IDENTIFICATION, CHARACTERISATION, AND MOLECULAR ANALYSIS OF THE ALTERNATIVELY SPLICED FORMS OF THE LUTEINIZING HORMONE RECEPTOR IN THE OVINE OVARY

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A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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ERRATA

At the time of submission of this thesis, the paper "Mutational analyses of the extracellular domain of the full-length Lutropin/Choriogonadotropin receptor suggests leucine-rich repeats 1-6 are involved in hormone binding" by Thomas, D., Rozell, T.G., Liu, X. and Segaloff, D.L. (1996) in Molecular Endocrinology 10:760-768, was unavailable to the author. This paper further defines the region of LH/CG receptor that is involved in high affinity binding to hCG and LH. *i.e.* from amino acids -26 to 206 (Braun *et al.*, 1991; *see Table 1.7*) to -26 to 164, with amino acids 165 to 211 being mildly important.

To Denise,

My Guiding Light

in the Darkest Hours

Nothing in this world can take the place of persistence.

Talent will not;
nothing is more common than unsuccessful men with talent.

Genius will not;
unrewarded genius is almost a proverb.

Education will not;
the world is full of educated derelicts.

Persistence and determination alone are omnipotent.

Calvin Coolidge

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CHAPTER TWO: GENERAL MATERIALS AND METHODS

SUMMARY

In the ovary, luteinizing hormone (LH) is one of the primary regulators of ovarian function. It controls follicular and luteal development, as well as initiating ovulation by binding to its cell surface receptor. In the sheep ovary, LH stimulates follicular oestrogen production by enhancing androgen secretion from the thecal layer. Late in follicular development, granulosa cells express LH receptors and respond to LH with an increased production of progesterone. The surge release of LH initiates ovulation and transformation of the thecal and granulosa cells into the small and large luteal cells of the corpus luteum. LH is necessary for maintenance of the corpus luteum *in vivo*. *In vitro*, LH stimulates progesterone production by the small luteal cells, but not by the large luteal cells, even though the large luteal cells have a similar number of LH receptors and they produce progesterone. The reason for this is unknown, but differences in LH receptor function between these cell types may be involved.

Cloning of the rat and the pig LH receptor cDNA in 1989, revealed the LH receptor is a single polypeptide consisting of three domains; a large extracellular domain, which binds LH or hCG with high affinity, a transmembrane domain, that spans the membrane seven times (typical of G protein associated receptors), and a small intracellular domain. Extensive alternative splicing of the gene has been demonstrated in every species examined, often resulting in transcripts which, if translated, would be able to bind the ligand but would not possess the transmembrane and/or intracellular domains. Northern analyses of mRNA encoding LHR in other species revealed multiple mRNA species in ovarian tissue, but do not distinguish between the full length (functional) form and the splice variants.

We demonstrated that ovine LH receptor mRNA expression, as revealed by Northern analyses was similar to that reported in other species. Reverse transcription of mRNA, amplification via the polymerase chain reaction (RT-PCR) and cDNA sequencing was performed to determine which alternatively spliced mRNA species were present in ovine ovarian follicles and corpora lutea. This was the first time that any regions of the ovine LH receptor cDNA had been cloned and sequenced. The development of LH receptor RNase protection assays, confirmed these results, and enabled us to determine the relative abundance of these splice variants. Three alternatively spliced forms of the ovine LH receptor were identified, in addition to the full length (A form). The other forms included the B form, which has been detected in all species examined, and two novel splice forms designated F and G. It was estimated that the ratios of the steady-state mRNA levels of the splice variant B form : the full length A form : G form : F form were 5-3.5 : 1 : 1 : 0.3. The overall level of expression of LHR mRNA was greater in corpora lutea than follicles, but the relative abundance of the splice variants was similar between follicles and corpora lutea.

To determine if the LH receptor is regulated by alternative splicing of the mRNA, we synchronised the oestrous cycle in ewes, and examined mRNA from corpora lutea and follicles from the follicular, early luteal and mid luteal phases by RNase protection assay. The ratios of the LH receptor B splice form : A form was approximately 4.5 : 1 at all stages. Therefore, the LH receptor was not regulated by alternative splicing throughout the oestrous cycle, and the level of LH receptor message was higher in CL from the mid luteal phase, than in regressing or recently formed CL.

The B splice form, which is the dominant LH receptor transcript, encodes for a putative protein that has the high affinity LH/CG binding domain, but no

transmembrane or intracellular domains. However, translation into a protein product of any of the alternatively spliced forms has not been demonstrated *in vivo* in any species. Therefore, to determine if some or all of the alternatively spliced forms are translated in vivo in the ewe, we raised polyclonal antibodies against three peptides based on the predicted amino acid sequence from our isolated clones. Peptide "LHR-ALL" was homologous to a region common to all the ovine LH receptor splice forms, peptide "LHR-A&F" was homologous to a region found only in the product of the A and F splice forms, and peptide "LHR-B&G" was homologous to a region unique to the translated product of the B and G splice forms. While the polyclonal antibodies raised against the "LHR-ALL" and "LHR -A&G" peptides did not specifically recognise LH receptors, the "LHR-B&G" antibody did recognise a protein of the predicted molecular weight (42 kDa) that occurs in ovine corpora lutea but not liver or kidney as detected by Western analyses. In addition, this protein localises to the cytosolic fraction, as predicted. This thesis provides the first evidence that the alternatively spliced LHR transcripts are translated *in vivo*, and consequently suggests the alternatively spliced products may play an important role in ovarian function.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED: DATE: 10-7-96

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PUBLICATIONS

Publications arising from the work in this thesis:

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CONFERENCE PRESENTATIONS

International meetings:

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AWARDS

1991-1995 The University of Adelaide Reproductive Medicine Postgraduate Scholarship.

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1995 Finalist: Endocrine Society of Australia Junior Scientist Award.

LIST OF ABBREVIATIONS USED IN THIS THESIS

A	-	androstenedione
aa	-	amino acid
bp	-	base pair(s)
cDNA	-	complementary DNA
CG	-	chorionic gonadotrophin
CL	-	corpora lutea or corpus luteum
cps	-	counts per second
dH_2O	-	distilled water
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
ddNTP	-	di-deoxynucleoside triphosphate
E_2	-	oestradiol
FSH	-	follicle-stimulating hormone
FSHR	-	follicle-stimulating hormone receptor
hCG	-	human chrorionic gonadotrophin
3β-HSD	-	3β-hydroxysteroid dehydrogenase
kb	-	kilobase
kDa	-	kilodalton
KLH	-	keyhole-limpet haemocyanin
LH	-	luteinizing hormone
LHR	-	luteinizing hormone receptor
µg	-	microgram
µl	-	microlitre
mRNA	-	messenger RNA
nt	-	nucleotide
o	-	ovine
OD_x	-	optical density at x nanometers
PCR	-	polymerase chain reaction
$PGF_{2\alpha}$	-	prostaglandin $F_{2\alpha}$
QEH	-	Queen Elizabeth Hospital
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcription-polymerase chain reaction
T	-	testosterone
TSH	-	thyroid-stimulating hormone
TSHR	-	thyroid-stimulating hormone receptor
UV	-	ultra-violet
v/v	-	volume per volume
w/v	-	weight per volume

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Luteinizing hormone (LH) is secreted by the anterior pituitary gland, and is involved in the regulation, differentiation and development of the ovary and testis. It exerts its effects on these organs by binding to specific receptors located on the plasma membrane of the theca and granulosa cells of preovulatory follicles, the corpus luteum, and interstitial cells in ovaries, and Leydig cells in testes. These receptors have also been reported in some non-gonadal tissues. In primates the LH receptor also binds the placental glycoprotein chorionic gonadotrophin (CG), which maintains corpus luteum function during early pregnancy. LH and hCG activate the receptor through the guanine nucleotide associated protein, Gs, which is responsible for adenylate cyclase activity, cAMP accumulation and cAMP dependent protein kinase A activity. There is also some speculation that it interacts with other G proteins, which activate protein kinase C activity. Activation of these secondary messenger systems enhances steroidogenesis, and in some cells types, differentiation.

Recent cloning of the LH/CG receptor in a number of species, has provided tools for further understanding of how the receptor is regulated. However, it has also revealed that in all species, many of the messenger RNA transcripts for the LH/CG receptor do not encode the full length receptor, having portions of the coding region removed due to extensive alternative splicing. The function and regulation of these alternatively spliced forms is not known, however many of them have been demonstrated to encode proteins capable of binding LH and hCG with high affinity, but do not contain the receptors transmembrane domain. It is the intention of this thesis to investigate their regulation and possible role in the ovary, using the sheep as an animal model. Three areas of research relevant to alternative splicing of the LH receptor in the ovine ovary will be discussed. Firstly, the relevant physiology of the ovary that relates to LH action. Secondly, the role and function of LH/CG receptors, and thirdly the development and current status of alternative splicing, with respect to the LH/CG receptor.

1.2 PHYSIOLOGY OF THE OVARY

The main function of the ovary is to produce and release functional eggs, and in mammals to assist in producing the endocrine environment necessary for implantation after fertilisation. In order to achieve this, various temporary endocrine organs are grown in the ovary to produce the correct steroid environment. Much of the developmental control and regulation of these endocrine organs are directly controlled by LH or hCG, via the LH/CG receptor.

1.2.1 From Primordial Germ Cells to Primordial Follicles

Primordial germ cells (PGCs) originate in the extra embryonic mesoderm (Ozdzenski, 1967; Clark and Eddy, 1975) and migrate to the gonadal ridge, increasing from less than 10 in day 7 mouse embryos (Ginsburg *et al.*, 1990), to more than 20 000 in each ovary, by the time the gonadal ridges are fully colonised, and are the sole source of the adult germ cells. The PGCs move into the cortex and give rise to the cortical sex cords in conjunction with the mesonephros and supporting epithelial cells. In some mammalian species (*e.g.* sheep, cow, pig, dog) the germ cells secrete steroids (Byskov, 1979). In the sheep, the germ cell cords are lined in a basal lamina and are clearly defined from the surrounding loose mesenchyme (Gropp and Ohno, 1966). When the germ cells begin meiosis, the germ cell chords degenerate, their steroid production ceases and it defines the transition of oogonia (proliferating germ cells) into oocytes.

During meiosis in most mammals (*e.g.* sheep, human, mouse and rabbit), the DNA of the oocytes undergoes condensation and homologous chromosomes pair, allowing recombination between maternal and paternal chromatids (Mauleon *et al.*, 1976). Approximately 10% of the oogonia reach the diplotene stage where meiosis arrests, with the rest of the oogonia degenerating (Baker, 1972). Meiosis is not completed until the

oocyte is ovulated and fertilised. However, for ovulation to occur, the formation and growth of follicles must initially take place.

Follicle formation begins in the inner part of the ovarian cortex, with the migration of mitotically dividing extra-ovarian mesonephric rete cells into the intra-ovarian rete (Stein and Anderson, 1979). These cells surround diplotene oocytes, differentiating into pregranulosa cells and forming primordial follicles (Gondos, 1970; Hoyer and Byskov, 1981). Each fully formed primordial follicle is surrounded completely by basement membrane. The time at which primordial follicles appear varies between species. In sheep, oogenesis begins at about 52 days after fertilisation, and is completed by day 82 *post coitum* (Mauleon and Mariana, 1976), while primordial follicle formation begins at about 65 days after fertilisation (Mauleon, 1967), and is completed by day 100 *post coitum* (Mauleon, 1969).

1.2.2 Follicular Development

Follicle growth begins with a series of mitotic divisions of the pregranulosa cells, which surround the oocyte, converting it into a multilayered preantral secondary follicle. The fibroblast like cells which surround the follicle then differentiate into thecal cells. Finally, formation of an antral cavity marks the transition of a secondary follicle into a tertiary follicle.

After primordial follicles have formed, their pregranulosa cells cease to divide and enter a period of senescence (Guraya, 1985), making up a large reserve of quiescent primordial follicles. Some follicles begin to grow as soon as they are formed, although most spend months or years in the quiescent stage. In the ewe, the start of follicular growth begins as early as day 95 *post coitum*, and the first antrum containing follicles appear around day

135 *post coitum*, 15 days before birth. The mechanism that triggers the initiation of primordial follicle development is unknown. There is some evidence that gonadotrophins are involved (Peters, 1969; de Wolff-Exalto, 1982), although this action is species specific, since healthy vesicular follicles can be found in long term hypophysectomised ewes (Dufour *et al.*, 1979).

The first sign of growth is the division and changing shape of the pregranulosa cells from flattened to a columnar shape. This is followed by a dramatic increase in the oocyte's volume (Lintern-Moore and Moore, 1979), reaching its maximum size by the time an antrum forms, approximately 80-120 μm in most domestic species (Wassarman, 1988). The zona pellucida begins to form around the oocyte about the time follicular growth begins, and appears to be secreted by the oocyte itself (Hadad and Nagai, 1977; Takagi *et al.*, 1989).

Whilst the oocyte is enlarging, the granulosa cells increase in number to surround the oocyte with several layers of granulosa cells. Granulosa cells from ovine follicles below 2 mm in diameter contain high concentrations of follicle stimulating hormone (FSH) receptors, though this concentration gradually decreases as follicles increase in diameter (Carson *et al.*, 1979a). Granulosa cells of preantral follicles do not contain LH binding sites (Carson *et al.*, 1979a).

During the course of folliculogenesis, cells indistinguishable from fibroblasts become aligned concentrically around the follicle, forming the thecal layer. The theca develops a blood supply, and in some mammals differentiates into the theca interna in which vascular development is particularly rapid, and a less vascularised theca externa which merges into the stromal tissue without clear boundaries. In the sheep, a well differentiated theca interna appears only when follicles are 2 to 3 mm (O'Shea *et al.*, 1978). It is made up of

two types of thecal cells, with the cells located just outside the follicular basement membrane rich in smooth endoplasmic reticulum, while the other theca interna cells are rich in rough endoplasmic reticulum (O'Shea *et al.*, 1978). The mechanism that regulates thecal cell differentiation is not known, but is assumed to occur under the influence of growth factors emanating from the oocyte-granulosa complex (Greenwald and Terranova, 1988). During ovine follicular growth, not only do the number of thecal cells increase, but the proportion of cells that are steroidogenically active also increases. Approximately 20% of thecal cells in follicles less than 3 mm in diameter steroidogenically active, compared to approximately 50% of thecal cells steroidogenically active in the largest follicles (Greenwald and Terranova, 1988). LH receptors are present on thecal cells at all stages of follicular growth (Zeleznik *et al.*, 1974; Linder *et al.*, 1977; Henderson *et al.*, 1984; Carson *et al.*, 1979a). During follicular growth in the ewe, the receptor concentration on thecal cells, as determined by hCG binding, decreases slightly (Carson *et al.*, 1979a). FSH binding to ovine thecal cells has only been detected at very low levels (Carson *et al.*, 1979a) and is thought to be due to granulosa cell contamination (Carson *et al.*, 1979b).

The transition of a preantral to an antral follicle is gradual and some species have follicles that never acquire antral cavities. The smallest antral follicle (approximately 0.2-0.3 mm diameter in the sheep) takes 25-35 days to become a mature preovulatory Graafian follicle (about 8 mm; Turnbull *et al.*, 1977). As the granulosa cells divide there is an increase in the production of fluid that leads to the formation of a follicular cavity (antrum). The accumulating fluid is also formed by filtration of thecal blood through the basal lamina which acts as a crude molecular weight sieve (Shalgi *et al.*, 1973).

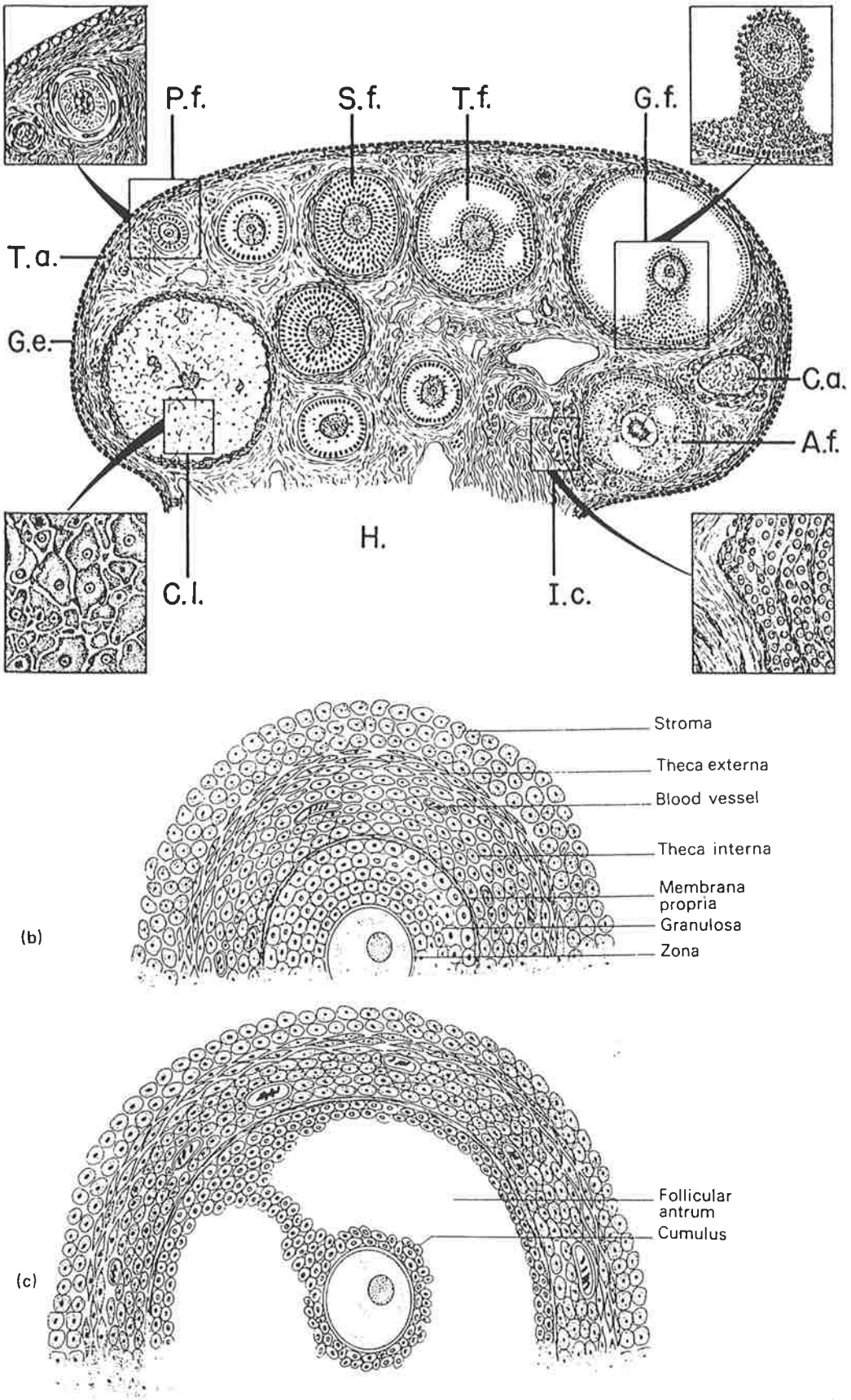

Figure 1.1 (A) Composite diagram of the mammalian ovary. Progressive stages in the differentiation of a Graafian follicle are indicated (*upper left to right*). The mature follicle becomes atretic (*lower right*) or ovulates and form a corpus luteum (*lower left*). A.f. (atertic follicle); C.A. (corpus albicans); C.l. (corpus luteum); G.e. (germinal epithilium); G.f (Graafian follicle); P.f. (primary follicle); S.f (secondary follicle); T.f. (tertiary follicle). From Byskov, 1989. **(B) A secondary follicle (C) A Graafian follicle.** From Johnson and Everitt, 1984.

The granulosa cells of the antral follicle also differentiate such that they are distinguishable by their location *i.e.* those that form the follicular wall (mural granulosa), are morphologically different from those that form the cumulus oophorus and the corona radiata *(see Figure 1.1).* Increasing differentiation of mural granulosa cells close to the basement membrane, makes them morphologically and functionally distinct. They cease dividing, their appearance changes from cuboidal to a columnar appearance (Hirshfield, 1991) and they develop gap junctions and LH receptors (Richards and Midgley, 1976; Weiss *et al.*, 1978; Carson *et al.*, 1979a). It has been suggested that gap junctions are important in LH receptor formation as well as in the dissociation of the hormone to its binding site (Albertini *et al.*, 1975).

FSH is the primary inducer of LH receptors on granulosa cells, and their acquisition is a late sign of follicular maturation; LH receptors do not appear on sheep granulosa cells in significant numbers until the follicle that they are part of reaches 4-6 mm in diameter (Carson *et al.*, 1979a). Their appearance indicates the follicles are competent to be stimulated by exogenous gonadotrophin to ovulate (Ying and Greep, 1971).

As expected from hCG binding studies not all granulosa cells contain the same amounts of LH receptor mRNA. While small follicles contain insignificant levels of LH receptor mRNA, the granulosa cells of medium-sized follicles (300-400 mm) undergoing maturation, reveale a stratified pattern of expression of LH receptor mRNA. The mural granulosa cells located next to the basement membrane contain the highest levels of LH receptor mRNA, at levels higher than the thecal cells at that time, with the LH/CG-R mRNA levels gradually decreasing in the granulosa cells which are closer to the antrum, such that cells surrounding the oocyte contained no LH receptor mRNA. This pattern of message distribution was less evident in fully grown follicles, although they contain more LH receptor RNA per granulosa cell (Peng *et al.*, 1991).

1.2.3 Ovulation

Preovulatory follicles will ovulate if exposed to a large gonadotrophin surge (begining when LH levels are at a concentration >5 ng/ml and reaches levels of about 200 ng/ml in the ewe). The large endogenous LH surge that triggers ovulation in sheep is initiated by a high LH pulse frequency which stimulates sufficiently high oestrogen levels and low progesterone levels (Baird and McNeilly, 1981) and will be discussed further in section 1.3.

The number of follicles that become preovulatory and ovulate varies widely between species. Shortly before follicle rupture the follicle rapidly increases in size, resulting in the follicle bulging from the surface of the ovary (the follicle size at this stage is about 5-10mm in diameter in sheep and approximately 16-18 mm in humans). Blood flow decreases and minute haematomas form, followed by disintegration of the surface epithelium (Blandau, 1967), the basement membrane and organisation of the theca layer (Byskov, 1967). Proteases, such as collagenase, are released by the thecal cells at this stage resulting in this dissolution of the extracellular matrices (Espey, 1980; Hirch *et al.*, 1993). Finally, the granulosa cells part, allowing the egg to escape into the periovarian space (Byskov, 1967). Meanwhile, blood flow to the theca interna increases (Moor *et al.*, 1975), and its capillaries become more permeable (Burr and Davies, 1951), with leukocytes moving to the extravascular spaces (Byskov, 1969; Espey, 1974).

In many mammals luteinisation of the granulosa cells precedes ovulation by hours or days (Bjorkman, 1962; Bjersing *et al.*, 1972; Baird *et al.*, 1975), causing enlargement of the cell, granular appearance of the cytoplasm, an increase of the smooth endoplasmic reticulum, spherical mitochondria with tubular cristae and the appearance of lipid inclusions (Peters and McNatty, 1981). The interval between the LH peak and ovulation

varies from about 12-15 hours in mice, rats and rabbits, through 25 hours in the sheep, 36 hours in humans, to about 42 hours in the pig.

1.2.4 Resumption of Meiosis

As ovulation approaches, the oocyte resumes meiosis. This resumption is induced by the LH surge, but the precise mechanism is open to debate, as many factors including interactions between follicle cells and the oocyte, gonadotrophins, cAMP, purines, calcium, calmodin, and growth factors (EGF, TGF), have been shown to affect the regulation of meiosis (Wassarman, 1988; Sirucusa *et al.*, 1990). A reduction division then takes place with homologous sets of chromosomes dividing *i.e.* no centromeric division, so that both cells have a haploid complement of chromosomes. The cytoplasm is then unequally divided so that one set of homologues receives most of the cytoplasm, and the other set extruded. The oocyte then enters its second meiotic division arresting at metaphase II, and ovulation occurs in most species. Meiosis does not continue unless fertilisation takes place. Upon fertilisation, each chromosome splits into sister chromatids by division of the centromeres (Peters and McNatty, 1980).

1.2.5 Atresia

The majority of follicles never ovulate but become atretic. There are two main stages after follicle organisation has taken place at which atresia occurs; (1) the degeneration of follicles that have not started to grow, of which little is known; and (2) the degeneration of follicles which have begun to develop and whose differentiation has become disrupted.

In the sheep, phagocytic cells representing transformed granulosa cells increase in number as atresia progresses (Hay *et al.*, 1976), and during stage II, atretic bodies develop from the fusion of many nuclei (Greenwald and Terranova, 1988). Gross identification of

atretic and healthy follicles is possible in the sheep in 95% of cases (Moor *et al.*, 1978), and is based primarily on vascularisation of the theca, thecal hypertrophy, integrity of the membrana granulosa and translucency of the follicle. In the ewe thecal cells undergo apoptosis with condensation and fragmentation and are ultimately phagocytosed by healthy thecal cells. Blood flow and capillary area to the theca layer are significantly reduced in atretic follicles.

LH and FSH binding by large atretic follicles (>4mm) does not differ from healthy follicles until the most advanced stage of atresia (Carson *et al.*, 1979a), though they do have reduced aromatase activity. However, the granulosa cells of atretic intermediate follicles (2-4mm) lose their ability to bind FSH before the loss of LH/hCG binding (Greenwald and Terranova, 1988). In the ewe, the molar ratio of oestrogen to testosterone and androstenedione is always >1 in the follicular fluid of normal and early atretic follicles, but as atresia progresses the ratio shifts in favor of the androgens, primarily due to the fall in oestradiol (Carson *et al.*, 1979a).

1.2.6 Follicular Steroidogenesis and its Control by Cell Type

Follicular steroid synthesis and metabolism occurs under gonadotrophic control in theca and granulosa cells in all animals. It also occurs in secondary interstitial cells derived from the theca of degenerated follicles, although these cells are not common in domestic ruminants. Oestrogen secretion by the preovulatory follicle depends on the coordination of theca and granulosa steroid output (Flack, 1959; Short, 1962a; Bjersing and Carstensen, 1967; Ryan *et al.*, 1968; Baird, 1977; Moor, 1977), controlled by FSH and LH (Armstrong and Dorrington 1979).

This "two cell, two gonadotrophin" model of oestrogen secretion by the follicles is based on 3 facts (1) LH binds to its receptor on theca interna cells which stimulate the synthesis of the precursor androgen, androstenedione, (2) the androgen crosses the basal lamina and enters granulosa cells, where (3) binding of FSH to its receptors results in stimulation of the aromatase enzymes complex, which convert androgens into oestradiol (Dorrington *et al.*, 1975, *see Figure 1.2*). The exogenous supply of androgens to the granulosa cells is the rate limiting factor *in vivo* and *in vitro* for oestrogen biosynthesis (Baird, 1977). Since LH is required for significant rates of androgen production by thecal cells, it is considered the primary regulator in controlling oestrogen secretion by all but the most immature of follicles.

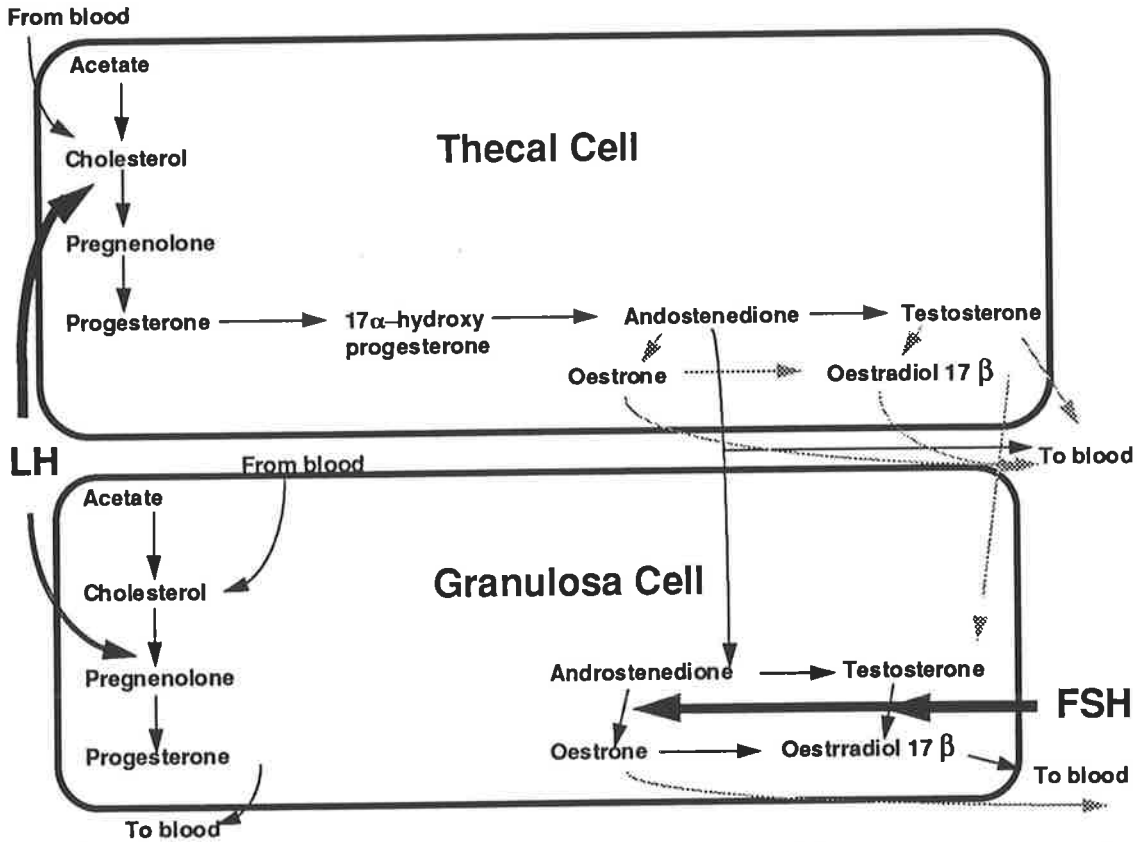

Figure 1.2. Principle steroidogenic pathway in developing follicular cells. Double lined arrow, indicates the effect of LH on granulosa cells of preovulatory and progressing into the luteal phase. Where alternative pathways exist, the minor pathway is indicated in gray arrows. Based on a figure in Johnson and Everitt, 1984.

LH stimulation of theca cells increases androgen secretion by increasing the activity of (1) the cholesterol side chain cleavage system and (2) the 17a-hydroxylase: C-17,20-lyase complex (Fukuda *et al.*, 1979; Aono *et al.*, 1981; Bogovich and Richards, 1982). These enzymes convert pregnenolone to androstenedione *(see Figure 1.2)*. When the 17α-hydroxylase: C-17,20 lyase complex is rate limiting the theca is a significant progestin source. However when C21 steroid synthesis is the rate limiting step, exogenous progestin from granulosa cells can be utilised by the theca cell to make androgen.

Lipoprotein from circulation provides the major source of cholesterol to the thecal cells, and *in vitro* steroidogenesis is limited in the absence of lipoprotein cholesterol (Dyer *et al.*, 1985). The preferred pathway for the conversion of cholesterol to androgens in the theca is via the 5-ene-3β-hydoxysteroid pathway (Aakvaag, 1969). DHEA is then metabolised to androstenedione. Androstenedione is the major aromatisable C19 steroid produced by isolated theca interna (Moor, 1977; Hamberger *et al.*, 1978; McNatty *et al.*, 1980; Evans *et al.*, 1981; McNatty *et al.*, 1984).

Thecal cells may also aromatise androgens directly, though this oestrogen production varies between species and follicular stage *e.g.* human (Ryan *et al.*, 1968; Batta *et al.*, 1980), sheep (Armstrong *et al.*, 1981), pig (Evans *et al.*, 1981) and cow (Lacroix *et al.*, 1974). In the sheep, oestrodiol production by thecal cells increases as the follicle matures.

Initially FSH, and later on as the follicle becomes medium sized (4-6 mm) LH bind to granulosa cells to increase activity of the aromatase enzyme complex (Armstrong *et al.*, 1981). FSH induces cholesterol side chain cleavage P_{450scc} (Nimrod, 1977; Jones and Hseuh, 1982; Toaff *et al.*, 1983) and the $\Delta^{5-3}\beta$-hydroxysteroid dehydrogenase: Δ^{5-4}-isominase enzyme complex production in granulosa cells (Zeleznik *et al.*, 1974), resulting in increased progesterone secretion (Nimrod, 1977; Dorrington and Armstrong, 1979;

McNatty *et al.*, 1979; Ball and Knuppen, 1980). However, this does not occur until after the preovulatory stages when the basal lamina breaks down and vascularisation of the granulosa occurs, as the cholesterol required for progestin production *in vivo*, comes via the blood supply.

Secreted oestradiol effects positive feed back control of pituitary LH release, and thereby stimulates its own formation (Yen *et al.*, 1975; Baird and McNeilly, 1981). This leads to a gradual increase in the integrated circulating LH level, and each pulse of LH delivered to the preovulatory follicle stimulates concurrent bursts of androstenedione and oestradiol into the ovarian vein (Baird *et al.*, 1981). Oestrogens promote granulosa cell development and responsiveness to gonadotrophins *in vivo* (Gaarenstrom and deJongh, 1946; Hisaw, 1947; Goldenburg *et al.*, 1973; Richards, 1980).

Androgen receptors on granulosa cells (Schreiber and Ross, 1976), allow LH induced thecal androgens to function, (in addition to aromatase substrates), as intercellular theca to granulosa regulators, mediating certain follicular requirements for stimulation by LH (Armstrong *et al.*, 1981; Hiller and deZwart, 1982; Campo *et al.*, 1984). Rat ovarian interstitial cells have LH receptors and constitutively express Δ^5-3β-hydroxysteroid dehydrogenase (Erickson *et al.*, 1985).

1.2.7 The Corpus Luteum

After the LH surge, corpus luteum begins to form and produce progesterone. The speed with which this luteinisation occurs is species dependent, (Peters and McNatty, 1980). After ovulation the basement membrane breaks down, and blood vessels from the theca interna invade the ruptured follicle. The wall of the follicle becomes convoluted, and the antrum fills with blood and lymph (*e.g.* primates, sheep, pigs). In the ewe, granulosa cells

start to luteinise after the LH surge, forming smooth endoplasmic reticulum for the synthesis of steroids. 5Δ-3β-hydroxysteroid dehydrogenase is then synthesised and accumulates within a few hours of ovulation (Hay and Moor, 1975), thus allowing progesterone production. By 48 hours after ovulation, gap junctions are no longer apparent, and by 72 to 120 hours, the granulosa cells have differentiated into the large steroidogenic luteal cells (McClellan *et al.*, 1975; O'Shea *et al.*, 1979). Ovine granulosa cells do not divide after ovulation (McClellan *et al.*, 1975).

In the ewe, cells from the theca interna migrate into the deepest parts of the luteal tissue as early as 24 hours after ovulation (O'Shea *et al.*, 1979), attaining close and extensive contact with the luteinised granulosa cells. Although cells from the theca interna do not undergo major changes in their internal structure, they do give rise to the small luteal cells and fibroblast populations of the corpus luteum. The cells in the theca externa remain as an encapsulating layer of flattened cells around the developing corpus luteum, and contribute only to a small extent to the central core of the infolding formed at and following ovulation. After ovulation the small and large luteal cells develop an increased LH receptor content and at least in the rat this appears to be in response to the actions of prolactin and oestradiol (Lee and Ryan 1974; Holt *et al.*, 1976; Richards, 1979; Segaloff *et al.*, 1990). Although initial reports by Niswender and associates claimed that there were very low numbers of hCG binding sites on large luteal cells (Fitz *et al.*, 1982; Niswender *et al.*, 1985), they later reported that there was approximately equal numbers of hCG bindng sites sites on large luteal and small luteal cells from CL's of non-pregnant ewes (Harrison *et al.*, 1987).

In most species the corpus luteum secretes mainly progesterone. The single most important endocrine factor regulating the synthesis of progesterone is LH. LH increases the synthesis and secretion of progesterone *in vivo* (Schomberg *et al.*, 1967) and when

incubated with luteal slices or luteal cells *in vitro* (Kaltenbach *et al.*, 1967; Simmons *et al.*, 1976; Cook *et al.*, 1967; Armstrong and Black, 1966). LH binds its receptors on small luteal cells, resulting in the activation of adenylyl cyclase and increased cAMP levels. This in turn, increases cAMP dependent protein kinase activity, leading to increased steroid secretion (Kuo and Greengard, 1969). LH may also affect steroidogenesis through the Ca^{2+}/inositol phosphate pathway, (see section 1.3.6.3).

In sheep and most mammals the majority of the progesterone is secreted by the large luteal cells (Niswender *et al.*, 1985). In large luteal cells secretion of progesterone is not regulated by the cAMP pathway. These cells do contain adenylate cyclase, which can be activated by forskolin or cholera toxin, and while it leads to increases in cAMP, progesterone production is unaffected (Hoyer *et al.*, 1984).

In cycling animals signs of regression start to appear soon after the corpus luteum has formed *i.e.* between 10-12 days post ovulation in the sheep (Deane *et al.*, 1966), with the luteal cells shrinking, and the chromatin clumping (Lennep and Madden, 1965). Connective tissue gradually invades the regressing corpus luteum, eventually resulting in a corpus albicans (Peters and McNatty, 1980).

The corpus luteum generally enlarges during gestation, due partly to cell hypertrophy and fluid accumulation. In many animals the corpora lutea of pregnancy persists for some time post partium. While the corpus luteum persists, no large antral follicles are seen in the cow or sheep ovaries. In non-lactating primates, the ewe and the cow, the corpus luteum of pregnancy has regressed by the 30th postpartum day.

1.2.8 Luteal Regression

Luteal regression is characterised by a rapid decline in steroidogenesis of the corpus luteum, and the gradual disappearance of the tissue itself. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted by the uterus, exerts a luteolytic effect on the corpus luteum of sheep (McCracken *et al.*, 1970; Thorburn and Nicol, 1971), cattle (Lauderdale, 1972; Liehr *et al.*, 1972), and pseudopregnant rodents *e.g.* mice (Bartke *et al.*, 1972) and rats (Pharriss and Wyngarden, 1969). Morphological changes include lipid droplet accumulation in the cytoplasm of luteal cells, degeneration of capillaries, and an increase in primary lysosomes (reviewed in Knickerbocker *et al.*, 1988).

$PGF_{2\alpha}$ works via a number of mechanisms including: (1) rapidly reducing luteal blood flow (Nett *et al.*, 1976), (2) decreasing LH receptor numbers (Behrman *et al.*, 1978), (3) uncoupling of LH receptors from adenylate cyclase (Fletcher and Niswender, 1982) and (4) a cytotoxic effect (Silvia *et al.*, 1984). However the effects of $PGF_{2\alpha}$, differ between species and between *in vivo* and *in vitro* experiments. In the ewe, it decreases blood flow (Niswender *et al.*, 1976), but has no effect on LH binding site numbers until progesterone levels decrease significantly, *i.e.* by 22.5, but not 7.5 hours after the administration of $PGF_{2\alpha}$ (Diekman *et al.*, 1978). *In vitro*, $PGF_{2\alpha}$ completely blocks LH induced cAMP and progesterone production, even though $PGF_{2\alpha}$ receptors are not found on the LH inducible small luteal cells (Fitz *et al.*, 1982). However, Fitz *et al.* (1982) also reported very low numbers of LH receptors on large luteal cells in comparison to small luteal cells, and so some doubt must be cast on the correct identification of cell types by these researchers.

In ewes, an interaction between $PGF_{2\alpha}$ and oestrogens is important for normal luteolysis, presumably the role of the oestrogens is to regulate luteal levels of $PGF_{2\alpha}$ receptors

(Karsch *et al.*, 1970; Gengenbach *et al.*, 1977; Niswender *et al.*, 1994). $PGF_{2\alpha}$ induced luteolysis of ovine luteal cells involves apoptosis of these cells (for a brief review see McGuire and Niswender, 1993).

The antisteroidogenic effects of $PGF_{2\alpha}$ are mediated through the protein kinase C second messenger pathway (Niswender *et al.*, 1994). Pharmalogical activation of PKC reduces progesterone production from large ovine luteal cells (Conley and Ford, 1989; Hoyer and Marion, 1989; Wiltbank *et al.*, 1991). This effect appears to inhibit cholesterol transport to cytochrome P450scc (Wiltbank *et al.*, 1993), however $PGF_{2\alpha}$ leads to a reduction in steady-state levels of mRNA encoding 3β-HSD (Hawkins *et al.*, 1993).

Surprisingly, $PGF_{2\alpha}$ has no effect on LH induced progesterone secretion *in vivo* (Weston and Hixon, 1980). However, when $PGF_{2\alpha}$ is administered to ewes simultaneously with hCG, it fails to reduce either luteal function or luteal weight of the corpus luteum (Bolt, 1979). A differential loss of large and small luteal cells occurs in the CL after $PGF_{2\alpha}$ administration (Braden and Niswender, 1985).

1.2.9 Luteal Function during Pregnancy

In most mammalian species progesterone must be secreted throughout pregnancy to maintain the required uterine environment. In most species *e.g.* sheep (Ricketts and Flint, 1980) the conceptus does not secrete progesterone until after the CL would have normally regressed. The mechanism by which the CL is maintained varies between species. In the pregnant ewe, the CL is maintained by the secretion of ovine trophoblast protein-1 (oTP-1; an embryonic interferon), by the conceptus. This stimulates the uterus to secrete prostaglandin E_2 (PGE_2), which protects the CL from the luteolytic effects of $PGF_{2\alpha}$ (reviewed in Flint *et al.*, 1991; Salamonsen *et al.*, 1991; Roberts *et al.*, 1992; and Bazer *et*

al., 1992). The small luteal cells from CLs of the pregnant ewe have significantly more LH binding sites than the large luteal cells (Harrison *et al.*, 1987).

In primates the placenta secretes chorionic gonadotrophin as early as 8 to 12 days *post coitum,* which extends the lifespan and function of the corpus luteum. Chorionic gonadotrophin and LH bind to the same receptor resulting in progesterone production. Serum levels of hCG peak around week 14 of gestation and then decline, however by week 20 the placenta secretes sufficient quantities of progesterone so that the CL is no longer required.

1.3 Luteinizing Hormone and Chorionic Gonadotrophin

Both luteinizing hormone and chorionic gonadotrophin bind to the LH/CG receptor. They are members of the family of glycoprotein hormones, together with follicle-stimulating hormone and thyroid-stimulating hormone. These hormones all consist of two dissimilar subunits, α and β, which associate non-covalently to form a heterodiamer. In any given species the alpha subunits of each hormone are identical. While the beta subunits differ, they are homologous for the different hormones. The heterodimer is required for receptor binding, and obviously it is the β subunit the confers specific activity of each hormone.

The alpha subunit of the glycoprotein hormones is encoded by a single gene (Fiddes and Goodman, 1981; Boothby *et al.*, 1991; Naylor *et al.*, 1983). The alpha subunit message encodes the 92 amino acid mature subunit, plus a 22 amino acid hydrophobic signal peptide that is cleaved from the precursor protein. The mature subunit has an approximate molecular weight of 14 900 daltons, with the protein portion representing 10 200 daltons and the remainder due to N-linked glycosylation. The presence of a carbohydrate side chain at Asn52 is critical for biological activity of LH and CG (Matzuk *et al.*, 1990).

The beta subunits of hLH and hCG share an 85% homology between the first 114 amino acids, with the hLH beta subunit consisting of 121 amino acids and the hCG beta subunit 145 amino acids. The extra 24 amino acids of the hCG beta subunit is thought to have arisen from a read-through mutation during the evolution of the hCG beta subunit. Both contain a signal peptide of 20 amino acids which is cleaved from the precusor protein. The hCG beta subunit has a molecular weight of approximately 23 000 daltons, 16 000 daltons comprising the amino acid component, with the remainder made up of both N-linked and O-linked glycosylation. In contrast, the hLH beta subunit has an approximate molecular weight of 19 100 daltons, 13 700 daltons comprising the amino acid component

and the remaining component made up of N-linked but not O-linked carbohydrate chains, the four O-linked glycosylation sites of the hCG beta subunit are found in the extra 24 amino acid tail. There is considerable heterogeneity in the size of the carbohydrate portion of the LH and hCG molecules both within and between individuals.

1.3.1 Regulaton of LH secretion

LH expression and secretion from the anterior pituitary is primarily under the control of pulsatile gonadotrophin releasing hormone (GnRH) stimulus. However oestradiol and inhibins result in a negative feedback of expression, especially of the beta subunit. These work together to communicate with the ovary to control follicular growth. As mentioned in *Section 1.2,* follicle growth is supported by hormones secreted from the anterior pituitary, including LH, FSH and prolactin. Early follicular growth requires only tonic (low) pulsatile secretion of gonadotrophins, from the pituitary. However cyclic (high) pulses are required for antral follicle growth, the final stages of follicular differentiation, and ovulation. The hypothalamus regulates the pulsatile release of LH and FSH through neurosecretions of gonadotrophin releasing hormone (GnRH) (Carmel *et al.,* 1976). The concentration of oestradiol regulates the release of LH and FSH from the pituitary, by increasing the pituitary's sensitivity to GnRH when oestradiol is low, and reducing its sensitivity to GnRH when oestradiol is high. Oestrogen is also responsible for stimulating the preovulatory surge of gonadotrophins (*see Figure 1.3*; Knobil, 1974). This positive feedback works only when the oestrogen stimulus is of the appropriate strength *e.g.* >200 mg secreted oestrogen/ day in women (Peters and McNatty, 1980), and there are sufficient stores of gonadotrophin (Yen *et al.,* 1975). Although progesterone has little effect on gonadotrophin secretion alone (Franchimont, 1971; Scaramuzzi *et al.,* 1971, Knobil, 1974), it does inhibit oestradiol's positive feedback (Knobil, 1974), and enhance its negative feedback of gonadotrophin secretion (Leyendecker *et al.,* 1972). Prolactin also suppresses LH secretion in the ewe, supposedly by inhibiting the positive feedback

mechanism of oestradiol (Kann *et al.*, 1977). The corpus luteum of domestic ruminants secretes progesterone but not oestrogen, thus the gonadotrophin levels remain high enough to allow normal healthy follicles to develop throughout the luteal phase (Salamonsen *et al.*, 1973; Baird *et al.*, 1975a). For a more detailed review of pituitary regulation of LH production in the ewe, see Fink, 1988; Nett *et al.*, 1990; Landerfeld 1990; Haisenleder *et al.*, 1990 and Mercer *et al.*, 1990.

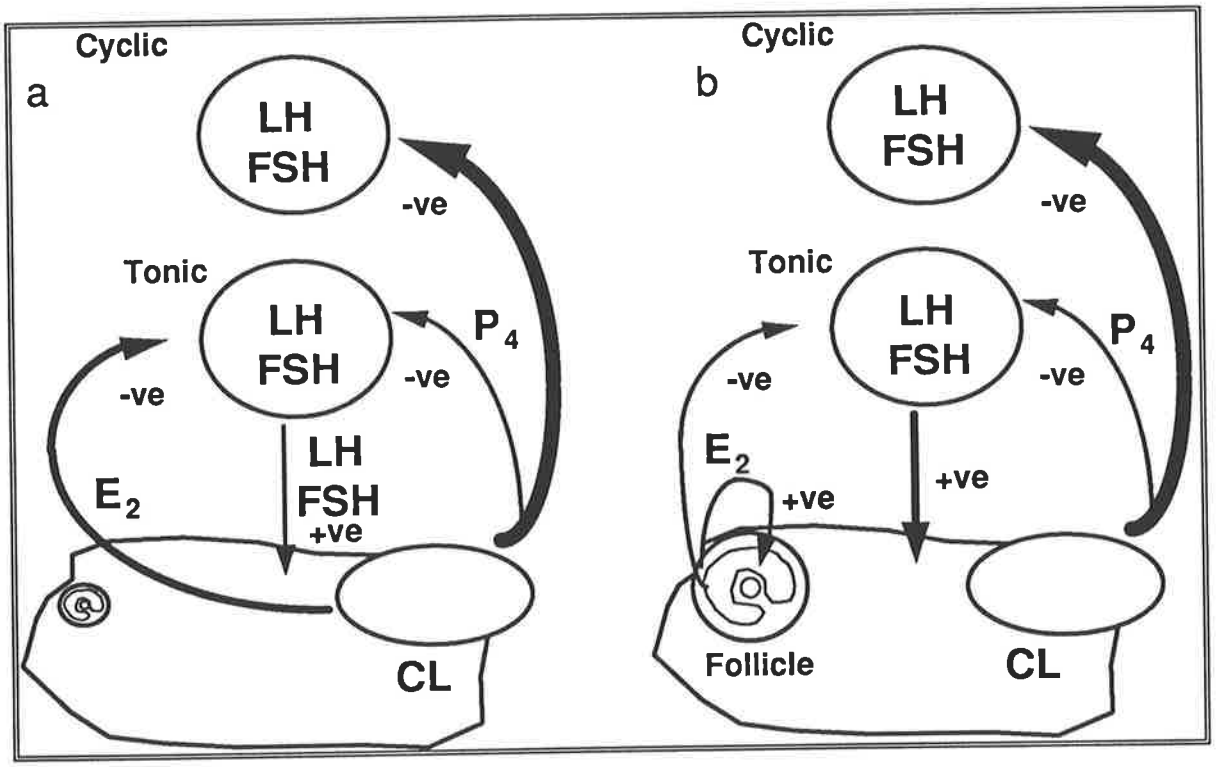

Figure 1.3 Hypothalamic-pituitary relationships in the luteal phase of the ovarian cycle in (a) women and (b) ewes. The width of lines indicates the magnitude of the effects. CL= corpus luteum. The positive effect (+ve) of oestradiol (E_2)in (a) and (b) is inhibited by secretion of progesterone (P_4) from the CL. The tonic secretion of gonadotrophins is controlled by the negative feedback (-ve) of oestradiol secreted by the follicles, which continue to develop throughout the luteal phase. Women control the tonic secreation of gonadotrophins through the negative feedback of oestradiol and the developing follicles become atretic. However, the CL in the ewe does not secrete oestradiol, and follicles continue to develop through the phase. (Modified from Baird *et al.*,1975a).

1.3.2 Regulation of Chorionic Gonadotrophin secretion

In primates (human, baboon, rhesus monkey *etc.*) the syncytiotrophoblastic cells of the placenta begin producing chorionic gonadotrophin by 8-12 days after conception. Chorionic gonadotrophin acts as a lutrophic factor taking over from the inadequate support provided by low levels of pituitary LH and extending the life span and function of the corpus luteum (Niswender and Nett, 1988). Levels of both α and β subunit expression steadily increase, peaking at the end of the first trimester in man, though there is more alpha subunit mRNA than beta subunit produced. By term, the alpha subunit mRNA levels are 10-20% of that seen at its peak, and the beta subunit mRNA is undetectable. Although expression of the subunits rise and fall together, the activation and repression of these genes are different (Jameson *et al.*, 1990). The placenta can secrete sufficient quantities of progesterone by the 20th week of conception so that continued maintenance of the corpus luteum is not required HCG has a half-life in blood of 300 minutes, in comparison to hLH's half-life of 30 minutes.

1.4 LH/CG Receptors

1.4.1 Location of the LH/CG Receptor

LH/CG receptors are found in various cell types of the ovary, the Leydig cells of the testis and have recently been reported in some non-gonadal tissues. The cell types of the ovary have been discussed in *Section 1.2*, however a brief description of non-ovarian cell types that reportedly express LH/CG receptors is given below.

1.4.1.1 The Testes

In the testis, androgen synthesis is controlled by LH stimulation of Leydig cells. Testosterone negatively regulates LH via the hypothalamic-pituitary axis (Williams-Ashman, 1988). As in the steroidogenic cell types of the ovary, binding of LH activates adenylate cyclase stimulating G protein, which results in increases in cAMP. LH may also act through another secondary messenger pathway, the Ca^{2+}/ inositol phosphate pathway, since low (physiological) concentrations of LH stimulates submaximal steroidogenic responses in Leydig cells, without any changes in cAMP concentrations (Catt and Dufau, 1973). These regulatory pathways will be discussed in *Sections 1.4.6.2* and *1.4.6.3*.

1.4.1.2 Non-Gonadal Tissue

LH/CG receptors have also been detected on non-gonadal tissues in humans and other animals, as summarised in Table 1.1. Attention should be drawn to the general lack of either cloning and sequencing or the use of a method such as RNase protection anaylsis of the so called LH/CG receptor from these tissues. This would demonstrate categorically that what has been claimed to be LH/CG receptors in fact corresponds to the full length functional receptor mRNA transcript. The methods so far used to detect these forms in non-gonadal tissues fail to distinguish the full length form from the alternatively spliced and alternatively polyadenylated forms of the LH/CG receptor, and related receptors *(see Sections 1.4.4 and 1.5.2)*.

Table 1.1 LH/CG receptors in non gonadal tissues.

Location of LH/CG receptors	Species	Method of detection	Reference
uterus	human	Immunocytochemistry Northern analyses, Western analyses	Reshelf *et al.*, 1990 Lei *et al.*, 1992a
	pig	hCG binding assays	Ziecik *et al.*, 1986. Ziecik, 1990.
	rabbit	hCG binding assays	Jensen and Odell, 1988 Sawitzke and Odell, 1991
	rat	hCG binding assays	Bonnamy *et al.*, 1990 Bonnamy *et al.*, 1993 Sawitzke and Odell, 1991
blastocyst	rabbit	hCG binding assays	Khan-Dawood *et al.*, 1984
placenta -cytotrophoblasts	human	Immunocytochemistry Immunocytochemistry Northern analyses Ab aggregation assay	Reshelf *et al.*, 1990 Shi *et al.*, 1993
fetal membranes	human	Immunocytochemistry	Reshelf *et al.*, 1990
decidua	human	Immunocytochemistry	Reshelf *et al.*, 1990
gestational trophoblastic neoplasms	human	*In situ* hybridisation, Immunocytochemistry	Lei *et al.*, 1992b
endometrial & myometrial blood vessels	human	*In situ* hybridisation, Immunocytochemistry	Lei *et al.*, 1992a
endometrium	human	*In situ* hybridisation	Lei *et al.*, 1992a Lincoln *et al.*, 1992
endometrial carcinoma	human	Northern Analyses, Western blots, Ligand blotting *In situ* hybridisation, Topical autoradiography, Immunohistochemistry	Lin *et al.*, 1994
umbilical cord	human	Northern Analyses, Western blots, *In situ* hybridisation, Topical autoradiography, Immunohistochemistry	Rao *et al.*, 1993

fallopian tubes	human	hCG binding assays *In situ* hybridisation Immunocytochemistry	Bailey-Pridham *et al.*, 1989 Lei *et al.*, 1993b
uterine arteries	human	Northern Analyses Western Analyses Ligand blots *In situ* hybridisation Immunocytochemistry	Toth *et al.*, 1994
Adenomyosis (invasive disease of the endometrium)	human	*In situ* hybridisation Immunocytochemistry	Lei *et al.*, 1993a
Brain	rat	Northern Analyses RT-PCR Western Analyses Ligand blots *In situ* hybridisation Immunocytochemistry	Lei *et al.*, 1993c
hippocampus	rat	*In situ* hybridisation Immunocytochemistry Topical autoradiography Dot blot analyses	Lei *et al.*, 1993c
cortex	rat	Immunocytochemistry Dot blot analyses	Lei *et al.*, 1993c
cerebellum	rat	*In situ* hybridisation Immunocytochemistry Topical autoradiography Dot blot analyses	Lei *et al.*, 1993c
brain stem	rat	Dot blot anayses	Lei *et al.*, 1993c
dentate gyrus	rat	*In situ* hybridisation Immunocytochemistry Topical autoradiography	Lei *et al.*, 1993c
ependymal cells of the 3rd & 4th ventricle	rat	Immunocytochemistry	Lei *et al.*, 1993c
ependymal cells and choroid plexus of the lateral ventricle	rat	Immunocytochemistry *In situ* hybridisation	Lei *et al.*, 1993c
area postrema	human	*In situ* hybridisation Immunocytochemistry	Lei *et al.*, 1993c

paraventricular nucleus, arcuate nucleus and preoptic area of the hypothalamus	rat	Immunocytochemistry Dot blot analyses	Lei *et al.*, 1993c
hypothalamus	cow	*In situ* hybridisation Immunocytochemistry	Lei *et al.*, 1993c
prostate	rat	Northern Analyses RT-PCR Western Analyses Ligand blots *In situ* hybridisation Immunocytochemistry Sequencing*	Tao *et al.*, 1995 Reiter *et al.*, 1995

Table 1.1 LH /CG receptors in non-gonadal tissues. Summary of non-gonadal tissues and the methods used in which LH/CG receptors have been reported. It is of intrest that in many of the western analyses, the size of the protein was ~50 kDa. *Sequencing has been reported by only one group, Reiter *et al.* (1995) and this was only of a 257bp RT-PCR fragment that amplified exon 8 to exon 10.

The function of the LH receptor in these tissues is not known, although many observations have been made that suggest they do have a function. *In vitro* rat uterine studies demonstrated that hCG stimulates cAMP and progesterone production (Bonnamy *et al.*, 1989). Ziecik *et al.* (1986) suggested LH/CG receptors play a role in the relaxation of the myometrium. Other possible functions include the regulation of steroid hormone synthesis (Troen, 1961; Varangot *et al.*, 1965; Cedard *et al.*, 1968; Genti-Raimondi *et al.*, 1981, Wolf *et al.*, 1985; Yagel *et al.*, 1989), prostaglandin synthesis (North *et al.*, 1991), increase cAMP formation (Menon and Jaffe, 1973; Demers *et al.*, 1973), and glycogen breakdown (Demers *et al.*, 1973; Cedard *et al.*, 1970), as well as possible involvement in hCG/prolactin regulation within the human feto-placental unit (Rosenberg and Bhatnager, 1984; Yuen *et al.*, 1986; Reshef *et al.*, 1990). LH/CG receptors may also play a role in the uterus in implantation of the blastocyst, given that cAMP induces blastocyst

implantation in ovarectomoised rats (Holmes and Bergström, 1975). Furthermore, hCG also regulates the differentiation of cytotrophoblasts in culture into syncytiotrophoblasts (Shi *et al.*, 1993).

In the brain, LH or hCG injected into the peripheral circulation results in alterations of the electrical activity of neurons in the hypothalamus and hippocampus (Terasawa *et al.*, 1969; Gallo *et al.*,1972), biochemical changes in the hypothalamus (Khul and Taubert, 1975), and decreases in pituitary and/or peripheral LH levels (Miyake *et al.*, 1976; David *et al.*, 1966; Molitch *et al.*, 1976). Other studies have demonstrated LH stereotaxically implanted in the hypothalamus leads to decreased pituitary and peripheral LH levels, and changes in associated reproductive events (Corbin and Cohen, 1966; Corbin, 1966; Hirono *et al.*, 1972; Kawakami and Sakuma, 1974; Sanghera *et al.*, 1978; Terasawa *et al.*, 1969; Gallo *et al.*,1972). HCG, through the LH/hCG receptors in the brain, may also effect some of the pregnancy related behaviours, for example morning sickness.

1.4.2 Primary Structure and Topology of the LH/CG Receptor

Up until 1989 and the cloning of the LH/CG receptor there was still a large amount of controversy over its size and structure, with its alleged sizes ranging from 24 kDa to over 300 kDa, consisting of one to many subunits (Ji *et al.*, 1990, Dufau, 1990). Determination of the size and structure of the LH/CG receptor involved chemical and photoaffinity cross-linking of the hormone receptor complex, or direct labelling and purification of the receptor, although some western analyses had been performed. The data from these studies has been summarised in the following two tables.

Tissue	Purification steps	Mr (kDa) of putative receptor	Remarks	Reference
Bovine corpora lutea	Gel filtration, zone electrophoresis	R_1= 85 R_2= 38	Subunits are disulphide bonded	Dattatreyamurty *et al.*, 1983
Pseudo-pregnant rat ovaries	Lectin chromatography Affinity chromatography	R_1=79 R_2= 66 R_3= 55 R_4= 47	Subunits are disulphide bonded	Bruch *et al.*, 1986
Porcine corpora lutea	Affinity chromatography	R_1= 68 R_2= 45	R_2 may be a contamination or degradation product	Wimalasena *et al.*, 1985
Pseudopregnant rat ovaries	Lectin chromatography Affinity chromatography	R=73		Kusuda and Dufau, 1986
Pseudo-pregnant rat ovaries	Affinity chromatography	R=90		Keinanen *et al.*, 1987
Rat testes	Affinity chromatography	R=90		Minegishi *et al.*, 1987

Table 1.2 Summary of studies that attempted to purify the LH/CG receptor to determine its size and structure. R_n= predicted size of the receptor or of the receptor subunit. (From Ascoli and Segaloff, 1989)

Table 1.3

Source of rececceptor	Method	Mr (kDa) of cross-linked product(s)	Proposed idenity	Mr (kDa) of putative receptor	Reference
MA-10 cells	CX	132 117 107	(αβ)R (β)R (α)R	79-85	Ascoli and Segaloff, 1986
MLTC-1 membranes	CX	158 123	(αβ)R (α)R	101-105	Rebois 1982
Rat testes membranes	CX	158 123	(αβ)R (α)R	101-105	Rebois 1981
Purified rat testes receptor	CX	145 105	(αβ)R (α)R	83-92	Minegishi *et al.*, 1987
Porcine granulosa cells	CX	134 110	(αβ)R (α)R	81-88	Ascoli and Segaloff, 1986

Purified rat luteal receptor	CX	130	$(\alpha\beta)R$	77	Metsikko and Rajaniemi, 1982
Purified rat luteal receptor	CX	130 95	$(\alpha\beta)R$ $(\alpha)R$	73-77	Kusuda and Dufau, 1986
Rat luteal membranes	CX	125	$(\alpha\beta)R$	72	Kellokumpu and Rajaniemi, 1985
Rat luteal membranes	PA	106 85 80	$(\alpha)R$	84	Rapoport *et al.*, 1984
Porcine granulosa cells	PA	106 96 88 76 83 73	$(\beta)R_1$ $(\alpha)R_1$ $(\beta)R_2$ $(\alpha)R_2$ $(\beta)R_3$ $(\alpha)R_3$	R_1=73-74 R_2=54-55 R_3=50-51	Ji and Ji, 1980 Ji and Ji 1981 Ji *et al.*, 1981
Porcine granulosa cells	CX	136 102 74 68	$(\alpha\beta)R_1$ $(\alpha\beta)R_2$ $(\alpha\beta)R_3$ $(\alpha\beta)R_4$	R_1=83 R_2=49 R_3=21 R_4=15	Shin *et al.*, 1981 Ji *et al.*, 1985
Rat luteal cells	CX	105 96 74 68 62	$(\alpha)R_1$ $(\alpha)R_2$ $(\alpha)R_3$ $(\beta)R_4$ $(\alpha)R_4$	R_1=83 R_2=74 R_3=52 R_4=35-40	Hwang and Menon, 1984a Hwang and Menon, 1984b
Purified rat luteal cells	CX	85 73 66	$(\alpha)R_1$ $(\alpha)R_2$ $(\alpha)R_3$	R_1=63 R_2=51 R_3=44	Bruch *et al.*, 1986
Rat Leydig cells	CX	144 106 75	$(\alpha)R_1$ $(\alpha)R_2$ $(\alpha)R_3$	R_2=49 R_2=49 R_2=49	Zhang and Menon, 1988

Table 1.3 Summary of studies using chemical or photoaffinity cross linking to determine the sizeand composition of the LH/CG receptor. CX=crosslinking; PA= photoaffinity labelling, α= hCG α subunit, β=hCG β subunit and R_n= predicted size of the receptor or of the receptor subunit. (From Ascoli and Segaloff, 1989)

Few antibodies against the LH/CG receptor were available before its cloning, and hence the use of western analyses were limited. However, Metsikko and Rajaniemi (1984) detected a 90 kDa band by western analysis of purified rat ovarian LH/CG receptor and immunoprecipitation of labelled purified ovarian membranes. Rosemblit *et al.*, (1988) identified the LH/CG receptor by western analyses of partially purified luteal receptors as a single 93 kDa protein. Monoclonal antibodies raised by Podesta *et al.*, (1983) were reported to bind to a 90 kDa protein by western analysis of LH/CG receptor partially purified from luteinisied rat ovaries (Podesta *et al.*, 1986)

Milgrom and associates, raised monoclonal antibodies to the LH/CG receptor (VuHai-LuuThi *et al.*, 1990), which enabled them to clone the LH/CG receptor cDNA sequence (Loosfelt *et al.*, 1989). Using these antibodies, they detected 3 bands by Western analyses of porcine testicular membrane extracts, or of partially purified receptor preparations. These consisted of a major band at 85 kDa, and minor bands at 68 and 45-48 kDa, with the 85 kDa band also seen in ovarian but not non gonadal membranes (VuHai-LuuThi *et al.*, 1990). Comparisons of receptors purified by immunoaffinity chromatography using either antireceptor or antihormone monoclonal antibodies demonstrated in both cases binding to the 85 kDa and 45 kDa species, but not the 68 kDa species, indicating that only the 85 and 45 kDa species are capable of binding the ligand (VuHai-LuuThi *et al.*, 1990).

Despite the reports of different sizes and compositions of the LH receptor summarised in Tables 1.2 and 1.3, all the investigators agreed that the LH/CG receptor is an oligomeric structure in the cell membrane (Ascoli and Segaloff, 1989). The size of the receptor isolated with non ionic detergents and in the absence of reducing agents is at least two times higer than any molecular weight listed in the above two tables. However, the

reported size of the receptor under these conditions also varies, ranging from 170 kDa (Crine *et al.*, 1984), 194 kDa (Dufau *et al.*, 1974) or 224 kDa (Ascoli, 1981) to as high as 305 kDa (Hwang and Menon, 1984a). It had been suggested it was (1) a non-covalently bound oligomer of a single peptide (see Ascoli and Segaloff, 1989 for a review) or (2) a more complex form(s) with at least three different subunits joined by non-covalent bonds (Ji and Ji, 1981; Zhang and Menon, 1988) or as many as four different subunits joined by disulphide bonds (Ji *et al.*, 1985; Hwang and Menon, 1984a; Bruch *et al.*, 1986).

Two major factors that have lead to these confusing and conflicting results (see Ascoli and Segaloff, 1989 for a review) are (1) the presence of endogenous proteases, which are capable of cleaving the free or occupied LH/CG receptor (Kellokumpu and Rajaniemi, 1985a; Kellokumpu and Rajaniemi, 1985b; West and Cooke, 1991), and (2) the presence of proteases that contaminate crude collagenase preparations which are capable of cleaving the LH/CG receptor (Ascoli and Segaloff, 1986; Kim *et al.*, 1987; Minegishi *et al.*, 1987). The action of these proteases on LH/CG receptors can be inhibited by some protease inhibitors *i.e.* N-ethylmaleimide, EDTA, and to some extent phenylmethyl sulphonylfluride (PMSF), as well as performing incubations at 4°C.

In addition to the possibility of proteases generating the smaller receptor subunits, alternative spliced transcripts of the LH/CG receptor have been revealed in all species examined, and if translated, could produce these lower molecular weight proteins, and will discussed in further detail later on. Alternatively the smaller receptor subunits could be unrelated polypeptides.

1.4.3 Cloning of the LH/CG Receptor

The cloning and expression of the complementary DNA of the LH receptor in 1989 was achieved independently by two groups. McFarland *et al.* (1989), cloned the LH receptor from a rat luteal cDNA library using a probe generated by the polymerase chain reaction (PCR), using degenerate primers based on the amino terminal amino acid sequence and several internal peptide sequences of purified LH receptor. Loosfelt *et al.* (1989), cloned the receptor from a porcine testes cDNA λgt11 expression library, using monoclonal antibodies.

The deduced amino acid sequence from these cDNAs, revealed that the LH/CG receptor is a single polypeptide of 674 amino acids in the rat (McFarland *et al.*, 1989), and 669 amino acids in the pig (Loosfelt *et al.*, 1989), with a predicted molecular weight of 75 kDa. The difference in molecular weight, compared with the expressed receptor in tissue (*i.e.* 93 kDa; Rosemblit *et al.*, 1988) is due to N-linked glycosylation and sialic acid residues. When the LH/CG receptor is deglycosylated either by treatment with endoglycosidase F, glycopeptidase F (PNGase F) or N-glycanase or inhibition of receptor glycosylation with tunicamycin, the size of the protein is decreased to approximately 63 kDa as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Keinänen, 1988; Minegishi *et al.*, 1989; Petäjä-Repo *et al.*, 1991; VuHai-LuuThi *et al.*, 1992). However, SDS-PAGE is considered "not very precise in the case of highly hydrophobic proteins", such as the LH receptor, and is probably an underestimation of its size (personal communication with Professor E Milgrom, 1994). Deglycosylation of LH/CG receptors from testis verses ovarian sources demonstrate that the (90 kDa *vs.* 85 kDa) size difference is solely due to N-linked glycoslyation (Dufau *et al.*, 1989).

