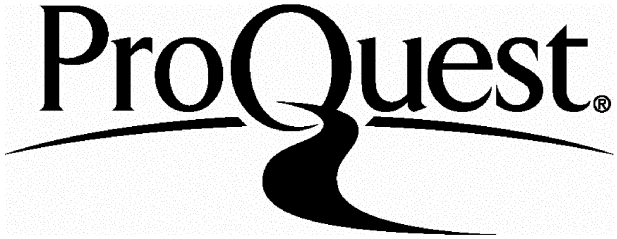
The Structure/Function Relationships of the Murine Leydig Tumour (MA10) Cell, Luteinising Hormone Receptor

by **Lara K. Monks**

A thesis submitted in fulfilment of the conditions
for the degree of Doctor of Philosophy
of the University of London

Department of Biochemistry and Molecular Biology
Royal Free Hospital School of Medicine
London NW3 2PF
UK

September 1997



To mum and dad

Acknowledgements

I would firstly like to thank Professor Brian Cooke for allowing me the opportunity to do a PhD in his department and for his valuable guidance throughout my work and during the writing of this thesis.

In addition, many thanks go to Dr Robert Abayasekara for his considerable help and advice and willingness to listen. To Dr Tony Michael for his support and excellent proof-reading abilities, and to Dr Katja Teerds (University of Utrecht, Netherlands) for her help with the immunohistochemisty experiments.

I would also like to thank the many friends and collegues of the Department of Biochemistry and Molecular Biology, without whom, I doubt I would've lasted the distance. In particular I would like to thank Helen Ramnath, Zac Pallikaros and Joe Atoniw for their enormous support and friendship. I am also very grateful to Rachel Helliwell for all her help during this PhD and for generally being a good laugh.

Outside of the department I would also like to thank those friends that have had to put up with me throughout my PhD. In particular I would like to acknowledge Treena Bacon and Helen Cooper, who have both heard far more than is healthy about testicles, and also to my 'beer mates' Lynda Fletcher and Becca Upton who have always managed to cheer me up no matter what. I must also acknowledge and thank my current collegues in the Department of Biological Sciences, University of Keele for all their support. In particular I wish to thank Professor Noel Morgan and Dr Sue Chan for their never-ending patience in waiting for me to finish.

Finally, I wish to thank my family for their unerring emotional support (and considerable financial!), and of course to Daf, for being there no matter what and for taking all the crap.

Declaration

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously mentioned. The work was carried out at the Department of Biochemistry and Molecular Biology, Royal Free Hospital School of Medicine, London.(October 1993 to December 1996).

The work described in this thesis has not been previously submitted for a degree at any other institution.

Contents

Figures

Chapter One

Chapter Three

Chapter Four

Abbreviations

A23187	calcium ionophore
aa	amino acid
α_2AR	α_2-adrenergic receptor
ACTH	adrenocorticotrophin
ADP	adenosine diphosphate
AIDS	autoimmunedeficiency sydrome
AMP-P(NH)P	adenyl-5'-yl imidodiphosphate
ANOVA	'analysis of variants'
aPKC	atypical protein kinase C (isoforms)
arg	arginine residue
ATP	adenosine triphosphate
AVP	arginine vasopressin
Bo	zero concentration (radioimmunoassays)
BAP	bovine alkaline phosphatase
ß$_2$AR	ß$_2$-adrenergic receptor
ßARK	ß$_2$-adrenergic receptor kinase
ßARK(2)	ß-adrenergic receptor kinase (type 2)
BCIP	5-bromo-4-chloro-3-indolyl phosphate
ß-gal	ß-galactosidase
3ß-HSD	3ß-hydroxysteroid dehydrogenase/3-oxosteroid-4, 5-isomerase
17ß-HSD	17ß-hydroxysteroid dehydrogenase
bp	base pairs
4ß-PMA	4ß-phorbol 12-myristate 13-acetate
8-Br-cAMP	8-bromo-cAMP
BSA	bovine serum albumin
°C	degrees centrigrade
C22-C20	covalent bond between carbon 22 and carbon 20
CaM kinase II	calcium/calmodulin kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CG	chorionic gonadotrophin
Ci	Curie
CNS	central nervous system
COS1 cells	African green monkey kidney cells transformed with SV40 virus
cPKC	classic protein kinase C (isoforms)
cpm	counts per minute
CSF	cerbrospinal fluid
C-terminal/terminus	carboxyl-terminal/terminus
CTP	cytosine triphosphate
D_2	Dopamine D_2 receptor

Da	Dalton
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
DBI	diazepam-binding inhibitor
dCTP	deoxycytosine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanine triphosphate
ddTTP	dideoxythymidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanine triphosphate
DMEM	Dulbeccos Modified Eagles Medium
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EC_{50}	median effective concentration
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid (disodium salt)
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
fmol	femtomole
FMPP	familial male precocious puberty
FSH(R)	follicle stimulating hormone (receptor)
FSH/LH-RL	FSH-LH-releasing hormone
FTAz	horse serum, Triton X-100 and sodium azide
293F cells	HEK 293 cells transfected with the FSHR
g	gram
GABA	γ-aminobutyric acid
$G_{\alpha i}$	inhibitory G-protein $_{\alpha}$ subunit
$G_{\alpha o}$	'other' G-protein $_{\alpha}$ subunit
$G_{\alpha olf}$	olfactory G-protein $_{\alpha}$ subunit
$G_{\alpha s}$	stimulatory G-protein $_{\alpha}$ subunit
$G_{\alpha s}$-GTP	stimulatory G-protein subunit bound to guanine triphosphate
$G_{\alpha q}$	$_{q}$G-protein $_{\alpha}$ subunit
$G_{\beta\gamma}$	G-protein $_{\beta\gamma}$ complexes
GDP	guanine diphosphate
GF109203X	PKC inhibitor
GH	growth hormone

G_i	inhibitory G-protein
GMP-P(NH)P	guanyl-5'-yl imidodiphosphate
GnRH(R)	gonadotrophin releasing hormone (receptor)
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide linked protein
GRK (2/5)	G-protein coupled receptor kinases (type 2/type5)
G_s	stimulatory G-protein
GTP	guanine triphosphate
GTPase	guanine triphosphatase
xg	times gravity
^3H	tritium
hCG	human chorionic gonadotrophin
HCV	hepatitis C virus
HEK cells	human embryonic kidney cells
HEPES	(*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid])
hFSH	human FSH
HS	horse serum
IBMX (MIX)	3-isobutyl-1-methylxanthine
IgG	immunoglobulin G
[^{125}I]-hCG	iodinated hCG
IP	inositol phosphates
IP_3	inositol triphosphate
IPTG	ß-D-isopropyl-thiogalactopyranoside
IU	international unit
JM109	high efficiency competent *E. coli* cells
Kb	kilobase
K_D	equilibrium dissociation constant
kDa	kilodalton
l	litre
LB agar	Luria Bertaini agar
LB broth	Luria Bertaini broth
LCH	Leydig cell hypoplasia
LDH	lipid dehydrogenase
LH	luteinising hormone
LH/CGR	luteinising hormone/chorionic gonadotrophin receptor
LRRs	leucine-rich repeats
293L cells	HEK 293 cells transfected with the LH/CG receptor
M	molar

mA	milliampere
MA10	murine Leydig tumour cells
mCi	milliCurie
MDRs	mitochondrial DBI receptors
mg	milligram
MIX (IBMX)	3-isobutyl-1-methylxanthine
ml	millitre
µl	microlitre
mLH/CG receptor wt	murine LH/CG receptor wild-type
mLH/CG receptor-ct628	murine LH/CG receptor C-terminally truncated at residue 628
MLTC-1 cells	murine Leydig tumour cells
mM	millimolar
µM	micromolar
M-MLV RT	Moloney-MurineLeukaemia Virus Reverse Transcriptase
mmol	millimole
MPC	magnetic particle concentrator
mRNA	messenger RNA
Mw	molecular weight
NADH	reduced nicotinamide dinucleotide
NADPH	reduced nicotinamide dinucleotide phosphate
NBT	nitroblue tetrazolium
ng	nanogram
NH_2-terminal	amino-terminal
N-linked	linked to a nitrogen atom
nPKC	novel protein kinase C (isoforms)
nm	nanometre
nmol	nanomole
NSB	nonspecific binding
N-terminal/terminus	amino-terminal/terminus
-OH	hydroxyl group
oligo.	oligonucleotide
O-linked	linked to an oxygen atom
$P\text{-}450_{aro}$	cytochrome P-450 aromatase
$P\text{-}450_{scc}$	cytochrome P-450 side chain cleavage enzyme
$P\text{-}450_{c17}$	cytochrome P-450 17α-hydroxylase
PBS	phosphate buffered saline
pBSLHR/59	full length rat LH/CG receptor cloned into bluescript plasmid
PCR	polymerase chain reaction
PDE	phosphodiesterase
PEG	polyethylene glycol
pg	picogram
PGB	pas gelatin buffer

PGE_1	prostaglandin E_1
Pi	inorganic phosphate
PI	phosphoinositol
PIP_3	phosphotidylinositol-3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PKI	heat stable inhibitor of PKA
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
pmol	picomole
PMSF	phenylmethylsulphonyl fluoride
PMSG	pregnant mares serum gonadotrophin
PNK	polynucleotide kinase
PPi	inorganic diphosphate
PRL	prolactin
pUC18	plasmid vector
RIA	radioimmunoassay
rLH/CG receptor-t631	rat LH/CG receptor C-terminally truncated at residue 631
RNA	ribonucleic acid
Rnase	ribonuclease
rNTP	ribonucleotide triphosphates
RRL	rabbit reticulocyte lysate derived cell-free system
RO 31-8220	PKC inhibitor
S	sedimentation coefficient
$S^{no.}$	serine residue (as designated by number)
SAP	steroidogenic activator protein
ScAMP-TME	2'-monosuccinyladenosine-3',5'-cyclic monophosphate tyrosyl methyl ester succinylated cAMP
SCP2	sterol carrier protein 2
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of mean
ser	serine residue
SIP	steroidogenesis-inducing protein
SOC medium	solution C medium
StAR	steroidogenic acute regulatory protein
SSC	sodium citrate buffer
t	student *t* test
Taq	DNA polymerase I isolated from *Thermophilus aquaticus*
TBE	Tris-borate-EDTA buffer

TBS	Tris buffered saline
TE	Tris-EDTA buffer
TEMED	*N,N,N', N'*-tetramethylethylenediamine
TnT	coupled transcription and translation cell-free system
tRNA	transfer RNA
TSH(R)	thyroid stimulating hormone (receptor)
TTBS	Tris buffered saline with Tween-20
U	unit
UTR	untranslated region
UV	ultra violet
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-galactopyranoside

Greek symbols

α	alpha
ß	beta
γ	gamma
δ/Δ	delta
ε	epsilon
ζ	zeta
η	eta
ϕ	theta
λ	lambda
μ	mu

Abstract

The LH/CG receptor is a member of the G-protein coupled receptor family. It is found expressed on testicular Leydig cells and ovarian granulosa-luteal and thecal cells. On binding its ligand LH, the activated LH/CG receptor initiates a cascade of intracellular signalling which results in the production of androgens and oestrogens. In this thesis the structural and functional relationships of the LH/CG receptor and in particular the process of LH/CG receptor desensitisation have been investigated.

One of the aims of this work was to establish whether, when desensitised, the LH/CG receptor is phosphorylated. In order to achieve this it was first necessary to establish an immunoprecipitation protocol using antibodies which had previously been developed in this laboratory (Pallikaros *et al.* 1995). We used two different methods of immunoprecipitation and different LH/CG receptor antibodies, however, no immunoprecipitation of the LH/CG receptors from a solubilized membrane preparation occurred. Subsequently it was demonstrated that the LH/CG receptor antibodies did detect the LH/CG receptor in Leydig cells and ovarian preparation using immuncytochemical and confocal techniques and Western blotting. However, variations in the sensitivity of detection were observed depending on the method of fixation, nature of the tissue and the LH/CG receptor antibodies used. It was concluded, therefore, that the lack of immunoprecipitation may have been due to the inaccessibility of the antigenic sites of the LH/CG receptor to the antibodies (e.g. because of aggregation or degradation).

Previous work from this laboratory had used an antisense strategy to produce C-terminally truncated LH/CG receptors in intact Leydig (MA10) tumour cells, some of which did not undergo ligand-stimulated desensitisation (West and Cooke 1991). We intended to use this antisense strategy to establish which regions of the LH/CG receptor underwent phosphorylation or were involved in coupling to G_s. However, because we could not reproduce the original results, we investigated the use of a cell free system. We successfully established that the rat LH/CG receptor could be synthesized in a coupled transcription/translation cell-free system. However, when the antisense oligonucleotides previously reported to cause truncated LH/CG receptors, were included in this cell-free system, they were found to have no effect on the size or level of LH/CG receptor synthesis. Preliminary studies indicated that antisense oligonucleotides targeted to the start site of translation may be more effective in preventing LH/CG receptor synthesis.

In parallel with the antisense work, we amplified and cloned the LH/CG receptor from the MA10 cells so that we could generate truncated cDNAs by the use of restriction enzymes. These truncated cDNAs could then be expressed in a mammalian cell line and used to establish whether truncated LH/CG receptors could undergo ligand-induced desensitisation. The amplification and cloning of the whole LH/CG receptor proved difficult and so the amplification reaction was divided into two. The amplification and cloning of the C-terminal region of the MA10 LH/CG receptor was successfully performed. Sequence analysis confirmed that it was identical to that of the murine LH/CG receptor. However, despite extensive optimisation procedures, it was not

possible to amplify the first 1.1kb of the receptor. Analysis of the predicted secondary structure formed by the mRNA indicated a considerable level of hairpin bend formation suggesting that this may have been responsible for preventing adequate primer hybridisation to the template.

The putative kinases responsible for phosphorylation of the activated LH/CG receptor have not, as yet, been identified. In order to investigate the possible role of PKC, the effects of three PKC inhibitors (staurosporine, GF109203X and RO 31-8220) on LH-, cholera toxin- and forskolin-stimulated cAMP production in MA10 cells were investigated. It was found that staurosporine markedly increased, and GF109203X and RO 31-8220 decreased, the cAMP levels stimulated by these compounds. The effects of staurosporine were not via effects on LH binding, phosphodiesterase or cell viability. It is concluded that because the GF and RO compounds are more specific PKC inhibitors than staurosporine, that PKC-mediated phosphorylation may not be involved in LH-induced desensitisation. It is suggested that staurosporine may act via inhibition of CaM-kinase II.

Chapter One: General introduction

1.1: The glycoprotein hormones

Luteinising Hormone (LH) is a glycoprotein and belongs to a family of anterior pituitary glycoprotein hormones (Pierce and Parsons 1981, Matzuk *et al.* 1988), which includes follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). Chorionic gonadotrophin (CG) is a glycoprotein hormone of placental origin and is related in structure to these pituitary hormones. This family of hormones contain covalently bound carbohydrate moieties, consisting of a number of sugars (Pierce and Parsons 1981) at one or more positions within their structures. Each of these glycoproteins is composed of two chains, termed the α- and ß-subunits, that are joined together by non-covalent forces. The common α-subunit consists of 92 amino acids and contains two oligosaccharides that are *N*-linked to asparagine residues. In the gonadotrophins (LH, CG and FSH), removal of these carbohydrate moieties causes an uncoupling of the receptor-adenylyl cyclase system in target cells of the testis, implying that the integrity of the α-subunit oligosaccharides are somehow involved in the cell signalling of these hormones (Hadley 1992). The ß-subunits also contain *N*-linked glycosylated asparagine residues. Studies where all the carbohydrate moieties have been removed from glycoprotein hormones have demonstrated that the half-life of these hormones in circulation, and therefore their biological potency, is reduced (Sairam 1983). In general, removal of *N*-linked carbohydrates does not interfere with receptor binding and has been shown to actually increase binding, however, deglycosylation does markedly decrease stimulation of adenylyl cyclase and steroidogenesis. This would indicate that glycosylation is required by both subunits for full biological activity (Keutmann *et al.* 1983). There is a high degree of structural conservation of the α-subunit among different species and within a species, the α-subunit is common to LH, FSH, TSH and CG (Sairam and Bhargavi 1985). The ß-subunit of each hormone however, is structurally distinct. Both subunits are required for binding to their respective receptors. It has been suggested that the α-subunit endows the hormone-specific ß-subunit with the correct conformation to enable binding to the receptor, whilst the α-subunit is necessary for the stimulation of adenylyl cyclase. It has also been proposed that domains on the α-subunit, in combination with the ß-subunit or alone,

are responsible for the interaction of the hormone with its receptor. In the latter case, conformational changes contributing to the specificity of binding could also be induced in the α-subunit by the ß-subunit (Sairam and Bhargavi 1985). Recombination studies using the α- and ß-subunits, have demonstrated that it is the ß-subunit that confers hormonal specificity to the protein. Despite being structurally distinct, the various ß-subunits do demonstrate areas of homology. This is most apparent in the conservation of 12 cysteine residues implying that ß-subunits generally have similar tertiary structures (Matsuk *et al.* 1988). Amino acid sequences that are identical in the ß-subunits have been suggested to represent contact sites between subunits, whereas the nonidentical residues contribute to interaction with target receptors and therefore provide hormonal specificity.

