes as well.

Another possibility presented by the existence of two transcription initiation sites in a gene is the existence of another translation start codon between the transcription start sites. Whether such an in-frame ATG codon exists in the as yet

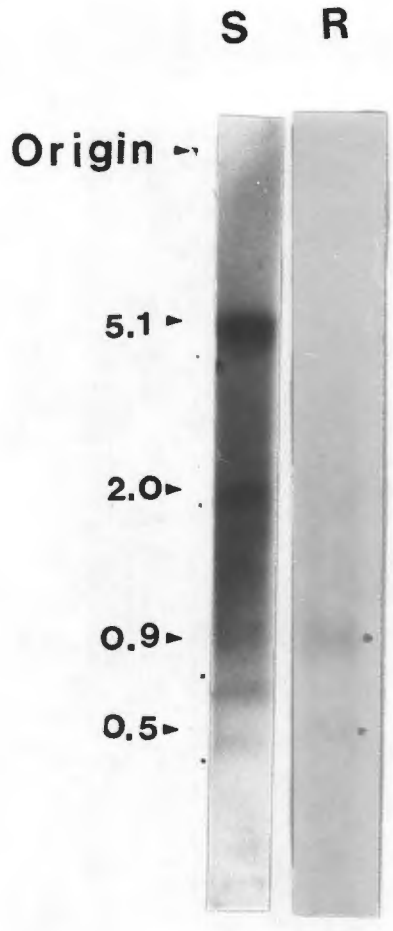

Fig. 5.5 **NORTHERN BLOT ANALYSIS OF 10µG OF RAT PREOPTIC AREA POLY-A RNA AND SHEEP HYPOTHALAMIC RNA**
The mixed probe L1 was used for hybridization. This mixture includes the correct sequence for the rat LH-RH gene. Two RNA species of ±500 and 900 bases were detected in the rat pre-optic area RNA while at least four were seen in the sheep RNA. These results are consistent with the interpretation of the primer extension studies and the observed inclusion of an intron transcript in some tissues, e.g. placenta and the breast tumour cell lines.

unsequenced 5′ end of the LH-RH gene deserves investigation. This possibility is supported by the demonstration described in this thesis and by the work of other investigators, of proteins with LH-RH-IR and which are larger than the 10 000 daltons predicted by the only translation start codon demonstrated to date (Adelman et al., 1986; Millar et al., 1977; Curtis and Fink 1983a, 1983b). However, translating the 5′ terminus of the human placental mRNA to amino acids in all three reading frames results in termination of the hypothetical protein prior to the proposed transcription initiation site proposed here. Thus, one would have to invoke the argument of sequencing errors in the published sequence in order to persist with the proposal of a larger protein precursor being translated from the mRNA transcribed from the upstream initiation site of the LH-RH gene.

The rat aldolase A gene has been shown to have three transcription iniation sites resulting in three mRNAs differing in the length of their 5′ untranslated regions (Joh et al., 1986). In the case of this gene, as well as in the mouse alpha amylase gene which has two transcription initiation sites, there are strong indications that the promotors for the different initiation sites are of different strengths and may have tissue specificity (Young et al., 1981; Joh et al., 1986; Breitbart et al., 1987). In the case of the LH-RH gene, the transcription start site described here appears to be driven by a stronger promotor than the upstream promotor, hence the more abundant 125 base primer extended product in all tissues tested (Fig. 5.3). The demonstration of enhancer elements in close proximity to the proposed CAAT box lends further support to this interpretation of the primer extension studies.

That genes have multiple transcription start sites which result in mRNAs differing in the length of the 5′ untranslated region may be a ubiquitous phenomenon, is supported by the recent demonstration of this fact in the insulin-like growth factor I gene (Lowe et al., 1987) and the beta-TSH gene (Wolf et al., 1987).

Transcription initiation at heterogeneous sites in the gene can give rise to defective or untranslatable mRNA. This is demonstrated in the case of the shorter POMC gene transcripts found in the testis which cannot give rise to the normal POMC peptides (Lacaze-Masmonteil et al., 1987).

RNA "fusion" studies have shown that labile sequences having short half lives (e.g. the 3′ untranslated region of the C-fos mRNA) can decrease the stability of a stable mRNA when fused to the 5′ terminus of the latter (e.g. the 5′ untranslated region of the V-fos mRNA) (Miller et al., 1984).

Whether the longer LH-RH gene transcripts are defective, untranslatable or marginally translatable and whether RNA turnover is significantly different in the messages arising from the two transcription initiation sites is deserving of investigation.

5.3 LH-RH Related Genes In Mammals

5.3.1 Introduction

Although conclusive evidence has been provided for the existence of multiple forms of LH-RH in other vertebrates (Millar and King, 1987), no peptide or gene sequencing data has been presented to establish this fact in mammals. Indeed Southern Blot studies by the group of Peter Seeburg have led them to

conclude that the LH-RH gene in the mammal is a single copy gene (Seeburg and Adelman, 1984; Adelman et al., 1986; Mason et al., 1986). Furthermore, no support for cross hybridising genes has been forthcoming using the LH-RH precursor cDNAs as probes in these Southern blot studies (Adelman et al., 1986).

It has to be borne in mind however, that the enkephalin precursor cDNA could not be detected with the pro-opiomelanocortin cDNA but by using oligonucleotide probes (Douglas et al., 1984). The frog skin TRH precursor cDNA was also an unsuccessful probe for the mammalian cDNA since only the repeating TRH encoding units are conserved (Lechan et al., 1986). In the well characterized globin family of genes an alpha globin-like gene escaped detection for a number of years (Marks et al., 1986; Proudfoot, 1986). The cloned glycoprotein hormone beta-subunit cDNAs were also not suitable as probes for many of their related beta-subunit cDNAs. The reason for this is that the conserved sequences within a family of genes may be very small and there may even be divergence at the level of codon usage within the part of the gene conserved at the level of amino acid sequence. Thus, although the amino acid sequence of the LH-RH decapeptide is identical in rats and humans, there are 3 differences at the nucleotide level. Thus, the use of an oligonucleotide as a probe for related genes could also be fruitless if there has been divergence within the sequence to which the oligonucleotide is directed.

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5.3.2 Methods

5.3.2.1 Southern Blots see 5.2.1.3 (f)

5.3.3 Results and Discussion

Many restriction endonuclease generated human DNA fragments hybridised to the oligonucleotide probes used in this study after low stringency washes of Southern blots. However, many of these were due to non-specific hybridisation to satellite DNA bands. At high stringency, probes L1 and L10 hybridized to only a single DNA band in the tracts of each of three restriction enzymes used. The size of this band was 11.5 Kb in the case of EcoRI digests, 5.0 Kb in the case of BglII digests and 1.2 Kb in the case of PstI digests. These results coincide directly with the DNA fragments expected from the published data (Seeburg and Adelman, 1984; Adelman et al., 1986).