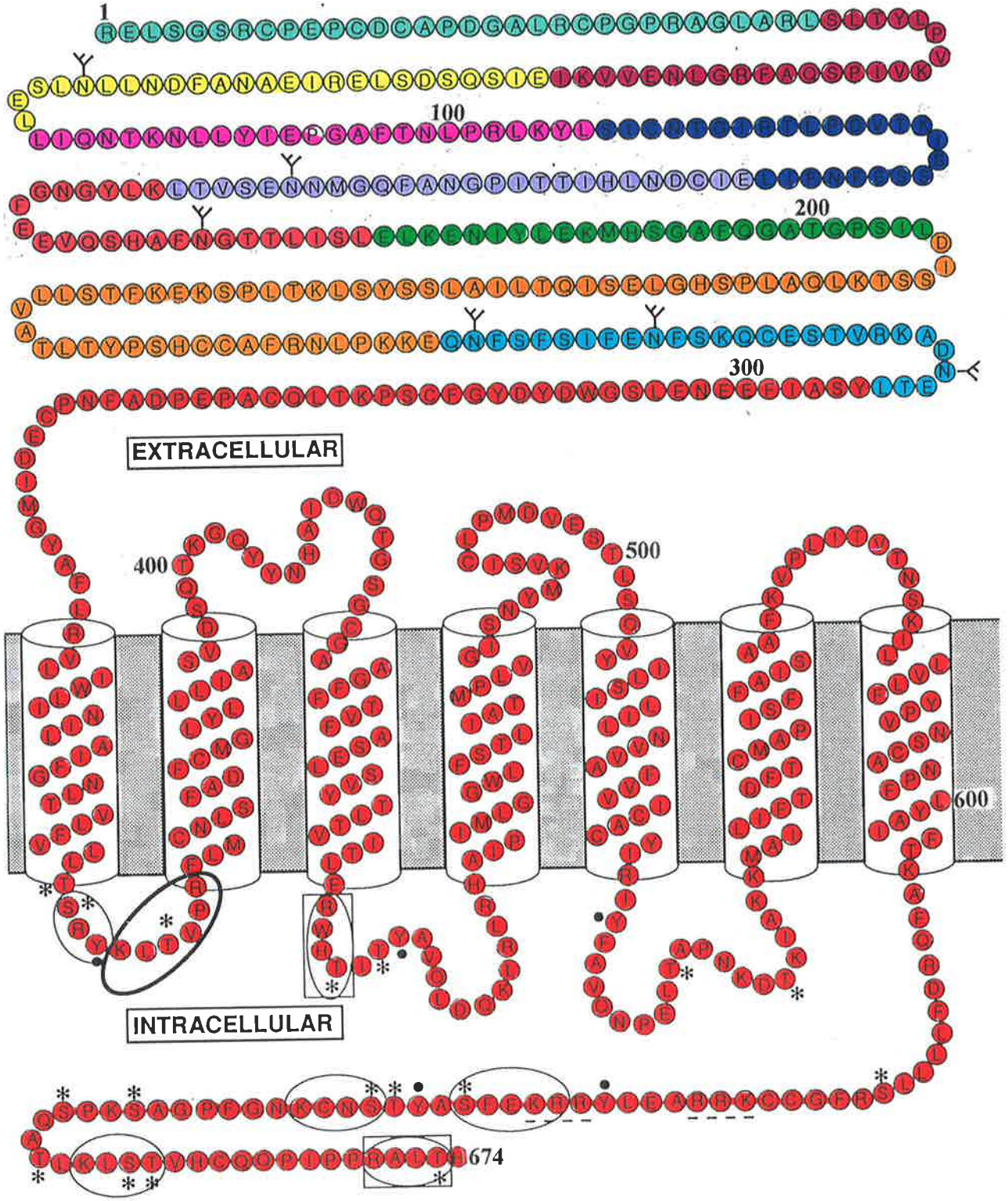

Figure 1.4 Amino acid sequence, orientation and supposed topology of the rat LH/CG receptor in the plasma membrane. The deduced amino acid sequence is taken from McFarland *et al.*, 1989. The overall orientation was deduced using site specific antibodies as described in Rodriguez and Segaloff (1990). The topology of the transmembrane regions is proposed based upon homology with other G protein coupled receptors. Potential sites for N-linked glycosylation are shown by the branch-like structure. The sequences underlined with dashes in the cytoplasmic tail indicate two possible tryptic cleavage sites. Potential intracellular sites for phosphorylation are denoted by asterisks (serine and threonine residues) or dark dots (tyrosine). The rectangles denote weak consensus sequences for cAMP-dependent protein kinase-catalysed phosphorylation. The light and heavy ovals denote weak and strong consensus sequences for C kinase-catalysed phosphorylation respectively. The different colors correspond to amino acids encoded by individual exons. Figure taken from Segaloff and Ascoli, 1993

The LH/CG receptor consists of three domains *(see Figure 1.4)*; (1) a large extracellular domain of 333 amino acids (pig (Loosfelt *et al.*, 1989) or 341 amino acids in the rat (McFarland *et al.*, 1989)), (2) a transmembrane domain of 266 amino acids (pig or 333 amino acids in the rat), that weaves in and out of the plasma membrane seven times, and is common to G-protein associated receptors, and (3) a 70 amino acid carboxyl terminal intracellular domain (pig or 68 amino acids in the rat). In addition there is a 27 (pig), or 26 (rat) amino terminal amino acid signal peptide which is cleaved from the mature receptor, and is thought to be involved in membrane insertion/secretion during synthesis (Von Heijne, 1986; Lewin, 1994). Rodriguez and Segaloff (1990), demonstrated the amino and carboxyl terminii were located extracellularly and intracellularly respectively, by site specific polyclonal antibodies directed against specific peptide sequences.

The extracellular domain contain six potential sites for N-linked glycosylation (Hubbard and Ivatt, 1981; McFarland *et al.*, 1989; Loosfelt *et al.*, 1989) with carbohydrate chains attached to Asn-173 and Asn-152, and at least one other carbohydrate chain at either Asn269, Asn-177 or Asn-291 (numbering based on the rat sequence; Zhang *et al.*, 1995), although controversy reigns on whether they are required for ligand binding, which will be discussed later. All potential glycosylation sites have been conserved between the rat, pig, mouse and human LH/CG receptors (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Minegish *et al.*, 1990; Gundermann *et al.*, 1992).

When comparing the amino acid sequence of the LH/CG of the four known species, all 12 cysteines located in the extracellular domain are conserved as well as the 8 cysteines in the transmembrane domains and connecting loops. The first two cysteines of the cytoplasmic tail and the cysteine at amino acid 663 in the rat *(see Figure 1.5)* are also conserved between species.

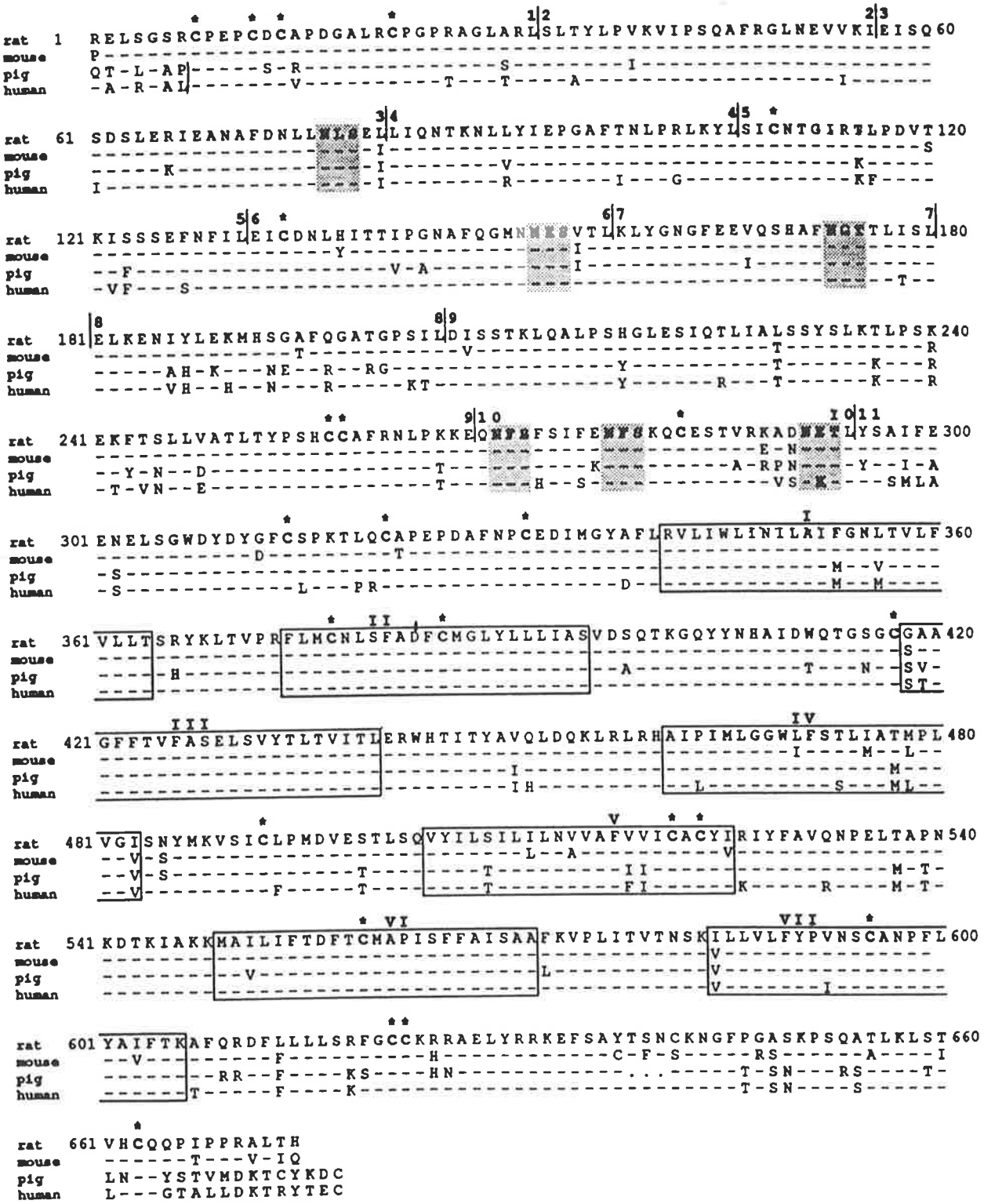

Figure 1.5 Alignment of amino acid sequence of the LH/CG receptor from the four known species. Amino acids are shown in single letter code. The dashes indicate identical amino acids. Conserved cysteines are shown in bold and are marked with asteriks. Consensus sequences for N-linked glycoslyation are shaded. The putative transmembrane regions are enclosed in rectangles and labelled I-VII. The sequences coded for by the different exons of the rat are delineated by the vertical bars and labelled 1-11. From Segaloff and Ascoli, 1993

The amino acid sequence homology of the LH/CG receptor from the four known species is very high, ranging from 84-85% (mouse/rat *vs.* human) to 95% (mouse *vs.* rat). The transmembrane domains and connecting loops have the highest homology, 95.8% (mouse *vs.* rat) 90.6% (mouse *vs.* human), while the lowest degree of homology conservation occurs in the C-terminal cytoplasmic tail (~56% mouse/rat *vs.* human/pig).

1.4.4 Structural Organisation of the LH/CG Receptor Gene

Two groups in 1991 independently cloned the rat LH/CG receptor genomic sequence (Koo *et al.*, 1991; Tsai-Morris *et al.*, 1991), revealing it to consist of eleven exons and ten introns, spanning over 60 kilobases *(see Figure 1.6)*. The first ten exons encode for the extracellular domain, while exon eleven encodes for 47 residues of the extracellular domain, the seven transmembrane domains and the intracellular domain. The majority of the exons, 2-8 and 10, are between 69 and 81 bp in length, while exons 1 and 9 are about 200 bp, and exon 11 is more than 1000 bp in length. Koo *et al.* (1991), demonstrated that the rat LH/CG receptor gene is a single copy gene based on restriction digest maps of the gene and gene dosage analysis.

The intron size varies from 88 base pairs to 14.7 kb *(see Figure 1.6.)*, though remarkably all of the introns of the rat LH receptor are in phase, *i.e.* the intron-exon boundaries all occur between the same two base position of a codon (phase 2; Koo *et al.*, 1991), which is rarely seen in multi-intron genes (Patthy, 1987). The DNA sequences around the exon/intron/exon boundries of the LH/CG receptor cDNA, are highly conserved between species, (Koo *et al.*, 1991), with the 3' junction sequence of the first ten exons being CT in eight of the exons (seven of the exons in mouse and pig and six of the exons in human; Koo *et al.*, 1991 Gundermann *et al.*, 1992;), AT in one, and GA in another, which

suggests the LH receptor gene structure in these species is highly conserved, if not identical.

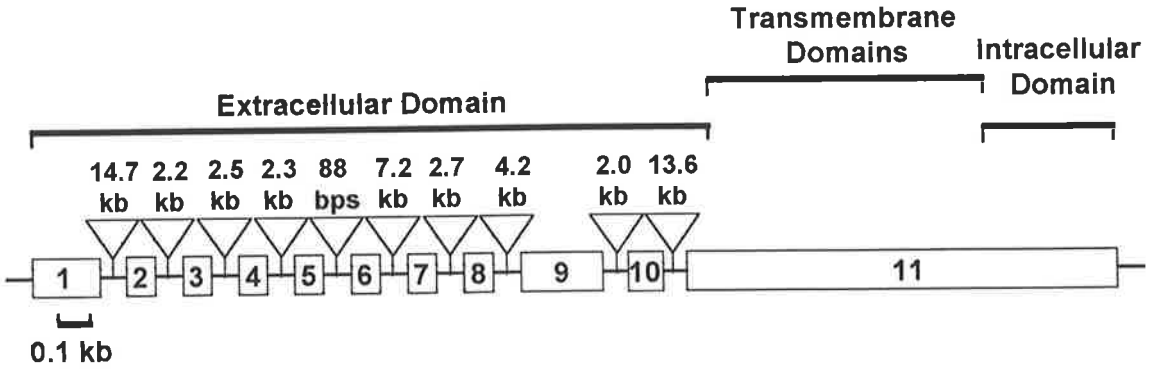

Figure 1.6 Organisation of the rat LH/CG receptor gene. Exons and introns are represented by rectangles and triangles respectively. The size of the different introns is indicated on the top of each triangle. Exons are drawn to scale indicated under exon 1. The first ten exons encode for the majority of the extracellular domain. Exon eleven encodes for 47 amino acids of the extracellular domain, the transmembrane regions and the intracellular domain. Based on a figure from Segaloff and Ascoli (1993).

It is believed that exons 1-9 arose by exon insertion and duplication, based on four pieces of evidence. Firstly, the sequence homology between the exon-intron boundaries; secondly, that all exons are in phase, thirdly the similarity in size of eight of the ten exons and lastly, the existence of imperfectly matching motifs common to exons 1-9 (Koo *et al.*, 1991). These motifs do not correspond to the leucine rich repeats of the LH/CG receptor, nor extend to the intron-exon boundries.

Exon eleven is similar to the intronless G-protein associated receptors *e.g.* β_2-adrenergic receptor *etc.* (Kobilka *et al.*, 1987; O'Dowd *et al.*, 1989), containing all seven membrane spaning domains, the entire C-terminal cytoplasmic domain and 47 amino acids of the extracellular domain. The first 140 bases 5' of exon eleven have a large degree of homology between species (rat *vs.* human 80%) and contains some interesting features. The rat sequence has, 32 bases 5' of exon 11, a translation initiation site which is in frame

with the coding sequence of exon eleven, while a short open reading frame is conserved within 100 bases upstream of exon 11 in both rat and human (Koo *et al.*, 1991). This region shows a high degree of DNA sequence homology with the promoter and regulatory regions of the other G-coupled receptors (Koo *et al.*, 1991), which suggests the LH/CG receptor gene arose by adding the first 10 exons, which may have encoded for a secreted protein, to the intronless gene of the other G-protein associated receptors.

The 5' flanking region of the LH/CG receptor gene has been cloned in the rat (Koo *et al.*, 1991; Tsai-Morris *et al.*, 1991; Wang *et al.*, 1992), and mouse (Huhtaniemi *et al.*, 1992). These regions share a high degree of homology between species (~75%; Huhtaniemi *et al.*, 1992), and both have multiple promoter sites and no TATA or CCAAT boxes close to the sites of transcription initiation. The first 160 bases of the 5' untranslated region is extremely G/C rich *i.e.* the G/C content between nucleotide -164 and -47 is ~79%, and contains four potential promoter specific transcription factor (Sp1) consensus sites (Mitchell and Tjian, 1989) in the rat (Tsai-Morris *et al.*, 1991; Wang *et al.*, 1992) and three in the mouse (Huhtaniemi *et al.*, 1992), though only one is conserved between species. Despite these similarities between the 5' untranslated region of the rat and mouse LH/CG receptor, the location of their transcription initiation sites are considerably different. These differences maybe species and/or tissue dependent, (pseudopregnant rat ovary, hormone induced rat ovary versus mouse testes; Tsai-Morris *et al.*, 1991; Wang *et al.*, 1992; Huhtaniemi *et al.*, 1992), as well as due to the lack of tight promoter control by a TATA box.

In the rat, the minimal promoter for the LH/CG receptor has been localised to the proximal 173-186 bp 5' from the initiation of translation, based on the effects of 5' and 3' deletions of this area on reporter expression in target and non-target cells (Wang *et al.*, 1992; Tsai-Morris *et al.*, 1993; Tsai-Morris *et al.*, 1994; Nelson *et al.*, 1994). This

promoter induces maximal basal transcriptional initiation, with repression of this activity goverened by regions upstream of -173 bp. However there are conflicting reports as to which regions are important in tissue specific expression (Tsai-Morris *et al.*, 1993; Nelson *et al.*, 1994; Tsai-Morris *et al.*, 1995).

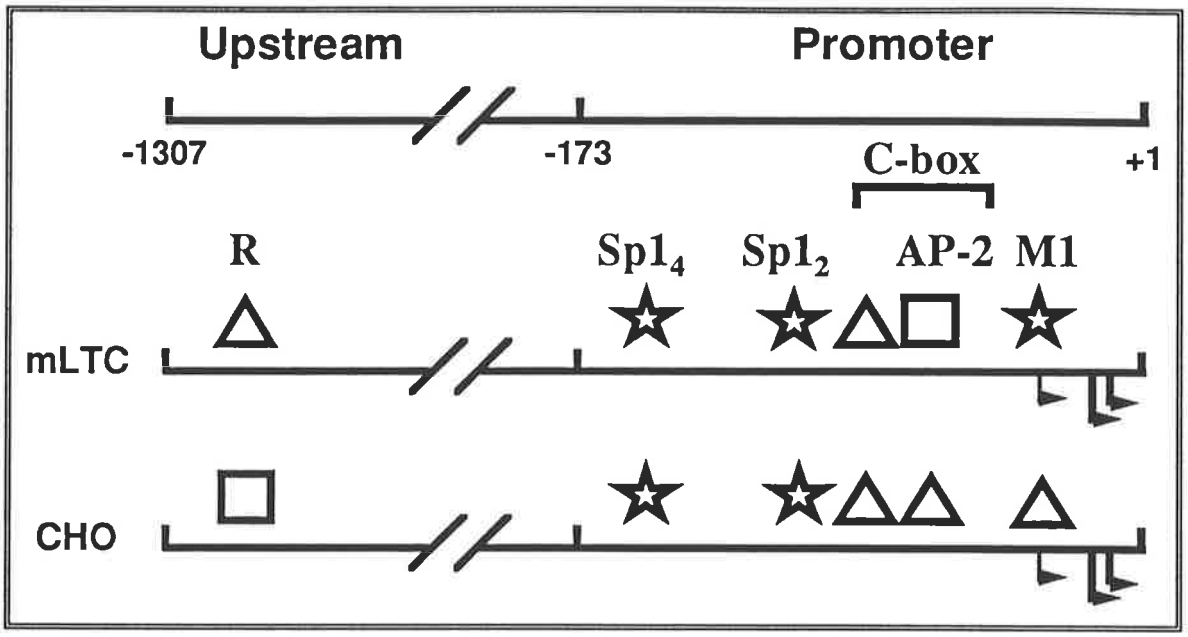

Figure 1.7. Functional activity of protein binding domains of the LH-R gene in the expressing mLTC and non-expressing CHO cell lines. ☆ represents activating trans factors, ❑ represents inhibitory trans factors and Δ represents neutral trans factors. ➤ indicate the transcription start sites, and these are within Inr consensus sequences. (Based on diagrams from Tsai-Morris *et al.*, 1994).

A number of functional regulatory elements within the basal promoter region of the LH/CG receptor gene have been identified, and are indicated in Figure 1.7. There are two potential Sp1 binding domains, $Sp1_2$ (-73 to -94 bp) and $Sp1_4$ (-135 to -154 bp), which are essential for basal promoter activity (Tsai-Morris *et al.*, 1993). Two other Sp1 consensus binding domains, $Sp1_1$ and $Sp1_3$, do not bind proteins and are therefore non-functional. An M1 protein binding domain (-24 to -42 bp) is present and appears to be involved in tissue specific regulation of the $Sp1_4$ promoter, as well as a C-box binding

domain (-43 to -73), that responds to cAMP with decreased transcription in Leydig cells but has little effect on transcription in CHO cells (Nelson *et al.*, 1994). Within the C-box binding domain is an activator protein-2 (AP-2) consensus site (-52 to -59) which binds Leydig cell-specific protein(s) that repress transcription. These proteins were not recognised by an antibody to AP-2 (Nelson *et al.*, 1994), and while proteins from non-target cells (*i.e.* MSC-1 and CHO cells) did bind, they had a neutral effect (Tsai-Morris *et al.*, 1994). Tsai-Morris *et al.* (1994) demonstrated that the C-box binds another protein, which interacts with the protein that binds the M1 domain in Leydig cells, to increase transcription, but does not interact with the M1 binding protein in CHO cells. The protein that is involved in the Leydig cell interaction does not bind the AP-2 site (Tsai-Morris *et al.*, 1994).

The rat LH/CG receptor promoter also has regulatory elements upstream of the basal promoter, which responds with a decrease in transcription, to epidermal growth factor and phorbol esters, however these elements are not found in the proximal 1371 bp of the 5' flanking region of the LH/CG receptor (Wang *et al.*, 1992). However, Tsai-Morris *et al.* (1994) identified an upsteam inhibitory domain, the R domain (-1266 to -1307), which was initially identified as a CHO-specific transcription inhibitory region. AP-2 factors that can potentially be modulated by cAMP and phorbol esters (Roesler *et al.*, 1988; Park and Kim, 1993) bind both the R and AP-2 domains (Tsai-Morris *et al.*, 1994).

The two major *in vivo* LH/CG receptor transcriptional start sites (at -14 and -19), and a minor site (at -33; Tsai-Morris *et al.*, 1991) fall within a region that has a large degree of homolgy with the Inr sequence of the terminal deoxynucleotidyltransferase gene (Smale *et al.*, 1990; Tsai-Morris *et al.*, 1994). However, as the Inr domains of the rat LH/CG receptor do not bind proteins (Tsai-Morris *et al.*, 1993), and that it overlaps the M1 protein binding domain, its contribution to expression cannot be separated from the M1

domain and is therefore questionable. No exact matches for AP-1 sites or oestrogen response elements were observed in the basal promoter.

In the mouse, the majority of transcripts are initiated 310 bp upstream of the initiation of translation, as determined by primer extension. (Huhtaniemi *et al.*, 1992). As with the rat no consensus cAMP, AP2 or steroid receptor binding sites were observed, although the LH receptor is regulated by these substances.

There are three functional polyadenylation domains in the rat LH/CG receptor genes 3' untranslated region, H1 (nt 2368-2491) and H2 (nt 5579-5768) and a minor putative polyadenylation site at approximately nt 4400 (Lu *et al.*, 1994; Hu *et al.*, 1994). Functional nuclear and cytoplasmic polyadenylation sites are also located within introns three, four and ten (Koo *et al.*, 1994), which account for some of the LH/CG receptor mRNA heterogeneity (*see Section 1.4.9*).

1.4.5 Homology of the LH/CG Receptor with the FSH and TSH Receptors

There is a large degree of homology between the glycoprotein receptors. All three have the same basic structure; a large extracellular domain which binds the ligand, a transmembrane domain, and a short cytoplasmic domain. The transmembrane domain, containing the seven transmembrane helices and connecting loops, is the most conserved, with amino acid homology between 60-70%, while the homology of the extracellular and intracellular domains is only 20-40% (Segaloff and Ascoli, 1993).

All the cysteines of the transmembrane domain are conserved between glycoprotein receptors, although only eight of the twelve are conserved in the extracellular domain, and only the first one in the cytoplasmic domain. All three glycoprotein receptors contain leucine rich repeats in their extracellular domain, although the TSH receptor

contains an extra two copies. Only the N-linked glycosylation site at residue 173, is conserved between the three receptors, however two others, at residue 152 and 278, are conserved between the LH and TSH receptors.

The gene structure of the glycoprotein receptors are very similar, with the transmembrane and intracellular domains being encoded by the last exon. The large extracellular domain is mostly encoded by the first ten exons in the LH receptor and the first nine in the FSH-R and TSH-R. The amino acid sequence of the first nine exons show relatively high homology between species (LH-R vs. FSH-R 47.5%; LH-R vs. TSH-R 45.2%) and the corresponding exons are of remarkably similar size. In addition, they are all in intron phase two, the same as the LH/CG receptor. The major difference in the gene structure of the glycoprotein hormone receptors is the extra exon, exon 10, of the LH receptor. This exon has a low degree of homology to the FSH receptor though some homology can be seen with the TSH receptor. Alternative splicing of mRNA transcripts has also been reported in all of these genes (Loosfelt *et al.*, 1989; Segaloff *et al.*, 1990; Tsai-Morris *et al.*, 1990, Bernard *et al.*, 1990; Misrahi *et al.*, 1990; Aatsinki *et al.*, 1992; Gromoll *et al.*, 1992; Kelton *et al.*, 1992, Khan *et al.*, 1993; Bacich *et al.*, 1994 and Themmen *et al.*, 1994; *see Section 1.5.2*).

Although there is no structural relationship between the 5' untranslated regions of these genes (Koo *et al.*, 1991; Tsai-Morris *et al.*, 1991; Gross *et al.*, 1991; Heckert *et al.*, 1992; Huhtaniemi *et al.*, 1992), they all have multiple transcription start sites. They also lack TATA and CCAAT consensus sequences close to their transcription initiation sites (Huhtaniemi *et al.*, 1992). The LH/CG and FSH receptor genes are located on chromosome 2p21 in man (Rousseau-Merck *et al.*, 1990; Gromoll *et al.*, 1994), while the TSH receptor gene is on chromosome 14 (Libert *et al.*, 1990). In the sheep the LH

receptor and FSH receptor genes have been mapped to chromosome 3p 22-17, which is homologous to chromosome two in man (Broad *et al.*, 1995; Montgomery *et al.*, 1995).

1.4.6 Functional Properties of the LH/CG Receptor

1.4.6.1 G Protein Activation

The LH/CG receptor is a G protein associated receptor. Guanine nucleotide coupling proteins (G proteins) consist of three subunits (α, β, γ). In mammals there are at least 15 different α subunits of G proteins, ranging in size from 39 to 52 kDa, at least 3 β subunits of approximately 35 kDa and at least 3 γ chains of approximately 7.8 kDa. Many heterotrimers can be created, as many, but not all of each type of subunit can combine with more than one version of the other two (Bourne, 1993). Signalling functions of some of these heterotrimers have been identified, such as Gs, the G protein that mediates hormonal stimulation of adenylyl cyclase (Birnbaumer, 1990; Gilman, 1987; Birnbaumer *et al.*, 1987), and that may regulate Ca^{2+} channels (Yatani *et al.*, 1988; Scott and Dolphin, 1987).

Binding of LH to its receptor triggers a conformational change and opens up the nucleotide binding site of Gs. In its unactivated state, the Gs nucleotide-binding site binds guanosine diphosphate (GDP). However, once activated by hormone binding, the Gs nucleotide binding-site releases GDP and binds guanine triphosphate (GTP), the most abundant guanine nucleotide present in cells. The α subunit of Gs is then released into the surrounding membrane to activate adenylyl cyclase. GTPase activity of the free α subunit of Gs rapidly converts GTP to GDP, thus halting further adenylyl cyclase activity, and allowing the GDP-liganded α-subunit to reassociate into an inactive α_S, β and γ trimer. Agents which inhibit adenylate cyclase do so by activating receptors that interact

with another G protein, Gi, which counteracts the stimulatory effects of Gs on adenylyl cyclase (Michell, 1989; for review see Hunzicker-Dunn and Birnbaumer, 1985).

LH and hCG activation of the phospholipase C pathway suggests that LH/CG receptors may also activate another G protein Gp, although direct proof of this is still lacking (Gudermann *et al.*,1992).

1.4.6.2 The cAMP Pathway

Activation of adenylyl cyclase, converts Mg.ATP to cAMP. Cyclic AMP binds to the regulatory subunit of cAMP-dependent kinase A (Flockhart and Corbin, 1982; Nimmo and Cohen, 1982; for a reveiw see Hunzicker-Dunn and Birnbaumer, 1985). Protein kinase A is a tetrameric complex comprising of two regulatory subunits, which binds cAMP, and two catalytic subunits with phosphotransferase activity (Beebe *et al.*, 1989). The phosphotransferase activity of the catalytic subunits is supressed by cAMP, allowing phosphorylation of protein substrates, and inducing transcription of cAMP-regulated genes (Kurten and Richards, 1989; *see Figure 1.8*). Increased protein kinase activity can influence the function of the steroidogenic cell via several mechanisms *(summarised in Figure 1.9)*. These include the regulation of various genes and gene products (Irby and Hall, 1971; Janszen *et al.*, 1977; Jungmann and Hunzicker-Dunn, 1978), the activation by phosporylation of cholesterol esterase (Trzeciak and Boyd, 1974; Caffrey *et al.*, 1979), and possibly other proteins (Caron *et al.*, 1975; Cooke, *et al.*, 1977; Downing and Dimino, 1979; Neymark, *et al.*, 1984; Strauss *et al.*, 1988), and its stimulation of cholesterol transport into the inner mitochondrial membrane (Hall, 1985; Ghosh *et al.*, 1987; Wiltbank *et al.*, 1993). In addition it may stimulate the transport of pregnenolone out of the mitochondria, and the uptake of low-density lipoprotein (LDL), thus increasing cholesterol for substrate (Niswender, 1988).

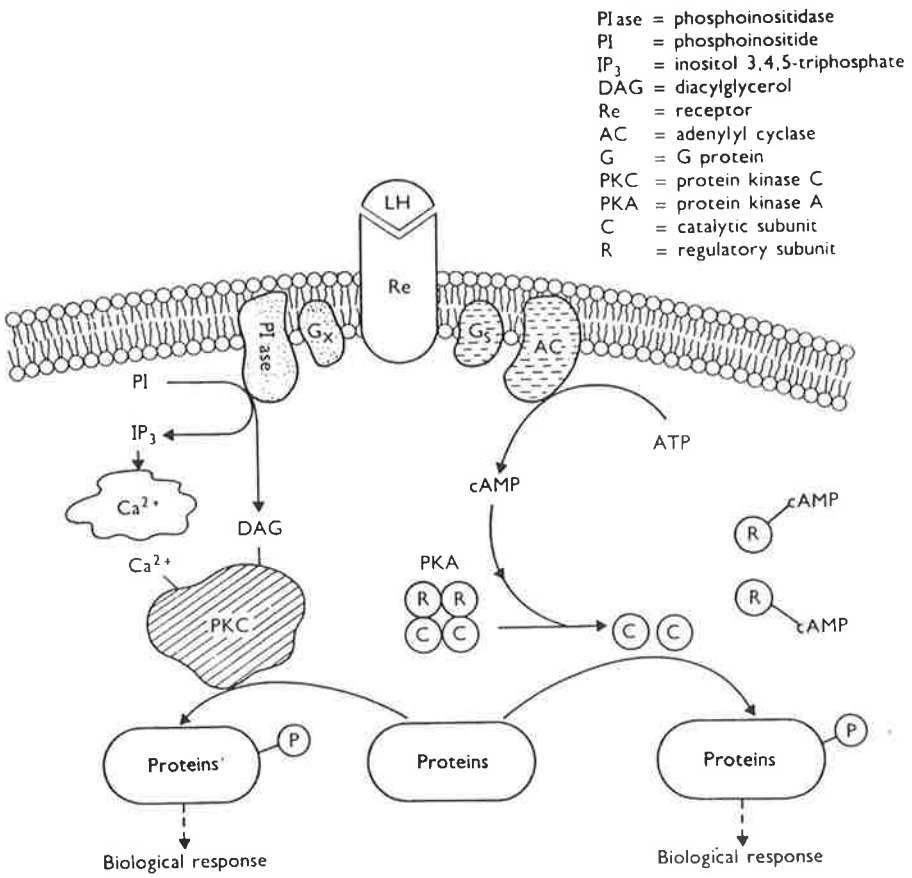

Figure 1.8 Second messenger systems activated by LH/hCG. Diagramatical representation of activation of both the cAMP and phospholipase C pathways

The cloning of the LH/CG receptor from a number of species, and its expression in transformed cell lines, demonstrated that binding of LH or hCG to its receptor, resulted in cAMP accumulation (McFarland *et al.*, 1989; Hipkin *et al.*, 1992; Gunderman *et al.*, 1992a; VuHai-LuuThi, *et al.*, 1992). However it has been sugested that cAMP is not an obligatory second messenger for luteinizing hormone activity (Theman *et al.*, 1985; Cooke, 1990), and there is strong evidence that their is another second messenger pathway of LH (Gunderman *et al.*, 1992a).

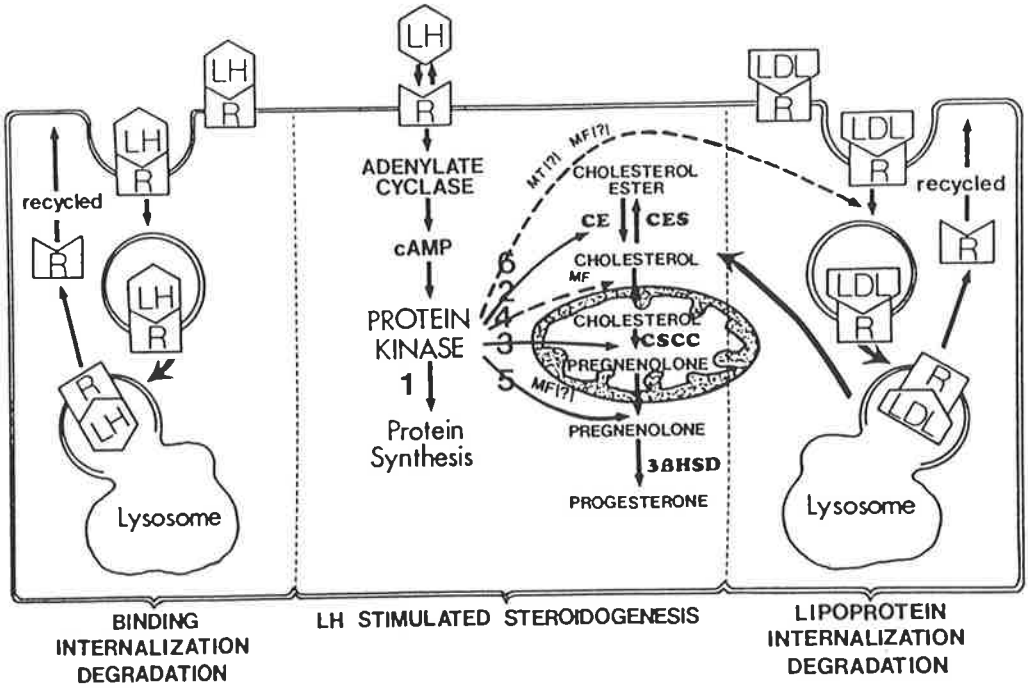

Figure 1.9 Intracellular events involved in LH/hCG stimulated steroidogenesis in luteal cells. The activated protein kinase A stimulates (1) the regulation of various genes and gene products (Irby and Hall, 1971; Janszen *et al.*, 1977; Jungmann and Hunzicker-Dunn, 1978), including a cholesterol-binding protein (Simpson *et al.*, 1978), and phosphorylation of ribosomes (Azhar and Menon, 1975), (2) the activation of cholesterol esterase by phosphorylation (Trzeciak and Boyd, 1974; Caffrey *et al.*, 1979), (3) the activation of the cholesterol side chain cleavage enzyme complex by phosphorylation of its cyctochrome P_{450} (Caron *et al.*, 1975; Downing and Dimino, 1979; Neymark *et al.*, 1984), (4) its stimulation of cholesterol transport into the mitochondria by microfilaments (Hall, 1985), (5) the phosphorylation of various proteins which correlate with steroidogenic responses to LH (Cooke, *et al.*, 1977; Neymark, *et al.*, 1984; Strauss *et al.*, 1988), (6) may stimulate the transport of pregnenolone out of the mitochondria, and/or (7) may stimulate the uptake of low-density lipoprotein (LDL), thus increasing cholesterol for substrate (Niswender, 1988). From Niswender and Nett, 1988.

Doubts about cAMP being the only second messenger, stems from observations that physiological levels of LH which cause at least 50% of maximum steroidogenesis in rat ovarian and Leydig cells, cause no detectable changes in intracellular cAMP levels (Moyle and Ratmachandran, 1973; Catt and Dufau, 1973; Rommerts *et al.*, 1973; Cooke

et al., 1976). It has been argued that this lack of change in cAMP levels maybe due to insensitivity of the assay, or localised increases in cAMP in a non-homogeneous cytoplasm, which is sufficient to activate protein kinase activity locally, amplifying the effects of LH without effecting the overall cAMP level. This argument is supported by findings that the amount of cyclic AMP bound to the regulatory subunit of protein kinase can increase, while no increases occur in overall cAMP within the cell (Dufau *et al.*, 1977; Dufau *et al.*, 1980).

In addition, deglycosylated LH and hCG has no or little effect on cAMP levels in ovarian or Leydig cells (Moyle *et al.*, 1975; Sairam and Schiller, 1979; Siaram, 1989), but can stimulate steroidogenesis (Platts *et al.*, 1988; Rose *et al.*, 1989). MA10 cells cannot be stimulated this way, however this may be due loss of some internal machinery in these Leydig tumor cells (Siaram, 1989). Another finding that suggests cAMP is not the only second messenger, is that a calmodium inhibitor, calmidazolium, at low doses (1-4 mM), reduces cAMP concentrations, while stimulating testosterone synthesis in Leydig cells (Choi, 1989; Cooke, 1990). Reports that LH stimulation of cyclic AMP production is affected by protein kinase C (Rose and Band, 1988), imply that the initial activation of adenylate cyclase may be dependent upon the activation of protein kinase C.

1.4.6.3 The Phospholipase C Pathway

LH and hCG also stimulate the inositol phospolipid/phosholipase C pathway, at least in a number of species and gonadal cell types (as summarised in Table 1.4 and Table 1.5). Activation of this pathway is measured by increases in inositol 1,4,5-triphosphate (IP3) and intracellular calcium (Davis *et al.*, 1987; Alila *et al.*, 1989).

Species	Cell type	Reference
Cow	Luteal cells	Davis *et al.*, 1981; Davis *et al.*, 1987 Allen *et al.*, 1988; Davis *et al.*, 1989a
Rat	Granulosa cells	Davis *et al.*, 1984; Davis *et al.*, 1986
Rat	Leydig cells	Lowitt *et al.*, 1982; Farese, 1984
Pig	Granulosa cells	Dimino *et al.*, 1987

Table1.4 Steroidogenic cell type and species that LH and hCG stimulate the inositol phospholipid/phospholipase C pathway.

Species	Cell type	Reference
Rat	Luteal cells	Schuler *et al.*, 1978; Lahav *et al.*, 1988
Human	Cultured granulosa-luteal cells	Davis *et al.*, 1989b
Mouse	MA-10 cells	Ascoli *et al.*, 1989

Table1.5 Steroidogenic cell types and species that LH and hCG do not stimulate the inositol phospholipid/phospholipase C pathway.

Gundermann *et al.* (1992a, 1992b) demonstrated conclusively that the LH receptor can directly activate the phospholipase C pathway, by expressing the murine LH-R in mouse L cells and xenopus oocytes. These cells bound hCG with high affinity, and in addition to stimulating cyclic AMP activity, increased the rate of phosphoinositide hydrolysis and intracellular Ca^{2+} levels independently of adenylyl cyclase activity, presumably activated through another G protein, independent of Gs. However, hCG resulted in lower maximal levels of inositol phosphate accumulation than induction by other phospholipase C pathway stimulators. Also, the level of hCG required to induce a half maximal accumulation of inositol phosphates was 20 to 100 times higher than those needed to induce adenylyl cyclase at half maximal levels. It has therefore been suggested that activation of this pathway occurs only during the preovulatory LH surge or pregnancy, as LH levels in the male and female at other times would be below its activation threshold if similar levels of LH were required for the activaction of the phospholipase C pathway *in*

vivo. The proposed effect of LH on the inositol phospholipid pathway is diagramatically represented in Figure 1.8.

Binding of the LH/CG receptor stimulates G-protein associated phospholipase C activity which converts phosphatidylinositol-4,5-bis phosphate (PIP_2) to inositol triphosphate (IP_3) and 1,2-diacyglyerol (DAG; Davis, 1991; Gudermann *et al.*, 1992a) . DAG has been shown to activate protein kinase C (PKC) (Nishizuka, 1989), of which multiple distinct forms exist (Coussens *et al.*, 1986; Nishizuka, 1989; Parker *et al.* 1989), while IP_3 stimulates intracelluar calcium mobilisation, resulting in raised intacellular levels of Ca^{2+} (Berridge and Irvine, 1989). Protein kinase C is a serine/theronine protein kinase, requiring Ca^{2+} and phospholipid for activity (Davis and Clark, 1983; Nishizuka, 1989; Wheeler and Veldhuis,1989). DAG and phorbol esters compete for the same binding site, resulting in activation of the enzyme at physiological Ca^{2+} levels.

PKC is involved in the regulation of phosphorylation and steroidogenesis in the ovine corpus luteum (Wiltbank *et al.*, 1989), with DAG and phorbol ester binding sites on both small and large ovine luteal cells. The effect of PKC activators appear to be dose, species, and cell dependent (Davis, 1991). In the bovine corpus luteum, phorbol esters stimulate progesterone synthesis in small luteal cells (Hansel and Dowd, 1986; Bruswig *et al.*, 1986; Benhaim *et al.*, 1987; Hansel *et al.*, 1987; Alila *et al.*, 1988; Benhaim *et al.*, 1990), but has no effects in large luteal cells (Hansel and Dowd, 1986; Hansel *et al.*, 1987; Benhaim *et al.*, 1990). However, in the rat and ovine corpus luteum, phorbol esters inhibit progesterone synthesis in large luteal cells and stimulated (LH , forskolin, or dBcAMP) small luteal cells (Baum and Rosberg,1987; Hoyer *et al.*, 1988; Wiltbank *et al.*, 1989). It is believed that this inhibition of progesterone synthesis results from inhibition of cholesterol esterase, or cholesterol transport to the side-chain cleavage

enzyme in ovine small luteal cells, and by inhibiting cholesterol side-chain cleavage activity in large luteal cells (Wiltbank *et al.*, 1989).

Recent cloning and expression of the TSH receptor, a calcitonin receptor, and an M2 muscarinic receptor, indicates that they are also able to activate adenylyl cyclase and phosphoinositide activity (Asmkenazi *et al.*, 1987; VanSande *et al.*, 1990; Chabre *et al.*, 1992). Other G protein coupled receptors have been reported to interact with more than one G protein, activating different effector systems (Fargin *et al.*, 1989; Cotecchia *et al.*, 1990; Vallar *et al.*, 1990).

1.4.7 Regulation of the Functional Properties and Numbers of LH/CG Receptors

Cells containing LH/CG receptors, undergo desensitisation in response to continual or repetitive stimulation with hCG or LH (Harwood *et al.*, 1978; Dufau and Catt, 1978; Catt *et al.*, 1979; Freeman and Ascoli, 1984). This involves the regulation of LH/CG receptors as well as the regulation of the activity of several key enzymes that are involved in steroid biosynthesis. Desensitisation of the LH receptor can occur by two independent mechanisms, uncoupling and down-regulation. Uncoupling refers to "an agonist induced change in the functional properties of the receptor without a change in the number of receptors" (Segaloff and Ascoli, 1993), while down regulation refers to "an actual reduction in the density of receptors". LH receptor down-regulation and uncoupling also occur in response to other receptors or second messenger pathways in gonadal cells (Ascoli, 1981; Rebois and Patel, 1985; Inoue and Rebois, 1989; Hafez and Ascoli, 1990).

1.4.7.1. Uncoupling of LH/CG receptors

Uncoupling of LH/CG receptors is homologous when responsiveness is only lost to LH or hCG and not other effectors, and heterologous when adenylyl cyclase activity becomes refractory to all effectors.

Heterologous Uncoupling of the LH/CG receptor

In some *in vivo* studies uncoupling of ovarian adenylyl cyclase appears heterologous and involved in reduction of Gs activity (Conti *et al.*, 1976; Harwood *et al.*, 1980; Kirchick *et al.*, 1983), as the maximal amount of steroid production is reduced by down-regulated cells when exposed to compounds that do not act through the LH/CG receptors, such as cholera toxin or cAMP analogs (Tsuruhara *et al.*,1977; Saez *et al.*, 1978; Cigorraga *et al.*, 1978; Dufau *et al.*, 1979; Nozu *et al.*,1981). In rat Leydig cells, uncoupling is largely due to an LH/hCG induced decrease in the activities of 17α-hydroxylase, C17-C20 lyase, and is mediated through oestrogens produced by the cells. Very high levels of hCG can cause early steroidogenic lesions (for review see Ascoli, 1985), which appear to involve reduced levels of intracellular cholesterol. However, the early lack of progesterone synthesis by down regulated rat granulosa cells is not due to either of these mechanisms (Azhar *et al.*, 1983; Ascoli, 1985). Other studies indicated limited heterologous uncoupling, as incubation of some gonadal cells with phorbol esters (Rebois and Patel, 1985; Inoue and Rebois, 1989) or epidermal growth factor (Hafez and Ascoli, 1990) also leads to uncoupling.

Homologous Uncoupling of the LH/CG receptor

Homologous uncoupling in gonadal tissues usually occurs within minutes to hours of addition of LH or hCG, without down-regulation of LH/CG receptors (Ekstrom and

Hunzicker-Dunn, 1989a; Segaloff and Ascoli, 1993). It is not mediated by cAMP, and appears to be due to a blockage in the interaction between the receptor and Gs proteins. In some *in vivo* studies significant receptor loss was also observed, although early time points were not sampled, and occupied receptors were not counted (Hunzicker-Dunn and Birnbaumer, 1976; Conti *et al.*, 1976; Kirchick *et al.*, 1983). *In vivo* and *in vitro* examples of homologous uncoupling of the LH/CG receptor include findings that FSH-, prostaglandin E_2-, and NaF-sensitive adenylyl cyclases are not impaired in various gonadal tissue and cell lines from different species, after administration of high doses of hCG (Lamprecht *et al.*, 1973; Zor *et al.*, 1976; Lamprecht *et al.*, 1977; Janhsen *et al.*, 1981; Hunzicker-Dunn, 1981; Dix *et al.*, 1982; Rebois and Fishman, 1984; Budnik and Mukhopadhyay, 1987; Ekstrom and Hunzicker-Dunn, 1989a).

LH/hCG mediated uncoupling of LH/CG receptors is concentration dependent; the concentration of hCG in the initial incubation must be above 0.1 nM for any uncoupling to occur and above 1 nM for full uncoupling to be observed (Rebois and Fishman, 1984), with adenylyl cyclase activity decreased by 30-50% in membranes from various gonadal cell types and species (Rebois and Fishman, 1984; Ekstrom and Hunzicker-Dunn, 1989a).

Uncoupling of the β-adrenergic receptor has been extensively studied, and it has been hypothesised by numerous investigators that LH receptor uncoupling occurs in a similar manner *i.e.* phosphorylation of the receptor (Dix, *et al.*, 1982; Bockaert, *et al.*, 1976; Salomon, *et al.*, 1981; Rebois and Patel, 1985). Uncoupling of the β-adrenergic receptor is catalysed by the cAMP-dependent (protein kinase A) and β2-adrenergic receptor kinases (reviewed in Dolhman *et al.*, 1991; Lefkowitz *et al.*, 1990; Palczewski and Benovic, 1991). The rat LH/CG receptor has been reported to be a substrate for protein kinase A-catalysed phosphorylation (Minegishi *et al.*, 1989), however the phosphorylation site is unknown, and two additional protein kinase A phosphorylation

consensus sequences exists in the extracellular domain. The intracellular regions of the rat, pig, mouse or human LH/CG receptor contain weak cAMP-dependent protein kinase-catalysed phosphorylation consensus sites, while the β-adrenergic receptor has two strong consensus sites in its intracellular regions. Interestingly, the vast majority of serine or threonine residues are not conserved between the β-adrenergic and LH/CG receptors, however two of the conserved sites correspond to the region that is phosphorylated by β-adrenergic receptor kinase (Lefkowitz *et al.*, 1990; Hausdorff *et al.*,1990). However, uncoupling of the LH receptor is not likely to involve phosphorylation by protein kinase A, as non LH/CG induced increases in cAMP do not uncouple the receptor (Lamprecht *et al.*, 1977; Hunzicker-Dunn and Birnbaumer, 1981; Rebois and Fishman, 1984).

Sanchez-Yagüe *et al.* (1992) demonstrated that the last 43 C-terminal amino acids are important in hCG induced uncoupling. These investigators compared membranes from human embryonic kidney 293 cells expressing rat wild type LH receptors *vs.* receptors lacking the terminal 43 residues and found that while the wild type lose up to half their LH/CG stimulated adenylyl cyclase activity, by ligand induced uncoupling (Sanchez-Yagüe *et al.*, 1992). While cells transformed with the truncated LH receptor, bound LH with high affinity, and uncoupled at a slower rate, with only a 20% reduction in adenylate cyclase activity. This region in the β-adrenergic receptor contains the serine/threonine residues that are phosphorylated by β-adrenergic receptor kinase (Hausdorff *et al.*, 1989; Lefkowitz *et al.*, 1990; Hausdorff *et al.*, 1990; Palczewski and Benovic, 1991; Dohlman *et al.*, 1991). This result is of increased significance, given that the mutant receptors were internalised at a faster rate (Rodriguez *et al.*, 1992), and therefore the loss of responsiveness to hCG at later times (*i.e.* after 30 minutes) may be due hCG-induced internalisation of the receptors.

The β-adrenergic receptor requires ATP for optimal uncoupling, while in the LH receptor some groups have reported that GTP is the nucleotide triphosphate required for optimal uncoupling (Ezra and Salomon, 1980; Ezra and Salomon, 1981; Ekstrom and Hunzicker-Dunn, 1989a; Ekstrom and Hunzicker-Dunn, 1989b). However, other groups have shown that in porcine ovarian membranes (Kuemmerle and Humzicker-Dunn, 1984), Leydig tumour cells and cell lines transformed with mutant LH receptor cDNA, ATP fulfills the nucleotide triphosphate requirement for optimal uncoupling (Sanchez-Yagüe *et al.*, 1993). These discrepant results are likely to be due to variations in experimental conditions, although species and/or cell differences may also play a role.

1.4.7.2. Up-Regulation of LH/CG Receptors

Up-regulation of LH/CG receptors has been reported mostly in follicles, though there are some reports of increases in luteal cells and neonatal Leydig cells. However, there are major differences in the mechanism and effect of LH receptor up regulation between cell type, species and cell lines. In the cell types of the ovary which contain LH receptors (granulosa from preovulatory follicle, theca, luteal cells and interstitial cells), both heterologous and homologous up and down-regulation of the LH receptors occurs.

In granulosa cells both oestradiol and FSH are required for LH receptor up-regulation, as neither can cause induction by itself in hypophysectomised immature rats (Segaloff *et al.*, 1990). Although FSH alone is required to induce LH receptors, and its mRNA in intact immature rats, this may be due to low levels of oestrogen in intact immature rat ovaries (Segaloff *et al.*, 1990). This results in both hCG binding sites and LH receptor mRNA levels being up-regulated, with an increase in concentration of all the LH receptor mRNA transcripts detectable by Northern analyses (Segaloff *et al.*, 1990; LaPolt *et al.*, 1990; Piquette *et al.*, 1991; Camp *et al.*, 1991). The up-regulation of rat LH receptor mRNA by FSH is attenuated by growth factors and GnRH and is summarised in Table 1.6.

Up-regulation studies have also been carried out on cultured porcine granulosa cells (see Table 1.6; Goxe *et al.*, 1992),and while there was a correlation between the amount of mRNA and receptor up-regulation, the amount varied significantly between inducers, which suggests different hormone-responsive elements, are probably involved (*see Section 1.4.4*), although some of these effects may be indirect. There also appears to be some post transcriptional regulation, or regulation by alternative splicing as the LH receptor/mRNA index changes between induction factors (Goxe *et al.*, 1992).

Cell Type	Factor	Effect	Reference
pig granulosa cells	0.6 nM FSH	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	5 ng/ml insulin	no effect	Goxe *et al.*, 1992
pig granulosa cells	5 μg/ml insulin	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	30 nM oestradiol	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	100 nM thyroxine	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	0.3 nM deoxycorticosterone	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	16 pM EGF	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	1 mM dbcAMP	↑ binding sites & RNA	Goxe *et al.*, 1992
pig granulosa cells	5.4 nM cortisol	no effect	Goxe *et al.*, 1992
rat granulosa cells	FSH+EGF	suppressed FSH effect	Piquette *et al.*, 1991
rat granulosa cells	FSH+ bFGF	suppressed FSH effect	Piquette *et al.*, 1991
rat granulosa cells	FSH+GnRH	suppressed FSH effect	Piquette *et al.*, 1991
sheep luteal cells	hCG	↑ in 1st 10 min then ↓	Niswender *et al.*, 1981
neonate rat testes	IGF-1	directly or indirectly[1]	Shan and Hardy, 1992
neonate rat testes	FGF	directly or indirectly[1]	Shan and Hardy, 1992
neonate rat testes	PRL	directly or indirectly[1]	Shan and Hardy, 1992
rat luteal cells	PRL	directly or indirectly[2]	Gafvels *et al.*, 1992

Table 1.6 Factors that up-regulate, or suppress up-regulation of LH receptors. [1]IGF-1, FGF and PRL may all stimulate LH receptor induction by increasing differentiation of the Leydig cells. [2] indicates may be due to either up regulation of the message or inhibition of luteolysis.

Studies on regulation of LH receptors on thecal cells are less common due to lack of purity and culture systems, and have generally been studied by *in situ* hybridisation (Camp *et al.*, 1991; Peng *et al.*, 1991). However, it appears that LH receptors and their corresponding mRNAs are also induced during follicular development (Segaloff *et al.*, 1990b; Camp *et al.*, 1991; Peng *et al.*, 1991). This increase is relatively small due to the higher levels in control cells (*i.e.* only a 2-3 fold increase; Segaloff *et al.*, 1990b; Camp *et al.*, 1991), while uninduced levels in theca are far greater than in uninduced granulosa cells. However, there is a large discrepancy of the relative levels of LH receptors between granulosa and theca cells, amongst various studies. Segaloff *et al.* (1990b) revealed thecal cells contain almost three times more LH receptor mRNA than granulosa cells in preovulatory cells, as determined by solution hybridisation, while Camp *et al.* (1991) demonstrated relatively equal amounts of LH receptor mRNA by *in situ* hybridisations, and Peng *et al.* (1991), showed mural granulosa cells contain significantly more LH receptor mRNA than theca cells both in medium and large follicles.

1.4.7.3. Down-Regulation of the LH Receptor

Down-regulation of LH/CG receptors by high concentrations of LH or hCG has been reported in follicular (Richard *et al.*, 1976; Rao *et al.*, 1977), luteal (Conti *et al.*,1976; Conti *et al.*, 1977a; Conti *et al.*, 1977b; Harwood *et al.*,1978; Dufau and Catt, 1978; Niswender, 1981) and testicular (Hseuh *et al.*, 1976; Hseuh *et al.*, 1977; Tsuruhara *et al.*,1977; Purvis *et al.*, 1977) tissue. However there are major differences in the mechanism and effect of LH receptor down-regulation between cell type, species and cell lines.

Down-Regulation of LH Receptors in Follicles

As mentioned earlier, a large dose of hCG or LH results in luteinisation of the preovulatory follicle. It also causes down-regulation of the LH/CG receptors in a time and dose-dependent manner (Richards *et al.*, 1976; Conti *et al.*, 1976; Richards and

Williams, 1976; Rao *et al.*, 1977, LaPolt *et al.*, 1990; Hu *et al.*, 1990; LaPolt *et al.*, 1991). The decreased receptor binding represents internalisation of receptors (Amsterdam *et al.*, 1979; Jaaskelainen *et al.*,1983), and/or postranslational receptor modification as well as a decrease in receptor mRNA synthesis (LaPolt *et al.*, 1990; Hu *et al.*, 1990; Segaloff *et al.*, 1990b; Hoffman *et al.*, 1991; Camp *et al.*, 1991; LaPolt *et al.*, 1991). In these studies it has been revealed that both hCG binding and LH/CG receptor mRNA declines, however depending on the study, significant decreases are not seen until 4 hours (Segaloff *et al.*, 1990b), 6 hours (LaPolt *et al.*, 1990), or 12 hours (LaPolt *et al.*, 1991). LaPolt *et al.* (1991) report a decline in the amount LH receptor mRNA preceding the decline in hCG binding, while LaPolt *et al.* (1990) report the opposite. These groups also report a far greater down-regulatory response of LH receptor mRNA, than the amount seen by Hu *et al.* (1990). Interestingly, all of the reports of LH receptor mRNA down-regulation in granulosa cells have demonstrated that the mRNA differs from Leydig cell RNA not only in size (*see Section 1.4.9*), but also in regulation, with all bands on Northern analyses from rat ovaries coordinately down-regulated (Hu *et al.*, 1990; LaPolt *et al.*, 1990; Segaloff *et al.*, 1990b; LaPolt *et al.*, 1991). When LH receptors are down regulated in Leydig cells the 1.8 Kb mRNA transcript is not coordinately regulated.

Previous studies by Schwall and Erikson (Schwall and Erikson, 1983a; Schwall and Erikson, 1983b) examined the *in vitro* down-regulation of LH/CG receptors in cultured rat granulosa cells, and concluded that it occurs in response to a increase in cAMP. *In vitro* studies have demonstrated that this down-regulation of LH/CG receptor mRNA is mimicked by forskolin (Segaloff *et al.*,1990b), confirming that down-regulation is caused, at least in part, by increases in cAMP (Segaloff *et al.*,1990b). Thus it appears that low concentrations of cAMP plus oestradiol up-regulate LH/CG receptor mRNA, while high concentrations of cAMP down-regulate LH receptor mRNA levels. The high correlation between receptor numbers and receptor mRNA levels confirms that this is a major regulating mechanism of up- and down-regulation in granulosa cells.

In thecal cells, down-regulation of LH receptor mRNA by ovulatory doses of LH or hCG is also not as great as that which occurs in granulosa cells (Camp *et al.*, 1991; Peng *et al.*, 1991), declining to levels approximately a third of untreated cells (Peng *et al.*, 1991). Interstitial cells also seem to act in a similar manner, and with similar concentrations of LH receptor mRNA to thecal cells (Peng *et al.*, 1991). Interestingly, Milgrom and associates reported zonation of the theca interna of porcine ovaries by immunohistochemistry for luteinizing hormone receptors (Meduri *et al.*, 1992). However the lack of LH binding to the innermost third of the theca interna (thecal cells closest to the basal lamina, maybe due to the high vascularisation of this region in porcine ovaries (King *et al.*, 1991), and not to a unique subset of thecal cells, which lack LH receptors.

Down-Regulation of LH Receptors in Luteal cells

After an ovulatory dose of hCG or LH, preovulatory follicles in rats ovulate, and start to form the new corpus luteum as discussed previously. During this formation an increase in LH/CG receptors and its mRNA occurs, in the luteal cells as well as a slight increase in interstitial cells (McFarland *et al.*, 1989; LaPolt *et al.*, 1990; Hu *et al.*, 1990; Nakamura *et al.*, 1990; Segaloff *et al.*, 1990b; Hoffman *et al.*, 1991; Camp *et al.*, 1991; LaPolt *et al.*, 1991). LH/CG receptor mRNA increases significantly in the first 1-4 days, reaching their maximum levels between days 7-15 in the rat. Luteolysis begins by day 19-23, and a decrease in both LH receptors and LH-R mRNA occurs (Segaloff *et al.*, 1990b).

Up-regulation of LH receptors occurs in ovine luteal cells after a single dose of oLH (Suter *et al.*, 1980). In ovine luteal cells, hCG significantly increases the number of LH receptors in the first ten minutes, however receptor levels return to control levels within 2 hours, and then decrease by 66% after 12 hours, returning to control levels within 48 hours (Niswender *et al.*, 1981).

The major difference between the data from ewes and rats is that in the ewe LH receptors are initially up regulated, and that although there is close agreement of the number of receptors occupied and lost, unoccupied receptors are still lost after circulating hCG has disappeared in sheep, while rats appear to lose a far greater amount of receptors than the number occupied (Conti *et al.*, 1977a). This suggests that homologous receptor down-regulation in ovine luteal cells is due to internalisation and degradation. However, the rate of receptor-hormone internalisation varies depending on the ligand bound, *i.e.* internalised receptor-hCG has a half life of 23 hours and receptor-oLH a half life of 0.4 hours (Mock and Neiswender, 1983).

Lakkakorpi *et al.* (1993), reported that a supraphysiological dose of hCG to primed pseudopregnant rats resulted in loss of hCG binding sites within 12 hours, followed by a partial recovery of binding by days 4 and 5. LH receptor mRNA transcripts were down-regulated within 12 hours of hCG treatment, with levels increasing on day 2 and a significant increase seen on days three and four (Lakkakorpi *et al.*, 1993; Lu *et al.*, 1993). The proportion of the smaller mRNA bands (2.8, 2.0, 1.4, and 1.1 kb) in relation to the 4.2 kb band increased during down regulation, and decreased during the up-regulation, although these differences were minor in comparison to the magnitude of the overall up- and down-regulation of the transcripts (Lakkakorpi *et al.*, 1993). It has been suggested that these different sized bands may be due to alternative splicing, *(see Section 1.5.2).* Lu *et al.* (1993), recently demonstrated that the down-regulation of LH receptor mRNA is not due to a decrease in its transcription, but due to an increase in is degradation *i.e.* a decrease in the mRNAs half life. Thus it appears that regulation of LH receptor mRNA plays an important role in up- and down-regulation of LH receptors, in addition to receptor internalisation (Conn *et al.*, 1978), and proteolysis of receptors (Kellokumpo, 1987).

Down-Regulation in Leydig Cells and Leydig Tumor Cells

Adult Leydig cells are not an ideal model for long term studies, as they lose their LH receptors after a few days in culture (Saez *et al.*, 1983; Dufau, 1988), and hence Leydig cell tumour lines, such as MA-10, MLTC-1 and R2C cells, are often used to study down-regulation. However, while they do share many similarities with Leydig cells they also behave differently in other ways. Down-regulation occurs in a time and concentration dependent manner, without a change in the affinity for the receptor (Freeman and Ascoli, 1981; Rebois and Fishman, 1983; Rebois and Fishman, 1984). In adult rat Leydig cells, low doses of LH up-regulate receptors and steroidogenic enzymes, while higher doses down-regulate receptors *in vivo* (Dufau and Catt, 1978), and *in vitro* (Nozu *et al.*, 1981). Down-regulation of neonatal and fetal rat testis LH/CG receptors varies from the adult, and are refractory to the uncoupling process (Huhtaniemi *et al.*, 1984; Warren, *et al.*, 1982; Tsai-Morris *et al.*, 1986; Tsuruhara *et al.*, 1977).

Homologous down-regulation of LH/CG receptors in neonatal rat testes, results in a significant decrease in receptor numbers by day one after hCG administration, but the receptor numbers quickly return to control levels by day three (Pakarinen *et al.*, 1990). Interestingly there is no effect on LH receptor mRNA levels within the first 24 hours, though levels increase five fold, after two days (Pakarinen *et al.*, 1990). When rat Leydig cells are separated by their stage of differentiation, adult-like Leydig cells have more LH receptor mRNA than progenitor Leydig cells, while immature Leydig cells have by far the most, suggesting that either these cells translate LH receptor mRNA more effectively, or the half life of the LH receptor is significantly greater in adult Leydig cells (Shan and Hardy, 1992). This is a surprising finding given that there is 10 fold more LH receptor mRNA in adult testes (Pakarinen *et al.*, 1990).

Administration of a supraphysiological dose of hCG induces adult rat Leydig cells, like sheep luteal cells, to initially increase the number of hCG binding sites due to presynthesised receptors moving to the surface (Dufau, 1988). LH receptors were then down-regulated, in a dose dependent manner,with the number of unoccupied receptors dropping to less than 10% of control levelon the first day, and to undetectable levels on days two and three, although only a 50% decrease in LH receptor mRNA levels was observed after day one (Pakarinen, 1990). However, LaPolt *et al.* (1991) reported hCG administration resulted in a 94% decrease in LH receptor mRNA 24 hours after hCG administration, with the decline in mRNA preceeding the decline in receptor binding and no significant loss of either LH receptors or LH receptor mRNA seen within the first 6 hours. The down-regulation of LH receptor mRNA was not coordinately regulated, with the 1.8 Kb transcript unaffected by down-regulation (LaPolt *et al.*, 1991).

A major difference between MA-10 cells and normal Leydig cells is the amount of occupied receptors required for maximal steroid synthesis; 60-70% in MA-10 cells in comparison to less than 1% in adult rat Leydig cells (Catt and Dufau, 1973; Mendelson *et al.*, 1975; reviewed in Ascoli, 1985)). This lack of excess receptors in MA-10 cells means that the loss of receptors by down-regulation is a far more important determinant in the ability of hCG to stimulate progesterone biosynthesis, than in normal cells (Freedman and Ascoli,1981; Freedman and Ascoli, 1984; Ascoli, 1985).

In MA-10 and MLTC-1 cells down-regulation occurs in two distinct phases. In the first phase, LH receptors are rapidly lost, with saturating concentrations of hCG decreasing LH receptor numbers to half maximal levels within one hour, and by at least 80% by 4 hours (Freeman and Ascoli, 1981, Wang *et al.*, 1991; Rebois and Fishman, 1984). This initial loss is due to loss of occupied receptors, and is inhibited by cyclohexamide both in MLTC-1 cells and rat leydig cells *in vivo* (Dix and Cooke, 1981; Saez *et al.*, 1978;

Rebois and Fishman, 1984) and appears due to cAMP dependent proteolytic cleavage, and results in the release of the extracellular domain (West and Cooke, 1991). Paradoxically, inhibition of LH receptor truncation with protease inhibitors, also inhibits cAMP production in mouse, but not rat, Leydig cells (West *et al.*, 1991). Interestingly, Kellokumpo and associates, demonstrated that rat luteal cells undergo proteolysis of their LH receptors on the cells surface, which releases two distinct receptor-^{125}I-hCG fragment complexes with mol wt of 96000 and 74000 daltons (Kellokumpo and Rajaniemi, 1985a; Kellokumpo and Rajaniemi, 1985b; Kellokumpo, 1987).

The second stage of LH receptor down-regulation appears to involve internalisation of receptor/hormone complexes and degradation (Conn *et al.*, 1978; Freeman and Ascoli, 1981; Ascoli, 1982; Ascoli, 1984; Rebois and Fishman, 1984; Bernier *et al.*, 1986; Ascoli and Segaloff, 1987), as well as a decrease of LH/CG receptor mRNAs (Cooke *et al.*, 1992; Wang *et al.*, 1991a; Wang *et al.*, 1991c). During the first 4 hours of down-regulation in MA-10 cells there is no significant loss of LH receptor mRNA (Wang *et al.*, 1991a). However in the next 4 to 8 hours LH receptor mRNA levels decrease to 40-60% of the initial level, with binding sites decreasing by another 10% to 5-10% of control levels (Wang *et al.*, 1991a). This second phase of down-regulation in MA-10 and MLTC-1 cells has been shown to be cAMP dependent, and is mimicked by cAMP analogs. Interestingly, deglycosylated hCG, which has a reduced ability to activate adenylcyclase, does not decrease the levels of LH/CG mRNA in MA-10 cells (Wang *et al.*, 1991c). Down-regulation of MA-10 cell LH receptor mRNA reduces the intensity of all the bands on Northern blots coordinately.

LH/CG receptor mRNA of MA-10 cells is also down-regulated by epidermal growth factor (EGF) or second messenger analogs such as phorbol myristate acetate (PMA) (Ascoli, 1981; Lloyd and Ascoli, 1983; Rebois and Patel, 1985; Wang *et al.*, 1991b), with

a similar time course to cAMP analogs *i.e.* a six hour lag time, before reduction of LH receptor mRNA levels. This reduction declines slowly to about 20-40% of initial levels within 40 hours, followed by receptor numbers decreasing to a similar extent (Wang *et al.*, 1991a; Wang *et al.*, 1991b). The reduction in mRNA is due to a reduction in transcription, and not a reduction in the mRNA half life. In fact, it seemed to increase its half life (Sanchez-Yagüe *et al.*, 1992; Nelson and Ascoli, 1992). The regulatory sequences for EGF and PMA are different from cAMP and are not found in the first 1370 bases upstream of the translation initiation site (Wang *et al.*, 1992; Wang *et al.*, 1991a).

Luteinizing hormone receptors on pig Leydig cells, have been reportedly down-regulated by 40% after only one hour after incubation with hCG (Ghinea *et al.*, 1992). Using antibodies which do not interfere with ligand binding, or induce receptor down-regulation, homologous down-regulation was studied. The LH receptor clustered in the coated pits at high concentrations of hCG at a faster rate, resulting in an eleven fold increase in the rate of internalisation (Ghinea *et al.*, 1992). Internalisation signals have been determined in several receptors (for review see Trowbridge, 1991), and in most cases involve a tyrosine residue located in a tight turn environment (Ktistakis *et al.*, 1990). This type of structure is conserved around tyrosine 632 (pig LH receptor) in pig, rat and human LH receptors. The pig does not appear to recycle its LH receptors to the Leydig cells surface (Ghinea *et al.*, 1992).