1.11: The gonadotrophins

The testes and the ovaries secrete steroid hormones that regulate the growth and development of a variety of target tissues. In the past, it was noted that when the pituitary gland of animals was removed, the gonads underwent atrophy [see Hadley 1992]. It was discovered that gonadal size and function could be restored upon the administration of pituitary gland extracts, thus implicating the pituitary gland in the control of gonadal function. Various fractions of the pituitary gland extracts were subsequently obtained. One of these fractions was found to stimulate testosterone secretion and the development of secondary characteristics in the male, whilst stimulating ovulation and corpora lutea formation in the female (Hadley 1992). This hormone is now referred to as luteinising hormone (LH). As previously described, LH is composed of an α- and ß-subunit which are individually inactive, but have full biological activity when combined together. LH and the placental hormone chorionic gonadotrophin (CG), share common biological properties and their ß-subunits have been shown to share a high degree of sequence homology, (85% in the first 114 amino acids (Talmadge *et al.* 1984)). This high degree of sequence homology allows LH and CG to bind to a common gonadal receptor (in most species), known as the LH/CG receptor. The main difference between the ß-subunit of LH and that of CG is the existence of a carboxyl terminal extension of the CG ß-subunit, which is rich in proline

and serine residues and which facilitates further *O*-linked glycosylation (Talmadge *et al.* 1984).

In the testis, LH stimulates testosterone synthesis by binding to its receptor found on the Leydig cells. Testosterone is required for the process of spermatogenesis in the post-pubertal testis. FSH, which was first discovered as a pituitary fraction separate from LH, increases the spermatogenic activity of the testes and stimulates follicular growth in the ovaries. Whilst spermatogenesis can be maintained in hypophysectomised adult animals by testosterone in the absence of gonadotrophins, FSH and testosterone are both required for the pubertal initiation of spermatogenesis (Matsumoto and Bremner 1989). However, recent work by some researchers (Zirkin *et al.* 1994, Tapanainen *et al.* 1997) questions whether FSH is essential for the latter. In the study by Tapanainen *et al.* (1997), males were characterised which were homozygous for an inactivating FSHR mutation. Suprisingly, none of these males demonstrated azoospermia or absolute infertility, although they did have variable degrees of spermatogenic failure. This data would suggest that FSH may not be essential for the initiation of spermatogenesis as was previously thought.

1.12: The control of hypothalamic-hypophysial hormone secretion

The pituitary gland (also known as the hypophysis) has often been referred to as the master endocrine gland of vertebrates because it appeared to function autonomously and yet seemed to control important endocrine glands such as the gonads, thyroid and the adrenals. The pituitary gland is now known to be subservient to hormonal stimuli derived from the brain and from other endocrine glands (Lechan 1987). The gland lies at the base of the brain and consists of two halves; the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary) (Kandel *et al.* 1991). The neurohypophysis contains neurones that secrete the peptide hormones, arginine vasopressin (AVP, also called antidiuretic hormone) and oxytocin. The adenohypophysis releases six principal hormones into the systemic circulation. These include adrenocorticotrophin (ACTH), growth hormone (GH, also called somatotrophin), prolactin (PRL), TSH and the gonadotrophins LH and FSH (Hadley 1992).

Early experiments noted that retransplanting an excised pituitary gland to an ectopic site still resulted in atrophy of the adrenal cortex, gonads and thyroid. Only when the pituitary gland was retransplanted back under the hypothalamus was target organ revascularisation and functional reactivation of the pituitary gland achieved. Thus, it was concluded that pituitary activity was dependent on hypothalamic activity. Crude hypothalamic extracts were found to contain factors that were either inhibitory or stimulatory to pituitary gland function [see Hadley 1992]. These extracts where further purified and found to yield subfractions that either inhibited somatotrophin release or stimulated TSH or LH and FSH secretion. These subfractions were termed hypophysiotropic factors and were found to be released into the hypophysial portal system from the median eminence of the hypothalamus. The median eminence is the point where the neurosecretory cells of the hypothalamus and the anterior pituitary gland converge. The hypophysial portal system functions as the vascular link between the anterior pituitary and the median eminence (Kandel *et al.* 1991) [see *Figure* ***1.1*** taken from Kandel *et al.* 1991]..

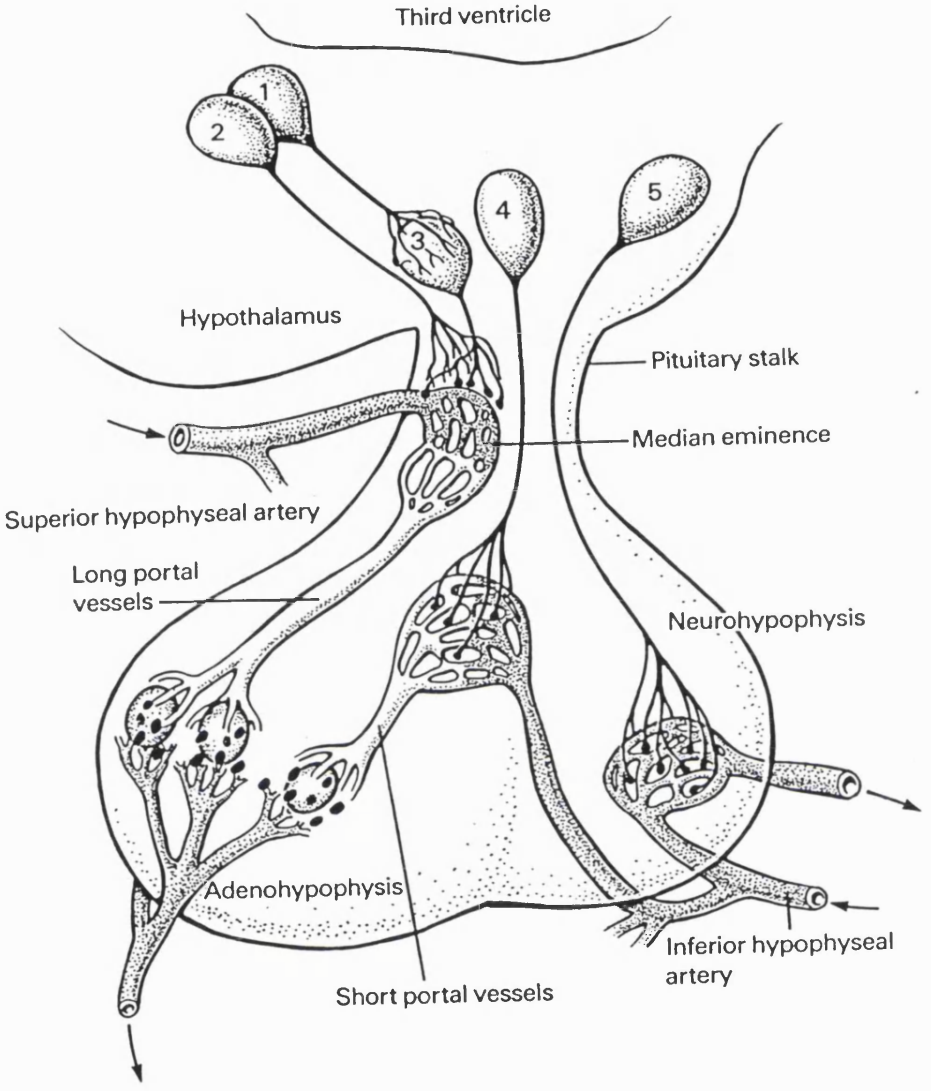


Figure 1.1: **Diagram showing the various functional elements which participate in the control of the pituitary gland by the hypothalamus.** *This diagram was taken from Hadley 1992.*

Thus, the secretion of anterior pituitary hormones occurs in response to stimulation of the gland by hypophysiotropic factors. Sensory neurones conduct stimuli through neuronal routes by the production of neurotransmitters at synapses, to the brain. Axons from these neurones project into the hypothalamus where they innervate with hypophysiotropic hormone producing cells (Kandel *et al.* 1991). Depending on the nature of the neuronal receptors, the neurotransmitters produced will either inhibit or stimulate the production of hypophysiotropic hormones from these cells. This will then be reflected in the enhanced or inhibited secretion of pituitary hormones. The control of LH and FSH secretion (and synthesis) from gonadotrophin producing cells of the *pars distalis,* located in the anterior pituitary, is regulated by the hypophysiotropic hormone, gonadotrophin releasing hormone (GnRH) (Redding *et al.* 1972).

1.13: The regulation of LH and FSH release by GnRH

Mammalian GnRH is a decapeptide whose primary structure appears to be similar between mammalian species. Whilst GnRH has been detected in the nerve fibre next to the portal vessels in the median eminence of the hypothalamus, the anatomical site of the cell bodies of these neurones has not been established. The role of GnRH in the control of pituitary gonadotrophin secretion has been confirmed by the use of immunisation techniques. Injections of antibodies to GnRH have been found to cause testicular atrophy and prevention of the preovulatory LH surge in rats. In addition, the administration of anti-GnRH serum to normal or castrated rats has been found to lower both LH and FSH levels, demonstrating the role of this hypophysiotropin in maintaining the secretion of these gonadotrophins (Hadley 1992).

The secretion of pituitary gonadotrophins in the male has been considered to be under a tonic regulatory control. However, there is evidence that suggests that LH and FSH undergo wide fluctuations in their circulating concentrations, over relatively short periods of time (Desjardins 1981). It has been established that GnRH is released into the hypophysial portal circulation from the median eminence of the hypothalamus in a pulsatile fashion. In the adult human male there is a pulsatile release of LH about every 90 minutes, although the precise pattern of this episodic release varies from day to day (Hadley 1992). The pulsatile release of GnRH from the hypothalamus is essential in

maintaining the responsiveness of the gonadotrophs to GnRH. Several studies using rat (Haisenleder *et al.* 1991), primate (see review Marshall and Kelsh 1986), and sheep (McIntosh and McIntosh, 1985) have demonstrated that a continuous GnRH infusion desensitises the gonadotroph cell to further GnRH stimulation and down regulates GnRH receptors (Clayton, 1982). These results have lead to wide clinical applications of GnRH in the treatment of gonadal-hormone-dependent disorders (Clayton 1989).

Despite the fact that in the post-pubertal female GnRH differentially stimulates FSH and LH secretion from gonadotrophs at different times throughout the ovulatory cycle, there is strong support for the existence of only one type of GnRH. As a result this hormone is sometimes referred to as FSH/LH-releasing hormone (FSH/LH-RH). However, it would seem that there is accumulating evidence suggesting that FSH and LH secretion are independently regulated and that there are separate secretory controls (possibly inhibin [see below] or steroid hormones/hypothalamic neurotransmitters), for each gonadotrophin at the level of both the pituitary gland and the hypothalamus (Hadley 1992, Kandel *et al.* 1991). The secretion of gonadotrophins from the anterior pituitary occurs by GnRH binding to its receptor, located on the gonadotroph membrane. Binding activates inositol trisphosphate production which triggers a complex calcium signalling process (Davidson *et al.* 1991, Stojilkovic and Catt 1992) [see section ***1.51***]. This in turn leads to the exocytosis of gonadotrophins into the circulatory system.

The observations that pituitary gonadotrophin secretion is enhanced after orchidectomy, whereas it is diminished after the administration of exogenous androgens, established that testicular androgens, in particular testosterone, exert a negative feedback on pituitary gonadotrophin secretion (Hadley 1992). Part of this feedback is directed at the hypothalamus and acts to inhibit GnRH secretion. However, it is still uncertain as to the exact cellular site(s) of testosterone feedback in the hypothalamus. FSH secretion is known to be regulated by the action of a hormone known as inhibin (Rhoades and Pflanzer 1989). Inhibin exists in two forms; inhibin A and inhibin B and functions to inhibit the secretion of FSH from the anterior pituitary (Woodruff *et al.* 1996). The inhibins are synthesised by the gonads. Whilst both forms of inhibin have been found in normally cycling female rats, in the male rat it has been reported that inhibin B appears to be the dominant form of FSH

regulating protein of the testes (Woodruff *et al.* 1996). Because FSH release, but not LH release, is altered, it has been postulated that inhibin might exert a selective inhibitory effect on the release of a hypothalamic releasing factor. This argument would seem to suggest then, that despite popular opinion, there may indeed be hypothalamic releasing hormones specific for both FSH and LH (Hadley 1992).

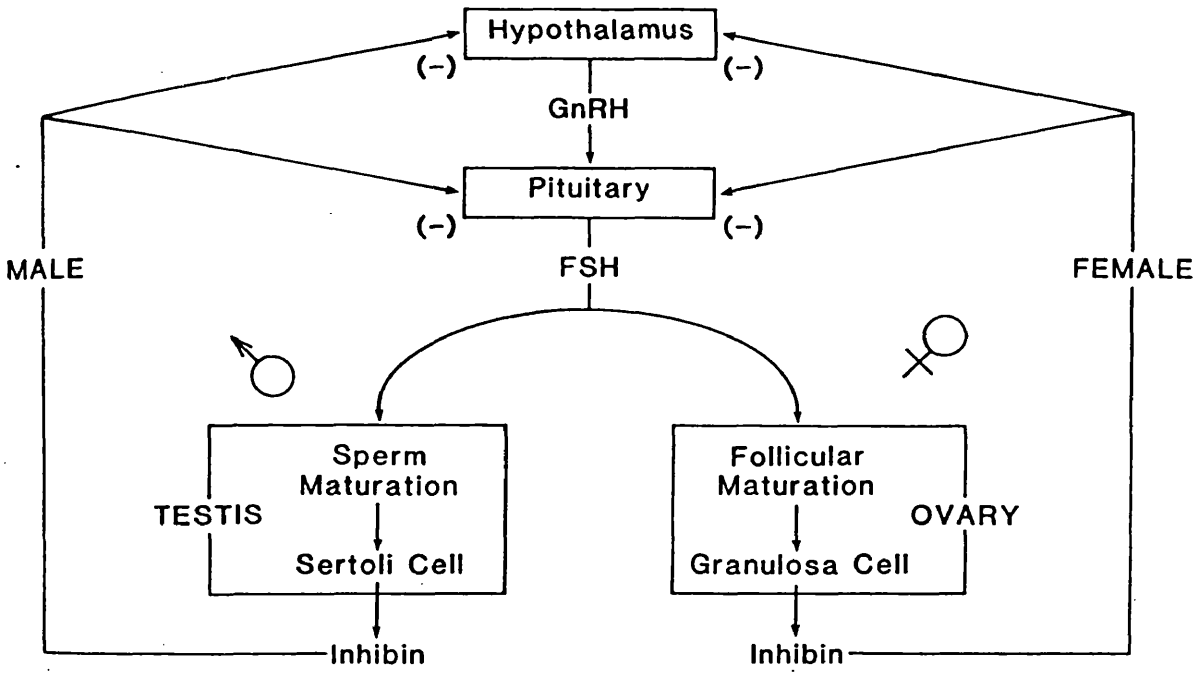


Figure 1.2: **A summary scheme of the roles of FSH and inhibin in the control of testicular (and ovarian) function.** *This diagram was taken from Hadley 1992.*

1.2: The structure and function of the testes

In the human fetus the indifferent gonads of the genetic male are induced to differentiate into testes. The fetal testes in turn, produce testosterone which is responsible for the differentiation and development of the male urogenital system. The testes remain quiescent until puberty when they become activated by the pituitary gonadotrophins (Hadley 1992).

The major functions of the adult testis are twofold; to provide an environment for the production of sperm and to make and secrete male androgens, in particular testosterone (see review Skinner 1991). In order to fulfil these two functions, the testis consists of two closely interlinked compartments. Spermatogenesis occurs in the compartment consisting of convoluted seminiferous tubules. In most mammals the seminiferous tubules account for over 90% of the volume of the testis. These tubules converge into the rete testis which then opens to the epididymis. The tail end of the epididymis connects to the vas deferens where mature sperm are stored prior to ejaculation. The outer sheet of the seminiferous tubules is made up of connective tissue and smooth muscle, the inner lining is composed of non-proliferating somatic cells, known as Sertoli cells. Within these cells are embedded spematogonia. These germ cells undergo successive mitotic and meiotic divisions, proceeding through the spermatocyte and spermatid stages before finally becoming mature spermatozoa and being released into the lumen of the seminiferous tubule. Thus, the seminiferous tubules contain millions of developing germ cells, all at various stages of maturity and in close association with the Sertoli cells. It has been shown from three-dimensional studies that each Sertoli cell is in contact with up to 5 other Sertoli cells at its base and up to 47 germ cells at different stages of development (see review Skinner 1991). The Sertoli cells form specialised "tight junctions" between them so that in essence they make a continuous barrier within every seminiferous tubule. This barrier is referred to as the blood-testis (or lymph-testis) barrier. The barrier divides the tubules into basal and adluminal compartments and excludes large molecules, such as proteins and certain other components of the lymph, from entering the tubule.

Between the seminiferous tubules are found the interstitial cells of Leydig. These cells represent the second compartment of the testis and are the site of androgen production, the main one being testosterone. In the rat testis, Leydig cells constitute 2.7% of testicular

volume, with each cubic centimetre of the testis containing about 22 million Leydig cells (Mori and Christensen 1980).