The only probe which consistently hybridized to more than one restriction endonuclease generated fragment of human DNA under high stringency conditions, was probe L10. This probe hybridized to at least three DNA fragments generated by each of three restriction enzymes (Fig. 5.6). Besides the 11.5 Kb EcoRI fragment known to contain the LH-RH encoding gene characterized by the Seeburg group, two other EcoRI fragments of 2.5 Kb and 570 bases hybridized to this probe. In the case of the Bgl II digests DNA fragments of 5.0 Kb, 2.3 Kb and 1.6 Kb hybridized to this probe, while three fragments of 5.2 K.b, 1.8 Kb and 1.2 Kb were seen with Pst I digests (Fig. 5.6). Since washing of the Southern blots was carried out at the highest possible stringency, the other DNA fragments hybridizing to this unique (non-degenarate) probe are more than likely to contain sequences related to the LH-RH prcursor encoding gene. This result is the

only positive indication to date of mammalian DNA sequences (reflecting other genes) which are related to the LH-RH precursor encoding gene. It should be noted that this probe did not hybridize to rat genomic DNA restriction endonuclease fragments under the hybridization conditions used. Examination of the rat LH-RH gene sequence corresponding to this probe revealed four base differences between it and the human gene at this locus, thus explaining this negative hybridization result.

This probe (L10) corresponds to amino acids 11-16 of the LH-RH precursor which includes the processing site residues Gly^{11}-Lys- Arg^{13}. This sequence is probably shared by numerous precursor proteins and thus the nine out of eighteen bases at the 3' end of probe L10 are likely to hybridize to a large number of genes. The Southern blot result obtained here could thus be partially explained by the expected high incidence of this sequence in the human genome. A search of the Genbank database for the entire sequence encoded by L10 did not reveal homology with other genes sequenced to date. However, in support of the existence of proteins related to the amino terminus of GAP, it was noted that there is remarkable sequence homology between amino acids 18-26 of the LH-RH precursor and amino acids near to the N-terminus of the inhibin family of polypeptides (Table 5.1). Since strong sequence homology exists between alpha inhibin and beta-inhibin subunits and beta-TGF (Mason et al., 1985b) the homology indicated in Table 5.1 between the stretch of N-terminal amino acids in GAP and the inhibin/beta-TGF family of polypeptides, strongly suggests that the LH-RH precursor gene is related to this gene family. The Southern blot data presented here could be

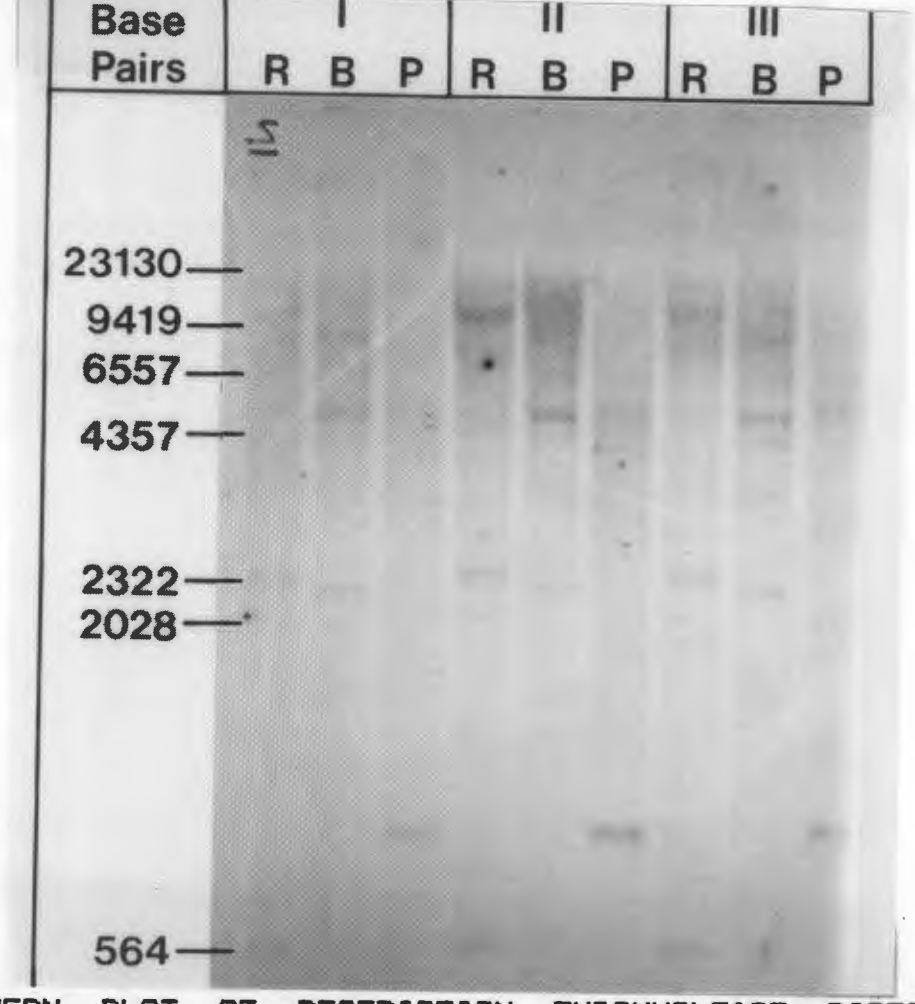

Fig. 5.6 **SOUTHERN BLOT OF RESTRICTION ENDONUCLEASE DIGESTED HUMAN DNA PROBED WITH OLIGONUCLEOTIDE L10**
Probe L10 is a complementary DNA oligonucleotide directed against the sequences encoding amino acids 11 to 16 of the LH-RH precursor (Table 4.7). 30 μg of restriction enzyme digested DNA was run on an agarose gel and blotted onto nitrocellulose. 5'-end-labelled L10 was hybridized to 3 DNA fragments in every tract. These included an 11.5 Kb EcoR1 (R) fragment, a 5.0 Kb BglII (B) fragment, and a 1.2 Kb Pst1 (P) fragment as expected for the LH-RH encoding gene. The other fragments probably represent fragments of related genes. DNA sample I was from a normal subject while sample II was from a person with Kallmann's Syndrome and sample III from a patient with hypogonadism. There was no difference in the size of the LH-RH gene fragment or the other two hybridizing fragments for all three patients with the three enzymes used.

H LH-RH	^{18}**Leu Ile Asp Ser Phe Gln Glu Ile** Val^{26}
R LH-RH	^{18}**Leu** Val **Asp Ser Phe Gln Glu** Met **Gly**^{26}
H αInhibin	^{235}**Leu** Asn Ile **Ser Phe Gln Glu** Leu **Gly**^{243}
H β_AInhibin	^{298}Phe Phe Val **Ser Phe** Lys Asp **Ile Gly**^{306}
H β_BInhibin	^{240}Phe Phe Ile Asp **Phe** Arg Leu **Ile Gly**^{248}
H βTGF	^{300}Tyr **Ile Asp** Phe Arg Lys Asp Leu **Gly**^{308}

Table 5.1 SEQUENCE HOMOLOGY BETWEEN THE HUMAN (H) AND RAT (R) LHRH PRECURSOR AND INHIBIN / TGF FAMILY OF POLYPEPTIDES.

indicative of other members of this gene family, i.e. related to the N-terminus of GAP but not sequenced to date.