West and Cooke recently reported that treatment of MA-10 cells with antisense oligonucleotides to regions corresponding to the third extracellular loop, the intracellular loop or part of the C-terminal end of the LH receptor prevented down-regulation of hCG binding sites (West and Cooke, 1991b). The actual mechanism of how these antisense oligonucleotides work is unknown. It is possible that they act as a stop codon thus truncating the receptor at the point the antisense oligonucleotide binds. Alternatively,

LH/CG receptor mRNAs lacking the regions complementary to the antisense oligonucleotides may already exist due to alternative splicing. Thus when the antisense oligonucleotides are added they may prevent the synthesis of the full length LH receptor and thus allow only the translation of the mRNAs for the truncated receptors (West and Cooke, 1991b; Cooke *et al.*, 1992).

1.4.8 Structural Functions of the LH/CG Receptor

There are 14 imperfectly repeated sequences of about 25 amino acids known as "leucine rich repeats" (McFarland *et al.*, 1989; Braun *et al.*, 1991) in the LH-R extracellular domain and are found at roughly two copies per exon in exons 2-8 (Segaloff and Ascoli, 1993). These repeats are also found in many proteins from different species, including the collagen binding proteoglycan PG40, human lucine-rich α2 glycoprotein (Takahashi *et al.*, 1985), α and β chains of the platelet glycoprotein IB (Lopez *et al.*, 1987; Titani *et al.*, 1987; Lopez *et al.*, 1988), and adenylyl cyclase from *Saccharomyces cerevisiae* (see Leong *et al.*, 1992 for a more complete list). The function of these leucine-rich repeats is unknown, though it may be involved in protein-protein interactions and cell-cell or cell-matrix adhesion by interacting between hydrophobic and hydophilic surfaces (Roth, 1991; Krantz *et al.*, 1991). Subsequently, they may be involved in interaction of the extracellular domain of the LH receptor with its transmembrane domain (Segaloff and Ascoli, 1993). The extracellular domain also contains a site homologous to part of the soybean lectin (residues 244-255 in the rat), though not the soybean lectin binding domain. The function of this site is unknown.

The transmembrane domain of the LH /CG receptor spans the plasma membrane seven times, a classic feature of G protein associated receptors. It contains a weak cAMP-

dependent protein kinase phosphorylation consensus site, and two weak and one strong protein kinase C phosphorylation consensus sequences sites *(see Figure 1.10)*.

The intracellular domain of the LH/CG receptor consists of 68 amino acids in the rat and 70 amino acids in the pig, with 22 potential phosporylation sites (serine, threonine, and tyrosine residues) through which cellular regulation of the receptor may occur (Sibley *et al.*, 1988). The one of the first two cysteines of the intracellular domain Cys_{621} and Cys_{622} which are conserved between species in the LH/CG receptor, is also conserved at comparative positions in all G protein associated receptors (Segaloff and Ascoli, 1993). In rodopsin and human β_2-andrenergic receptors either both or just one of the cysteine residues resepectively are palmitoylated (Ovchinnikov *et al.*,1988; O'Dowd *et al.*, 1989). The intracellular domain also contains four weak consensus sequences for C-kinase catalysed phosphorolation and a weak consensus site for cAMP-dependent protein kinase-catalysed phosphorylation (*see Figure 1.10*; Segaloff and Ascoli, 1993).

A. Hormone binding

1. Amino Acid Determinants

The LH/CG receptor, and the other glycoprotein hormone receptors contain a large extracellular domain and bind large glycoprotein hormones, in contrast with the other G protein associated receptors which have a relatively small extracellular domain and bind small ligands, interacting with amino acids in the transmembrane helices (reviewed in Dohlman *et al.*, 1991; Savarse and Fraser, 1992). It has been proposed that the large extracellular domain would be responsible for high affinity binding of the glycoprotein hormones. Evidence to confirm this hypothesis come from two main approaches; (1) *in vitro* expression systems which have been transfected with LH receptor cDNA lacking

various regions of the gene, and (2) the use of overlapping peptides of the LH receptor to determine which regions are important in LH or hCG binding.

Numerous groups have expressed parts of the rat LH/CG receptor in transfected cell lines, resulting in proteins capable of binding LH and hCG with high affinity (see Table 1.7) Based upon which part of LH/CG receptor was expressed, and its binding affinity (Kd) for hCG relative to the full length receptor, the regions that are important for high affinity binding have been determined. It was demonstrated that the extracellular domain (the first 341 amino acids) can bind hCG with the same affinity as the full length receptor by a number of groups (Tsai-Morris *et al.*, 1990; Xie *et al.*, 1990; Moyle *et al.*, 1991; Ji and Ji, 1991a). Braun *et al.* (1991) demonstrated that the majority of the high affinity binding for hCG is found in the first 146 aminio acids (see Table 1.7). Some of the amino acids between 273 and 294 appear to be important in binding (see Table 1.7; Tsai-Morris *et al.*, 1990; Braun *et al.*, 1991), and maybe some residues between 6 to 33 (see Table 1.7; Ji and Ji, 1991a; Ji and Ji, 1991b). Segaloff and Ascoli (1993) demonstrated the region homologous to part of the soybean lectin binding domain (residues 244-255) is not important for high affinity binding (see Table 1.7). Whether it is involved in adenylyl cyclase activity is still unknown, as it was not expressed on the cell surface.

Interestingly, when forms encoding exon 1 and exon 11 or forms with most of the extracellular domain deleted were expressed, they recognised hCG with a low affinity but stimulated cAMP production (see Table 1.7; Ji and Ji, 1991a; Ji and Ji, 1991b). These were membrane bound, and suggest a second low affinity hormone binding site, possibly in the interconnecting loops of the transmembrane region (see Table 1.7; Ji and Ji, 1991a; Ji and Ji, 1991b). However, it is possible that the low binding affinity is a residual of the first 32 or 5 residues, given that these are within the area that binds hCG with high affinity.

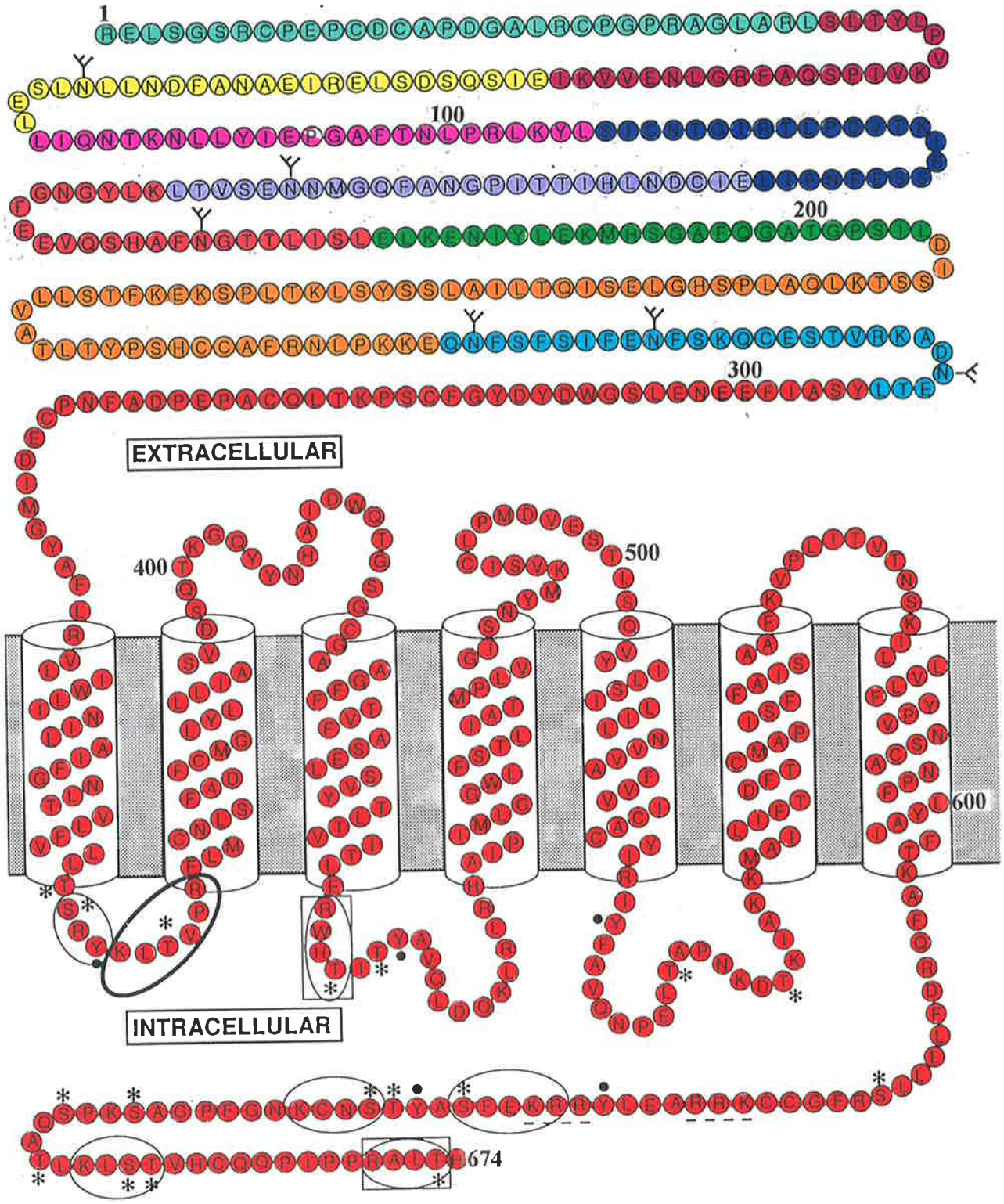

Figure 1.10 Amino acid sequence, orientation and supposed topology of the rat LH/CG receptor in the plasma membrane. The deduced amino acid sequence is taken from McFarland *et al.*, 1989. The overall orientation was deduced using site specific antibodies as described in Rodriguez and Segaloff (1990). The topology of the transmembrane regions is proposed based upon homology with other G protein coupled receptors. Potential sites for N-linked glycosylation are shown by the branch-like structure. The sequences underlined with dashes in the cytoplasmic tail indicate two possible tryptic cleavage sites. Potential intracellular sites for phosphorylation are denoted by asterisks (serine and threonine residues) or dark dots (tyrosine). The rectangles denote weak consensus sequences for cAMP-dependent protein kinase-catalysed phosphorylation. The light and heavy ovals denote weak and strong consensus sequences for C kinase-catalysed phosphorylation respectively. The different colors correspond to amino acids encoded by individual exons. Figure taken from Segaloff and Ascoli, 1993.

Receptor	Cell Type	Kd (nM)	Location	References
rLH-R (splice form A)	see footnote	0.18-0.52	cell surface & intracellular	see footnote
rLH-R (-26➡81) (splice form I-3)	293 cells	25	intracellular	Koo *et al.*, 1994
rLH-R (-26➡106) (splice form I-4)	293 cells	30	intracellular	Koo *et al.*, 1994
rLH-R (-26➡206)	293 cells	0.72	intracellular	Braun *et al.*, 1991
rLH-R (-26➡273)	293 cells	0.71	intracellular	Braun *et al.*, 1991
rLH-R (-26➡294+1) (splice form I-10)	293 cells	0.24	intracellular	Koo *et al.*, 1994
rLH-R (-26➡294+22) (splice form B)	COS 1	0.28	secreted	Tsai-Morris *et al.*, 1990
rLH-R (-26➡294+22) (splice form B)	293 cells	0.17	intracellular	Koo *et al.*, 1994
rLH-R (-26➡294+22) (splice form B)	COS 1 Sf9 cells	0.28	intracellular intracellular	Zhang *et al.*, 1995
rLH-R (-26➡294) exons 1-10	COS 7A	0.49	detergent extract	Ji and Ji, 1991a
rLH-R (-26➡310)	293 cells	0.50	intracellular	Braun *et al.*, 1991
rLH-R (-26➡338)	293 cells 293 cells	0.22 0.52	intracellular intracellular	Xie *et al.*, 1990; Braun *et al.*, 1991
rLH-R (-26➡354+ n*Lys) (splice form E-11B)	293 cells	0.16	intracellular	Koo *et al.*, 1994
rLH-R (-26➡341 aa)	COS 7	0.25	intracellular	Moyle *et al.*, 1991
rLH-R (-26➡616 aa)	293 cells	0.27	intracellular	Rodriguez *et al.*, 1992
rLH-R (-26➡631 aa)	293 cells	0.53-0.58	cell surface	Rodriguez *et al.*, 1992; Sanchéz-Yagüe *et al.*, 1992
rLH-R (-26➡653 aa)	293 cells	0.44	cell surface	Rodriguez *et al.*, 1992
rLH-R (Δ6-297)	COS 7A	1068	cell surface	Ji and Ji, 1991b
rLH-R (Δ6-336)	COS 7A	1036	cell surface	Ji and Ji, 1991b
rLH-R (Δ33-294) exons 1 & 11	COS 7A	651	cell surface	Ji and Ji, 1991a
rLH-R (Δ244-255)	?	0.22	intracellular	Segaloff and Ascoli, 1993
rLH-R (Δ404-409)	?	0.16	intracellular ?	Segaloff and Ascoli, 1993
rLH-R (Δ410-414)	?	0.15	intracellular ?	Segaloff and Ascoli, 1993
rLH-R (Δ493-496)	?	0.36	intracellular ?	Segaloff and Ascoli, 1993
rLH-R (Δ576-578)	?	0.24	intracellular ?	Segaloff and Ascoli, 1993

rLH-R (-26➡146 aa) + rFSH-R(148➡C aa)	293 cells	0.62	cell surface	Braun *et al.*, 1991
rLH-R (-26➡40 aa) + rFSH-R(348➡C aa)	293 cells	0.52	cell surface	Braun *et al.*, 1991
rLH-R (-26➡341 aa) + hβ_2AR(29➡C aa)	COS 7	0.28	intracellular	Moyle *et al.*, 1991
rFSH-R(-26➡257 aa) + rLH-R (257➡C aa)	293 cells	none detected	cell surface	Braun *et al.*, 1991
hLH/CG-R splice form A	CHO	0.14	cell surface	Igarashi *et al.*, 1994
rLH-R (Asp^{383}➡Asn)	COS 7A	120	cell surface	Ji and Ji, 1991c
rLH-R (Asp^{383}➡Asn)	293 cells?	0.53	cell surface	Quintina *et al.*, 1993
rLH-R (Asp^{410}➡Asn)	COS 7A	0.42	cell surface	Ji and Ji, 1991c
rLH-R (Glu^{429}➡Gln)	COS 7A	0.51	cell surface	Ji and Ji, 1991c
rLH-R (Glu^{441}➡Asp)	293 cells	0.20	74% cell surface	Wang *et al.*, 1993a
rLH-R (Glu^{441}➡Gln)	293 cells	0.13	11% cell surface	Wang *et al.*, 1993a
rLH-R (Cys^{8}➡Ser)	COS-1	ND	ND	Zhang *et al.*, 1996
rLH-R (Cys^{12}➡Ser)	COS-1	ND	ND	Zhang *et al.*, 1996
rLH-R (Cys^{14}➡Ser)	COS-1	ND	ND	Zhang *et al.*, 1996
rLH-R (Cys^{22}➡Ser)	COS-1	ND	ND	Zhang *et al.*, 1996
rLH-R (Cys^{109}➡Ser)	COS-1	*24 & 21*[a]	cell surface	Zhang *et al.*, 1996
rLH-R (Cys^{134}➡Ser)	COS-1	*22 & 26*[a]	cell surface	Zhang *et al.*, 1996
rLH-R (Cys^{257}➡Ser)	COS-1	*ND & 77*[a]	intracellular	Zhang *et al.*, 1996
rLH-R (Cys^{258}➡Ser)	COS-1	*ND & 101*[a]	intracellular	Zhang *et al.*, 1996
rLH-R (Cys^{282}➡Ser)	COS-1	*50 & 96*[a]	cell surface	Zhang *et al.*, 1996
rLH-R (Cys^{314}➡Ser)	COS-1	*95 & 98*[a]	cell surface	Zhang *et al.*, 1996
rLH-R (Cys^{321}➡Ser)	COS-1	*ND & 75*[a]	intracellular	Zhang *et al.*, 1996
rLH-R (Cys^{331}➡Ser)	COS-1	*ND & 89*[a]	intracellular	Zhang *et al.*, 1996
rLH-R (Cys^{417}➡Ser)	COS-1	*ND & 97*[a]	intracellular	Zhang *et al.*, 1996
rLH-R (Cys^{492}➡Ser)	COS-1	*ND & 4.1*[a]	intracellular	Zhang *et al.*, 1996
mLH-R Splice form A	L cells	0.15	cell surface	Gudermann *et al.*, 1992a
pLH-R Splice form A	COS-7 L cells	~ 0.71	65% cell surface 26% cytosolic	Loosfelt *et al.*, 1989; VuHai-LuuThi *et al.*, 1992
pLH-R (-27➡289)+13 Splice form B	COS-7	N.R.	43% secreted 55% cytosolic	VuHai-LuuThi *et al.*, 1992
pLH-R (-27➡289)+15 Splice form C	COS-7	~ 0.77	58% secreted 40% cytosolic	VuHai-LuuThi *et al.*, 1992
pLH-R (Δ290-601) Splice form D	COS-7	~ 0.71	33% secreted 65% cytosolic	VuHai-LuuThi *et al.*, 1992

pLH-R Splice form A	COS	0.32	cell surface	Remy *et al.*, 1993
pLH-R (-27➡270)	COS	N.S.	intracellular	Remy *et al.*, 1993
pLH-R (sp+271➡669)	COS	~3.5	cell surface	Remy *et al.*, 1993
pLH-R (-27➡270) + pLH-R (271➡669)	COS	0.37	~30% cell surface	Remy *et al.*, 1993
rLH-R Splice form A	293 cells	hCG 0.56 hLH 0.24 oLH 10 & 370	cell surface	Thomas and Segaloff, 1994
rLH-R (-26➡338)	293 cells	hCG 0.51 hLH 0.20 oLH 57	intracellular	Thomas and Segaloff, 1994

Table 1.7. hCG binding affinity and cellular location of several LH/CG receptor truncations, deleations alternative splice forms and chimeras. Truncations have the resides present in the brackets. Deletions are denoted by a Δ followed by the residue numbers that were deleted. Alternative splice forms are denoted as the above, however many alternative splice forms contain a 3' unique tail indicated by + the number of unique residues. Chimeras are denoted by the receptor name and the amino acid residues that it contributes to the chimera. Numbering of amino acids begins with the methionine which intiates translations as 1. [a] refers to percentage binding activity of these cells where the wildtype receptor binding is equal to 100% (the first number is the binding on intact cells and the second value is binding in cell homogenate). * indicates the low and high values of all determinations in the rat references cited in the table. Based on a table in Segaloff and Ascoli, 1993.

Roche *et al.* (1992), determined which regions of the LH/CG receptor are involved in hormone binding, by synthesising a series of overlapping peptides that replicate the entire extracellular domain as well as peptides derived from the three extracellular connecting loops. These peptides were then tested for their ability to compete for binding of labelled hCG to rat luteal membranes. Four potential hormone contact sites were identified, three in the extracellular domain (residues 21-38, 102-115, and 253-272) and one in the third extracellular loop (residues 573-583). Their potencies to inhibit hCG binding (EC_{50}) were 9.67±2.06 mM, 40.1±8.46 mM, 39.8±4.52 mM, and 232±45.9 mM respectively. These findings are in general agreement with the expression studies, and the inhibition of hCG binding by peptides to the third extracellular loop presents further evidence for a

second low affinity binding site as suggested by Ji and co-workers (Ji and Ji, 1991a; Ji and Ji, 1991b).

A number of sites within the transmembrane domain, which are highly conserved between the G-protein coupled receptors were mutated to determine their effect on hCG binding and hCG induced cAMP production. Ji and Ji (1991c) expressed forms of the LH receptor in which transmembrane residues 383, 410, 429 or an intracellular residue 556 were mutated. They reported this had no effect on the binding affinity of these forms for hCG or their ability to induce cAMP by hCG, except for the mutation to residue 383 (aspartic acid→asparagine which dramatically decreased its hCG binding affinity (Kd of 120 nM). This result could not be repeated by Quintana *et al.* (1993), who demonstrated that its hCG affinity and maximal hCG binding was comparable to the wild type receptors, and is not surprising given that forms completely lacking this region still bind hCG with high affinity (Tsai-Morris *et al.*, 1990; Xie *et al.*, 1990).

Recently, Zhang *et al.* (1996) reported that replacement of any of the cysteine residues with serine residues in exon one (*i.e.* Cys^{8Ser}, Cys^{12Ser}, Cys^{14Ser} or Cys^{22Ser}) abolishes hormone binding activity (*see Figure 1.10* and Table 1.7). Replacement of Cys^{109Ser} and Cys^{134Ser} from exons 5 and 6 causes a 75% decrease in binding activity (see Table 1.7). However, replacement of Cys^{257Ser} or Cys^{258Ser} (exon 9), Cys^{321Ser} or Cys^{331Ser}, of the extracellular domain (exon 11) or (Cys^{417Ser} or Cys^{492Ser} of the extracellular loops I and II respectively (exon 11)) had no effect on ligand binding but prevented expression of these forms of the LH/CG receptor on the cells surface membrane (*see Figure 1.10* and Table 1.7). $Cysteine^{282Ser}$ (exon 10) and Cys^{314Ser} (exon 11) were demonstrated to be nonessential for either hormone binding or plasm membrane insertion (see Table 1.7). Thus all cysteine N-terminal to exon 7 influence hormone binding (Zhang *et al.*, 1996).

Thomas and Segaloff (1994), demonstrated that while the extracellular domain may be responsible for high affinity binding of hCG and human LH and involve only one binding site, binding of ovine LH appears to involve two binding site in the full length receptor. However, receptors which do not express the transmembrane or intracellular domains, appear to have only one functional binding site, with a Kd midway between that of the high and low binding sites of the full length receptor (see Table 1.7).

2. Carbohydrate Determinants

The effects of carbohydrate moieties of the LH/CG receptor on binding to its ligand, has been determined by two different approaches; (1) the treatment of membrane bound receptors with glycososidases to remove various carbohydrates, and (2) the expression of mutant receptors in which certain bases have been substituted so N-linked glycosylation can not occur. The first approach has been used by a number of groups (Kusuda and Dufau, 1988; Keinänen, 1988; Minegishi *et al.*, 1989; Ji *et al.*, 1990b; Petäjä-Repo *et al.*, 1991; Zhang *et al.*, 1995), using endoglycosidase F and/or *N*-glycanase, which removes *N*-linked carbohydrates or treatment of LH/CG receptor expression cells with Tunicamycin, which prevents *N*-linked glycosylation Both treatments reduced the size of the receptors, though three of the groups reported that the deglycoslyation of the receptors had no effect on its ability to bind hCG (Keinänen, 1988; Petäjä-Repo *et al.*, 1991; Ji *et al.*, 1990b; Zhang *et al.*, 1995). In contrast, Dufau's group reported that the deglycosylated receptor was unable to bind hCG (Kusuda and Dufau, 1988; Minegishi *et al.*, 1989; Zhang *et al.*, 1995), while Ji *et al.* (1990b), found that treatment with *N*-glycanase had no effect on ligand binding, and that Tunicamycin treatment abolished binding, suggesting that glycosylation was important in transport of the receptor to the cell surface but not involved in ligand binding. Zhang *et al.* (1995) found that the rLH-R B splice variant does not bind hCG after treatment with *N*-glycanase or Tunicamycin, but does with treatment with endoglycosidase F, suggesting that the proximal *N*-acetylglucosamine linked to Asn is essential for high affinity binding.

Receptor	Cell Type	Kd (nM)	Location	References
rLH-R (Asp^{556}➡Asn)	COS 7A	0.45	cell surface	Ji and Ji, 1991c
rLH-R (Asn^{77}➡Gln)	COS 1	0.13	cell surface	Zhang *et al.*, 1991
rLH-R (Asn^{77}➡Gln)	293 cells	0.17	79% cell surface	Liu *et al.*, 1993
rLH-R (-26➡294+22) (Asn^{77}➡Gln)	Sf9	0.20	intracellular	Zhang *et al.*, 1995
rLH-R (Asp^{152}➡Gln	COS 1	0.16	cell surface	Zhang *et al.*, 1991
rLH-R (Asp^{152}➡Gln)	293 cells	0.54	61% cell surface	Liu *et al.*, 1993
rLH-R (-26➡294+22) (Asp^{152}➡Gln)	Sf9	ND	intracellular	Zhang *et al.*, 1995
rLH-R (Thr^{154}➡Ala)	293 cells	0.37	73% cell surface	Liu *et al.*, 1993
rLH-R (-26➡294+22) (Ser^{154}➡Ala)	Sf9	0.18	intracellular	Zhang *et al.*, 1995
rLH-R (Asp^{173}➡Gln)	COS 1	ND	cell surface	Zhang *et al.*, 1991
rLH-R (Asp^{173}➡Gln)	293 cells	≥20	11% cell surface	Liu *et al.*, 1993
rLH-R (-26➡294+22) (Asp^{173}➡Gln)	Sf9	ND	intracellular	Zhang *et al.*, 1995
rLH-R (Thr^{175}➡Ala)	293 cells	0.49	15% cell surface	Liu *et al.*, 1993
rLH-R (-26➡294+22) (Thr^{175}➡Ala)	Sf9	ND	intracellular	Zhang *et al.*, 1995
rLH-R ($Asp^{269,277}$ ➡Gln)	293 cells	0.22	63% cell surface	Liu *et al.*, 1993
rLH-R (Asp^{269}➡Gln)	COS 1	0.24	cell surface	Zhang *et al.*, 1991
rLH-R (Asp^{277}➡Gln)	COS 1	0.19	cell surface	Zhang *et al.*, 1991
rLH-R (Asp^{291}➡Gln)	COS 1	0.22	cell surface	Zhang *et al.*, 1991
rLH-R (Asp^{291}➡Gln)	293 cells	0.24	68% cell surface	Liu *et al.*, 1993
rLH-R ($Asp^{77,152,173}$,269,277,291➡Gln)	293 cells	≥20	25% cell surface	Liu *et al.*, 1993
rLH-R ($Asp^{77,152,269,277,291}$ ➡Gln, Thr^{175}➡Ala)	293 cells	0.53	4% cell surface	Liu *et al.*, 1993

Table 1.8 hCG binding affinity and cellular location of several LH/CG receptors containing deleations and substitutions. Truncations have the resides present in the brackets. Alternative splice forms are denoted as the above, however many alternative splice forms contain a 3' unique tail indicated by + the number of unique residues. Base substitutions are indicated with the substituted base first, with its position in superscript and the base that it has been substituted to after the arrow. Numbering of amino acids begins with the methionine which intiates translations as 1. Based on a table in Segaloff and Ascoli, 1993.

Site directed mutagenesis of the potential *N*-linked glycosylation sites has been reported by two groups (see Table 1.8; Zhang *et al.*, 1991; Liu *et al.*, 1993; Zhang *et al.*, 1995), again producing conflicting findings. Dufau and associates introduced Asn to Gln mutations in each of the six consensus potential *N*-linked glycosylation sites in the extracellular domain of the LH receptor, and expressed these forms (See Table 1.8; Zhang *et al.*, 1991). Five of these form had no effect on binding hCG with high affinity, however mutation of the asparagine residue at position 173 to glutamine, failed to bind hCG, and the mutations at Asn^{77Gln}, and Asn^{152Gln} resulted in significantly lower numbers of functional binding sites at the cell surface than the wild type, backing up their original findings. However, a subsequent study by Segaloff and associates (Liu *et al.*, 1993), mutated the six consensus potential *N*-linked glycosylation sites to Gln residues, and the Thr reside at position 175 to Ala, thus preventing *N*-linked glycosylation through the asparagine residue at position 173. This revealed similar results to the findings of Zhang *et al.* (1991) for the asparagine mutants, however mutation of the threonine residue at position 175 produced a receptor which bound hCG with a similar affinity to the wild type receptor, though it was trapped primarily intracellularly (see Table 1.8; Liu *et al.*, 1993). It appeared that reduced binding of the asparagine 173 mutants is not due to the prevention of potential glycosylation at the site, but due to the asparagine residue at position 173 having a critical role in high affinity hCG binding.

Recently, Zhang *et al.* (1995) expressed the rLH-R B splice form, which encodes for most of the extracellular domain and binds hCG with high affinity, and mutated forms of the B splice variant similar to those expressed by Liu *et al.*, 1993 (to remove N-linked glycosylation sites) in Baculovirus infected insect cells. This study found (1) that mutation of Asp^{152Gln} resulted in complete loss of hCG binding, and mutation of Ser^{154Ala} resulted in marked reduction of binding, in contrast to their own earlier data (Zhang *et al.*, 1991) and that of Liu *et al.* (1993); (2) that mutation of Thr^{175Ala} resulted

in complete loss of hCG binding in contrast to the findings of Liu *et al.* (1993); and (3) that while Asn^{77} is not glycosylated mutation of Asn^{77Gln} results in reduced binding activity, again in contrast to the findings of Liu *et al.* (1993). It is possible that the differences between the above studies are a result of the cell type and splice variant expressed. How they relate to the situation *in vivo* is also quite speculative, given that LH receptors from ovarian sources appear to have different amounts of glycosylation to LH receptors from testicular sources (Dufau, 1990).

B. Effector Activation

Experiments to determine which residues of the LH/CG receptor are important in effector activation (*i.e.* adenylyl cyclase or phospholipase C activation), have been generally unsuccessful (Ji and Ji, 1991b; Ji and Ji, 1991c; Rodriguez *et al.*, 1992; Segaloff and Ascoli, 1993). Many of the mutated forms were not expressed on the plasma membrane, but remained within the cytoplasm, and hence unable to interact with G-proteins. It has been suggested that this is in part due to truncation of part of the cytoplasmic tail, as truncations at residue 616, which removes 84% of the cytoplasmic tail, resulted in little or no surface binding, though detergent solubilised cells revealed that these forms were located intracellularly (see Table 1.7; Rodriguez *et al.*, 1992). However, truncation of the rat LH receptor after residue 631, which removes ~65% of the cytoplasmic tail, results in receptors being located mostly at the cell surface, indicating that regions between 616 and 631 are required for proper insertion and/or targeting of the receptor into the plasma membrane (see Table 1.7; Rodriguez *et al.*, 1992). The cells expressing receptors truncated at residue 631, responded to hCG with a two fold greater cAMP production than the wild type receptor, and internalised and degraded hCG faster (Rodriguez *et al.*, 1992) which indicates that the carboxyl terminal 43 amino acids are not required for hCG internalisation or adenylyl cyclase activation. Interestingly, receptors truncated at residue 631 are not uncoupled by a preincubation of hCG, demonstrating that the last 43 amino

acids are involved in agonist induced uncoupling (Sanchez-Yagüe *et al.*, 1992). Supporting this finding is a case of familial Leydig cell hypoplasia, where a mutation in the LH/CG receptor at position 1635 leads to a stop codon at residue 545, which results in reduced expression of the receptor on the cell surface, a lower affinity for hCG and lack of cAMP inducibility (see Table 1.9; Laue *et al.*, 1995a).

The extracellular domain is not important for adenylyl cyclase actitivation, as cells expressing truncated receptors containing the first 5 and last 5 amino acids of the extracellular domain and the rest of the rat LH receptor were capable of binding hCG resulting in the stimulation of cAMP synthesis (Ji and Ji, 1991b). Receptors with mutations to Glu^{410Gln}, Glu^{441Asp}, or Asp^{556Asn}, sites which are highly conserved between G protein associated receptors, activate adenylyl cyclase activity like the wildtype (Ji and Ji, 1991c; Wang *et al.*, 1993a). However, mutations to Asp^{383Asn} suggest that this site is important in activation of cAMP production (see Table 1.7; Ji and Ji, 1991c), though this is in contrast to the findings of Quintina *et al.* (1993).

Expression of mutated receptors, in which the mutations or deletions occur in the extracellular and/or transmembrane domain follow no clear pattern, with some mostly expressed on the cells surface and others being retained internally (see Table 1.7). It has been suggested that since some mutated receptors are not expressed on the cell surface in transformed cell lines, that a similar mechanism (*i.e.* a single amino acid substitution), could be responsible for some forms of reduced fertility or familial male limited precocious puberty (Segaloff and Ascoli, 1993). Table 1.9 summarises the literature on families with mutations in their LH receptor gene which result in these problems. It appears that residues Ile^{542} to Cys^{581} are very important in interacting with the Gs protein as mutaions in this region leeds to adenylyl cyclase activation independent of ligand binding (*see Figure 1.11;* Laue *et al.*, 1995b).

hLH/CG-R mutation	Location	Cell Type	Effects	Reference
Cys^{545}➡Stop	TM helix 5	293 cells	↓ expression on cell surface, ↓ Kd for hCG and lack of cAMP inducibility	Laue *et al.*, 1995a
Ile^{542}➡Leu	TM helix 5	293 cells	FMPP, hCG unresponsive	Laue *et al.*, 1995b
Asp^{564}➡Gly	CP loop 3	293 cells	FMPP	Laue *et al.*, 1995b
Met^{571}➡Ile	TM helix 6	293 cells	FMPP	Kremer *et al.*, 1993 Shenker *et al.*, 1994 Laue *et al.*, 1995b
Ala^{572}➡Val	TM helix 6	COS 7	FMPP, ↑ basal IP3 and ↑ hCG affinity for hCG	Yano *et al.*, 1995
Thr^{577}➡Ile	TM helix 6	293 cells	FMPP	Shenker *et al.*, 1994 Laue *et al.*, 1995b
Asp^{578}➡Tyr	TM helix 6	293 cells	FMPP	Laue *et al.*, 1995b
Asp^{578}➡Gly	TM helix 6	COS 7 293 cells	FMPP ↑ in IP3 at high conc. of hCG but not basal or low levels	Shenker *et al.*, 1993 Kremer *et al.*, 1993 Boepple *et al.*, 1994 Yano *et al.*, 1994 Laue *et al.*, 1995b
Cys^{581}➡Arg	TM helix 6	293 cells	FMPP and unresponsive to hCG	Laue *et al.*, 1995b
Ala^{593}➡Pro	TM helix 6	293 cells	Male pseudohermaphroditism, binds hCG but no ↑ in cAMP	Kremer *et al.*, 1995

Table 1.9. Summary of mutations in the LH/CG receptor leading to familial male precocious puberty (FMPP) or male pseudohermaphroditism with Leydig cell hypoplasia.. Cys^{581}➡Arg indicates that the normal cysteine residue at position 581 has been replaced in the mutated receptor to an arginine *etc.* Based on a table from Laue *et al.*, 1995b

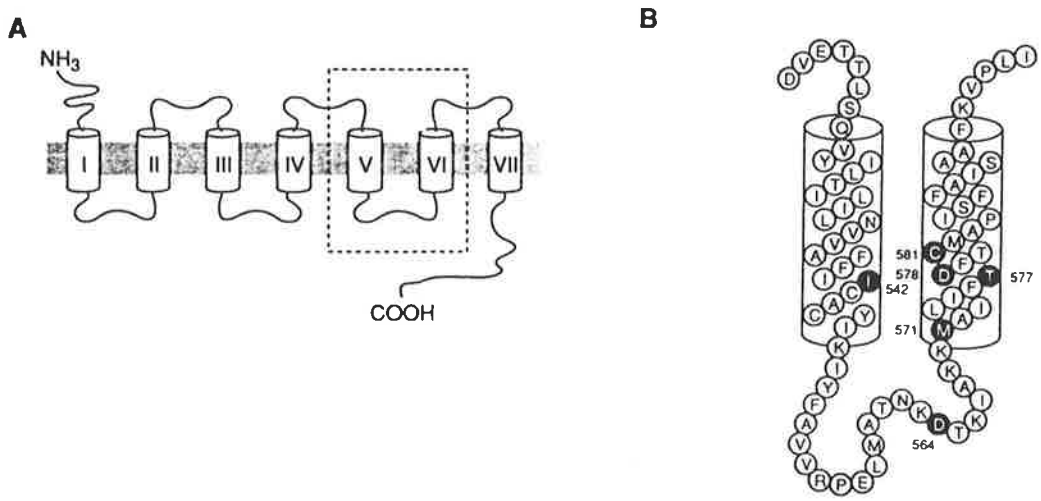

Figure 1.11 (A) Schematic representation of the membrane topology of the LH/CG receptor. (B) Position of mutations described in Table 1.8. Based on a figure from Laue *et al.*, 1995b.

1.4.9. Heterogeneity of LH/CG Receptor Messenger RNA Transcripts

Multiple species of LH receptor mRNA transcripts of different sizes have been observed by Northern analyses (Minegish *et al.*, 1990; McFarland, *et al.*, 1989; Loosfelt *et al.*, 1989; Wang *et al.*, 1991b; LaPolt *et al.*, 1990, Hu *et al.*, 1990, Lu *et al.*, 1993). Currently these LH receptor transcripts are classified by size only, since very little is known about the sequence of each transcript. In rat ovaries mRNA species of 6.7, 4.3, 2.6 and 1.2 kb are seen, the 6.7 kb band being the most abundant. Northern analyses on rat testes reveal all these transcripts, as well as a 1.6-1.8 band (this band is not down-regulated). Mouse testes and Mouse Leydig MA-10 tumour cells have multiple LH receptor transcripts of 7.7, 4.3, 2.6, 1.9, 1.6, and 1.2 kb in length. However all of these cells express functional 85-93 kDA receptors. Since the LH/CG receptor gene is a single copy gene (Koo *et al.*, 1991), the diversity seen by Northern analyses must be due to either alternative transcription start sites, alternative RNA splicing, or the use of alternative polyadenylation sites. Clearly some of the diversity is due to alternative RNA splicing (LaPolt *et al.*, 1991; Bhalla *et al.*, 1992; Vihko *et al.*, 1992; Sokka *et al.*, 1992). The possibility of alternative transcription initiation sites of the LH receptor gene, given that no TATA or CCAAT boxes have been identified (Koo *et al.*, 1991) has also been suggested (Huhtaniemi *et al.*, 1992). This seems unlikely, due to the lack of alternative transcription initiation sites, detected by nuclear run on experiments *(see Section 1.4.4)*, which are significantly distant from each other to account for the differences in the size of the heterogeneity of the LH receptor transcripts detected by Northern analyses. Alternative polyadenylation sites (Lu and Menon, 1994; Koo *et al.*, 1994) and different lengths of poly(A) tails (Wang *et al.*, 1990b; LaPolt *et al.*, 1991) have also been observed.

Evidence from Lu and Menon (1993; 1994), suggested that the largest LH receptor mRNA transcripts detected by Northern analyses of rat was due to a longer 3' untranslated region that had not been previously identified. Importantly this region had

multiple polyadenylation sites (*see Section 1.4.4*). The use of alternative polyadenylation sites in a long 3' untranslated region could account for the higher molecular weight forms. It is possible that, some of the larger transcripts were due to unspliced or incompletely spliced pre-mRNA species, however these have not been identified by other methods in the gonads (Hu *et al.*, 1990), though a form containing introns was identified from a human thyroid cDNA library (Frazier *et al.*, 1990). The identity of 1.2 kb band in Northern blots of rat gonads (Wang *et al.*, 1991b) and mouse testis derived MA-10 cells and of a 1.7 kb band in Northern blots of rat testis (Wang *et al.*, 1991b; LaPolt *et al.*, 1991), was recently determined with the discovery by Ji *et al.* (1994) of alternative polyadenylation sites in introns 3, 4, and 10 (Ji *et al.* 1994). LH receptor mRNA using these polyadenylation sites are found in the 0.6, 1.2, 1.8, 2.5, and 4.2 kb bands in rat ovarian Northern analyses. They also demonstrated that these forms make up the majority of LH receptor mRNA. Strangely these forms were not initially identified from cDNA libraries. The fact that the lower sized bands on Northern blots of MA-10 cells (Wang *et al.*, 1991b) and rat testes (LaPolt *et al.*, 1991) and maybe in rat ovaries (Wang *et al.*, 1991b), would not hybridise to LH receptor probes consisting only of the transmembrane domain, but did bind probes from the extracellular domain, gives added support that these bands are made up of the transcriptsusing the alternate polyadenylation sites found in introns 3, 4 and 10.

A paper generated from some of the work performed in this thesis, Bacich *et al.* (1994), reports for the first time that Northern analyses performed on RNA from ovine ovaries identifies, as in other species, numerous LH receptor mRNA transcripts of various sizes. To date no other papers reporting the analysis of ovine LH receptor mRNA has been performed, although Yarney *et al.* (1993) cloned and demonstrated the expression of the ovine follicle stimulating hormone receptor.

1.5 ALTERNATIVE SPLICING

1.5.1. Mechanisms of Alternative Splicing

Alternative pre-mRNA splicing is a widespread device for gene regulation and for generating isoform diversity. It can lead to crucial changes of function such as the cellular localisation of the encoded protein, deletion or alteration of the protein sequence, modification of protein activity, novel protein activities, and changes in RNA stability and translational efficiency (see Smith *et al.* 1989).

Splicing requires introns to posses three identifiable regions; the 5' splice site junction or splice donor site, which contains a consensus sequence of C/AAG⇓GURAGU (where R= a purine base, and ⇓= the intron exon boundary); the 3' splice junction or splice acceptor site, which contains the consensus sequence of $Y_nNYAG⇓$ (where Y= a pyrimidine and N= any base); and finally at some point greater than 17 nucleotides upstream of the 3' splice site there is a sequence referred to as the branch piont. This has the loose consensus of YNCUG**A**C in which the **A** residue is involved in the formation of the lariat intermediate of splicing *(see Figure 1.12)*. Splicing therefore requires the consensus sequences being recognised and acted upon by a set of ribonucleo-protein particles (snRNPs).

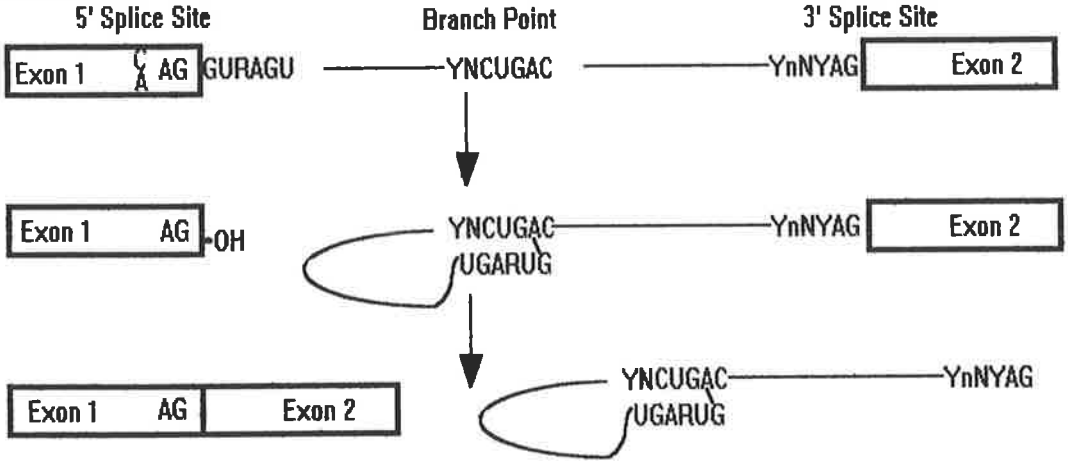

Figure 1.12 A schematic presentation of the events involved in RNA splicing. Boxes represent exons, with the consensus sequences in their relative positions.

Alternative splicing can arise from a number of possible splicing events. The vast majority of events are depicted in *Figure 1.13*. Many of these alternative splicing events have been reported to occur with the LH/CG receptor mRNA *(see Section 1.5.2)*. In addition, combinations of the various splicing events can also occur.

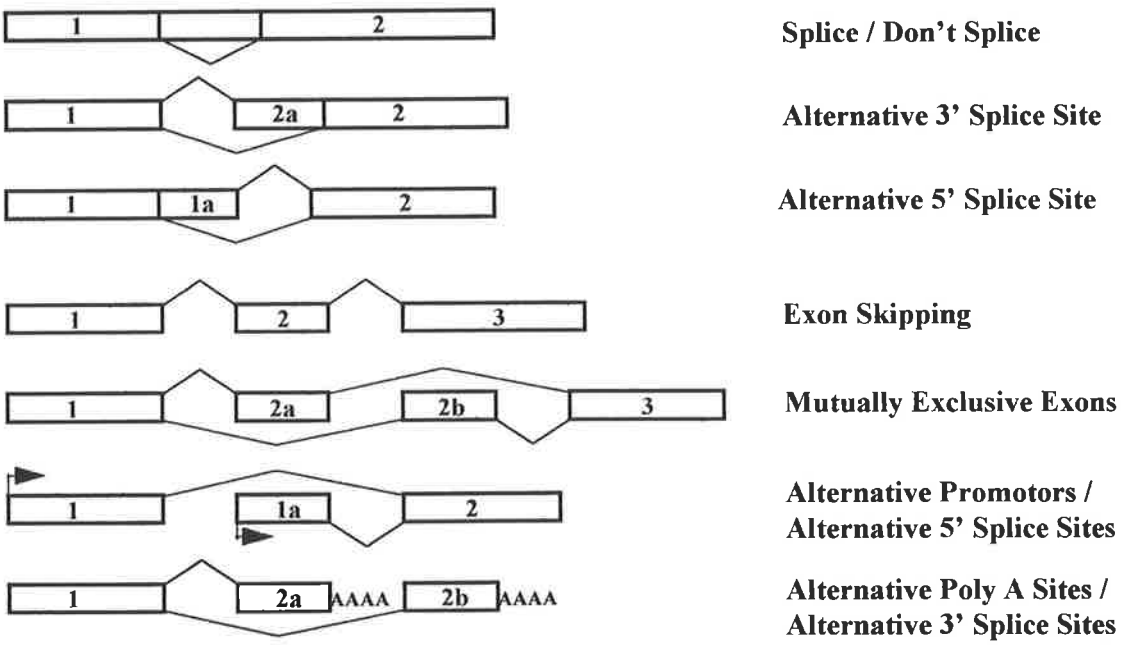

Figure 1.13 Types of alternative splicing. Various simple types of alternative splicing events are diagrammed such that only the particular events are shown. Boxes represent sequences that can be found in "mature" RNAs while the lines represent splicing out of intervening sequence. Alternative events are represented above and below the line of exons. From a Figure in McKeown, 1990.

1.5.2. Alternative Splicing of the LH/CG Receptor Gene

Loosfelt *et al.* (1989), described four alternatively spliced cDNA transcripts when they cloned the porcine LH receptor cDNA sequence. The cDNA transcript which they proposed encoded for the full length mature functional receptor, they called the A splice form, while they named the other three alternativly spliced forms that they identified the B, C, and D splice forms. The A splice form appears to use identical splice donor and acceptor sites in pig, rat, mouse, and man *(see Section 1.4.4)*. Alternatively spliced forms have only been examined in the rat, and pig. Alternative splicing of the LH/CG receptor

has been reported in humans but the specific alternatively spliced products were not described (Minegish *et al.*, 1990; Denker *et al.*, 1994).

The B isoform is common to pig (Loosfelt *et al.*, 1989), human (personal communication with Dr Richard Rohan), and rat (also called B4 (Segaloff *et al.*, 1990)), and splices to an alternative 3' acceptor site in exon XI of the coding region of the full length A form, at position 1214 from the initiation of translation in the pig A form cDNA sequence. The sequence at this site is UUUGCAG⇓A, which differs slightly from consensus 3' acceptor sites. In the rat and pig a conserved putative branch point sequence of UCCUGAC is located 57-63 nucleotides upstream of this alternative 3' splice site (Aatsinki *et al.*, 1992). The C and D splice forms have only been reported in the pig, and also splice into the coding region of exon XI, although downstream from the B splice acceptor site *(see Figure 1.12)*. The use of alternative 3' splice sites is common, and occurs in many LH receptor splice forms reported in rats; B1, B2, B3, B4, and EB forms (*see Figure 1.14*).

Splice forms which retain parts of introns, and splice from regions within the intron to splice intron/exon boundaries have also been reported in the rat; C1 and C2 (Segaloff *et al.*, 1990). Many of the alternatively spliced forms splice out of frame, and if translated would result in unique amino acid sequences from the alternative splicing event onwards, resulting in significantly truncated receptors.

Three other alternatively spliced forms have been reported in the rat, rLHR1950, rLHR1759, and the E splice form, which have entire exons spliced out (Segaloff *et al.*, 1990, Bernard *et al.* 1990, Aatsinki *et al.*, 1992). This is a common alternative splicing event, and is referred to as exon exclusion. Therefore not only is alternative splicing of the LH receptor gene common in all species examined, but some splice variants, particularly the B form, appear to be conserved.

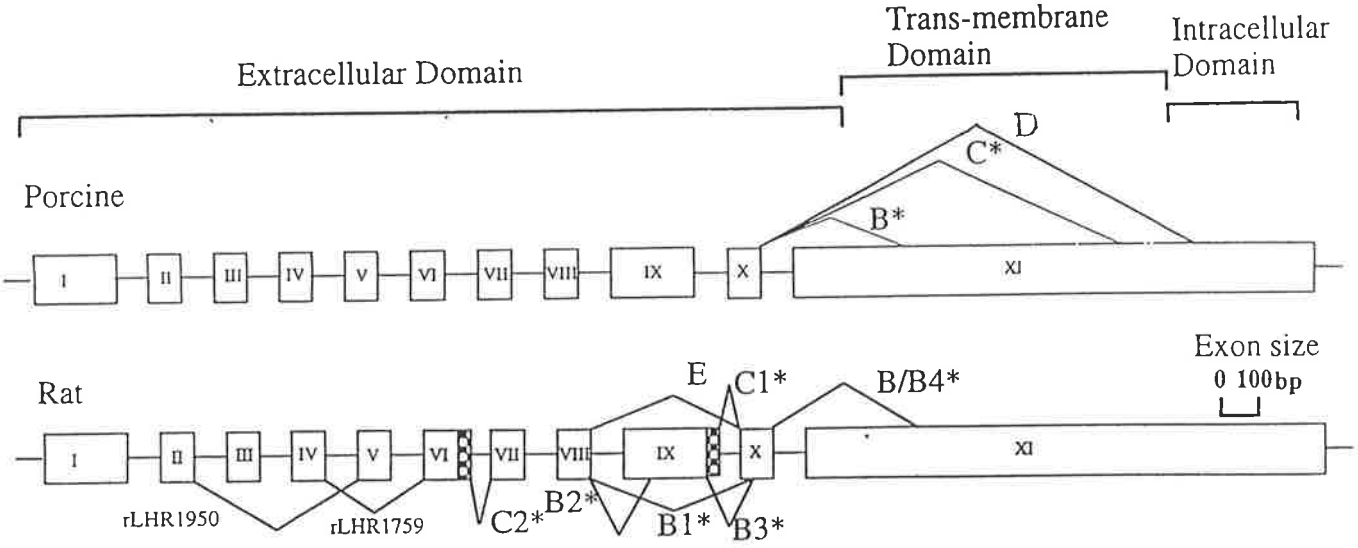

Figure 1.14 Comparisons of the splice variants in the pig and rat LH receptor genes. The genomic structure is that of the LH receptor of the rat, with the exons ofthe full length form shown as *boxes*, and introns (of the A form) shown as *horizontal lines* (not to scale). Slice variants that have been sequenced are depicted by lines that delineate the extent of the region that is removed by splicing. Each variant is labelled by its standard nomenclature. All splice forms identified with an *asterisk* result in a frame shift and, thus, early termination of the putative translated protein generated by these forms. *Checkered boxes* denote presumed intronic regions that are retained in some of the splice variants.

There is considerable evidence that the splicing pattern of the LH receptor is regulated based upon Northern and RT-PCR analyses. For example Bhalla *et al* (1992) demonstrated that different sizes of LH receptor mRNA transcripts are produced by different fractions of Leydig cells separated by size, suggesting differential regulation by cell type occurs. It has also been suggested that regulation of LH receptor mRNA splicing occurs during development of the rat testis (Vihko *et al.*, 1992; Sokka *et al.*, 1992) and further evidence of regulation of differential splicing of the LH receptor in rat testes and ovaries in response to hormonal down regulation has also been reported (LaPolt *et al.*, 1991), although this could also be explained by differential regulation of the half lives of these LH receptor mRNA isoforms.

Although numerous alternative LH receptor mRNA splice variants have been reported in every species examined, quantitation of the relative steady-state levels of these splice variants has been harder to achieve. The existing data comes indirectly from the frequency of isolation of cDNA clones, RT-PCR and Northern analyses. In the pig testis, the frequency of isolated clones suggested that less than 60% of LH receptor transcripts were the A form (Loosfelt *et al.*, 1989). Similarly in the rat corpus luteum less than 50% were the A form (Bernard *et al.*, 1990), and that the B (or B4 (Segaloff *et al.*, 1990)) form (Bernard *et al.*, 1990) made up over 50% of the transcripts. Thus clearly the B splice variant was a major form of the LH receptor mRNAs. In the rat some forms (rLHR1950, rLHR1759, EB) have been detected only by using RT-PCR (Aatsinki *et al.*, 1992; Bernard *et al.*, 1990), and others (C1, C2, B1, and B3), which were detected by screening a rat luteal cDNA library, have only been observed by a single group of researchers (Segaloff *et al.*, 1990). As such, these forms may not make up a significant proportion of the rat LH receptor mRNA. While it is likely that splicing of the LH receptor shares some characteristics across mammalian species, to date the molecular analyses of the LH receptor have not been carried out in the sheep, apart from the current study.

The fate of the LH receptor mRNA splice variants in the cell is unknown, although much has been postulated about them. The majority of the splice variants so far reported do not encode the entire transmembrane domain except for the E, rLHR1950 and rLHR1759 forms in rat (Segaloff *et al.*, 1990, Bernard *et al.* 1990, Aatsinki *et al.*, 1992), leading to speculation that if translation of the splice variants lacking the transmembrane domain occurs, then the encoded proteins would be secreted by the cells and be capable of binding LH and hCG. Expression of these splice forms in mammalian cell lines (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992), or of artificially altered forms which also lack the transmembrane domain (Xie *et al.*, 1990; Braun *et al.*, 1991), revealed that

all had hCG binding affinities comparable to that of the A form (see Table 1.7). However, there are conflicting findings regarding the secretion of these forms. Artificial expression studies have resulted in the secretion of the protein translated from the B splice variant from both the rat and the pig (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992) and the porcine C and D. However, two later studies, one also by Dufau's group, were unable to repeat these results with the rat B splice form (Koo *et al.*, 1994; Zhang *et al.*, 1995). In addition, artificial expression studies with artificially truncated forms which are almost almost identical to the B splice form but lack the unique amino acids generated by the frame shift, were not secreted (Xie *et al.*, 1990; Braun *et al.*, 1991; Thomas and Segaloff, 1994). Further experiments are needed to determine if these splice forms are translated *in vivo,* and if so if they are secreted.

The expression of porcine splice variants in transfected cells produced immunoreactive proteins of similar size (both glycosylated and deglycosylated), to forms isolated from porcine testes (VuHai-LuuThi *et al.*, 1992), suggesting that these variants may be translated *in vivo*. Unfortunately the molecular weight of the alternatively spliced forms are similar to the size of the extracellular domain of the functional A form. As stated earlier, fragments of this domain can be cleaved by proteases activated by binding of the ligand (West and Cooke, 1991a), and also in some of the crude collagenase preparations used in extracting the receptors (Ascoli and Segaloff, 1986). Hence, the origin of the smaller products with LH receptor immunoreactivity may be from either or both sources described above.

Alternatively spliced transcripts of both the FSH and TSH receptors have also been reported (FSH-R (Gromoll *et al.*, 1992; Kelton *et al.*, 1992; Khan *et al.*, 1993; Themmen *et al.*, 1994); TSH-R (Goodwin *et al.*, 1990; Pascke *et al.*, 1994; Hunt *et al.*, 1995; Paschke and Greenen, 1995)). As with the LH/CG receptor splice variants, the function

of the FSH receptor alternatively spliced forms is unknown, although there is speculation that these could be translated into soluble receptors that do not contain a trans-membrane domain. The expression of the alternatively spliced FSH-R transcripts has been examined at differential developmental stages (testis) and stages of the cycle (ovary), and it closely follows the expression of the full length FSH receptor mRNA (Themmen *et al.*, 1994). Many of the alternatively spliced transcripts of the TSH receptor gene also encode for proteins without a membrane spanning domain (Hunt *et al.*, 1995), and their association with polysomes suggests that they are translated *in vivo*. In addition to being expressed in the thyroid, some of the TSH-R alternatively spliced transcripts have also been detected in retro- and extro-ocular muscle, peripheral blood mononuclear cells, fat and fibroblasts. Although the function of these forms is unknown it has been suggested that they may act as a nonfunctional autoantigen in Graves disease (Paschke *et al.*, 1994; Pascke and Greenen 1995), or a source of soluble TSH receptors (Hunt *et al.*, 1995).

Messenger RNAs encoding truncated receptors without a transmembrane/extracellular domain are not limited to the gonadotrophin receptors. Soluble receptors have been described in biological fluid and blood for other receptors including the growth hormone (Leung *et al.*, 1987; Baumbach *et al.*, 1989; Edens *et al.*, 1994), prolactin receptor (Postel-Vinay *et al.*,1991), the epidermal growth factor receptor (Carpenter, 1987), interleukin-4 receptor (Mosley *et al.*, 1989) interleukin-7 receptor (Takeshita *et al.*, 1992), N-CAM (Gower et al., 1988), IgM (Gough, 1987) and MHC antigens (Gussow and Pleogh) resulting from alternative splicing of the receptor gene. The function of expressed spliced variants remains a puzzle, there is some evidence that secreted, soluble receptor ligand binding domains can act as inhibitors of hormone action by sequestering the ligand away from the receptor *i.e. in vitro* studies with soluble IL-4 receptors demonstrate that they are able to inhibit IL-4's effects (Mosley *et al.*, 1989). In addition, growth hormone in serum circulates complexed with a growth hormone binding protein

(GHBP). In rats and mice GHBP arises from alternative splicing of the receptor, while in humans and rabbits they arise from proteolytic cleavage of the receptor (Baumbach *et al.*, 1989; Edens *et al.*, 1994; Leung *et al.*, 1988; Trivedi and Daughaday, 1988). However, a free ligand-binding domain of a truncated receptor might act to present the hormone to the receptor resulting in a more efficient signal, and if the receptors are not secreted than intracellular role may exist.

Although the function of the alternatively spliced LH/CG receptor forms is not known, expression studies have demonstrated that they have some enhancing effect on the activity of the full-length receptor (VuHai-LuuThi *et al.*, 1992). Cells co-transfected with either the C or D splice variant and the A splice form responded with increased maximal hormone-induced stimulation of adenylate cyclase above that of cells only transfected with the functional A splice form (VuHai-LuuThi *et al.*, 1992). However there was no effect on the amount of hormone required to elicit a half maximal stimulation. Interestingly, VuHai-LuuThi *et al.* (1992) demonstrated that soluble hormone-receptor complexes were monomeric, thus either the bonds between receptor monomers are unstable, or the reactions that occur in the cell membrane can not be observed in solubilised receptors. Remy *et al.* (1993), demonstrated that co-expression of the extracellular domain, and the transmembrane-cytoplasmic domain of the LH receptor results in functional receptors, therefore one could postulate that alternatively spliced forms may interact with other receptors, or the full length LH receptor and subsequently lead to LH or hCG stimulation.

1.6 Hypothesis and Aims of the Thesis

Alternatively spliced forms of the LH/CG receptor mRNA have been observed in all species so far examined, with some of these forms apparently conserved between species. This suggests that they may have some regulatory or physiological function. The existence of alternatively spliced LH receptor mRNA forms has not been reported in sheep, however this is an ideal animal model, due to the wealth of knowledge concerning the control of its reproduction, and ease of isolation and identifying the cell types of the ovary. Using this model we intend to investigate if the ovine LH receptor gene is alternatively spliced, if there is regulation of this alternative splicing between tissues, and within tissue at different times of the estrus cycle. In addition, if the ovine LH receptor is alternatively spliced, we intend to determine if these alternatively spliced forms are translated *in vivo*, and if so are the relative levels of the products equivalent to the levels of the mRNA spliced forms, or is there differential post-translational processing, the localisation of the alternatively spliced translated forms and hopefully a possible role or function such as are they involved in the inability of LH or hCG to stimulate adenalate cyclase in LH binding sites found on large luteal cells.

Therefore the aims of this thesis are:

(1) determine if alternative splicing of the LH/CG receptor mRNA occurs in the ovine ovary,

(2) set up a method to determine the relative abundance of the alternatively spliced forms is in comparison to the full length receptor

(3) determine if they are alternatively regulated in a cell dependent manner,

(4) determine if they are alternatively regulated at different stages of the oestrus cycle

(5) Set up a method to determine if they are translated into proteins *in vivo,* and if so what their relative abundance is in comparison the full length receptor protein, if there concentration mimics that of the mRNA,and whether they are secreted

Hopefully these aims will reveal what regulatory or physiological function these alternatively spliced forms have in the ovine ovary.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.0 Reagents and Solutions

All reagents were of analytical grade, and unless otherwise stated, were obtained from the following distributors; Ajax Chemicals (Regency Park, SA, Australia), Amersham Australia (North Ryde, NSW, Australia), Amrad-Pharmacia Biotech (Australia), Bayer Australia Ltd. (Botany, NSW, Australia), BDH Laboratory Supplies (Poole, England), Bio-Rad Laboratories Pty Ltd. (North Ryde, NSW, Australia), Boehringer-Mannheim Australia (North Ryde, NSW, Australia), Bresatec (Thebarton, SA, Australia), CSL (Parkville, Vic, Australia), Difco (Detroit, MI), Eastman Kodak (Rochester, NY), Gibco-BRL Life Technologies (Gaithersburg, MD), Merck (Kilsyth, Vic, Australia), New England Biolabs (Genesearch, Brisbane, Australia), Progen Industries Ltd. (Darra, Qld, Australia), Promega (Madison, WI), Qiagen Inc. (Chatsworth, CA), Selby (Australia), Sigma Chemical Co. (St Louis, MO) and U.S. Biochemical Corp (Cleveland, OH).

2.1 Solutions for Molecular Biology

Sterilisation of solutions was by autoclaving for 20 minutes at 15 lb/sq inch. Filter sterilisation involved passing the solution through either a 0.45 μm disposable filter (Amicon), or a 0.22 μm disposable filter (Millipore).

2.1.1 Molecular Biology Stock Reagents and Buffers

Alkaline Lysis Buffers for Mini Preparations of Plasmid DNA :

Solution A was 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8.0, and was prepared as 100 ml batches which were autoclaved and stored at 4°C.

Solution B was 0.2 N sodium hydroxide and 1% SDS and was prepared just prior to use.

Solution C was 3 M potassium acetate, pH 4.8, and 2 M acetic acid and was stored at 4°C.

5.7 M Caesium Chloride (CsCl) Solution

Caesium chloride (191.94 g), and 3 M sodium acetate (1.67 ml; pH 5.2) were added to 40 ml of distilled water. The volume was adjusted to 200 ml with distilled water, and diethylpolycarbonate (200 µl) added, the solution was shaken for 1 hour, before autoclaving. The solution was weighted before and after autoclaving and the difference in weight, due to evaporation, was adjusted for with DEPC-treated water. This ensured that the solution was actually 5.7 M when used.

10x Calf Intestinal Alkaline Phosphatase buffer

Calf intestinal alkaline phosphatase buffer (10x) consisted of 500 mM Tris-HCl, pH 9.0, 10 mM magnesium chloride, 1 mM zinc chloride, and 10 mM spermidine. The solution was stored at -20°C, for up to one year.

Deionised Formamide

Formamide was deionised by mixing formamide with approximately 10% of the volume of mixed-bed resin AG® 501-X8(D) 20-50 mesh beads (Biorad) with constant agitation for at least 30 minutes. The formamide was then filter sterilised and stored at -20°C in 500 µl aliquots. Thawed aliquots were used immediately or discarded.

50x Denhardt's solution

To 400 ml DEPC treated water, 5 *g* of Ficoll, 5 *g* of polyvinyl pyrrolidine (PVP) and 5 *g* of bovine serum albumin (Pentax fraction V; Sigma) were added and dissolved and then the volume adjusted to 500 ml with DEPC treated water. The solution was filter sterilised, and stored in 50 ml volumes at -20°C.

Diethylpyrocarbonate (DEPC) treated H_2O

To 100 mls of distilled H_2O, 100 µl of diethylpyrocarbonate (Sigma) was added. The solution was shaken vigorously every 10 minutes for 1 hour, before autoclaving.

1 M Dithiothreitol (DTT)

DTT (3.09g) was dissolved in 18 ml of 10 mM sodium acetate, pH 5.2. The solution was adjusted to 20 ml with 10mM sodium acetate, pH 5.2, filter sterilised and stored at -20°C in 500 µl aliquots.

6x DNA loading buffer

The gel loading buffer contained 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol, and was stored at 4°C. This loading buffer was used when running DNA on non-denaturing agarose gels.

40 mM dNTP solution

A 40 mM stock solution of dNTPs containing approximately 10 mM of each nucleotide was prepared. 50 mg of each dNTP (2'-deoxy-adenosine-5'-triphosphate disodium salt; 2'-deoxy-cytidine-5'-triphosphate disodium salt; 2'-deoxy-guanosine-5'-triphosphate disodium salt and 2'-deoxy-thymidine-5'-triphosphate tetrasodium salt; Boehringer-Mannheim), were pooled and dissolved in 9 mls of DEPC treated water. The pH of the solution was adjusted to pH 7.0 by drop-wise addition of 1 M Tris-base, after which the final volume of the solution was adjusted to 10 mls. This was further diluted to working solution aliquots of 2 mM dNTP's with DEPC treated water and stored at -20°C.

Ethidium Bromide

A 10 mg/ml solution was made by adding one gram of ethidium bromide to 100 ml of water and stirring on a magnetic stirrer overnight to ensure all solutes were dissolved.

The bottle containing the ethidium bromide was then wrapped in aluminium foil and stored at room temperature.

0.5 M Ethylenediaminetetra Acetic Acid (EDTA), pH 8.0

To 186.1g of ETDA.$2H_2O$ sodium salt, 800 ml distilled water was added and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 by the addition of NaOH pellets, and once the solutes were completely dissolved, the volume was adjusted to 1 litre and the solution sterilised by autoclaving.

3.1 M GTC solution

This solution consisted of 3.1 M guanidinium isothiocynate (378.24 g/l), 6.25 mM Tris-HCl (pH 7.4), and 6.25 mM EDTA. This solution was sterilised by filtration through a 0.45 μm filter, and then 3% of the volume of β-mercaptoethanol added. The solution was stored in a light proof container at room temperature. The solution was discarded if it started to turn yellow.

Isopropylthio-β-D-galactoside (IPTG)

A 200 mg/ml stock solution was prepared by dissolving 2 *g* of IPTG in 8 ml of distilled water. The volume was adjusted to 10 mls with distilled water and sterilised by filtration through a 0.22 μm disposable filter (Millipore Australia). The solution was stored as 1 ml aliquots at -20°C.

X-gal

A 20 mg/ml solution of X-gal was prepared by dissolving 50 mg of X-gal (Boehringer Mannheim) in 2.5 ml of dimethylformamide. The X-gal solution was then wrapped in aluminium foil and stored at -20°C.

Ligase 10x buffer

Ligase buffer (10x) consisted 300 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP. The solution was stored at -20°C, for up to six months.

Maxiprep Lytic Mix

This consisted of 0.1% Triton X-100, 62 mM EDTA, pH 8.0, and 50 mM Tris-HCl, pH 8.0.

Northern Blot Prehybridisation mixture

Prehybridisation solution for Northern analyses consisted of 50% deionised formamide, 5x Denhardt's solution, 5x SSPE, 0.1% SDS and 100 μg/ml of herring sperm DNA. This solution was centrifuged at 1000 x *g*, to remove any precipitate, and stored at 4°C.

Northern Blot Stripping Solution

Northern blot stripping solution consisted of 0.1x SSC, 1 mM EDTA and 0.1% SDS. Blots were prevented from drying out by wrapping them in cling wrap until they were stripped.

Phenol

Phenol (Ajax Chemicals) was melted at 68°C, an equal volume of 0.5 M Tris-HCl, pH 8.0 was added and the solution vigorously mixed at room temperature. The phases were allowed to separate and the aqueous phase removed. Tris-HCl (100 mM), pH 8.0, was added in equal volumes, mixed vigorously and allowed to separate. The aqueous phase was removed and the pH of both layers checked. This was repeated with 100 mM Tris-HCl, pH 8.0, until the pH of the phenol reached approximately 7.6. Finally, 100 mM Tris-HCl, pH 8.0 containing 0.2% β-mercaptoethanol was added to the equilibrated phenol and stored in a light excluding bottle at 4°C.

Phenol : Chloroform : Isoamyl alcohol

Preparations of phenol : chloroform : isoamyl alcohol were prepared as a 25 : 24 : 1 ratio and stored at 4°C.

Proteinase K Buffer

Proteinase K digestion buffer consisted of 200 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 300 mM NaCl and 2% SDS. This was sterilised by passing through a 0.45μm filter and stored at room temperature.

Qiagen Buffers for Large Scale Plasmid Preparations

Buffer P1 contained 100 μg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0.

Buffer P2 contained 200 mM NaOH, 1% SDS

Buffer P3 contained 3.0 M potassium acetate, pH 5.5

Buffer QBT contained 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, and 0.15% Triton X-100.

Buffer QC contained 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0.

Buffer QF contained 1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5.

RNase (for general use)

Pancreatic RNase (RNase A, Sigma) was dissolved to produce a final concentration of 10 mg/ml in 10 mM Tris-HCl, pH 7.5 /15mM NaCl and heated to 100°C for 15 minutes. The sample was cooled to room temperature, aliquotted and stored at -20°C. This RNase was used for mini prep etc., but was not used for the RNase protection assays.

10x RNA loading buffer

RNA loading buffer (10x) contained 50% glycerol, 1 mM EDTA, pH 8.0, and 4 mg/ml bromophenol blue. Following autoclaving, the buffer was aliquotted into 1 ml batches, and stored at -20°C. This loading buffer was used for non-denaturing agarose gels.