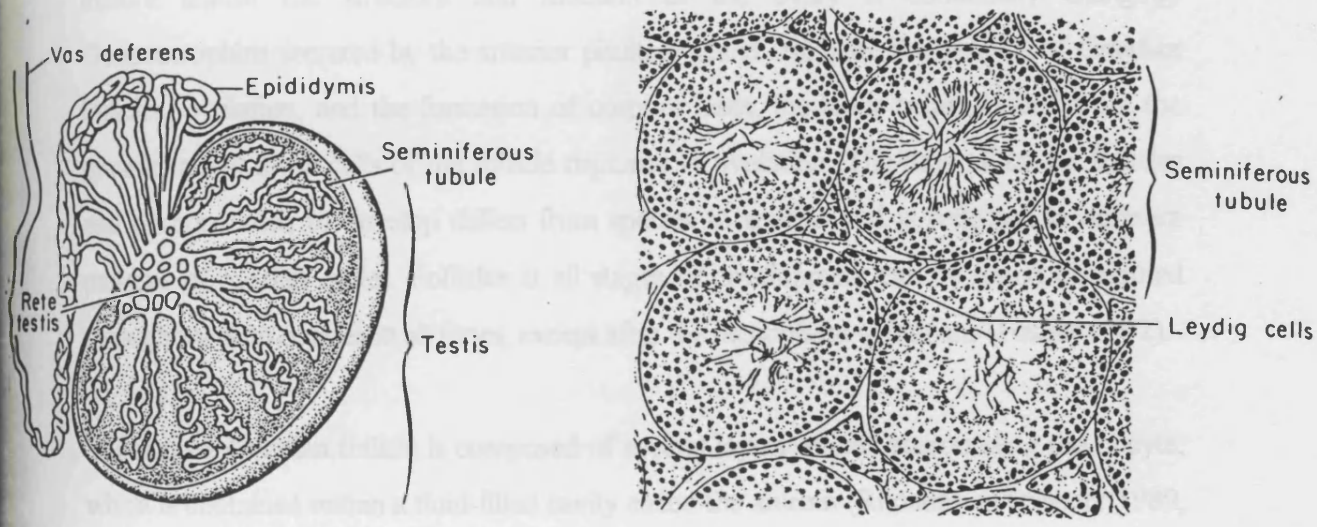


Figure 1.3: **Diagram showing the gross and microscopic anatomy of the human testes.** *This diagram was taken from Hadley 1992.*

1.21: The structure and function of the ovaries

Like the testis, the ovary can also be divided into different tissue components: the follicles, corpora lutea and interstitial/stromal tissue. There is a clear distinction between the developmental programme of gametes in the ovary and the testes. In the ovary, oogenesis is complete before birth, whereas in the testis there is a continuous proliferation of germ cells and the production of spermatozoa throughout adult life. The endocrine function of the ovary ensures the regular production of healthy oocytes at a time when they will have a maximum chance of being fertilised (Rhoades and Pflanzer 1989, Hadley 1992). In the mature animal the structure and function of the ovary is continually changing. Gonadotrophins secreted by the anterior pituitary gland stimulate the growth of Graafian follicles, ovulation, and the formation of corpora lutea, the latter being formed from the granulosa and theca cells of the follicle ruptured at ovulation. The time taken for follicles and corpora lutea to develop differs from species to species and is reflected in different patterns of ovarian cycles. Follicles at all stages of development can be found distributed throughout both ovaries at all times, except after the menopause in humans (Hadley 1992).

The mature Graafian follicle is composed of several layers of cells surrounding the oocyte, which is contained within a fluid-filled cavity called the antrum (Rhoades an Pflanzer 1989, Hadley 1992). The outermost layers, the theca externa and theca interna, are formed from the adjacent stromal cells. They are supplied by a rich network of capillaries and separated by a basement membrane from the avascular granulosa cells which line the follicular cavity (Hadley 1992). The granulosa cells are comparable to the Sertoli cells of the testis and are important in maintaining the very specialised conditions within the antral cavity which permit development of the oocyte (Hadley 1992). The population of granulosa cells is probably not homogeneous, since those lining the basement membrane (mural granulosa cells) and those surrounding the oocyte (the cumulus oophorus) serve different functions (Richards 1994). For example, there are microtubular connections between the cumulus and oocyte which are probably important for transferring nutrients and hormonal signals to the oocyte (Salustri *et al.* 1992).

The effects of gonadotrophins on follicular growth, ovulation, and luteinisation have been shown to be associated with differences in the numbers of luteinising hormone (LH) and

follicle stimulating hormone (FSH) receptors. Iodinated LH binding to the ovarian theca cells increases during follicular development, especially in the preovulatory follicles (Richards 1994). The endogenous LH surge and administration of an ovulatory dose of LH or human chorionic gonadotrophin (hCG) causes a down regulation in the LH receptor content of ovulatory follicles. During luteinisation and corpus luteum formation, there is resynthesis of LH receptors (Richards 1994). In the corpus luteum, the binding of human LH increases from the early luteal phase to the midluteal phase and decreases towards the late luteal phase (Misrahi *et al.* 1993).

LH has several distinct actions on the ovary. The most important action of LH however, is to increase the synthesis of steroids by those cell types in the ovary which have receptors for LH, i.e. the stroma, theca interna and granulosa cells of pre-ovulatory follicles and later the corpus luteum (Richards 1994).

1.3: Testicular steroidogenesis

Almost all testicular androgens are synthesised and secreted by the Leydig cells. In addition to androgens, the testis also produces small amounts of oestrogens (Weinstein *et al.* 1974), the main site of synthesis of which, in the mature pig (Raeside and Lobb 1984) and human testis (Payne *et al.* 1976), is the Leydig cells. The main substrate for steroid synthesis in Leydig cells, as in all steroidogenic tissue, is cholesterol. In order for steroidogenesis to begin, cholesterol has to be delivered to the outer membrane of the mitochondria. There are three potential sources of cholesterol for the Leydig cell. Firstly, cholesterol which has been stored in Leydig cells in the form of lipid droplets can be utilised during androgen synthesis (Stocco 1996). Alternatively, cholesterol can be synthesised *de novo* from acetate or lastly, it can be brought into the cell from plasma lipoproteins (Saez 1994). In the mouse Leydig tumour (MA10) cell the majority of the cholesterol for steroid synthesis probably originates from lipid droplet stores (Stocco 1996), but it has also been reported that the plasma membrane is an important source of cholesterol (Freeman 1989).

The main immediate effect of steroidogenic hormones such as LH and FSH binding to their specific receptors, is to stimulate the delivery and transfer of cholesterol into the mitochondria. This is achieved by increasing the amount of cholesterol in the cytosol and by

enhancing the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where enzymes involved in steroidogenesis are located. This intramitochondrial transfer is thought to be the rate-limiting step in steroidogenesis and protein synthesis inhibitor studies have indicated that this process (Stocco 1996) is mediated by one or more newly synthesised proteins. The identity of this labile protein has been the subject of many studies over recent years and several candidates have been put forward.

1.31: Potential candidates for the labile protein involved in the intramitochondrial transfer of cholesterol

Many putative 'labile proteins' have been identified. Studies by Pedersen and Brownie, resulted in the isolation of a steroidogenic activator protein (SAP), from rat adrenal cortex (1983) and rat Leydig cells (1987). This protein is a 30 amino acid peptide and demonstrates a large homology with the C terminus of the minor heat shock protein, glucose regulatory protein 78. As a result, it has been suggested that SAP may arise from the cotranslational proteolysis of this heat shock protein when it is transferred from the ribosome into the lumen of the endoplasmic reticulum.

The role of a 13kDa protein, known as the sterol carrier protein 2 (SCP2), has also been studied as a potential candidate for the labile protein. SCP2 was first isolated from liver (Vahouny *et al.* 1985), and has been demonstrated to enhance the transfer of cholesterol from lipid droplets to mitochondria in adrenal cells. It has also been shown to weakly stimulate steroidogenesis in adrenal mitochondria.

Another candidate for intramitochondrial transfer is the endogenous peptide ligand known as diazepam binding inhibitor (DBI). DBI is present in many tissues, particularly in the adrenal cortex, Sertoli cells, Leydig cells and interstitial fluid (Rheaume *et al.* 1990, Garnier *et al.* 1993). DBI recognises the GABA (γ-aminobutyric acid)-regulated chloride channel, but has also been shown to recognise receptors located on the mitochondria of testis, adrenal and glial cells (Papadopoulos *et al.* 1992, Garnier *et al.* 1993). These receptors have subsequently been termed mitochondrial DBI receptors (MDRs). When benzodiazepine agonists have been added to these cells, and also to mitochondria isolated

from these cells, they have been found to stimulate steroidogenesis (Papadopoulos *et al.* 1990, Papadopoulos *et al.* 1992). Conversely, a DBI antagonist was shown to inhibit hormone-stimulated steroidogenesis in both adrenal Y-1 and MA10 cells (Papadopoulos *et al.* 1991).

Steroidogenesis-inducing protein (SIP) is another protein potentially involved with the regulation of steroidogenesis. This protein was first isolated from human ovarian follicular fluid and was found to stimulate Leydig, ovarian and adrenal cells (Stocco 1996). SIP is an interesting protein as it has been demonstrated to stimulate steroidogenesis in MA10 Leydig tumour cells via a second messenger pathway independent of cAMP (Stocco and Khan 1992). Whilst Leydig cell signal transduction is discussed further in sections ***1.5*** and ***1.51***, studies such as this suggests the existence of more than one signal transduction pathway in steroidogenic cells. The exact mechanism of how SIP participates in steroidogenesis is still unclear and is the subject of ongoing research (Stocco 1996).

The final candidate for the labile protein to be discussed here is the 'StAR' protein. In recent years this protein has been largely studied by Stocco and co-workers in MA10 cells. StAR is a member of a family of cyclohexamide sensitive mitochondrial proteins, which are approximately 30kDa in size and are found in many steroidogenic tissues (Stocco and Clark 1996). In a study by Clark *et al.* (1994), a full length cDNA clone of the StAR protein was used to stably transfect MA10 cell lines. The results from this work demonstrated that the transfected cells possessed a significantly increased level of steroid production. In addition, it was observed that non-steroidogenic monkey COS1 kidney cells could undertake steroidogenesis if transfected with the cloned protein and also the mitochondrial enzymes involved in converting cholesterol to pregnenolone. As a result of these experiments, the protein was named the steroidogenic acute regulatory (StAR) protein.

Based on the results of many studies, a model for the mechanism whereby StAR functions has been proposed (Stocco and Clark 1996). It suggests that on Leydig cell stimulation by LH, a precursor protein to StAR is rapidly synthesised in the cytoplasm and transported to the outer mitochondrial membrane. Here it interacts with a specific mitochondrial receptor and becomes inserted into the membrane to undergo two cleavage reactions which result in

the production of the mature 30kDa StAR protein. The insertion of the precursor protein into the membrane results in the formation of contact sites between the inner and outer mitochondrial membranes. At this point, cholesterol is able to transfer from the outer to the inner mitochondrial membrane. The mature 30kDa StAR protein, produced by the cleavage of the precursor protein, can no longer function in the intramitochondrial transfer of cholesterol and the whole process begins again. Thus, it is hypothesised that the processing of precursor StAR to mature StAR results in the transfer of cholesterol to the inner mitochondrial membrane, the site of the mitochondrial enzymes involved in converting cholesterol to pregnenolone.

1.32: The steroidogenic pathways of testosterone production

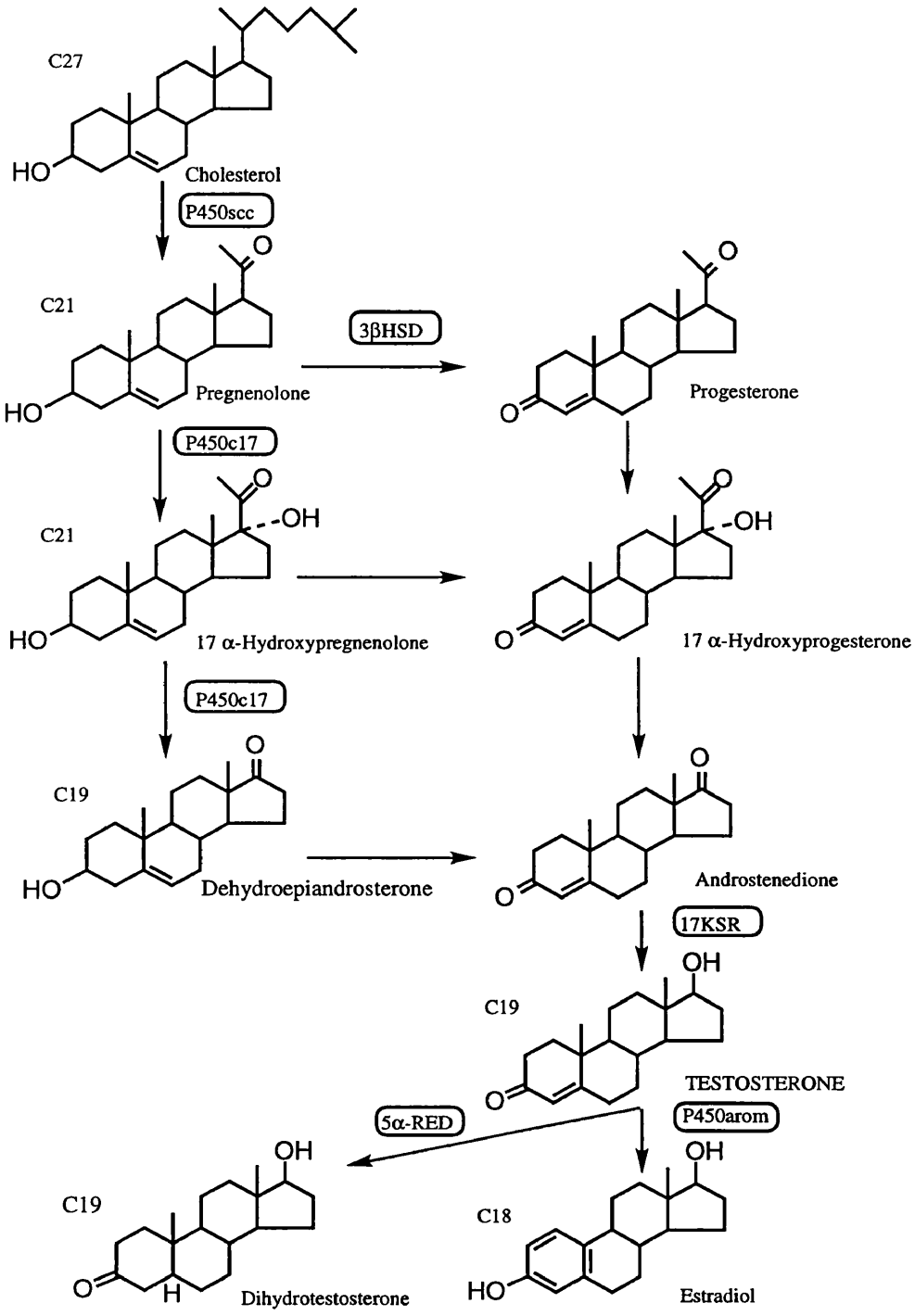
Pregnenolone is the precursor to all steroid hormones. After cholesterol has entered the mitochondrial membrane, the first reaction to occur is the side chain cleavage of cholesterol resulting in its conversion to pregnenolone. This conversion requires the enzyme cytochrome P-450 side-chain cleavage ($P\text{-}450_{scc}$), and two electron carriers, adrenoferrodoxin and adrenoferrodoxin reductase. These electron carriers convey electrons from reduced NADPH to $P\text{-}450_{scc}$ and molecular oxygen, resulting in the sequential hydroxylation of carbon 22 and 20 of cholesterol, to yield the intermediates 22R-hydroxycholesterol and 20α, 22R-dihydroxycholesterol (Dixon *et al.* 1970, Burstein and Gut 1976). Cleavage of the C22-C20 bond results in the conversion of hydroxylated cholesterol to pregnenolone and isocaproaldehyde (later oxidised to isocaproic acid). The pregnenolone that results from these reactions is rapidly expelled from the mitochondria as its hydroxyl group produces repulsive forces at the hydrophobic active site of $P\text{-}450_{scc}$. The mechanisms by which pregnenolone leaves the mitochondria are unknown, but it is thought to be a rapid process since the level of pregnenolone in the mitochondria is low. A pregnenolone binding protein has been isolated from the cytosol of guinea pig adrenal cortex (Strott 1977). However, the role of this protein is still unknown and it is not found to be present in Leydig cells (Whitnall *et al.* 1990).

After this point, there are two biosynthetic pathways by which testosterone can be synthesised, termed the Δ^5 pathway and the Δ^4 pathway respectively. Which pathway is used is likely to be species- and age-dependent (Payne and Shaughnessy 1996). The Δ^4

pathway is preferentially used in the rat, whereas in human, pig, rabbit and dog testis, the Δ^5 pathway appears to be the most significant (Yanaihara and Troen 1972, Weuesten *et al.* 1987, Payne and Shaughnessy 1996).

The Δ^5 pathway proceeds as follows; pregnenolone is converted to progesterone by the enzyme 3ß-hydroxysteroid dehydrogenase/3-oxosteroid-4, 5-isomerase (3ß-HSD). There are two types of 3ß-HSD expressed in human, but it is type II that is the form predominantly expressed in the human testis (Payne and Shaughnessy 1996). The next reaction is common to both Δ pathways. Either progesterone or pregnenolone must be converted from steroids which consist of 21 carbon atoms, into steroids which consist of only 19 carbon atoms. This is acheived in two stages and is carried out by a single enzyme called cytochrome P-450 17α-hydroxylase ($P\text{-}450_{c17}$). Firstly, carbon 17 of either progesterone or pregnenolone is hydroxylated and then secondly, the C17-C20 bond is cleaved. These reactions result in the conversion of progesterone to androstenedione via 17-hydroxyprogesterone (17-OH progesterone) and pregnenolone to dihydroepiandrosterone via 17-hydroxypregnenolone (17-OH pregnenolone). The final stages of testosterone production are carried out by the enzyme type 3 17ß-hydroxysteroid dehydrogenase (17ß-HSD). This enzyme catalyses the conversion of androstenedione to testosterone. The conversion of androstenedione and testosterone into the oestrogens, oestrone and oestradiol-17ß respectively, is catalysed by a cytochrome P-450 enzyme called aromatase (P-450aro) (Saez 1994, Payne and Shaughnessy 1996).

Δ^5–3β–HYDROXYSTEROIDS Δ^4–3–KETOSTEROIDS



P450scc, cytochrome P450 cholesterol side chain cleavage; P450c17, cytochrome P450 17 α-hydroxylase/C_{17-20}lyase; 3βHSD, 3β–hydroxysteroid dehydrogenase/ Δ^5-Δ^4 isomerase; 17 KSR, 17 ketosteroid reductase (17β-hydroxysteroid dehydrogenase); 5α-RED, 5α-reductase; P450 arom, cytochrome P450 aromatase.

***Figure 1.4*: The Δ^4 and Δ^5 steroidogenic pathways found in Leydig cells.**

This diagram was reproduced from Payne and Shaughnessy (1996).