Pro LH-RH 14-26 and pro LH-RH 17-37.NH_2 have been shown to have LH- and FSH-releasing activity (Millar et al., 1986; Milton et al., 1987). Inhibin alpha-beta-dimers inhibit FSH release from the pituitary (de Jong and Roberts, 1985 and Mason et al., 1985) while beta-dimers of inhibin stimulate FSH release (Ling et al., 1986 and Vale et al., 1986). The homologous amino-acids in pro-LH-RH 18-26 and the inhibin monomer precursor sequences shown in table 5.2 are more than likely to be the residues required for these biological effects on pituitary gonadotropes.

Another feature of the result obtained here is the fact that there is no difference in the size of the DNA fragments that hybridize to probe L10 in normal subjects or patients suffering from Kallmann's Syndrome or Hypogonadal Hypogonadism (Kallman et al., 1944; Santoro et al., 1984). The LH-RH gene defect in these disorders is thus not due to any major deletions or insertions within the gene. Similar investigations carried out with Dr Mali Kelly (Columbia University Medical School) on several patients with these disorders and using the same probe have confirmed this conclusion. The defect in the human LH-RH gene is thus unlike that of the hypogonadal mouse where a major deletion of 33.5 Kb results in the exclusion of the last two exons of the gene (Mason et al., 1986). Further characterisation of the transcription units in the LH-RH gene should make it possible to investigate whether aberrations at these loci are responsible for defective gene expression in these human disorders.

5.4 LH-RH Gene Regulation Studies

5.4.1 Introduction

Ovariectomy results in high serum levels of LH in the female rat (for review see Fink, 1979). Estrogen replacement therapy reverses this effect of gonadectomy. This experimental model has established the feedback role of the steroid target hormone at the level of the pituitary and the hypothalamus (Arakis et al., 1975; Fink, 1979). The frequency of LH-RH pulses in the hypophyseal portal blood in rats (Fink, 1979) and primates (Carmel et al., 1976; Neill, 1980) is similar to the frequency of LH pulsatility in the intact rat. However, continuous administration and or high doses of LH-RH ablates the LH response in a "paradoxical" manner (Belchetz, 1983; Bex and Corbin, 1984). It does not follow therefore that extremely elevated serum levels of LH are necessarily due to high portal blood levels of LH-RH. Furthermore, the initial negative feedback of estradiol at the level of the pituitary (decreased LH pulse amplitude) is accompanied by no change in LH-RH pulse frequency. This initial effect of estradiol is followed by an increase in LH-RH pulse frequency (positive feedback) with concomitant changes in LH pulses and increased LH pulse amplitude (i.e. positive feedback at the pituitary level). Progesterone acts synergistically with estradiol to decrease LH-RH pulse frequency and to reinforce the negative and positive effects of estradiol at the pituitary level. These results were obtained in the ewe (Clarke, 1984), in the female rat *in vivo* (Sarkar and Fink, 1979; Sherwood and Fink, 1980) and in female rat hypothalami *in vitro* (Kim and Ramirez, 1985).

Immunohistochemistry and more recently in situ hybridisation have demonstrated that the LH-RH neurons have their cell bodies in the diagonal band of Broca and in the preoptic area of the hypothalamus (King and Anthony, 1983; Silverman, A.J. et al., 1978, 1979; Shivers et al., 1986). The LH-RH immunoreactive neurones do not appear to be directly affected by estradiol since they do not concentrate this steroid in their nuclei (Shivers et al., 1983).

Since as few as 1000 forebrain neurons have been shown to synthesize LH-RH (Shivers et al., 1983) and since large amounts of Poly A RNA are required to visualize the LH-RH message on a Northern Blot, it was decided that the highly sensitive mRNA detection systems of in situ hybridisation and S1 nuclease mapping would have to be employed to study LH-RH gene expression in the ovariectomized-steroid replaced rat model system. Also, an experimental protocol most likely to establish a measurable difference in LH-RH mRNA was decided on. Thus, estradiol replacement was effected 14 or 21 days after ovariectomy (when serum LH values are extremely high). Estradiol was replaced by subcutaneous implants of silastic capsules containing estradiol at 260 ug/ml in sesame oil. 1 cm of silastic capsule (outer diameter 0.125 in; inner diameter 0.062 in) was implanted per 100 g of rat weight. This resulted in serum estradiol levels of ± 100 pg/ml by constant release over the 1 to 4 day period. The hypothalamic LH-RH mRNA content was measured one or four days after the commencement of replacement (when serum levels of LH have reached those of the intact animal).

5.4.2 Methods

5.4.2.1 In Situ Hybridisation

(a) Sectioning

Prior to the collection of hypothalami, rats were anaesthetized with pentobarbitol and sytemically perifused with cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Hypothalami were dissected from decapitated rats, embedded in Lipshaw M-1 embedding matrix and frozen in liquid nitrogen. Ten micrometer sections were cut on a cryostat and mounted on polylysine coated slides. Sections either in the vertical (sagittal) or horizontal planes were catalogued according to Paxinos' stereotaxic atlas of the rat brain and stored dessicated at -70^{o}C.

(b) Prehybridisation

Thawed sections were incubated for 10 min in 5 ug/ml proteinase K, washed in 2 x SSC (RNase-free) and overlayed with prehybridisation buffer (2.5 M NaCl, 0.5 M Tris (pH 7.5), 3% Ficoll, 3% PVP, 3% BSA, 125 mM EDTA, 5 mg/ml sonicated salmon sperm DNA, 10 mg/ml yeast total RNA, 25 mg/ml yeast tRNA and 50% formamide). Prehybridization was at 50^{o}C for 2h in a humidified box containing Whatmans 3 M paper saturated with 4 x SSC in 50% Formamide.

(c) Probe Synthesis

A complementary RNA probe was transcribed off the rat LH-RH cDNA in the pGem Vector using SP6 RNA polymerase. 2 ug of template DNA in a solution of 40 mM Tris/HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidue, 500 uM rCTP, rGTP, and rATP, 0.7 mg/ml

DTT, 30 units RNasin and containing 500 pmoles of [alpha-^{35}S]-UTP was used to make the cRNA probe by incubation for 20 min at 37^{o}C with 15 units of SP6 RNA polymerase. The UTP concentration was increased to 500 uM and the reaction continued for a further 40 min. The DNA template was digested with RNase-free DNase and the solution was extracted with phenol:chloroform and precipitated from 2M NH_4Ac with 2.5 vol of ethanol. The labelled RNA pellet was washed with 70% ethanol dried and stored at -20^{o}C in TE.

d) Hybridization and washing

The probe was heated to 60^{o}C for 10 min and added to the tissue in hybridization buffer at a concentration of 2 50 cpm/ul. Hybridization buffer was identical to prehybridization buffer except for the presence of 10% Dextran sulphate.