RNase Protection Hybridisation buffer

RNase Protection Hybridisation buffer was made up freshly and added directly to the samples. The buffer contained 400 mM sodium chloride, 40 mM 1,4-piperazinediethansulfonic acid (PIPES; pH 6.4), 1 mM EDTA (pH 8.0) and 80% deionised formamide. The NaCl, PIPES and EDTA solutions were made up as a 5x solution, sterilised by autoclaving and stored at room temperature. The formamide was either deionised within an hour of making the RNase Protection Hybridisation buffer *(see 2.1.1)*, or came from frozen stocks.

RNase Protection Digestion mixture

RNase Protection Digestion Mixture contained 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 200 units/ml RNase T1 (Boehringer Mannheim) and 20 μg/ml RNase A. This solution was made just prior to use, as it can not be stored.

RNase Protection Formamide Loading buffer

RNase Protection Formamide loading buffer contained 80% freshly deionised formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol and 1 mg/ml bromophenol blue. This solution was made just prior to use as it can not be stored.

10x RNA Transblot Buffer

The buffer consisted of 100 mM Tris, 50 mM sodium acetate and 5 mM EDTA. The buffer was adjusted to pH 7.8 with 5 N HCl.

RNA Methylene Blue stain

This stain consisted of 0.5 M sodium acetate pH 5.2 and 0.04% methylene blue. The solution was filtered through 3 mm Whatman filter paper and stored at room temperature.

20x SSC

This stock solution consisted 3 M sodium chloride (175 g/l) and 0.3 M sodium citrate (88 g/l), and adjusted to a pH of 7.0 with 1 N HCl.

20x SSPE

The 20x SSPE solution consisted of 3.6 M sodium chloride, 0.2 M sodium phosphate, and 0.2 M EDTA. The solution was adjusted to pH 7.4 using 5 N NaOH, and autoclaved.

STE Buffer

STE buffer consisted of 0.1 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0.

10x Tris-Acetate-EDTA (TAE) Buffer

To 800 ml of distilled water, 48.4 *g* of Trizma base, 11.42 ml glacial acetic acid and 20 ml 0.5 M EDTA, pH 8.0 were added and stirred until all the solutes had dissolved. The volume was adjusted to 1 litre with distilled water and stored at room temperature.

10x Tris-Borate-EDTA (TBE) Buffer

To 800 ml of distilled water, 108 *g* of Trizma base, 55 *g* boric acid and 40 ml 0.5 M EDTA, pH 8.0 were added and stirred until all the solutes had dissolved. The volume was adjusted to 1 litre with distilled water and stored at room temperature.

2.1.2 Bacterial Media

Ampicillin

A 100 mg/ml stock solution of ampicillin, was filter sterilised through a 0.22 μm disposable filter (Millipore) and stored at -20°C.

1 M $CaCl_2$ Stocks

$CaCl_2.2H_2O$ (29.4g) was dissolved in 200 ml of distilled water and sterilised by filtering through a 0.22 μm disposable filter (Millipore).

20% Casamino acids

Casamino acids (20g; Difco), were dissolved in 80 mls of distilled water. The volume was adjusted to 100 ml, autoclaved and the solution stored at 4°C.

1 M $MgCl_2$ Stocks

In 800 ml of distilled water, 203.3 *g* $MgCl_2.6H_2O$ was dissolved, the volume adjusted to 1 litre and the solution sterilised by autoclaving.

1 M $MgSO_4$ Stocks

In 800 ml of DEPC treated water, 246.48 *g* $MgSO_4.7H_2O$ was dissolved. The volume was adjusted to 1 litre and sterilised by autoclaving.

10x M9 Salt Solution

To 400 ml of distilled water, 30 *g* Na_2HPO_4, 15 *g* KH_2PO_4, 5 *g* NH_4Cl and 2.5 *g* NaCl were added. The solution was mixed thoroughly to completely dissolve all solutes, and the pH adjusted to 7.4 by the addition of either 10 N HCl or 5 N NaOH. The volume was increased to 500 ml and the solution sterilised by autoclaving.

Glucose/Minimal Medium Agar Plates

To 450 ml of distilled water, 7.5 *g* agar, 50 ml 10x M9 salts, 0.5 ml 1 M magnesium sulphate and 500 µl 0.1 M calcium chloride were added and the solution autoclaved. Thiamine-HCl (500 µl of 1M stock solution) and 5 ml of a pre-autoclaved 20% glucose solution were added to the agar and the solution cooled to approximately 50°C before plates were poured.

Luria Bertani (LB) Medium

To 900 ml of distilled water, 10 *g* bacto-tryptone (Difco), 5 *g* bacto-yeast extract and 10 *g* NaCl were added. The solution was mixed thoroughly to completely dissolve all solutes, and the pH adjusted to 7.5 by the addition of 5 N NaOH. The volume was increased to 1 litre and sterilised by autoclaving.

Superbroth Medium

To 900 ml of distilled water, 12 *g* bacto-tryptone (Difco), 24 *g* bacto-yeast extract and 4 *g* glycerol were added. After autoclaving the broth was cooled to 60°C and 100 ml of sterile 0.17 M KH_2PO_4 / 0.72 M K_2HPO_4 added. The medium was stored at 4°C.

Special Broth Medium

To 434 ml of distilled water 50 ml of LB medium, 50 ml 10x M9 salts, 500 µl 1 M magnesium sulphate, 50 µl 1 M calcium chloride, 5 ml 20% Casamino acids, 5 ml 20% glucose, 500 µg thiamine and 100 mg ampicillin were added and the broth was used within 24 hours.

SOB Medium

To 400 ml of distilled water, 10 *g* bacto-tryptone, 2.5 *g* bacto-yeast extract, 290 mg sodium chloride and 90 mg potassium chloride were added and the solution autoclaved.

1 M magnesium chloride (5 ml) and 5 ml of 1M magnesium sulphate were added and the solution made up to 500 ml. The solution was filter sterilised and stored at 4°C.

SOG Medium

To 10 ml of SOB medium, 180 µl of 20% glucose solution was added.

TFB buffer

TFB medium contained 10 mM K-MOPS, 100 mM rubidium chloride, 45 mM manganese chloride, 10 mM calcium chloride and 3 mM cobalt chloride. The pH of the media was adjusted to 6.15 with 1 N potassium hydroxide, then filter sterilised and stored at 4°C.

Preparation of LB plates with ampicillin

Agar (15 *g*; Difco) was added to 1 litre of LB medium and autoclaved. When the medium had cooled to 50°C, ampicillin stock (100 mg/ml) was added to a final concentration of 60 µg/ml. In a biohazard hood approximately 35 mls of medium was poured into 85 mm petri dishes. Air bubbles were flamed and cooled plates were stored at 4°C for up to one month.

Converting LB Amp plates into X-gal plates

To a pre-made LB agar plate containing ampicillin, 40 µl of X-gal (20 mg/ml) and 4 µl of IPTG (200 mg/ml), were spread over the entire surface of the plate with a sterile glass spreader. The plates were then incubated at 37°C until all of the fluid had been absorbed. These plates were used within a few days.

2.2 MOLECULAR BIOLOGY TECHNIQUES

2.2.1 Plasmid Purification

Two methods were used to isolate plasmids from transformed bacterial cells, depending on the quantity and quality of the plasmid required. Small scale plasmid preparations were used when small amounts of plasmid were required for restriction endonuclease analyses, while large scale plasmid preparations were performed when a large amount of highly purified plasmid was required (*e.g.* for sequencing and *in vitro* transcription).

2.2.1.1 Small Scale Plasmid Preparation : Alkaline Lysis

Small scale plasmid preparations were based on the method by Sambrook *et al.* (1989), and were performed on colonies to determine positive transformants. The colonies were grown overnight at 37°C in 2 ml of LB medium containing 100 µg ampicillin, with vigorous shaking. The overnight cultures were pelleted by centrifugation at 12 000 x *g* for 1 minute, and the supernatant discarded. The pellet was resuspended in 100 µl of ice cold solution A (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 4 mg/ml lysozyme (Boehringer Mannheim)), and left at room temperature for 5 minutes. Two hundred microlitres of solution B (0.2 M NaOH, 1% SDS) buffer was added and the sample mixed by inversion and left for a further 5 minutes on ice. Protein, chromosomal DNA and high molecular weight RNA were precipitated by the addition of 150 µl solution C (3 M potassium acetate, 2 M acetic acid, pH 4.8), gentle vortexing and incubating on ice for 5 minutes before centrifugation at 12 000 x *g* for 5 minutes. The supernatant was extracted with an equal volume of phenol, and the resulting aqueous phase was collected and extracted once with phenol:chloroform:isoamylalcohol (25:24:1), and finally with 500µl of chloroform:isoamylalcohol (24:1). Plasmid DNA was precipitated with two volumes of absolute ethanol, and pelleted by centrifuging at 12 000 x *g* for 5 minutes. The DNA pellet was washed in 70% ethanol, vacuum dried, resuspended in distilled water and stored at -20°C.

2.2.1.2 Large Scale Plasmid Preparations

Two methods were used to isolate large amounts of high quality plasmid. The CsCl gradient centrifugation method was used to generate plasmid for subcloning, sequencing and some *in vitro* transcription of RNA, however the Qiagen DNA purification method also produced plasmid DNA of sufficient quality to use for *in vitro* translation and as PCR template.

Large Scale Plasmid Preparation and Purification by CsCl gradient centrifugation

A 10 ml culture of transformed *E. coli*, either JM109 or DH5α, in LB medium containing 100 μg/ml of ampicillin and 0.4% glucose, was grown overnight at 37°C with shaking, and used to spike 500 ml of either special broth *(see Section 2.1.2)*, LB medium *(see Section 2.1.2)* or Superbroth medium *(see Section 2.1.2)*, containing 100 μg/ml ampicillin, in a 2 litre flask for a further 24 hours at 37°C with orbital shaking. The bacteria were harvested by centrifugation at 10 444 x *g* for 10 minutes at 4°C using a Sorvall GSA rotor. The bacterial pellet was washed with 100 ml of STE buffer *(see Section 2.1.2)*, and centrifuged again. The pellet was resuspended in 20 ml of STE, and centrifuged at 1 090 x *g* for 10 minutes at 4°C using a Beckman JA-20 rotor. The pellet was resuspended in 3.5 ml buffer 1 (25% sucrose, 50 mM Tris-HCl, pH 8), and then 1.5 ml lysozyme (2.5 mg/ml) and 0.7 ml of 0.5 M EDTA added, and incubated on ice for 20 minutes. Lytic mix (5.5 ml; *see Section 2.1.1*) was added, mixed by pippetting up and down three times, and incubated for 20 minutes on ice. The supernatant was collected following centrifugation at 48 400 x *g* for 1 hour at 4°C in a Beckman JA-20 rotor. The supernatant was extracted twice with an equal volume of phenol : chloroform : isoamyl alcohol, and then once with chloroform : isoamyl alcohol. One gram of CsCl per ml of aqueous phase was added, and when dissolved, 800 μl 10 mg/ml ethidium bromide solution was added per 10 ml of CsCl solution.

Centrifugation at 183 252 x *g* of the DNA/CsCl solution was performed in a Beckman Ti 50 rotor for 36 h at 25°C. The lower supercoiled DNA band was collected using a 21 gauge needle and syringe, and the ethidium bromide extracted six times with an equal volume of water saturated butanol. Caesium chloride was removed by dialysis against 3 changes of 1 litre TE at room temperature in a Sartorius microcollodion bag (Sartorius AG, Goettingen, Germany). The DNA was ethanol precipitated, washed twice in 70 % ethanol, vacuum dried and resuspended in distilled water, then stored at -20°C.

Maxi Preparation of Plasmid DNA : Qiagen Method

Plasmid purification was carried out on a 100 ml bacterial culture using a Qiagen tip-100, according to the manufacturer's instructions. Briefly, the bacteria were pelleted by centrifugation at 15 000 x *g*, at 4°C for 10 minutes. The bacterial pellet was resuspended in 4 ml of buffer P1. Following the addition of 4 mls of P2 buffer, the mixture was incubated at room temperature for 5 minutes. Chilled buffer P3 (4 ml) was added, and the mixture incubated on ice for 15 minutes. Bacterial debris was pelleted by centrifuging the mixture at 30 000 x *g*, at 4°C for 30 minutes. The supernatant was immediately removed and centrifuged again (30 000 x *g* at 4°C for 30 minutes) to pellet any remaining bacterial debris. A Qiagen tip-100 was equilibrated with 4 mls of buffer QBT. The supernatant containing the plasmid was then applied to the tip, and the tip washed twice with 10 mls of buffer QC. The plasmid DNA was then eluted from the tip with 5 mls of buffer QF, and precipitated with 0.7 volumes of isopropanol (Sigma), by centrifugation at 15 000 x *g* at 4°C for 30 minutes. The resultant DNA pellet was washed with 5 mls of cold 70% ethanol and air dried before dissolution in 50 µl of TE.

2.2.2 Restriction Endonuclease Digestion

Plasmid DNA was analysed for appropriately sized cDNA inserts by restriction enzyme cleavage. Unless otherwise stated, digestions were performed on 0.5-1 µg of plasmid

DNA with the appropriate restriction enzymes (2-10 U of enzyme per μg of DNA at 37°C, for 2 h) in the enzyme buffer supplied by the manufacturer, and the products of digestion analysed by electrophoresis *(see Section 2.2.8).*

2.2.3 General procedures for RNase-free conditions

Ribonuclease (RNase)-free conditions were maintained for materials, chemicals and solutions used in the isolation and generation of RNA. Precaution against RNase contamination included the use of disposable latex gloves through out the experiments, and when preparing solutions, reagents and apparatus, using disposable plastic pipettes and tubes where possible, diethyl pyrocarbonate (DEPC, Sigma) treatment of water and solutions not containing Tris, and soaking electrophoresis equipment in 3% hydrogen peroxide overnight prior to use.

DEPC-treatment of solutions

Solutions not containing Tris-HCl had DEPC adding to them at a final concentration of 0.1% and stirred overnight at room temperature. The solutions were then autoclaved to break down any remaining DEPC.

2.2.4 Preparation of Total RNA

Two methods of RNA isolation were performed. RNA was isolated from the vast majority of samples by a modification of the CsCl method described by Sambrook *et al.* (1989). However some of the RNA samples used for *in vitro* translation were obtained using a modification of the method described by Ilaria *et al.*, (1985) and is referred to as the Morrison method.

RNA Isolation by CsCl Gradient Centrifugation

The tissues were homogenised in at least ten volumes of 3.1 M GTC solution *(see Section 2.1.1)*, using a Kinematica GmbH polytron (Littau, Switzerland) with a 7 mm probe for 30-45 sec on ice. The homogenate was spun at 3 000 x *g* for 10 minutes to pellet the connective tissue. The supernatant was mixed with caesium chloride and N-sarcosine to a final concentration of 220 mg/ml and 5.5 mg/ml respectively, and this solution was layered on top of a 5.7 M CsCl solution cushion *(see Section 2.1.1)* in a Beckman Quick-Seal polyallomer tube, and the tube heat sealed.

The tubes were then centrifuged at either 92 677 x *g* for 18 h at 20°C in a Beckman Ti 50 rotor, or at 245 000 x *g* for 2.5 h at 20°C in a Beckman TLA-120.2 rotor. The supernatant was then decanted and the RNA pellet resuspended in DEPC treated water. RNA was then precipitated with ethanol and the RNA pellet washed with 70% ethanol. The pellet was then dried under vacuum, resuspended in DEPC treated water and stored at -20°C.

RNA Isolation by the Morrison Method

Tissue was homogenised in at least 10 volumes of 3.1 M GTC solution using a Kinematica GmbH polytron (Littau, Switzerland) with a 7 mm probe for 30-45 sec on ice. The homogenate was spun at 3 000 x *g* for 10 minutes to pellet the connective tissue. The homogenate was then centrifuged at 12 100 x *g* for 15 minutes in a Beckman JA-20 rotor at 20°C. While vortexing the supernatant, a half volume of 100% ethanol was added to the solution, and then incubated at -20°C for 1 hour. The supernatant was then centrifuged at 12 100 x *g* for 15 minutes in a Beckman JA-20 rotor at 4°C, and the supernatant discarded. The pellet was resuspended in 3 M GTC solution, and a half volume of 100% ethanol added before incubating at -20°C for 1 hour once more. After

centrifugation at 12 100 x *g* for 15 minutes in a Beckman JA-20 rotor at 4°C, the supernatant was discarded and the RNA pellet resuspended in Proteinase K buffer *(see Section 2.1.1)*, containing freshly prepared proteinase K (400 µg/ml). The RNA solution was then incubated at 37°C for 15 minutes, before extracting twice with an equal volume of phenol : chloroform : isoamyl alcohol. The RNA was precipitated with twice the volume of ice-cold 100% ethanol at -80°C for 30 minutes, and then centrifuged at 12 100 x *g* for 15 minutes at -10°C in a Beckman JA-20 rotor to pellet the RNA. The pellet was then washed twice in 70% ethanol, dried under vacuum and resuspended in DEPC treated water.

2.2.5 Quantitation of DNA and RNA

To quantitate DNA and RNA, 5 µl of sample was diluted in 1000 µl of water and the optical density at 260 nm and 280 nm was determined using a Shimadzu UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan). DNA and RNA was considered pure if their OD_{260}:OD_{280} ratio was approximately 1.8 and 2.0 respectively. The concentration of DNA and RNA was based on one OD_{260} unit being equivalent to 50 µg of DNA per ml, and 40 µg of RNA per ml of solution respectively. In addition, 200 ng of RNA was electrophoresed through a 1x TBE, 1% non-denaturing agarose gel. The gel was stained in a 5 µg/ml solution of ethidium bromide for 10 minutes, and then destained in water for one hour. 18S and 28S ribosomal RNA bands were then visualised by subjecting the gel to UV (245 nm) light.

2.2.6 Polymerase Chain Reaction (PCR)

Primer Design

Primers were designed with the assistance of the Primer (version 0.5), computer program, (copyright 1991, Whitehead Institute for Biomedical Research). Criteria requested by the program were set to the following:

Optimal primer length:	24
Minimum primer length:	22
Maximum primer length:	26
Optimal primer melting temperature:	70°C
Minimum acceptable primer melting temperature:	65°C
Maximum acceptable primer melting temperature:	75°C
Minimum acceptable primer GC%	20
Maximum acceptable primer GC%	80
Salt concentration (mM)	65
DNA concentration (nM)	50
Maximum number of unknown bases (Ns) allowed in primer	0
Maximum acceptable primer self-complementarity (no. of bases)	12
Maximum acceptable 3' end primer self-complementarity (no. of bases)	8
GC clamp- how many 3' bases	0
Product range	variable

Synthesis and isolation of oligonucleotides

Oligonucleotides were synthesised by four different sources. Oligonucleotides LHR-S1 and LHR-AS2, were synthesised by the Flinders Medical Centre Oligonucleotide Synthesis Service. The oligonucleotides were cleaved from their support using 1.4 ml 28% NH_4OH which was passed through the column over 4-5 hours using, two, 2.5 ml syringes on either end of the column. The ammonia solution was then heated to 55°C

overnight in a sealed screw capped microfuge tube. The oligonucleotides were dried in a Speedi Vac, resuspended in DEPC treated water and centrifuged for 5 minutes 15 000 x *g* to pellet the benzamide. The oligonucleotides were then extracted four times with a half volume of water saturated butanol and the resultant pellet dried under vacuum.

Oligonucleotide LHR-AS5 was synthesised on a Pharmacia-LKB Gene Assembler Plus DNA synthesiser by Mr Terence Gooley at The Queen Elizabeth Hospital. Initially excess synthesis reagents were removed from the column by microfuging in 1.5ml Sarsdedt tubes for 1 minute at 1 000 x *g*. The oligonucleotide was cleaved from its support using 28% NH_4OH; the column was placed in 1.5mls of -80°C NH_4OH, and centrifuged again for 1 minute at 1 000 x *g* to soak the column. Cleavage was achieved by incubation at 37°C overnight. The ammonia solution was then removed and vacuum dried for approximately 2 hours, after which time the remaining oligonucleotide pellet was resuspended in 1 ml of DEPC treated water.

Oligonucleotide LHR-AS6 was obtained desalted from Bresatec (Thebarton, South Australia). Oligonucleotides LHR-S7, LHR-AS8 and LHR AS9 were obtained from Macromolecular Resources (Fort Collins, CO), and had been purified by low pressure reverse phase purification. All oligonucleotides were resuspended in DEPC treated water, diluted and stored at -20°C as 1 ml 2.5 µM aliquots.

The β-actin oligonucleotides were a gift from Dr Rhys Williams, (Dept. Haematology, Flinders Medical Centre, S.A. Australia).

Oligonucleotide concentration was determined as described for DNA *(see Section 2.2.5)*, with the exception that one OD_{260} unit was assumed to represent an oligonucleotide concentration of 33 µg/ml.

Standard amplification conditions

Unless stated otherwise, all PCR reactions were carried out as follows. Target DNA (50 ng) or cDNA (one twentieth of the reverse-transcription) was placed in a 0.5 ml reaction tube in a total reaction volume of 25 µl containing 250 nM of each of the oligonucleotide primers to be used, 200 µM of each dNTP (Boehringer Mannheim), 0.5 units of *Taq* DNA polymerase (Promega), 160 mM $MgCl_2$ in 1x Promega PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9 at 25°C), 1% Triton X-100). The reaction was overlayed with mineral oil (Sigma), to prevent evaporation. The DNA was then denatured by heating the reaction to 94°C for 5 minutes, before amplification was achieved by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes. To assess whether contamination occurred, negative controls (without cDNA or DNA) were included. Generally, PCR was performed in an OmniGene Temperature Cycler (Hybaid Ltd, Teddington, UK).

2.2.7 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as described *(see Section 2.2.4)*. Reverse-transcription was then accomplished as follows: RNA (1µg) was mixed with 0.5 µg of random-hexamers (Pharmacia) in a total volume of 20 µl of DEPC treated water. The RNA solution was then denatured by heating to 60°C for 5 minutes, followed by cooling on ice for 3 minutes. Twenty microlitres of a reaction master-mix, containing 400 Units of MMLV reverse-transcriptase (Gibco-BRL), 2x first strand buffer (Gibco-BRL), 20 mM DTT (Gibco-BRL) and 4 mM dNTPs, was then added and the reaction incubated at 37°C for one hour. The reaction was then diluted with 60 µl of sterile (but not DEPC treated) water, and stored at -20°C. RT-PCR was carried out using 5 µl of the cDNA mix as target, under the same amplification conditions described in section 2.2.6 of this chapter.

2.2.8 Gel Electrophoresis

Agarose gel electrophoresis

PCR products were routinely resolved by mixing with 6x DNA loading buffer *(see Section 2.1.1)* and electrophoresis through 1-1.5% agarose (Progen), 0.5x TBE horizontal slab gels, containing 100 ng/ml of ethidium bromide (Biorad). Bands were visualised by subjecting the gel to UV (254 nm) light.

Polyacrylamide gel electrophoresis

PCR products were occasionally resolved on 8% vertical polyacrylamide gels (37.5:1, acrylamide:bis ratio). A 30% stock solution of acrylamide (37.5:1; acrylamide: *bis*-acrylamide ratio), obtained from Biorad, was diluted in 1x TBE buffer, to which 420 µl of a 10% solution of ammonium persulfate (Biorad) and 10 µl of TEMED (Ultrapure), was added. Amplified fragments were visualised by staining the gel in ethidium bromide (5 µg/ml) and subjecting the gel to UV (254 nm) light.

2.2.9 Synthesis of RNA probes

<u>In vitro</u> transcription of RNA probes

RNA probes were generated from two sources, either linearised plasmid or directly from PCR product. CsCl purified DNA plasmids LHR-2&3, LHR-2, LHR-3 or ACT-1 were linearised by digestion with *Hind* III, *Xho* I, *Xho* I or *Xba* I respectively by the method described *(see Section 2.2.2)*. Linearised DNA (1µg) was electrophoresed *(see Section 2.2.8)* to check that digestion was complete, and the remaining DNA was extracted with phenol : chloroform : isoamyl alcohol. The Riboprobe® kit (Promega), was used to generate RNA probes incorporating α-^{32}P-UTP (Bresatec) by the *in vitro* transcription method. One microgram of linearised plasmid or PCR product (both of which contained an RNA polymerase promoter site), was incubated with 15 units of T7, T3 or SP6 RNA polymerase (Promega), and 50-100 µCi (approximately 3000 Ci/mmol) α-^{32}P-UTP

(Bresatec) in a final volume of 20 µl containing 1 U/ ml RNasin (Promega), 10 mM DTT, 250 µM ATP, 250 µM CTP, and 250 µM GTP pH 7.6, 10 µM UTP and 1x RNA Transcription buffer (Promega). In some experiments "cold" transcript was generated by substituting 250 µM UTP in the place of ^{32}P-UTP. The reaction was incubated at 37°C for 1 h, diluted with 78 µl of DEPC treated water. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM if the probe was required for Northern analysis, or by heating to 55°C for ten minutes if the probe was to be used in RNase protection experiments.

Determining ^{32}P-UTP incorporation

The percentage incorporation of ^{32}P-UTP into RNA probes was determined before using the probes for Northern or RNase Protection analyses. Briefly, this involved spotting 1 µl of probe onto two pieces of DE81 filter (Whatman), one marked "total", the other "washed", and allowing them to dry. The "washed" filter was then washed 4 x 5 minutes in 500 mM Na_2HPO_4 on a rotating platform. Both filters were then washed 2 x 2 minutes in water, once in 70% ethanol and allowed to dry. Scintillation fluid (4 ml) was added to the filters before counting in a β-counter (LS 3801, Beckman Instruments (Australia) Pty. Ltd.), and percentage incorporation determined by ("washed" counts / "total" counts) x 100%, with values between 55% and 85% incorporation acceptable.

2.2.10 RNA Analysis by Northern Blotting

Preparation of samples

Total RNA was isolated and quantitated as described *(see Section 2.2.4 and 2.2.5)*, and then electrophoresed through a denaturing formaldehyde gel as described by Sambrook *et al.* (1989). Briefly, this involved drying the required amount of total RNA under vacuum, and resuspending the pellet in 1x MOPS running buffer, 2.2 M formaldehyde (pH >4.0), and 50% deionised formamide. The sample was incubated at 55°C for 15

minutes and then RNA loading buffer added to give a final concentration of 1x loading buffer.

RNA electrophoresis for Northern blotting

The denatured RNA samples were loaded onto a 1.0% agarose gel (dimensions 15 x 15 x 0.5 cm, 15 x 7.5 x 0.5 cm or 7 x 7.5 x 0.5) containing 2.2 M formaldehyde, and 1x MOPS running buffer. The RNA was then separated by electrophoresis in 1x MOPS buffer, 2.2 M formaldehyde, at 3-4 V/cm until the bromophenol blue marker had migrated two thirds of the total distance.

Electrotransfer of RNA to nylon filters

Transfer of RNA from agarose gels to nylon membrane (Hybond N; Amersham) was performed by electroblotting based on the method described by Reed and Mann (1985). Briefly this involved sandwiching between the grates of a protein electroblotter, mesh soaked in 1x RNA transblot buffer, three sheets of 3 mm Whatman filter paper, the gel, a piece of Hybond N nylon filter, three more pieces of filter paper and then mesh, ensuring all air bubbles were removed. Both the filter paper and the nylon filter were cut to a slightly larger size than the gel and soaked in 1x RNA transblot buffer *(see Section 2.1.1.)* before assembly of the sandwich. The sandwich was then electrophoresed in a TE series Transphor electrophoresis unit (Hoefer Scientific Instruments) at >30 volts, 4°C overnight.

Staining nylon filters for RNA

Lanes containing 0.1-1 μg of total RNA were generally run as 18S and 28S ribosomal RNA markers, and after the RNA had been transblotted to Hybond N, these lanes were excised, air dried and soaked in 5% acetic acid for 15 minutes. The lanes were then

stained in methylene blue stain *(see Section 2.1.1)* and rinsed in water for 5 to 10 minutes.

Filter hybridisation

Filter hybridisation was based on the method described in Sambrook *et al.* (1989). Briefly this involved the following. Prior to hybridisation, filters were baked at 80°C for 2 h, then incubated for 10-18 h at 45°C in prehybridisation mixture (50% formamide, 0.1% SDS, 5x SSPE and 5x Denhardt's solution) to which 100 μg/ml of sheared and denatured herring sperm DNA was added as a blocking agent. The prehybridisation mixture was then removed and fresh prehybridisation mixture/herring sperm DNA, containing 1 x 10^6 cpm/ml ^{32}P-labelled cRNA probes (previously denatured by heating to 100°C for 5 minutes), was added. Hybridisation was carried out by incubating the filter overnight at 60°C or 50°C, depending on the particular probe used.

Washing the filter and autoradiography

Following hybridisation, the membrane was washed three times in 3x SSC (450 mM NaCl, 450 mM sodium citrate), 1 mM EDTA at room temperature for ten minutes. The membrane was then washed three times with 2x SSC, 0.1% SDS at 60°C for 15 minutes, three times with 0.5x SSC, 0.1% SDS at 60°C for 20 minutes and three times with 0.1x SSC, 0.1% SDS at 60°C for 20 minutes. The membrane was attached to a blotting paper support, wrapped in cling wrap and placed in a light-proof cassette next to XAR-5 film (Eastman Kodak), and exposed at -80°C for up to 72 hours. The blotting paper mount had been marked with radioactive ink, to help with the alignment of the blot after autoradiography. The film was developed in an automatic developer (Eastman Kodak).

Re-use of blots

Membranes could be stripped and re-probed by immersing them 2 x 20 minutes in boiling Northern blot stripping solution *(see Section 2.1.1)*. For stripping to be effective it was important that the blots were not permitted to dry out.

2.2.11 Cloning and subcloning of DNA fragments

Isolation of DNA fragments from TAE gels and from solution

DNA fragments to be cloned or subcloned fell into two categories; (1) plasmid inserts which were digested with restriction endonucleases to produce smaller fragments which could be subcloned, and (2) PCR products that were to be cloned directly, both required isolation of the DNA fragments from contaminants before cloning. DNA fragments to be cloned were isolated with the Geneclean® plus kit either directly from solution or from a TAE agarose gel, depending upon the size of the contaminating DNA.

PCR products were digested with the appropriate restriction endonuclease *(as described in section 2.2.2)*. Primers, nucleotides, non-specific products and the small restriction endonuclease digestion by-products were removed using the Geneclean plus kit (Bresatec) as described below. The concentration of PCR product was estimated by comparing the intensity of 5 µl of PCR product with 500 ng of pUC19 cut with *Hpa* II molecular weight markers (Bresatec), electrophoresed on an agarose gel, stained with ethidium bromide and viewed with a UV (254 nm) light source. The PCR products were then purified by adding 2.5 volumes of Geneclean® NaI stock solution. GLASSMILK® (5-8 µl/µg of DNA) was added and the solution vortexed over 10 minutes. The DNA/GLASSMILK® slurry was then centrifuged for a few seconds to pellet the GLASSMILK®. The pellet was washed three times in the supplied ethanol wash solution, and the DNA eluted from the GLASSMILK® by resuspending the pellet in 5-8 µl of water, and incubation at 50°C for five minutes. The DNA was isolated by pelleting

the GLASSMILK® and collecting the supernatant. Elution of the DNA was performed twice and the supernatants combined. DNA was extracted with phenol : chloroform : isoamyl alcohol to remove restriction enzyme activity before ligation into a vector.

The oLHR-2&3 insert was excised from the vector by restriction endonuclease digestion *(as described in Section 2.2.2)*, and the insert isolated from the vector by electrophoresis through a low melting point 1% agarose, 1x TAE gel *(as described in Section 2.2.8)*. The gel was stained with ethidium bromide and viewed under long wave length UV light, before cutting the band corresponding to the insert from the gel. The DNA was then extracted from the gel slice using the Geneclean® kit as described above, with the exception that 2.5 volumes of Geneclean® NaI stock solution was added to the mashed gel slice and the solution incubated at 55°C for 30 minutes to dissolve the gel, before adding the GLASSMILK®.

End filling restriction enzyme generated 5' overhangs

The 5' sticky end, or overhang, that is generated after digestion of DNA with some restriction endonucleases, can be filled to give a blunt end. This is used for two purposes; (1) when a blunt end is necessary for blunt ended ligation, and (2) when labelling the DNA fragments. Both use the following method, with the only exception that labelled bases are used in the end fill reaction for labelling reaction. Digested DNA (1 μg), containing 5' overhangs, was incubated with 50 μM of the appropriate dNTP's (or 1 μl of the appropriate labelled dNTP [10μCi/μl; 4000Ci/mM]) in 1x PCR buffer (2mM $MgCL_2$, 67 mM Tris, 16.6 mM $(NH_4)SO_4$, 0.45% Triton X-100, and 100 μg/ml gelatin) with 5 units of Klenow enzyme at room temperature for 10 minutes. The filled DNA was phenol : chloroform : isoamyl alcohol extracted and the DNA precipitated with two and a half volumes of ethanol.

Dephosphorylation of linearised vector DNA

Vector (500 ng) was incubated in 1x calf intestinal alkaline phosphatase buffer *(see Section 2.1.1)* and 0.5 units of calf intestinal alkaline phosphatase (Promega) in a final volume of 50 µl at 37°C 1 hour. The reaction was terminated by extracting with phenol : chloroform : isoamyl alcohol.

Ligation of DNA

Vector (50 ng) and a variable amount of purified DNA (PCR product or digested insert; in a 1:3 molar ratio respectively) were incubated overnight at 15°C, in ligase buffer with one Weiss unit of T4 DNA-ligase enzyme (Promega), in a total reaction volume of 10 µl. The reaction was terminated by heating to 72°C for 10 minutes. Ligation of polylinkers (Pharmacia) was carried out via the same method with the exception that the molar ratio of insert to polylinker was approximately 1:100.

2.2.12 Transformation of *E. Coli* cells

Two methods of transformation of *E. coli* cells were performed. The initial transformations of the ACT-1 and LHR-1 PCR products and subcloning of the LHR-A26 clone, were performed using the Hanahan's transformation method of JM109 cells. All other transformations involved transforming competent DH5α cells by electroporation.

Hanahan's Transformation of E. coli JM109

The *E. coli* JM109 cells were incubated at 37°C in 10 mls of SOB buffer *(see Section 2.1.2)* under constant shaking, until the cell density was 4-7 x 10^7 cells per millilitre (*i.e.* the solution had an optical density at 550 nm of 0.5-0.7). Cells were then incubated on ice for 10 minutes before pelleting (3 000 x *g*, 12 minutes at 4°C), washed in 3.3 ml TFB buffer *(see Section 2.1.2)* and incubated again on ice for 10 minutes. The cells were re-pelleted, then resuspended in 0.8 ml TFB buffer, and dimethyl sulphoxide (DMSO)

added to a final concentration of 3.5%, before incubating on ice for 5 minutes. DTT (28µl of a 1M stock solution) was added and the cells incubated on ice for 10 minutes, before more DMSO was added to increase the concentration to 7%, and the cells incubated on ice for another five minutes.

One quarter of the competent cells were mixed with to 2 µl of the ligation mix, containing approximately 10 ng of vector DNA, and the cells incubated on ice for 30 minutes. The cells were then "heat-shocked" by placing them at 42°C for 90 seconds, before incubating on ice for 2 minutes. The cells were resuspended in 800 µl of SOG buffer *(see Section 2.1.2)* and incubated at 37°C for 60 minutes under orbital shaking. One fifth of the cells were plated out on Luria Bertani (LB) plates containing 50 µg/ml ampicillin *(see Section 2.1.1)*, and incubated at 37°C overnight.

Preparation of competent DH5α E. coli cells for electroporation

The DH5α *E. coli* cells were cultured in 500 ml of LB at 37°C with shaking (200 rpm) to an OD_{550} between 0.5 and 0.7 before placing on ice for 10 to 15 minutes. The cells were then pelleted (4 000 x *g* at 2°C for 15 minutes), the supernatant decanted and the cells resuspended in 250 mls ice cold water. This was repeated twice more and then the cells resuspended in 20 ml of 10% sterile glycerol. The cells were re-centrifuged at 4 000 x *g* at 2°C for 15 minutes, the glycerol removed and resuspended in 2 ml of 10% sterile glycerol. The resuspended cells were then transferred to microfuge tubes in 80 µl aliquots and placed on dry ice for 10 minutes before storing at -70°C .

Transformation of competent DH5α E. Coli cells by electroporation

The competent cells were transformed with 2 µl of ligation mix, or the equivalent of 10 ng of vector DNA. An aliquot of cells were also transformed with supercoiled plasmid DNA (*eg.* pBluescript) as a positive control. Briefly, 40 µl of competent cells were

mixed with the DNA before they were loaded into a chilled 0.2 cm electrode gap, Gene Pulser cuvette (Bio Rad, Hercules, CA). The cuvette was then placed in the electroporation apparatus and electroporated (2.5 mV). Resistance of between 4000-6000 ohms indicated efficient electroporation. The cells were then washed with 1 ml SOC medium *(see Section 2.1.2.)* and incubated at 37°C for 60 minutes with shaking. The transformed cells were plated onto agar plates containing ampicillin (50 μg/ml), 0.5 mM IPTG and 80 μg/ml X-Gal, and incubated overnight at 37°C.

2.2.13 Analysis of Transformants

Standard colour selection allowed white (recombinant) colonies to be differentiated from colonies containing the parent vector (blue). The colonies were picked from the plate using sterile toothpicks, which were then used to inoculate firstly 5 mls of LB broth containing ampicillin (50 μg/ml), and secondly PCR reaction mix containing T7 and T3 promoter primers (see below). The inoculated cultures were incubated overnight at 37°C with constant shaking.

Screening bacterial colonies for recombinant plasmids using PCR

The standard PCR conditions described in section 2.2.6 were used to detect the presence of recombinant plasmids from freshly picked bacterial colonies, with the following exceptions. The initial denaturation of 5 minutes was followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 minutes, and 72°C for 30 seconds. The primers used for this reaction were the T7 promoter and T3 promoter sequencing primers (Promega), which span the multi-cloning sites of the pBluescript II SK (Stratagene, La Jolla, CA) and pBS (Stratagene) vectors. The size of the products were estimated from 1.5% agarose gels. Parent pBluescript II SK vector produced a 164 bp fragment, while vector plus insert produced an appropriately larger fragment.

2.2.14 Sequencing

Denatured plasmids (5 μg), were sequenced by the Sanger Sequencing method (Sanger *et al.*, 1977) using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH), according to the manufacturer's instructions.

Sequencing gels

Sequencing reactions were resolved on 8% polyacrylamide, 1x TBE gels containing 7M urea. A TBE solution containing 8% polyacrylamide (19:1; acrylamide: *bis*-acrylamide ratio), 40% stock obtained from Bio Rad, and 50% urea (w/v; Gibco BRL-Life Technologies Inc.) was heated to 50°C to allow the urea to dissolve. The solution was cooled to room temperature before adding 600 μl of 10% ammonium persulfate (Bio Rad) and 100 μl of TEMED (Gibco BRL-Life Technologies Inc.), and the acrylamide solution poured between the sequencing plates . Electrophoresis was carried out at 55 watts for 2-3 hours, after which time the gel was soaked in 10% acetic acid, 10% methanol to remove the urea. The gel was then gently attached to Whatman 2 mm blotting paper, and dried under vacuum at 80°C. The dried gel was exposed to XAR-5 film (Eastman Kodak,), overnight.

2.2.15 RNase Protection assays

Complementary RNA probes were generated as described in 2.2.9, diluted in twice the volume of DEPC treated water and incubated with 1.5 units of RNase free DNase 1 (Boehringher Mannheim), at 37°C for 15 minutes. The DNase 1 was then inactivated by extracting with an equal volume of phenol : chloroform : isoamyl alcohol. The percentage ^{32}P-UTP incorporation was then determined as described in 2.2.9, and 2 μl of the probe run on an 1 x TBE, 8% acrylamide gel containing 7M urea, to determine if at least 90% of the probe was a full length transcript *(see Section 2.2.14)*.

If the probe was determined to be of adequate quality, 1.2 µl was dried with the appropriate amount of RNA in a centrifugal evaporator, for less than 15 minutes (no heat). The probe/RNA pellet was then resuspended in 30 µl of hybridisation buffer *(see Section 2.1.1)*, denatured by heating it to 85°C, and then incubated in an oven at the appropriate temperature for at least 12 hours but not exceeding 20 hours.

The samples were digested with 350 µl of RNase digestion mixture *(see Section 2.1.1)*, containing 70 units of RNase T_1 (Boehringer Mannheim) and 7 µg of RNase A (Boehringer Mannheim), by incubating at 30°C for 40 minutes. Proteinase K (100µg) and 20 µl of 10% SDS was added and the reaction incubated at 37°C for 15 minutes, before termination of the reaction by extracting with an equal volume of phenol : chloroform : isoamyl alcohol. The aqueous phase was ethanol precipitated, centrifuged at 15 000 x *g* , and the pellet washed in 70 % ethanol before drying the pellet in a centrifugal evaporator, with the heat off and for no longer than 20 minutes. The pellet was then resuspended in 10 µl of RNase protection formamide loading buffer *(see Section 2.1.1)*, and heated to 85°C for 5 minutes to ensure the RNA was single stranded and to remove any secondary structure of the RNA. The tubes were then placed on ice and 4.5 µl of the sample loaded onto a pre-warmed 1 x TBE, 7.5% acrylamide gel containing 7M urea *(see Section 2.2.14)*. The gel was electrophoresed at between 2.5 and 1.8 kV until the xylene cyanol marker was within 15 cm of the bottom of the gel. The gel was then either washed to remove the urea if it contained lanes with ^{35}S on it *(see Section 2.2.14)*, or transferred directly onto Whatman 2 mm blotting paper, and dried under vacuum at 80°C. The dried gel was either exposed to XAR-5 film (Eastman Kodak) at -70°C, or placed under a phosphorimager screen (Molecular Dynamics Pty. Ltd, Melbourne, Vic., Australia) for an appropriate amount of time (*i.e.* 3-72 hours).

Analysis of RNase Protection Assays

RNase protection assay autoradiographs were analysed using a Bio-Rad GS-670 Imaging Densitometer with Molecular Analyst software version 1.01 (Bio-Rad, Hercules, CA). The RNase protection assay phosphorimages were analysed using a Molecular Dynamics Phosphorimager with Molecular Dynamics ImageQuaNT™ software version 4.1.

2.3 Solutions for Polyclonal Antibody Production

2.3.1 Polyclonal Antibody Production Stock Solutions

Phosphate Buffered Saline (PBS)

Phosphate buffered saline consisted of 139 mM NaCl, 8.2 mM Na_2HPO_4, 3 mM KCl and 1.5 mM KH_2PO_4, pH 7.4 in distilled water. The solution was sterilised by autoclaving and stored at 4°C .

Normal Saline

Normal physiological saline contained 0.9% (154 mM) NaCl.

Antibody Assay Buffer

The assay buffer contained 40 mM sodium phosphate (pH 7.4), 500 KIU/ml Aprotinin (Bayer Australia), 205 mM NaCl, 100 mM EDTA and 0.25% bovine serum albumin (RIA grade; Sigma Chemical Co.).

2.4 Polyclonal Antibody Production Techniques

2.4.1 Peptide design

Based upon the predicted amino acid sequence derived from ovine ovarian LH receptor cDNA splice variants (Bacich *et al.*, 1994), three peptides were designed, peptide "LHR all", peptide "LHR A&F", and peptide "LHR B&*G*". The location in LHR from which the amino acid sequences of the peptides were derived is discussed in chapter 5. The

peptides were synthesised by standard PIN technology by Chiron Mimitopes Pty Ltd (Clayton, Vic., Australia). Peptides used for immunisation were purified by HPLC to greater than 87% and cross-linked to Keyhole Limpet Hemocyanin via the cysteine residues using 6-maleimido-carpronic acid N-hydroxysuccinimide ester (Lee *et al.*, 1985). Peptides for iodination and determining antibody titres etc. were purified by HPLC to greater than 93% purity. All purification and cross-linking was performed by Chiron Mimotopes Pty Ltd.

2.4.2 Animals

Six adult male New Zealand White rabbits were obtained from and housed in the Flinders Medical Centre's Animal House. Ethical approval number 368/93 for the raising of polyclonal antibodies in these rabbits was obtained from the Flinders Medical Centre Animal Ethics Review Committee.

2.4.3 Immunisation Protocol

New Zealand White adult male rabbits (N=2 per peptide) were bled (20 ml) for pre-immune serum prior to the initial immunisation. The initial immunisation consisted of peptide conjugated to Keyhole Limpet Hemocyanin (KLH), (equivalent to 100 μg peptide), dissolved in 1 ml saline; (peptide "LHR B&*G*" had a small amount of propylene glycol (FMC pharmacy, Bedford Park S.A.) added to aid solubilisation), and mixed with an equal volume of Freunds complete adjuvent (Sigma Chemical Co., St. Louis, MO). The initial immunisation was sonicated for 30 seconds then injected subcutaneously via four sites on the rabbits' shaved backs. The rabbits were also immunised with 0.5 ml of Diphtheria, Tetanus and Pertussis toxin (25 LF units purified diptheria toxoid, 5 LF units purified tetanus toxoid and 20 000 million killed *B.pertussis* organisms; CSL, Parkville, Vic, Australia) intramuscularly into a single site on the leg, in order to further stimulate the immune response. Booster injections with sonicated conjugated peptide were made

every 4-8 weeks (equivalent of 100 μg peptide) using Freunds incomplete adjuvent (Sigma Chemical Co.; 0.5 ml peptide conjugate in saline plus 0.5 ml of adjuvent; intramuscularly). Blood samples (10 ml) were taken before every booster injection as well as 2-6 weeks after each injection. After approximately six months the rabbits were bled out, with at least 100 ml of blood per animal collected at this point. Rabbits were administered 1 ml of local anaesthetic intramuscularly (Acepromazine 10 mg/ml, Delta Vet Laboratory, Hornsby, NSW, Australia) 10-30 minutes prior to bleeding.

2.4.4 Collection of Serum

The blood samples were incubated at 37°C for 1 hour to ensure maximum clotting. The samples were then ringed, to prevent the clot sticking to the side of the tube, and placed at 4°C overnight. The clot was pelleted by centrifugation at 3 000 rpm for 15 minutes (Damon/IEC PR-6006, Paton Industries, Stepney, SA, Australia), and the serum removed. This was re-centrifuged, and the serum decanted and stored at -20°C or -80°C.

2.4.5 Iodination of peptides

Iodogen™ coating of tubes

Tubes were coated with Iodogen™ by the method of Fraker and Speck (1978), which entailed evaporating a 0.2% solution of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (100 μl), in a 1.5 ml microfuge tube overnight, before storing at 4°C.

Iodination of peptides

Unconjugated peptides were dissolved in 0.1 M acetic acid at 1 mg/ml and 5 μg iodinated with ^{125}Iodine by the Iodogen™ method of Fraker and Speck (1978). Briefly, this involved adding 5 μg of resuspended peptide to 30 μl of 0.5 M sodium phosphate buffer, pH 7.2, and 500 μCi Na^{125}I (Amersham Australia, North Ryde, NSW, Australia) in Iodogen™ coated tubes, and incubating at room temperature for 20 minutes.

Purification of the iodinated peptides

Unincorporated $Na^{125}I$ was removed using Sep-pak C18 cartridges (Waters Millipore, Milford, MS). Cartridges were activated by initially washing with 1 ml (80% methanol, 0.1% trifluroacetic acid (TFA)), and then equilibrating with 5 ml 1% TFA. Iodinated peptides were loaded onto the cartridges in 0.1% trifluroacetic acid and washed, to elute free $Na^{125}I$, with 0.1% TFA (5 ml). Peptides were eluted by washing the column in a series of 5 by 1 ml washes containing 0.1% TFA and increasing amounts of methanol (30%, 40%, 60% and 80%) and collecting 1 ml fractions. The amount of $Na^{125}I$ in each sample was determined using a gamma counter (LS 3801, Beckman Instruments Australia Pty. Ltd.) and the tubes containing the labelled peptide stored at -20°C. After the elution pattern was determined for each peptide, a two stage elution method was employed to isolate the radioactive peptide from unbound $Na^{125}I$ for subsequent iodinations, consisting of five by 1 ml washes of 0.1% TFA and then five 1 ml eluting washes of 0.1% TFA and the following amount of methanol (60% for "LHR all" and "LHR A&F"; 80% "LHR B&*G*"). These had the following specific activities respectively, 19.87 μCi/μg, 20.25 μCi/μg, and 22.39 μCi/μg.

2.4.6 Isolation of IgG

IgG was prepared from rabbit sera with the aid of caprylic (octanoic) acid (Sigma) as described by Steinbuch and Audran (1969). The sera was brought to room temperature, and its pH reduced to 4.8 by the drop-wise addition of 1 N acetic acid. Caprylic acid (74 μl per ml of serum) was added in a drop-wise manner, while the serum was stirred vigorously. After stirring for 30 minutes, the precipitate was removed by centrifugation 10 000 x *g* at room temperature and the supernatant containing the IgG fraction dialysed against PBS at room temperature initially, and then overnight at 4°C.

2.4.7 Determining Antibody Titre

The iodinated peptides were diluted in antibody assay buffer *(see Section 2.3.1)* to a concentration of approximately 15 000-25 000 cpm/100 µl. Diluted sera (100 µl), in antibody assay buffer, was mixed with tracer (100 µls) and antibody assay buffer (100 µls), and incubated overnight at 4°C. Unbound peptide was separated from peptide bound to antibodies in the sera by precipitation of the bound fraction. This was accomplished by mixing the sera with 100 µl of antibody assay buffer containing 8 mg human immunoglobulin/ml, 1.125 mg/ml glycine, and 2% thrimersol (w/v; CSL). Antibodies bound to the peptide were precipitated with 1.25 ml of 17.6% polyethylene glycol 6000 (PEG; BDH Laboratory Suppliers) at room temperature for 30 minutes. Tubes were then centrifuged (4°C, 3 400 rpms, 30 minutes; Damon/IEC PR-6006, Paton Industries, Stepney, SA, Australia), before the aspiration of the supernatant and the pellets counted on a gamma counter. The antibody titre was determined as the dilution of the antisera that gave 50% maximal binding.

2.5 Protein Analysis Solutions

2.5.1 Protein Analysis Stock Solutions

Transblot buffer

This consisted of 20% methanol, 20 mM Tris and 150 mM glycine.

Acrylamide gel stain

This consisted of 25% methanol, 10% acetic acid and 0.1% Coomassie® Brilliant Blue R-250 (Bio-Rad Laboratories). The stain was filtered through 2 mm Whatman filter paper and stored at room temperature.

Acrylamide gel destain

This consisted of 33% methanol and 10% acetic acid, and was stored at room temperature.

Acrylamide gel preservative

NaI_3 (30mg) was dissolved in 285 ml of distilled water and 125 ml of methanol and the solution stored at room temperature.

SDS-Acrylamide Gel Running Buffer

One litre of SDS-Acrylamide Gel Running Buffer contained 6 *g* Trizma® base, 28.8 *g* of glycine, 1 *g* of sodium dodecyl sulphate and 670 mg of EDTA dissolved in distilled water.

2x Denaturing Sample Buffer

This contained 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2 mM EDTA, 0.01% bromophenol blue and 2% SDS. The solution was sterilised by autoclaving and β-mercaptoethanol added to a final concentration of 360 mM. The 2 x denaturing sample buffer was stored at room temperature.

4x SDS Running Gel Buffer

This contained 1.5 M Tris-HCl (pH 8.8), 8 mM EDTA (pH 8.0) and 0.4% SDS. The pH was adjusted to 8.8 with 1 N HCl, and the solution was stored at room temperature.

4x SDS Stacking Gel Buffer

This contained 500 mM Tris-HCl (pH 6.8), 8 mM EDTA (pH 8.0) and 0.4% SDS. The pH was adjusted to 6.8 with 1 N HCl, and the solution was stored at room temperature.

Tris-Tricine SDS-PAGE Anode buffer

This consisted of 200 mM Tris-HCl (pH 8.9). This solution was stored at room temperature.

Tris-Tricine SDS-PAGE Cathode buffer

This consisted of 100 mM Tris-HCl (pH 8.25), 100 mM Tricine, and 0.1% SDS. This solution was stored at room temperature.

3x Tris-Tricine SDS-PAGE Gel buffer

This consisted of 3 M Tris-HCl (pH 8.45) and 0.3% SDS. This solution was stored at room temperature.

Nitrocellulose Protein Stain

This consisted of 45% methanol, 10% acetic acid and 0.05% napthol blue black stain (Sigma Chemical Co.) and the solution was stored at room temperature.

Nitrocellulose Protein Destain

Nitrocellulose protein destain contained 45 % methanol and 10 % glacial acetic acid. The solution was stored at room temperature.

Homogenisation Buffer A

This contained 100 mM $NaPO_4$, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% β-mercaptoethanol, 0.1% SDS and 1% Triton X-100. The solution was stored at room temperature, and just prior to use the following protease inhibitors were added at final concentrations of 5 mM N-ethylmaliamide (NEM), 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 μg/ml pepstatin A, 100 μg/ml bacitracin, 35 μg/ml aprotinin and 0.2

mM leupeptin (all protease inhibitors were obtained from Sigma with the exception of aprotinin, which was obtained from Bayer).

Homogenisation Buffer B

A 250 mM sucrose solution was buffered with 2 mM Tris-HCl, pH 7.5. This solution was stored at room temperature, and just prior to use the following protease inhibitors were added at final concentrations of 5 mM NEM, 1 mM PMSF, 1 μg/ml pepstatin A, 100 μg/ml bacitracin, 35 μg/ml aprotinin and 0.2 mM leupeptin.

Homogenisation Buffer C

This contained 100 mM $NaPO_4$, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% β-mercaptoethanol and 15% glycerol. The solution was stored at room temperature, and just prior to use the following protease inhibitors were added at final concentrations of 5 mM NEM, 1 mM PMSF, 1 μg/ml pepstatin A, 100 μg/ml bacitracin, 35 μg/ml aprotinin and 0.2 mM leupeptin.

Western Blot Buffers

Buffer A contained 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40 (Sigma) and 5% BSA.

Buffer B contained 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40 (Sigma), 0.25% deoxycholic acid and 0.1% SDS.

Buffer C contained 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl.

Bouins Fixative Solution

To 75 ml saturated picric acid solution, 25 ml 40% formamide and 5 ml glacial acetic acid was added. This solution was stored at room temperature.

2.6 PROTEIN ANALYSIS

2.6.1 Protein Isolation

General Homogenisation Method

Frozen and fresh tissues were homogenised in at least ten volumes of homogenisation buffer A *(see Section 2.5.1)* using a Kinematica GmbH polytron (Littau, Switzerland) for no longer than 30 seconds on ice. Samples were centrifuged at 15 142 x *g* for 10 minutes and the supernatants used for further analysis.

Microsomal Preparation

Tissues were homogenised in at least ten volumes of sucrose homogenisation buffer (homogenisation buffer B) or homogenisation buffer A containing 15% glycerol. The homogenised solution was then subjected to two passes of the potter-elvehjam. The homogenate was then centrifuged at 1 088 x *g* and the supernatant taken, this removes the connective tissue. The supernatant was centrifuged at 20 000 x *g* for 10 minutes at 4°C which pellets the nuclear fraction. The supernatant was then centrifuged at 100 000 x *g* for 60 minutes at 4°C, this pellets the microsomal/membrane fraction. The supernatant was considered the cytosolic fraction. Pellets were resuspended in homogenisation buffer, and re-pelleted to ensure clean preparations.

2.6.2 Quantitation of proteins

The concentration of proteins were determined using Bio Rad Protein Assay (Bio Rad), which is based on the Bradford Assay. Briefly, this involved creating a standard curve using proteins of known concentration (BSA) diluted in the same homogenisation buffer as the samples to be measured. Diluted protein (80µl) was mixed with Bio Rad Protein Assay dye reagent concentrate (20µl), placed in a well of a 96-well ELISA tray and analysed in a plate reader at 575 nm within 90 minutes of preparation. Protein

concentration was determined by comparison with the results obtained for the standard curve.

2.6.3 Iodination of Goat anti-Rabbit Antibodies

Two hundred micrograms of goat anti-rabbit immunoglobulin *G* and 20 µg lactoperoxidase in PBS, pH 7.2, was mixed with 2 mCi $Na^{125}I$ (100 mCi/ml; Amersham Australia, North Ryde, NSW, Australia) and 0.0225% hydrogen peroxide and incubated at room temperature for 5 minutes. The reaction was halted by adding a few grain of NaI_3, and the solution applied to a Sephadex *G*-50 column pre-equilibrated with PBS containing 0.1% BSA. Fractions containing the iodinated antibody were collected and the activity of the antibody determined using a gamma counter.

2.6.4 SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970), using the Protean II Gel Apparatus (Bio-Rad). Generally samples were electrophoresed through gels containing a 10% denaturing running gel (10% polymerised polyacrlyamide (acrylamide:bis-acrylamide 37.5:1) in 1x SDS Running Gel buffer), and a 5% denaturing stacking gel (5% polymerised polyacrlyamide (acrylamide:bis-acrylamide 37.5:1) in 1x SDS Stacking Gel buffer). Samples were prepared (reduced and/or alkylated) as described by Fairbanks *et al.* (1971). This involved mixing an aliquot of the sample with an equal volume of 2 x denaturing sample buffer *(see Section 2.5.1)*, then boiling the samples for five minutes before loading onto the stacking gel. Gels were run overnight at a constant current of 15 mA at 4°C in 1 x running buffer.

Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out as described by Schagger and von Jagow (1987) in an attempt to resolve low molecular

weight proteins *i.e.* proteins below 20 KDa in size. This involved electrophoresis of the samples through a three tiered gel. The separating tier consisted of a 16.5% denaturing running gel (16.5% polymerised polyacrylamide (acrylamide:bis-acrylamide 16.7:1) in 1x Tris-Tricine SDS-PAGE Gel buffer and 13% glycerol). The spacer tier consisted of a 10% denaturing spacer gel (10% polymerised polyacrylamide (acrylamide:bis-acrylamide 37.5:1) in 1x Tris-Tricine SDS-PAGE Gel buffer). The stacking tier consisted of a 4% denaturing running gel (4% polymerised polyacrylamide (acrylamide:bis-acrylamide 37.5:1) in 1x Tris-Tricine SDS-PAGE Gel buffer). Cathode buffer was placed in the upper tank and anode buffer in the bottom tank. Samples were prepared (reduced and/or alkylated) as described by Fairbanks *et al.* (1971). This involved mixing an aliquot of the sample with an equal volume of 2 x denaturing sample buffer *(see Section 2.5.1)*, then boiling the samples for five minutes before loading onto the stacking gel. Gels were run overnight at a constant current of 15 mA at 4°C in 1 x running buffer.

Staining Acrylamide Gels

Acrylamide gels that were not to be analysed by Western analyses were stained for at least 1 hour in acrylamide gel stain *(see Section 2.5.1)* and then destained in several changes of gel destain *(see Section 2.5.1)* overnight. These gels were then stored for up to three months in gel preservative *(see Section 2.5.1)*.

2.6.5 Protein Analysis by Western Blotting

Transfer of Proteins to Nylon

Proteins from SDS-polyacrylamide gels were transferred to Hybond™-C extra, using a TE series Transphor Electrophoresis Unit (Hoefer Scientific Instruments), as described by Gershoni and Palade (1982). This involved sandwiching the SDS-polyacrylamide gel and a sheet of Hybond™-C extra between 2 stacks of 3 pieces of 3 mm Whatman chromatography paper cut to the exact size of the gel, each soaked in 1x transblot buffer

(see Section 2.5.1) and ensuring that there were no bubbles present. The transfer was then subjected to 30 mAmp, 175 V at 4°C in 1x transblot buffer, for at least 12 hours.

Detection of Proteins

The lane containing the molecular weight markers was removed from the rest of the Hybond™-C Extra blot, and stained in nitrocellulose protein stain *(see Section 2.5.1)* for 1 hour, before destaining in several changes of nitrocellulose protein destain buffer *(see Section 2.5.1)* over 1 hour. The nylon filters containing protein to be immunodetected were incubated in Buffer A *(see Section 2.5.1)* at 37°C, with constant agitation for 45 minutes. Buffer A was removed, replaced with Buffer A (25 ml) containing the primary antibody (5 µl) and incubated for 2.5 hours at room temperature with constant agitation. The primary antibody solution was then removed and the blot washed three times in Buffer B, for 15 minutes each, and then rinsed in Buffer C. The blot was then incubated with 25 ml buffer A containing the ^{125}I iodinated goat anti rabbit antibody (2.5×10^7 cpm), for 45 minutes at room temperature with constant agitation. The second antibody was removed and the blot washed four times for 10 minutes each wash in buffer B. The blot was dried and either exposed to XAR-5 film (Eastman Kodak) at -70°C, or placed under a phosphorimager screen (Molecular Dynamics Pty. Ltd., Sunnyvale, CA) for an appropriate amount of time. The film was developed in an automatic developer (Eastman Kodak).

2.6.6 Immunoprecipitation

Immunoprecipitation of homogenised samples, as well as labelled products from *in vitro* translation reactions used the same basic method as described by Anderson and Blobel (1983). Incubation of homogenised samples or labelled product with washed *Staphylococcus aureus* cells (30 µl) at 4°C for 30 minutes was carried out, before centrifugation for 3 minutes at 1 200 x *g*. The pellet was discarded, and 4 µl of

preimmune sera added to the supernatant and the mixture incubated at room temperature for 60 minutes. *Staphylococcus aureus* cells (30 µl) were added, the solution mixed, and incubated for a further 30 minutes before centrifugation for 3 minutes at 1 200 x *g*. The supernatant was collected, and incubated with the antibody of interest (4 µl) overnight at 4°C. The next day, *Staphylococcus aureus* cells (30 µl) were added, and incubation carried out for 30 minutes at room temperature, before centrifugation (3 minutes at 1 200 x *g*). The supernatant was discarded and the pellet washed 6 times in PBS containing 0.1% SDS. Finally, the pellet was resuspended in 30 µl of 2x loading buffer.

2.6.7 *In vitro* Translation

In vitro translation of *in vitro* transcribed RNA transcripts, as well as mRNA extracted from various tissues was carried out using Promega's rabbit reticulocyte lysate system. Briefly 17.5 µl of Rabbit Reticulocyte lysate, 20 units of RNasin® Ribonuclease inhibitor (Promega), 20 µM amino acid mixture (minus methionine; Promega), [^{35}S]methionine (1,200Ci/mmol; Amersham International) at a final concentration of 0.8 mCi/ml, and either 20 µg of *in vitro* translated RNA or 50 µg of total RNA, were mixed in a 25 µl reaction and incubated at 30°C for 60 minutes. Sometimes non-radioactive reactions were performed, these were as above, with the exception that 40 µM total amino acid mix (Promega) was substituted for [^{35}S] methionine and amino acid mix minus methionine.

2.6.8 Immunohistochemistry

Fixing of Tissues

Tissues were collected fresh from the abattoir, and placed in Bouins fixative solution *(see Section 2.5.1)* for 24 hours, but not more than 48 hours. The Bouins solution was then decanted and the tissues washed in 70% ethanol, before being sent to the Histology Dept, (Flinders Medical Centre) to be placed into paraffin wax blocks.

Preparing the Slides

Microscope slides were thoroughly washed in Decon 90 and rinsed extensively in distilled water before soaking in a 0.01% poly-L-ornithine solution (Sigma) for 1 hour. Excess solution was drained and slides dried at room temperature overnight.

Mounting the Sections

Paraffin wax blocks were cut into 5 μm sections, with a microtome and mounted on the treated slides.

Immunohistochemistry

The paraffin wax was removed from the slides by heating them to 57°C for 10-15 minutes, followed by two 5 minute xylene washes. Next, the slides were rinsed in ethanol (2x 100% followed by 1x 90%), followed by 2x 5 minutes washes in water. Endogenous peroxidase activity was blocked by incubating the slides for 30 minutes at room temperature in 2% hydrogen peroxide, 98% methanol. The slides were rinsed with water (3x 5 minutes), followed by two 5 minute washes in PBS. Blocking of non-specific binding was achieved by incubating the slides in 3% goat serum in PBS (blocking solution), for 20 minutes at room temperature, in a humid box. Excess blocking solution was then removed from the slide, and 30-50 μl of the first antibody diluted (1:50) in blocking solution added to the section, before incubation for 30 minutes at room temperature in a humid box. The first antibody was washed away with PBS (3x 5 minutes). The slide was dried with a tissue, and 30-50 μl of biotinylated goat anti-rabbit secondary antibody (1:300 in blocking solution) added. Following a 30 minute incubation at room temperature in a humid box, the second antibody was washed off in the same manner as the first antibody. The slide was then incubated with avidin-biotin-horseradish peroxidase complex (1:300; Vectastain ABC Kit; Vector laboratories, CA). After a 30 minute incubation at room temperature the complex was removed by washing

twice for 5 minutes in PBS. The slide was then incubated in DAB (Sigma) for 6 minutes in a humid box, and washed in PBS (2x 5 minutes). Some slides were counter-stained with haemotoxylin, and washed in 3% ammonia followed by a rinse in water. The sections were dehydrated by washing them in ethanol (1x 90% for 2 minutes followed by 2x 100% for 2 minutes). Finally, the slides were washed in xylene (10 dips), and allowed to air dry before they were mounted with a coverslip using DPX mountant.

CHAPTER THREE

ANALYSES OF LH RECEPTOR mRNA IN THE OVINE OVARY

3.0 INTRODUCTION

Complementary DNA clones encoding the luteinizing hormone receptor were recently isolated from pig (Loosfelt *et al.*, 1989), rat (McFarland *et al.*, 1989), human (Minegish *et al.*, 1990), and mouse (Gundermann *et al.*, 1992) testes or ovaries. Many of the LHR cDNAs isolated from these species encoded incomplete and therefore possibly inactive forms of the LHR (Loosfelt *et al.*, 1989, Bernard *et al.*, 1990, Minegish *et al.*, 1990, Segaloff *et al.*, 1990, Tsai-Morris *et al.*, 1990, and Aatsinki *et al.*, 1992). The major incomplete cDNAs were due to alternative splicing of the full length LHR cDNA. Northern analyses of mRNA encoding LHR in these species revealed multiple mRNA species in ovarian tissue, but were unable to distinguish between the full length (functional) form and the splice variants. The ovine LH receptor cDNA sequence and the expression of its RNA transcripts in the ovine ovary was unknown. Therefore the aim of the experiments described in this chapter was to clone various parts of the ovine LHR and to determine if alternative splicing of the LHR gene occurs in the sheep, and if this does occur, to determine the relative abundance of the alternatively spliced forms.

3.1 Cloning of Regions of the Ovine LH Receptor

Ovine LH receptor cDNA clones were obtained from two sources. Clone oLHR-1 was generated by using RT-PCR to amplify a region of the ovine LH receptor mRNA transcript. The other ovine clone, oLHR-2&3, was obtained as an uncharacterised clone from Dr Richard Rohan (University of Maryland, Baltimore, U.S.A.) which had been partially sequenced to enable rudimentary identification. The clone was sequenced and subsequently sub-cloned to produce the clones oLHR-2 and oLHR-3.

3.1.1. Clone oLHR-2&3

Clone oLHR-2&3 was generated by RT-PCR of total RNA from a pool of small ovine ovarian follicles, using the primer pair LHR-S3 and LHR-AS4. These primers were designed based on the consensus exon nine and eleven cDNA sequences of the rat, pig and human luteinizing hormone receptor (McFarland *et al.*, 1989; Loosfelt *et al.*, 1989; Minegish *et al.*, 1990). The primers have a high degree of affinity with the homologous regions of the rat follicle stimulating hormone receptor cDNA sequence (Sprengel *et al.*, 1990), and the human, rat and canine thyroid stimulating hormone receptor cDNA sequences (Parmentier *et al.*, 1989; Akamizu *et al.*, 1990; Libert *et al.*, 1990)*(see Figure 3.1)*.

Clones of both the ovine LH receptor and the ovine FSH receptor were generated from these experiments. Dr Rohan partially sequenced the clones to confirm their identity, and also generated a partial restriction enzyme map of the insert.

```
LHR-S3        5'-ATAGTCGACTACCCCAGCCACTGCTGTGCTTT-3'
Rat FSHR          .GCC..AC.......................tgcaaacttgaag
Rat LHR           .CGC.GAC................C..C..cagg..t...cc.
Pig LHR           .C.T.GACT.......................ag....c..cca
Human LHR         .CGT.GACT.......................ag.......cca
Ovine FSHR        .GCC..AC.....................C..c.......g..g.
Human TSHR        GACC.TTCT.....A.................aag..tca....
Canine TSHR       GGCC TT.T.......................aag..t.a....
Rat TSHR          GGCC TTCT.....A..T.............. aag....a....

LHR-AS4       5'-AGCGAATTCCCTGTTTGCCAGTCAATGGC-3'
Rat FSHR          CAGCCTGCT.....................atagttgtggtactggc
Rat LHR           CAGCC.C.......C........T.....g.g...a.a........
Pig LHR           CA.CC.........C........T.....g.g...a.aa.......
Human LHR         CA.CC.C.......C........T.......g...a.a........
Ovine FSHR        CAGCCTGCT..A.....................................
Human TSHR        CA.CC.GGG.....C........G.......g.....a.....cag
Canine TSHR       CA CC GGG.....C........T.......g.....a........
Rat TSHR          CAGCC GGG..C..C..............g.g.....a........
```

Fig 3.1. Oligonucleotide primers LHR-S3 and LHR-AS4, and their homology to related species trophic hormone receptors. The primers are aligned with the homologous sequence of pig, rat, human and mouse LHR cDNA (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Minegish *et al.*, 1990; Gundermann *et al.*, 1992), and the homologous region from the rat and ovine FSH (Sprengel *et al.*, 1990; Yarney *et al.*, 1993) and the canine, human and rat TSH receptor cDNA sequences (Libert *et al.*, 1989; Nagayama *et al.*, 1989;). The sense primer, LHR-S3, binds to bases 831-857 of the rat LHR cDNA sequence (McFarland *et al.*, 1989). The anti-sense primer, LHR-AS4, binds to bases 1300 to 1321 of the rat sequence (McFarland *et al.*, 1989). Homologous bases are indicated with a dot, while mismatches are indicated with the appropriate letter. The sequence 3' to the peptide is indicated in lower case letters. The bold bases in LHR-S3 and LHR-AS4 correspond to *Eco*R1 and *Sal*1 restriction endonuclease sites that were artificially created for ease of cloning. Three random bases were added to the 5' end of each oligonucleotide to aid in digestion of the restriction endonuclease sites.