1.33: Mouse Leydig tumour (MA10) cells

MA10 cells are a clonal strain of mouse tumour Leydig cell adapted to culture in the laboratory of Dr. M. Ascoli (Ascoli 1981). These cells were derived from a transplantable tumour that originated spontaneously in C57B1/6 mouse. MA10 cells contain 10000-20000 LH/CG receptors per cell which show a high affinity for LH/CG (K_D= 10^{-9}-10^{-10}M). Like mouse testis Leydig cells, MA10 cells have the capacity to convert cholesterol into steroid hormones and respond to LH/CG with increased steroid biosynthesis. However, a comparison of the steroids produced by freshly isolated mouse Leydig cells and MA10 cells reveals some differences in the steroidogenic pathway. The most obvious of these are an increase in the ability of the cultured cells to synthesise 20α-dihydroprogesterone and a decrease in the activity of $P450_{C17}$ enzyme in MA10 cells. This is the enzyme which is involved in the conversion of progesterone to androstenedione and thus, the major steroid produced by MA10 cells is progesterone rather than testosterone (Ascoli 1981). Steroidogenic cholesterol in MA10 cells is derived from the plasma membrane (Freeman 1989) and from intracellular stores of lipid droplets (Stocco 1996).

The MA10 cell system is ideally suited to study gonadotrophin actions and regulation of the expression of differentiated functions of Leydig cells. The advantages of using these cells are twofold. Firstly, tumour cells are generally easier to grow in culture than normal cells and secondly, the retention of differentiated function could be easily detected by measuring hormone binding and/or steroid production. However, it must be noted that these cells are of neoplastic origin and may therefore sustain molecular features that are different from those found in normal Leydig cells. In addition it is known that the continuous passaging of cells such as these can result in karyotype changes leading to alterations in cellular activity.

1.4: The structure and function of the luteinising hormone/chorionic gonadotrophin receptor (LH/CG receptor).

The LH/CG receptor is a member of the G-protein coupled receptor family (GPCR). GPCRs are integral membrane proteins whose amino acid sequences are characterised by seven hydrophobic, alpha-helical domains, which have been demonstrated to span the cellular membrane (Baldwin 1993). Receptors from this family have been found in a wide range of organisms and they are believed to be involved in the transmission of signals across

membranes. The receptors bind a signalling molecule on the extracellular side of the membrane and then, following activation, bind and interact with a heterotrimeric guanine nucleotide-binding protein (G-protein) on the intracellular side, which results in a cascade of second messenger signalling [see section ***1.5***].

In recent years, the LH/CG receptor gene has been cloned and sequenced from several species. The first LH/CG receptor gene to be cloned was that from rat ovaries (McFarland *et al.* 1989) and porcine testis (Loosfelt *et al.* 1989). This was followed by the human ovarian LH/CG receptor (Minegishi *et al.* 1990), the rat testis LH/CG receptor (Tsai-Morris *et al.* 1991) and the mouse ovarian LH/CG receptor (Gudermann *et al.* 1992). Sequence alignment of the LH/CG receptor genes from different species has revealed a large amount of homology. For instance, the human ovarian LH/CG receptor gene has been shown to share, overall, 85% sequence homology with the rat ovarian LH/CG receptor gene and 87% sequence homology with the porcine ovarian LH/CG receptor gene (Minegishi *et al.* 1990). In addition, knowledge of the nucleotide sequence of these genes made it possible for the amino acid sequence to be deduced. From this it was revealed that the LH/CG receptor shares considerable homology with other members of the GPCR family. For example, the transmembrane regions of the LH/CG receptor demonstrate an amino acid homology of up 26% when aligned with the transmembrane regions of rhodopsin-like GPCRs (McFarland *et al.* 1989).

Hydropathy plots from the sequence data of LH/CG receptor clones suggested a possible model for the organisation of the LH/CG receptor protein. The protein model consisted of a large NH_2-terminal part, approximately 333 amino-acids long, that was thought to be the extracellular ligand-binding domain of the receptor (Loosfelt *et al.* 1989, Minegishi *et al.* 1990, McFarland *et al.* 1989). This putative extracellular domain contained six potential, *N*-linked glycosylation sites and was followed by a region of approximately 266 amino-acids that suggested seven possible transmembrane segments (Loosfelt *et al.* 1989). The LH/CG receptor protein model ended in a relatively short cytoplasmic tail (see review Segaloff *et al.* 1990). This putative model was confirmed by using antibodies directed to sequences in the amino-terminal (amino acid residues 194-207) and the carboxyl-terminal sequence (residues 660-674). Using these site-specific antibodies in immunofluorescence studies in

intact cells, the postulated orientation of the receptor was confirmed. Immunofluorescence was detected with the amino-terminal antibody on the surface of intact luteal cells, whereas immunofluorescence was only detected with the carboxyl-terminal antibody when the cells were permeabilised (Rodriguez and Segaloff, 1990).

In spite of the structural and functional similarities with other GPCRs, the LH/CG receptor, in common with the TSHR and FSHR, have several distinctive features unique to glycoprotein hormone receptors. They are approximately twice the size of other cloned GPCRs and possess a large N-terminal extracellular domain (approximately 340 amino acids) that is *N*-glycosylated. This extracellular domain in the LH/CG receptor, which is reported to be responsible for the binding of LH and CG (Keinanen and Rajaniemi 1986), is glycosylated at six sites. Whilst some studies have suggested that these glycosylation sites are essential for hormone binding and/or the correct folding of the LH/CG receptor (Minegishi *et al.* 1989), others have suggested that *N*-linked carbohydrates are not essential for hormone binding (Keinanen 1988, Tapanainen *et al.* 1993, Davis *et al.* 1997). In the LH/CG receptor the N-terminal extracellular domain accounts for approximately half of the polypeptide chain. The most striking feature of this extracellular domain is a 14-fold imperfect repeat sequence of approximately 25 residues (Keutmann 1992). These motifs are termed 'leucine-rich repeats' (LRRs) and form amphipathic helices which may allow the protein to interact with both hydrophobic and hydrophilic surfaces. The results from studies involving charge inversion, site-directed mutagenesis of these repeat regions, has suggested that many of the amino-acids located in the LRRs are involved in hormone binding (Bhowmick *et al.* 1996).

The C-terminal half of the LH/CG receptor includes the seven transmembrane domains and is equivalent in size to the whole protein when compared to other GPCRs such as the adrenergic receptors (Lefkowitz and Caron 1987). In adrenergic receptors, an extended third cytoplasmic loop seems to be required for specific interactions with the G-protein (Kobilka *et al.* 1988, Strader *et al.* 1989, Cheung *et al.* 1991). In addition the N-terminal segment of this loop has been found to be important in G-protein recognition and activation in a number of GPCRs including the muscarinic receptors (Bluml *et al.* 1994). In a study of the dopamine D_2 receptor, Malek *et al.* (1993) demonstrated the existence of two specific

sites in this third cytoplasmic loop which are involved in functional G-protein-mediated coupling to adenylyl cyclase. Thus, it would appear, in light of the evidence from these GPCRs, that the third intracellular region plays an important role in G-protein activation. It would seem likely then, that this region may also be involved in LH/CG receptor signal transduction. It must be noted however, that unlike the $ß_2$ adrenergic receptor ($ß_2$AR), the LH/CG receptor does not have an extended third cytoplasmic loop, nor does it demonstrate significant sequence homology with the G-protein-coupling domain of the $ß_2$AR (McFarland *et al.* 1989, Loosfelt *et al.* 1989). The third cytoplasmic loop and cytoplasmic tail of GPCRs have also been implicated in the uncoupling of receptors from their respective G-proteins (Bouvier *et al.* 1988, Sanchez-Yague *et al.* 1992). The intracellular regions of the LH/CG receptor carboxyl-terminal contains consensus sequences for phosphorylation by cAMP-dependent kinase (PKA) and Ca^{2+}-dependent protein kinase (PKC). Studies of the $ß_2$AR have demonstrated that these regions are phosphorylated after receptor activation and that this phosphorylation is involved in the control of $ß_2$AR activity. In recent years some studies have demonstrated that the LH/CG receptor can be phosphorylated in response to hormone binding (Hipkin *et al.* 1993, Hipkin *et al.* 1995, Wang *et al* 1996, Wang *et al.* 1997) and more recently the identification of residues S^{635}, S^{639}, S^{649} and S^{653} have been implicated as the major loci for phosphorylation (Hipkin *et al.* 1995, Wang *et al.* 1996, Wang *et al.* 1997) [see section ***1.62***].

Many GPCRs contain one or more cysteine residues located in their cytoplasmic tails which are highly conserved within the GPCR family. These cysteine residues are believed to represent the consensus site for palmitoylation (Probst *et al.* 1992). Palmitoylation of cysteine residues has been demonstrated in rhodopsin (Morrison *et al.* 1991, Papac *et al.* 1992, Karnik *et al.* 1993), the $ß_2$AR (O'Dowd *et al.* 1989, Mouillac *et al.* 1992, Moffett *et al.* 1993), the α_{2A}-adrenergic receptor (Kennedy and Limbird 1993, Eason *et al.* 1994) and the LH/CG receptor (Kawate and Menon 1994, Zhu *et al.* 1995, Kawate *et al.* 1996). The palmitate present at this location is expected to insert into the plasma membrane, thus forming a fourth cytoplasmic loop, which may be important for receptor function. Studies where palmitoylation was prevented in the $ß_2$AR resulted in a loss of G-protein activation (O'Dowd *et al.* 1989), whilst similar studies in the α_{2A}-adrenergic (Kennedy and Limbird 1993), the M_2 muscarinic receptors (van Koppen and Nathanson 1991) and rhodopsin

(Karnik *et al.* 1993) did not demonstrate a loss in G-protein activation. However, the removal of palmitate by hydroxylamine was not found to impair the binding of transducin to rhodopsin, but rather to enhance its activation (Morrison *et al.* 1991). The prevention of palmitoylation by mutagenesis in the LH/CG receptor had no effect on the binding affinity of hCG when measured using intact cells or detergent extracts, compared to the wild type receptor. However, the mutations did result in the expression of only 6% of the mutated receptors at the cell surface compared to the wild type receptor, indicating that much of the mutated LH/CG receptor remained trapped intracellularly (Zhu *et al.* 1995). A more recent study by Kawate *et al.* (1997) found that when cells expressing either the wild type LH/CG receptor or the mutated LH/CG receptor to similar levels were stimulated with hCG, the mutated receptors demonstrated an increase in down-regulation [see section ***1.64***] compared to the wild type. Furthermore, the prevention of palmitoylation by mutagenesis showed no effect on the efficiency of the mutated receptors to couple to the G_s-protein (Kawate *et al.* 1997).

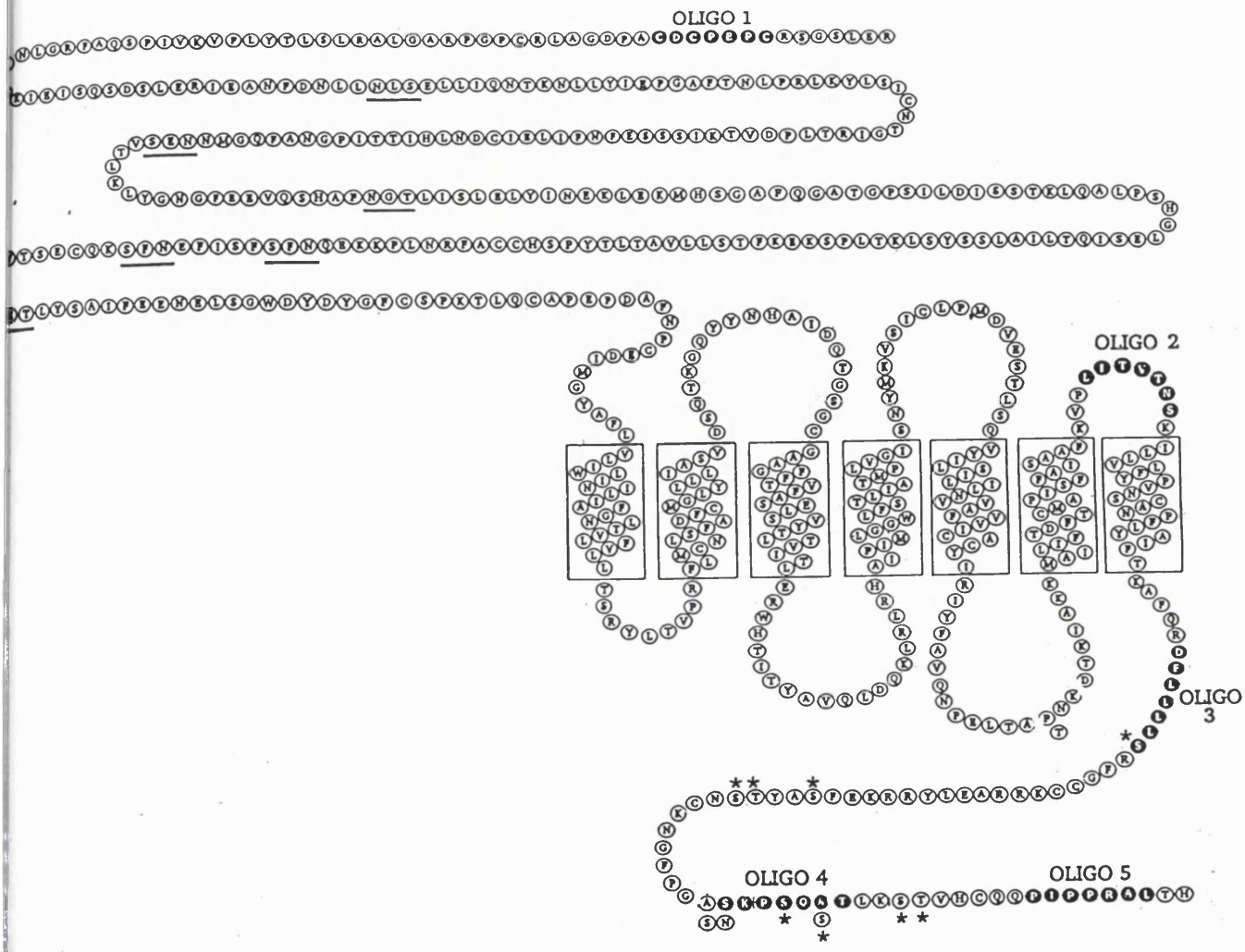


Figure 1.5: **The amino acid diagram of rat LH/CG receptor**. This diagram shows the transmembrane as boxed areas. The underlined parts of the receptor represent glycosylated regions on the extracellular domain. The stars indicate intracellular serine and threonine residues (potential phosphorylation sites). (This diagram also demonstrates the regions to which antisense oligonucleotides were targeted on the LH/CG receptor mRNA [see *Chapter Five*]. These regions are indicated by the darker circles corresponding to "OLIGO 1/2/3/4/5").

In addition to examining the LH/CG receptor DNA for clues to its structure, other studies have focused on the examination of the receptor *per se*. For example, several investigations have been undertaken to isolate the LH/CG receptor from a variety of species and tissues, including rat ovaries (Kusuda and Dufau 1986, Keinanen *et al.* 1987), rat testes (Dufau *et al.* 1975), bovine ovaries (Saxena *et al.* 1986), porcine ovaries (Wimalasena *et al.* 1986) and murine Leydig tumour cells (Kim *et al.* 1987). These studies have yielded conflicting results indicating the receptor to be made up of 1-4 noncovalently or covalently linked homo- or heteropolypeptide chains. The reported molecular size of the LH/CG receptor has varied between 12-300kDa. The current consensus of opinion is that across different species and sexes, the LH/CG receptor occurs primarily as a monomer with a molecular weight of between 70-92kDa (Bahl and Sojar 1990). The smaller molecular components reported are now thought to have been either the products of proteolytic degradation of the LH/CG receptor or contaminating proteins, both of which were likely to be as a result of the collagenase method used for gonadal cell dispersal (Kim *et al.* 1987). The higher molecular weight forms were attributed to LH/CG receptor aggregation.

1.41: The structural organisation of the LH/CG receptor gene

The structure of the rat LH/CG receptor gene has been determined by the isolation and sequencing of LH/CG receptor genomic clones (Tsai-Morris *et al.* 1991, Koo *et al.* 1991). The LH/CG receptor gene exists as a single copy and spans a total of 95.6kb, extending from 23kb downstream of the translation start site, extending 13kb upstream of the stop codon. There appears to be a large 5' untranslated region (5'UTR), (approximately 2kb), downstream from the start site of translation. This region has not been found to contain any consensus motifs such as TATA or CCAAT boxes, that are close enough to the coding region of the gene to be involved in the start site of transcription, (Tsai-Morris *et al.* 1991, Koo *et al.* 1991, Segaloff and Ascoli 1993). Additional sites that may mediate the transcriptional response to hormones and second messenger analogues have been found in the 5'UTR of the LH/CG receptor gene, but it is not known whether any of these are involved in the mediation of transcription (Wang *et al.* 1992).

The coding region of the LH/CG receptor gene consists of 11 exons and 10 introns. The first 10 exons encode the N-terminal, extracellular half of the LH/CG receptor, while exon 11 encodes the C-terminal half, (encompassing the seven transmembrane domains and intracellular tail), of the LH/CG receptor [see *Figure 1.6*]. The overall DNA sequence of some of these exons show remarkable similarities to one another, and exons 3, 6, 7, and 8 show as much as 40% homology in some regions. Since DNA sequence homologies are rare compared to those found in the sequence of amino-acids, this DNA sequence homology between some exons suggests that not only has the LH/CG receptor evolved through a series of exon shuffling and duplications, but that the exons themselves have evolved from a common ancestral piece of DNA (Koo *et al.* 1991). In contrast, exon 10 does not align well with the other exons and is unique in that it encodes three consensus sequences for *N*-glycosylation.