Hybridization was allowed to proceed by overnight incubation in the humidified box at 50^{o}C. The slides were washed at room temperature for 30 min with 2 x SSC. RNase A at 30 ug/ml in 0.5 M NaCl, 10 mM Tris/HCl (pH 8.0) was added to the tissue to digest unhybridized probe by incubating at 37^{o}C for 45 min. The slides were washed for 1 h in 4 l of 2 x SSC at 50^{o}C and for 3 h in 4 l of 0.1 x SSC, 0.05% NaPPi and 14 mM beta-mercaptoethanol. The solution was cooled to room temperature and washing continued overnight. The sections were then dehydrated through 50%, 70% and 90% ethanol containing 0.3 M NH_4Ac for 2 min each.

e) Visualization

Slides were dried in a dessicator overnight and then dipped in Lipshaw NTB2 photographic emulsion at 42^{o}C in the dark.

Slides were allowed to dry in the dark for 2 h and stored at 4°C for 14 days in a light box. The slides were developed and the distribution of silver grains analysed on a Leitz "TAS Plus" image analysis system by an independent investigator who did not know the experimental details nor the arrangement of the coded slides.

5.4.2.2 S1 Nuclease mapping using oligonucleotide L11

Poly A RNA was prepared from various tissues and individual rat hypothalami as outlined in 5.2.1.1 and 5.2.1.2. Oligonucleotide L11 was labelled as outlined in 5.2.1.3(e). 1 ug of test Poly A RNA, 10 ng of yeast tRNA and 5 x 10^5 cpm of the oligonucleotide were precipitated from 0.5 M NH_4Ac with ethanol in an eppendorf tube, washed with 70% ethanol and evacuated dry. The pellet was dissolved in 30 ul of hybridization soltuion containing 400 mM NaCl, 400 uM Na_2PIPES (pH 6.5), 1 mM EDTA and 80% deionized formamide. The solution was then incubated at 72°C for 15 min followed by 45°C for 18 h.

Unprotected RNA and unhybridized oligonucleotide were digested with 100 units of S1 nuclease added in 100 ul of S1 buffer containing 0.25 M NaCl, 30 mM Na OAc (pH 4.6, 1 mM $ZnSO_4$ and 20 ug/ml sonicated salmon sperm DNA. Digestion was allowed to proceed at 30°C for 40 min. The solution was extracted with phenol: chloroform. 10 ug of carrier tRNA was added, followed by 300 ul of Ethanol. The tubes were incubated on crushed CO_2 for 10 min and microcentrifuged for 1 h at 4°C. The pellet was washed with 70% ethanol, evacuated dry and redissolved in 10 ul of sequencing gel dye. 5 ul of sample was electrophoresed on a 16% polyacrylamide, 50% urea, 1 x TBE denaturing gel in 1 x TBE

until the xylene cyanol migrated to a position 2/3rds down the length of the gel. The gel was sandwiched between two sheets of gel bond backing and directly autoradiographed at -70°C in the presence of Cronex amplifying screens.

5.4.3 Distribution of silver grains

The distribution of silver grains representing the presence of LH-RH mRNA containing cells on the tissue sections examined, followed the pattern expected for LH-RH neurones. Thus cell bodies in the diagonal band of Broca and in the pre-optic area were detected. A few labelled cells were located in the basal hypothalamus, ventral and lateral to the ventromedial nucleus. No labelled cells were observed in the arcuate nucleus. The low background distribution of silver grains indicated that the unhybridized probe had been successfully digested by RNase. Tissue sections through the hypothalamus of three ovariectomized rats (14 days) and three ovariectomized 4-day estradiol-replaced rats were examined by an independent investigator from Amersham USA. The silver grain count representing LH-RH mRNA content and the position of the tissue section examined is presented in Table 5.2. Fig. 5.7 shows the concentration of silver grains over a pre-optic area LH-RH neurone.

The number of cells labelled in comparable sections in the two experimental groups was not different, but the average number of grains per cell was significantly higher in the steroid replaced group. This was true in case of the LH-RH cell bodies in the diagonal band of Broca, but the increase was greater in the case of the pre-optic area LH-RH neurons. The average grain count per cell increased by 83% overall in the steroid replaced

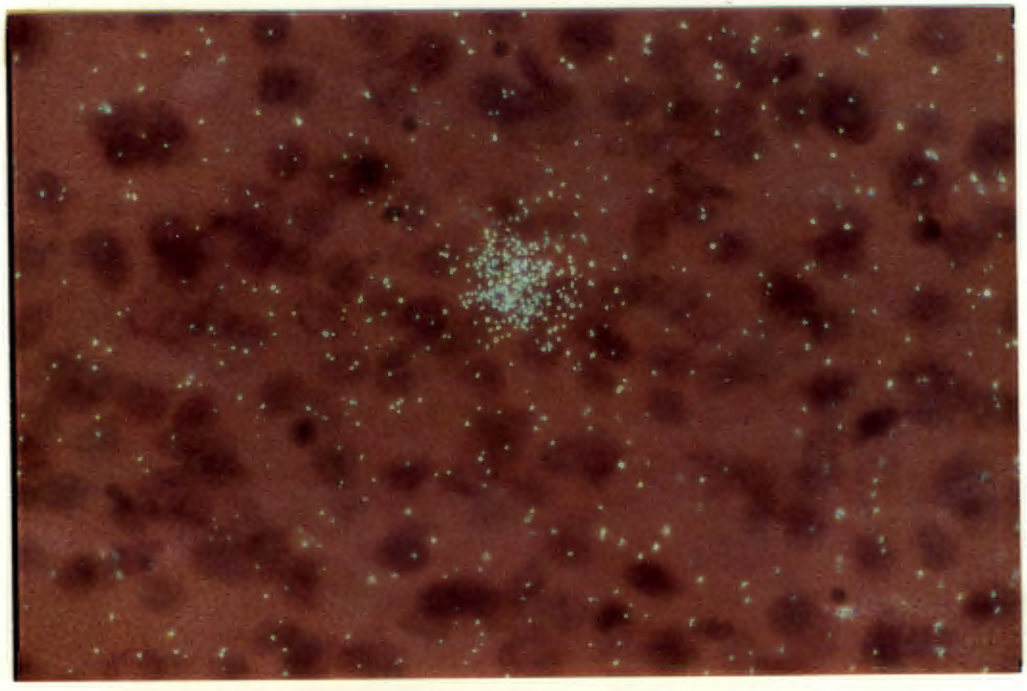

Fig. 5.7 **IN SITU HYBRIDIZATION USING A RAT LH-RH PRECURSOR cDNA PROBE ON RAT PRE-OPTIC AREA SECTIONS**
10 micron frozen sections of rat hypothalamus were used to detect LH-RH mRNA containing neurones by in situ hybridization with a ^{35}S-labelled cRNA probe. The diagonal band of Broca and the pre-optic area contained distinct cell bodies with fluorescent granules when examined under epiluminescence indicating the presence of LH-RH-cRNA hybrids. A neurone in the pre-optic area is shown.