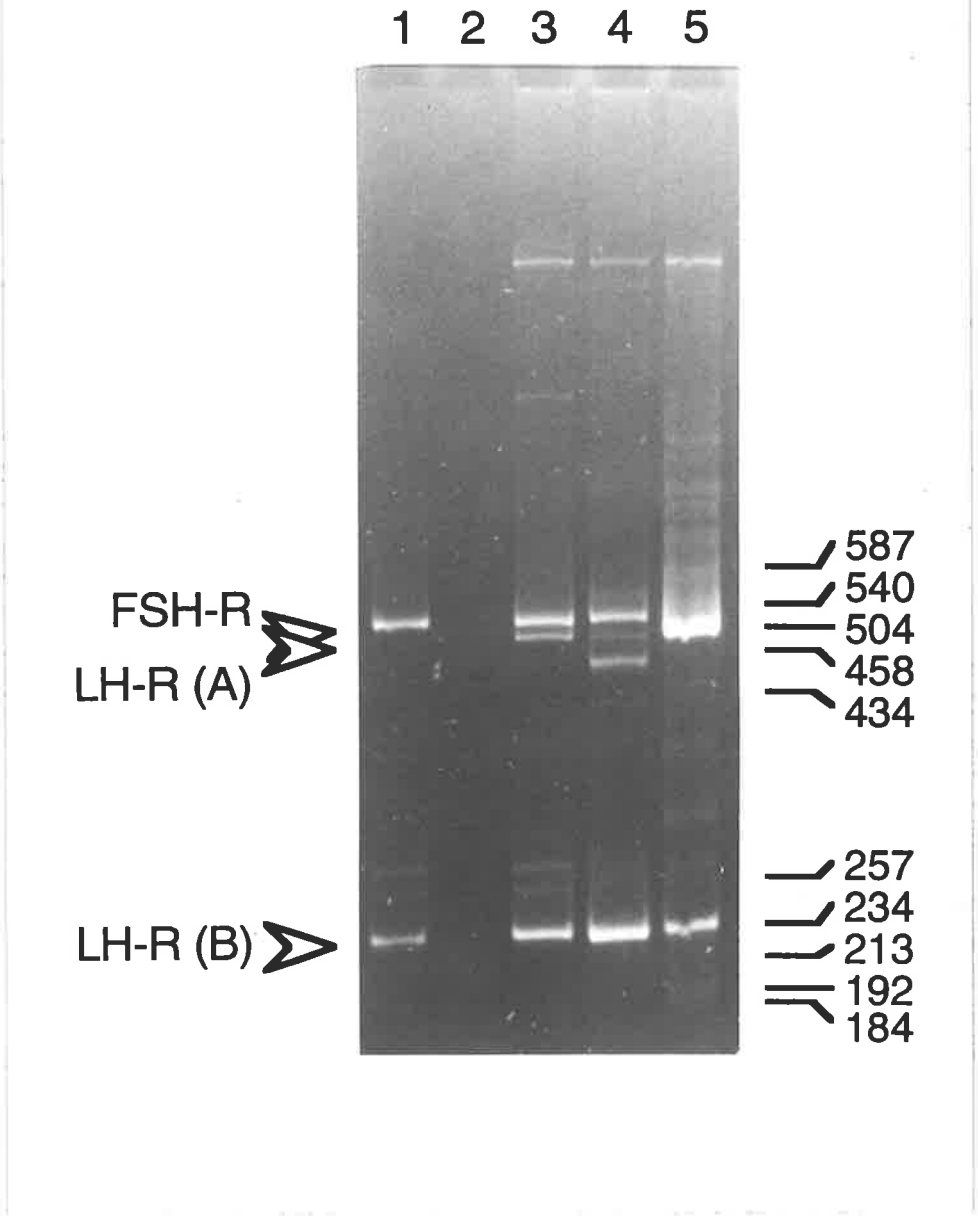

Figure 3.2 Amplification by RT-PCR of gonadotrophin receptors in ovarian RNA from rat, sheep and human. Oligonucleotide primers (LHR-S1 and LHR-AS2) specific for gonadotrophin receptors were used to amplify by PCR cDNA generated from (1) immature rat granulosa cells, (2) no target (negative control), (3) immature rat (25 day old) whole ovary, (4) a pool of 2-3mm ovine follicles collected on the day of oestrus, and (5) human ovary. Amplified PCR products were resolved on a 4% acrylamide gel, amplification of FSH receptor RNA from rat is expected to give a product of 521 bp (Sprengel *et al.*, 1990), whereas amplification of rat full length LH receptor is expected to give a 503 bp product (McFarland *et al.*, 1989). The size of the PCR product from the B splice variant of the rat LH-R is expected to be 237 bp (Bernard *et al.*, 1990). Figure courtesy of Richard Rohan.

Transformation of E.coli JM109 with oLHR-2&3.

DNA from the oLHR-2&3 plasmid, A26, was transformed into *E.coli* strain JM109 by the Hanahan method *(see Section 2.2.12)*, and positive transformants detected using ampicillin selection *(see Section Section 2.1.2)*. Small scale plasmid preparations were performed on a number of transformed colonies *(see Section 2.2.1.1)*, and the insert verified by restriction endonuclease digestion *(see Section 2.2.2)*, before selecting one colony and performing a large scale plasmid preparation and purification by CsCl gradient centrifugation *(see Section 2.2.1.2)*.

3.1.2 The bovine cholesterol side-chain cleavage cytochrome P-450 clone (T33.2)

As a positive control for Northern analyses, RNA was hybridised with probes generated from a clone of the bovine cholesterol side-chain cleavage cytochrome P-450 ($P\text{-}450_{scc}$), the rate limiting enzyme in the conversion of cholesterol into steroid hormones. The clone was obtained from Dr Ray Rodgers, and contained approximately 240 bases of the bovine $P\text{-}450_{ssc}$ cDNA as published by Morohashi *et al.* (1984). This clone was generated by subcloning the 5' 240 bps, of a larger (approximately 650 bp) bovine cholesterol side-chain cleavage cytochrome $P\text{-}450_{ssc}$ cDNA clone (pBSCC-2; John *et al.*, 1984), into pBluescript II SK+ (Stratagene) via the *Pst*1 restriction endonuclease site. The plasmid pBSCC-2 has been used to detect cholesterol side-chain cleavage cytochrome P-450 mRNA in ovine ovaries by Northern analyses (Rodgers *et al.*, 1987), and as such it was assumed that the smaller fragment of coding region also would detect ovine cholesterol side-chain cleavage cytochrome P-450 mRNA.

3.2 Northern Analyses of Ovine Ovarian RNA.

3.2.1 Sample Preparation

The majority of the ovine tissues was collected from cyclic ewes obtained from the local abattoir. Corpora lutea, follicles and stroma were frozen on dry ice within 20

minutes of death. Some corpora lutea were excised from ovaries at the laboratory after transportation in ice-cold Earle's Balanced Salt solution buffered with HEPES (pH 7.4; Sigma Chemical Company). RNA was prepared as a modification of the method described by Sambrook *et al.*, (1989; *see Section 2.2.4*), and its concentration and purity determined by spectrometry *(see Section 2.2.5)*. In addition, the RNA was electrophoresed through a 2% 0.5 x TBE , 1.5% agarose gel, and the 18S and 28S ribosomal RNA bands visualised by staining the gel with ethidium bromide, before viewing under ultraviolet illumination *(see Section 2.2.5)* to verify both the concentration of the RNA and that it was not degraded.

3.2.2 Generation of cRNA probes

^{32}Phosphorous-labelled complementary RNA probes were generated from oLHR-2&3 (linearized with *Hind*III) or from the bovine cholesterol side-chain cleavage cytochrome P-450 clone, T33.2, (linearized with *Bam*H1) using T7 RNA polymerase *(as described in Section 2.2.9)*. The percentage of ^{32}P-UTP incorporated into the probes was determined by the percentage of bound ^{32}P to DE81 filters *(as described in Section 2.2.9)*.

3.2.3 Northern blotting

Northern blots were prepared using total RNA from stroma, pooled follicles and corpora lutea *(described in Section 2.2.10)*. Marker lanes were removed and stained in methylene blue stain *(see Section 2.2.10)*, and the remaining lanes blocked with prehybridization mixture at 45°C for 18 hours *(as described in Section 2.2.10)*. The filter was then hybridized under the same conditions with ^{32}P-labelled LHR-2&3 and T33.2 cRNA probes at 10^6 cpm/ml for 18 hours at 60°C and 50°C respectively (*as described in Section 2.2.10*). The filters were then washed at 60°C and 50°C for the ovine and bovine probes respectively *(as described in Section 2.2.10)*, and autoradiography performed for up to 72 hours *(see Section 2.2.10)*.

3.3 Results and Discussion

Analyses of Northern blots of RNA from ovine ovarian stroma, pools of follicles and corpora lutea using the LHR-2&3 cRNA probe identified four bands of approximately 1.5, 2.5, 4.0, and 7 kb. As a control, the RNA from these tissues was hybridized with a cRNA probe for cholesterol side chain cleavage cytochrome P-450$_{scc}$. The expected sizes and relative amounts were obtained for side chain cleavage cytochrome P-450$_{scc}$, (Rodgers *et al.*, 1987), confirming the integrity of the RNA.

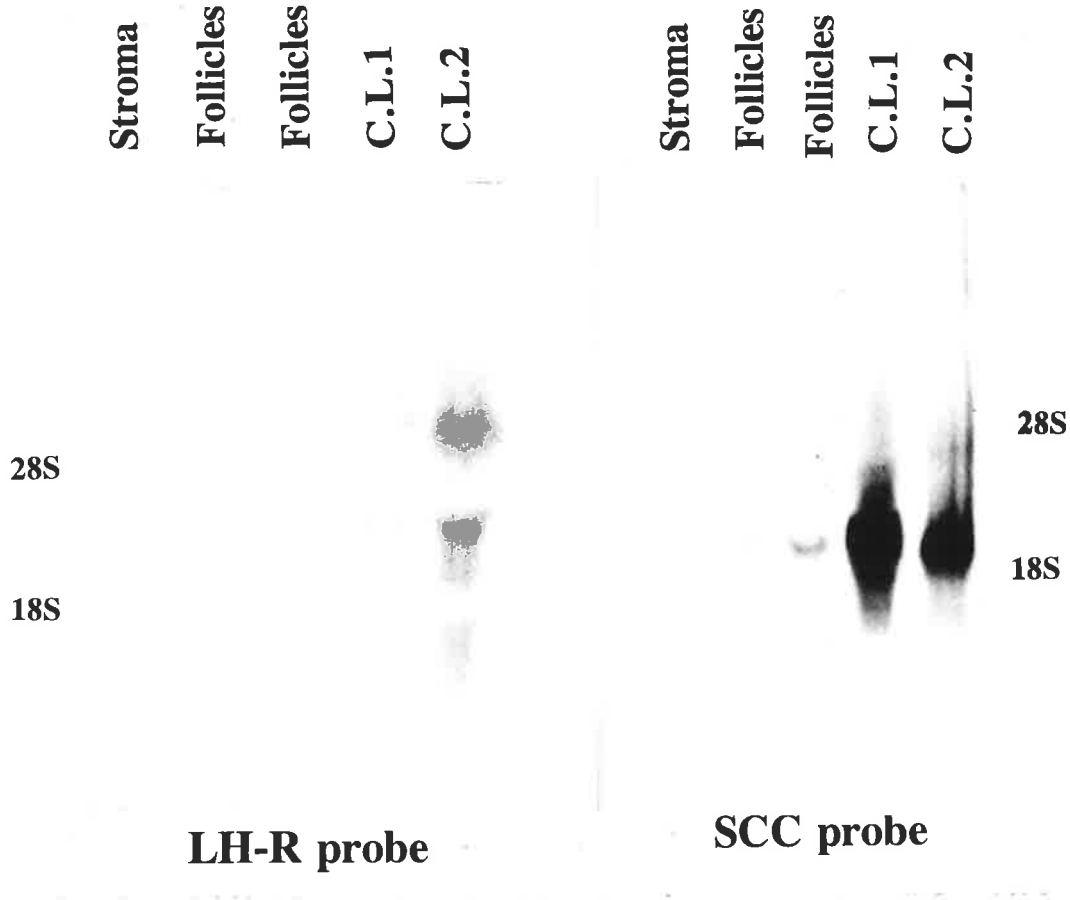

Figure 3.2b Northern blot analyses of LH receptor and cytochrome P450 cholesterol side-chain cleavage (SCC) mRNAs in ovine ovarian tissues. Ovaries were collected, and RNA was isolated from ovarian stroma (lane 1), pools of follicles (4-6mm; lanes 2 and 3), and corpora lutea in the midluteal phase (C.L. 1) and from a pregnant ewe (C.L. 2). Total RNA (20μg) was analysed by Northern blot analyses using cRNA probes for LHR (probe LHR-2&3) and cytochrome P450 cholesterol side-chain cleavage enzyme (scc probe). The autoradiograms were exposed for 72 and 24 hours respectively, at -70°C using intensifying screens. The locations of 28S and 18S ribosomal RNA band (indicated) were determined by staining a marker lane with methylene blue *(see Section 2.2.10)*.

The LH receptor Northern analyses revealed similar results in both the size and the number of bands to those reported from Northern analyses of the LH receptor of other species *e.g.* rat, pig, human (LaPolt *et al.*, 1990; Wang *et al.*, 1991; Loosfelt *et al.*, 1989; Minegish *et al.*, 1990). However, as in the other species, the different sized bands identified by Northern analyses can not be accounted for by alternative splicing alone. The majority of alternatively spliced forms of the LH receptor transcripts have at most a couple of hundred base pairs extra spliced out in comparison to the full length A form. The differences seen in the size of the bands detected by Northern analyses is far greater than 200 base pairs *(see Figure 3.3)*. Clearly, Northern analyses fail to distinguish the various splice forms of the LH receptor. Consequently, any quantitation of transcripts by Northern analyses to determine regulation of the LH receptor, is of limited value for two reasons. Firstly, it is not known which bands in Northern analyses correspond with which splice forms, and in particular the full-length A form. Secondly, if alternative splicing of the LH receptor mRNA transcripts is regulated independently of transcriptional regulation of the LH receptor gene, then changes in the amount of LH receptor mRNA present would not be a true indication of the changes occurring in the amount of full length A form mRNA alone. Due to the fact that the alternatively spliced forms of the LHR could not be distinguished from the full-length A form using Northern analyses, RNase protection analyses for ovine LHR were developed.

3.4 Cloning and Sub-Cloning of Regions of the Ovine LH Receptor

Ideally, the size of probes used for RNase protection assays should be between 150 and 400 bp in length. This helps in both the generation of full length probes and in distinguishing protected fragments from artefacts such as undigested probe (Dr. Ray Rodgers, personal communication). As the clone oLHR-2&3 was 488 bp, it was subcloned to form two smaller clones (oLHR-2 and oLHR-3). In addition, the smaller sized subclones assisted in sequencing of the entire clone from both directions.

A region of the ovine LH receptor cDNA which, by analogy with other species was not expected to be highly involved in alternative splicing, was also cloned (oLHR-1; *see Figure 3.3*). The primers used to generate this clone were also used in RT-PCRs to confirm the presence of the LH receptor transcript. The primers were localised in exons encoding the extracellular domain of the gene, and as such, any confusion that may have arisen due to the alternatively spliced regions or amplification of related gene transcripts (*e g.* FSH-R or TSH-R) was avoided.

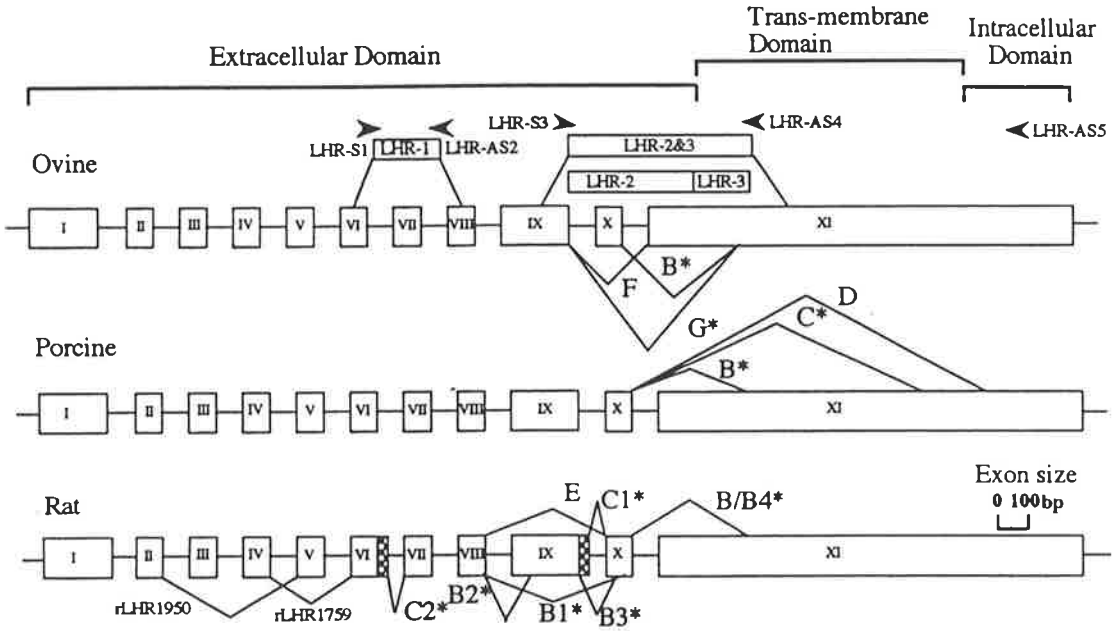

Figure 3.3 Comparisons of splice variants in the sheep, pig and rat luteinizing hormone receptor (LHR) genes. The known genomic structure of the LHR of the rat (Koo *et al.*, 1991; Tsai-Morris *et al.*, 1991) with exons of the full length A form shown as boxes, and introns (of the A form) shown as dotted lines (not to scale). Although unknown, the genomic structure of the pig and sheep LHR are depicted in similar fashion since the positions of introns 1-9 are identical in the rat LHR (Tsai-Morris *et al.*, 1991), rat FSH receptor (Heckert *et al.*, 1992) and the human TSH receptor (Gross *et al.*, 1991) and are therefore likely to be conserved in all mammals. Splice variants that have been sequenced [rat (Bernard *et al.*, 1990; Segaloff *et al.*, 1990; Aatsanki *et al.*, 1992), pig (Loosfelt *et al.*, 1989)] are depicted by lines that delineate the extent of the region that is removed by splicing. Each alternative splice event is labelled by its standard nomenclature. All splice forms identified with an asterisk result in a frame shift, and early termination of the putative translated proteins generated by these forms. Checkered boxes denote presumed intronic regions that are retained in some of the splice variants. The relative positions of the oligonucleotide primers (LHR-S1, LHR-AS2, LHR-S3, LHR-AS4, and LHR-AS5) used for RT-PCR are indicated on the ovine LHR map. LHR-1 and LHR-2&3 are the RT-PCR products that were cloned, sequenced and subsequently used as probes. LHR-2 and LHR-3 are subclones of LHR-2&3 that were also used as probes.

For use as a positive control for the RNase protection assays, a region of the ovine β-actin gene was cloned (oACT-1). The β-actin gene was chosen as it is ubiquitously and constitutively expressed and commonly used as a positive control in many Northern and RNase protection analyses.

3.4.1 Subcloning of the oLHR-2&3 clone

Partial sequencing of the oLHR-2&3 clone was required to identify a suitable restriction enzyme site for subcloning. This was performed via a modification of the Sanger sequencing method *(described in Section 2.2.14)*. Initial sequencing data was generated and a *Mae*III restriction enzyme site was identified approximately 150 bp from the *Eco*RI cloning site of the insert *(see Figure 3.4)*. The oLHR-2&3 insert was excised with the restriction enzymes *Hind*III and *Eco*RI, and purified *(as described in Section 2.2.11)*. The purified insert was then digested with the restriction enzyme *Mae*III, and the sticky ends end-filled with Klenow enzyme. Restriction enzyme polylinkers for *Eco*RI were then ligated to the fragments, and the ligated fragments digested with *Eco*RI. pBluescript SK+ was linearized by digestion with *Eco*RI, and the ends phosphorylated with calf alkaline phosphatase to prevent recircularization of the vector. The fragments were then ligated into the linearized pBluescript SK+ *(see Section 2.2.11)* and the ligated products were used to transform *E.coli JM109* cells by the Hanahan's transformation method *(see Section 2.2.12)*. Positive clones were detected using ampicillin and blue/white colour selection *(see Section 2.2.13)*.

Small scale plasmid preparations were carried out on transformed colonies *(see Section 2.2.1.1)*. The isolated plasmid DNA was then digested with the restriction enzymes *Xba*I and *Xho*I to determine which clones contained the correct inserts. At least five of each of the two different clones were sequenced in both directions *(see Section 2.2.14)*. A clone that contained 318 bp of the 5'-end of the oLHR-2&3 insert was designated oLHR-2, and a clone containing 150 bp of the 3'-end of the oLHR-2&3 insert was designated oLHR-3.

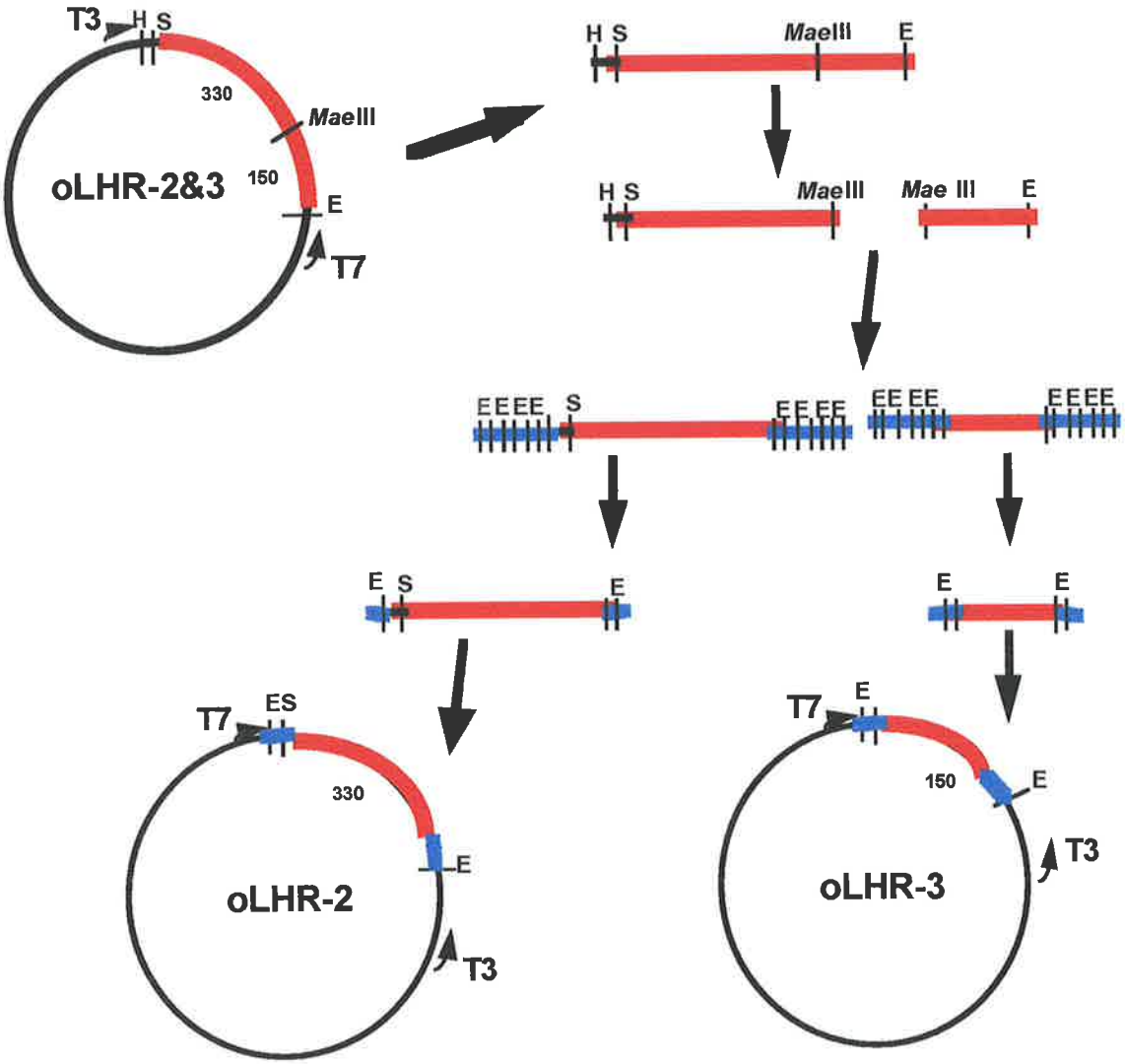

Figure 3.4 Strategy used to subclone oLHR2&3. The oLHR-2&3 insert (shown in red) was excised with the restriction enzymes *Hind*III and *Eco*RI. The purified insert was then digested with the restriction enzyme *Mae*III, and the sticky ends filled. *Eco*RI polylinkers (shown in blue) were then ligated to the fragments, and the ligated fragments digested with *Eco*RI. pBluescript SK+ was linearized by digestion with *Eco*RI, and the ends phosphorylated. The fragments were then ligated into the linearized pBluescript SK+ (shown in black).

3.4.2 Cloning of oLHR-1

Oligonucleotide primers (LHR-S1 and LHR-AS2) for use in RT-PCR were designed to amplify a region of the ovine LH receptor cDNA that was not likely to be involved in alternative splicing. As the ovine LH receptor cDNA sequence was not known, the primer design was based on regions of homology in the species that it is known, e.g. rat (McFarland *et al.*, 1989), pig (Loosfelt *et al.*, 1989) mouse (Gundermann *et al.*, 1992) and human (Minegish *et al.*, 1990).

When considering the potential primer sequences, regions containing amino acids that can be coded for by more than four codons were deliberately avoided. This was based on the premise that selection pressure on the LH receptor would only be exerted at the protein level. As such, amino acids that are encoded for by more than four codons could accommodate greater divergence at the DNA level with out changing the amino acid sequence than amino acids encoded by fewer codons. In addition, oligonucleotides were selected to generate a PCR product between 100 and 200 bp in length.

It was also important that the primers not amplify the related trophin receptors, i.e. TSHR (Libert *et al.*, 1989; Nagayama *et al.*, 1989) and FSHR (Sprengel *et al.*, 1990). Potential primer sequences were compared with the known cDNA sequences of these receptors to ensure a lack of homology, especially at the 3' end of the oligonucleotide from which extension occurs in the polymerase chain reaction.

It was also necessary to ensure that at least one intron be present between the primer binding sites, so that PCR product generated from cDNA and DNA target could be distinguished by its size. The genomic structure of the LH receptor is only known for the rat, however it is assumed that the LH receptor would be of a similar genomic structure in other mammalian species, given that the bases at the putative intron/exon boundaries appear to be highly conserved across species (Koo *et al.*, 1991).

The final consideration taken into account when designing this set of primers, was that the region to be amplified should have a similar GC content as that found in oLHR-2&3, so that the hybridisation temperatures for all the probes would be similar. The program Primer Designer was used *(as described in Section 2.2.6)* to assist in selection of the oligonucleotide sequences

```
LHR-S1      5'-AGCGTCGACGGAAATGCTTTYCAAGGGATGAA-3'
Pig LHR     5'-.C...ACCA.C.........C...........ta-3'
Rat LHR     5'-.C.A.ACC...G........C...........ta-3'
Human LHR   5'-.C.A.ACCA...........T...........ta-3'
Mouse LHR   5'- C.A.ACCA..G..C.....C...........ta-3'
Rat FSHR    5'-CATCGTTG.CAGG.ACTCC.T..T....CTG.gt-3'
Ovine FSHR  5'-CA.AGTTGAAAG..ATTC..T..T...GCT..gt-3'
Human TSHR  5'-TCAA..CCT.TG........T..G..AC.ATGca-3'

LHR-AS2     5'-GTAGAATTCGGAAGGCNYCRYTGTGCATCTT-3'
Pig LHR     5'-TGGCCCC.........................ctt-3'
Rat LHR     5'-TGGCCCCCT.......................ctc-3'
Human LHR   5'-TGGCCCCA........................ctc-3'
Mouse LHR   5'-TGGCCCCCT......T................ctc-3'
Rat FSHR    5'-AGGCTCCCT....AA.......AG...AT.Cttc-3'
Ovine FSHR  5'-AGGCTCCCT....AA.......AG...GT.Cttc-3'
Human TSHR  5'-TATACTCCTCC..ATG.ATC..TGT..ATAActc-3'
```

Figure 3.5. Oligonucleotide primers LHR-S1 and LHR-AS2. The primers are aligned with the homologous sequence of pig, rat, human and mouse LHR cDNA (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Minegish *et al.*, 1990; Gundermann *et al.*, 1992), and the homologous region from the rat and ovine FSH (Sprengel *et al.*, 1990; Yarney *et al.*, 1993) and the human TSH receptor cDNA sequences (Libert *et al.*, 1989; Nagayama *et al.*, 1989). Homologous bases are indicated with a ".", while mismatches are indicated with the mismatched base. The sequence 3' to the peptide is indicated in lower case letters. The bold bases in LHR-S1 and LHR-AS2 correspond to *Sal*1 and *Eco*R1 restriction endonuclease sites that were artificially created for ease of cloning

The primers chosen were termed LHR-S1 and LHR-AS2. The primer sequences relative to the homologous regions of the rat (McFarland *et al.*, 1989), porcine (Loosfelt *et al.*, 1989), human (Minegish *et al.*, 1990), and mouse (Gundermann *et al.*, 1992) LH/CG receptor cDNA sequences, and the rat and ovine FSH receptors

(Sprengel *et al.*, 1990; Yarney *et al.*, 1993) and human TSH receptor (Libert *et al.*, 1989; Nagayama *et al.*, 1989) cDNA sequences are shown in *Figure 3.5*. LHR-S1 and LHR-AS2 bind to the porcine LHR cDNA sequence at positions 496-518 and 657-634 respectively.

Total RNA was isolated from a number of ovine tissues (corpus luteum, pooled ovarian follicles, testis, kidney, liver, spleen and heart) by CsCl centrifugation *(as described in Section 2.2.4)*, and its concentration determined (*as described in Section 2.2.5)*. The RNA (1 μg/sample) was then reverse transcribed using random hexamers *(as described in Section 2.2.7)*. Polymerase chain reaction amplification of the ovine LHR region was then carried out using 5 U Taq DNA polymerase (Bresatec, Thebarton, Australia), 200 pM each of LHR-S1 and LHR-AS2 primers, one fifth of the reverse transcription reaction was used as template and 1x Bresatec PCR buffer (2mM $MgCl_2$, 67 mM Tris, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 100μg gelatin/ml, and 200 μm deoxy-NTPs) per 25 μl reaction total. Amplification consisted of 40 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and on the final cycle 72°C for 5 minutes. In some instances the PCR products were then incubated at 37°C with DNA polymerase-1 (Klenow fragment; Pharmacia) to ensure full extension of all DNA products.

One fifth of the polymerase chain reaction (5 μl) was subjected to electrophoresis (2% agarose, 1x TBE gel; *see Section 2.2.8*), to confirm that DNA of the correct predicted size had been amplified *(see Figure 3.7)*. The PCR product generated from corpus luteum cDNA was then digested with the restriction enzymes *Eco*R1 and *Sal*1 *(see Section 2.2.2)*, before ligating into linearized Bluscript SK+ (Stratagene Cloning Systems; *see Section 2.2.11*).

E.coli JM109 cell were transformed with the ligated products using the Hanahan's transformation method *(as described in Section 2.2.12)*. Positive transformants were

analysed by performing small scale plasmid preparations, followed by restriction endonuclease digestion *(as described in Sections 2.2.1.1 and 2.2.2)*, as well as analysis of colonies by PCR using T7 and T3 promoter primers (Promega; *as described in Section 2.2.13*). Large scale plasmid preparations were performed on colonies containing the insert, and plasmid DNA purified by CsCl gradient centrifugation *(see Section 2.2.1.2)* before sequencing.

3.4.3 Cloning of oACT-1

Oligonucleotide primers that amplify part of the β-actin cDNA of other species were obtained from Dr Rhys Williams, Department of Haematology, Flinders Medical Centre, Bedford Park, South Australia. The primer sequences were based on consensus regions of the β-actin cDNA between various species *(as indicated in Figure 3.6)*.

ACT-S1	5'-AT**GGATCC**GCCAACACAGTGCTGTCTGG-3'
Human β-Actin	5'-.CCTG.A.....gg-3'
Chick β-Actin	5'-..CTG.AT.....tg-3'
Rat β-Actin	5'-.CCTC.AT.....tg-3'
Mouse β-Actin	5'-.CCTC.AT.....tg-3'

ACT-AS2	5'-GC**GAATTC**TACTCCTGCTTGCTGATCCA-3'
Human β-Actin	5'-A.TCG.CA.....ca-3'
Chick β-Actin	5'-A.TCG.CA.....gc
Rat β-Actin	5'-A.TCG.CA.....gc
Mouse β-Actin	5'-A.TCG.CA.....gc

Figure 3.6 Sequence homology of ACT-S1 and ACT-AS2 with various species. The actin oligonucleotide primers are aligned with the homologous regions of the human, chicken, rat, and mouse β-actin sequence (Ponte *et al.*, 1984; Kost *et al.*, 1983; Nudel *et al.*, 1983; Tokunaga *et al.*, 1985). Homologous bases are indicated with a ".", while mismatches are indicated by the mismatched base. Bold type indicates the bases artificially introduced to generate *Bam*H1 and *Eco*R1 restriction endonuclease sites. The sequence 3' to the peptide is indicated in lower case letters.

Primers ACT-S1 and ACT-AS2 bind to the human β-actin cDNA sequence at positions 883-902 and 1085-1066 and are located within exons five and six, respectively. It was calculated that amplification by PCR using these primers would generate a 203 bp fragment from human or rat cDNA, and 316 bp or 328 bp fragment from human or rat genomic DNA.

RT-PCR using these primers and subsequent cloning of the PCR product was performed as described for the oLHR-1 clone *(see Section 3.4.2)*, with the following exceptions. The amplification reaction was carried out for 40 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute with the final cycle 72°C for 5 minutes. The PCR products generated from corpus luteum cDNA were then digested with restriction enzymes *Eco*RI and *Bam*HI *(see Section 2.2.2)*, before ligation into linearized Bluscript SK+ (Stratagene Cloning Systems; *see Section 2.2.11*).

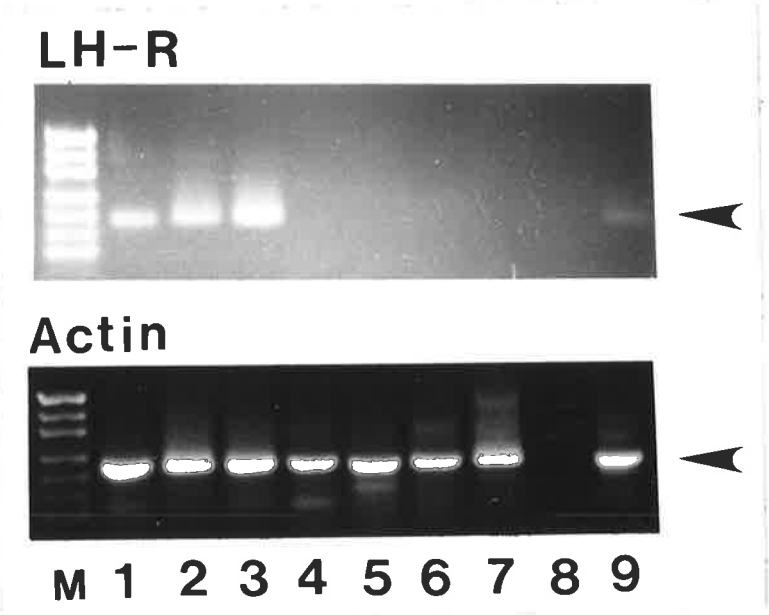

Figure 3.7 Amplification of ovine LHR and actin by RT-PCR. Oligonucleotide primers LHR-S1 and LHR-AS2 (see Figure 3.5), specific for the LHR (upper panel), and ACT-S1 and ACT-AS2, specific for actin (lower panel), were used to amplify cDNA generated from tissues of cycling ewes and testis from adult rams. These tissues included corpus luteum (lane 1), a pool of small (1-3 mm) follicles (lane 2), a pool of large (4-6 mm) follicles (lane 3), kidney (lane 4), liver (lane 5), spleen (lane 6) and testis (lane 8). Lane 7 is the negative control (no template). Amplified PCR products were resolved on a non-denaturing 2% agarose gel. The PCR products of correct predicted sizes for LHR and actin are indicated (arrows). The molecular weight markers (M) are pUC19 digested with *Hpa* II.

3.5 Sequencing of the Ovine LH Receptor Clones

At least three different clones of oLHR-1, oLHR-2 oLHR-3 and oACT-1 were sequenced completely in both directions via a modification of the Sanger sequencing method using T7 and T3 promoter primers (Promega), and Sequenase® (DNA polymerase, USB; *as described in Section 2.2.14*). The sequencing products were then resolved on a denaturing 1x TBE, 8% acrylamide gels containing 7M urea *(as described in Section 2.2.14)*, and autoradiography performed. The sequencing data was obtained before using the program MacVector to analyse sequence homology at both the cDNA and predicted amino acid level with other species *(see Figures 3.8, 3.9, and 3.10)*. The DNA sequences were then submitted to GenBank under accession numbers U08203 (oLHR-1), U08204 (oLHR-2&3) and U08283 (oACT-1).

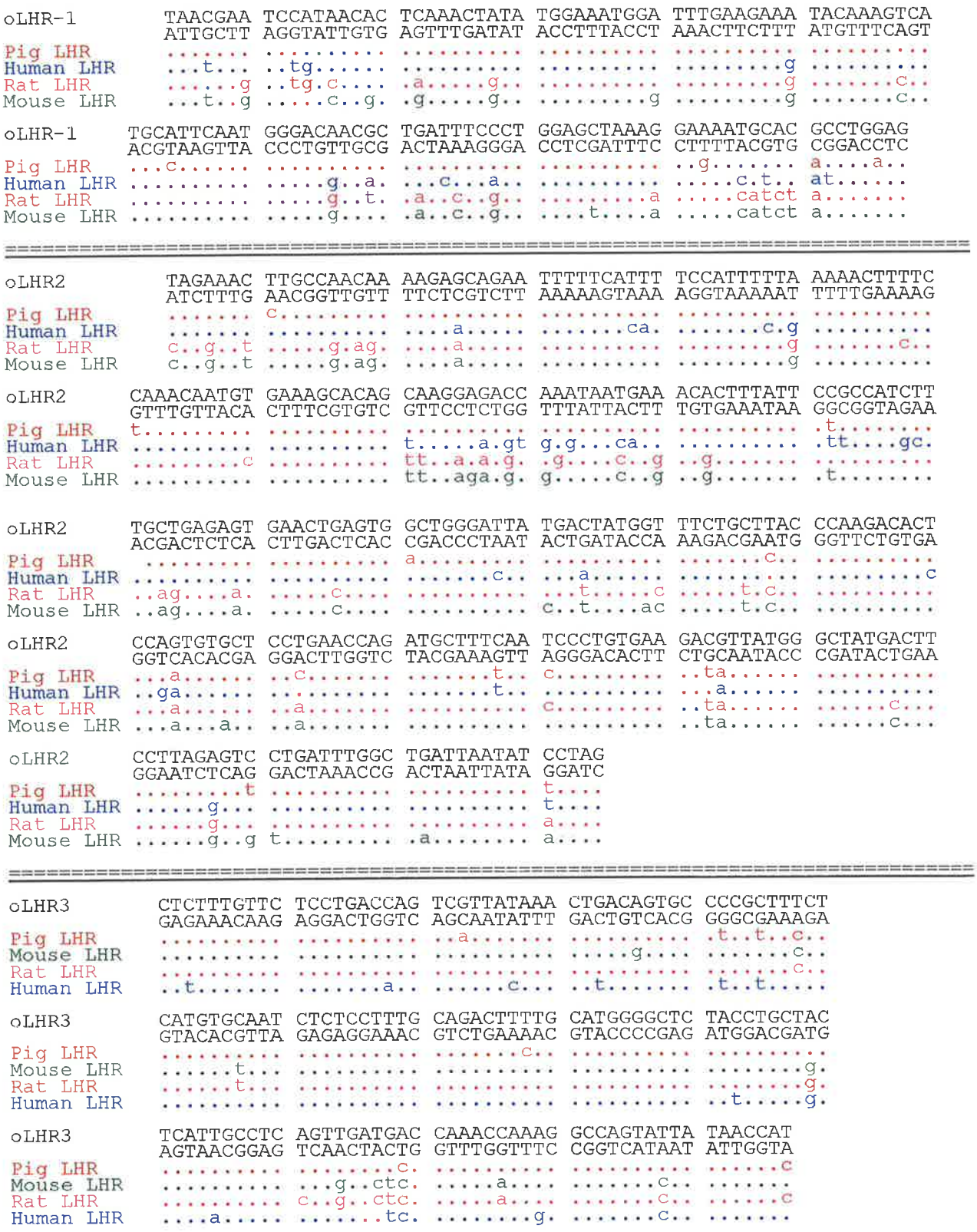

Figure 3.8 cDNA sequence comparison of oLHR-1, oLHR-2, and oLHR-3 with the complementary region of LHR cDNA sequence in other species. Homologous bases are indicated with a ".", while mismatches are indicated by the mismatched base.

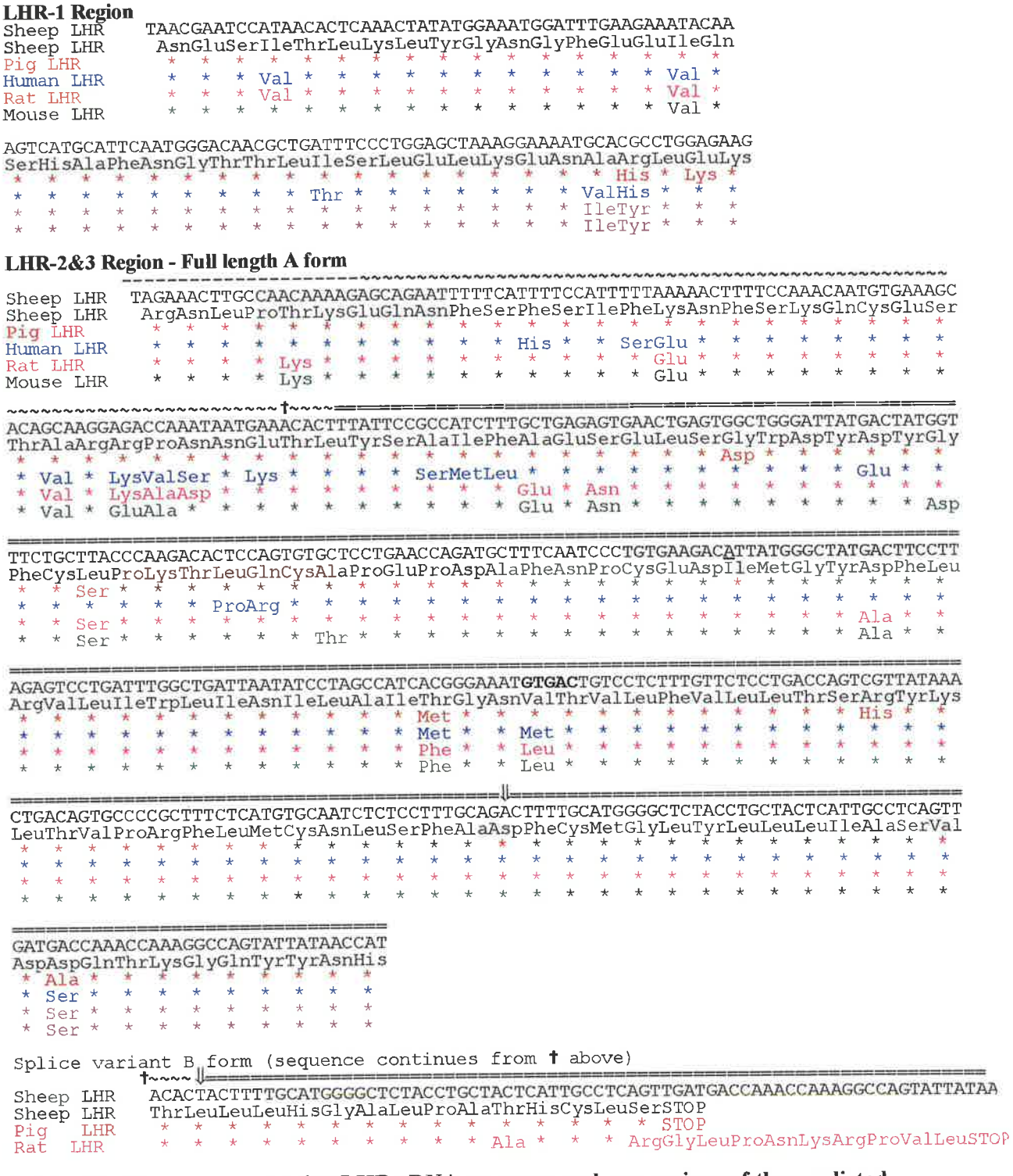

Figure 3.9 The consensus ovine LHR cDNA sequence and comparison of the predicted ovine amino acid sequences with LHR sequences from other species. Complementary DNA sequence and amino acid sequence of the regions corresponding to LHR-1, and LHR-2&3 in sheep (see Figure 3.3), aligned to those of the pig (Loosfelt *et al.*, 1989), rat (McFarland *et al.*, 1989), human (Minegish *et al.*, 1990) and mouse (Gundermann *et al.*, 1992) amino acid sequences. Asterisks represent homologous amino acids. For region LHR-2&3 both the full length A form and the common splice variant B form sequences are shown. ---- represents exon IX, ~~~~ represents exon X, and ===== exon XI. The ⇓ represents the 3' splice site in exon XI for the B splice variant. The *Mae* III restriction endonuclease site used in subcloning LHR-2&3 into LHR-2 and LHR-3 is shown in bold lettering. The underlined A represents the consensus sequence, whereas a PCR generated A to G substitution occurred in the original LHR-2&3 clone A26.

```
Sheep    TGGCACCACCATGTACCCTGGCATCGCCGACAGGATGCAGAAGGAGATCACCGCG
Sheep     GlyThrThrMetTyrProGlyIleAlaAspArgMetGlnLysGluIleThrAla
Human β    *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Human γ    *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

CTGGCTCCGAGCACGATGAAGATCAAGTTTATCGCTCCTCCCGAGCGCAAGTACTCGGTGTGG
LeuAlaProSerThrMetLysIleLysPheIleAlaProProGluArgLysTyrSerValTrp
 *  *  *  *  *  *  *  *  * Ile *  *  *  *  *  *  *  *  *  *  *
 *  *  *  *  *  *  *  *  * Ile *  *  *  *  *  *  *  *  *  *  *

ATCGGAGGCTCCATCCTGGCCTCCCTGTCCACCTTCCAGCAGATG
IleGlyGlySerIleLeuAlaSerLeuSerThrPheGlnGlnMet
 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
```

Figure 3.10 Ovine actin cDNA sequence and comparison of its amino acid sequence with that of the human. Complementary DNA sequence generated by RT-PCR of the region corresponding to ACT-1 in sheep and amino acid alignment to the human β and γ actin sequences (Ponte *et al.*, 1984; Erba *et al.*, 1986). Asterisks represent homologous amino acids.

3.6 Ovine LHR and Actin RNase Protection Assays

Development of the RNase protection assays for the ovine LHR made it possible to determine the abundance of the alternatively spliced forms relative to the full length mRNA that encodes the functional LH receptor. This had two distinct advantages over RT-PCR. Firstly, RT-PCR using the primers LHR-S3 and LHR-AS4, amplified the FSHR mRNA transcript as well as the LHR transcripts *(see Figure 3.2)*, leading to confusion in the interpretation of results. There has been reports of alternative splicing of the FSHR as well as the LHR in most mammalian species. At the time these experiments were carried out, it was not known if the sheep LHR or FSHR was subject to alternative splicing events. This uncertainty would have caused major problems with analysis of the results. Although expression of functional FSH receptors has not been reported in either corpora lutea or thecal cells, it was possible that low levels of FSHR mRNA transcripts or non-functional (alternatively spliced) FSHR transcripts could be detected by the sensitive technique of RT-PCR in these ovine cell types. These problems are avoided by using the RNase-protection assay, as even one or two base pair mismatches between the probe and target RNA can result

in RNA digestion, making this technique far more specific than RT-PCR. Therefore, if the LHR cRNA probe bound to FSHR mRNA, there would be insufficient homology to protect a large fragment from RNase digestion.

The other advantage of using the RNase protection assay in preference to RT-PCR to examine LHR mRNA is that the RNase protection assay is quantitative. Determining the relative level of expression of the alternatively spliced transcripts by RT-PCR would have been extremely unreliable, as amplification of smaller molecular weight forms is generally favoured in the polymerase chain reaction. The alternatively spliced forms would be significantly smaller than the full length A form, and as such the relative abundance of these forms would probably be over-estimated.

Furthermore, another advantage of RNase protection analysis over RT-PCR is that in the latter if one of the primers is complementary to a region that is spliced out in an alternatively spliced form, then this transcript would not be amplified, even if it made up a significant proportion of the ovine LH receptor transcripts.

3.6.1 Strategic Design of the RNase Protection Assays

The RNase protection assays were designed so that different sized protected fragments would be produced depending upon which splice variant of the LHR mRNA hybridised to the probe. The predicted sizes of protected fragments expected from the known forms described in the other species for the oLHR-2 and oLHR-3 probes are shown in Table 3.1, and graphically represented in Figure 3.11. In addition, these RNase protection assays had the potential to identify any novel splice forms that are alternatively spliced in these regions, but which have not been reported in other species. The RNase protection assays using the oACT-1 probe were expected to generate only one protected fragment of 203 nucleotides in length.

LHR splice form	oLHR-2	oLHR-3
A	316	178
B/B4	127	96
C	127	0
D	127	0
E/A2	270	178
EB	81	96
B1	266	178
B2	316	178
B3	266 & 46	178
C1	270 & 46	178
C2	316	178
rLHR1759	316	96
rLHR1950	316	178

Table 3.1. Predicted sizes of protected fragments for the known variants of the LH receptor in RNase protection assays using the probes oLHR-2 and oLHR-3.

The size estimation of the protected fragments of the RNase protection assay was based on DNA sequencing ladders or labelled DNA markers. Unfortunately, under the conditions normally used (polyacrylamide/7M urea gel; 40-45V/cm), RNA runs approximately 5-10% more slowly than single-stranded DNA of the same size (Sambrook *et al.*, 1989). Therefore, although the molecular weight markers were useful, it was necessary to take into account the inherent inaccuracies of comparing the sizes of DNA and RNA fragments.

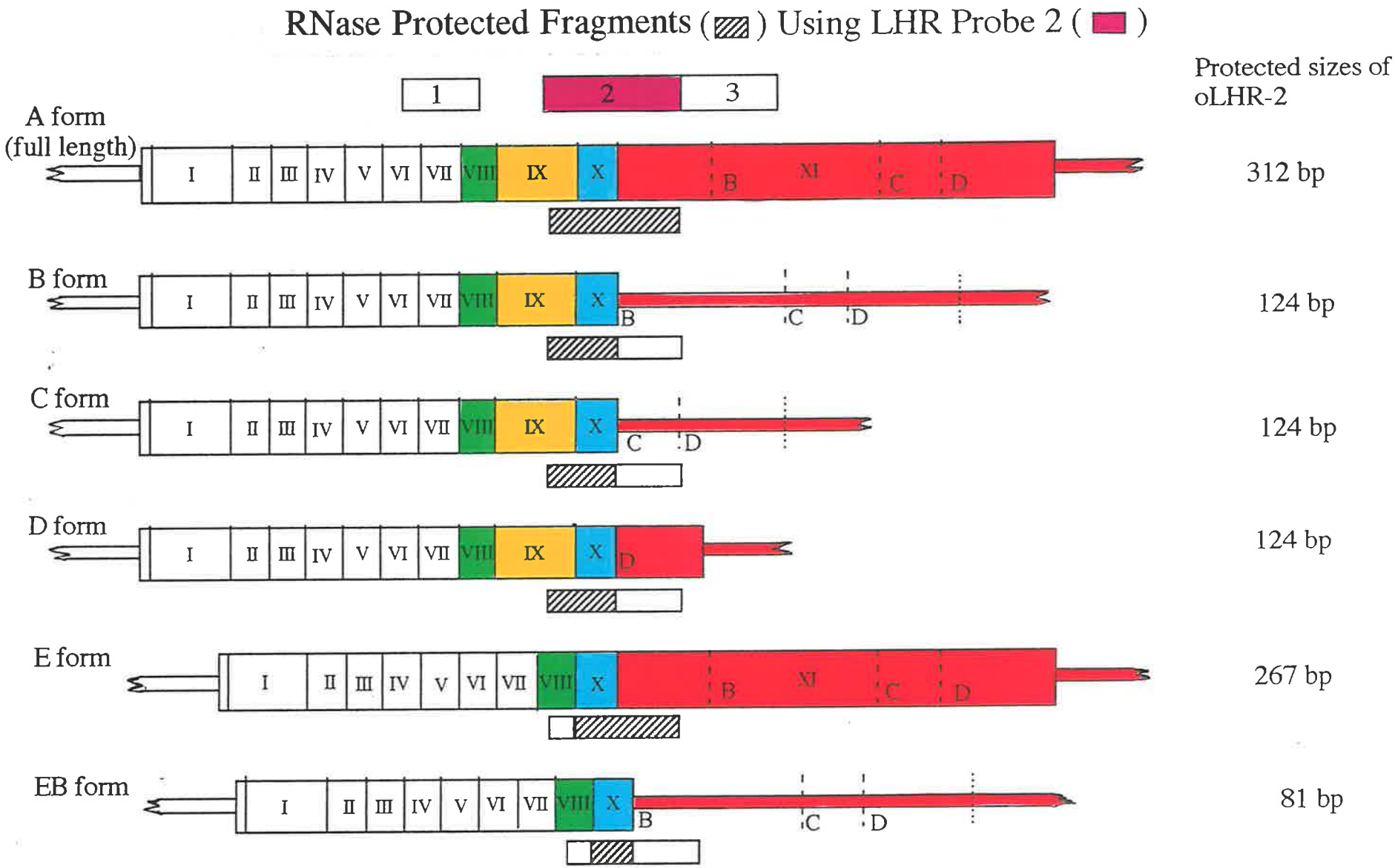

Figure 3.11 Graphical representation of predicted protected fragments in the RNase protection assay using the probe oLHR-2.

3.6.2 RNase Protection assays using oLHR-2, oLHR-3 and oACT-1

Complementary RNA probes were generated incorporating [α-^{32}P]UTP (194 Ci/mmol) using either T7 or T3 RNA polymerases and a Promega riboprobe kit *(as described in Sections 2.2.9 and 2.2.15)*. ACT-1 was linearized with *Xba* I (probe size 267 nucleotides), LHR-2 with *Xho* I (probe size 453 nucleotides) and LHR-3 with *Xho* I (probe size 290 nucleotides). RNase protection assays were then performed *(as described in Section 2.2.15)*. Briefly, this involved hybridising denatured DNA-free RNA probes (1-2x10^6 cpm) and total RNA from each of the tissues for 16 h at 50°C, before digesting the hybridized products with 2 µg/ml RNase T1 and 40 µg/ml RNase A for 1 h at 30°C *(as described in Section 2.2.15)*. The protected products were analysed by electrophoresis on a 7 M urea/polyacrylamide (6%) gel using 1xTBE running buffer *(see Section 2.2.15)*. Sequencing reaction products were used as molecular weight markers, as well as DNA molecular weight markers (Bresatec Ltd), [α-^{32}P]-dCTP end-filled pUC 19 originally cut with *Hpa* II *(see Section 2.2.11)*. Analysis of RNase protection assays involved either autoradiography or phosphoimagery *(as described in 2.2.15)*.

3.6.3 Qualitative Results of the oLHR-2 RNase Protection Assay

RNase protection assays were carried out with RNA extracted from pools of follicles (n=10), and individual corpora lutea (n=25), with representative results shown in Figures 3.12. Protected fragments of approximately 312, 242, 184, 169, and 124 nucleotides (nt) were observed *(see Figure 3.12)*. Allowing for small differences between the DNA molecular weight markers and the the RNA protected fragments, the 312-nucleotide fragment corresponded well with the expected size of 316 nucleotides for the A form. The lack of any protected fragment of approximately 270 nt or 94 nt in length indicated that the E or EB splice forms respectively are not found at any detectable levels in these tissues in the sheep. This is perhaps not totally suprising, as the E and EB forms have only been reported in the rat (Bernard *et al.*, 1990). The 124-nucleotide protected fragment presumably arose from either the B, C

and/or D forms (expected size of 127 nucleotides). While the B splice form has been detected in every species examined so far, the C and D splice forms have only been reported in the pig testis (Loosfelt *et al.*, 1989). The extra protected bands of 242, 184 and 169 nucleotides which were highly repeatable, did not correspond with any known alternatively spliced forms of the LH receptor in any species, and may be explained by novel alternative splice forms unique to the sheep.

Interestingly, all of the protected fragments were found in RNA from both corpora lutea and pools of follicles, but not the negative control, t-RNA or liver (data not shown), at approximately the same ratios in both cell types, although there was approximately ten times the amount of total LHR mRNA per gram of total RNA in corpora lutea as there was in follicles. This will be discussed in further detail in Section 3.7.

The major protected fragment, the 124 nucleotide band, could have corresponded to either the B, C and/or D splice forms, and based on the oLHR-2 RNase protection assay it would be impossible to identify which form had generated the fragment. However, by combining the results from both the oLHR-2 and oLHR-3 assays, it was possible to determine if this band was in fact generated from the B splice form, as the oLHR-3 probe detects a 96 nt fragment if the B form is present, and no fragment is protected from the C or D forms. Nevertheless, the presence of the B splice form in the oLHR-3 RNase protection does not rule out the presence of the C and D splice forms.

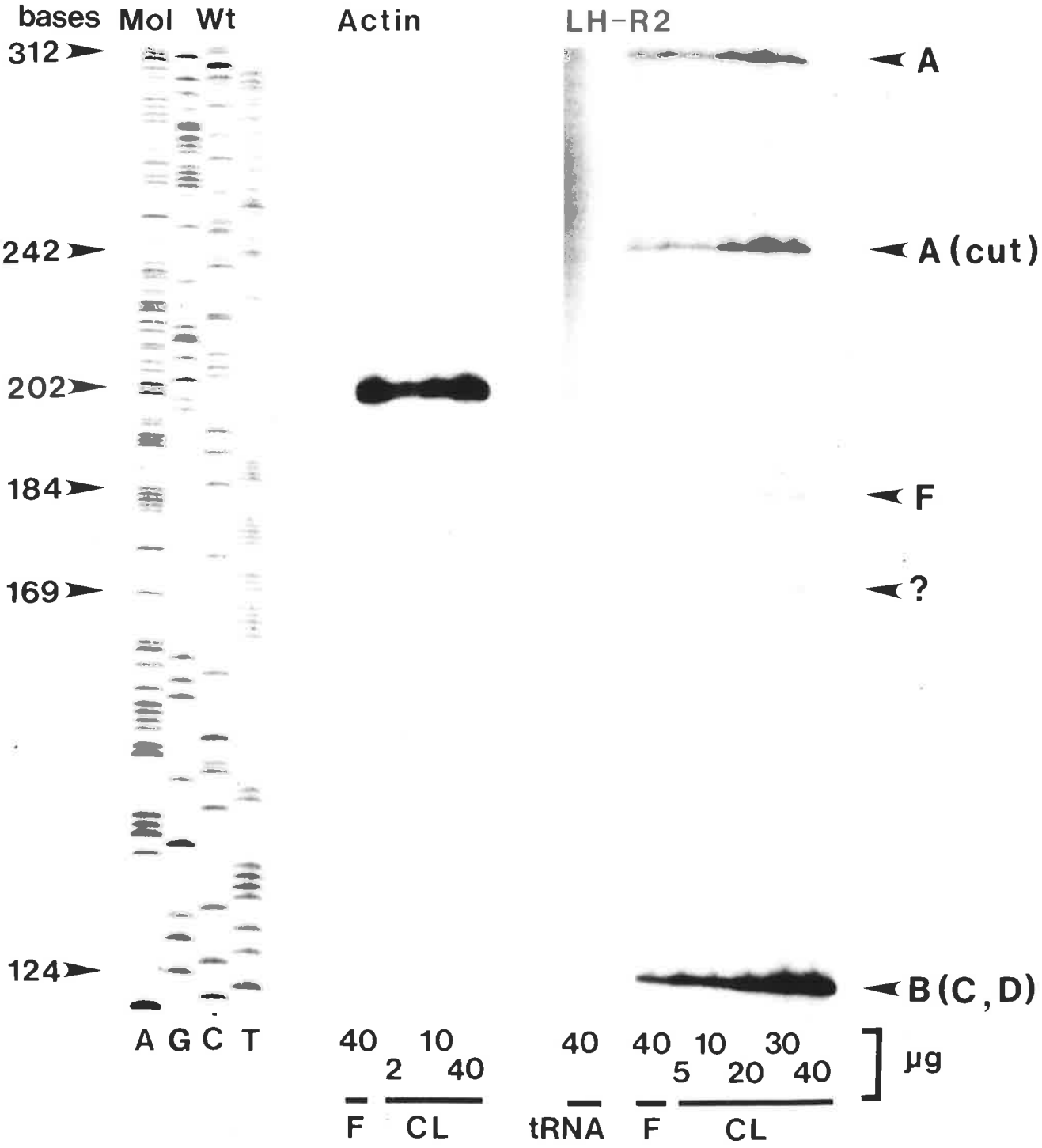

Figure 3.12 oLHR-2 and oACT-1 RNase protection assays of RNA from an ovine corpus luteum and a pool of follicles. Samples of total RNA (amounts indicated) from an ovine corpus luteum (CL), a pool of follicles (F), or transfer RNA from yeast were examined using RNase protection assays with either oLHR-2 or oACT-1 probes. RNase-protected fragments were resolved on an 8% polyacrylamide-urea gel. Five different sizes of protected fragments of 124, 169, 184, 242, and 312 nucleotides were produced with the oLHR-2 assay, and three major fragments of 201, 202, and 203 nucleotides were produced with the actin RNase protection assay. An oLHR-2 sequencing reaction using the -20 universal primer (17-mer) was used to determine the sizes of the protected fragments, and these are indicated on the left side by arrows. The identities of the protected fragments in the oLHR-2 RNase protection assay are indicated on the right side.

3.6.4 Qualitative Results of the oLHR-3 RNase Protection Assay

RNase protection assays were carried out with RNA extracted from pools of follicles (n=6) and individual corpora lutea (n=18), with representative results shown in Figure 3.13. Protected fragments of approximately 178, 164, 96, and 80 nucleotides were observed *(see Figure 3.13)*. Allowing for small differences between the DNA molecular weight markers and the the RNA protected fragments, the 178 nt fragment corresponded well with the expected size of 167 nt for the A form The 96 nt protected fragment corresponded well with the size expected for the B and EB splice forms, 94 nt. However the lack of an 81 nt protected band in the oLHR-2 RNase protection assay indicated that there was no EB LHR forms present in the RNA samples.

There were also two other protected bands of 164 and 80 nt, that could not be accounted for by any known LHR alternatively spliced forms reported in any other species. Given that these bands were quite intense relative to the 178 and 96 nt protected bands, and they both appeared 14-16 nt lower than the expected A and B forms, it was possible that the small protected fragments (164 and 80 nt) may have arisen due to a mismatch in oLHR-3 approximately 15 nt from the 3' end (to account for both the 164 and 80 nt fragments, as the B splice form has no homology with the 5' end). The oligonucleotide primer LHR-AS4, which was used for RT-PCR and was incorporated into the oLHR-3 probe, was originally designed to be homologous to the FSH receptor as well as the LHR, and as such contained a T base 15 bases from the 3' end, while an A base was found at this location in the LHR cDNA sequences from pig (Loosfelt *et al.*, 1989), rat (McFarland *et al.*, 1989), human (Minegish *et al.*, 1990) and mouse (Gundermann *et al.*, 1992; *see Figure 3.2*). Cleavage at this mismatch would generate protected fragments 15 nucleotides smaller than those expected, and account for the 164-nucleotide protected fragment from the A form and the 80-nucleotide fragment from the B form.

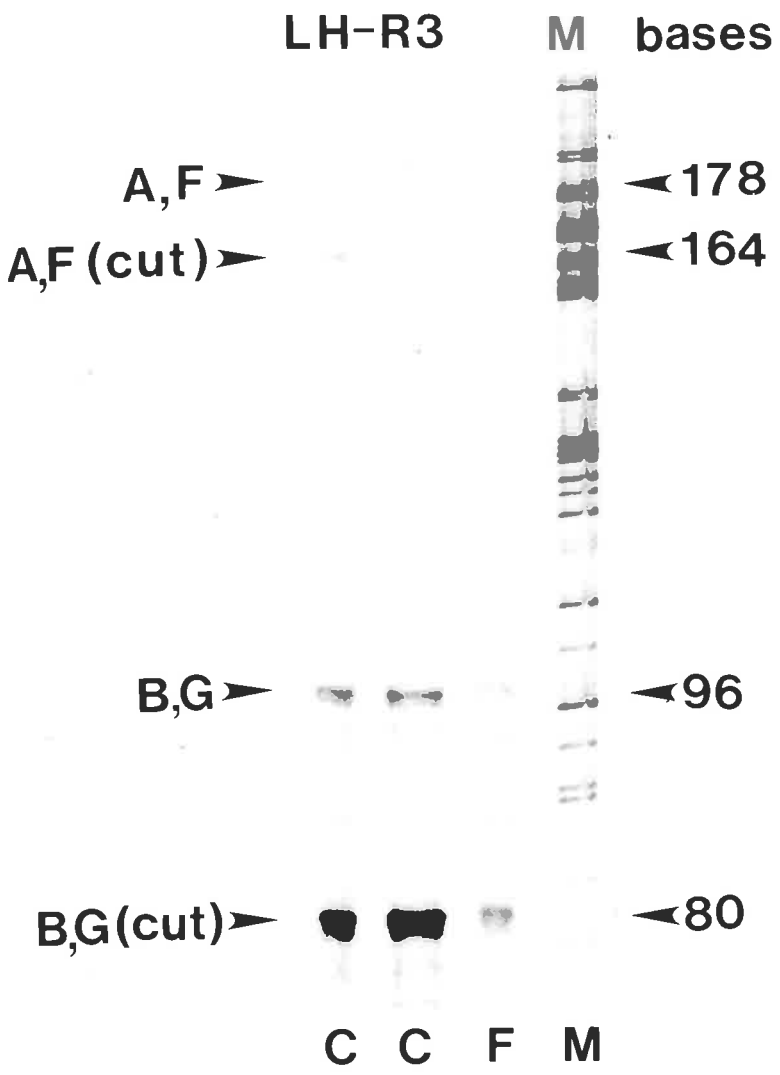

Figure 3.13 oLHR-3 RNase protection assays of RNA from ovine corpora lutea (C) and a pool of follicles (F). Samples of total RNA (15 μg) from corpora lutea and a pool of follicles were examined by RNase protection analyses using the oLHR-3 probe. RNase protected fragments were resolved on an 8% polyacrylamide-urea gel. Four different sizes of protected fragments of 80, 96, 164, and 178 nucleotides were produced. The t termination reaction of an oLHR-2 sequencing reaction using the T7 promoter primer (17-mer) (M) was used to determine the size of the protected fragments, and these are indicated on the right side by arrows. The identities of the protected fragments are indicated on the left side.

3.6.5 Qualitative Results of the oACT-1 RNase Protection Assay

RNase protection assays were carried out with RNA extracted pools of follicles (n=10) and individual corpora lutea (n=25), with representative results shown in Figure 3.12. Protected fragments of 203, 202, and 201 nucleotides were observed *(see Figure 3.12)*. These protected fragments corresponded well with the expected protected fragment of 203 nucleotides. The appearance of three bands instead of just one is probably due to partial hybridisation of the region of the probe that corresponded with the artificial restriction enzyme sites *(see Figure 3.6)*. Some of the oACT-1 RNase protection assays were carried out using Boehringer Mannheim RNase T1 which was suspended in 3M ammonium acetate, as opposed to RNase T1 from Sigma which we resuspended in DEPC treated H_2O, which was used for most of

the assays. The assays carried out using the Boehringer Mannheim RNase T1 resulted in a salt pellet forming at the end of the procedure (following precipitation), and only one protected fragment, of approximately 203 nucleotides, was observed. Presumably the different salt concentrations of the RNase T1 affects the stringency of the hybridisation step, lending further support to the hypothesis that the three protected bands were due to partial hybridisation to the restriction enzyme site.