A recent study by Zhang *et al.* (1997) was involved in the cloning and functional expression of the LH/CG receptor from the marmoset monkey testis. Interestingly, sequence analysis of the monkey LH/CG receptor cDNA revealed the absence of exon 10. Whilst exon 10 appeared to be present in the LH/CG receptor gene, it was found to be spliced out of the primary transcript. Functional studies using transfected COS cells demonstrated that this LH/CG receptor displayed similar ligand binding affinities and hCG-stimulated cAMP and IP_3 production as COS cells transfected with the human LH/CG receptor. Thus the workers conclude that exon 10 is apparently not necessary for LH/CG receptor function.

All the introns of the rat LH/CG receptor gene are in the same phase (phase 2) and the sequences of DNA at the exon/intron junctions are virtually identical throughout the gene. These in-phase introns and homologous junction sequences are thought to be preferred for exon insertion and duplication (Patthy 1987). Similarly, the exon/intron structure appears to be highly conserved across species (Koo *et al.* 1991).

1.42: Multiple mRNA transcripts of the LH/CG receptor gene

During the cloning of the porcine (Loosfelt *et al.* 1989) and rat (McFarland *et al.* 1989) LH/CG receptor genes, the existence of variant forms of the LH/CG receptor cDNA was documented. These findings were confirmed in many subsequent reports (Minegishi *et al.*

1990, Wang *et al.* 1991, VuHai-LuuThi *et al.* 1992, Koo *et al.* 1994, Bacich *et al.* 1994, Lu and Menon 1994).

In the porcine LH/CG receptor (Loosfelt *et al.* 1989), all the variant forms had identical N-termini with divergence occurring at the same point (corresponding to amino-acid 316 of the precursor protein). Two of the variants underwent a frameshift after this point of divergence, resulting in cDNAs that lacked the coding sequence for the putative transmembrane and intracellular domains. The relative frequencies of the different variants was estimated by hybridising LH/CG receptor clones from a nonamplified cDNA library with probes specific for each variant. The full-length form was the most frequent (60%), followed by the three variant forms (20%, 15% and 5% respectively). Blot analyses of polyadenylated RNAs obtained from tissue preparations from various organs were then performed. From the testicular and ovarian preparations similar profiles were obtained. The major mRNA species in both tissues was 4.7kb. This was followed by mRNA of 6.7kb which was a major species in the testis but only a minor species in the ovary. Minor species of 5.8kb, 4kb, 2.6kb and 1.4kb were detected in both testes and ovaries. No LH/CG receptor mRNA was detected in tissue from liver, lung or muscle. It would seem from studies of the exon/intron boundaries in the rat LH/CG receptor gene, that two of the three variants could be explained by the alternate splicing of exon 10 with exon 11 (Koo *et al.* 1991).

Whilst multiple forms of mRNA have been documented in other species, it is important to note that there is a heterogeneity regarding the sizes and abundance of those found. For example, in the MA10, the most abundant mRNA species was found to be 1.2kb in size, whilst in the rat ovary the most abundant form was 6.7kb in size (Wang *et al.* 1991). Despite this heterogeneity of mRNAs across species, all the gonadal cells have been found to express a 70-92 kDa protein as the most abundant cell surface LH/CG receptor protein.

The open reading frame of the LH/CG receptor gene is approximately 2.1kb in size. Many of the mRNA transcripts reported have coded for proteins much larger or smaller than the mRNA predicted from the full length cDNA. The variant forms of mRNA are thought to arise from alternative splicing of the precursor RNA. Evidence for this hypothesis has come from the results of PCRs, where transcripts both larger and smaller in size than the 2.1kb

mRNA predicted from the full length cDNA, have been amplified (Sokka *et al.* 1992). In addition to alternative splicing, the larger transcripts may arise through different sites of polyadenylation, or indeed through different lengths of polyadenylation. Alternatively, larger transcripts could be the result of different transcriptional start sites being used by the transcription complex (Segaloff and Ascoli 1993).

The functional significance of these multiple transcripts has not yet been determined. Various mRNA transcripts are expressed in gonadal tissue during fetal development (Sokka *et al.* 1992, Zhang *et al.* 1994), and after ethane dimethane sulphonate (EDS) treatment in adult rats (Tena-Sempere *et al.* 1994). It has also yet to be established whether the smaller mRNA transcripts (< 2.1kb) are translated and expressed. If this were the case, proteins lacking the transmembrane regions might be expected to be secreted and act as soluble forms of LH/CG receptor. There is however, little evidence to suggest that this might occur, since cells transfected with truncated cDNAs encoding for such proteins, (Xie *et al.* 1990, Moyle *et al.* 1991, Braun *et al.* 1991, Ji and Ji 1991, Zhu *et al.* 1995), produce proteins that bind LH but that remain trapped inside the cell as opposed to being secreted.

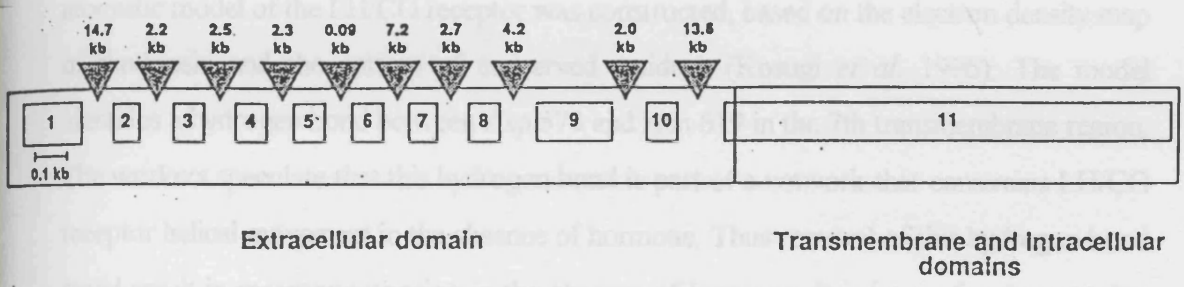


Figure 1.6: Diagram of the organisation of the LH/CG receptor gene. Exons and introns are represented by the open rectangles and shaded triangles respectively. The size of the introns is indicated on the top of each triangle. The exons are drawn to scale indicated under exon 1. The first 10 exons encode for the majority of the extracellular domain. Exon 11 encodes for a small portion of the extracellular domain, all the transmembrane regions, and the entire C-terminal cytoplasmic tail. *This diagram was taken from Segaloff and Ascoli 1993.*

1.43: Mutations of the LH/CG receptor gene

Sequence analysis has been fundamental in locating the cause of many inherited genetic disorders. Several inherited human diseases have recently been attributed to mutations that alter the function of GPCRs. Shenker *et al.* (1993) reported the identification of a single base change (asparagine to glycine at position 578 in the 6th transmembrane domain) in the LH/CG receptor gene from males affected with a disorder called Familial Male Precocious Puberty (FMPP). This is a disease that is inherited in an autosomal dominant, male-limited pattern and is characterised by males generally exhibiting signs of puberty by the age of 4. The base change is thought to render the LH/CG receptor constitutively active, resulting in autonomous, LH-independent Leydig cell activity. In agreement with this theory, an atomistic model of the LH/CG receptor was constructed, based on the electron density map of rhodopsin and the pattern of conserved residues (Kosugi *et al.* 1996). The model identifies a hydrogen bond between Asp 578 and Asn 619 in the 7th transmembrane region. The workers speculate that this hydrogen bond is part of a network that constrains LH/CG receptor helical movement in the absence of hormone. Thus removal of this hydrogen bond could result in receptor activation in the absence of hormone. It is interesting to note that mutations in the 3rd (Parma *et al.* 1993) and 6th (Kosugi *et al.* 1994) transmembrane domain of the TSHR have also been documented. These mutations are thought to render the TSHR constitutively active resulting in hyperfunctioning thyroid adenomas.

A nonsense mutation resulting in the production of a stop codon (at position 545 in the 5th transmembrane domain) has been documented in two related cases of Leydig Cell Hypoplasia (LCH) (Laue *et al.* 1995). In contrast to FMPP, LCH is a form of male pseudohermaphroditism, in which affected 46 XY males have a female phenotype associated with low basal and hCG-stimulated levels of serum testosterone and elevated levels of LH. The disorder results from failure of the fetal Leydig cells to differentiate and the workers postulated that such a mutation in the LH/CG receptor gene might result in loss of function of the human LH/CG receptor. At first sight it would seem that the transmembrane regions of GPCRs are especially sensitive to mutations, which if they occur, often result in phenotypic changes. More recent work by Laue *et al.* (1996a) however, has documented the deletion of exon 8 of the LH/CG receptor gene in a case of LCH. Exon 8 encodes for an extracellular region which forms part of a high-affinity hormone binding site. The deletion of exon 8

renders the LH/CG receptor inactive. Similarly, in a case of sporadic male precocious puberty (SMPP) no mutations have been identified within the LH/CG receptor gene (Laue *et al.* 1996b). This lead the researchers to conclude that either the constitutively activating mutations in this case were not constrained to the coding sequence of the LH/CG receptor gene, or that the SMPP was caused by a mutation in another gene.

1.5: The LH/CG receptor and activation of the adenylyl cyclase second messenger pathway

As mentioned previously, the LH/CG receptor is a member of the G-protein coupled receptor family. Members of this family, when activated by hormone binding, are assumed to undergo a conformational change that allows them to interact with a guanine nucleotide-binding protein (G-protein). The G-protein in turn interacts with the membrane protein adenylyl cyclase and other enzymes and ion channels to modulate the activity of these effectors which are responsible for the generation of second messengers.

Molecular cloning techniques have revealed that G-proteins are a complex and diverse set of highly homologous proteins. They consist of three heterologous subunits termed $_{\alpha}$, $_{\beta}$ and $_{\gamma}$. Currently there are at least 16 known $_{\alpha}$-subunit genes, 4 $_{\beta}$-subunit genes and 5 $_{\gamma}$-subunit genes (Simon *et al.* 1991, Birnbaumer 1992). It is the $_{\alpha}$-subunit that confers the distinction among different G-proteins (Birnbaumer 1987). There are three main types of $_{\alpha}$-subunit; $G_{\alpha s}$ (stimulatory to adenylyl cyclase), $G_{\alpha i}$ (inhibitory to adenylyl cyclase) and $G_{\alpha o}$ (other). The hormone activated LH/CG receptor presumably alters the conformation of the intracellular loops of the receptor and promotes interaction with G-proteins, including G_s. The inactive G-protein is bound to GDP. However, when activated by receptor interaction, the G-protein releases GDP in favour of binding GTP. This results in the dissociation of the G-protein to yield $G_{\alpha s}$-GTP and $G_{\beta\gamma}$. The $G_{\alpha s}$-GTP subunit interacts with the integral membrane protein adenylyl cyclase which results in the activation of the enzyme, catalysing the synthesis of cAMP from ATP. The formation of cAMP is fundamental to cell signalling as it initiates a cascade of protein activation solely by the stimulation of cAMP-dependent kinase (PKA). This activated kinase then phosphorylates other proteins and so signal transduction proceeds. The $G_{\alpha s}$-subunit has an intrinsic GTPase activity which allows it to terminate its activation of adenylyl cyclase by hydrolysing the bound GTP to GDP. This

results in the reassociation of $G_{\alpha s}$ with the $_{\beta\gamma}$-subunit and the reformation of the inactive heterotrimer (Simon *et al.* 1991, Birnbaumer 1992) [see *Figure 1.7*]. Research in the last few years has suggested that the $_{\beta\gamma}$-subunit may also play a role in the adenylyl cyclase activation. In a review by Tang and Gilman (1992), the $_{\beta\gamma}$-subunit has been found to regulate certain subtypes of adenylyl cyclase.

It has been known for several years that GTP is necessary for hormonal activation of adenylyl cyclase. This fact had previously been largely overlooked because the ATP used in assays of the enzyme often contained sufficient GTP as a contaminant to fulfill this requirement (Morgan 1989). However, it is now clear that the requirement for GTP reflects the presence of a transducer protein which carries a signal between the activated hormone receptor and the catalytic subunit of the enzyme. Thus, unlike many reactions which require ATP, the role of GTP is *not* one in which the hydrolysis of the terminal phosphate is required as part of the activation mechanism. Since non-hydrolysable analogues of GTP inhibit the process of desensitisation, it would seem that hydrolysis of the γ-phosphate of GTP is involved in terminating the stimulatory process (Morgan 1989).

A study by Ekstrom and Hunzicker-Dunn (1989b), demonstrated that the LH-sensitive adenylyl cyclase has a specific requirement of GTP, in place of ATP, for hormone-stimulated desensitisation. Previous work by this group, using pig ovarian follicular membranes, had demonstrated that GTP was necessary for the cell-free desensitisation of LH-stimulated adenylyl cyclase to take place (Ekstrom and Hunzicker-Dunn 1989a). In this study it was reported that GTP alone could fulfill the nucleotide requirement for LH-stimulated desensitisation. Whilst other nucleotides could support desensitisation they were reported to be 100-1000 times less potent than GTP. A non-hydrolysable analogue of GTP, guanyl-5'-yl imidodiphosphate [GMP-P(NH)P], was found to inhibit desensitisation that was supported by the other deoxynucleotide triphosphates, whilst the equivalent non-hydrolysable analogue of ATP, adenyl-5'-yl imidodiphosphate [AMP-P(NH)P], was not found to be an inhibitor of desensitisation. These findings were in agreement with earlier studies by Ezra and Salomon (1980 and 1981).

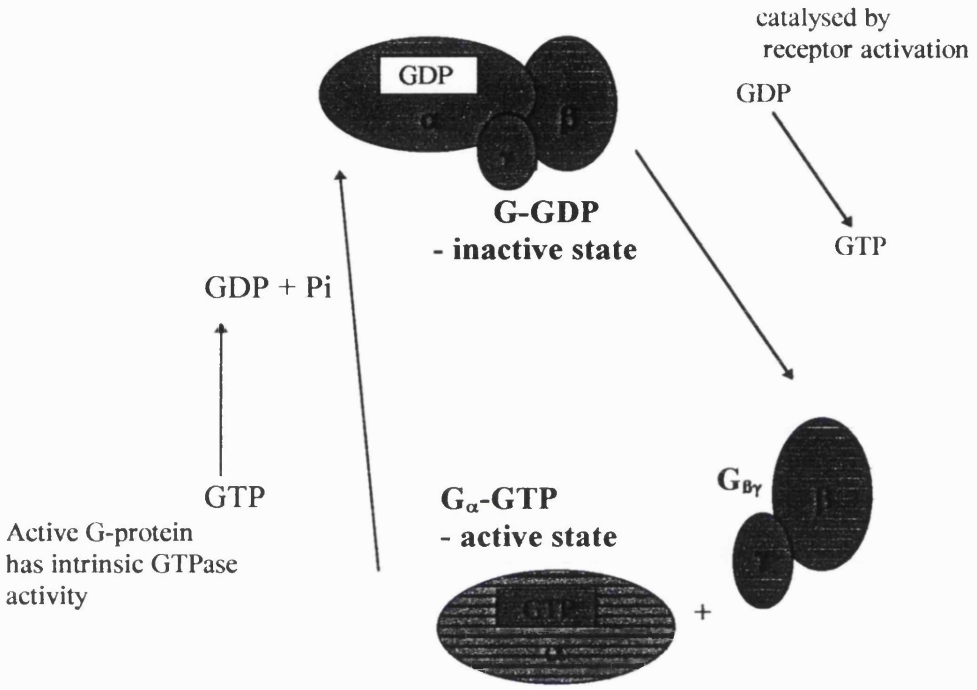


Figure 1.7: **G-protein activation.** G-proteins interconvert between an inactive GDP form and an active GTP form. The exchange of bound GDP for GTP is catalysed by receptor activation. $G_{\alpha s}$-GTP activates adenylyl cyclase leading to the production of cAMP, but other G-proteins activate different effectors. Hydrolysis of bound GTP reverts the active G-protein to the inactive state.

The adenylyl cyclase system of gonadal cells like those of other cell types (see review Birnbaumer 1990), can also be negatively regulated by the inhibitory GTP binding protein, G_i (Platts *et al.* 1988). However, the mechanism by which G_i-subunits lower intracellular cAMP levels is still unclear (Simon *et al.* 1991) G_i is present in Leydig cells and is therefore involved in the negative modulation of cAMP and steroidogenesis (Platts *et al.* 1988).

The study of G-proteins has been considerably aided by the use of bacterial toxins such as cholera toxin and pertussis toxin and also the naturally occuring diterpene, forskolin. The bacterial toxins catalyse the covalent modification (ADP-ribosylation) of particular G_α-subunits. Cholera toxin modifies an arginine residue in $G_{\alpha s}$ which results in the inhibition of the intrinsic GTPase activity. This leads to the constitutive activation of the $G_{\alpha s}$-subunit and therefore the continuous activation of adenylyl cyclase and cAMP production (see review Moss and Vaughan 1994). Hence adenylyl cyclase is persistently activated in the absence of hormone. Cholera toxin has been demonstrated to increase cAMP production and steroidogenesis in intact Leydig cells (Cooke *et al.* 1977, Dufau *et al.* 1978). Pertussis toxin ADP-ribosylates $G_{\alpha i}$ and $G_{\alpha o}$ on a cysteine residue near the carboxy-terminus which results in the uncoupling of the G-protein from the receptor (Simon *et al.* 1991). Forskolin was first described in 1981 as a cardioactive drug that reversibly stimulated adenylyl cyclase *in vitro* and *in vivo*, resulting in increased intracellular cAMP (Seamon *et al.* 1981). Forskolin is known to bind to high-affinity sites associated with complexes of the adenylyl cyclase catalytic subunit and the $G_{\alpha s}$-subunit. Thus, in the absence of hormone activation, adenylyl cyclase can be activated by forskolin (Laurenza *et al.* 1989).