	Tissue section	No. of labeled cells	Ave. no. silver grains per cell	Total silver grains
OVX (n=3)	a	8	123	991
	b	31	171	5303
	c	27	101	2752
	total	66	137	9046
OVX+E (n=3)	a	5	150	750
	b	28	285	7980
	c	19	237	4503
	total	52	254	13233

Table 5.2 **GnRH mRNA MEASURED IN RAT HYPOTHALAMIC SECTIONS BY IN SITU HYBRIDIZATION**

Cells were labelled with ^{35}S cRNA. After 14 days exposure, slides were developed and silver grains analysed on a Leitz "TAS Plus" image analysis system. The sections analysed transversed the diagonal band of Broca and the pre-optic area, where GnRH containing cells are located. The average silver grain count per cell increased by 83% overall when OVX rats were replaced with estradiol.

a Anterior hypothalamic sections (plates 12-13, Paxinos Stereotaxic Atlas of Rat Brain).
b Pre-optic area sections (plates 14-15, op cit).
c Horizontal sections (plates 54-56, op cit).
OVX Animals ovariectomized for 18 days.
OVX+E Animals ovariectomized for 14 days and estrogen replaced for 4 days.

Still ovariectomized for the next 4 days

14 days plus 4D vehicle

ovariectomized rats. This could be interpreted as almost a doubling in the LH-RH RNA content of the cells after steroid replacement.

In order to validate these results, the second method of RNA analysis was employed, viz. a sensitive S1 nuclease mRNA assay. Fig 5.8 shows that LH-RH mRNA was detectable by this assay in tissues known to contain the decapeptide. Longer exposure of this particular gel showed that shorter fragments were protected in the sheep hypothalamic poly A tract indicating that the sheep mRNA probably differs from the rat, human and mouse sequence in this region of the message. The high amounts of LH-RH mRNA in the ZR-75-1 cells as detected by this method of analysis confirms the result of the primer extension study (Fig. 5.3).

Fig. 5.9 shows that within each experimental group of rats, similar levels of LH-RH mRNA were present. The rats replaced with estradiol for 1 day showed a significantly increased level of the LH-RH message above the level seen within the control group which received sesame oil implants. The increase in the LH-RH mRNA was even greater in the group of rats which received estradiol for 4 days. Thus the S1 assay results complemented the <u>in situ</u> hybridization results.

It appears from these results that LH-RH mRNA levels increased while serum LH levels decreased in this model system. One possible explanation for this is that the high serum LH levels in the ovariectomized animal feed back at the hypothalamic level and inhibit LH-RH synthesis in an attempt to overcome the excessive LH release due to the removal of the negative feedback of estrogen at the pituitary. This short loop feedback is insufficient to overcome the removal of the negative effect of

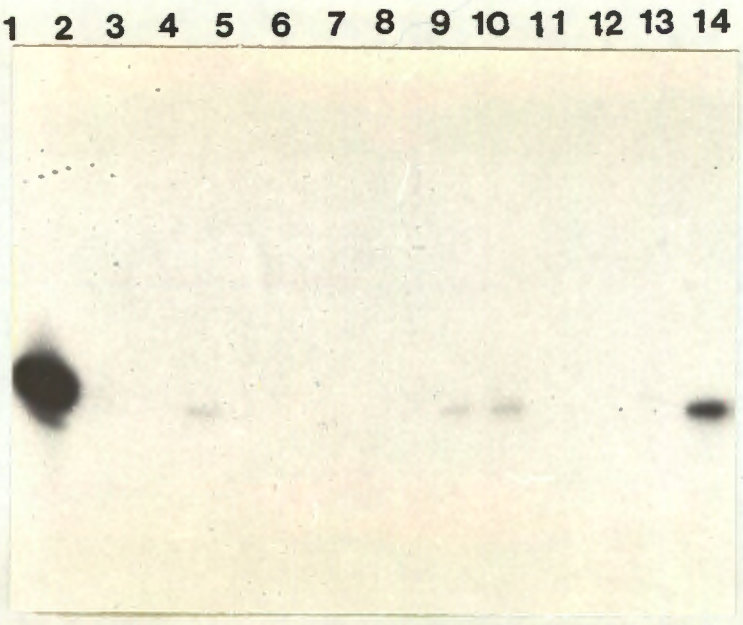

Fig. 5.8 **S1 NUCLEASE SOLUTION PHASE HYBRIDIZATION ASSAY FOR THE LH-RH PRECURSOR mRNA USING PROBE L11**
LH-RH precursor encoding RNA was detected in the rat and human hypothalamus and the two breast tumour cell lines. The tracts are:

1. The 34-mer probe, L11, loaded directly onto the gel.
2. Zero RNA control.
3. E. coli RNA.
4. Human hypothalamic RNA.
5. Human pituitary RNA.
6. Human placental RNA.
7. Frog brain RNA.
8. MDA-MB-231 RNA (Prep 1).
9. MDA-MB-231 RNA (Prep 2).
10. Rat pre-optic area RNA.
11. Sheep hypothalamus RNA.
12. Sheep pituitary RNA.
13. Human liver RNA.
14. ZR-75-1 RNA.

1 μg of poly-A RNA was used in the assay for all the above tissues except for E. coli RNA where 10 μg total RNA was used.