3.6.6 RT-PCR to determine if the C and D LHR splice forms are present in ovine ovaries.

The RNase protection assays using oLHR-2 and oLHR-3 indicated that there was a significant amount of the LHR B splice form mRNA present in ovine corpora lutea and follicle pools. However, the possibility that the C and/or D splice forms of the LH receptor mRNA exist in these tissues was not excluded by these experiments. Therefore to determine if these forms exist, another LHR RT-PCR was designed. This would also enable identification of any other alternatively spliced forms of the LHR that may exist in the ovine ovary, which was a distinct possibility given the extra protected bands seen with the oLHR-2 RNase protection assay.

The new RT-PCR used primer LHR-S1 *(see Figure 3.5)* as its forward primer and an antisense primer (LHR-AS5) was designed on the 3' side of the 5' acceptor site for the D splice variant. The sequence of the LHR-AS5 oligonucleotide primer is shown in Figure 3.14, and was based on consensus sequences of the pig (Loosfelt *et al.*, 1989), rat (McFarland *et al.*, 1989), human (Minegish *et al.*, 1990), and mouse (Gundermann *et al.*, 1992) LHR cDNA and the homologous region from the rat (Sprengel *et al.*, 1990) and sheep (Yarney *et al.*, 1993) FSH and the human TSH receptor cDNA sequences (Libert *et al.*, 1989; Nagayama *et al.*, 1989), and is complementary to nucleotides 1999-1980 of the porcine LHR sequence. The porcine C and D splice forms splice to nucleotide 1658 and 1884, respectively.

```
LHR-AS5     5'-TCCAGTGAAGCCATTTTTGC-3'      .
Pig LHR     5'-.....AGTAAG-3'
Rat LHR     5'-...T.G.....TG.-3'
Human LHR   5'-.....T.G-3'
Mouse LHR   5'-..TT.GA.....G......G...TG.-3'
Rat FSHR    5'-...T..A..T.TGGGC.....T.TCA-3'
Ovine FSHR  5'-...TTG..T.AA.G..G..GGCAGT.-3'
Human TSHR  5'-GGGT...GGTAACC.....A.CCTGA-3'
```

Figure 3.14. Oligonucleotide primer LHR-AS5. The primer sequence is aligned with the homologous sequence of pig, rat, human and mouse LHR cDNA (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Minegish *et al.*, 1990; Gundermann *et al.*, 1992), and the homologous region from the rat (Sprengel *et al.*, 1990) and ovine (Yarney *et al.*, 1993) FSH receptor and the human TSH receptor cDNA sequences (Libert *et al.*, 1989; Nagayama *et al.*, 1989).

The expected sizes for the alternatively spliced forms of the LHR mRNA amplified by RT-PCR using primers LHR-S1 and LHR-AS5 are shown in Table 3.2.

LHR Splice Form	Expected PCR Product Size
A	1512
B/B4	1246
C	802
D	580
E/A2	1326
EB	1060
B1	1321
B2	1486
B3	1507
C1	1607
C2	1578
rLHR1759	1246
rLHR1950	1512

Table 3.2 Predicted RT-PCR product sizes of the known alternatively spliced forms of the LHR using primers LHR-S1 and LHR-AS5. The predicted RT-PCR product sizes are based on the known cDNA sequence from the species in which the alternatively spliced forms exist.

Total RNA was isolated from ovine corpus luteum, pooled ovarian follicles, and liver by CsCl centrifugation *(as described in Section 2.2.4)*. Total RNA (1 μg/sample) was reverse transcribed, using random hexamers *(as described in Section 2.2.7)*. Polymerase chain reaction using the primers LHR-S1 and LHR-AS5 was carried out *(as described in Section 2.2.6)*, with the exception that ^{32}P-deoxy-ATP (μCi/ml; Bresatec) was added per 25 μl reaction. Amplification conditions consisted of 94°C for 5 minutes, followed by 30 cycles of 94°C for 50 sec, 59°C for 50 seconds to anneal the primers to the template, and 72°C for 2 minutes to extend the primers, and, on the final cycle 72°C for 5 minutes. One fifth of the PCR product (5 μl) was subjected to electrophoresis (3% polyacrylamide-urea gel), with SPP1 bacteriophage DNA digested with *Eco*RI (Bresatec) radio-labelled (*described in sectiomn 2.2.11)* for use as molecular weight markers. After electrophoresis autoradiography was performed at -80°C with an intensifying screen overnight *(see Figure 3.15; pools of follicles and liver RT-PCRs not shown)*.

RT-PCR of the LH receptor mRNA from both ovine corpora and pools of ovarian follicles using the primers LHR-S1 and LHR-AS5 only produced bands greater than 980 bp in molecular weight *(see Figure 3.15)*. The band sizes corresponded with the sizes expected for the A and B splice forms. However, extra bands were observed both at molecular weights greater and less than that expected for the B splice form, suggesting that other splice forms of the ovine LH receptor exist. No bands at 802 and 580 bp were observed, as expected if the C and D forms, respectively, were amplified. As the PCR reaction generally favours amplification of smaller molecular weight forms, and amplified C and D forms would be smaller than the other forms amplified, it was concluded that C and D forms were not present in ovine corpora lutea or follicle pools. On the basis of these results, the protected fragment of 124 nt observed in the oLHR-2 RNase protection assay was attributed solely to the B form.

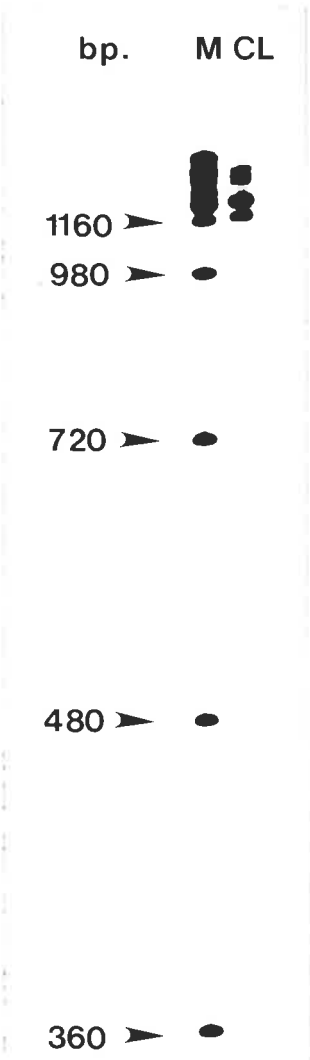

Figure 3.15 Amplification of LHR splice variants by RT-PCR from an ovine corpus luteum. Oligonucleotide primers LHR-S1 and LHR-AS5 specific for the LHR were used to amplify by PCR , cDNA generated from a mature ovine corpus luteum. ^{32}P-deoxy-ATP was incorporated during amplification of the PCR products. The products were resolved on a 3% polyacrylamide-urea gel and the gel dried, before autoradiography was performed overnight at -80^{o}C with an intensifying screen. The molecular weight markers (M) are *Eco*RI-digested SPP1 phage DNA (Bresatec), end filled with [^{32}P]-deoxy-ATP. bp=basepairs.

3.6.7 Sequencing of the alternatively spliced variants of the ovine LH receptor RNA.

The additional bands generated from the RT-PCR using the primers LHR-S1 and LHR-AS5 suggested that there were other alternatively spliced LHR mRNA variants in the ovine ovary in addition to the A and B forms. This hypothesis was supported by the findings of the oLHR-2 RNase protection assay, which generated unexplained additional protected fragments. To determine if the additional fragments in both experiments were in fact due to novel LHR splice variants, a RT-PCR using the

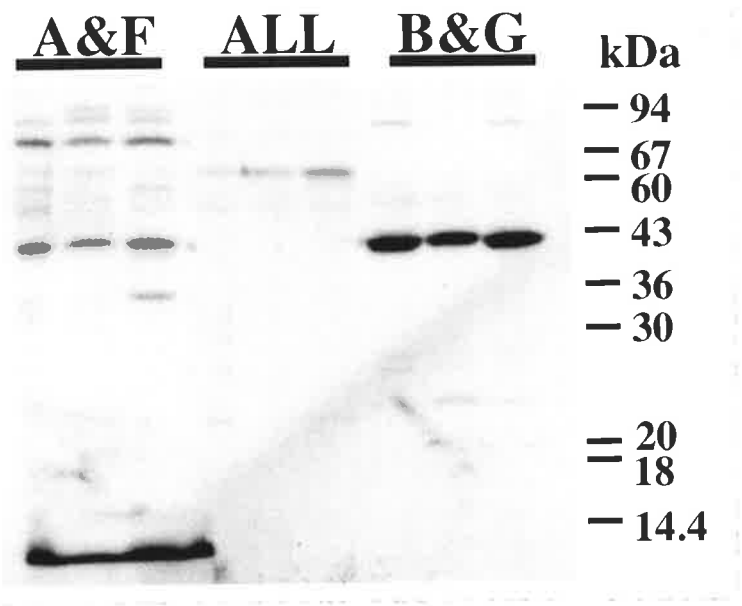

Figure 4.16 Western Analysis of three ovine corpora lutea probed with (A) peptide LHR-A&F, (B) peptide LHR-ALL, and (C) peptide LHR-B&G. Corpora lutea were collect from three cycling ewes and the tissue homogenised as described in section 2.6.1. The homogenised tissues (100 µg/sample) were investigated by Western analyses using either; (A) LHR-A&F anti sera, (B) LHR-ALL anti sera, or (C) LHR-B&G anti sera, respectively. The autoradiograms were exposed for 24 hours at -70°C using intensifying screens. The molecular weights were "high" and "low" protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

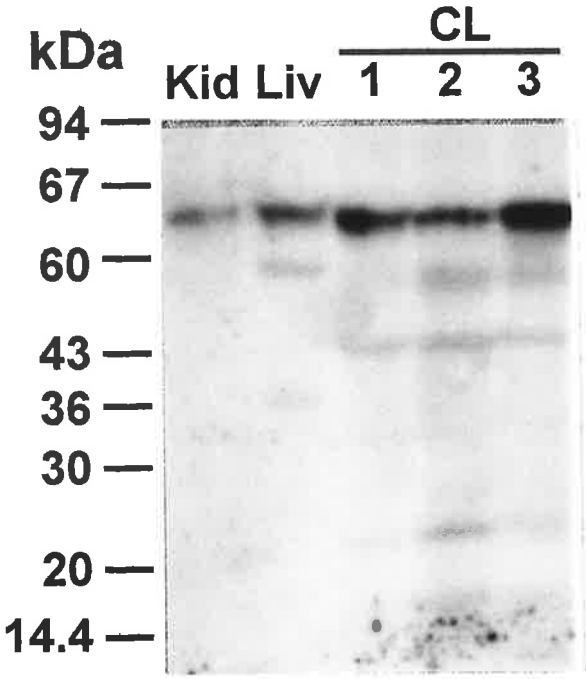

Figure 4.17 Western blot analysis of corpora lutea and liver samples, probed with antisera to peptide LHR-ALL. The autoradiograms were exposed for 24 hours at -70°C using intensifying screens. The molecular weights were "high" and "low" protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

The expected size of the glycosylated ovine functional LH receptor is approximately 90 kDa for the functional form, or 68 kDa for the high mannose precursor of the full length receptor that has been reported in other species (VuHai-LuuThi *et al.*, 1992). As no 90 kDa band was observed in these experiments, it was concluded that the antisera can not recognise the full length receptor. Although binding of the antisera to a protein of approximately 65 kDa, could have indicated the identification of the high mannose precursor of the full length receptor (expected size in pigs 68 kDa; VuHai-LuuThi *et al.*, 1992), the fact that this band was also seen in the liver samples indicates it does not correspond to the LH receptor. In addition, the size of the proteins identified by the antisera to the peptide LHR-ALL did not correspond with the proteins identified by Western analyses using antisera to the other two peptides (LHR-A&F and LHR-B&G). This suggests that the anti- LHR-ALL antibody does not identify either the functional LH receptor, or any translated products generated from alternatively spliced forms.

4.5.3 Western Analyses using antisera to peptide LHR-A&F

Western analyses of proteins from corpora lutea using the antisera raised against peptide LHR-A&F identified numerous bands *(see Figures 4.16 and 4.18)*. The strongest band ran just below the 14.4 kDa molecular weight marker, while the second most intense band occurred at approximately 42 kDa, and a band at approximately 38 kDa appeared in some samples. Three faint bands were observed in some Western blots at approximately 55, 60 and 65 kDa. Two bands occurred at 85 and 87 kDa *(appearing as one strong band in Figure 4.16)*, with a couple of minor bands of larger than 95 kDa in some samples. The anti-LHR-A&F antibody was expected to identify the translated A and F splice forms; bands of 90 kDa and 68 kDa were expected from the A form (based upon the porcine receptor; VuHai-LuuThi *et al.*, 1992) with smaller bands expected for the putative translated F form.

Comparison of Western analyses on ovine corpora lutea, liver and kidney samples *(see Figure 4.18)* revealed that most of the recognised protein bands also occurred in the liver and kidney samples, and therefore these bands are not represented solely by LH receptors or the translated products of the splice form. We expected any full length LH receptors to be either approximately 90 kDa (functional receptor) or 68 kDa (precursor form, based upon the porcine receptor). While proteins were observed at these sizes, they were common between tissues, although they were more intense in the corpora lutea sample. However, the 55 kDa protein that was detected appears to be unique to corpora lutea, although it was not at the expected molecular weight for either the A or F splice form LH receptor protein products, and as such is unlikely to correspond to the protein product of either of these transcripts.

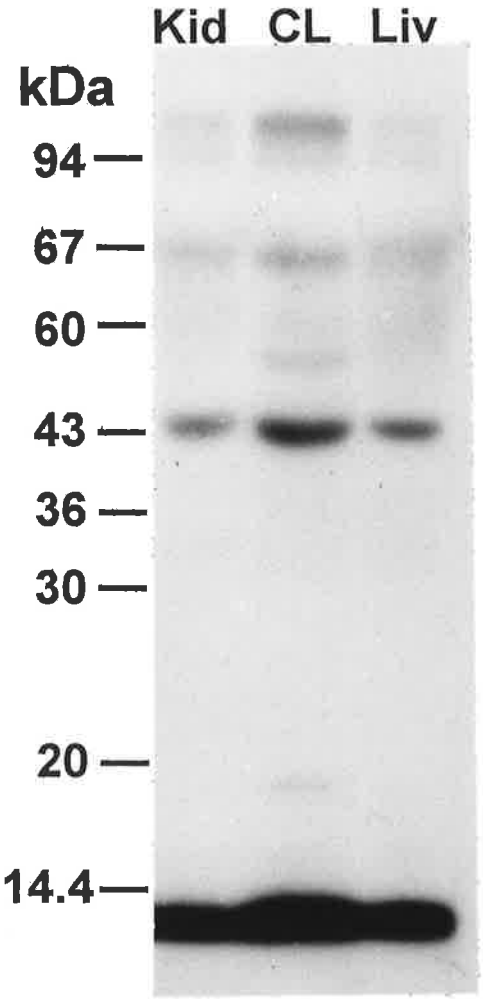

Figure 4.18 Western analysis of ovine corpora lutea, liver and kidney samples using the anti-LHR-A&F sera. The autoradiograms were exposed for 24 hours at -70^{o}C using intensifying screens. The molecular weights were "high" and "low" protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

4.5.4 Western Analyses using antisera to peptide LHR-B&G

Western analyses using the LHR-B&G antisera generated a major band of approximately 42 kDa with very faint bands at approximately 82-86, 65, and 57 kDa *(see Figures 4.16 and 4.19; the 65 and 57 kDa proteins can not be seen Fig 4.16).* In some Western analyses, faint bands were also observed at approximately 120 kDa and 24-27 kDa *(see Figure 4.16)*, however these were not repeatable in most Western analyses performed with this antisera on ovine corpora lutea. If the antisera contained antibodies that recognise the translated B and G splice forms, and if these forms were translated *in vivo* in the corpus luteum, then based upon expression studies with the porcine splice forms, a band of approximately 46 kDa should be identified (VuHai-LuuThi *et al.*, 1992). Clearly the major protein detected with this antisera corresponds well with the size expected for the translated B splice form, the slight variation in size could be due to either altered glycosylation, or possibly differences in gel composition.

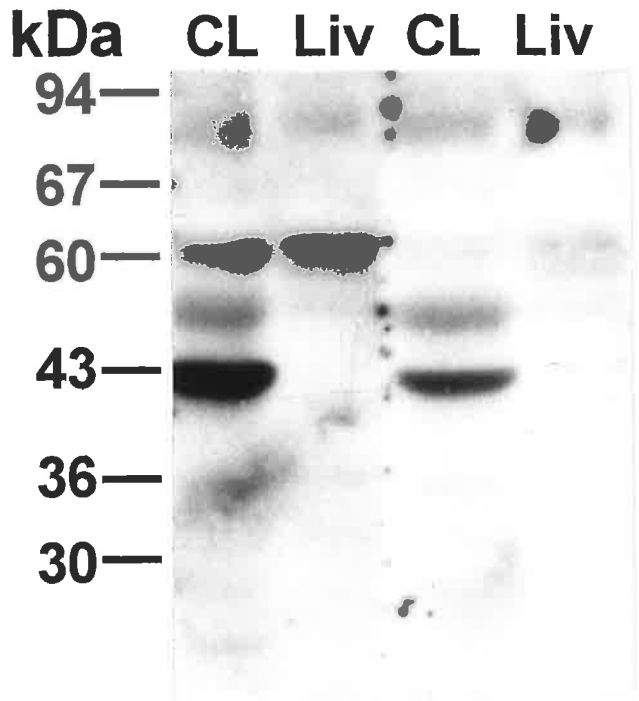

Figure 4.19 Western analysis of ovine corpora lutea, and liver samples using the anti-LHR-B&G sera. CL refers to corpora lutea samples (100 µg) and LIV refers to liver samples (100 µg). Western analysis of lanes 1 and 2 was performed with the antisera LHR-B&G at a dilution of 1:5000. Western analysis of lanes 3 and 4 was performed with the antisera LHR-B&G at a dilution of 1:25000. The autoradiogram was exposed for 24 hours at -70^{o}C using intensifying screens. The molecular weights were high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

Western analyses were then performed with the anti-LHR-B&G antisera against corpora lutea samples and liver samples *(see Figure 4.19)*. These revealed that while faint bands were observed at 82-86 and 63 kDa in both samples, the minor band at 55 kDa and the major band at 42 kDa were unique to the corpora lutea samples. These results strongly indicate that the LHR-B&G antisera recognises a protein of the expected molecular weight of the translated B splice form. Furthermore, this protein is expressed in corpora lutea but not in liver, which is the same as the mRNA expression pattern that was observed for the LHR B splice form *(see Chapter Three)*. However, additional verification was needed to prove that this protein was in fact the translated product of the LHR B splice form transcript. Elucidation of the subcellular localisation of the proteins identified by Western analyses, especially for the antibodies to the B and G forms, appeared to be a more informative measure to determine if the immunoreactive proteins bound are in fact the translated products of the B and G splice forms.

4.6 Western Analyses after Subcellular Fractionation

The subcellular localisation of the putative protein products of the alternatively spliced forms was predicted based on their amino acid structure, and reports of their localisation in transformation experiments. It was expected that none of the LHR forms, *i.e.* the full length A, the B, the F, or the G isoforms would be localised to the nucleus. Proteins that localise to the nucleus of a cell often have specific peptide sequences located in their carboxyl tail that are important in the transport of the proteins from the golgi apparatus to the nucleus *i.e.* lysine/arginine clusters, or the DDDED sequence for nuclear transport signals (Imamoto-Sonobe *et al.*, 1990; Ramotar *et al.*, 1993). As expected, neither the full length receptor or the potential products generated by the alternatively spliced transcripts contain these sequences.

It was expected that full length receptor would be localised primarily in the membrane fraction. Clearly, functional LH receptors are located on the plasma

membrane, anchored by the transmembrane domain. However, some of the A form receptor might be found in the cytoplasm of the cells, as when the porcine A form was expressed in transformed cells, 26% of the receptors were located in the cytoplasm, although 65.5% were located in the membrane fraction (VuHai-LuuThi *et al.*, 1992). The localisation of the F form is difficult to predict, as the sheep is the only species in which this form has been reported, and hence there are no expression studies from other species. This form would contain an intact transmembrane domain and intracellular domain identical to the A form, and therefore it is likely that it would be expressed on the plasma membrane if translated. Although the F splice form would not contain the potential N-linked glycosylation sites at Asn-269, Asn-277, and Asn-291 (rat LHR sequence numbering; Loosfelt *et al.*, 1989), these do not contribute to either hormone binding activity or membrane transport and insertion of the full length A form in the rat (Zhang *et al.*, 1991; Liu *et al.*, 1993). Based upon these findings, the cellular location of the F form should be very similar to that observed for the full length receptor. However the cellular localisation of the 63 kDa high mannose precursor of the full-length receptor is unknown.

The subcellular localisation of the translated protein of the LHR B splice form transcript, appears to be mostly cytoplasmic in other species. Expression of the porcine B isoform in transfected cell lines revealed 55% was located in the cytosol, 43% secreted, and only 2% in the cellular membrane fraction (VuHai-LuuThi *et al.*, 1992). Expression of the rat B isoform in transformed cells by a number of groups, indicated that this protein was either secreted or located in the cytoplasm (Tsai-Morris *et al.*, 1990; Ji *et al.*, 1991; Zhang *et al.*, 1995). Although little is known about the G splice form, it can be assumed that its subcellular localisation would be similar to that of the B isoform as it is identical to this form, except that like the F splice form, it is missing the 27 amino acids encoded by exon ten.

4.6.1 Subcellular protein isolation

Isolation of homogenised tissue into nuclear/mitochondrial, membrane/microsomal and cytosolic fractions was based on a combination of three methods; two based on rat liver microsomal preparations and one on porcine LHR splice forms from transformed cell lines (CSinti, *et al.*, 1972; Amar-Costesec *et al.*, 1974; VuHai-LuThi *et al.*, 1992). Briefly, the method involved homogenisation of the tissue in homogenisation buffer B or C, before centrifugation initially at 1 088 *x g* to remove the connective tissue, before centrifugation at 20 000 *x g* which pellets the nuclear/mitochondrial fraction, and then a further centrifugation at 100 000 *x g* to pellet the microsomal/membrane fraction. The remaining supernatant was considered the cytosolic fraction *(see Section 2.6.1 for more details)*. The protein concentration of the individual fractions was determined by a Bradford assay *(see Section 2.6.1)*, and Western analyses performed on the samples with the anti-LHR-A&F and anti-LHR-B&G antibodies *(as described in Sections 2.6.3 and 2.6.4)*. Western blots were visualised by autoradiography *(see Section 2.6.4)*, and typical results are shown in *Figures 4.20 and 4.21*.

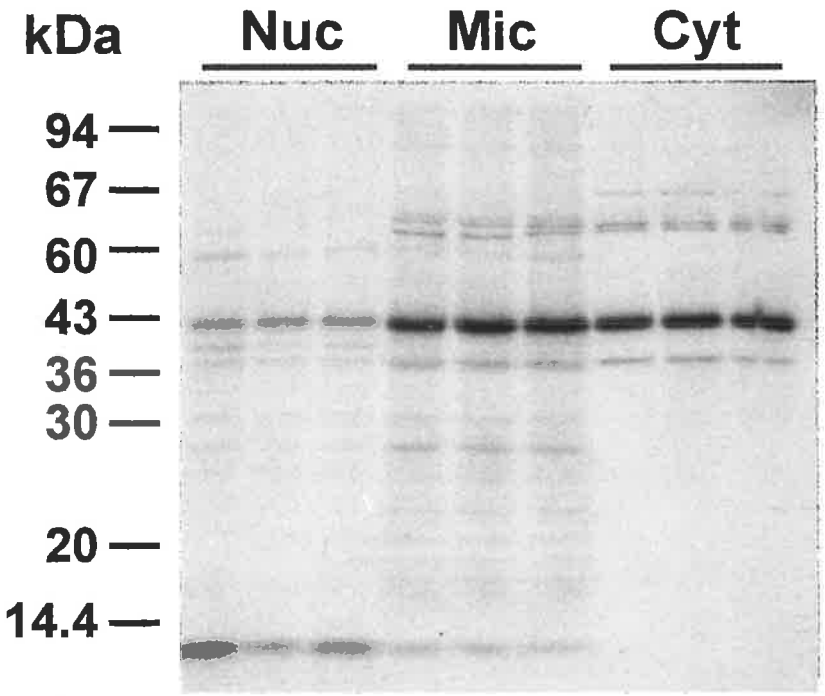

Figure 4.20 Western Analyses of ovine corpora lutea proteins separated into nuclear/mitochondrial, microsomal and cytosolic fractions using the anti-LHR-A&F antibody.

Western analysis of corpora lutea proteins that had been separated into nuclear/mitochondrial, microsomal/membrane-bound and cytosolic fractions by the anti-LHR-A&F antibody, revealed that the 14.4 kDa and 38 kDa bands localised mostly with the nuclear/mitochondrial fraction, and the 14.4 kDa band to a lesser extent the microsomal/membrane fraction. The 42 kDa band seemed to localise with the cytosolic fraction, while the strong band that runs at approximately 58 kDa appears in all three fractions. The relatively intense bands that occurred at 85 and 87 kDa appeared to localise exclusively within the microsomal/membrane and cytosolic fractions, while the band greater than 95 kDa appears only in the cytosolic fraction.

It would be assumed that the location of the functional LH receptor would be mostly in the microsomal/membrane fraction, with some receptor found in the cytoplasmic fraction (VuHai-LuuThi *et al.*, 1992). The results obtained from the subcellular localisation experiments indicate that the 85 and 87 kDa bands could in fact correspond to the A form, and even the F isoform. However, this seems unlikely given the results of the CL versus liver and kidney Western analyses with anti-LHR A&F. It is also unlikely that the bands localising to the nuclear/mitochondrial fraction indicate LH receptors. However, given that so little is known about the expression of the LHR isoforms, it is difficult to categorically rule out any band as a product of these forms

Western analyses of corpora lutea proteins that had been separated into nuclear/mitochondrial, microsomal/membrane-bound and cytosolic fractions using the anti-LHR-B&G antibody revealed the major band of approximately 42 kDa localised to the cytosolic fraction. The 82-86 and 65 kDa bands localised to the nuclear/mitochondrial fraction, and hence are unlikely to be related to the LHR isoforms. The faint band at approximately 57 kDa was found in all three subcellular fractions, again suggesting that this protein is not likely to be related to the LHR or its isoforms. The localisation of the 45 kDa band to the subcellular fraction that the B

splice form was expected to localise in if expressed, further supports the suggestion that this protein of the correct molecular weight, is in fact the translated product of the B splice form of the ovine LH receptor.

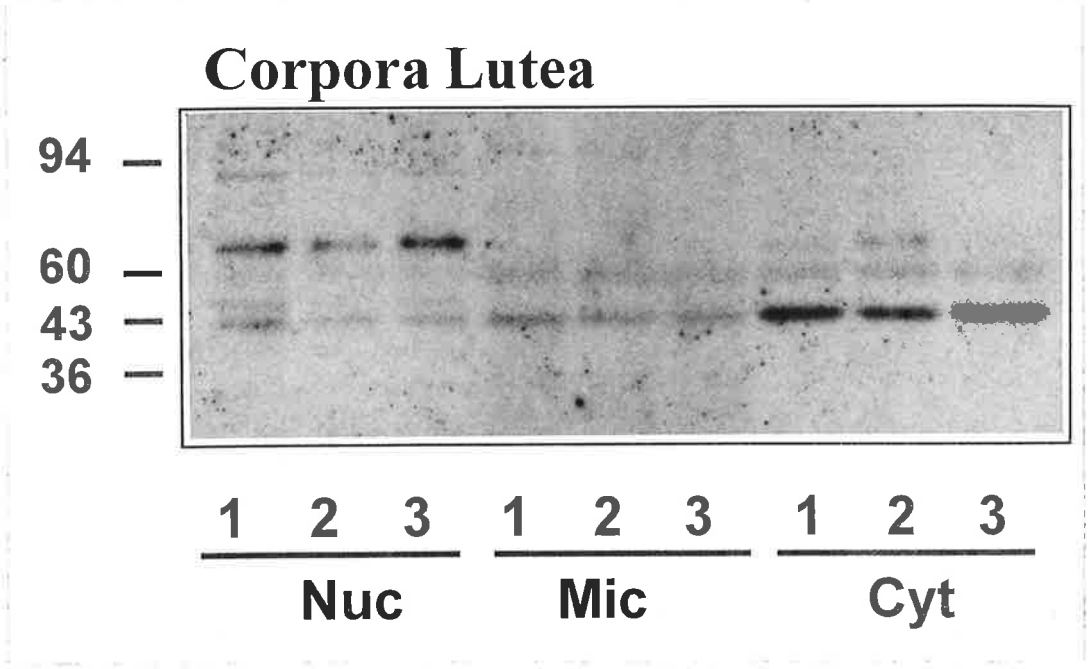

Figure 4.21 Western Analyses of ovine corpora lutea proteins separated into nuclear/mitochondrial (Nuc), microsomal (Mic) and cytosolic (Cyt) fractions using the anti-LHR-B&G antibody.

4.7 WESTERN ANALYSES USING COOKE'S ANTIBODIES

Towards the end of this project three polyclonal antibodies raised against peptide sequences of the rat LH receptor were provided as a kind gift from Professor Brian Cooke, who is based in the Department of Biochemistry and Molecular Biology, at the Royal Free Hospital School of Medicine, the University of London, UK. The antibodies had been raised in rabbits against synthetic peptides corresponding to regions within both the extracellular N-terminal domain (Cooke antibody LHR-1 and LHR-2 against residues 48-65 and 187-206, respectively) and the cytoplasmic C-terminal domain (antibody LHR-3 against residues 622-636 of the rat LH receptor (Pallikaros *et al.*, 1995)). These antibodies have been characterised (Pallikaros *et al.*, 1995) and were shown to bind specifically to the peptide against which the rabbits

were immunised, and also to a protein of 95 000 to 100 000 daltons, isolated from membrane preparations prepared from superovulated rat ovaries, mouse Leydig tumour (MA10) cells, and rat testes. The two N-terminal antibodies also inhibited ^{125}I-hCG binding to this protein on ligand blots, and ^{125}I-hCG binding to MA10 cells. In addition, antibody LHR-1, and to a lesser degree antibody LHR-2, significantly inhibited LH-stimulated cAMP and progesterone production (Pallikaros *et al.*, 1995).

Cooke's antibodies were raised against peptides corresponding to regions of the rat LHR that remain unknown in the sheep. As such, it was not known if there was sufficient homology between the rat and sheep LHR sequence in these regions for antibodies to recognise the ovine LH receptor. However, given that all three antibodies recognised the rat LHR, it was hoped that at least one would also recognise the ovine LHR, and provide additional evidence that the protein recognised by our anti-LHR-B&G antibody is the translated B splice form of the LH receptor. Clearly, this could potentially only occur with either Cooke's antibody LHR-1 or LHR-2, as the peptide that the LHR-3 antibody was raised against corresponds to a region in the cytoplasmic tail, and as such a region of the LH receptor that the B splice form does not contain.

Cooke's antibodies have not been used for immunohistochemical studies by any investigators, and therefore the optimal conditions were unknown, even for rat samples. Given that the antibodies were only received late in the project, and that immunohistochemical studies using these antibodies on ovine tissues would not verify anything about the protein that our anti-LHR-B&G antibody recognises, we decided that there was insufficient time available to carry out any such experiments.

4.7.1 Western analyses using Cooke's antibodies

Western analyses using Cooke's anti-LHR-1, -2 and -3 antibodies were performed under the same conditions as the Western analyses using the anti-LHR-B&G

primers LHR-S3 and LHR-AS4 was performed on RNA isolated from corpora lutea from mature ewes. These primers were those used to generate the clone oLHR-2&3. As FSH receptors have not been reported in corpora lutea, it was assumed that there would be no FSHR cDNA target, and therefore the primers, which can also amplify the FSH receptor, would specifically amplify LHR cDNA.

This experiment was performed by Dr Rohan, under the same conditions as described in Section 3.1.1, and generated four bands *(as seen in Figure 3.16)*. These four bands were all cloned into the pBS vector (Stratagene), using the *Eco*RI and *Sal*I restriction enzyme sites present in the primers *(as described in Section 3.1.1)*.

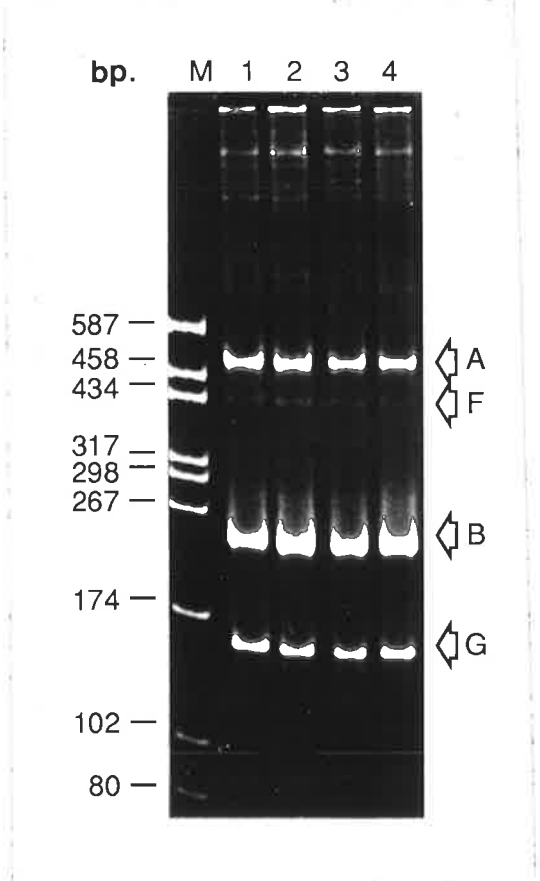

Figure 3.16 Amplification of LHR splice variants by RT-PCR of RNA from ovine corpora lutea. Oligonucleotide primers (LHR-S3 and LHR-AS4) specific for gonadotrophin receptors were used to amplify by PCR cDNA generated from corpora lutea of cycling parous ewes on various days of the oestrus cycle (lane 1, day 14; lane 2, day 5; lane 3, oestrus; lane 4, day 11). Amplified PCR products were resolved on a 5% acrylamide gel and stained with ethidium bromide. The DNA products corresponding to the predicted sizes of mRNA forms A, B, F, and G are indicated. The molecular weight marker (M) was *Hae*III-digested pBS (Stratagene Cloning Systems), and the indicated sizes are in basepairs (bp).

The resulting clones were sequenced, and four different products identified. Sequence analysis was carried out using the computer program MacVector®. One clone was identified as the full length A form, and the others were the splice variant B, and two previously unreported splice variants, designated F and G. The splicing of the B clone was identical to that of the B splice variant observed in rats (Bernard *et al.*, 1990, Segaloff *et al.*, 1990) and pigs (Loosfelt *et al.*, 1989), splicing from the 3' end of exon X into the coding region of exon XI and out of frame *(see Figures 3.3, 3.9 and 3.17)*. The newly identified F form was spliced from the 3'-end of exon IX to the 5'-end of exon XI in frame, deleting exon X *(see Figures 3.3, and 3.17)*. The G form, also newly identified, spliced from the 3'-end of exon IX to the same acceptor site as the B form in the coding region of exon XI, out of frame *(see Figures 3.3, 3.9 and 3.17)*.

In addition to identifying these extra alternatively spliced forms in the ovine ovary, the sequencing identified that the original oLHR-2&3 clone (clone A26), contained an A to G transition from the consensus ovine LHR sequence *(Figure 3.9)*. This PCR-generated mutation was located 244 nucleotides from the 5'-end of the A form protected fragment, and thus RNase cleavage at this mismatch was the likely cause of the 242 nucleotide protected fragment. The identification of the additional alternatively spliced forms, F and G, and the identification of the A to G transition in oLHR-2 enabled the RNase protections to be analysed further, and the relative abundance of the various splice forms calculated.

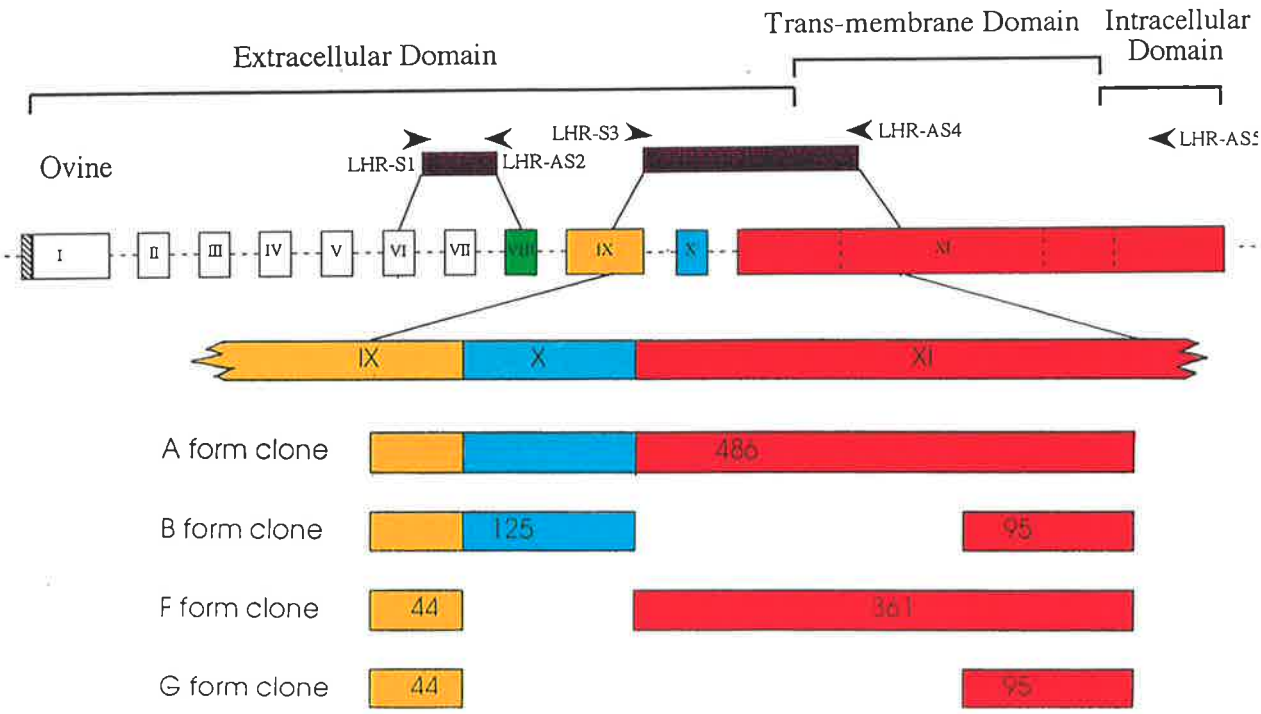

Figure 3.17 Sequence comparison betwen the ovine A, B, F, and G splice forms

3.6.8 Quantitative Results of the oLHR-2 and oLHR-3 RNase Protection Assays

Taking into account the newly identified F and G splice forms and the A to G PCR generated mutation present in the oLHR-2 riboprobe, all but one of the protected fragments were accounted for in the RNase protection assays *(see Table 3.3)*. The 169 nucleotide protected product seen in the oLHR-2 RNase protection assay could not be accounted for by any known splice variants and may correspond to another alternatively spliced form. It is possible that more variants of the LHR exist, which were not detected in the RT-PCR using the primers LHR-S3 and LHR-AS4; presumably one of the primers binds to the region removed during splicing. Furthermore, as six bands were amplified by the RT-PCR using LHR-S1 and LHR-AS5, there may be at least two more uncharacterised splice forms of the ovine LHR mRNA.

Ovine LHR Splice Forms	Protected sizes using oLHR-2		Protected sizes using oLHR-3	
	Uncut	Cut[1]	Uncut	Cut[2]
A	316	245 & 70	178	161 & 16
B	127	127	96	79 & 16
F	189 & 46	118, 70 & 46	178	161 & 16
G	46	46	96	79 & 16

Table 3.3 Predicted RNase protected fragment sizes found in the ovine corpora lutea using probes oLHR-2 and oLHR-3. The predicted sizes of the protected bands for the splice forms of the LHR using probe oLHR-2 are given for both cut and uncut, with the Cut[1] lane referring to the limited amount of digestion of the oLHR-2 probe at the A to G PCR generated mutation. The Cut[2] protected fragments for the oLHR-3 probe refers to the limited amount of digestion that occurs due to the presumed mismatch in the LHR-AS4 primer that was used to generate the oLHR-2&3 fragment that oLHR-3 was subcloned from. The 70 and 46 nucleotide protected fragments would be too small to clearly resolve on the gels used.

Identification of the major protected bands allowed the relative abundance of the alternatively spliced forms to be determined in the various tissues examined *i.e.* corpora lutea, pools of large follicles and pools of small follicles. The relative abundance of each protected fragment was determined by densitometry, and correction was made for the different UTP content of each protected fragment. The results were validated by demonstrating that the intensities of the RNA protected bands were linearly related to the amount of RNA analysed *(see Figure 3.12)*. The amount of RNA analysed was confirmed by carrying out the control actin RNase protection *(see Figure 3.12)*. The integrity and relative concentration of the RNA was analysed by visual examination on non-denaturing gels *(data not shown)*.

The full length A form mRNA comprised only 10-15% of the LHR mRNA detected in this assay. The F form mRNA was 3-4 fold less abundant, in keeping with the RT-PCR results *(see Figures 3.12 vs 3.16)*. The vast majority of mRNA species (nearly 75%) appeared to be spliced at the end of exon X. Since splice forms C and D could

not be detected in ovine tissue *(see Section 3.6.6)*, this protected fragment must have been entirely due to the B form mRNA. In order to assess the amount of G form mRNA, identical samples were hybridised with oLHR-2 and oLHR-3 probes of similar specific activities, electrophoresed on the same gel and exposed to X-ray film for identical times. Under these circumstances, the 124 nucleotide protected fragment (probe oLHR-2; indicating the B form) was slightly lower in density than the sum of the 96 and 80 nucleotide protected fragments (probe oLHR-3; indicating the B and G forms), suggesting that the amount of the G form was nearly equivalent to the amount of the A form. The steady state levels of LHR mRNA in follicles was much lower than in corpora lutea, but the splice variant expression pattern was similar between the both tissues. It was estimated that the mean relative abundance of the B : A : G : F forms was 5-3.5 : 1 : 1 : 0.3. It was unclear whether or not the variation in the relative amount of the B form to the A form was due to the sensitivity of the assay, or due to a real shift between individual corpora lutea or follicles at different stages of development.

3.7 SUMMARY

Using Northern analyses, we observed many different sized LHR mRNA transcripts in adult sheep ovaries. This was not surprising, as similar results have been reported in every species examined to date (McFarland *et al.*, 1989, Loosfelt *et al.*, 1989, Minegish *et al.*, 1990, Frazier *et al.*, 1990, LaPolt *et al.*, 1990, Hu *et al.*, 1990, Wang *et al.*, 1991, Lu *et al.*, 1993). Currently these LHR transcripts are classified by size only, since very little is known about the sequence of each transcript. The main mechanism that generates the diversity that is seen in LHR transcript size on Northern blots is probably the use of alternative polyadenylation sites *(see Section 1.4.10)*, and as such the size of LH receptor transcripts observed on Northern analyses can not be related back to the known alternatively spliced forms.

To circumvent this problem and determine if alternative splicing of the LH receptor gene transcripts occurs in the adult ovine ovary, we used RT-PCR and RNase protection assays to show the existence of the full length A form and a number of splice variants of LHR mRNA. These are similar to the A and B forms of other species (Loosfelt *et al.*, 1989, Bernard *et al.*, 1990, Segaloff *et al.*, 1990). In addition, two new forms (designated F and G, *see Figure 3.17*) were identified, however no C, D, E, EB, B1, B3, C1 or C2 forms, that have been found in other species, were detected *(see Table 3.4 for a summary of experimental evidence supporting these conclusions)*.

The RNase protection assays also enabled us to determine the relative abundance of these forms. The relative amount of the steady state levels of mRNA for the B form : A form : G form : F form were estimated to be 5-3.5 : 1 : 1 : 0.3. Both follicles and corpora lutea had similar expression patterns, but the steady state levels of LHR mRNA were greater in corpora lutea.

LHR Splice Form	Evidence that the form exists/does not exist	Tissue type(s) examined
A, B, F and G	These forms exist as proven by RT-PCR, Sequencing, and oLHR-2 and oLHR-3 RNase protections	C.L., large and small follicles
C	RT-PCR using LHR-S1 and LHR-AS5; no 802 bp band	C.L. and follicles[1]
D	RT-PCR using LHR-S1 and LHR-AS5; no 580 bp band	C.L. and follicles[1]
E	oLHR-2 RNase protection assay; no 270 nt band	C.L. and follicles[2]
EB	oLHR-2 RNase protection assay; no 81 nt band	C.L. and follicles[2]
B1	oLHR-2 RNase protection assay; no 266 nt band	C.L. and follicles[2]
B3	oLHR-2 RNase protection assay; no 270 nt band	C.L. and follicles[2]
C1	oLHR-2 RNase protection assay; no 270 nt band and RT-PCR using LHR-S3 and LHR-AS4; no 598 bp band	C.L. and follicles[2]
C2	RT-PCR using LHR-S1 and LHR-AS2; no 227 bp band	C.L. and follicles[2]
B2	Unable to determine if these forms are present as the experiments did not examine the region of the gene that is spliced out in these forms	
rLHR1759		
rLHR1950		

Table 3.4 Summary of the evidence that proves which LHR splice forms are present in ovine ovary. The LHR splice forms indicated by bold lettering represent forms detected in the ovine ovary. C.L. is corpora lutea; [1] refers to RNA from pools of follicles 4-6 mm in diameter; [2] refers to RNA from pools of small follicles (1-3 mm in diameter) and large follicles (4-6 mm in diameter).

The evidence for the existence of the A, B, F, and G splice forms in ovine corpora lutea, and large and small follicles came from a number of experimental sources including RT-PCR, cloning and sequencing of these PCR products, and two RNase protection assays. Evidence that the other splice forms reported in other species do not occur in the ovine ovary is summarised in Table 3.4, and involved either the lack of amplification of the various forms by RT-PCR using various LHR primers, or a lack of protected fragments in the RNase protection assays at sizes expected if these forms existed in this species. However, the experiments performed were not designed to identify the B2, r1950 and r1759 splice forms, and as such the existence of these forms in the ovine ovary is unknown. These forms were reported to occur in a cDNA

library from pseudopregnant rat ovaries at a rate of 15%, 1% and 1% respectively (Segaloff *et al.*, 1990; Aatsinki *et al.*, 1992). Evidence for the existence of the EB splice form in the ovine ovary relied on detecting an 80 nucleotide protected fragment in LHR-2 RNase protection assays. While an 80 nucleotide protected fragment was not observed in RNA from corpora lutea or follicle pools, the size of this fragment is below the optimal range for protected fragments for this assay, and as such we can not conclusively exclude the existence of the EB splice variant in ovine ovarian tissues.

The A isoform is regarded as the full length mature form of the LHR mRNA, and in the sheep it appears to use identical splice donor and acceptor sites to those of the pig, rat, and man. The B isoform in sheep is homologous to the B form in pig (Loosfelt *et al.*, 1989) and rat (Bernard *et al.*, 1990; also called B4 (Segaloff *et al.*, 1990)), and this and the G form splice to the same alternative 3' acceptor site. This 3' acceptor site is within exon XI of the coding region of the full length A form. The sequence at this site is UUUGCAG/A (/=splicing site) and differs slightly from consensus 3' acceptor sites (Y_nNYAG/C, Y=C or U, N=U, A, C, or G; see Smith *et al.*, 1989). In the sheep a conserved putative branch point sequence (YNYURAY, R=A or G; Aatsinki *et al.*, 1992, Smith *et al.*, 1989) is located 57-63 nucleotides upstream of this alternative 3' splice site (UCCUGAC; *see Figure 3.9*). The F splice form deletes exon X, splicing from the 5' donor site in exon IX to the 3' acceptor site of exon XI. By splicing in frame, the F form would translate into a protein identical to the A form, but missing the sequence encoded by exon X. Thus not only is alternative splicing of the LHR gene transcripts common in mammals, but some splice variants, particularly the B form, appear to be conserved.

Although numerous alternative LHR mRNA splice variants have been reported in every species examined, quantitation of the relative steady-state levels of these splice variants has been harder to achieve. The existing data comes indirectly from the

frequency of isolation of cDNA clones, PCR and Northern blotting. Our data, using RNase protection assays, found that in ovine follicles and corpora lutea the B splice variant was between three and a half fold to five fold more abundant than the full length A form. In the pig testis, the frequency of isolated clones suggested that less than 60% of LHR transcripts were the A form (Loosfelt *et al.*, 1989). Similarly, in the rat corpus luteum less than 50% were the A form (Bernard *et al.*, 1990), and the B form (Bernard *et al.*, 1990) made up over 50% of the transcripts. Clearly, the B splice variant is a major form of the LHR mRNAs. In the rat some forms (rLHR1950, rLHR1759 and EB) have only been detected by PCR techniques (Bernard *et al.*, 1990, Aatsinki *et al.*, 1992), and others, (C1, C2, B1, and B3), were detected by screening a rat luteal cDNA library, and have only been observed by a single group of researchers (Segaloff *et al.*, 1990). Consequently, these forms may not make up a significant proportion of the rat LHR mRNA.

The putative LHR protein encoded by the B splice variant would be shorter than the full length A form, not only because part of the coding region has been spliced out, but also because the splicing is out of frame, producing a premature stop codon and an alternative carboxyl terminus. The carboxyl terminus on the B form splice variant in sheep is identical to the carboxyl tail in the B form in the pig, and it differs from that of the rat by only two amino acids (out of thirteen amino acids), although the rat form is nine amino acids longer *(see Figure 3.9)*. This high degree of conservation across species, both in the conservation of the splice sites and the amino acid sequences, may be due to the imposed conservation of the amino acids of the second transmembrane domain of the A form, in which all amino acids are conserved between species (Loosfelt *et al.*, 1989, McFarland *et al.*, 1989, Minegish *et al.*, 1990, Gundermann *et al.*, 1992). However, analysis of the DNA sequence in this region revealed that the sequence could potentially have diverged considerably due to degeneracy in codon usage, whilst keeping the amino acid sequences of the A form conserved. There is just one base difference between the sheep and the pig, of the 39

bases that encode for the unique 13 amino acid tail of the B splice form, and this does not result in an amino acid substitution. Similarly, only two of 39 bases in the rat differ from the sheep sequence. The fact that these sequences are highly conserved suggests that there has been selection pressure to preserve the amino acid sequence of the B form. It is therefore possible that the B form may have some biological function. However it does not encode a product with a protein kinase C phosphorylation consensus site, identified in the A form and in the B2 form rat splice variant. The function, if any, of the highly expressed B form remains to be determined.

In summary, we have identified and determined the relative abundance of the LHR mRNA transcripts in sheep ovarian follicles and corpora lutea. The vast majority of the transcripts in both follicles and corpora lutea do not encode for the full length receptor, but encode for a putative protein containing the LH and hCG binding domain. It remains to be determined whether the alternatively spliced LHR mRNA transcripts are translated, and if so, if their products have a physiological role.

CHAPTER FOUR

PROTEIN ANALYSES OF THE OVINE LH RECEPTOR SPLICE FORMS

4.0 INTRODUCTION

Cloning of the luteinizing hormone receptor (LHR) messenger RNAs from pig, rat, mouse, and human testes or ovaries revealed many of the LHR transcripts encoded alternatively spliced, and therefore possibly inactive, forms of the LHR. In the ovine ovary, we identified three alternatvely spliced LHR mRNA transcripts in addition to the full length A form *(see Chapter Three)*. This included the B form, which uses the same alternative splice site as the B splice forms that have been identified in pig testes (Loosfelt *et al.*, 1989), rat ovary (Bernard *et al.*, 1990), rat testes (Segaloff *et al.*, 1990), and human ovary (Rohan and MacKoul, personal communication). In addition, two novel splice forms, which we designated the F splice form and the G splice form, were also identified. Examination of mRNA from corpora lutea and follicles revealed that the B splice form is approximately five times more abundant than the full length A form transcript, with similar levels of the A and G form transcripts present, and approximately four times more A form than F form. The function, if any, of the alternatively spliced forms of the LH receptor is unknown; it is not even known if *any* of these forms are translated *in vivo,* in *any* species.

The A form encodes for the functional LH receptor, containing a large extracellular domain, a transmembrane domain that weaves in and out of the plasma membrane seven times, and a small intracellular domain. The F form encodes a putative protein which, if translated, would be very similar to the A form. It would contain transmembrane, intracellular and extracellular domains identical to the A form, with the exception that the extracellular domain of the F form would be missing amino acids 268 to 294 (based on the rat sequence) of the A form. Based on binding studies in other species, the F form protein should still be able to bind LH or hCG with high affinity. Interestingly, the missing region contains three conserved potential N-linked glycosylation sites *(see Figure 1.4)*, of which one is thought to bind a carbohydrate chain (Zhang *et al.*, 1995), although this does not seem to be important in either hormone binding or subcellular localisation (Zhang *et al.*, 1991).

The B and G splice forms encode putative proteins that have the high affinity LH/CG binding domain, but no transmembrane or intracellular domains. Both the B and the G forms are homologous to the N-terminal 86.2% and 78.1% amino acids of the A form extracellular domain, resepectively. However, due to the 3' alternative splicing site both transcripts utilise, there is a shift in the reading frame, resulting in the same unique thirteen amino acid carboxyl tail for both putative proteins. Translation of any of the alternatively spliced forms into a protein product has not been demonstrated *in vivo* in any species, although transfection of the LHR B form cDNA into heterologous cells does result in the production of a truncated LHR capable of binding hCG with high affinity (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992). Whether or not the putative proteins arising from these alternatively spliced forms are secreted remains controversial, with some groups reporting that they are secreted *in vitro* (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992), and other groups reporting that they are not (Ji *et al.*, 1990; Koo *et al.*, 1994; Zhang *et al.*, 1995). To add to this confusion, the Tsai-Morris *et al.* (1990), and the Zhang *et al.* (1995) reports are from the same research group, and presumably using the same stock of cell lines.

In addition, there is some *in vivo* evidence that indicates the alternatively spliced LHR mRNA transcripts might be translated. Numerous reports of proteins of different sizes that bind hCG and LH, or which are recognised by antibodies to the LH receptor, have been reported. Most of these proteins are significantly smaller than the mature or high mannose precusor form of the full length LH/CG receptor (VuHai-LuuThi *et al.*, 1992), and are the approximate size of the putative protein products from the alternatively spliced LHR mRNA transcripts. However, there have also been numerous reports of proteolytic cleavage of the LH/CG receptor during both normal function, and in the isolation of the receptors, and this could be the origin of these smaller than expected proteins (Hwang and Menon, 1984; Rosemblit *et al.*, 1988; West and Cooke 1991).

Given the lack of literature concerning translation of the alternatively spliced LHR forms, the aim of the experiments described in this chapter was to develop a method to specifically identify any protein products encoded by these forms in the ovine ovary, and to determine if they are translated *in vivo*. To achieve this, polyclonal antibodies were generated, which would theoretically enable the B and G form protein products to be distinguished from those of the A and F forms..

4.1 Raising of Antibodies to Detect LHR Splice Variants.

To determine if the alternatively spliced ovine LH receptor mRNAs are translated into proteins, there were two possible strategies. The approach not undertaken involved isolating RNA transcripts in the process of being translated in the cell. These transcripts are associated with polysomes as part of the translation process. Therefore by isolating the polysomes, the translated subset of transcripts could be analysed for alternatively spliced LHR transcripts. Since this approach would not reveal any information about the location of the putative protein products in the cells, we opted to generate antibodies which specifically detected the translated products of the alternatively spliced LHR mRNAs. This allowed us not only to determine if the spliced forms were translated, but also the location of any such proteins..

4.1.1 Peptide design strategy for raising polyclonal antibodies which identify the putative protein products of the alternatively spliced ovine LHR mRNAs

Three peptides were designed such that the antibodies raised against them would be specific to the various translated products of the LHR mRNA transcripts; peptide "LHR ALL", peptide "LHR A&F", and peptide "LHR B&G". Peptide "LHR ALL" was designed as an immunogen to raise antibodies that would recognise translated products from all of the known ovine LH receptor mRNAs, *i.e.* the A, B, F, and G forms. The LHR A&F peptide was designed so the antibodies raised against it would identify the translated products from the A and F forms, but not the B and G forms.

Finally, the LHR B&G peptide was designed to raise antibodies that detect the translated products of the B and G alternatively spliced LH receptor forms, and not the products of the A and F forms *(see Figure 4.1)*.

The most common practice of raising antibodies against peptides, is to choose a 10-15 residue peptide and to chemically cross link it to a carrier molecule such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). If an internal sequence (*i.e.* not the amino or carboxyl terminals) is required, the selection of the peptide is based on algorithms that predict potential antigenic sites. These methods are based on predictions of hydrophobicity (Kyte and Doolittle, 1982). Other factors that were taken into consideration when designing the peptides included:

(1) The regions upon which the peptides were based were located in the extracellular domain. This presumably allows the antibodies to bind to the ligand in intact cells. If the peptide was designed to represent regions in the transmembrane domain, the ability of the antibodies to interact with the antigen sites may be severely restricted. Similarly, if regions of the intracellular domain were chosen, lysis of the cells may be required to enable the antibodies to gain access to the ligands.

(2) The peptides were designed to represent regions on the outside of the protein. By observing the amino acid sequence of the LH receptor in the sheep and other species (rat (McFarland et al., 1989), pig (Loosfelt et al., 1989) human (Minegish et al., 1990) and mouse (Gundermann et al., 1992)), it was determined that certain regions had a higher probability of appearing on the external faces of the protein. As antibodies can only interact with sequences that they contact, it was important to know which regions were on the outside of the protein. This is especially important when examining non-denatured proteins, *i.e.* for immunohistochemistry.

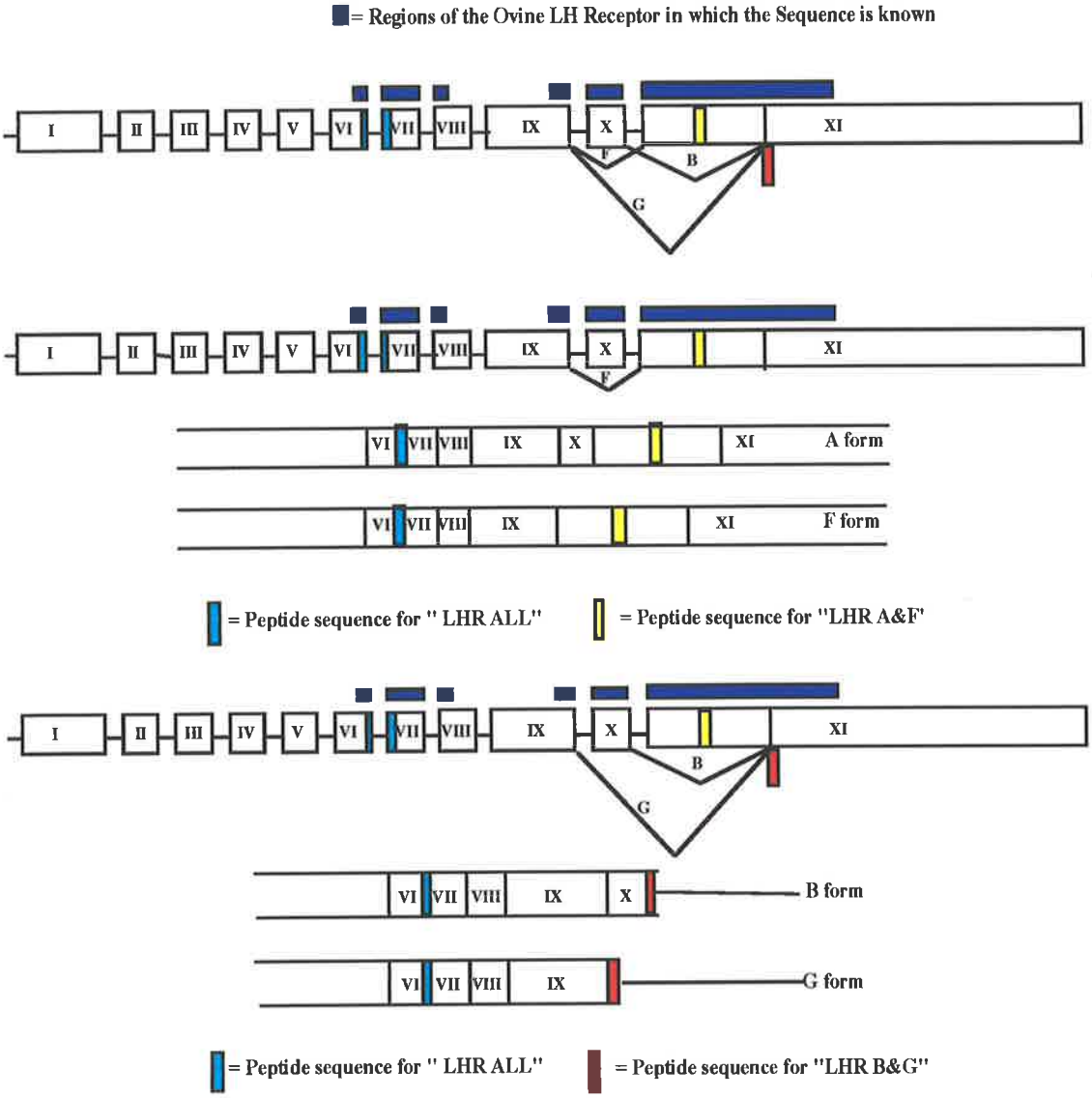

Figure 4.1. Predicted amino acid sequence of the ovine LH receptor based on the cloned regions oLHR-1, and oLHR-2&3. Diagramatical representation of the genomic structure of the ovine LHR, based on the known rat structure. oLHR-1 and oLHR-2&3 are the RT-PCR products that were cloned and sequenced. The amino acid sequences of oLHR-1 and oLHR-2&3, with exon boundaries are indicated by the dark blue boxes. The light blue, yellow and red boxes indicate the regions that peptides were based on for the production of antibodies LHR-ALL, LHR-A&F, and LHR-B&G respectively *(see Sections 4.1.2, 4.1.3 and 4.1.4)*.

(3) Peptides were designed to represent regions found in the sheep LH receptor and the translated spliced forms. Since only limited regions of the ovine LH receptor sequence were known, for us to be assured that we had the correct ovine sequence, the sequences of the peptides could only be derived from a limited amount of ovine LH receptor sequence *i.e.* the regions we amplified and cloned as described in the previous chapter.

(4) To allow iodination of the peptides, it was necessary that tyrosine residues were present. It was not vital that these residues be part of the native peptide sequences of the ovine LH receptor, as they can be added to the end of the peptides. This allows iodination without interfering with the ability of the peptide to generate antibodies that will recognise the native sequence.

(5) The peptide sequences chosen were, if possible, relatively homologous between species. This would allow the antibodies to detect the alternatively spliced products in other species. However the peptides were designed so that they would not detect other proteins *i.e.* the FSH or TSH receptor. A region just prior to the first transmembrane domain (amino acids 325-342 of the rat sequence) is conserved (82% homology) between the LH, FSH and TSH receptors, and as such could not be considered for raising antibodies, as these antibodies would be expected to cross react with the FSH and TSH receptors. Although there is another highly conserved region betwen the LH, FSH and TSH receptors (at amino acids 253-262 of the rat sequence; 90% homology), this is encoded by the bases that primer LHR-S3 binds and as such the ovine sequence for this region is not known.

4.1.2 Peptide LHR ALL

The region for a possible peptide that met the above criteria for peptide LHR-ALL was narrowed down to the sequence encoded for by the probe oLHR-1, but not including the PCR primers *(see Figure 4.1)*, and theoretically the last part of exon IX cloned in oLHR-2&3. However, after eliminating the amino acids encoded by the PCR primers (the ovine sequence for these regions is not known), this region was only seven amino acids in length. As such, it was not of sufficient length for raising antibodies *(see Section 4.1)*. After observing the hydophilicity, surface probability, flexibility, and antigenic index of this region predicted using MacVector *(see Figure 4.2)*, the following sequence was chosen:

Ac-y**SLELKENARLE**c-OH

with the tyrosine (Y) and cysteine (C) residues (shown in lower case), added to the native sequence (shown in bold). The cysteine residue was added to allow coupling of the peptide to KLH, and the tyrosine residue added to allow iodination of the peptide. The sequence from which this peptide was derived is indicated in *Figure 4.1*.

4.1.3 Peptide LHR A&F

The sequence from which this peptide was derived was narrowed down to the amino acids encoded by exon XI cloned in oLHR 2&3 *(see Figure 4.1)*, but occurring before the first transmembrane helix, as the amino acids in the transmembrane domain highly conserved between the other gonadotrophin and G-protein associated receptors *(see Section 1.4.5)*. The hydophilicity, surface probability, flexibility and antigenic index of this region was predicted using the computer program MacVector *(see Figure 4.3)*, and indicated that the following sequence complied with the majority of criteria for generating effective antibodies. This sequence was:

Ac-**AESELSGWDYDYG**c-OH.

A cysteine residue (shown in lower case) was added to the native sequence (shown in bold) to allow coupling of the peptide to KLH. The sequence from which this peptide was derived is indicated in *Figure 4.1*.

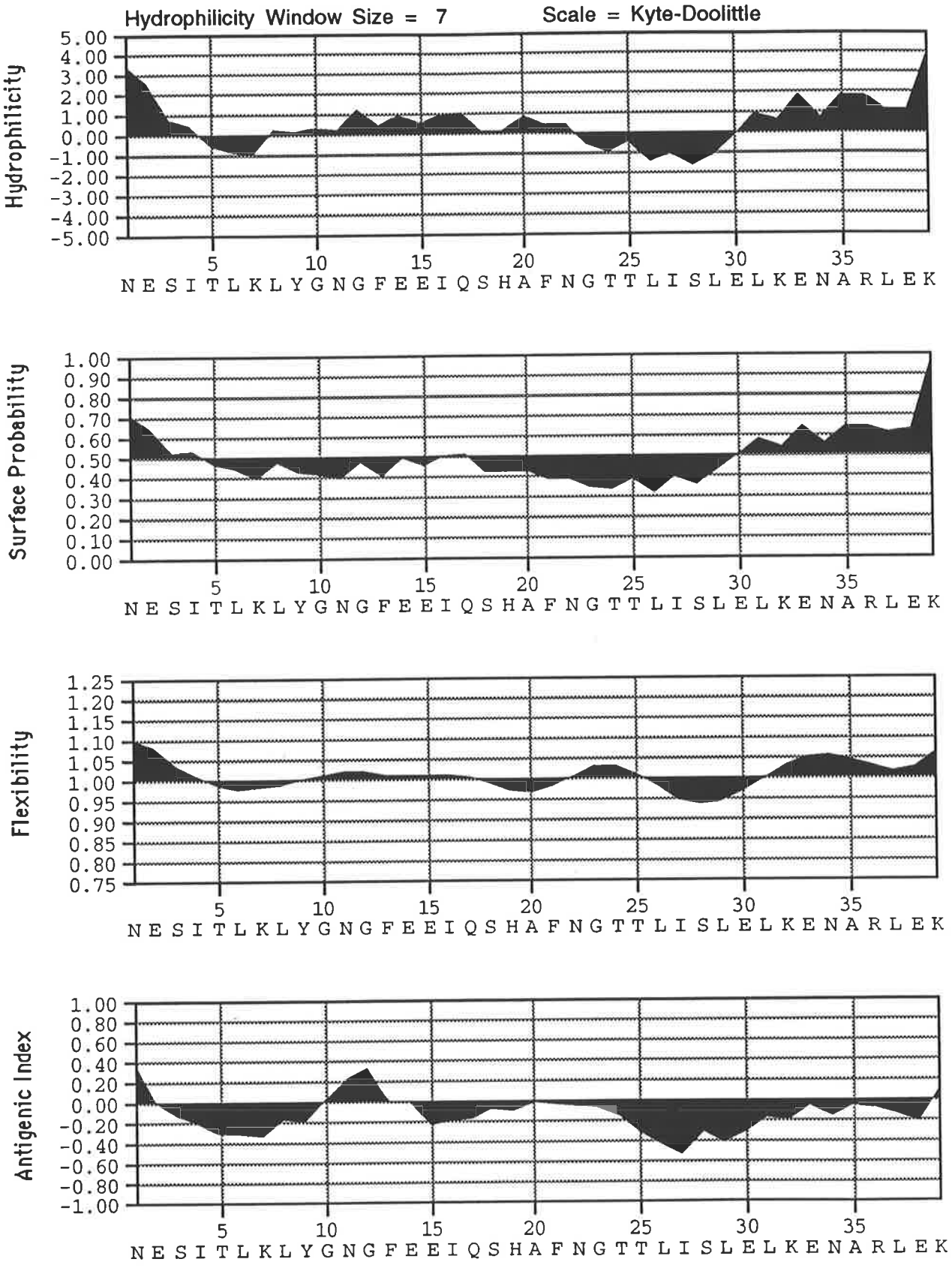

Figure 4.2 The hydophilicity, surface probability, flexibility, and antigenic index of the amino acid sequence generated from the predicted translation of oLHR-1. These graphs were determined using the program MacVector® (International Biotechnologies, New Haven, CT).