1.51: The LH/CG receptor and activation of other second messenger pathways

cAMP is generally regarded as the major second messenger in LH/CG receptor signal transduction, primarily because it satisfies most of the criteria devised by Sutherland (1972) (see review by Rommerts and Cooke 1988). These criteria were established because it was noted that many hormones interact with receptors to activate adenylyl cyclase, which then generates a physiological response. It was therefore suggested that certain criteria should first be satisfied before concluding that a particular effect of a hormone is mediated through cAMP (Sutherland 1972).

In addition to the cAMP second messenger pathway, a large body of evidence exists that suggests that the LH/CG receptor may be coupled to other pathways which are subsequently involved in mediating the cellular actions of LH and CG. Davis *et al.* (1986 and 1987) reported the involvement of inositol 1, 4, 5 trisphosphate (IP_3) and diacylglycerol (DAG) phosphate in LH/CG receptor signal transduction. IP_3 and DAG are generated by the action of phospholipase C (PLC) on phosphotidylinositol 4, 5 bisphosphate and can act either independently or together, leading to the production or mobilisation of other messengers and modulators. For example, IP_3 can stimulate the release of Ca^{2+} from the sarcoplasmic reticulum and calcisomes. This resulting rise in intracellular Ca^{2+} concentration, coupled with the generation of DAG, results in the synergistic activation of calcium-dependent protein kinase (PKC) and its translocation to the cell membrane. Several G_s-coupled receptors have been found to stimulate PLC and there is evidence to suggest that the LH/CG receptor may also be coupled to PLC, generating the production of IP_3 and DAG and/or calcium mobilisation when activated. This evidence comes from studies such as those by Gudermann and co-workers where the LH/CG receptor cDNA was transfected into 293L cells (Gudermann *et al.* 1992a) and *Xenopus* oocytes (Gudermann *et al.* 1992b). Hormone stimulation of these transfected receptors was found to activate PI hydrolysis in addition to adenylyl cyclase. However, it was found that considerably more hormone (EC_{50} 2400pM human CG) was required to stimulate PI hydrolysis than was required for adenylyl cyclase activation (EC_{50} 50-100pM human CG). Later studies suggested that the ability of G_s-coupled receptors to stimulate PLC was dependent on the levels of receptor expression (Zhu *et al.* 1994). In this work PLC activation was measured by the mobilisation of intracellular calcium. It was demonstrated that 10µg/ml of hCG induced a calcium signal in cells expressing the LH/CG receptor at 4000 sites/cell, 12000 sites/cell and 40000 sites/cell. Among these, the cells expressing the highest receptor density (40000 sites/cell), also had the largest increase in calcium concentration (from a basal level of 50nM to 150nM). To confirm that the increase in intracellular calcium levels was due to the stimulation of PLC by hCG in the LH/CG receptor-expressing cell lines, the workers measured the accumulation of IPs. Increases in IP accumulation over basal were found in all cell lines expressing the LH/CG receptor at 4000 sites/cell and above.

The percentage increase was found to be higher as receptor density increased. At 40000 sites/cell, the accumulation of IPs increased by 2.3 fold over the basal value in response to hCG.

Studies of this nature would appear to indicate that under experimental conditions, the LH/CG receptor can be seen to be coupled to at least two signal transduction pathways involving adenylyl cyclase/cAMP and PLC/IP_3. However, it would appear that this is not necessarily the case for all LH/CG receptors, since PLC activation induced by LH/CG binding cannot be demonstrated in *all* cell lines expressing the LH/CG receptor. For example, a study by Ascoli *et al.* (1989) failed to demonstrate LH/CG-stimulated IP_3 accumulation in MA10 cells.

Therefore, whilst some studies cannot demonstrate LH/CG receptor/PLC coupling, it does appear likely that in certain systems at least, the stimulated LH/CG receptor can activate PLC. Indeed some studies have even located regions of the LH/CG receptor that are important in the coupling to G-proteins that activate PLC (Hirsch *et al.* 1996, Gilchrist *et al.* 1996), whilst others have suggested that the coupling of the LH/CG receptor to both adenylyl cyclase and PLC is via distinct G-proteins, G_s and G_i respectively (Herrlich *et al.* 1996). A report by Gutowski *et al.* (1991) indicated that another distinct subfamily of G_α-subunits, termed $G_{\alpha q}$, was involved in the coupling of hormonal receptors to PLC. In addition, there are six known mammalian isozymes of adenylyl cyclase, each of which appears to differ in their mode of regulation. In a report by Lustig *et al.* (1993), it is suggested that whilst all the forms are stimulated by $G_{\alpha s}$-GTP, they also respond to other regulators. For example, $G_{\alpha q}$ is suggested to activate adenylyl cyclase type II via a PKC-dependent pathway. What is less certain however, is the role, if any, the PLC/IP_3 pathway plays under physiological conditions. It is well established that full steroidogenesis is attained at very low concentrations of cAMP. Therefore even if the LH/CG receptor is coupled to the PLC pathway in Leydig cells, it is unlikely that it plays a role in steroidogenesis (Cooke 1996). In addition, the stimulation of PLC by these receptors is relatively inefficient, requiring considerably more ligand to activate it compared to that required to activate the adenylyl cyclase pathway, and stimulation of PLC also requires a high receptor density.

The density of transfected LH/CG receptors in the studies described, is far higher than is found in testis Leydig cells (approximately 4000/cell) (Cooke 1996). If the effects of LH or hCG on PI hydrolysis is affected by cell density, as is suggested (Zhu *et al.* 1994), it is unlikely that in Leydig cells, which contain relatively low numbers of LH/CG receptors, that ligand-mediated PI hydrolysis would occur. It is possible however, that PI hydrolysis has a role to play in pregnancy and ovarian systems where CG levels are high, and where in luteal membranes from ovulating animals, LH/CG receptors reach a level capable of stimulating PLC (400-700 fmol/mg of membrane protein) (Kirchick and Birnbaumer 1983, Kirchick *et al.* 1983). As is reviewed in Richards (1994), there is evidence that the processes of ovulation and luteinisation involve the activation of both adenylyl cyclase and PLC/IP_3 pathways.

1.6: Desensitisation in GPCRs

Continuous exposure of cells to hormone agonists often results in a rapid loss of receptor responsiveness. This phenomenon is called desensitisation which can be defined as a decreased cellular response to a stimulus of a constant strength. The function of desensitisation is thought to prevent the excessive stimulation of cells by the persistent activation of signal transduction pathways of GPCRs. Thus desensitisation acts as a feedback loop that serves to dampen the signal initiated by receptor activation. Desensitisation processes can be subdivided according to the causative stimulus, namely homologous (agonist-specific) desensitisation or heterologous (agonist-non-specific) desensitisation. Homologous desensitisation refers to the agonist activation of a receptor resulting in the desensitisation of that specific receptor. Heterologous desensitisation on the other hand, refers to the activated receptor causing desensitisation of other types of receptor as well (Lohse *et al.* 1990, Lohse 1993). Desensitisation can also be subdivided according to the time-frame within which desensitisation takes place. For example, 'rapid' desensitisation occurs within minutes and can be exemplified by the processes of receptor/G-protein uncoupling and receptor sequestration. 'Slow' desensitisation takes place over hours or days and involves the loss of membrane receptors. Slow desensitisation is also known as down-regulation (Lohse 1993) [see section ***1.64***].

The molecular basis of desensitisation has been well studied in the β_2AR (Hausdorff *et al.* 1990, Dohlman *et al.* 1991) and rhodopsin (Stryer 1991), and therefore these proteins are often used as model systems by which to study desensitisation processes that may apply to other receptors.

1.61: Mechanisms of desensitisation

Two rapid mechanisms have been observed to correlate with β_2AR desensitisation and have been proposed to be responsible for it. The first to be discussed here, is that of receptor uncoupling whereby the receptors become functionally uncoupled from their effector system.

Receptor uncoupling

In both the β_2AR and the rhodopsin receptor, desensitisation is brought about by the phosphorylation of the receptor by cytoplasmic protein kinases (Hausdorff *et al.* 1989, Lohse *et al.* 1990, Lefkowitz *et al.* 1990). Phosphorylation of the β_2AR by PKA directly uncouples the receptor from the G_s-adenylyl cyclase system, which in turn mediates the activity of the β_2AR specific kinase, termed βARK (rhodopsin kinase in the retinal system) (Lorenz *et al.* 1991). These GPCR-specific kinases (GRKs) recognise the activated conformation of the receptor and phosphorylate the receptors on multiple serine and threonine residues located in the distal portion of the C-terminal cytoplasmic tail. These phosphorylation reactions promote the interaction of the phosphorylated receptor with a second cytosolic protein, termed β-arrestin (Hausdorff *et al.* 1990, Palczewski and Benovic *et al.* 1991), (arrestin in the retinal system (Wilden *et al.* 1986)). Arrestins bind to the phosphorylated forms of the appropriate receptors and appear to then sterically interdict their interaction with their G-proteins, thus leading to functional deactivation of the systems. Most, if not all, of the βARK phosphorylation sites of the β_2AR are located in the C-terminal tail of the molecule and are thought to involve serine and threonine residues. The sequence Arg-Arg-Ser-Ser appears twice in the β_2AR, representing PKA phosphorylation consensus sequences. It has been previously demonstrated in the β_2AR that deletion of the last 48 amino acids from the C-terminal cytoplasmic tail results in a delay in the onset of agonist-induced desensitisation (Bouvier *et al.* 1988).

The second mechanism involves the activated receptor becoming sequestered away from the cell surface and G-proteins into a membrane-associated compartment (Hausdorff *et al.* 1989, Lohse *et al.* 1990, Lefkowitz *et al.* 1990, Hertel *et al.* 1990), as described below.

It is clear from the work just described that there is a distinct, causal link between $ß_2$AR phosphorylation and its desensitisation. Despite the similarity between the $ß_2$AR and other GPCRs however, it is as yet unclear as to whether this model as it stands can be applied to other GPCRs. For example, it is still unclear as to whether the LH/CG receptor undergoes desensitisation as a result of receptor phosphorylation [see section *1.62*].

Receptor sequestration

In addition to the functional uncoupling of $ß_2$ARs from the G-protein, agonists can also cause rapid physical uncoupling, which is effected by translocation of the receptors to intracellular sites such that they can no longer interact with their hydrophilic agonists, or the G-protein (Hausdorff *et al.* 1989, Lohse 1993). This receptor sequestration is initiated within minutes of $ß_2$AR activation and therefore plays an important role in desensitisation. As yet, no general amino acid recognition sequence(s) for internalisation has been identified, although the intracellular loops, and in particular the cytoplasmic tail of several GPCRs including the $ß_2$AR, have been shown to be involved in internalisation (Hertel *et al.* 1990, Rodriguez *et al.* 1992, Nussenveig *et al.* 1993, Findlay *et al.* 1994, Stefan and Blumer 1994). A recent study by Ruiz-Gomez and Mayor (1997), suggests the direct involvement of ßARK in $ß_2$AR internalisation. In this study, 293L cells were stably transfected with epitope tagged ßARK and $ß_2$AR cDNA. Incubation with agonist resulted in an increased rate and extent of $ß_2$AR internalisation when it was coexpressed with ßARK, compared to when it was transfected into cells alone. In addition, double immunofluorescence studies using confocal microscopy demonstrated the extensive colocalisation of ßARK and $ß_2$AR in intracellular vesicles, in response to receptor stimulation.

Like other cell surface receptors, the activated LH/CG receptor undergoes receptor sequestration, resulting in the endocytosis and internalisation of receptor/ligand complex. Thus, the binding of ligand to testicular Leydig cells or to ovarian granulosa or luteal cells results in the intracellular accumulation of the hormone and its subsequent degradation, whilst the receptors are thought to be recycled [see section *1.64*]. Truncation studies using mutant rat LH/CG receptors (Rodriguez *et al.* 1992) and analysis of sequences put forward by other GPCR studies (Cranfield *et al.* 1991), have suggested that the regions important for mediating receptor internalisation are located on the cytoplasmic tail, generally within 30 amino acids from the plasma membrane. In addition, in studies where palmitoylation of the LH/CG receptor has been prevented by mutagenesis (Kawate *et al.* 1997) and where C-terminal truncations have been made for the study of LH/CG receptor phosphorylation (Wang *et al.* 1996, Wang *et al.* 1997), the mutated receptors have demonstrated an increase in ligand-induced sequestration. The results from the latter two studies would indicate that residues between 632-653 of the rat LH/CG receptor are important to the process of LH-stimulated receptor internalisation.

It must be noted however, that not all LH/CG receptors demonstrate internalisation on receptor activation. In a study using 293L cells which had been stably transfected with the murine LH/CG receptor (Gudermann *et al.* 1995), receptor internalisation, as measured by the binding of [^{125}I]-hCG, was not found to play a significant role in the homologous desensitisation of the LH/CG receptor when expressed in these cells.

1.62: The role of the LH/CG receptor C-terminus in desensitisation

As has been described, the cytoplasmic tail of the LH/CG receptor and of other GPCRs is implicated as being important in the two processes which lead to receptor desensitisation.

Studies by Bouvier *et al.* (1988), demonstrated that the deletion of the last 48 amino acids from the C-terminal cytoplasmic tail of the β_2AR, resulted in a delay in the onset of agonist-induced desensitisation. Evidence suggesting that the cytoplasmic tail is involved in the process of LH/CG receptor desensitisation comes from a study

performed by Sanchez-Yague *et al.* (1992). These workers created a number of mutated rat LH/CG receptors using the PCR to splice out or alter regions of the C-terminal tail. The mutated LH/CG receptors were transfected into 293 human embryonic kidney cells (293 HEK), and subjected to desensitising treatments of hCG. Adenylyl cyclase assays were performed to establish what effects the C-terminal deletions had had on the process of desensitisation. A mutant receptor that lacked the last 43 amino-acids (rLH/CG receptor-t631), was found to have enhanced intrinsic activity and did not undergo hCG-induced desensitisation. Since this mutant receptor lacked several of the potential phosphorylation sites, these results suggested that phosphorylation of certain residues within the C-terminal tail was linked to LH/CG receptor desensitisation.

A similar study published after the above however, produced conflicting evidence (Zhu *et al.* 1993). In this work a murine LH/CG receptor was mutated so as to lack the last 46 amino acids of the C-terminal tail (mLH/CG receptor-ct628). This truncation removed the same serine and threonine residues as were lacking in rLH/CG receptor-t631, although an additional tyrosine residue was also removed. In this work, clonal cell lines were established that expressed either the wild-type LH/CG receptor (mLH/CG receptor wt) or mLH/CG receptor-ct628. When LH or hCG induced adenylyl cyclase activity was assayed, both mLH/CG receptor wt and mLH/CG receptor-ct628 were found to respond to stimulation by undergoing desensitisation to a very similar degree. These results create a conflict with those previously reported (Sanchez-Yague *et al.* 1992) as they suggest that the LH/CG receptor desensitisation response does not require the participation of the cytoplasmic tail, including the 9 serine, threonine and tyrosine potential phosphorylation sites. The authors of this study suggest that a possible explanation for these discrepant results could be due to the formation of a tyrosinyl-arginyl-arginine terminus in the rLH/CG receptor-t631 mutant, which due to its bulkiness and positive charge density, interfered in a non-specific manner with the desensitisation process.

Later work following on from that of Sanchez-Yague *et al.* (1992) demonstrated that hCG and 4ß-phorbol 12-myristate 13-acetate- (4ß-PMA) induced phosphorylation of

the LH/CG receptor mapped to serines S^{635}, S^{639}, S^{645} and S^{652} in the cytoplasmic tail (Hipkin *et al.* 1995). In addition, the progressive truncations of the cytoplasmic tail which sequentially removed these residues was found to prevent hCG- or 4ß-PMA induced phosphorylation, impair hCG- or 4ß-PMA-induced desensitisation and enhance hCG-induced down-regulation [see section ***1.64***] (Wang *et al.* 1996).

In a recent report by Wang *et al.* (1997), stably transfected cell lines were made that expressed either the wild-type rat LH/CG receptor (rLHR) or a full-length rLHR in which the putative major loci for phosphorylation, S^{635}, T^{638}, S^{639}, S^{649} and S^{653} (Hipkin *et al.* 1995, Wang *et al.* 1996), had been simultaneously mutated to alanine residues. The mutated receptor was found to bind hCG with high affinity, leading to cAMP and inositol phosphate accumulation comparable in magnitude to those elicited by the wild-type receptor. Whilst cells expressing the wild-type receptor responded to stimulation by hCG or 4ß-PMA with an increase in rLHR phosphorylation, the phosphorylation of the mutated receptor was severely blunted, but not abolished completely. Likewise, after preincubation with 4ß-PMA, 4ß-PMA-induced desensitisation was evident in cells expressing the wild-type rLHR when they were rechallenged with hCG. In contrast, desensitisation was significantly reduced in cells expressing the truncated receptor. After preincubation with hCG, hCG-induced desensitisation was evident in cells expressing the wild-type rLHR when they were rechallenged with hCG. However, desensitisation was delayed, although not abolished, in cells expressing the mutated receptor. These results suggest that phosphorylation of rLHR is necessary, but not sufficient, for uncoupling of the receptor from adenylyl cyclase. In addition, the internalisation of [^{125}I]-hCG was found to be slower in cells expressing the mutated receptor than in cells expressing the wild type [see section ***1.64***].

1.63: LH/CG receptor phosphorylation and the roles of PKA and PKC

It is well established that the catecholamine GPCRs such as the αARs, ßARs and m_2-muscarinic receptors, become phosphorylated when exposed to their respective agonists. In addition, it is clear from the studies described earlier that there is a distinct, causal link between $ß_2$AR phosphorylation and its desensitisation, and that this

phosphorylation is dependent, in part, on PKA. Such a causal link between phosphorylation and desensitisation has yet to be established in the LH/CG receptor and if such a link is found, it is still uncertain as to the identity of the protein kinase(s) involved. Recent work in transfected 293L cells has indicated that the LH/CG receptor is phosphorylated in response to ligand binding (Hipkin *et al.* 1993). Since the LH/CG receptor has been demonstrated to couple to second messenger pathways that activate both PKA and PKC, it is conceivable that one or both of these proteins could mediate LH/CG receptor phosphorylation. Alternatively, these kinases could phosphorylate other proteins which could then interact with the receptor, resulting in its phosphorylation.