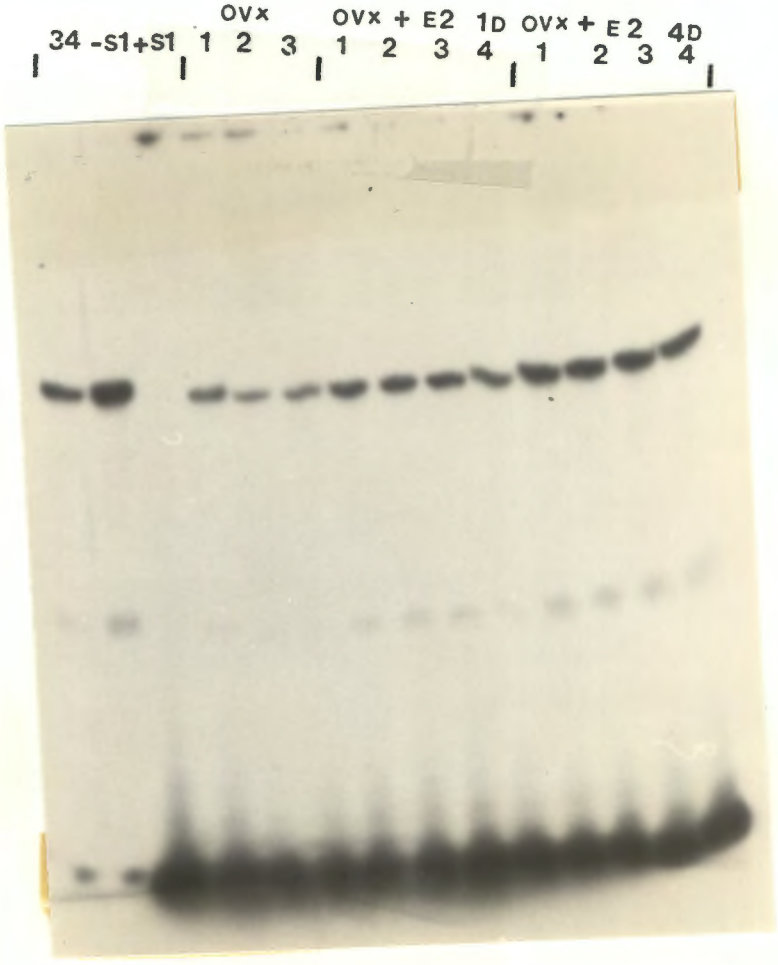

Fig. 5.9 **QUANTITATION OF RNA FROM OVARIECTOMIZED (OVX) AND OVARIECTOMIZED-ESTRADIOL REPLACED (OVX+E2) RATS.** Adult female Sprague-Dawley rats were ovariectomized for 21 days. Three rats were given implants of silastic capsules containing sesame oil (controls) while eight rats received estradiol-containing silastic capsule implants. Four of the latter group were sacrificed after 1 day of replacement while the remaining 4 were sacrificed together with the 3 control rats after 4 days of replacement. 1 μg of poly-A RNA from the pre-optic area of individual rats was assayed for LH-RH mRNA by the S1 nuclease protection assay using probe L11. LH-RH mRNA levels were elevated in the estradiol replaced rats. Since LH levels in the serum had decreased in these animals, the LH-RH mRNA seems to be reciprocally regulated relative to the serum LH levels in this model system.

estrogen i.e. high serum levels of LH persist while LH-RH mRNA (? LH-RH synthesis) has decreased. Evidence supporting this explanation comes from a single experiment carried out by Dr M. Blum (in the laboratory of Dr J. Roberts, Columbia University) where estradiol replacement in ovariectomized, hypophysectomized rats did not result in an increase in LH-RH mRNA. Thus some pituitary factor is required for steroid replacement to cause an increase in hypothalamic LH-RH mRNA.

The results obtained here were also obtained in the laboratory of Dr D.W. Pfaff (Pfaff, 1986). The difference in GnRH mRNA after steroid treatment of gonadectomized rats is in accordance with hypothalamic precursor levels and peptide content (Millar et al., 1977b, 1981) and LH-RH release (Goddard et al., 1981) in this model system. These studies indicate that steroids (estradiol in females and testosterone in males) have a positive feedback effect on LH-RH synthesis and secretion but an over-riding negative feedback on LH-production and release.

Experiments will have to be executed in other model systems to establish the relationship of LH-RH mRNA (and hypothalamic LH-RH precursor levels, LH-RH peptide content and LH-RH release) to pituitary LH synthesis and release. This will establish whether the inverse relation of LH-RH mRNA levels to circulating LH is a general phenomenon. Kinetic experiments should be particularly useful in this respect.

It has recently been demonstrated that pro TRH mRNA levels undergo an almost two fold increase in hypothyroid animals (low pituitary TSH and low circulating Thyroid hormone levels). This increase is obliterated by levothyroxine treatment, suggesting an inverse relation between circulating Thyroid Hormone (and

therefore pituitary TSH) and pro TRH mRNA (Segerson et al., 1987). Whether there is a general inverse relation between circulating levels of pituitary hormones and the mRNA of their releasing factors needs to be further investigated.

CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 INTRODUCTION

The idea that polypeptide hormones exist in more than one molecular form was first formulated in 1902 when prosecretin was postulated as a precursor of secretin (Bayliss et al., 1902). It was only in 1964 that chemical evidence was provided for molecular variation in polypeptide hormones, when two gastrin heptadecapeptides differing in the presence or absence of a sulphate group on the tyrosine were described (Gregory et al., 1964). This theme has now been established for a wide variety of peptide hormones. When this thesis was commenced, there was no evidence for a LH-RH family of peptides. A number of vertebrate hypothalamic LH-RHs have since been desribed. This thesis describes immunological chromatographic and nucleic acid hybridization studies confirming that a number of LH-RH-like peptides and genes/mRNAs exist in a variety of mammalian tissues.

In order to investigate the biosynthesis of LH-RH, *in vitro* translation, oligonucleotide hybridization, primer extension, S1 nuclease assay and cDNA library construction and analysis techniques were employed. To address the issue of LH-RH gene copy number and gene structural diversity, genomic restriction endonuclease fragments were screened for LH-RH precursor encoding sequences.

6.2 LH-RH: PEPTIDE DISTRIBUTION AND MOLECULAR HETEROGENEITY

This study confirms a wide tissue distribution for LH-RH and structurally related peptides. The extrahypothalamic brain, the gut and the gonads are regions now known to share a wide variety of identical or structurally similar peptides (Yalow et al., 1981). The extensive distribution of LH-RH-IR in these and other rat tissues would seem to support Niall's "second rule of peptide evolution", viz. that "everything is made everywhere" (Niall, 1982).

Indications that the LH-RH-IR in some of the tissues investigated is structurally distinct from the hypothalamic decapeptide, come from the fact that non-parallel displacement curves are obtained in the radioimmunoassay of extracts of these tissues.

The different estimation of the LH-RH-IR in gonadal and HEp-3 tumour extracts by antibodies directed at different parts of the LH-RH peptide, underscores the structural dissimilarity between the peptides in these tissues and the hypothalamic peptide. The multiple molecular forms of LH-RH-IR seen in HPLC elution prophiles of the testicular or HEp-3 extracts indicate that they contain LH-RH-like peptides which are distinct from the known vertebrate forms. HEp-3 extracts do, however, contain a peptide that co-elutes with the mammalian hypothalamic peptide, while gonadal extracts have a peptide that elutes in a position similar to chicken II LH-RH.

6.3 LH-RH BIOSYNTHESIS mRNA STUDIES

LH-RH immunoreactivity is associated with membrane bound ribosomes as is the case with other secreted polypeptides. The

injection of hypothalamic poly A mRNA into Xenopus oocytes results in the translation and export of immuno-assayable LH-RH into the incubation medium.(87) Northern blot analysis of RNA from sheep hypothalami, rat hypothalami, total rat brain, and the HEp-3 tumour with oligonucleotide probes for LH-RH coding sequences, indicate the presence of a number of mRNAs which hybridize to the probes. Primer extension assays, the DNA sequence of a rat hypothalamic cDNA clone and the human placental LH-RH cDNA sequence are consistent with 2 transcription sites within the LH-RH gene. The LH-RH mRNA in the ovariectomized-steroid replaced rat model appears to be reciprocally regulated relative to the serum LH levels.