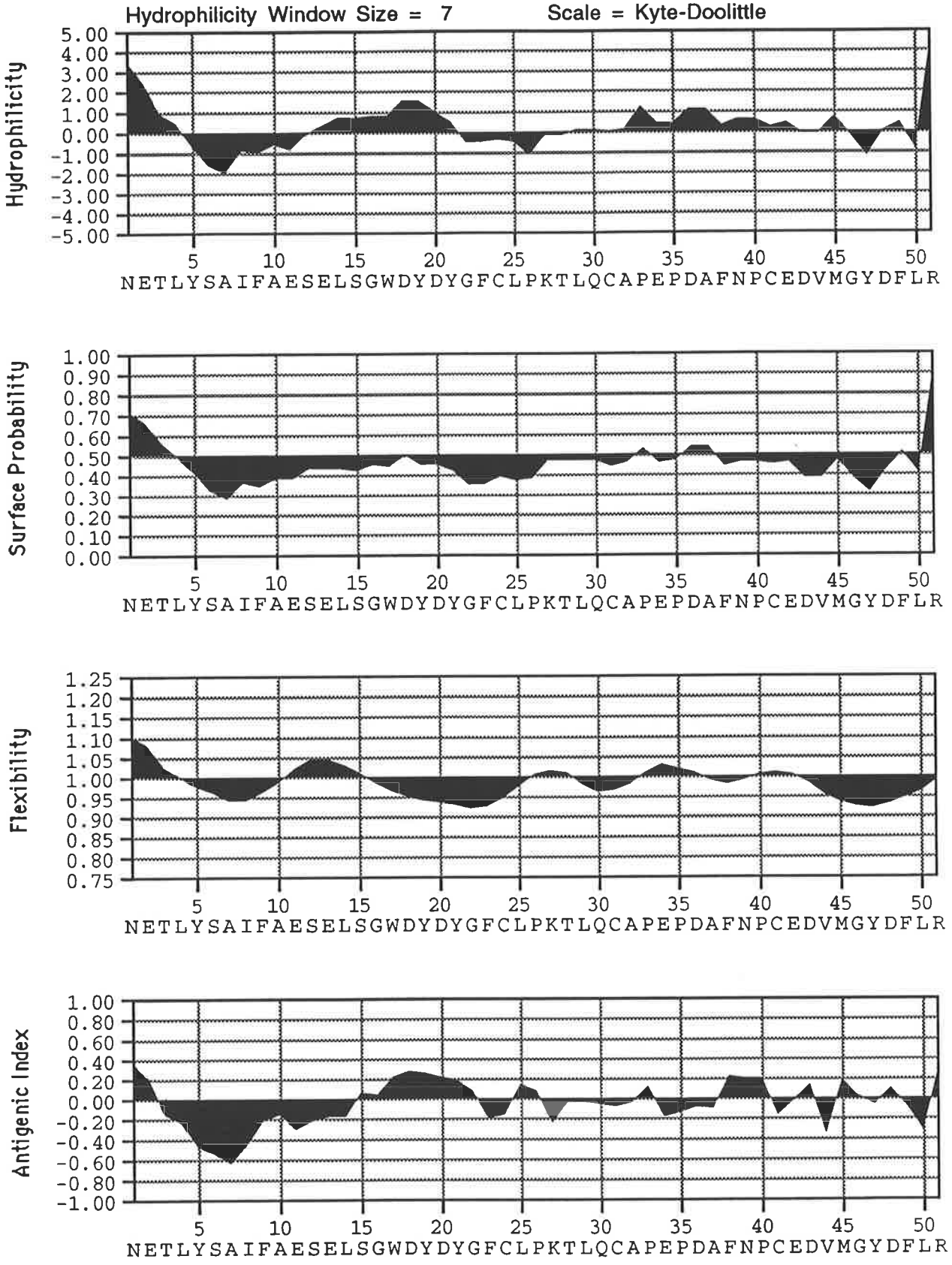

Figure 4.3 The hydophilicity, surface probability, flexibility and antigenic index of the amino acid sequence generated from the predicted translation of oLHR-2&3.

4.1.4 Peptide LHR B&G

This peptide was designed based on the predicted amino acid sequence derived from the cDNA sequence of the LHR B and G splice variants isolated from ovine ovarian cDNA (Bacich *et.al.*, 1994 and Chapter Three of this thesis). The peptide was derived from sequence downstream of the out-of-frame splicing event into exon XI in the B and G forms *(see Figure 4.1)*. The sequence for this peptide was limited, and consisted of the unique thirteen amino acid tail that was generated by the frame shift of the B and G splice forms. The advantage of using this region was that this sequence was completely unique to the LH receptor B and G splice forms, and therefore antibodies raised against it would not cross react with the A or F forms (Bacich *et al.*, 1994). The hydophilicity, surface probability, flexibility and antigenic index of this region was predicted using MacVector *(see Figure 4.4)*, and indicated that the following sequence complied with the majority of criteria for generating effective antibodies. The following peptide sequences were chosen:

Peptide 1 Ac-**ETLLLHGALPATHCLS**-OH

Peptide 2 Ac-y**ETLLLHGALPATHCLS**-OH

The glutamic acid (E), threonine (T) and first leucine (L) residues are common with the ovine LHR-A form. The sequence from which this peptide was derived is indicated in *Figure 4.1*. Peptide 1 was coupled to KLH via the cysteine residue (C) found in the native sequence. Peptide 2 was obtained to determine the antibody titres against peptide 1, in antibody binding assays *(see Section 2.4.7)*. It is identical to peptide 1, except that it contains a tyrosine residue (shown in lower case), through which labelled iodine was subsequently coupled, and it was not cross linked to KLH and not used to generate antibodies.

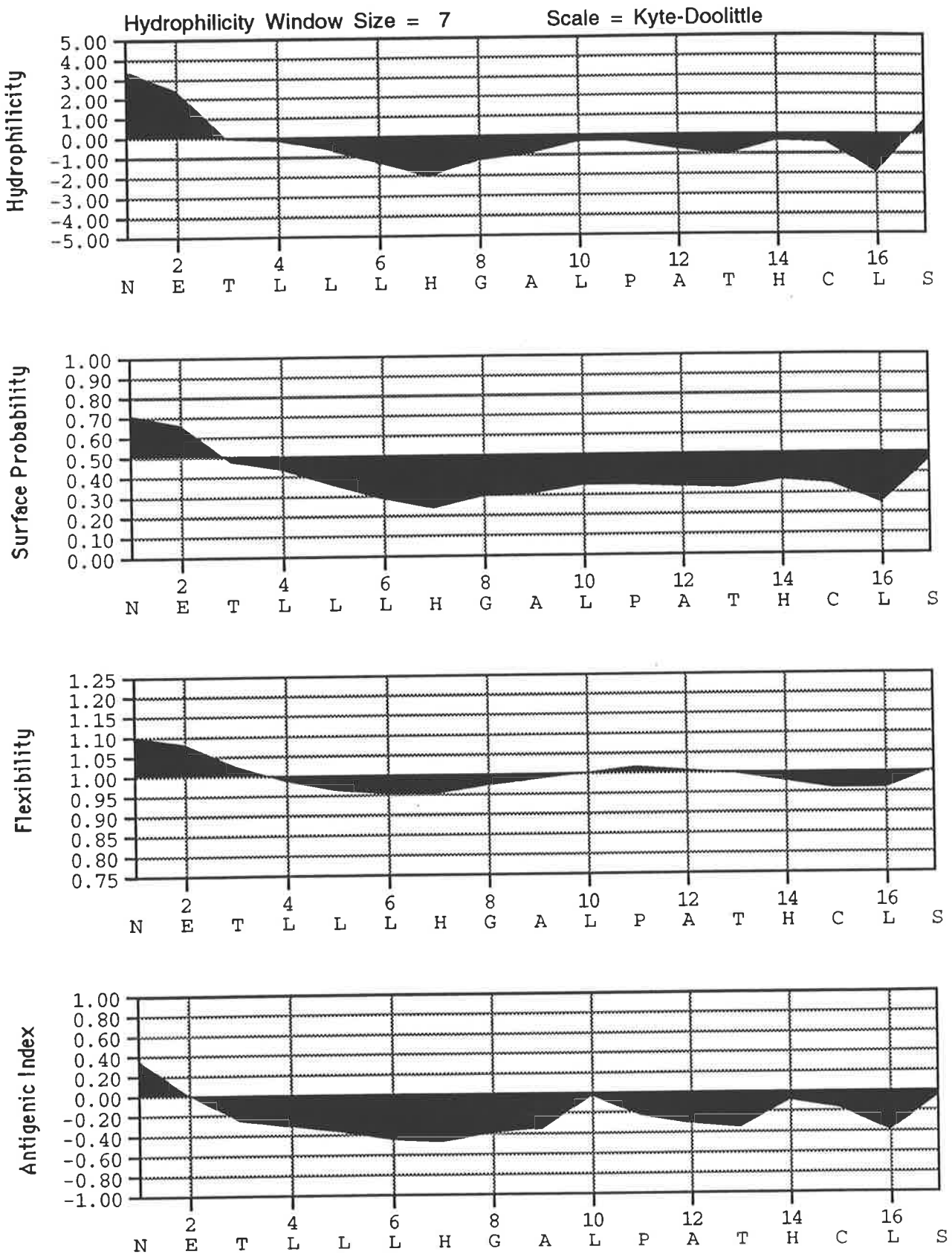

Figure 4.4 The hydrophilicity, surface probability, flexibility and antigenic index of the amino acid sequence generated from the predicted translation of oLHR-2&3 (for the B splice form).

4.1.5 LHR antigens

The LHR peptide sequences were synthesised using standard PIN (sic) technology by Chiron Mimotopes Pty. Ltd. (Clayton, Vic, Australia). The peptides were used for two purposes; firstly, to immunise rabbits, and secondly, for use in binding assays to determine the antibody titres. The peptides that were used for immunisation were obtained coupled to KLH. The other peptides were not coupled to KLH, but contained a tyrosine residue through which radioactive iodine was able to be coupled for use in the antibody binding assays. All of the peptides were acetylated at the N-terminal. Approximately one milligram of each peptide was coupled to KLH via the cysteine residue using 6-maleimido-capronic acid N-hydroxysuccinate ester (Lee *et al.*, 1985). The peptides were purified by high pressure liquid chromatography (HPLC)[1]. Purification and crosslinking to KLH was performed by Chiron Mimotopes Pty Ltd.

4.1.6 Immunisation Protocol.

Generation of the antibodies was carried out as described in Section 2.4.3. Briefly, two adult male New Zealand white rabbits per peptide were injected subcutaneously with peptide conjugated to KLH (equivalent to 100 μg peptide), dissolved in 2 ml of 50% saline, and 50% Freunds complete adjuvant (Sigma Chemical Co., St. Louis, MO). The rabbits were designated R11 and R12 (immunised with peptide LHR A&F), R21 and R22 (immunised with peptide LHR ALL) and R41 and R42 (immunised with peptide LHR B&G). The rabbits were also immunised with 0.5 ml of Diphtheria, Tetanus and Pertussis toxin *(see Section 2.4.3)*. Intramuscular booster injections were carried out every 4-8 weeks *(see Figure 4.5 for exact times that bleeds*

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[1] The purity of the LHR ALL, LHR A&F and LHR B&G peptides coupled to hemocyanin were 87%, 88% and 97% respectively, and the purity of the uncoupled peptides were 96%, 93% and 98% respectively.

and injections were performed), and contained the equivalent of 100 µg peptide dissolved in 1ml of 50% Freunds incomplete adjuvent (Sigma Chemical Co., St. Louis, MO, USA) and 50% saline. Blood samples (10 ml) were taken before every booster injection as well as 2-6 weeks after each injection. After approximately six months, the rabbits were bled out, with at least 100 ml of blood per animal collected at this point. Serum was isolated from the blood samples on the day that phlebotomy was carried out *(as described in Section 2.4.4)*, and stored at -20°C. Immunoglobulin type G was prepared from the rabbit sera with the aid of caprylic (octanoic) acid (as described Steinbuch and Audran, 1969; *see Section 2.4.6 for details*).

4.2 Antibody Screening

To determine the antibody titre of the serum obtained from the rabbits, unconjugated peptides were iodinated *(as described in Section 2.4.5)*, and antibody binding assays for each peptide designed *(as described in Section 2.4.7)*. Briefly, this involved diluting the sera in antibody assay buffer *(see Section 2.3.1)* and incubation overnight with 10 000-25 000 cpm/100 µl of iodinated peptide at 4°C. Bound peptide was then separated from unbound peptide by precipitation with polyethylene glycol 6000 and human immunoglobulin *(see Section 2.4.7)*. The bound precipitated pellets were then counted in a gamma counter, and the antibody titre determined by the dilution of the antisera that gave 50% of maximal binding

4.2.1 Antibody Titres

Initially, antibody titres were estimated from sera that was diluted 1 in 400 with antibody assay buffer, and sera for each bleed from all rabbits were examined for binding to iodinated peptide *(as described in Section 2.4.7)*. This enabled estimation of which bleeds had the highest antibody titres, and to compare antibody production between the rabbits. The antibody binding assays for all of the LHR peptides demonstrated a high degree of binding for almost all bleeds, excluding the pre-immune samples in which binding was negligible. The first post-immunisation bleed

(7 weeks after the initial immunisation) demonstrated relatively low immunogen binding in some of the animals. Based upon these results (data not shown), an individual sample from each rabbit which demonstrated a high level of binding was selected. A wide range of 1 in 4 serial dilutions (ranging from a 1:25 to a 1:102600 dilution), were made on the selected sera to estimate antibody titres. These diluted sera were then analysed in triplicate using the antibody binding assay.

4.2.2 Results

The serial dilution of sera from all three peptides and all six rabbits demonstrated that all antisera had a high antibody titre specific to the peptide against which the rabbit was immunised. Analyses of the binding data revealed that at a dilution of 1 in 25, all of the sera were very close to maximal binding. The binding titre for each rabbit's sera to the peptide against which it was immunised was determined as the dilution at which 50% maximal binding was be achieved.

The antibody titres of the sera isolated from bleed no 5 (*i.e.* 19 weeks after the initial immunisation) from the rabbits immunised against peptide LHR-ALL were very similar. Fifty percent maximal binding occured at a dilution of 1 in 8000 for rabbit R21 and 1 in 6000 for rabbit R22. Sera from each bleed was then diluted to 1 in 8000 in antibody binding buffer, and antibody binding assays performed on the samples in triplicate *(see Figure 4.6)*. The antibody titres of sera isolated from bleed no 5 from the rabbits immunised against peptide-A&F were similar to each other, but significantly lower than the antibody titres for antibodies raised against peptide LHR-ALL. Fifty percent maximal binding occurred at a dilution of 1 in 2000 for rabbit R11 and 1 in 1600 for rabbit R12. Sera from each bleed was then diluted to 1 in 3000 in antibody binding buffer and antibody binding assays performed on the samples in triplicate *(see Figure 4.7)*. The antibody titres of the fifth bleed from the rabbits immunised against peptide LHR-B&G, were considerably different between the two rabbits. Rabbit R41 displayed 50% maximal binding at a dilution of 1 in 200, while

for rabbit R42 this occurred at a dilution of 1 in 1500. All bleeds were diluted, 1 in 400 for rabbit R41 and 1 in 2000 for rabbit R42, and antibody binding assays performed on the samples in triplicate *(see Figure 4.8)*.

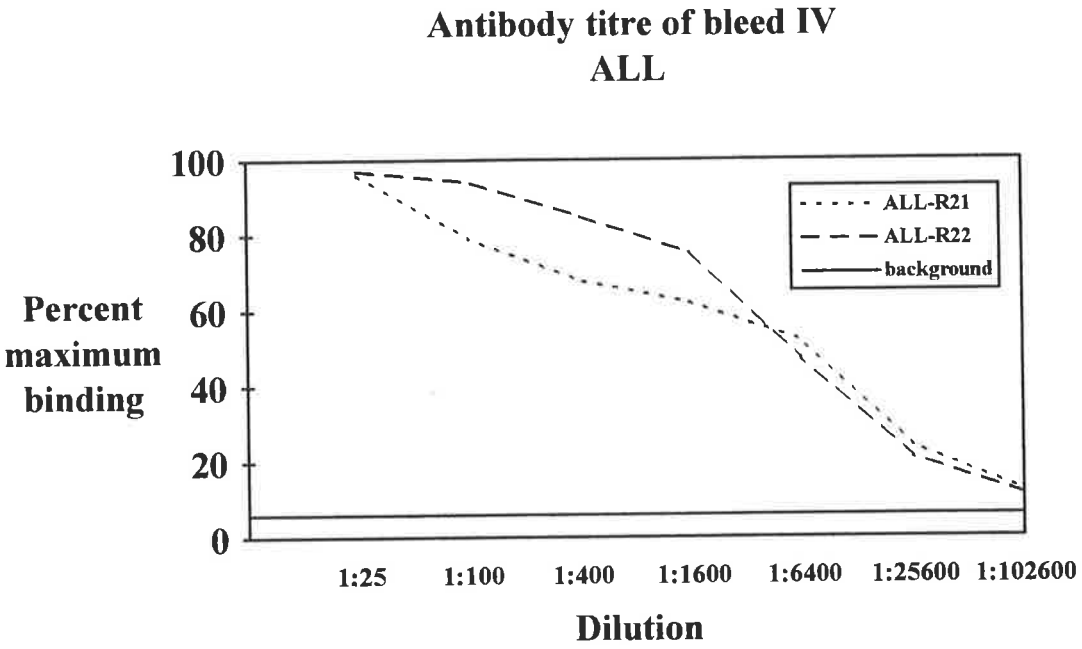

Figure 4.6 Antibody binding assay for sera from rabbits R21 and R22. Analysis of antibody binding of serial dilutions of sera from rabbits R21 and R22 revealed that 50% maximal binding occurred at dilutions of 1:8000 and 1:6000 respectively.

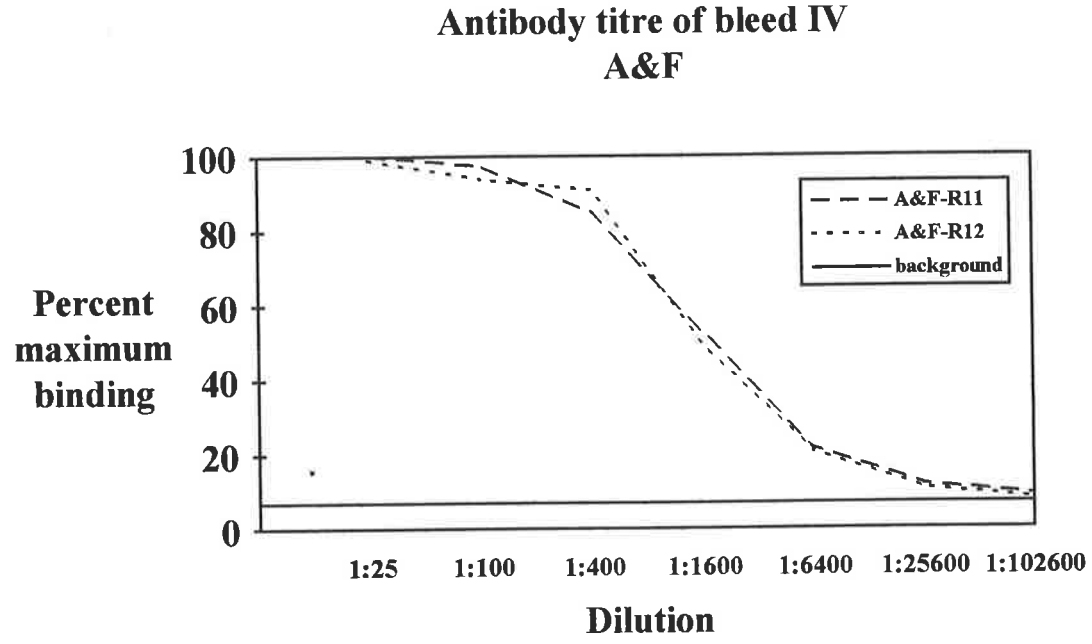

Figure 4.7 Antibody binding assay for sera from rabbits R11 and R12. Analysis of antibody binding of serial dilutions of sera from rabbits R11 and R12 revealed that 50% maximal binding occurred at dilutions of 1:3000 for both.

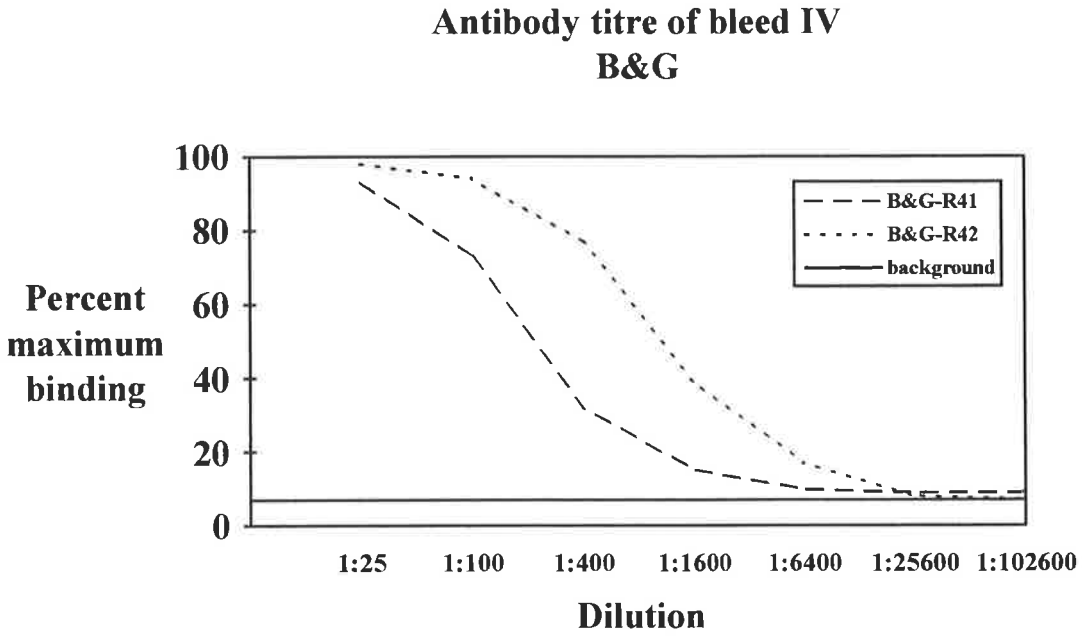

Figure 4.8 Antibody binding assay for sera from rabbits R41 and R42. Analysis of antibody binding of serial dilutions of sera from rabbits R41 and R42 revealed that 50% maximal binding occurred at dilutions of 1:200 and 1:1500 respectively.

All bleeds, except the pre-immune and the first bleed after immunisation, demonstrated a high amount of binding against the peptide that the rabbit was immunised. These results revealed which bleeds had the highest concentration of antibody specific to the peptide, and as a result this bleed was used for future experiments *i.e.* bleed 2 for rabbit R21 and bleed 5 for R22. The exception to this was with rabbits R42 and R41, where the final bleeds were used as they demonstrated very similar binding titres to the highest bleed from each rabbit.

Cold Competition Antibody binding assays

Specificity of the antisera to the peptide against which the rabbits were immunised was demonstrated by two approaches. Firstly, as a negative control, the sera were incubated with iodinated peptides other than the one used to immunise the particular rabbit. These experiments used the antibody binding assay *(see Section 2.4.7)*, and routinely demonstrated binding similar to the pre-immune and background (*i.e.* no

sera) tubes. These experiments demonstrated that the antibodies were sufficiently specific that they would not cross-react with the peptides against which the other rabbits had been immunised, *i.e.* sera from the rabbits immunised against LHR-ALL would not bind the peptides LHR-A&F or LHR-B&G and *vice versa.*

Secondly, the specificity of the antibodies was confirmed by a modification of the antibody binding assay. This involved competing out the binding of the antibody for the labelled peptide with excess unlabelled peptide. If the binding was specific, then the amount of bound labelled peptide should be considerably less than when no excess cold peptide was added. However, if it was non-specific, the addition of unlabelled peptide to the antibody binding assay would result in little or no change in the amount of bound labelled peptide.

The results of the cold competition experiments for LHR-ALL, LHR-A&F and LHR-B&G antisera from all the rabbits *(see Figure 4.9 for an example)* clearly demonstrated that the antisera were specific for the peptide against which the rabbit was immunised. The amount of labelled peptide binding steadily decreased with the increased amount of unlabelled peptide present. Interestingly, the values never reached the level of background or pre-immune binding, although the values were very close to this at the higher levels of excess peptide, *i.e.* 800 fold excess *(see Figure 4.9).*

The above experiments demonstrated that our intial aim had been achieved. Antibodies were raised against the peptides against which the rabbits were immunised. The antibody titres were significantly high for the sera to be potentially useful in antibody binding assays, immunohistochemical approaches, Western analyses and immunoprecipitations. In addition, we also demonstrated that the antibodies bound specifically to the peptide against which they were immunised, and

could be competed out with unlabelled target. Importantly, the antibodies were shown not to cross-react with the other peptides.

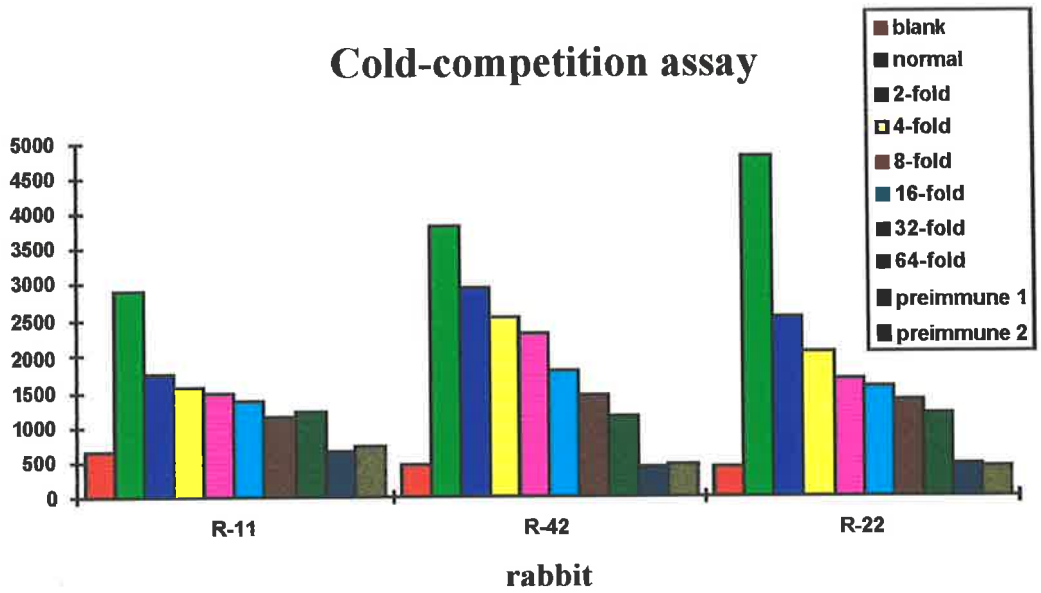

Figure 4.9 Cold-competition assay. Graphical representation of the results obtained for the cold-competition assay described in Section 4.2.2. Specificity of the antibodies to the peptide against which the particular rabbit was immunised is indicated by reduced binding of labelled peptide with the addition of increased unlabelled peptide.

4.3 Immunohistochemistry using the Ovine LHR Antibodies

The localisation of hCG and LH binding sites has been extensively documented in ovarian tissues of both ovine and other species (McClellan *et al.*, 1975; Carson *et al.*, 1979; O'Shea *et al.*, 1980; Rodgers and O'Shea, 1982; Visintin and Luborsky 1989; Lakkakorpi *et al.*, 1991; Bukovsky *et al.*, 1993) These cell types include thecal cells from all stages of follicles, granulosa cells from preovulatory follicles, and on both large and small luteal cells of the corpora lutea. The aim of these experiments was to use immunohistochemistry to confirm these findings, and to determine the temporal and spatial expression patterns of the LH receptor isoform proteins in the various cell types. In addition, it was anticipated that this technique would be useful in localising the putative proteins to subcellular fractions or individual cells within the tissues, resolving some of the controversy; (1) are the alternatively spliced forms of the LHR translated *in vivo*, and (2) if so, are these forms secreted as has been suggested by

some expression studies (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992), or trapped intracellularly as suggested by other expression studies (Ji *et al.*, 1990; Koo *et al.*, 1994; Zhang *et al.*, 1995).

4.3.1 Method

Ovine ovaries were collected from from cycling ewes obtained from a local abattoir, and placed in Bouin's Fixative Solution *(see Section 2.5.1)* for 24 hours, but not more than 48 hours. The ovaries were then washed in 70% ethanol for at least 24 hours, before being sent to the Histology Department (Flinders Medical Centre) and placed into paraffin wax blocks. Sections (5 μm) were cut from the wax blocks using a microtome *(see Section 2.6.7)*, and mounted onto slides that had been treated with poly-L-ornithine *(see Section 2.6.7)*. Slides with tissue sections were generally used within one week of cutting, although some were stored for up to two months at room temperature. In addition to immunohistochemical analysis of the tissue sections, general staining of the sections with hematoxylin and eosin was performed on a sample of the tissue sections *(see Section 2.6.7)*. This allowed for easy identification of the cell types within the tissue sections *(data not shown)*.

Immunohistochemical staining with either the purified immunoglobulin G fraction or whole (untreated) antisera was performed *(as described in Section 2.6.7)*. Briefly, this involved reducing non-specific binding by blocking sections with normal goat serum (1:50 dilution in PBS, pH 7.4, for one hour), before incubating with the primary antisera (at the appropriate dilution) for three hours. The sections were then washed, and incubated with the secondary biotinylated antisera (1:300 dilution of goat anti rabbit serum), before washing and incubating with avidin-biotin-horseradish peroxidase complex (diluted 1 in 300; Vectorstain®, ABC Kit). Peroxidase activity was localised using 3,3'-diaminobenzidine (DAB; Sigma Chemical Company) as the substrate, and the sections washed and mounted *(as described in Section 2.6.7)*. Sections were then examined and photographed using light microscopy.

Initally, the sera were diluted 1:10, 1:100 and 1:1000 to determine an optimal antibody dilution. Four controls were present for each immunohistochemical experiment. These consisted of; (1) pre-immune serum from the animal that the post-immunisation sera came from, to help identify any non-specific binding, (2) a section in which the primary antibody (*i.e.* anti-LHR-ALL, anti-LHR-A&F or anti-LHR-B&G) was not added so that only the secondary antibody (goat anti-rabbit antibody) was used. This control was carried out to identify any proteins and/or cells that the secondary antibody bound non-specifically. The third control was the use of no antibodies at all. This identifies any cells which contain endogenous alkaline phosphatase activity, which would react the same way as the horseradish peroxidase used for detection, and as such could generate false positives. The final control was the use of an antibody that was known to be positive for proteins found in some of the cells found in the sections. This control antibody detects the enzyme 3β-hydroxy-steroid dehydroxylase and was a kind gift from Dr Ian Mason. This antibody binds to luteal cells in the corpus luteum in ovine ovaries (Lorence *et al.*, 1990).

4.3.2 Results and Discussion

Regardless of the dilution of the antisera used for the immunohistochemical experiments (from 1:10 to 1:1000 dilutions), there were low levels of non-specific binding for all the bleeds of all rabbits *(see Figures 4.10 and 4.11)*, with one exception, the sera from rabbit R12. All sera produced very light staining of some of the endothelial cells found in the ovary. This was non-specific as even the pre-immune rabbit sera lightly stained these cells. Although some large cells in the stromal regions of the ovary appeared yellow, these cells were obviously pigmented yellow as they appeared this colour even in sections that had not been treated.

Sera from rabbit R12, that had been immunised against peptide LHR-A&F, displayed high levels of specific binding to the thecal cells of the theca interna of antral follicles, presumably thecal cells from the remains of some atretic follicles and

relatively low levels of binding to luteal cells (at least large luteal cells and possibly small luteal cells) within the corpus luteum *(see Figure 4.11)*. Unfortunately this result was also seen in the pre-immune sera from this rabbit *(see Figure 4.11)*, and as such it was concluded that the strong staining seen was not a result of antibodies raised against the LHR-ALL peptide that the rabbit was immunised against.

The polyclonal antibody to 3β-hydroxysteroid dehydrogenase that was used as a positive control generated very clear results, with very heavy binding to the cells of the corpus luteum, and very low levels of background *(see Figure 4.11)*. The negative controls were all negative. These results suggest the immunohistochemical technique was functioning, at least with the 3β hydroxysteroid dehydrogenase antibodies.

One explanation for the lack of binding observed with the LHR-B&G antisera is that these forms are not translated into a protein *in vivo*. However, this explanation can not account for the lack of binding of the anti-LHR-ALL and anti-LHR-A&F antisera. It is well established that LH receptors are present in the ovine ovary, and therefore if the anti-LHR-ALL and anti-LHR-A&F antisera recognised the native A form receptor, specific binding with these sera would be expected. As none of the LHR antibodies produced specific binding (with the exception of that from rabbit R12 as previously discussed), it was concluded that they were not useful for immunohistochemical studies.

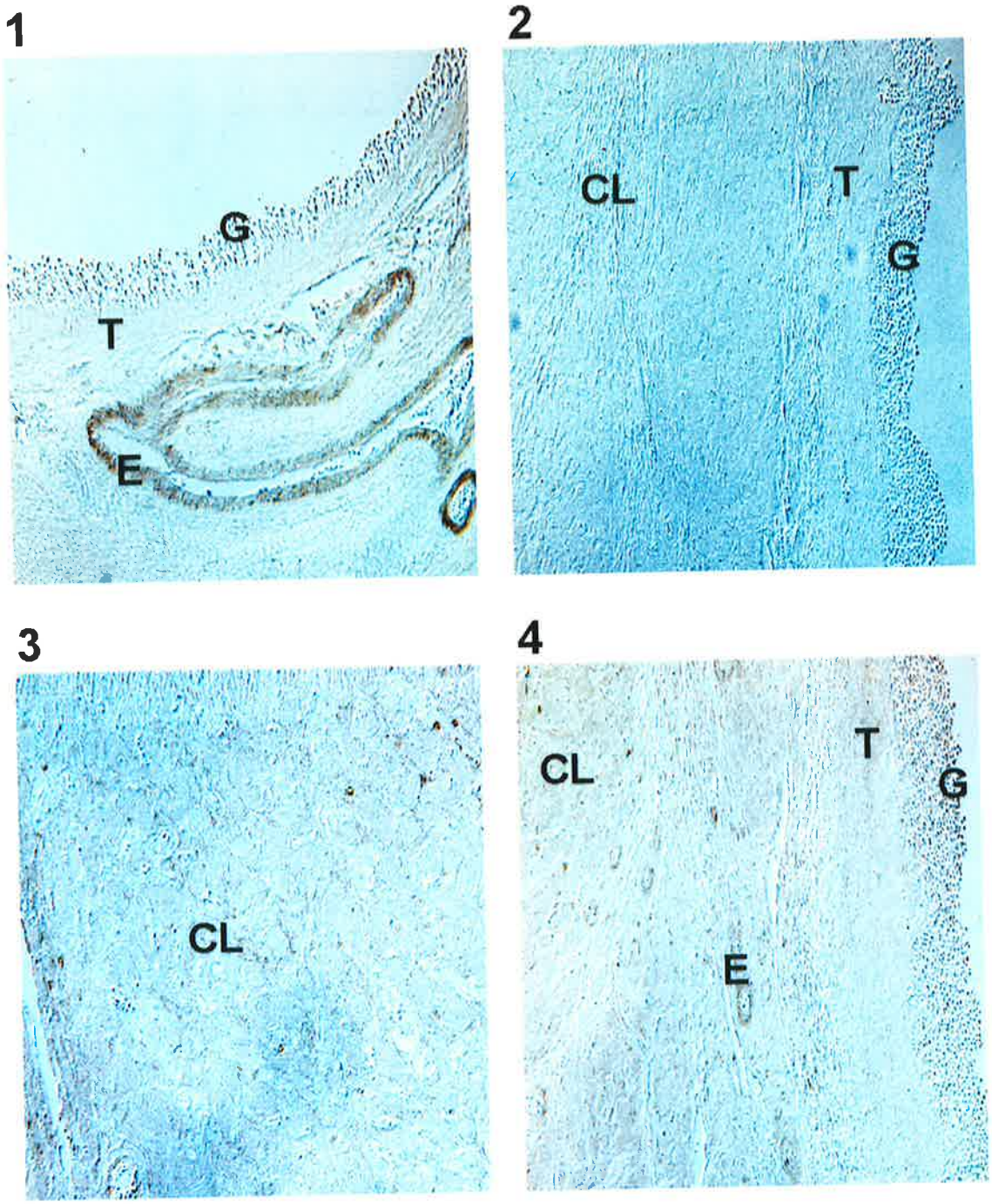

Figure 4.10. Immunohistochemical analysis of ovine ovaries. (1) Representative slide of preimmune staining; note light endothelial cell staining (2) Low level background staining of follicle and CL using anti-LHR-ALL antisera. (3) Low level background staining of large luteal cells in a CL using anti-LHR-A&F antisera. (4) Background staining of CL and stroma; note the light staining of endothelial cells using anti-oLHR-B&G antisera. T indicates theca, G indicates granulosa cells, CL indicates corpora lutea and E indicates endothelial cells.

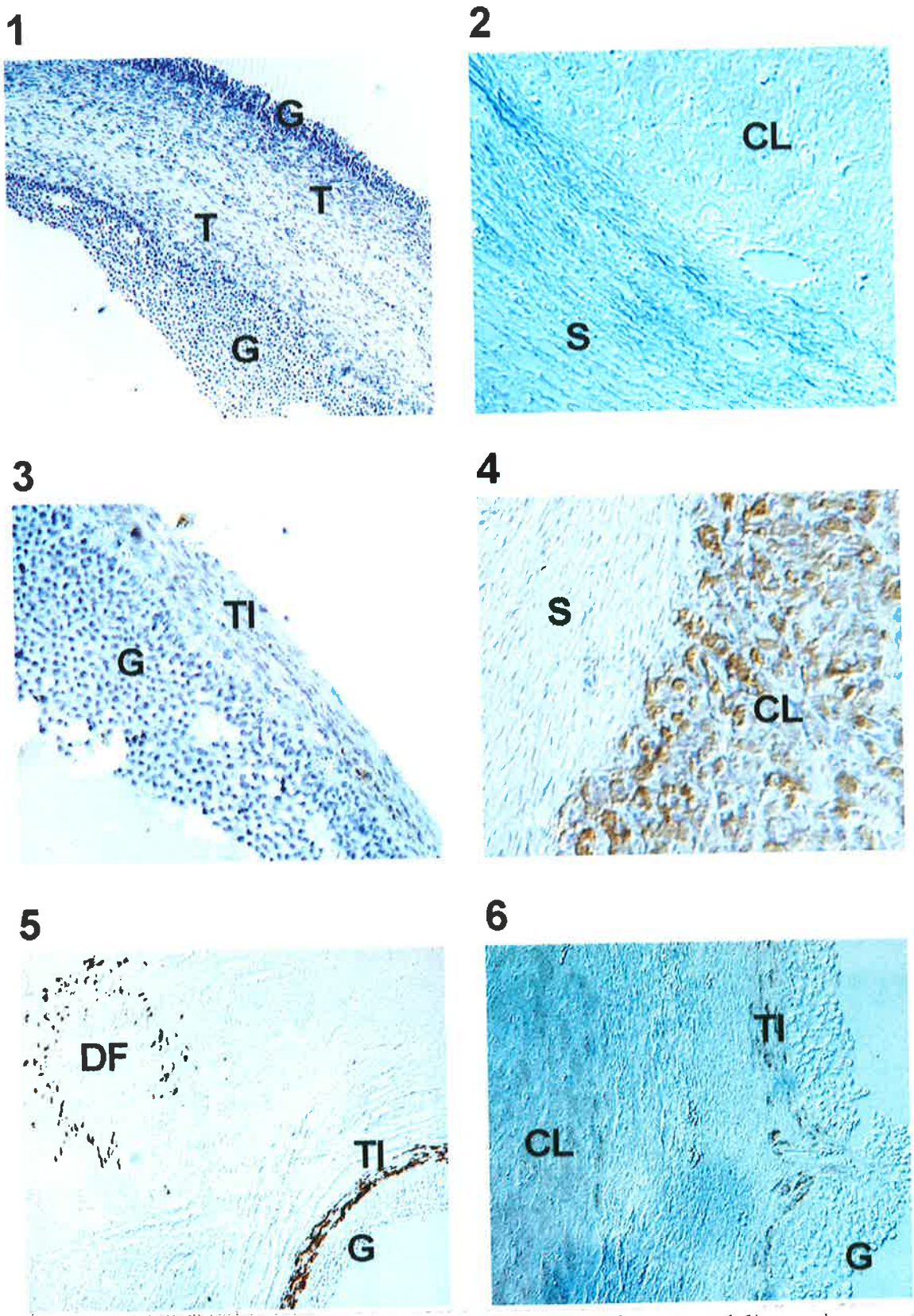

Figure 4.11. Immunohistochemical analysis of ovine ovaries. (1 and 2) negative control - no primary antibody. (3 and 4) 3β–HSD staining over CL, and weaker staining over thecal cells of an antral follicle. Both the preimmune (5) and the postimmune (6) sera of rabbit R12 resulted in dark staining over the theca interna, and presumably the remnants of thecal cells from degraded follicle with light staining over luteal cells of a CL. T indicates theca, G indicates granulosa cells, S indicates stroma, TI indicates theca interna, CL indicates corpora lutea and DF indicates degenerate follicle.

There are at least two possible explanations for the lack of results produced by the immunohistochemical studies. The most likely reason was that the antigens were not recognised by the antibodies in their native, non-denatured form. As the rabbits were immunised with a small peptide, the antigenic epitope that the antibodies were raised against would have had little, if any, defined tertiary structure. The targeted epitope in the LH receptor isoforms would form part of a much larger protein, and therefore there would be considerable opportunity for various tertiary structures of the epitope. The different tertiary structures would affect antibody recognition of the epitope sequence. Alternatively, the antigenic sites may have been hidden by the non-denatured protein, preventing the antibodies from reaching their antigenic target due to steric hindrance. From the outset of these experiments there was a strong possibility that differences in tertiary structure would inhibit the use of these of the antibodies for immunohistochemical studies. However, this problem could be considerably lessened by the use of denatured proteins for experiments (*i.e.* Western analyses).

Another explanation for the lack of positive results from the immunohistochemistry experiments is that the processing of the sections prior to labelling may have left the antigen non-reactive. There are many ways that tissue can be prepared *e.g.* frozen versus paraffin sections, and/or fixed *e.g.* Bouin's, paraformaldehyde, formaldehyde and so forth. It was hoped that the tissue sections that had been prepared would be adequate, as they bound the 3-β HSD antibody. If the proteins were treated with different fixative reagents etc. then they may have had a different tertiary structure, and hopefully one that the antibodies would recognise as all three antisera were designed to bind the same type of nascent proteins (LHR isoforms).

There a number of modifications to the standard immunohistochemical techniques that could be attempted to optimise these studies by increasing access to the antigen (such as cryosection, microwaving the sections etc). However, at this stage it was not

known whether the antibodies, which were raised against denatured peptides, could in fact recognise the denatured native protein. If the denatured protein could not be detected, optimisation of the immunohistochemical protocols would be a fruitless exercise. Western analyses provide an easy and convenient method for determining if antibodies can detect denatured proteins.

4.4 *In Vitro* Expression of the Partial Ovine LHR Clones

The Western analyses were designed to answer two questions; firstly, do the antibodies recognise the ovine LH receptor products? Secondly, are the alternatively spliced forms of the LH receptor translated *in vivo*? Unfortunately, the initial problem could not be answered unless the spliced forms of the LHR are translated *in vivo*. It is obvious that the full length A splice form is translated *in vivo*. However, to verify that the antibodies could bind the LHR and the translated products of its alternatively spliced transcripts, pure translated splice form products, without contamination with the other forms were required. The best way of achieving this would be to force expression of the alternative LHR cDNA transcripts *in vitro*. This has been done in a number of species in transfection experiments (for example; rat, pig, human and mouse; see Table 1.7). Unfortunately the entire cDNA sequence for the ovine LHR and its alternatively spliced transcripts is unknown, and therefore unavailable for these type of experiments.

Alternatively, it was possible to use the partial cDNA clones from which the peptide sequences were originally derived. RNA transcribed from these cDNA sequences could be translated to produce a protein containing the amino acid sequence that the antibodies were raised against. Although these proteins would be significantly smaller than the putative protein products of the alternatively spliced forms, they would be considerably larger than the peptides. Subsequently, the tertiary structure of the artificially translated proteins should more closely represent that of the LHR isoforms (should they exist), than that of the peptides.

There were two ways that these proteins could be generated. The ovine clones generated from RT-PCR (using primers LHR-S3 and LHR-AS4) of the different splice forms could have been ligated into an expression vector, and then used to transform a cell line. A major problem with this approach was that none of the clones contained a Kozak sequence (Kozak, 1989) or translation initiation codon at the beginning of the cDNA sequence. It was also considered important that the proteins be expressed in a mammalian cell system, so that glycosylation and folding of the proteins would be similar to that occurring *in vivo*. Unfortunately, most of the expression systems available were for bacterial cell systems *i.e.* pGEX, pQE *etc.*, and the eukaryotic expression systems that were available to us (pCMV5 or p91023[B]), were designed for full length cDNA sequence and did not contain a Kozak sequence, an initiator of translation sequence or a polyadenylation signal. While it was possible to ligate the partial cDNA sequences to the 3' end of another gene to generate a chimeric protein containing the region of the splice forms that the peptides were raised against, it was decided that the second approach was simpler.

This approach involved *in vitro* translation by SP6 RNA polymerase mediated transcription of PCR products, and is similar to the method described by Titlo *et al.* (1992). Oligonucleotide primers were designed to amplify the cloned region of the splice forms. Within the sense oligonucleotide primer there was an artificial SP6 RNA polymerase promoter sequence, followed by a Kozak sequence and translation initiation site. The 3' end of the sense primer contained a sequence homologous to the 5' end of the cloned sequence of the splice form for use in the PCR amplification process. Obviously, the Kozak sequence and translation initiation site were designed so that they were in the same reading frame as the cDNA clone. The antisense primer was designed to bind to the 3' end of the cDNA clones, and also contained a stop codon.

The PCR product generated from these primers would be specific for each splice form that was used as a template. RNA transcripts could then be transcribed from this product using SP6 RNA polymerase, and these transcripts could then be translated into a protein product in an *in vitro* translation system *e.g.* rabbit reticulocyte lysate or wheat germ extract systems.

4.4.1 Primer Design

When designing the oligonucleotide primers for the above experiment it was important to generate a product as large as possible, so that the translated protein would be able to be resolved using SDS-PAGE. For this reason, it was necessary that the sense primer bind adjacent to the 5' end of the cDNA clones. In addition, this allows all the splice variant clones to be amplified using the same primers. Furthermore, the sense primer could not end in a base triplet, as this could result in a shuffling effect in amplification resulting in some of the products not remaining in frame after the end of the primer. Therefore a sequence almost identical to primer LHR-S3 was chosen. However, to reduce amplification of the ovine FSH receptor (should this set of primers ever be used to amplify ovarian cDNA) the sense primer also contained three of the 5' most bases of the ovine cDNA sequence. The sequence of primer LHR-S7 is displayed in *Figure 4.12.*

```
LHR-S7     5'-GAATATTTAGGTGACACTATAGAAGCCACCATGGCCAGCCACTGCTGTGCTTTTAG-3'
LHR-S3                          5'-AT..T.GA.TACC.....................    -3'
Rat LHR                           ACGCTG...TACC.............C..C..c..
Pig LHR                           ACGTTG..TTACC.....................
Human LHR                         ACGTTG..TTACC.....................
Ovine FSHR                        .GCC..AC.....................C..cgc
```

Figure 4.12 Oligonucleotide primer LHR-S7 and its homology with primer LHR-S3 and related species trophic hormone receptors. The LHR-S7 primer is aligned with the homologous sequence of primer LHR-S3 and the rat, pig, and human LHR cDNA (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Minegish *et al.*, 1990) and the homologous region from the ovine FSH receptor cDNA sequence (Yarney *et al.*, 1993). **Bold** bases in primer LHR-S7 correspond to the SP6 RNA polymerase promoter sequence, the bases *italicised* correspond to the Kozak sequence which includes the ATG for translation initiation, and the underlined bases correspond to the sequence which is homologous to the ovine LHR sequence. Homologous bases are indicated with a dot, while non-homologous bases are indicated with the appropriate letter for the alternative base which is capitalised if it occurs in the region found in primer LHR-S3 and in lower case if in the region of the cloned ovine LHR cDNA sequence.

The antisense primer (LHR-AS8) was chosen using the computer program Primer Designer *(using the criteria described in Section 2.2.6)*, and binds to a region of the ovine cDNA sequence that is 3' of the B and G splice acceptor site, and 5' of the primer LHR-AS4 binding site *i.e.* the LHR-AS4 sequence was excluded so that the LHR-AS8 primer would be an exact match to the sequence[1] . A stop codon "TAA" was added to the 5'-end of the sequence homologous to the splice forms, so that the translation of the A and F forms (that would still have an open reading frame), would terminate at this point. In addition a *Hind*III restriction enzyme site was introduced in the 5' end to aid in any possible cloning of fragments that may occur in the future. The sequence of the primer LHR-AS8 is displayed in *Figure 4.13* and binds slightly 5' of the region that the primer LHR-AS4 binds.

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[1] The primer LHR-AS4 contains mismatches to the ovine LHR sequence, as determined by the RNase protection assay using the clone oLHR-3 (see Chapter 3)

LHR-AS8	5'-CCA**AAGCTT**AGGCATGGTTATAATACTGGC-3'
Ovine LHR	G.C.GT.AAT.....CCT
Ovine FSHR	.. G.C.GT.AAT.....A...G.GG.....TTT

Figure 4.13 Oligonucleotide primer LHR-AS8 and its homology with ovine LHR and FSHR cDNA sequence. The LHR-AS8 primer is aligned with the homologous sequence from the ovine LHR (Bacich *et al.*, 1994) and FSH receptor cDNA sequence (Yarney *et al.*, 1993). **Bold** bases in primer LHR-AS8 correspond to the HindIII restriction enzyme site, the italicised bases the artificial stop codon, and the underlined bases corresponds to the sequence which is homologous to the ovine LHR sequence. Homologous bases are indicated with a dot, while non-homologous bases are indicated with the appropriate letter for the alternative base.

Polymerase chain reaction amplification of the region spanned by these primers was performed *(as described in Section 2.2.6)* with primer LHR-S7 (844 ng/ml) and primer LHR-AS8 (825 ng/ml) and the four ovine LHR-2&3 splice form clones *i.e.* the A, B, F, and G, as target DNA. Amplification conditions were as described *(see Section 2.2.6)*, with the exception that the annealing temperature was 62°C instead of 60°C. One fifth of the PCR reaction was then subjected to electrophoresis (2% agarose , 1x TBE gel; *see Section 2.2.8*), to confirm that DNA of the correct predicted molecular weight had been amplified *(see Figure 4.14)*.

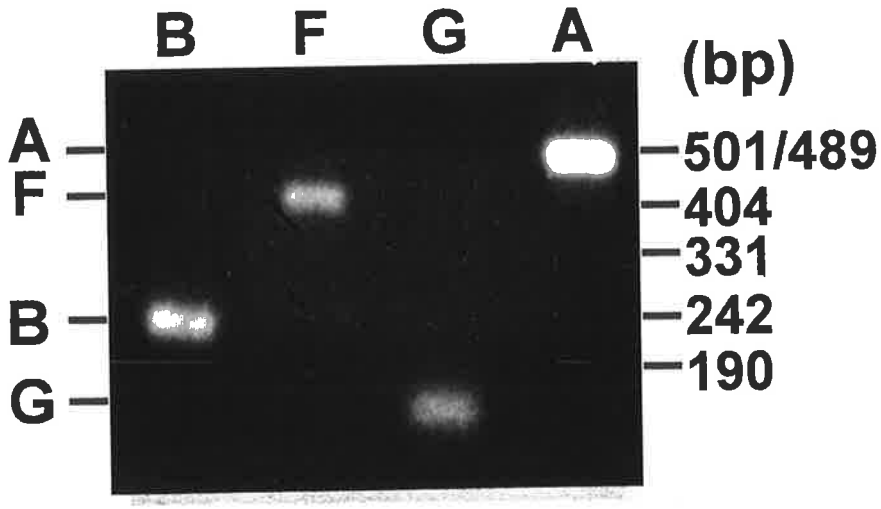

Figure 4.14 Amplification of ovine LHR A, B, F and G splice forms by PCR. Oligonucleotide primers LHR-S7 and LHR-AS8 *(see Figures 4.12 and 4.13)* were used to amplify by PCR DNA from partial clones of (A) the ovine LHR A, (B) the ovine LHR B, (F) the ovine LHR F and (G) the ovine G splice forms *(see Section 3.5.1)*. Amplified DNA was resolved on a non-denaturing 2% agarose gel. The DNA products of correct predicted sizes for the splice forms are indicated (arrows). The molecular weight markers (M) are *Hpa*II digested pUC19

4.4.2 *In vitro* translation of cRNA from the LHR alternatively spliced forms.

Complementary RNA transcripts were transcribed *in vitro* from the PCR products using SP6 RNA polymerase *(as described in Section 2.2.9)*, with the exception that 250 μM of cold UTP was added in the place of ^{32}P-UTP. Controls for the *in vitro* transcription reactions included incorporating ^{32}P-UTP to replicate tubes, and the generation of RNA transcripts with SP6 RNA polymerase from a clone containing an SP6 promoter *e.g.* pSP6-GCSF[3] . The *in vitro* transcription reactions were terminated by heating the reactions to 65°C for 10 mins.

In vitro translation of the cRNA products was performed according the technical manual that came with the Rabbit Reticulocyte Lysate System kit (Promega). Briefly, the rabbit reticulocyte lysate was mixed with 20 μM amino acid mixture, and [^{35}S] labelled methionine with *in vitro* transcribed RNA and the mixture incubated at 30°C for 60 minutes *(see Section 2.6.6 for details)*. In the initial *in vitro* translation experiments only the luciferase positive control RNA that came with the kit produced a protein product. Optimisation of the method revealed that the concentration of the RNA that the Rabbit Reticulocyte Lysate technical manual claimed was necessary for efficient translation, was approximately 10 to 160 fold less than was in fact required, and similar to the concentration supplied in the luciferase RNA positive control *i.e.* the manufacturer suggested 0.125 - 2μg of *in vitro* transcribed RNA per reaction, whereas approximately 20 μg per 25 μl reaction was in fact required.

The size of the potential *in vitro* translated proteins were based upon the cDNA sequence of the PCR products. The potential protein fragment translated from the A form would be 17.6 kDa, 6.2 kDa for the B splice form, 13.9 kDa for the F splice form and 2.5 kDa for the G splice form. The small sizes of these protein products, especially for the B and G splice forms were a major problem for separation and

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[3] This clone was a kind gift from Dr Nick Gough and contained an 829 bp insert mouse GCSF cDNA.

detection by normal Tris-Glycine-SDS-PAGE. However, it was thought that the use of Tris-Tricine-SDS-PAGE (Schägger and von Jagow, 1987; *see Section 2.6.3)* of these proteins would enable them to be separated and identified. Although the protein products of the A and F splice forms could be separated and distinguished by electrophoresis through the Tris-Tricine-SDS-Polyacrylamide gels *(see Figure 4.15)*, the protein products from the B and G transcripts could not, despite attempts to optimise the technique by using different ratios of bisacrylamide to acrylamide. These experiments were further complicated by the lack of commercially available very low weight molecular weight markers.

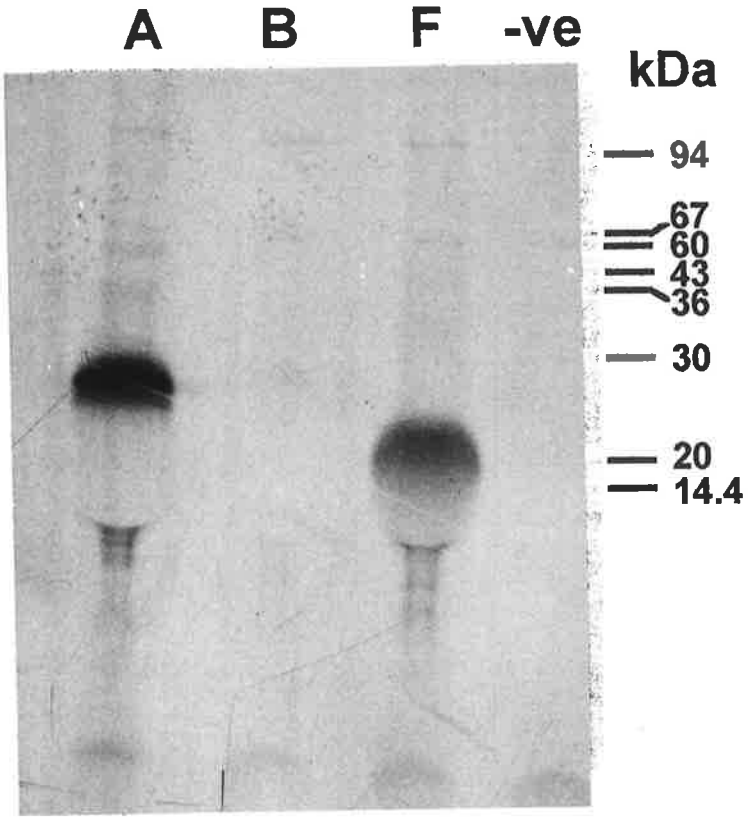


Figure 4.15 *In vitro* translation of cRNA from the A, B and F splice forms.

Based upon the assumption that corpora lutea would contain full length RNA transcripts of the LHR A, B, F and G splice forms, and that translation of these transcripts in the *in vitro* translation system would generate immunoreactive proteins to the antibodies, *in vitro* translation of total RNA and poly A+ RNA from ovine corpora lutea and liver samples were attempted, but produced unsatisfactory results *(data not shown)*. Both immunoprecipitation experiments *(see Section 2.6.5)* and Western analyses of the reactions using the LHR antibodies *(see Section 2.6)* failed to confirm the presence of the LH receptor. This was probably due to lack of LHR RNA per reaction *(see the amount of RNA needed for efficient in vitro translation)*. Unfortunately, due to time restrictions in this project, the generation of LHR alternatively spliced protein products by *in vitro* translation to determine the immunoreactivity of the antibodies raised could not be pursued. In an effort to determine if the antibodies were in fact going to be useful, it was decided that Western analyses of ovine ovarian tissue should be carried out.

4.5 Western Analyses of the Alternatively Spliced Forms of the Ovine LH Receptor

Given the strong possibility that the antibodies raised against the LHR peptides would only recognise denatured proteins, Western analyses of ovine corpora lutea were carried out to determine if the antibodies could recognise any protein products that are encoded by the luteinising hormone receptor gene. Although the exact molecular weights of the ovine LH receptor and the putative proteins generated from its alternatively spliced forms could not be predicted (because the complete cDNA sequence of the ovine LHR gene is not known), they were estimated from sizes of the LHR proteins translated in cells transfected with the porcine alternatively spliced forms of the gene (VuHai-LuuThi *et al.*, 1992).

Although the predicted amino acid sequences of the porcine LHR splice forms are known, the predicted molecular weight of the forms based upon these sequences can be considerably different to the size of the protein as determined by SDS-

polyacrylamide gel electrophoresis (SDS-PAGE). One example of this is the LHR full length A splice form in the pig; the predicted molecular weight based upon the amino acids sequence is 75 025 daltons (Loosfelt *et al.*, 1989), and the size of the deglycosylated receptor as determined by the rate that it electrophoreses by SDS-PAGE is 63 000 daltons (VuHai-LuuThi *et al.*, 1992). This is because SDS-PAGE is not an accurate method for estimating size in the case of highly hydrophobic proteins such as the LH receptor (Professor E. Milgrom, personal communication). However, unlike the A and F forms, the putative protein products from the B and G splice forms are probably not highly hydrophobic, as they forms do not encode the hydrophobic transmembrane domain. In the pig the predicted molecular weight of the translated B splice form based upon its amino acid sequence is 33 387 daltons, which is much closer to the molecular weight of the almost identical deglycosylated C splice form when electrophoresed on SDS-PAGE *i.e.* approximately 34 000 daltons (VuHai-LuuThi *et al.*, 1992). The size of the amino acid component of the F and G forms (based upon the porcine sequence), was estimated at 71 838 and 30 208 daltons respectively.

In addition, the predicted size as determined by the amino acid sequence does not take into account any increases in molecular weight due to glycosylation. It is more than likely that the LHR proteins found in corpora lutea samples would be highly glycosylated, as there are six potential N-linked glycosylation sites on the extracellular domain of the LH receptor (Hubbard and Ivatt, 1981; McFarland *et al.*, 1989; Loosfelt *et al.*, 1989), and it is known that at least three sites are glycosylated in the A form (Zhang *et al.*, 1995). Therefore, the alternatively spliced porcine forms that have been expressed in transfected cells (VuHai-LuuThi *et al.*, 1992), allow for the best estimations of the molecular weight of the homologous ovine forms expressed *in vivo*, even though these forms may be considerably different in size because, (1) they come from a different species, and (2) they are being expressed in different cell types, which is likely to effect their glycosylation patterns.

4.5.1 Western Analyses Methods

Tissues were collected from cycling ewes from the local abattoir and transported to the laboratory either in ice-cold Earles Balanced Salt Solution buffered with HEPES (pH 7.4), or frozen on dry ice within 20 minutes of death. The tissues were homogenised in homogenisation Buffer A *(as described in Section 2.6.1)*, and their protein content determined by a Bradford assay *(as described in Section 2.6.1)*. Aliquots of the samples were then prepared (as described by Fairbanks *et al.*, 1971; *see Section 2.6.3*), before electrophoresis through an SDS polyacrylamide gel *(see Section 2.6.3)* and transfer to a nylon filter (Hybond-C extra, Pharmacia) by electroblotting *(see Section 2.6.4)*. Proteins that were immunoreactive to the antisera were detected by initially hybridising the nylon filter with diluted primary antibody (*i.e.* the LHR antisera), before hybridising with an iodinated goat anti-rabbit secondary antibody *(as described in Section 2.6.4)*, and autoradiography or phosphorimagery performed.

A number of controls were carried out for the Western analyses. These included using tissue samples in which LH receptors were considered unlikely to occur *i.e.* ovine liver and kidney, and therefore any binding to these tissues would be due to either non-specific binding, or binding to a protein that shares a similar epitope. The second control involved incubating the antisera with an excess of the peptide that the antibodies were raised against, before adding the now stripped antisera to the nylon filters. This should remove any antibodies that are specific to the peptide, and as a result any binding of protein by the stripped antisera must be due to non-specific antibodies. However, if the sera is not completely stripped of antibodies against the peptide, the intensity of the specific bands should be significantly reduced.

The third Western blot control that was performed was the use of increasingly diluted primary antibody. This selects for proteins in which high affinity binding is seen, in

contrast to protein that exhibit a low affinity binding to the antibodies. In addition, the amount of protein for the samples were also diluted, again selecting for proteins in which high affinity binding is seen.

The final control for the Western analyses consisted of using the pre-immune sera and comparing any bands detected with those detected when the post-immunisation antisera was used. As the pre-immune sera could not contain any antibodies raised against the peptide, then any binding of proteins by this sera must be due to non-specific binding. The use of the above controls help rule out most non-specific binding or binding to unrelated proteins with similar epitopes.

4.5.2 Western analyses using the LHR-ALL antisera

Western analyses of ovine corpora lutea proteins using the anti-LHR-ALL antisera revealed a strong band at approximately 65 kDa, and very faint bands at approximately 58 kDa (not seen in all samples), 45 kDa, and 25 kDa *(see Figures 4.16 and 4.17)*. Unfortunately, Western analyses of liver and kidney samples with this antisera revealed binding to proteins of approximately 65 kDa, and a faint band at approximately 58 kDa *(see Figure 4.17)*. Due to the 65 kDa band occuring in the corpora lutea, liver and kidney samples, it was concluded that these bands correspond to the same protein. Given that the band was detected in liver and kidney, it is highly unlikely to correspond to the LH receptor or its alternatively spliced protein products. Although there were a number of faint lower molecular weight bands unique to the corpora lutea samples, these were of a size considered too small for the full length functional A splice form, which we know must be present in this tissue.

antibody, with the exception that a 1:1000 dilution in Western blot buffer A was used for Cooke's antibodies. Proteins were isolated by subcellular fractionation from ovine corpora lutea, liver and testis samples, as described in *Section 4.6*, and subjected to SDS-PAGE and Western analyses.

Antibodies to Cooke's LHR-1 peptide faintly recognised a protein at 42 kDa in all samples and all fractions *(see Figure 4.22)*. A protein was also weakly recognised at approximately 57 kDa in the cytosolic fractions, however this protein was located in all the tissues examined. As no proteins were recognised at approximately 90 kDa, and no bands were detected exclusively in corpora lutea, it was concluded that this antibody did not recognise the ovine LH receptor or its splice forms.

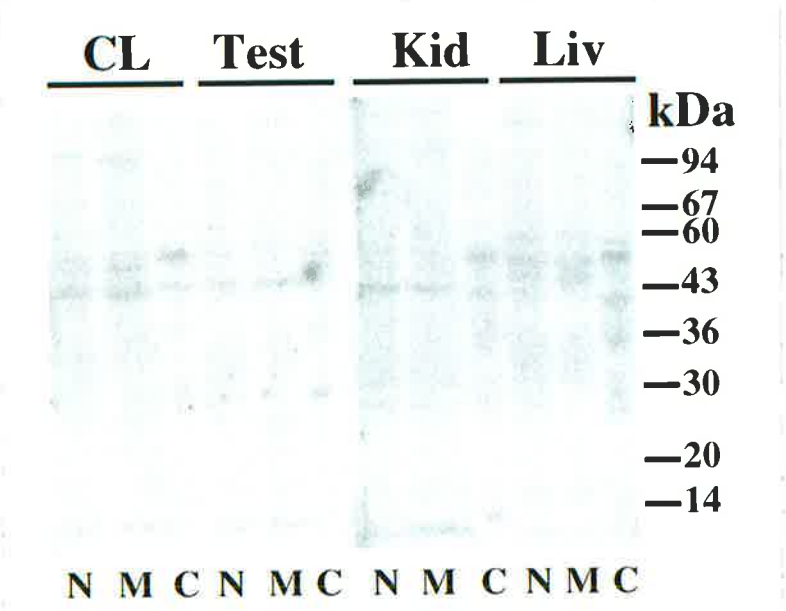


Figure 4.22 Western analysis with Cooke's anti-LHR-1 antibody of ovine corpora lutea (CL) , testis (Test), kidney (Kid) and liver (Liv) samples fractionated into nuclear/mitochondrial (N), membrane/microsomal (M) and cytosolic (C) fractions. Fractionated proteins (25 μg per lane) were investigated by Western analysis with Cooke's anti-LHR-1 antibody at a concentration of 1:1000. The molecular weight markers are high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

Western analyses using antibodies to Cooke's LHR-3 peptide, revealed numerous bands. In the corpora lutea samples, the most prominent protein identified was approximately 23 kDa in size, and was located in the nuclear/ribosomal and microsomal/membrane fractions *(see Figure 4.23)*. This protein was also found in these subcellular fractions in both liver and testis tissue, indicating that it did not correspond to the ovine LH receptor or the translated F splice form. Additional proteins that this antibody recognised in the ovine corpora lutea cytoplasmic fraction were approximately 25, 39 and 64 kDa, although the 39 and 64 kDa bands also appeared in the liver sample. A faint band at 45 kDa was observed in both the corpora lutea nuclear and microsomal fractions, again a similar sized band was recognised in the ovine liver sample *(see Figure 4.23)*. No protein of approximately 90 kDa was recognised by this antibody in the corpora lutea sample, and most of the proteins that were recognised in the corpora lutea sample were also found in the non corpora lutea samples, indicating that this antibody does not recognise the ovine LH receptor.

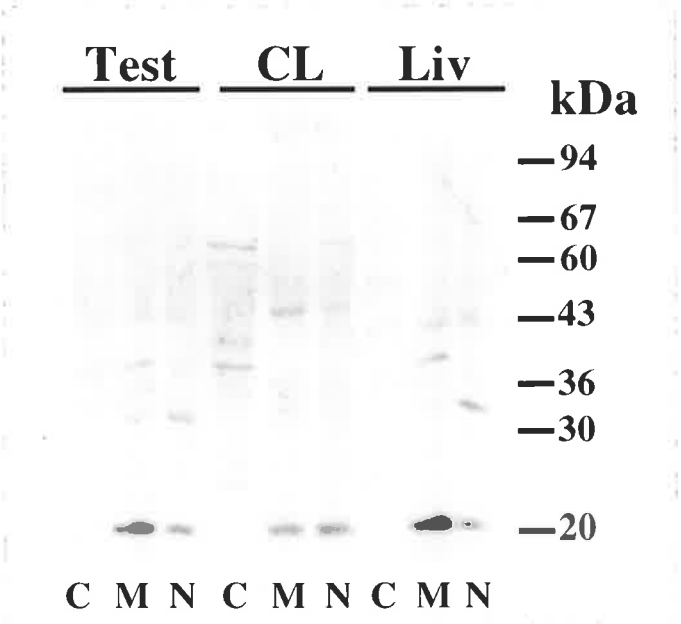


Figure 4.23 Western analysis with Cooke's anti-LHR-3 antibody of ovine corpora lutea (CL) , testis (Test) and liver (Liv) samples fractionated into nuclear/mitochondrial (N), membrane/microsomal (M) and cytosolic (C) fractions. Fractionated proteins (25 µg per lane) were investigated by Western analysis with Cooke's anti-LHR-3 antibody at a concentration of 1:1000. The molecular weight markers are high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

However, Western analysis of subcellular fractionated proteins from ovine corpora lutea, liver and testis samples using Cooke's antibodies to peptide LHR-2, strongly recognised a 40-42 kDa protein located exclusively in the cytosolic and microsomal/membrane fractions of the corpora lutea samples. In addition, a much weaker band was observed in these fractions at 36 kDa *(see Figure 4.24)*. Interestingly, very faint bands at 40-42 kDa and 36 kDa were observed with this antibody in the cytosolic and microsomal fractions from the ovine testis sample *(see Figure 4.24)*. As mentioned in *Section 4.6.1* this was the expected size and subcellular fraction for the translated protein product of the ovine LH receptor B splice form. This lends further support to the hypothesis that the B splice form of the LH receptor is translated *in vivo* in the ovine corpus luteum, and possibly the ovine testis.