Using a cell line stably transfected with the LH/CG receptor and employing immunoprecipitation techniques, Hipkin *et al.* (1993) demonstrated that the LH/CG receptor is phosphorylated in a basal state and that this phosphorylation is increased in response to hCG binding to the receptor. When 293L cells were co-transfected with a cDNA encoding for the rat cAMP phosphodiesterase, the LH/CG receptor phosphorylation was found to be greatly diminished, indicating that cAMP-dependent protein kinase (PKA) was likely to be involved in this phosphorylation. It is noted by the authors however, that the phosphorylated residues were not identified in this work. As is discussed in Segaloff and Ascoli (1993), additional extracellular PKA consensus sequences are evident in the rat LH/CG receptor, which if phosphorylated, are not likely to be involved in LH/CG receptor uncoupling from its G-protein.

In a similar study by Quintana *et al.* (1994), which investigated FSHR phosphorylation in a stably transfected cell line (293F), the FSHR was found to be phosphorylated in a basal state, with the addition of increasing amounts of FSH resulting in a dose-dependent increase in FSHR phosphorylation. It was noted however, that when 293F cells were co-transfected with a cDNA encoding for the rat cAMP phosphodiesterase, the cAMP response of these cells to a saturating concentration of hFSH was found to be only 10% of that obtained with the wild type. This response was found to be completely restored with the addition of a phosphodiesterase inhibitor. Despite the 90% reduction in the cAMP response to hFSH however, the degree of hFSH-

stimulated receptor phosphorylation was similar to that induced in the wild type cells. In addition, stimulation of the PKA pathway with other agonists such as 8-Br-cAMP resulted in little or no increase in the phosphorylation of the FSHR in ^{32}P-labelled 293F cells. These results would indicate, contrary to the results seen with the LH/CG receptor (Hipkin *et al.* 1993), that the involvement of PKA in FSHR phosphorylation is minimal. The results from these two studies indicate that despite being closely related, the LH/CG receptor and the FSHR may mediate the process of signal transduction in different ways.

A study by Gudermann *et al.* 1995 used 293L cells which had been stably transfected with the murine LH/CG receptor. In this work, pretreatment of these cells with 8-Br-cAMP did not result in a decrease in hCG-stimulable adenylyl cyclase activity. Pretreatment with another agonist, prostaglandin E_1 (PGE_1), which had been previously demonstrated to induce LH/CG receptor phosphorylation (Hipkin *et al.* 1993), was found to elicit a large increase in cAMP accumulation. However, despite this preincubation with PGE_1, no decrease in hCG-stimulated adenylyl cyclase activity was seen. In this study, LH/CG receptor desensitisation was also monitored across a Mg^{2+} concentration range. In the $ß_2$AR, PKA-induced desensitisation of adenylyl cyclase is only seen at low (submillimolar) concentrations (Lohse *et al.* 1990). However, in this study, hCG-stimulated LH/CG receptor desensitisation of adenylyl cyclase activity was detected at both low and high (10mM) Mg^{2+} concentrations. Thus, in contrast to the results obtained by Hipkin et al (1993), these results indicate that LH/hCG-stimulated desensitisation of the LH/CG receptor is not due to the phosphorylation of the receptor by PKA.

In the aforementioned study by Hipkin *et al.* (1993), it was demonstrated that in 293L cells, hCG (100ng/ml) led to a 3-fold increase in IP_3. In addition, when the cellular PKC activity was stimulated by the addition of 4ß-PMA, it was found that LH/CG receptor phosphorylation was increased over and above the basal rate. This increase in phosphorylation could not be mimicked by the addition of a calcium ionophore. However, 4ß-PMA induced phosphorylation of the LH/CG receptor did not prevent hCG-stimulable adenylyl cyclase activity in isolated membranes. In addition, a later

study by Hipkin *et al.* (1995), demonstrated that down-regulation of PKC activity did not prevent hCG-induced phosphorylation. These results would indicate that whilst both hCG and PKC can phosphorylate the LH/CG receptor, hCG-induced phosphorylation is not mediated via PKC. (Similar results were obtained when PKC was activated in a cell line stably transfected with the FSHR (Quintana *et al.* 1994)). The conclusions from this study are in agreement with those from earlier work by Inoue and Rebois (1989). In this study, murine Leydig tumour (MLTC-1) cells were pretreated with 4ß-PMA or DAG. Both these compounds are activators of PKC and were found to cause desensitisation of the hCG-stimulated cAMP response. Thus, PKC was implicated as being involved in LH/CG receptor desensitisation. However, since PKC is normally activated when phosphoinositides are metabolised to DAG and inositol phosphates, the workers measured the level of IP accumulation in cells treated with hCG. Incubation with hCG was found to neither increase the level of PKC or IP, but could induce desensitisation. Therefore, these results suggest that although PKC can desensitise the gonadotropin response, hCG does not cause desensitisation by activating PKC.

In the aforementioned study by Gudermann *et al.* (1995) which used 293L cells transfected with the murine LH/CG receptor, it was demonstrated that activation of PKC by prestimulation with 4ß-PMA did not result in an adenylyl cyclase desensitising response when cells were subsequently treated with hCG. In addition, down-regulation of PKC by long-term pretreatment with 4ß-PMA did not prevent hCG-stimulated adenylyl cyclase desensitisation. Thus, despite the 4ß-PMA activation of PKC being demonstrated to induce LH/CG receptor phosphorylation (Hipkin *et al.* 1993), it would appear that PKC does not play a significant role in the homologous desensitisation of the mouse LH/CG receptor expressed in 293L cells.

In a study by Lamm and Hunzicker-Dunn (1994), porcine follicular membranes and immunoprecipitation techniques were used to study whether the desensitised LH/CG receptor was phosphorylated. Under conditions that promoted LH/CG receptor desensitisation, there was no apparent phosphorylation of the receptor by endogenous

membrane associated protein kinases using [γ-^{32}P]-GTP or [γ-^{32}P]-ATP as phosphate donors.

In addition to the work described, it would appear that there may be differences between species with regards to the putative kinases involved in LH/CG receptor desensitisation. For example, it has been demonstrated that rat Leydig cells can undergo desensitisation, as measured by cAMP production, when stimulated by activators of PKC, whilst activators of PKA have not been demonstrated to affect desensitisation (see review by Rommerts and Cooke 1988). In contrast to these results, studies by West and Cooke (1991a) and West *et al.* (1991) have indicated that under the same experimental conditions, mouse Leydig cells can undergo desensitisation in response to stimulation with activators of both PKC and PKA. This desensitisation was found to be complete, in that it affected both cAMP levels and steroid production. It would seem from studies such as these then, that there could be distinct differences in the way in which different species undergo the desensitisation process. It was suggested by the authors that these differences may be as a result of potential variances in the number or type of phosphorylation consensus sequences found in the LH/CG receptor of rat and mouse Leydig cells.

From the studies described above, the involvement of PKA and/or PKC in LH/CG receptor desensitisation appears to be a contentious issue. The view that PKA is not involved in LH/CG receptor desensitisation is supported by research from groups which have shown that porcine follicular membranes (Bockaert *et al.* 1976), rat Leydig tumour cells (Dix *et al.* 1984), MLTC-1 cells (Rebois and Fishman 1986) and bovine luteal cells (Budnik and Mukopadhyay 1987), undergo desensitisation in a cAMP-independent manner. In light of what is known regarding desensitisation in the β_2AR, it would be of interest to establish whether a protein analogous to ßARK may be involved in LH/CG receptor desensitisation.

1.64: LH/CG receptor down-regulation

Desensitisation is an acute response to receptor activation. Whilst desensitisation results in a period of refractoriness, whereby further exposure to agonist cannot

reactivate the receptor, this refractoriness is relatively short-lived. In contrast, the prolonged exposure of cellular receptors to agonists results in a decrease in ligand binding in a process termed down-regulation, the 'recovery' period of which is much longer than that for desensitisation. Down-regulation is regarded as a slow response to continued receptor activation as it occurs over hours. This process reflects the internalisation of receptors away from the plasma membrane, resulting in the lowering of the total number of receptors in the cell. Down-regulation can involve modulation of the rates of receptor synthesis or degradation and can be cAMP dependent or independent (Lefkowitz *et al.* 1990).

The ability of LH/CG to modulate the number of LH/CG receptors was first demonstrated by Sharpe (1976 and 1977) and Hsueh *et al.* (1976 and 1977). These investigators found that the binding of radiolabelled hCG to rat testis decreases as a function of time after injecting the rats with either LH or hCG (homologous down-regulation). Other experiments by Hsueh *et al.* (1976), Tsuruhara *et al.* (1977) and Huhtaniemi *et al.* (1978 and 1981) have demonstrated that the homologous down-regulation of testicular LH/CG receptors is sometimes preceded by a phase of homologous up-regulation. In these studies, the magnitude of homologous up- and down-regulation was found to be dependent on the dose of hormone injected. In addition to the ability of exogenous LH or hCG to regulate testicular LH/CG receptors, elevations in the endogenous levels of LH induced by single injections of GnRH also induces down-regulation of rat testicular LH/CG receptors (Catt *et al.* 1979a, Dufau *et al.* 1979). The effects of homologous up- and down-regulation of LH/CG receptors has been confirmed to be as a result of changes in the real number of receptors expressed at the cell surface and not simply as a result of changes in the affinity of the receptors for hormone (Tsuruhara *et al.* 1977, Huhtaniemi *et al.* 1981).

Down-regulation of activated LH/CG receptors is thought to occur via receptor mediated endocytosis. In *in vitro* studies, the LH/CG receptor has been demonstrated to be in a highly dynamic state, being continually internalised into endoplasmic vesicles and recycled back to the cell surface during short term exposure (up to 24 hours) of Leydig cells to LH (Catt *et al.* 1979b, Cooke *et al.* 1986). The process of receptor mediated internalisation is temperature dependent and does not occur at 4°C (Habberfield *et al.* 1986). Recycling of

the LH/CG receptor has been demonstrated in rat (Habberfield *et al.* 1986) and porcine (Genty *et al.* 1987) Leydig cells. In MA10 cells LH/CG receptors were found to be continually synthesised and not recycled (Lloyd and Ascoli 1983). Salesse *et al.* (1989) demonstrated that LH and hCG are both internalised by the same pathway.

1.7: The non-gonadal expression of the LH/CG receptor gene/protein

Recent advances in molecular biological techniques have allowed for the detection of mRNA in tissues that were not previously thought to express the specific mRNA in question. LH/CG receptors which were once thought to be present only in the gonads have now been found in several non-gonadal tissues and cells. This raises questions about the physiological roles that LH or CG may have in addition to those currently identified.

Tao *et al.* (1995) demonstrated that rat prostate tissue contained several LH/CG receptor transcripts. These same workers also found that in human tissue isolated from normal prostate glands, benign prostatic hypertrophic tissue and prostatic cancer tissue, both the LH/CG receptor protein and the mRNA transcripts were expressed. This suggests that LH may directly regulate prostate functions. Growth and regression of the prostate gland had been shown to be associated with castration (see review Cunha *et al.* 1987). Previously, these growth changes in the prostate were assumed to be due to changes in androgen levels. However, since LH/CG receptors are present in the prostate, it is possible that these growth changes could at least partly be due to changes in LH levels. Hence LH/CG receptors may have relevance to both physiologic and pathologic regulation of prostate functions.

Recent studies have demonstrated LH/CG receptor mRNA in the brains of adult rats (Lei *et al.* 1993), in fetal rat brain (Al-Hader *et al.* 1997a) and in neonatal rat brains (Al-Hader *et al.* 1997b). Prior to this, LH/CG receptors had been detected in low levels in the cerebrospinal fluid (CSF) and central nervous system (CNS) of a variety of mammals (Antunes *et al.* 1979, Hostetter *et al.* 1981, Emanuele *et al.* 1981, Emanuele *et al.* 1983). The CSF concentrations of these hormones in humans have been shown to increase when the brain contains LH or hCG producing tumours (Bagshawe and Harland 1976,

Braunstein *et al.* 1981, Harris *et al.* 1988). The presence of hormones in CSF and the CNS raises the possibility that they may act locally if the receptors are present in brain

Shi *et al.* (1993) also found that in human placental trophoblasts, hCG regulates its own synthesis by promoting the differentiation of cytotrophoblasts (which make little hCG) into syncytiotrophoblasts (which make considerable amounts of hCG) (Hoshina *et al.* 1985, Kliman *et al.* 1986, Daniels-McQueen *et al.* 1987). It has also been showed previously that cytotrophoblasts and syncytiotrophoblasts in human placental tissue express LH/CG receptors (Reshef *et al.* 1990, Lei and Rao 1992).

LH/CG receptors have also been demonstrated in human endometrial and myometrial cells and uterine vascular endothelial cells (Lei *et al.* 1992) and in human uterine arteries (Toth *et al.* 1994). These findings suggest that LH/CG may directly regulate blood flow in the human uterus and other target tissues, perhaps by decreasing vascular resistance and/or increasing vascular endothelial cell proliferation.

More recent work has also demonstrated the presence of the LH/CG receptor in lactating rat mammary glands (Tao *et al.* 1997), human adrenal glands (Pabon *et al.* 1996a) and human skin (Pabon *et al.* 1996b).

1.8: The LH/CG receptor and antisense technology

In recent years, the study of many genes and proteins has made use of a relatively new area of biological research known as antisense technology. This science involves blocking the transcription or translation of genes by introducing a short piece of DNA or RNA (known as oligodeoxyribo- or oligoribonucleotides respectively) which are complementary to part of the gene of interest. The potential for such a seemingly simple idea was immediately realised by pharmaceutical companies and the like, as a real way forward into the treatment of diseases such as AIDS and cancer, and genetic diseases such as cystic fibrosis. Antisense technology has also provided a potentially powerful tool for biological research, allowing the study of fundamental molecular biology. As the years have progressed however, the technique has run into some unforeseen problems and it is now being realised that whilst the potential for antisense

technology is still high, the initial simplicity associated with this technique was misleading. For example, difficulties have been experienced in getting antisense oligonucleotides into target tissues and side effects such as increased blood pressure and cardiovascular problems have shown up in early animal studies (Gura 1995). One of the biggest concerns for researchers in recent years has been that many antisense oligonucleotides simply do not appear to be working in the way they were once thought to. This has resulted in some human trials being halted as the research is thought by some to be too preliminary to warrant human testing. Despite this, some oligonucleotide-based therapies have reached clinical trials. These studies suggest that the organs of the reticuloendothelial system (liver, spleen, kidneys and lungs) may be good sites for oligonucleotide efficacy as much of the oligonucleotide administered, by almost any route, appears to end up in these tissues [see Akhtar and Agrawal 1997]. However, this preferential accumulation of oligonucleotides within these organs could precipitate adverse effects. Modifications of the oligonucleotide backbone in order to impart increased resistance to nuclease attack [see *Chapter Five*], have been associated with causing renal failure, major haematological effects, massive splenomegaly, fatal hypotension as well as many more minor conditions in some animal studies [see Akhtar and Agrawal 1997]. However, recent reports have indicated that these side-effects may be minimised by altering the nature of the oligonucleotide backbone (Zhao *et al.* 1993, Agrawal *et al.* 1995). When addressing the issue of toxicity however, providing the presence of the oligonucleotide is effective, the negative side-effects must surely have to be balanced with the severity of the condition being treated.

1.81: The mechanisms of an antisense strategy

Antisense RNA or DNA strands have the potential to block any of the steps of gene expression. The mechanisms of inhibition could involve transcription, interfering with RNA processing, preventing mRNA transport to the cytoplasm, altering mRNA stability and preventing or altering mRNA translation into protein (Helene and Toulme 1990, Baertschi 1994).

One way of inhibiting translation of endogenous mRNAs has been established which involves the stable transformation of a cell. cDNA coding for a fragment of the protein

of interest is cloned into an expression vector but is inserted in the antisense orientation. The expression vector is then used to transform the cell. Incorporation of the cDNA into genomic DNA results in the expression of antisense RNA, often several thousand bases in length, which hybridises to the complementary mRNA, potentially having an effect at the level of translation by blocking the passage of the ribosome (Persaud and Jones 1994). However, because the cDNA is incorporated into the nucleus, antisense RNAs could theoretically hybridise with nascent RNA and the effect on gene expression could therefore take place in the nucleus as opposed to the cytoplasm. By hybridising to nascent RNA, the antisense RNA could interfere with processing events such as intron splicing, 5' capping and 3' polyadenylation which in turn could prevent the mRNA being transported into the cytoplasm. Similarly, the antisense RNA has the potential to hybridise to the gene itself by locating the locally opened transcription bubble created by RNA polymerase. In this way then, antisense RNAs could theoretically prevent the transcription of the gene by physically blocking the binding site, or passage of the transcriptional complex. This phenomenon is sometimes known as triplex formation since the addition of the oligonucleotide creates a third strand when it hybridises with the DNA duplex (Helene and Toulme 1990, Hogan 1993).

Much of the recent work utilising antisense technology has used 'presynthesised' antisense oligonucleotides, often DNA in nature as opposed to RNA, and added them in various ways to cells. Such antisense oligonucleotides are targeted to specific regions of mRNA and are thus thought to assert their effects by either hybridising to the start site of translation or blocking the binding or assembly of the 80S ribosomal subunits and/or initiation factors (Liebhaber *et al.* 1992, Persaud and Jones 1994). Similarly, at regions downstream of the translational start site, the antisense oligonucleotide could prevent the passage of the ribosome along the mRNA or it could initiate RNAse H activity (Minshall and Hunt 1986, Helene and Toulme 1990, Probst and Skutella 1996). RNAse H is a ubiquitous enzyme which is involved in DNA replication, transcription and repair. It recognises RNA/DNA heteroduplexes and hydrolyses the RNA component of the heteroduplex. Thus, RNAse H activity can be activated by certain types of antisense oligonucleotide when they hybridise with

mRNA, resulting in the cleavage of mRNA and the production of truncated mRNA transcripts.