6.4 LH-RH: GENE ANALYSIS

Southern blots of restriction endonuclease digested human DNA probed with a number of oligonucleotides showed hybridizing fragments identical in size to those initially detected by the group of Peter Seeburg. An 18-mer probe derived from the amino acid sequence immediately C-terminal to the LH-RH decapeptide in the human LH-RH precursor hybridized to at least two other DNA fragments for each restriction enzyme. These results are consistent with the existence of other genes carrying coding sequences for a peptide similar to LH-RH. The inhibin, betaTGF family of genes was seen to share sequence homology with the N-terminus of GAP (GnRH Associated Peptide).

	PEPTIDE/PROTEIN:	RESIDUES	AMINO ACID SEQUENCE:
1	LH-RH	1 - 10	Q H W S Y G L R P G
2.	Chicken I LH-RH	1 - 10	Q H W S Y G L Q P G
3	Salmon LH-RH	1 - 10	Q H W S Y G W L P G
4	Chicken II LH-RH	1 - 10	Q H W S H G W Y P G
5	Lamprey LH-RH	1 - 10	Q H Y S L E W K P G
6.	Bovine Gamma Crystallin	140 - 149	R G R Q Y L L R P G
7.	RSV, AMV, ALV Gag Protein Pr76	357 - 366	Q G Q A A E L R P G
8.	Bovine Protein C	2 - 11	N S F L E G L R P G
9	Adenovirus Hexon Assoc. Protein	140 - 149	V S S S L G L R P D
10	Human Prothrombin	291 - 300	G E A N C G L R P L
11.	E. coli Outer Protein 1e	263 - 272	Y Q F D F G L R P S
12.	Rhodomicrobium Cytochrome c2	36 - 45	F G Q K A G A R P G
13	Phi X 174 Gene J Protein	3 - 12	G K K R S G A R P G
14.	Human Muelin Basic Protein	115 - 124	S W G A E G Q R P G
15.	Mouse Leukaemia Virus Prot Pr76	901 - 910	G T R V R G H R P G
16.	RSV, AMV, ALV Gag Protein Pr76	551 - 560	R D G N Q G Q R P G
17.	Yeast ATPase Protein 3 or 6	179 - 188	R A I S L G L R L G
18.	E coli Beta Galactosidase	929 - 938	F P S E N G L R C G
19	Streptomyces Protease A	97 - 106	S G S T T G L R S G
20.	Yeast Enolase	362 - 371	A D L V V G L R T G
21.	Human IgG Lambda Chain VI	73 - 82	T L G I T G L R T G
22.	E. coli Lac Repressor	243 - 252	A I T E S G L R V G
23.	Mouse Cytochrome Oxidase III	73 - 82	P I V Q K G L R Y G
24.	Human Cytochrome Oxidase III	73 - 82	P P V Q K G L R Y G
25.	Human Skin Collagenase Alpha 2	3 - 12	B G K C V G L G P G
26.	Streptomyces Macromycin	19 - 28	T V S A T G L T P G
27	Bovine Cetolase	254 - 263	E D P D Y G L R D L
28.	Phage Lambda Bet Gene Protein	50 - 59	V A N Q Y G L N P W
29.	pBR322 Hypothetical Protein	82 - 91	F R R G Y G G R P V
30.	MSV Transforming Protein	317 - 326	A V V A Y N L R P S
31.	Semliki Forest Virus Coat Prot.	688 - 697	V Q Y Y Y G L Y P A
32.	Rat Group Specific Protease	135 - 144	D P T S Y T L R E V
33.	Bovine Kappa Casein	34 - 43	R Y P S Y G L N Y Y
34.	Human Glycophorin A	89 - 98	L L I S Y G I R R L
35.	B licheniformis Penicillinase	250 - 259	G A A S Y G T R N D
36.	Plasmid R 100 Replication Protein	53 - 62	H A R S R G L R R R
37.	B. subtilis Flagellin	28 - 37	E K L S S G L R I N
38	Human Glutemate Dehydrogenase	390 - 399	N H V S Y G R L T F
39	Human Carbonic Anhydrase	2 - 11	H H W G Y G K H N G
40.	Bakers Yeast Cytocrome b	95 - 104	M H M A K G L Y Y G
41.	Mouse Cytochrome b	96 - 105	L H V G R G L Y Y G
42	Human Cytochrome b	96 - 105	L H I G R G L Y Y G
43	RSV Envelope/Coat Protein	550 - 559	V H L L K G L L L G
44	Mouse Delta Chain (Ig) C Region	196 - 205	Q T W S V L R L P V
45	Human Plasminogen	277 - 286	Q H W S A Q T T H T
46	Myxobacter Beta Lytic Protease	121 - 130	Q H W S L K Q N G S
47	Yeast Alpha Mating Factor	1 - 9	W H W L Q - L K P G
48	Human Prolactin	147 - 154	P V W S - G L - P S
49	Chicken Gastrin Releasing Peptide	1 - 7	A L Q P G G

Table 6.1 POLYPEPTIDES HAVING SEQUENCE HOMOLOGY WITH MAMMALIAN LH-RH

PEPTIDE/PROTEIN:	AMINO ACID SEQUENCE:
1. Cauliflower Mosaic Virus Coat Protein	Q A E K L G L Q P I
2. Pig Lactate Dehydrogenase	Q H G S L F L Q T P
3. Bakers Yeast ATPase Protein 3/6	R A I S L G L R L G
4. Pig Pancreatic Alpha Amylase	Q L W S G T L Q T G
5. Adenovirus 2 Hexon Assoc. Protein VIII	V S S S L G L R P D
6. Adenovirus Early 55K Protein	Q L T T Y W L Q P G
7. Bakers Yeast Cytochrome C	A K E S T G F K P G
8. Thiocapsa High Potential Fe-S Protein	Q A D S G A W R P G
9. Clostridium Flavodoxin	L F G S Y G W G S G
10. Dogfish Lactate Dehydrogenase	Q H G S L F L H T A
11. Human, Bovine, Chicken Glutamate Dehydrogenase	N H V S Y G R L T F
12. Human Caerulosplasmin	N G F M Y G N Q P G
13. Tigersnake Phospholipase A2	Q C A N M G K R P F
14. E. coli Beta Galactosidase	I I W S L G N E S G
15. Myxobacter Beta Lytic Protease	Q H W S L K Q N G S
16. Human IgG C Chain	T Q K S L S L S P G
17. Bakers Yeast/Bovine Actin	L G K S Y E L P D G
18. Semliki Forest Virus Coat Protein	V Q Y Y Y G L ▼ P A
19. Human Mitochondrian Cytochrome b	L H I G R G L ▼ Y G
20. Human Corticotropin Precursor	E P R S D G A K P G
21. RSV Envelope Protein	L L Y S D I T R P G
22. RSV Envelope Protein	V H L L K G L L L G
23. Mouse/Bakers Yeast Cytochrome b	L H V G R G L ▼ Y G
24. E. coli ATPase	K P V S L G L R L F
25. Aspergillus Cytochrome b	L H I G R G L ▼ Y G
26. E. coli Cephalosporinase	Q N W Q P A W A P G
27. pBR Hypothetical Protein E-152	R H L S Y E L H D K

▩ Mammalian LH-RH
□ Chicken I LH-RH
◎ Salmon LH-RH
○ Lamprey LH-RH
▼ Chicken II LH-RH

Table 6.2 **POLYPEPTIDES WITH SEQUENCES HOMOLOGOUS TO VERTEBRATE LH-RHs**

6.5 LH-RH-LIKE SEQUENCES IN KNOWN POLYPEPTIDES

Five vertebrate hypothalamic LH-RHs have been isolated to date. Table 6.1 shows that the LH-RH of the primitive agnathan vertebrate, the lamprey, is most divergent from the mammalian peptide, having 5 different amino acids. The avian peptide Gln^8-LH-RH is the least different and a peptide with similar chromatographic properties has recently been described in the ovine pineal gland (Millar et al., 1986).