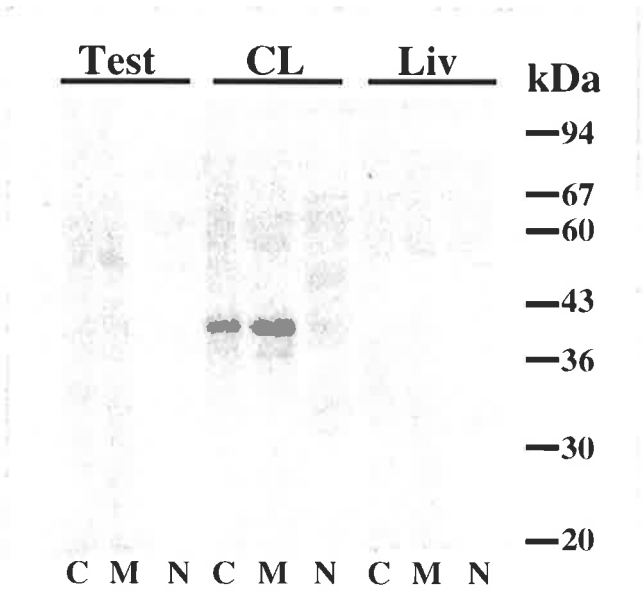


Figure 4.24 Western analysis with Cooke's anti-LHR-2 antibody of ovine corpora lutea (CL) , testis (Test) and liver (Liv) samples fractionated into nuclear/mitochondrial (N), membrane/microsomal (M) and cytosolic (C) fractions. Fractionated proteins (25 μg per lane) were investigated by Western analysis with Cooke's anti-LHR-2 antibody at a concentration of 1:1000. The molecular weight markers are high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

Verification that the protein that Cooke's anti-LHR-2 antibody recognised was the same as the protein recognised by our antibody anti-LHR-B&G, was deduced by Western analysis. Ovine corpora lutea proteins that had been subcellular fractionated were electrophoresed side by side on the same gel, and analysed by Western blotting using both Cooke's anti-LHR-2 antibody and our anti-LHR-B&G antibody *(see Figure 2.25)*. This revealed that these antibodies do in fact bind to the same sized protein, and that its subcellular localisation is the same with both antibodies.

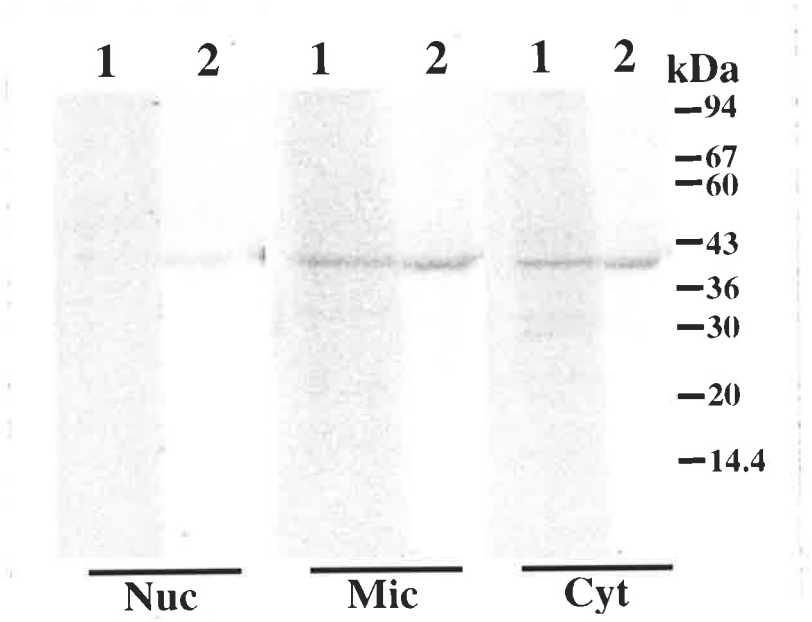


Figure 4.25 Western analyses comparing our anti-oLHR-B&G (1) and Cooke's anti-LHR-2 (2) antibody on ovine corpora lutea samples fractionated into nuclear/mitochondrial (Nuc), membrane/microsomal (Mic) and cytosolic (Cyt) fractions. Lanes containing fractionated proteins (100 μg) were cut into two strips before investigating these proteins by Western analysis with Cooke's anti-LHR-2 antibody and our anti-oLHR-B&G antibody. These strips were then realigned before phosphorimagery. The molecular weight markers are high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

It was intriguing that while Cooke's anti-LHR-2 antibody bound to the translated B or G splice form in the ovine corpora lutea sample, no bands corresponding with the A form at 90 kDa were observed. It is possible that the full length A form is expressed

at considerably lower levels than the B splice form, although this is unlikely given that the RNase protection assays indicated that the B form is expressed only 4.5 fold greater than the A form. However, the rate of post-transcriptional processing of these forms may differ. Another possible explanation is that the antibody recognised the epitope on the B splice form, but not the A splice form. While this is possible, it is also unlikely, as one would expect the tertiary structure of the extracellular domain to be very similar, given that the primary sequence of both forms is the same in this region. Another explanation for the lack of binding to the A form may be that the LH receptor is lost during isolation of the subcellular components. Unlike rodent corpora lutea, ovine corpora lutea samples are quite lipid rich (personal communication Dr. Ray Rodgers), and as a result, after homogenisation of the tissue with either homogenisation buffer B or C *(see Section 2.5.1)*, of which both do not contain any detergents, a fatty scum sticks to the tube and is lost during the pellet isolation involved in the subcellular isolation process. Thus it is possible that the full length form of the LH receptor, which contains the hydrophobic transmembrane domain, may associate with the fatty scum mentioned above, and be inadvertently lost. Although conceivable, this is unlikely as homogenisation buffer C contains 15% glycerol, presumably making these receptors more soluble in this buffer.

The most likely explanation for the lack of binding of the Cooke's anti-LHR-2 antibody to the full length A form receptor is that the receptor may have undergone proteolytic cleavage during the preparation of the samples. This is quite likely, despite the effort to reduce this with the use of a large spectrum of protease inhibitors, and may result in a recognisable protein product the size of the B splice form *i.e.* consisting of the extracellular domain only *(see Section 1.4.2)*. In addition, when Western analysis was performed using Cooke's anti-LHR-2 antibody on mature adult cycling Sprague-Dawley rat ovaries (16 weeks of age), but no proteins at 95 kDa as described by Pilankaros *et al.* (1995) were observed, however a protein of approximately 42 kDa was identified in the microsomal and cytosolic fractions *(see*

Figure 4.26). Therefore, it was concluded that Cooke's anti-LHR-2 antibody was binding to the proteolytically cleaved A form and/or the B form. Pilankaros *et. al.* (1995) isolated LH receptors from pseudopregnant rat ovaries using a different method to us, and as such did not detect the proteolytically cleaved A form product, or the putative translated protein product of the B form, should it exist in rat ovaries.

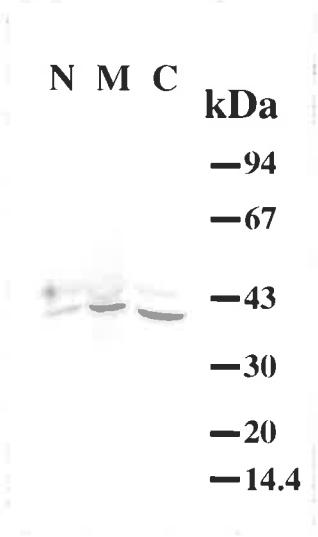


Figure 4.26 Western analyses using Cooke's anti-LHR-2 antibody on mature rat whole ovary samples fractionated into nuclear/mitochondrial (N), membrane/microsomal (M) and cytosolic (C) fractions. Fractionated proteins (40 µg per lane) were investigated by Western analysis with Cooke's anti-LHR-2 antibody at a concentration of 1:1000. The molecular weight markers are high and low protein molecular weight markers from Pharmacia *(see Section 2.6.4)*.

In conclusion, the use of Cooke's antibodies provided additional evidence that the 42 kDa protein identified by our anti -LHR-B&G antibody, does in fact recognise the translated product of the LHR B and/or G splice form. This evidence involved an antibody that was raised against a peptide sequence of the rat LH receptor, which has been characterised to bind the LH receptor in the rat, also recognising the same protein as the anti-LHR-B&G antibody. These antibodies were raised against entirely different peptide sequences, and hence it is assumed that the protein that they regognise contains these sequences, as expected for the translated product of the LHR B or G splice forms.

4.8 SUMMARY

This chapter describes our attempts to develop a method to specifically identify the protein products encoded by the alternatively spliced ovine LH receptor transcripts that we identified in corpus luteum and follicles *i.e.* the A, B, F, and G splice forms. This involved the raising of polyclonal antibodies to specific peptides which were designed to mimic antigenic structures that occur either in all of the translated ovine LHR isoforms (*i.e.* the LHR-ALL antisera), or to potential antigenic structures unique to a subset of these forms (*i.e.* the LHR-A&F or the LHR-B&G antisera). Antibodies raised against these peptides recognised them specifically and with a high affinity, as demonstrated by the high titres of the antibodies to the peptides and the cold competition experiments.

Unfortunately, these antibodies did not identify any LH receptors or protein products generated from the alternatively spliced transcripts by immunohistochemical methods. This was possibly due to a number of factors, including accessibility of the antigenic sites in the non-denatured proteins, or even the existence of the antigenic site in the non-denatured receptors. To determine if the antigenic structures that the antibodies were raised against correctly mimicked those found in the LH receptor or its isoforms, Western analyses were performed on ovine corpora lutea, and liver samples.

The Western analyses clearly demonstrated that the polyclonal antibodies recognised a number of denatured proteins. The antibodies raised against the LHR-ALL peptide did not bind to any proteins of the size expected for the mature ovine LH receptor (approximately 90 kDa), and the 65 kDa protein identified was common to both corpora lutea and liver samples, and as such it was highly unlikely to be the LH receptor or one of its isoforms. Western analyses using the anti-LHR-A&F antibody produced anomalous results, with many proteins being identified. Clearly most of them were not LH receptors, as they also appeared in the liver and kidney samples.

From the results of the Western analyses and the subcellular localisation experiments, it is unlikely that this antibody identifies the A and F isoforms.

Western analyses using the anti-LHR-B&G antibodies, strongly indicated that at least the B splice form and possibly the G splice form is translated *in vivo* in the ovine ovary. This is based on three facts: (1) A protein of the expected molecular weight for the B isoform binds to the antibody that was raised against a sequence that is presumably unique to the B and G isoforms, (2) this protein was detected in the tissues that the B splice form mRNA transcript has been detected in, and not in tissue that it is known not to be expressed in *(see Chapter Three)*, and (3) this protein localises to the subcellular fraction that the B isoform has been localised to in transfected cells, and as such the fraction that it would be localised in if it was translated *in vivo*. An additional piece of evidence to support this conclusion was supplied by the use of antibodies raised against peptide sequences based upon the rat LH receptor. Western analysis with one of these antibodies, Cooke's anti-LHR-2 antibody, also recognised the same sized protein, in the same subcellular location as the anti-LHR-B&G antibody. This indicates that this protein contains some regions homologous to the LH receptor, and is almost certainly the translated protein product of the B and/or the G splice forms. While it is feasible that the protein identified corresponds solely to the G splice form, this was considered unlikely, given that the B splice form mRNA was approximately 4.5 times more abundant than the G splice form mRNA.

In conclusion, we have shown that the B splice form, and possibly the G form of the ovine LH receptor is translated *in vivo*. This is the first time in any species that it has been demonstrated that the alternatively spliced forms of the LHR are translated *in vivo*. It remains to be determined if the alternatively spliced forms are alternatively regulated at different stages of the oestrous cycle; either at the transcriptional or translational level.

CHAPTER FIVE

ANALYSES OF THE ALTERNATIVELY SPLICED LH RECEPTOR FORMS IN THE OVINE OVARY THROUGHOUT THE OESTROUS CYCLE

5.0 INTRODUCTION

Gene regulation by alternative splicing is a common mechanism, and can occur in a number of ways (*see Chapter 1;* see Smith *et al.,* 1989 and McKeown, 1990 for detailed reviews). The LH receptor gene is highly spliced in every species so far investigated, and there have been reports of differential regulation by alternative splicing of this gene during development of the ovary and testis in the rat. Consequently, it was decided that examination of alternative splicing of LH receptor mRNA throughout the oestrous cycle may reveal that the gene is regulated by alternative splicing. Furthermore, the cell types of the corpus luteum that express LH receptors, the large and small luteal cells, differ in their response to the addition of LH or hCG, *i.e.* the binding of LH or hCG to small luteal cells induces an increase in progesterone production by these cells, while there is little, if any increase in progesterone production by large luteal cells upon binding of LH or hCG *(as reviewed in Chapter One).*

This distinction may be due to differential regulation of the LH receptor on these cell types via alternative splicing. If this is true, then as the proportion of small luteal cells to large luteal cell in an ovine corpora lutea decreases throughout the oestrous cycle (based on volume density; Farin *et al.,* 1986), regulation of the LH receptor by luteal cell type may be examined by comparing corpora lutea taken from different stages of the oestrous cycle.

The methods developed during the term of this project *(see Chapters Three and Four)*, allowed us to investigate whether the LH receptor is regulated throughout the oestrous cycle by alternative splicing, either at the post-transcriptional level, the translational or post-translational level. Therefore the aim of the experiments described in this chapter was to determine if the LHR mRNA transcripts or their

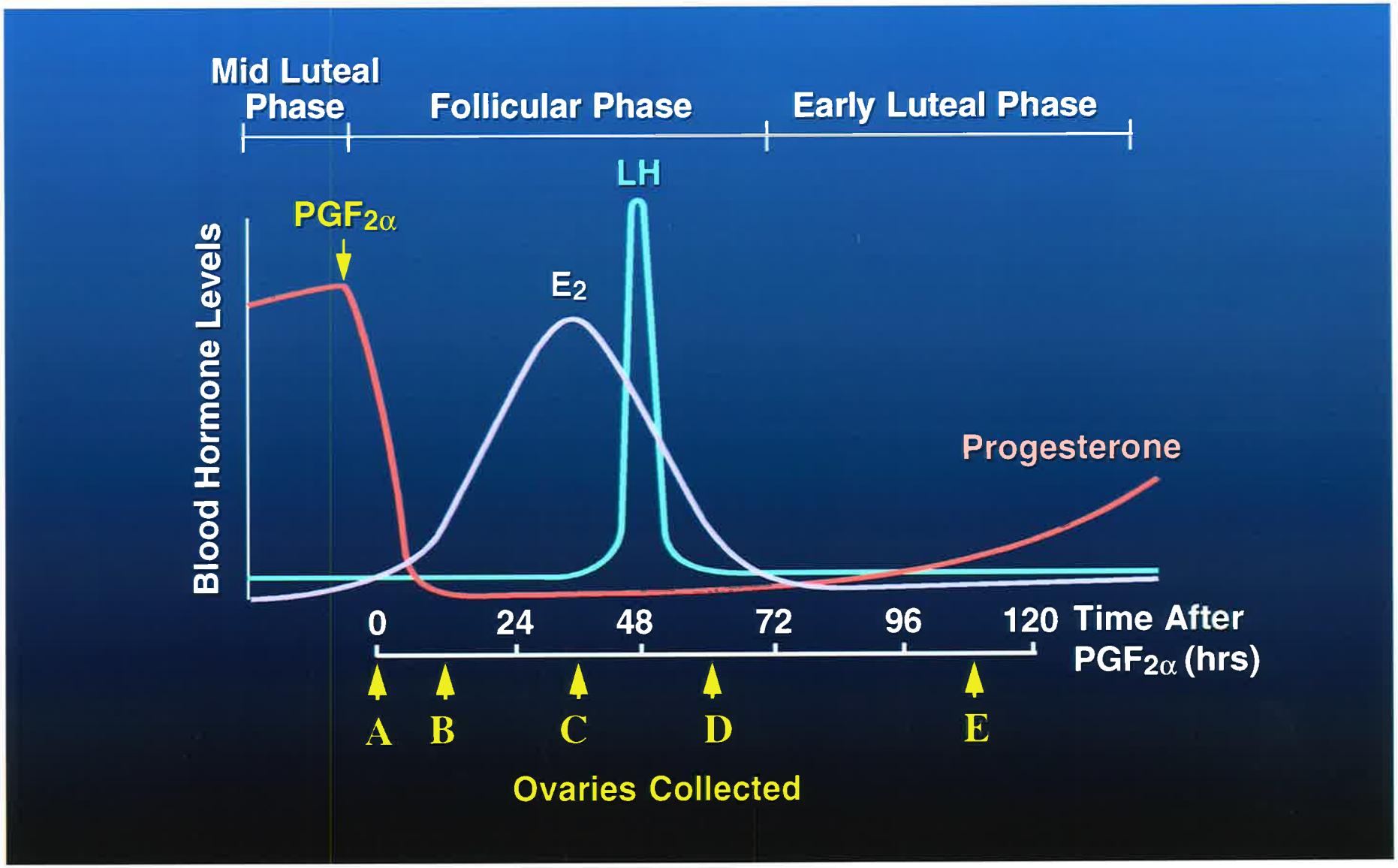
protein products are regulated at different stages of the oestrous cycle by alternative splicing.

5.1 Synchronisation of the Ewe Oestrous Cycle

5.1.1 Background

To obtain ovaries from ewes from all stages of the oestrous cycle, *i.e.* during the follicular, early-, mid-, and late-luteal phases there were two options. The first option was to determine at what stage in the oestrous cycle the individual ewes were (by either plotting their serum hormone levels or determining when they were in oestrus by seeing if they would present for a ram), and then performing ovarectomies at an appropriate time. The second option available, and the one that was opted for due to time considerations and technical ease was to artificially synchronise the oestrous cycle of the ewes.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered to the ewes, to induce regression of any corpora lutea present in their ovaries (Knickerbocker *et al.*, 1988). Therefore, any animal in the luteal phase of the oestrous cycle (approximately 14-15 days out of a 16-17 day cycle), would be affected, and its cycle brought forward to early follicular phase. If an animal was in the follicular phase of its oestrous cycle at the time of $PGF_{2\alpha}$ administration, it would be at the most 36 hours further advanced in the oestrous cycle than an animal treated in the luteal phase. A second dose of $PGF_{2\alpha}$ was administered to all ewes 10 days after the initial injection (*i.e.* at which stage all animals would have been in mid-luteal phase), excluding those whose ovaries were to be removed at the mid-luteal phase. This second synchronisation ensured that the ewes were all at the beginning of the follicular phase of the cycle.


Mid Luteal Phase
Follicular Phase
Early Luteal Phase
LH
PGF2α
E2
Blood Hormone Levels
Progesterone
0
24
48
72
96
120
Time After PGF2α (hrs)
A
B
C
D
E
Ovaries Collected

5.1.2 Synchronisation and Ovariectomy of the Ewes

Cycling merino ewes (25), that had been grazing on open pasture were synchronised by injecting them with Cloprostenol (a $PGF_{2\alpha}$ analog; generic name PG-estrumate, 250 μg intramuscular, Jurox, Silverwater, NSW, Australia) on Day -10. Ten days later, all sheep excluding those designated as group A, were injected with a further 250 μg of Cloprostenol. Bilateral ovariectomy was performed on the ewes at 10, 34, 58, and 108 hours after the second injection *(designated groups B, C, D and E respectively; see Figure 5.1)*, and 240 hours after the first injection for those ewes in group A *(see Figure 5.1)*. Briefly, this involved giving the ewes an initial anaesthetic injection of 7-10 ml of Pentothal (500 mg Pentothal (sodium thiopentone; Boehringer Ingelheim, Armaton NSW, Australia) in a 60 mg/ml solution of Nembutal (Sodium Pentobarbitone; Abbott Laboratories Pty Ltd, Sydney, Australia) in 10% ethanol. The ewes were maintained in an anaesthetised state using Fluothane (halothane; ICI Pharmaceuticals, Macclesfield, Cheshire, England) oxygen mixture. The injections and surgery were performed by Dr. Colin Earl of the Straun Research Centre, assisted by Professor David Armstrong of the Department of Obstetrics and Gynaecology, the University of Adelaide.

Large follicles and corpora lutea were carefully dissected from the ovaries within 15 minutes of ovariectomy. The corpora lutea were weighed, and follicular fluid collected from most of the follicles, before all tissues and fluids were frozen on dry ice.

Total RNA was isolated from the follicle samples and approximately 80% of each corpora lutea sample *(as described in Section 2.2.4)*. The RNA was then quantitated *(as described in Section 2.2.5)*, before RNase protection assays and RT-PCR were performed *(see Section 2.2.15 and 2.2.7)*. The remaining 20% of each corpora lutea

sample was homogenised in Homogenisation buffer A and used in Western analyses *(as described in Section 2.6.5)*.

5.1.3 Verification of oestrous cycle synchronisation

Synchronisation of the ewes was verified in two ways, (1) by observation of the size, weight, and structures of the ovaries of the various groups at ovariectomy (according to Diekman *et al.*, 1978), and (2) by determining the relative 3β-hydroxysteroid dehydrogenase (3β-HSD) concentrations in the corpora lutea samples using Western analyses (according to Jeungel *et al.*, 1994; *see Figure 5.2*).

Observational results

The morphological data obtained from observation of the collected ovaries has been summarised in Table 5.1, and was consistent with that expected for the particular phase of the cycle predicted for that animal (Diekman *et al.*, 1978).

The ovaries from the sheep in group A (*i.e.* 10 days after the first injection), showed large corpora lutea which were well vascularised. The majority of follicles seen were below 4 mm in diameter, and most of these were below 2 mm, however there were four follicles in the range of 6 to 8 mm. These results are consistent with those expected of ewes in the mid-luteal phase.

It was expected that the sheep in group B (*i.e.* 10 hours 25 minutes-11 hours 25 minutes after the second $PGF_{2\alpha}$ injection; early follicular phase), would have CL that were beginning to regress and some antral follicles, although no pre-ovulatory follicles or new CL. This compares well with what was observed for this group.

Group C, the ovaries of the ewes in late follicular phase (*i.e.* 35 hours 45 minutes-37 hours 30 minutes after the second $PGF_{2\alpha}$ injection), contained considerably

regressed CL, weighing relatively less than those observed from the mid-luteal phase ewes. In addition, large pre-ovulatory follicles 8-10 mm in diameter were present. Unexpectedly, one of the ovaries from a ewe in this group had recently ovulated (animal C13, CL1).

The ewes in group D were expected to be in early luteal phase (*i.e.* 58 hours 20 minutes-60 hours after the second $PGF_{2\alpha}$ injection). Ovaries from these sheep contained both very regressed CL (presumably from the previous cycle), of considerably lesser size and weight of those observed, including those of group C and also some recently formed CL. Large antral follicles (5-7 mm in diameter) were also seen, possibly due to some loss of tight synchronisation by this stage.

Finally, group E, the ewes that were subjected to the second injection 108 hours 20 minutes to 109 hours 45 minutes prior to ovariectomy, were expected to be in the early luteal phase. Notably regressed CL were observed, in addition to newly formed CL. All but one of the follicles observed were under 5 mm in diameter.

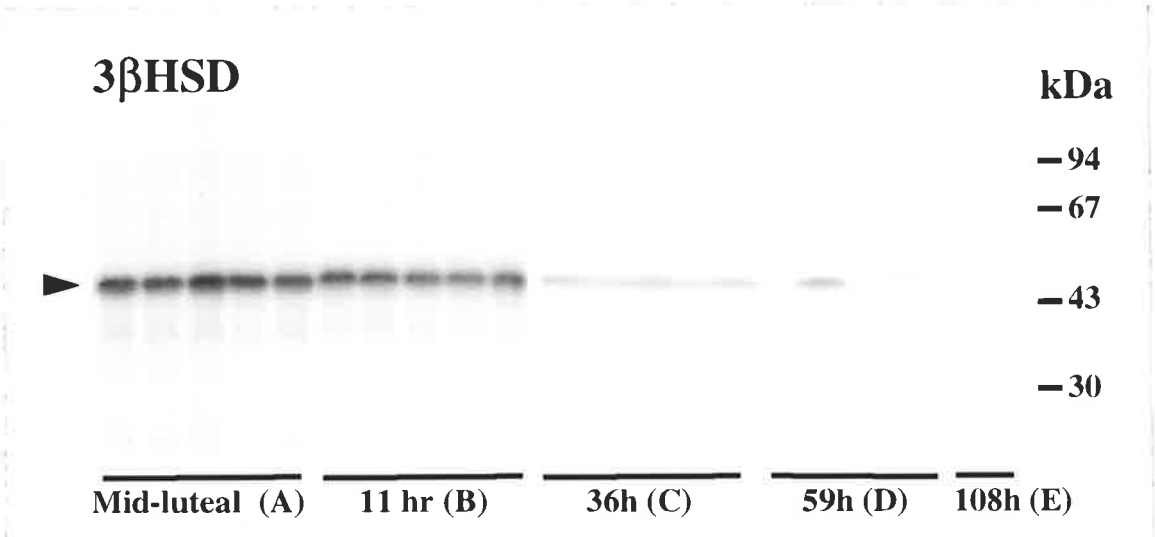


Figure 5.2 Western analyses of corpora lutea from different stages of the oestrous cycle. The amount of 3βHSD (indicated by the arrow-head) decreases as the time after $PGF_{2\alpha}$ administration increases. The results seen are consistent with the mRNA expression profile of 3βHSD throughout the oestrous cycle (Hawkins *et al.*, 1993; Jeungel *et al.*, 1994).

Time since PG administered	Animal ID	Mid-luteal & Regressing CL	Follicles	New Corpora Lutea
Group A 240 hours post PG	A25	(CL1) 312 mg (CL2) 367 mg	F1=6 mm	
	A26	(CL1) 463 mg (CL2) 473 mg	F1=7 mm; (FF#10)	
	A27	(CL1) 475 mg	F1=8mm; (FF#11)	
	A28	(CL1) 689 mg		
Group B $10h^{25}$-$11h^{25}$ post PG	B7	(CL1) 570 mg (CL2) 569 mg	F1=2*2 mm	
	B8	(CL1) 548 mg		
	B9	(CL1) 472 mg	F1=4 mm F2=3 mm	
	B10	(CL1) 991mgs[1]	F1=4 mm;(FF#1) F2=3 mm; (FF#2)	
Group C $35h^{45}$-$37h^{30}$ post PG	C13	(CL2) 168 mg		(CL1) 54+58 mg.
	C14	(CL1) 322 mg	F1=9 mm; (FF#3)	
	C15	(CL1) 274 mg.	F1=10mm;(FF#4)	
	C16	(CL1) 258 mg	F1=9 mm (FF#5) F2=6 mm (FF#7)	
	C17	(CL1). 90 mg	F1=8 mm (FF#8)	
	C18	(CL1) 220 mg (CL2) 201mgs	F1=7 mm (FF#9) F2=6 mm	
Group D $58h^{20}$-$60h^{04}$ post PG	D19.	(CL4) 101mg[2]	F1=7 mm F2=4 mm (FF#13)	(CL1) 182 mg (CL2) 216 mg (CL3) 253 mg
	D20	(CL1) 109 mg	F1=6 mm (FF#12) F2=5 mm (FF#14)	
	D22			(CL1) 34 mg
	D23	(CL1) 144 mg (CL2) 176 mg (CL3) 80 mg	F1=7 mm (FF#15) F2=5 mm (FF#16)	
	D24	(CL1) 131 mg (CL2) 132 mg	F1=7 mm (FF#17) F2=4 mm (FF#18	
Group E $108h^{20}$-$109h^{45}$ post PG	E1		F1=5 mm (FF#19)	
	E2	(CL2) 119 & 84 mg		(CL1) 71 mg
	E3	(CL2) 35 mg		(CL1) 85 mg
	E4	(CL2) 76 mg	F1=4 mm (FF#20)	(CL1) 105 mg
	E5	(CL2) 150 mg		(CL1) 54 mg

Table 5.1 Summary of the morphological data collected from tissues dissected at ovariectomy. [1] indicates that there was probably two CL, as it contained a double cavity, [2] indicates that there were three pooled regressing CL. FF means follicular fluid.

Steroid assays

To distinguish between healthy and atretic follicles, follicular fluid collected at the time of ovariectomy was analysed for testosterone, androstenedione and oestradiol concentrations. These analyses were carried out by Alan Gilmore, the Department of Obstetrics and Gynaecology at the Queen Elizabeth Hospital, Woodville, SA. As these assays are developed primarily for testing human serum steroid levels, it was necessary to dilute the (ovine) follicular fluids for analysis. Dilutions of 1:50, 1:125 and 1:250 (testosterone and oestradiol) and 1:25 and 1:125 (androstenedione) were assayed. The molar ratio of oestradiol : testosterone + androstenedione was calculated; less than 1.0 was taken to signify that the follicle was atretic, as described by Carson *et al.*, 1981. The results are tabulated in Table 5.2.

Follicular Fluid #	Group	Oestradiol (E_2)	Testosterone (T)	Androstenedione (A)	Molar Ratio E_2:(T+A)
1	B	134	<20	<7.5	>1 (H)
2	B	138.5	<20	<7.5	>1 (H)
3	C	695	<20	20	>1 (H)
4	C	82	<20	<7.5	>1 (H)
5	C	281	<20	<7.5	>1 (H)
7	C	80	<20	<7.5	>1 (H)
8	C	3.5	<20	<7.5	inconclusive
9	C	245	75	110	>1 (H)
10	A	4	45	57.5	0.04 (At)
11	A	42	<20	<7.5	>1 (H)
12	D	8	<20	<7.5	inconclusive
13	D	5.5	50	7.5	0.01 (At)
14	D	18.5	<20	<7.5	inconclusive
15	D	<3.5	<20	<7.5	inconclusive
16	D	18	<20	<7.5	inconclusive
17	D	<3.5	<20	<7.5	inconclusive
18	D	<3.5	85	75	<1 (At)
19	E	<3.5	20	77.5	<1 (At)
20	E	104	<20	<7.5	>1 (H)

Table 5.2. Concentrations (nM) of oestradiol-17β (E_2), testosterone (T), and androstenedione (A) in the fluid of individual follicles. Molar ratios of E_2:(T+A) less than one indicate that the follicle is atretic (At), and greater than one that the follicle is healthy (H). Values less than 3.5 (E_2), 20 (T) and 7.5 (A) are outside the accurate range of the assay.

5.2 LHR RNase Protection Assay of Timed Tissues

To determine if the LH receptor is regulated by alternative splicing throughout the oestrous cycle, we performed RNase protection analyses on RNA from the timed tissues. Any significant changes in the proportion of the splice forms relative to each other would indicate that there is regulation of the LH receptor by alternative splicing. In addition, this would also reveal changes in the overall expression of the ovine LH receptor mRNA throughout the oestrous cycle, with respect to $PGF_{2\alpha}$ administration, and the subsequent regression of the corpus luteum that occurs.

RNase protection analysis of the RNA from the timed tissue was performed *(as described in Section 3.6)*, with the exception that the complementary RNA probe was generated from a PCR product designated oLHR-4. This avoided the problems that occurred previously with the oLHR-2 and oLHR-3 RNase protections (*i.e.* additional bands due to PCR generated mismatches; *see Section 3.6.6*). To generate a PCR product from which the complementary RNA probe could be transcribed, it was necessary to design an oligonucleotide primer that contained an SP6 RNA polymerase promoter sequence. The primer sequence (designated LHR-AS9*; see Figure 5.3*) was based on LHR-AS8, as this primer had worked well in previous PCRs.

```
LHR-AS9      5'-GAATATTTAGGTGACACTATAGAATACTGGCATGGTTATAATACTGGC-3'
LHR-AS8                              5'-CCAAAGCTTAGGCATGGTTATAATACTGGC-3'
Ovine LHR                                  ...AT.....................CTT
Ovine FSHR                           ...G.C.GT.AAT.....A...G.GG.......TTT
```

Figure 5.3 Oligonucleotide primer LHR-AS9 and its homology with primer LHR-AS8 and the ovine LH and FSH receptors. The LHR-AS9 primer is aligned with the homologous region of primer LHR-AS8, and the ovine LH and FSH receptor cDNA sequences (Yarney *et al.*, 1993). **Bold** bases in primer LHR-AS9 correspond to the SP6 RNA polymerase promoter sequence, and the underlined bases correspond to the sequence which is homologous to the ovine LHR sequence. Homologous bases are indicated with a dot, while non-homologous bases are indicated with the appropriate letter for the alternative base.

Amplification by PCR was performed using LHR-AS9 (100 nM) and the T3 promoter primer (1µg/ml; Promega; generally used for sequencing) using an oLHR-2&3 clone that does not contain the PCR generated point mutation, as template *(under the conditions described in Section 2.2.6)*. This generated an amplified product 560 bp in length, from which ^{32}P-labelled cRNA probes were generated *(as described in Section 2.2.9)*. These probes were then used in RNase protection analyses. The estimated sizes of the protected fragments using the LH receptor RNase protection assay for the A, F, B, and G forms are shown in Table 5.3. RNase protection analyses of the timed samples using the oACT-1 probe were also performed to ensure accurate quantitation of the RNA *(see Figure 5.4; described in Section 3.6.2)*.

oLHR Splice Form	RNase Protected Product (nt)
A	466
B	124 & 78
F	344 & 43
G	78 & 43

Table 5.3 Predicted sizes of RNase protected products for the A, B, F, and G splice forms of the ovine LH receptor using the probe oLHR-4.

The LH receptor RNase protection assay on the corpora lutea samples collected from various stages of the oestrous cycle, revealed three protected fragments at approximately the correct molecular weights expected for the A, B and F splice forms *(see Figure 5.4)*. It was hoped that we would have been able to resolve the 78 nt protected band generated from the B and G splice forms, but the resolution of the RNase protection assay at this size was unreliable *i.e.* numerous band were seen around this size most probably due to incomplete digestion of the probe and/or artefact. In addition to the protected bands, some undigested probe (seen at approximately 543 nt), was observed in all samples.

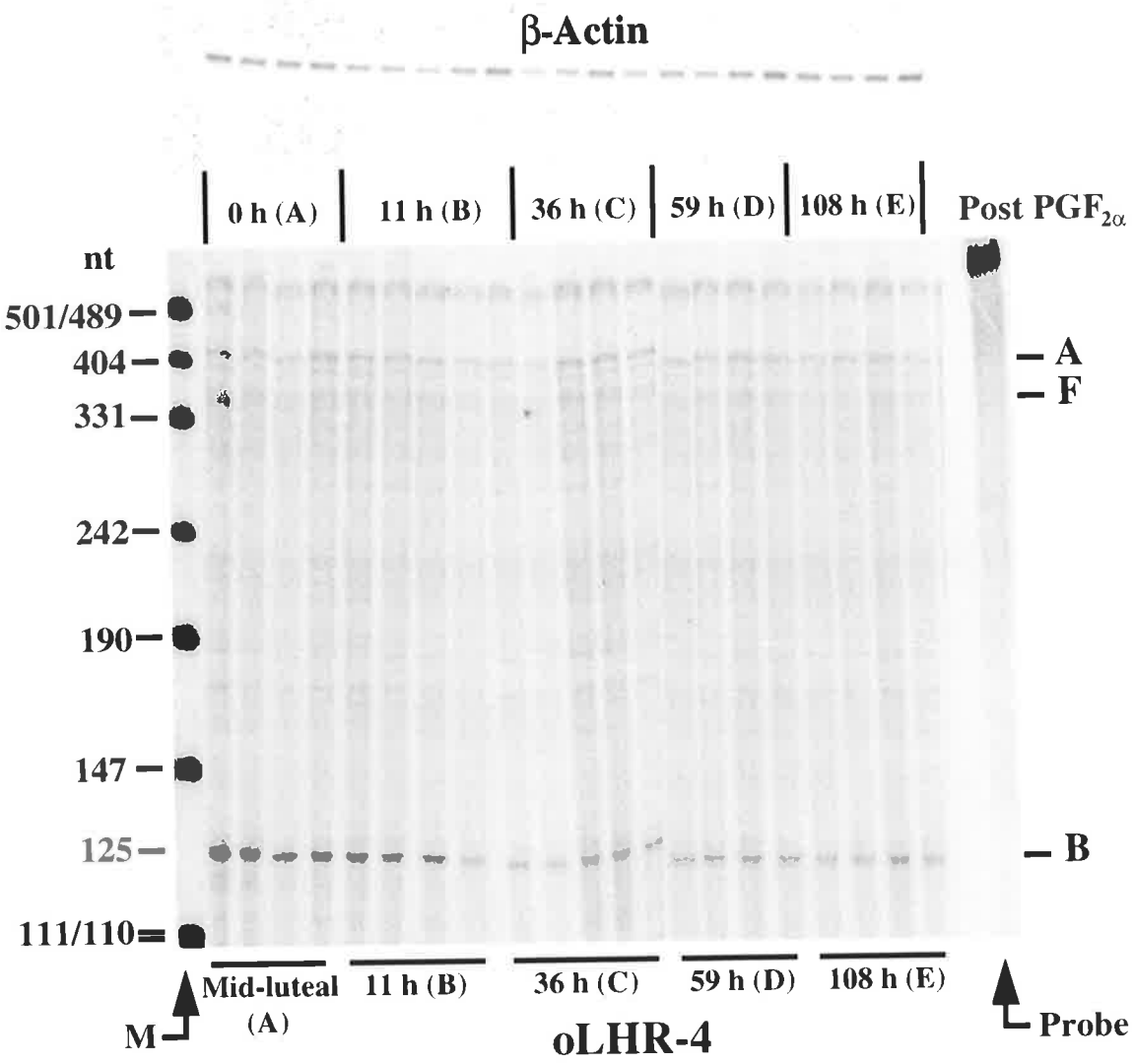


Figure 5.4 oLHR-4 and oACT-1 RNase protection assays. Corpora lutea samples collected at various times after $PGF_{2\alpha}$ administration indicated protected bands corresponding to the LHR A, F and B splice forms. While an overall decrease in the amount of LHR mRNA is evident, there does not appear to be any gross changes in the relative amounts of the splice forms throughout the cycle. Molecular weight markers (M) were radio-labelled pUC19 digested with HpaII. The probe lane refers to undigested oLHR-4 probe.

The ratio of the protected A to B fragments remained constant as the CL regressed with no significant differences[1]. Molar ratios of the splice forms based upon the protected bands indicated that there was approximately 4.5 times more B splice form transcripts present than those of the full length A form *(see the graphical representation of these results in Figure 5.5)*. Similar results were observed with the RNase protection assay results from the follicles. That is, there were no significant differences seen in the proportion of B splice form to the A form between follicles and corpora lutea *(see the graphical representation of these results in Figure 5.5)*.

The RNase protection data was also examined for changes in the overall amount of LHR mRNA throughout the cycle *(see the graphical representation of these results in Figure 5.5)*. Although it appears that there was a relatively greater amount of the A form in CL from the mid-luteal phase in comparison to preovulatory follicles, the newly forming CL, and regressing CL, it was not statistically significant[1]. However, the number of animals in each group was low, and we were at the limits of sensitivity for the assay with some of the lower values seen (*i.e.* for the detection of the A form in the preovulatory and newly formed CL). In contrast, there was a significant difference in the amount of B splice form present in the mid-luteal CL, compared with all of the other groups, reflecting what has been reported in the literature for overall LHR mRNA expression *(see the graphical representation of these results in Figure 5.5)*. This is important, as the vast majority of regulation studies of the LH receptor gene have only examined total LHR mRNA, assuming that there is a direct relationship between the overall expression of the gene and the proportion of the full length A form present.

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[1] Statistical analyses of the RNase protection assay results were by an unpaired student t-test.

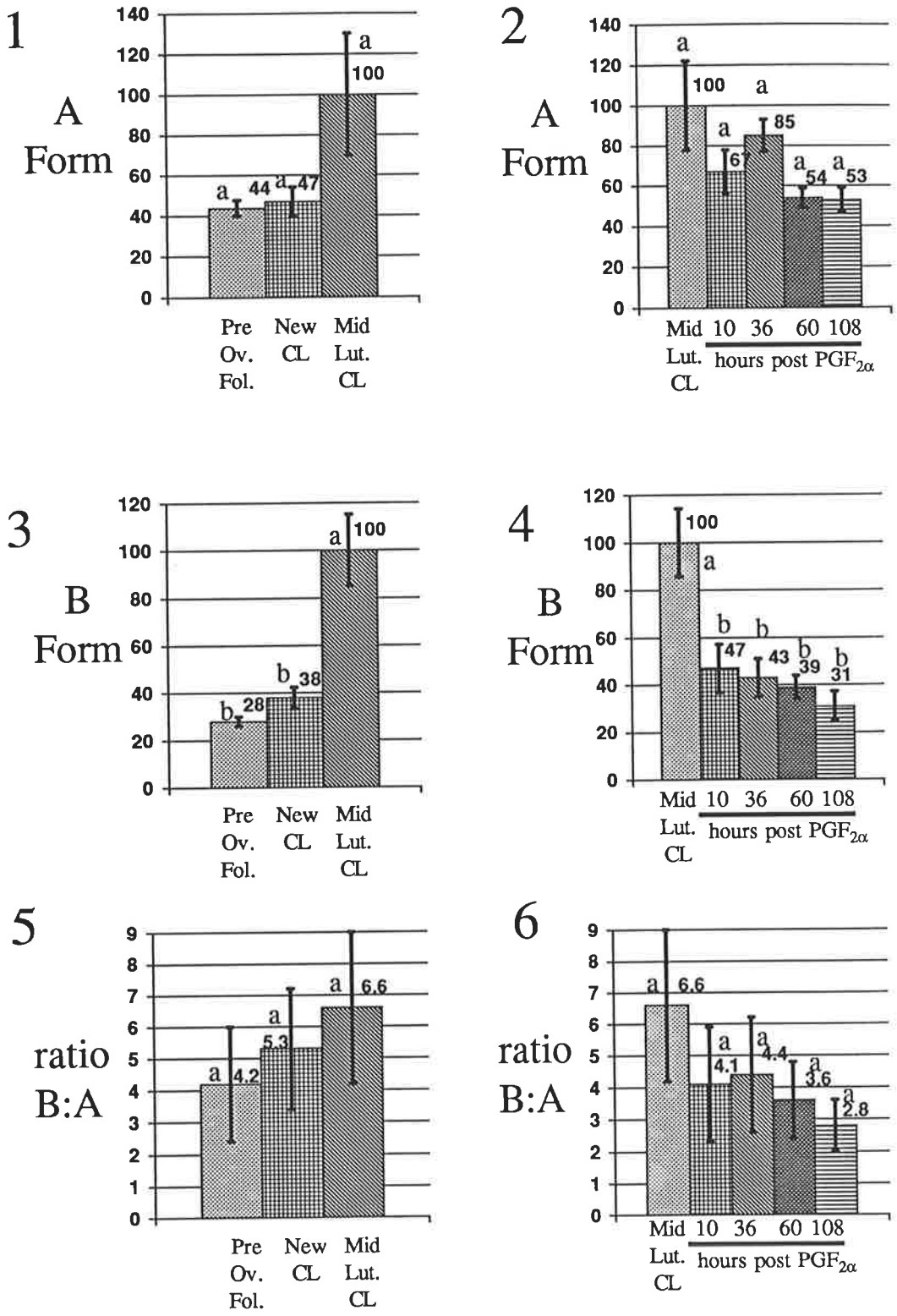


Figure 5.5 Analysis of LHR splice variant expression across the oestrous cycle. Graphs 1 and 2 show the relative amount of A form at various stages after $PGF_{2\alpha}$ administration, versus the amount of A form found in the mid-luteal sample. Graphs 3 and 4 show the relative amount of B form at various stages after $PGF_{2\alpha}$ administration, versus the amount of B form found in the mid-luteal sample. Graphs 5 and 6 show the relative ratio of B:A form at various stages after $PGF_{2\alpha}$ administration. Graphs 1, 3 and 5 compare pre-ovulatory follicles with mid-luteal and new CL. Graphs 2, 4 and 6 compare mid-luteal CL with increasingly regressed CL. "a" indicates no significant difference between groups ($P > 0.05$); "b" indicates a significant difference between groups ($P<0.05$). Statistical analysis was based on an unpaired student t-test. All figures represent the mean and the standard error of the mean.

Unfortunately, the RNase protection assays could not give conclusive results in some cases, as the assay was at the limits of its sensitivity (*i.e.* the bands were so faint that they barely registered above background using densitometry). This occurred with approximately 40% of the follicle samples, two regressing CL from group D and 2 CL from group E. The limited amount of tissue in these samples precluded the RNase protection assays from being repeated using greater amounts of RNA. However, the intensity of the protected B band for all of these samples was well above background, and it was the A and F protected bands for which the results were at the limits of the assay.

5.3 LHR RT-PCR OF TIMED TISSUES

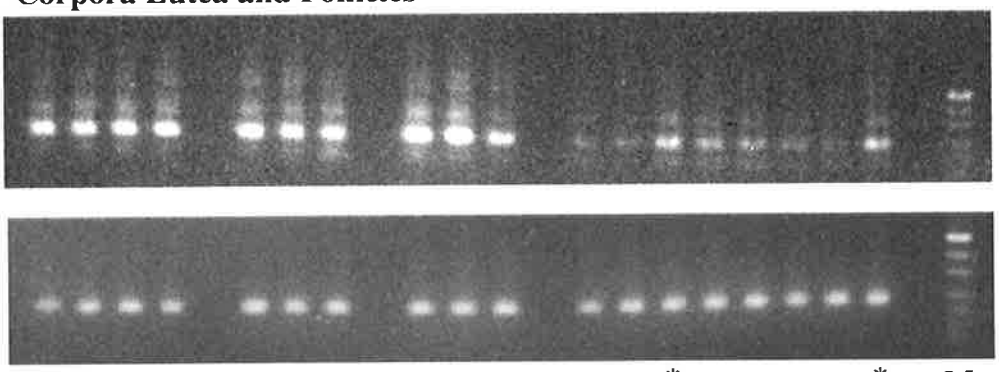
Given the limited amount of timed tissue available, the most sensitive method for identification of the alternatively spliced transcripts of the LH receptor, is the RT-PCR method described in Chapter Three with the modifications described in Chapter Four. In addition, this method would allow us to detect the G form, which could not be resolved by the RNase protection assay. Although this RT-PCR method is not quantitative, it was assumed that any major shifts in the relative amounts of the spliced forms, such as complete loss of expression of a form, would be detected. Amplification of the cDNA with β-actin primers *(described in Section 3.4.3)*, was performed as a positive control.

Reverse transcription-polymerase reaction amplification products of the ovine cDNA using the primers LHR-S7 and LHR-AS8 *(see Section 4.4.1)* were electrophoresed on a 1x TBE, 1.5 % agarose gel and stained with ethidium bromide *(see Section 2.2.8)* as shown in *Figure 5.6.* The β-actin PCR control reactions all amplified strongly staining products of the expected size of 203 bp, confirming that the reverse transcription reactions had been successful.

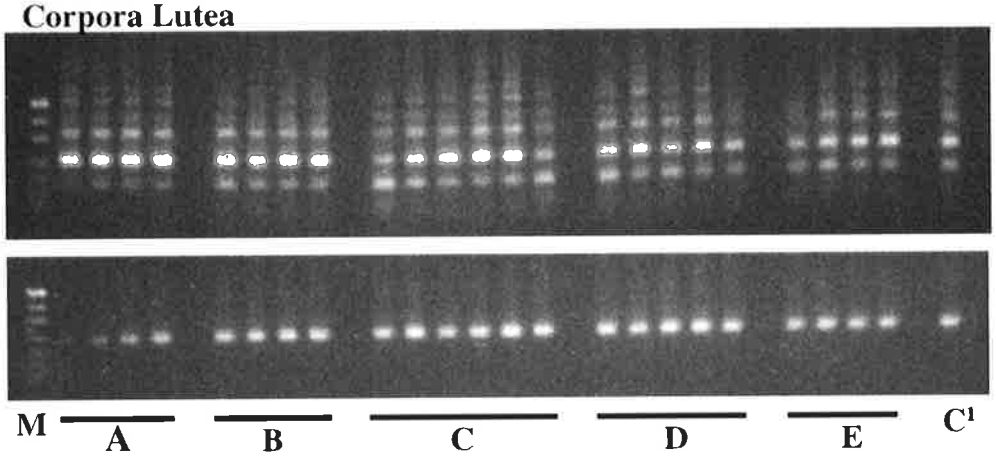
It was expected that the A, F, B and G forms of the LH receptor would generate amplified products of 498, 417, 234 and 153 bp in length respectively. While products of these sizes were observed, an unexpected additional product was also observed at approximately 365 bp in all of the samples. The size of this product does not correspond to the expected amplification size of any splice variant of the LH receptor described in any species. It was however, possible that this band was a heteroduplex formed from the two most abundant fragments, those that corresponded to the A and B forms, as the band ran approximately half-way between them.

To determine if the 365 bp fragment was a new splice variant of the LHR, or in fact a heteroduplex generated from the A and B form PCR products, two of the products were radio-labelled, and electrophoresed on a denaturing (5M urea) 8% polyacrylamide gel. The PCR product (15 µl) was labelled by adding 10 µCi of α-^{32}P-dCTP and 25 µl of fresh PCR reaction mixture, followed by a further six cycles of amplification. Following autoradiography of the electrophoresed product *(see Figure 5.7)*, it was clear that the 365 bp band was no longer present. As such, it was concluded that the band was in fact a heteroduplex formed from hybridisation of the complementary regions of the PCR fragments produced from the A and B splice form mRNAs. Electrophoresis of such PCR products on a non-denaturing gel allows the different sized strands of DNA to remain bound to each other at their complementary regions, and the heteroduplex so formed migrates more slowly than a homoduplex of its shorter strand, and more quickly than a homoduplex of its longer strand. However, electrophoresis of PCR products on a denaturing gel does not permit the retention of the hetero- (or homo-) duplex form, and as such all PCR products are single stranded and heteroduplexes can be distinguished from legitimate PCR products.

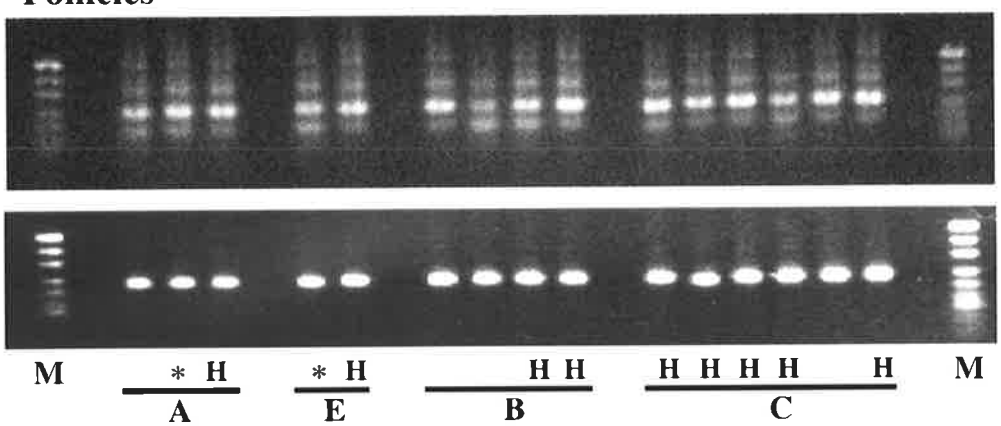
Corpora Lutea and Follicles



Corpora Lutea



Follicles



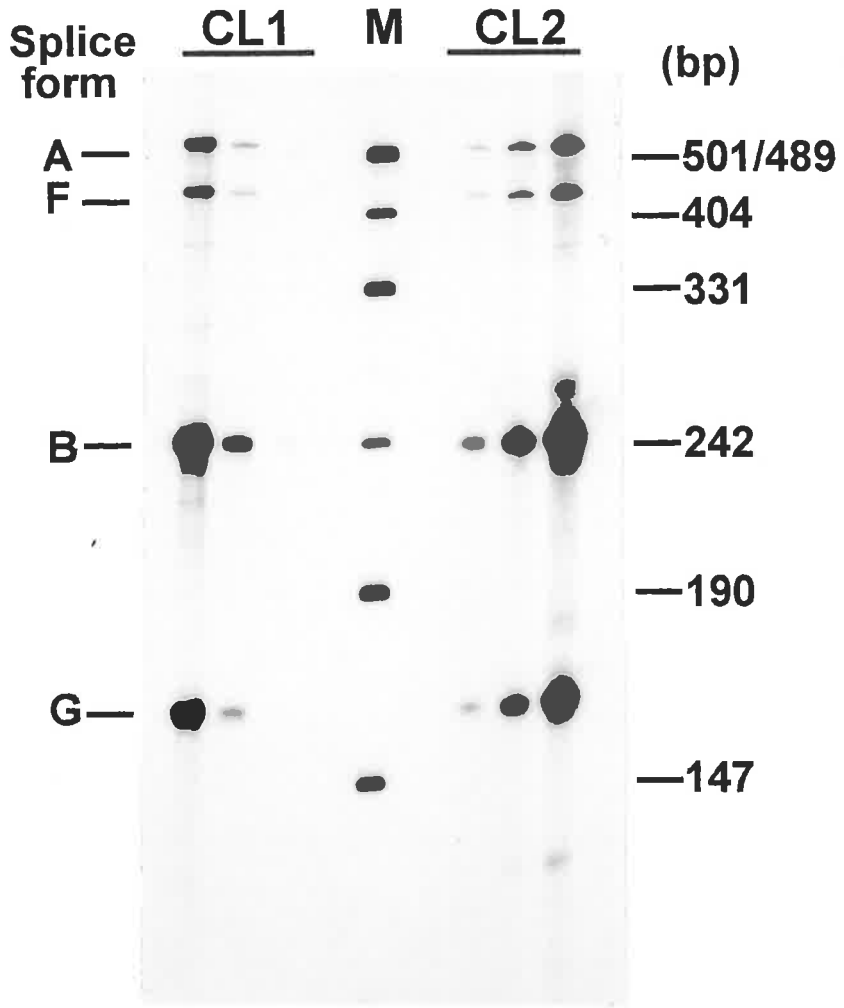


Figure 5.7 LHR RT-PCR electrophoresed on a denaturing gel. Radio-labelled RT-PCR products generated using the primers LHR-S7 and LHR-AS8 were electrophoresed on a denaturing gel. CL1 indicates the same product as seen in lane B1, panel 2a, Figure 5.6, (animal B7, CL1) in various concentrations (neat, 1:10 and 1:100 dilutions), while CL2 is the same product as seen in lane C5, panel 2a, Figure 5.6 (animal C16, CL1) in various concentrations (neat, 1:5 and 1:25 dilutions). While the presence of the 365 bp band in these samples is obvious in Figure 5.6 (a non-denaturing, agarose gel), it is clearly no longer present when the same samples are electrophoresed on a denaturing (5M urea/acrylamide) gel. Therefore, it must be concluded that the 365 bp product seen in Figure 5.6 is in fact due to heteroduplex formation. Molecular weight markers (M) are end-filled pUC19 digested with *Hpa*II.

The relative levels of the amplified products, correlating with the different splice forms of the LH receptor remained fairly constant across the oestrous cycle. However, it was observed that cDNA of CL from the mid-luteal and early follicular phases (groups A and B), generated relatively more amplified products than cDNA

from either the very regressed CL, or the newly formed CL (groups D and A; *see Figure 5.6; no quantitive analyses were performed on this data, due to the non-quantitative nature of RT-PCR*), consistent with the results of the RNase protection analyses. Some samples exhibited an altered ratio of the B : G forms *(e.g. see lanes C1 and C6 in the corpora lutea sample in Figure 5.6)*, relative to the majority of samples, however these altered results were variable, and were more likely a representation of the variability intrinsically associated with using PCR for relative quantitation.

5.4 Western Analyses of Timed Tissues

Both the RNase protection assays, and the RT-PCR analyses of the LHR indicated that the receptor is not regulated by alternative splicing throughout the oestrous cycle. It was however still possible that the receptor is regulated by post-translational modification of one or more of the variants. Unfortunately, although every attempt was made to obtain an LHR antibody that would detect the ovine full length receptor, we were unable to do so *(see Chapter Four)*. Therefore, Western analyses were only carried out using the anti-LHR-B&G antibody that was generated during the course of this project. Furthermore, there was insufficient material available to study some of the extremely regressed CL, the very new CL, and the follicles. Nevertheless, in an attempt to see if there was any change in translation of the B form throughout the cycle, Western analyses were performed as described in *Section 4.5.4*, the results are shown in *Figure 5.8.*

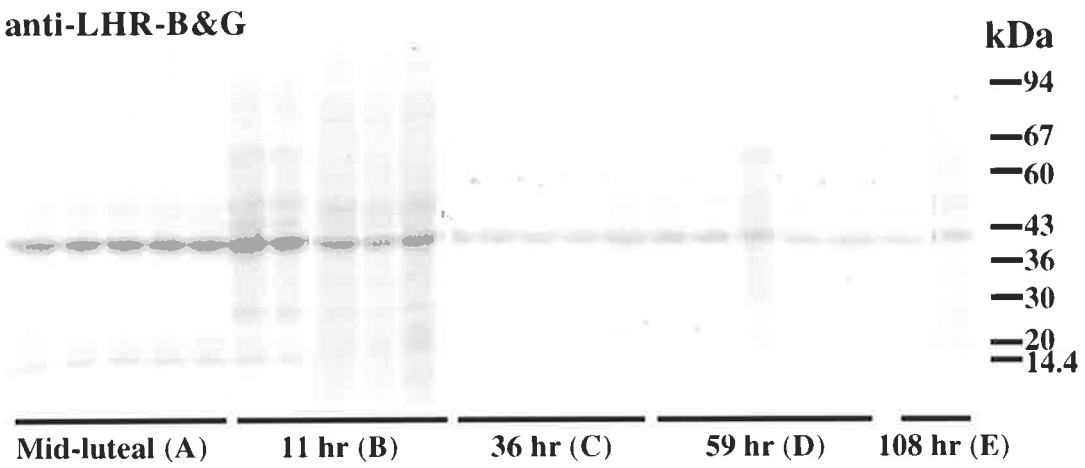


Figure 5.8 Western analyses using the anti-LHR-B&G antibody of CL from across the oestrous cycle. A predominant band of approximately 42 kDa corresponding to the translated B form can be seen to decrease with increasing time after $PGF_{2\alpha}$ administration.

Analysis of the results obtained from Western analyses of timed CL using the anti-LHR-B&G antibody revealed similar results to those obtained using the RNase protection assay and RT-PCR analyses. That is, a relative decrease in the amount of binding of the anti-LHR-B&G antibody as the CL regressed. This reduction was even greater when estimated per CL rather than per microgram of protein.

5.5 SUMMARY

The aim of the experiments described in this chapter was to determine if the LH receptor is regulated by alternative splicing throughout the oestrous cycle. This was examined in ewes whose oestrous cycles were synchronised with $PGF_{2\alpha}$. Three methods were used to examine LHR regulation; at the transcriptional level using RNase protection assays and RT-PCR, and at the translational level using Western analyses with the anti-LHR-B&G antibody. While it seems that the LHR is neither regulated by alternative splicing or at the translational level (at least not during

$PGF_{2\alpha}$ induced luteal regression), the overall amount of LHR mRNA, and translated B form protein product decreased as there was increased regression of the CL. The maximum amount of LHR mRNA and translated B form protein product was seen in mid-luteal phase CL, as might have been expected. Incidentally, the fact that the results of the Western analyses of the timed CL mirror the expression pattern of the LHR B splice form mRNA, provides additional compelling evidence that the protein detected by the anti-LHR-B&G antibody produced as part of this study, is in fact the translated B form protein product.

CHAPTER SIX

CONCLUDING DISCUSSION

The major aim of this study was to determine if alternative splicing of the LH/CG receptor gene transcript occurs in the adult ovine ovary. In particular, we wished to investigate whether the LH receptor gene is regulated by alternative splicing in the adult ovine ovary, and if the alternatively spliced transcripts are translated into proteins *in vivo*.

We identified three alternatively spliced transcripts of the ovine LH receptor in the ovary, in addition to the transcript that encodes for the full length functional receptor. One of the alternatively spliced forms, the B splice form, has been identified in every mammalian species examined. The other two alternative splice forms, which we have named the F and G forms, have never been reported in any other species, and at this stage appear to be unique to the sheep. These forms were identified by RT-PCR , cloning/sequencing and RNase protection assays. In addition, this was the first time that any regions of the ovine LH receptor cDNA had been cloned and sequenced. The sequencing data indicated that the smaller forms (the B, F and G forms), arose by alternative splicing, as the regions that are deleted in these forms correspond to either whole exons that have been described previously in the rat LH receptor gene, or to regions of homology to intron/exon boundaries, and in regions in which alternative splicing has been reported to occur in LH receptor transcripts from other species.

The high degree of conservation of the B splice form across species, both of the splice sites and the amino acid sequences, may be due to the imposed conservation of the second transmembrane domain of the A form, in which all amino acids are conserved between species (Loosfelt *et al.*, 1989, McFarland *et al.*, 1989, Minegish *et al.*, 1990, Gundermann *et al.*, 1992). However, the DNA sequence in this region could potentially have diverged considerably due to degeneracy in codon usage, whilst keeping the amino acid sequences of the A form conserved. The fact that the DNA sequence is highly conserved between mammalian species, suggests that there has

been selective pressure to conserve the amino acid sequence of the B form. It is therefore possible that the B form may produce a biologically functional protein.

To determine the relative abundance of the various LH receptor transcripts we developed an RNase protection assay that would quantitatively distinguish the alternatively spliced forms. This revealed that the relative abundance of the B:A:G:F form transcripts in both corpora lutea and follicles was 5-3.5:1:1:0.3. This was the first time that the relative abundance of the alternative splice forms has been examined in specific ovarian organs (*i.e.* follicles and corpora lutea), or corpora lutea at different stages of the oestrous cycle in any species. In addition, nearly all other reports of alternative splicing of the LH receptor have involved only semi-quantitative methods.

To determine if these splice forms were in fact translated *in vivo*, polyclonal antibodies were raised against specific peptide sequences, with the intention that these antibodies would identify the different protein products encoded by the LH receptor gene. Unfortunately two of the antibodies, LHR-ALL and LHR-A&F, despite recognising their target peptide, were not able to recognise the LH receptor in Western analyses of ovine ovaries. However, the third polyclonal antibody, LHR-B&G, which was designed to recognise the protein products encoded by the B and G splice variants, recognised a protein by Western analyses with all the expected characteristics of a protein encoded by either the B or G splice form. This antibody binds a protein of (1) the expected molecular weight for the B isoform, (2) was detected only in the tissues in which the B splice form mRNA transcript has been detected in, (3) localised to the subcellular fraction in which the B isoform has been localised to in *in vitro* experiments, and as such, is in the fraction that it would be expected to be localised in if it was translated *in vivo,* and (4) this protein shares the same characteristics as (*i.e.* molecular weight and localisation), and appears to be the same as a protein that is recognised by another LHR polyclonal antibody. This other

antibody was raised against a peptide sequence within the rat LH receptor extracellular domain, and is known to bind the rat LH receptor (Cooke's rat LHR-2 antibody; Pillikaros *et al.*, 1995). Based on these findings, it was concluded that the 42 kDa protein recognised by the LHR-B&G antibody was the translated product of the B splice form.

The LHR-B&G antibody enabled us to demonstrate that the B splice form transcript is translated *in vivo* in the ovine ovary. This is the first time in any species that the alternatively spliced forms of the LH receptor have been categorically shown to be translated *in vivo*. While other investigators have reported immunoreactive/ligand binding protein products of similar size to the translated B form, they have been unable to confirm that these products were not due to proteolytic cleavage of the full length receptor. In fact, some other groups have reported that "collagenase preparation treatment" is important in the generation of these fragments, and other groups have demonstrated that proteolytic cleavage of the extracellular domain is involved in ligand binding. However, the use of our LHR-B&G antibody circumvents these problems, as the sequence that this antibody recognises is unique to the putative proteins encoded by the B and G splice variants, and hence not found in the full length A form sequence.

There is evidence that the splicing pattern of the LH receptor is regulated in some animals during foetal development of Leydig cells and ovaries (Sokka *et al.*, 1992; Vihko *et al.*, 1992). Given the possibility that expression of the LH receptor is regulated by alternative splicing, at least at some stages of development, we decided to assess whether it is similarly regulated throughout the oestrous cycle. The expression of the LH receptor gene across the oestrous cycle was examined using the techniques developed during this project, *i.e.* the LHR RT-PCR, the LHR RNase protection assay, and Western analyses using the oLHR-B&G antibody. It was shown by RT-PCR that full length A form, and the three LH receptor alternatively spliced

transcripts, the B, F, and G forms, were present in all large follicles and corpora lutea throughout the oestrous cycle. The relative levels of expression of the LH receptor A and B splice form transcripts were determined by RNase protection analyses for both follicles and corpora lutea across the oestrous cycle. The concentration of LH receptor mRNA per milligram of total RNA varied significantly throughout the cycle, such that mid-luteal corpora lutea had higher levels than either newly formed corpora lutea, or regressing corpora lutea. This is consistent with the number of LH receptors present as predicted by binding studies. However, expression of the LH receptor is not regulated by alternative splicing, as the ratio of the A:B transcripts remained constant (~1:4.5), throughout the oestrous cycle.

The expression of the protein encoded by the B splice form was also examined in follicles and corpora lutea throughout the oestrous cycle. Its expression was concordant with that seen for the B form mRNA. Although no significant difference was seen per microgram of protein, a large decrease was seen in the amount of LHR B splice form per corpora lutea as the corpus luteum regressed.

The fate of the LHR mRNA splice variants in the cell is unknown, although much has been postulated about them. The majority of LHR splice variants reported so far do not encode the entire transmembrane domain, except for the E and rLHR1950 forms in rat (Bernard *et al.*, 1990, Segaloff *et al.*, 1990, Aatsinki *et al.*, 1992), and the F form in sheep, leading to speculation that if translation of the splice variants lacking the transmembrane domain occurs, then the encoded proteins would be secreted by the cells and be capable of binding LH and hCG. As reviewed in Chapter One, expression in mammalian cells transformed with the B, C or D splice forms (Tsai-Morris *et al.*, 1990; VuHai-LuuThi *et al.*, 1992), or of artificially altered forms which also lack the transmembrane domain (Xie *et al.*, 1990; Braun *et al.*, 1991) found that they had binding affinities for hCG comparable to that of the A form. Interestingly, these studies demonstrated that the translated products of the B, C, and D splice

variants were secreted, although the artificially constructed forms were not. Nevertheless, these findings should be taken with caution, as one of these groups, and another, have since attempted *in vitro* expression of the B splice variant, and were unable to detect secretion of the B splice form protein product (Koo *et al.*, 1994; Zhang *et al.*, 1995).

Soluble receptors resulting from alternative splicing of receptor genes have been described in biological fluid and blood for other receptors, including the growth hormone receptor (Leung *et al.*, 1987; Baumbach *et al.*, 1989; Edens *et al.*, 1994), the prolactin receptor (Postel-Vinay *et al.*,1991), the epidermal growth factor receptor (Carpenter, 1987), the interleukin-4 receptor (Mosley *et al.*, 1989) the interleukin-7 receptor (Takeshita *et al.*, 1992), and the N-CAM receptor (Gower et al., 1988). As discussed in chapter one, there is some evidence that secreted, soluble receptor ligand binding domains can act as inhibitors of hormone action by sequestering the ligand away from the receptor (Mosely *et al.*, 1991).

Alternatively, a free ligand-binding domain of a truncated receptor such as the translated LHR B form, might act to present the hormone to the receptor resulting in a more efficient signal. Although the functional importance of the leucine-rich repeats found in the extracellular domain of the LH receptor is unknown, it has been hypothesised that by forming amphipathic helices or β-sheets, this structure may be able to interact with both hydrophobic and hydrophilic surfaces (Segaloff and Ascoli, 1993). This would theoretically allow the free extracellular ligand-binding domain to interact with the transmembrane domain of the full length receptor.

This theory is supported by the fact that expression studies have demonstrated alternatively spliced LH/CG receptor proteins enhance the activity of the full-length receptor (VuHai-LuuThi *et al.*, 1992). Cells co-transfected with either the C or D splice variant and the A splice form responded with increased maximal hormone-

induced stimulation of adenylate cyclase above that of cells only transfected with the full length functional A form (VuHai-LuuThi *et al.*, 1992). However, there was no effect on the amount of hormone required to elicit a half maximal stimulation. Interestingly, VuHai-LuuThi *et al.* (1992) demonstrated that soluble hormone-receptor complexes were monomeric, thus either the bonds between receptor monomers are unstable, or the reactions that occur in the cell membrane can not be observed in solubilised receptors. Remy *et al.* (1993), demonstrated that co-expression of the extracellular domain, and the transmembrane-cytoplasmic domain of the LH receptor results in functional receptors. Therefore it could be postulated that the translated B form (or the other alternatively spliced forms, if they are translated), may interact with other receptors, or the full length LH receptor, and subsequently lead to LH or hCG stimulation. One could hypothesise that such mechanisms might be important to allow LH or hCG stimulation of cell types that do not express the full length functional receptor, perhaps eliciting a response other than cAMP stimulation. This could also explain the reported presence of LH receptors on non-gonadal tissues.

Further investigation is required to determine the role and function of the alternatively spliced forms of the LH receptor, and in particular if the translated B form is secreted. This could be achieved by the establishment of a two site assay using our anti-LHR-B&G, and Cooke's rat anti-LHR-2 antibodies. In addition, the anti-LHR-B&G antibody should be further characterised, by examining if the protein it recognises can also bind LH or hCG. Clearly, if this form is secreted, and it is capable of binding LH or hCG, then investigation of its role as a binding protein would be essential.

From the results presented here, alternative splicing is not regulating expression of the functional receptor in the adult sheep ovary. However, it is certainly intriguing that the vast majority of the LH receptor gene transcripts encode the B splice form, and not the full length A form. The finding that the B splice form is translated *in vivo* is

an exciting step forward and may represent an as yet uncharacterised mechanism for stimulation by LH and/or hCG.

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