A search of the Margaret Dayhoff protein sequence data bank revealed a large number of proteins ranging from a hypothetical plasmid protein to a number of human proteins which have at least four amino acids co-incident with those of LH-RH (Table 6.1). The high scores achieved by some of these proteins in the mutation data matrix search programme is suggestive of a common ancestral origin with LH-RH. Table 6.2 demonstrates that when compared with the other vertebrate LH-RHs there is even more homology between some of these proteins and the hypothalamic decapeptide.

In view of the large number of mammalian proteins having structural homology with LH-RH, and since it is known that at least three amino acids are required for an immunogenic site, it is quite possible that some of the proteins listed in Tables 6.1 and 6.2 may cross-react significantly with certain LH-RH antisera when mammalian extrahypothalamic tissue extracts are assayed.

These findings have important implications regarding the possible biological activity of some of these proteins and peptides in systems analogous or comparable to the release of pituitary gonadotropins by LH-RH. Yeast alpha mating factor has been shown to not only bind specifically to rat pituitary cells

but also to stimulate LH-release (Loumaye et al., 1982). Chicken gastrin-releasing peptide stimulates the release of LH from chicken pituitary cells to a greater degree than does yeast alpha mating factor (King, J.A. unpublished). Also, prolactin has been shown to compete, albeit at relatively high concentrations, in a rat pituitary LH-RH receptor binding assay (Millar, R.P. unpublished). The fact that LH-RH-like peptide sequences are found in the simplest life forms has relevance to the current theories regarding the evolution of the neuroendocrine system. In 1969, Pearse proposed that the endocrine system had developed as an "outpouch" of the brain (Pearse, 1969). Thus, the cells of the diffuse neuroendocrine system" had their origin in the neuroectoderm (the nervous system therefore being a precursor of the endocrine system). An alternate theory, which is more consistent with the fact that the common biochemical elements of the nervous and endocrine system occur in some unicellular organisms, is that the two systems have a common early evolutionary origin (Leroith et al., 1982). The occurence of the LH-RH-like peptide sequences in the simplest organisms is more consistent with the latter theory.

6.6 OTHER BIOLOGICAL ACTIVITIES OF LH-RH

The wide distribution of LH-RH and LH-RH receptors outside the hypothalamo-hypophyseal system is highly suggestive that the peptide and/or its structural analogues have other biological functions.

6.6.1 Central Nervous System Roles

The extrahypothalamic LH-RH neuronal pathways suggest neurotransmitter/neuromodulator roles for the peptide. Further support for such roles come from the following observations. Excitatory effects have been observed for iontophoretically applied LH-RH in the arcuate nucleus and other brain regions (Moss et al., 1978). Also, stimulation of frog sympathetic preganglionic fibres results in a slow excitatory post-synaptic potential that can be mimicked by exogenous LH-RH and blocked by LH-RH antagonists (Jan et al., 1979).

6.6.2 Reproductive System Roles

At the level of the gonad, LH-RH receptors have been demonstrated on ovarian granulosa and luteal cells, and testicular Leydig cells. The presence of a LH-RH-like peptide in ovarian granulosa cells and testicular Sertoli cells suggests a paracrine role for the peptide in the regulation of gonadal function. The direct effects of exogenous LH-RH on gonadal LH- and FSH receptors, steroidogenic enzymes and steriodogenesis implicate the locally produced peptide at all these levels of gonadal funcation (Hsueh et al., 1981). LH-RH has dramatic effects on the growth of accessory sex organs in both males and females. In the male this is due to an antiandrogenic effect of the peptide (Pedroza et al., 1980). Thus, androgen-dependent prostate tumour growth is also inhibited by LH-RH (Labrie et al., 1986). In females inhibition of uterine growth may be due to direct effects on the uterus (Sundaram et al., 1981). The possibility thus exists for a growth inhibitory role of the endogenous peptide at the level of the accessory organs.

In patients with bone metastases of prostate tumours, treatment with LH-RH brings about relief of bone pain. Whether the endogenous gonadal peptide can influence steroid production to influence pain pathways under normal circumstances is also a matter of speculation.

6.6.3 Roles in Other Physiological Systems

In the placenta the LH-RH located in the cytotrophoblasts may stimulate the release of hCG from the syncytiotrophoblasts (Hsueh et al., 1981).

At the level of the adrenal where LH-RH receptors occur, LH-RH has been shown to inhibit angiotensin II binding to the adrenal and some LH-RH agonists increase aldosterone production (Capponi et al., 1979). Receptors in the adrenals suggest the presence of a local peptide which may regulate steroidogenesis at this nonreproductive organ (Bernado et al., 1978). Such a peptide has been described in the frog adrenal medulla (Eiden et al., 1980). The LH-RH in milk has been implicated as an exocrine hormone, which is transported through the mother's milk to affect sexual development of neonates (Hazum, 1983). Since LH-RH receptors are found in mammary carcinomas and since growth of these tumours is inhibited by LH-RH analogs, the peptide may function as either a paracrine regulator of normal mammary function or an autocrine growth regulator (Eidne et al., 1975). In this regard it is interesting to note that bombesin which shares homology with the C-terminal chicken gastrin-releasing peptide is an autocrine growth regulator of small cell lung carcinomas (Cuttitta et al., 1985). LH-RH, on the other hand,

shares homology with the N-terminus of chicken gastrin-releasing peptide (Table 6.1).

LH-RH also has biological activities in the islets of the pancreas. Here the peptide is known to inhibit insulin release (Klier et al., 1980). The endogenous peptide may thus be involved in the control of glucose metabolism.

The fact that LH-RH is present in the retina and the olfactory bulbs, and since patients with the LH-RH deficiency disease, Kallmann's syndrome, sometimes present with colour blindness and anosmia may implicate the peptide in these two sensory systems. (But the gene is not defective!)

The above observations provide significant support for a variety of biological functions for endogenous, widely distributed LH-RH-like peptides distinct from the gonadotropin releasing activity of LH-RH in the hypothalamic-pituitary system. The structurally distinct peptides described in this thesis may therefore be endogenous regulators of some of these biological systems. The functional relevance of the LH-RH family of peptide described here is an area of research deserving further investigation.

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