1.82: The use of cell-free systems in which to study antisense strategies

In order to escape the constraints imposed by using antisense oligonucleotides in cell culture, many preliminary antisense experiments are performed in cell-free systems. This bypasses the problems that can be experienced in cellular uptake and greatly reduces the problem of oligonucleotide degradation by nucleases. There are several types of cell-free systems available but they are usually based around the use of reticulocyte lysates or wheat germ extracts. Both types have their advantages and disadvantages. The reticulocyte system is favoured when large proteins are to be generated (> 60kDa) and/or when co-translational processing products such as microsomal membranes are to be added. Because these types of systems are generated from nonnucleated reticulocytes, it used to be assumed that the level of endogenous RNAse H activity was low or absent. In order to study the effectiveness of antisense oligonucleotides in directing RNAse H activity, either RNAse H was added to the reticulocyte systems or a wheat germ system was used. The wheat germ systems are generated from the grinding of wheat germ in an extraction buffer and were thought to contain considerably more RNAse H activity than cell-free systems generated from rabbit reticulocytes. However, a comparison of the RNAse H activities of both these systems has now demonstrated that both contain RNAse H activity and that under certain conditions, the wheat germ system may only have approximately three fold higher activity compared to the rabbit reticulocyte system (Cazenave *et al.* 1993). The wheat germ system is often favoured when mRNA transcripts are thought to contain large amounts of secondary structure.

The cell-free systems can be used for the translation of mRNA or the systems can be coupled allowing DNA to be added to the system and resulting in the synthesis of the respective protein. The rabbit reticulocyte lysate (RRL) coupled system was used in the work described in this thesis.

1.83: mRNA targeting: Are all regions on the mRNA equally effective in preventing translation?

In work reported by Mizutani *et al.* (1995), three different antisense oligonucleotides were tested in cultured cells, in an attempt to discover which areas of the mRNA were best targeted in order to prevent translation of the hepatitis C virus (HCV). Antisense oligonucleotide 1 was targeted downstream of the start site of translation, whilst antisense oligonucleotide 2 was targeted to cover the AUG site and extend into the 5' untranslated region (5'UTR). Antisense oligonucleotide 3 was targeted to a region within the 5' UTR. It was found that both antisense oligonucleotides 2 and 3 had strong inhibitory effects on the translation of the HCV, whilst antisense oligonucleotide 1 had only a mild effect.

Similar results had been obtained in an earlier, distinct study, again using HCV as a model (Wakita and Wands 1994). Once again, antisense oligonucleotides that were targeted to regions within the 5' UTR and to the AUG codon resulted in the arrest of mRNA translation, whereas oligonucleotides targeted to regions downstream of the AUG were ineffective.

In a cell-free study using the rabbit reticulocyte lysate system (Blake *et al.* 1985), it was found that antisense oligonucleotides targeted to the 5' UTR and initiation codon regions of the ß-globin mRNA prevented the translation of both α and ß-globin mRNAs whereas antisense oligonucleotides targeted to coding regions had little or no effect. It was suggested by these authors that a helix-destabilising activity associated with the reticulocyte ribosomes was able to disrupt the secondary structure during the elongation step but not during the initiation step of translation. Thus oligonucleotides bound to the coding region of the mRNA would be expected to be unable to prevent translation in the reticulocyte systems. Conversely, when the same antisense oligonucleotides were used in the cell-free wheat germ system, despite the oligonucleotides targeted to the AUG region being more efficient inhibitors of translation, all the oligonucleotides, regardless of their binding site on the mRNA, were effective at preventing translation of the globin mRNAs. It has been suggested by the authors that the ability of antisense oligonucleotides targeted to coding regions of the

mRNA to inhibit translation in the wheat germ system and not the reticulocyte system, reflects some difference in the mechanism of translation between the two systems. Possibly the wheat germ ribosomes, unlike the reticulocyte ribosomes, have a reduced ability to disrupt oligonucleotide-mRNA complexes during the ribosome elongation step of translation. This could be in part due to the lower temperature at which wheat germ translation reactions are carried out compared to those in the reticulocyte system (25-30°C in the wheat germ system compared with 37°C in the reticulocyte system). Although not alluded to in the above study, it is possible that in the wheat germ system, which is sometimes found to contain a higher level of RNAse H activity than reticulocyte derived systems, the antisense oligonucleotides targeted to the coding region of the mRNA were able to direct RNAse H activity, resulting in mRNA cleavage and subsequent degradation.

RNAse H activity has also been shown to play a role in studies using *Xenopus* oocytes as an environment in which to study the effects of antisense oligonucleotides (Cazenave *et al.* 1987). Upon injection of antisense oligonucleotides targeted to the coding region of exogenous rabbit ß-globin mRNA, the ß-globin transcripts were found to disappear. Truncated transcripts were rarely detected due in part to the instability of the RNA fragments that were subsequently processed by RNAses. The presence of RNAse H and antisense oligonucleotides together, can lead to irreversible effects as once cleaved, mRNA can no longer support translation (Cazenave *et al.* 1987, Helene and Toulme 1990). Since RNAse H does not cleave the oligonucleotide, provided it is not itself degraded by other nucleases, it can proceed to induce the degradation of multiple transcripts. RNAse H would seem to act therefore, as an amplifier of the antisense effect.

From the studies described above, it would appear that the site to which antisense oligonucleotides should be targeted in order to prevent translation, can depend to a certain extent on which system is being used for study. In all cell-free systems, the most effective region to which antisense oligonucleotides should be targeted in order to prevent mRNA translation, appears to be the area upstream and surrounding the AUG start site of translation. In rabbit reticulocyte systems, oligonucleotides which are

directed to the coding regions of the mRNA are ineffective in preventing translation. This suggests that the unwindase activity possessed by ribosomes is only efficient during the elongation phase of translation and during initiation. Conversely, in the wheat germ system antisense oligonucleotides targeted within the coding region of the mRNA, may also be effective in preventing protein expression. This may be due, in part, to the higher level of RNAse H activity that is sometimes reported to exist in these cell-free systems. If RNAse H activity is the reason why such antisense oligonucleotides prevent mRNA translation, it is via an indirect mechanism. Rather than by preventing translation *per se*, mRNAs cleaved by RNAse H are degraded and are therefore not available for translation (Cazenave *et al.* 1987, Helene and Toulme 1990).

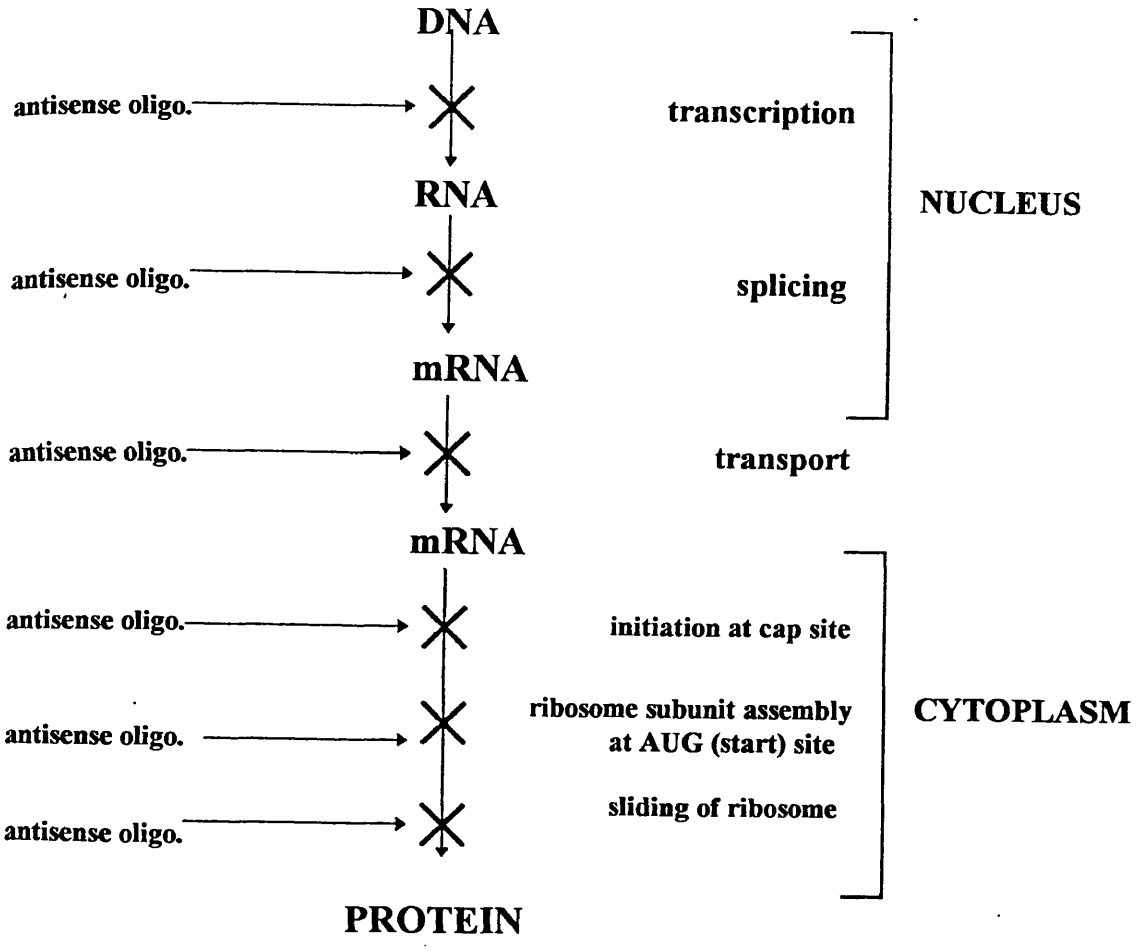


Figure 1.8: **Diagram demonstrating the events which can be interrupted by the use of antisense oligonucleotides.** The arrows indicate the possible sites of action of the antisense oligonucleotide (ODN). *This diagram was taken from Baertschi et al. 1994.*

1.84: Ribozymes

In recent years there has been a growing interest in using ribozymes to cleave target RNA (Woolf 1995). Ribozymes are naturally occuring RNA molecules that possess catalytic activity and can cleave either themselves or other RNA molecules. Ribozymes therefore represent an alternative to using antisense oligonucleotides that activate RNAse H activity as a way of cleaving target RNA sequences. In a report by Haseloff and Gerlach (1988), parameters have been suggested for the design of synthetic ribozymes with new sequence-specific endoribonuclease activities which could be used as an alternative to antisense oligonucleotides. In self-cleaving ribozymes the cleavage reaction results from RNA conformation bringing reactive groups into close proximity. The sites of cleavage are specific and associated with domains of conserved sequence and secondary structure (Haseloff and Gerlach 1988). Self-catalysed cleavage of these RNAs is generally an intramolecular reaction, that is to say a single molecule contains all the RNA-encoded functions required for cleavage. The self-cleaving domain is separate from the substrate domain. The cloning and *in vitro* mutagenesis of the self-cleaving domain has resulted in an RNA molecule which does not possess a substrate region and will therefore only cleave other RNA molecules which contain the correct substrate region (Haseloff and Gerlach 1988). Thus, mutation of the cleavage domain results in the cleavage of a selected region of target RNA. Because in naturally occurring self-cleavage, the site in the RNA substrate is preceeded on the 5'side by a GUC sequence which is highly conserved, this sequence must be located in target RNA (Haseloff and Gerlach 1988).

1.9: Aims and objectives of this project

The aim of this project was primarily to investigate the structure/function relationships of the LH/CG receptor, with a particular emphasis on understanding more fully the process of desensitisation of this receptor in testicular Leydig cells. The work was to be carried out using a murine Leydig tumour cell line (MA10). It was intended to approach this project from several different angles, using a number of methods. Some of these techniques were not routinely used in the department prior to this project and had first to be established.

Despite the similarity between the β_2AR and other GPCRs, it was unclear as to whether the LH/CG receptor underwent desensitisation as a result of receptor phosphorylation. Previous work in this department had been successful in raising antipeptide antibodies to the rat LH/CG receptor (Pallikaros *et al.* 1995). It was therefore proposed to firstly establish an immunopurification protocol using these antibodies. With this achieved, the second aim was to subject MA10 cells to various treatments in order to desensitise (or mimic desensitisation) in the presence of [^{32}P], and using immunopurification techniques, to establish whether the MA10 LH/CG receptor was phosphorylated when desensitised. It would also be possible to ascertain whether this receptor was co-immunopurified with other proteins and if so, the phosphorylation status of these proteins. In addition to immunopurification analysis of the MA10 LH/CG receptor, these antibodies were examined for the ability to recognise the respective antigens in immunocytochemistry and immunohistochemistry experiments. If the antibodies did prove successful in antigen recognition, these techniques would provide information regarding the expression of the LH/CG receptor in different cells and tissues. It could also be possible to study the intracellular trafficking of the LH/CG receptor during processes such as desensitisation and down-regulation by confocal microscopy.

Because rat Leydig cells and mouse Leydig cells have been reported to undergo desensitisation in response to activators of PKC and PKA differently (see review by Rommerts and Cooke 1988, West and Cooke 1991a, West *et al.* 1991), it was postulated that differences may exist between the phosphorylation consensus sequences of rats and mice. Whilst the mouse ovarian sequence data had been published (Gudermann *et al.* 1992a), the sequence of the MA10 LH/CG receptor was not known. Because the MA10 cell was neoplastic in nature, it was proposed to confirm that the receptor sequence was similar to that found in the murine ovarian LH/CG receptor, if not identical, particularly with respect to the postulated phosphorylation consensus sequences. It was important that we could be certain that the MA10 LH/CG receptor would respond as other murine LH/CG receptors in later desensitisation studies. The cloning of the MA10 LH/CG receptor would also provide a convenient way in which we could create truncated LH/CG receptors. These truncated receptors were required in order that the effects of putative truncations

predicted to result from the use of an antisense strategy [see *Chapter Five*] could be mimicked. This would provide a way in which to control for the potential, non-specific effects of incubating cells with antisense oligonucleotides and would also provide information as to how antisense strategies work.

Previous work in the department had established an antisense strategy which had been reported to generate truncated LH/CG receptors in MA10 cells (West and Cooke 1991b). Another of the aims of this project was to continue the antisense work, as the generation of mutated LH/CG receptors would provide information about the structure/function relationships of this receptor. Of particular interest was to establish which regions of the LH/CG receptor were important for functions such as G-protein coupling and desensitisation and/or phosphorylation. Mutations which were predicted to be caused by the presence of antisense oligonucleotides targeted to particular regions of the LH/CG receptor would be recreated by digesting the cloned MA10 LH/CG receptor cDNA with restriction enzymes and transfected into a mammalian cell line. In this way we could confirm that the effects of the antisense strategy were as a result of creating LH/CG receptor truncations and not through other non-specific mechanisms. However, due to problems that were encountered during preliminary experiments, it was realised that another experimental system would have to be designed before this work could progress further. To this end, the aim of the work evolved to include the establishment of cell-free methods in which to first test antisense oligonucleotides for effectiveness. Once suitable antisense oligonucleotides had been established as effective in the cell-free systems, it was intended to return to using the cultured MA10 cells to ascertain whether the results could be repeated.

Finally, the identity of the protein kinase(s) anticipated to be involved LH/CG receptor phosphorylation/desensitisation were yet to have been elucidated. Evidence was accumulating to suggest that other GPCRs could couple to more than one signal transducing system (Liu *et al.* 1992, Chabre *et al.* 1992 Van Sande *et al.* 1993, Gudermann *et al.* 1992 a and b), and it had been suggested that the LH/CG receptor itself may also be coupled to the PLC/ IP_3 pathway in addition to the adenylyl cyclase/cAMP second messenger system. Since activation of the PLC/IP_3 was known

to involve PKC activity, it was proposed that inhibitors of PKC should be used in cell culture experiments, in order to establish if this protein had a role in Leydig cell signal transduction/desensitisation.

Summary of aims

1. To develop an immunopurification protocol such that the phosphorylation state of the desensitised MA10 LH/CG receptor could be established.

2. To assess whether existing LH/CG receptor antibodies had the potential to be used in immunocytochemistry experiments and therefore also confocal microscopy. This would provide a way of following the LH/CG receptor during processes such as desensitisation and down-regulation.

3. To clone and sequence the MA10 LH/CG receptor to establish the sequence of putative phosphorylation consensus sequences and to permit the synthesis of truncated LH/CG receptors for clarification of the results anticipated from the use of an antisense strategy.

4. To continue work using the antisense strategy in order to learn more about the structure/function relationships of the LH/CG receptor and also the mechanisms by which an antisense strategy is thought to work. (This rapidly became modified to include the establishment of a cell-free system of LH/CG receptor synthesis in which to first assess the effectiveness of antisense oligonucleotides in preventing LH/CG receptor synthesis).

5. To investigate the putative role of protein kinases such as PKC in LH/CG receptor phosphorylation/desensitisation via the use of protein kinase inhibitors.

Chapter Two: Materials and Methods

2.1: Materials

General materials

Sterile plasticware and plastic coverslips were purchased from Nunc (Nunclon, Denmark). Dextran T500, sephadex G-25 and PD-10 columns were purchased from Pharmacia Biotech Ltd (Herts. UK). Centriprep-30 concentrators were purchased from Amicon, Inc. (MA0195, USA). Bio-Rad dye reagent and Tween-20 were purchased from Bio-Rad Laboratories Ltd (Herts. UK). Scintillation fluid (Ultima Gold) was purchased from Canberra Packard Ltd (Berks. UK). ScAMP-TME and MIX were purchased from Sigma (Leicestershire, UK). All other reagents were purchased from Sigma-Aldrich Chemical Company Ltd (Dorset, UK).

Cell culture and experiment materials

Mouse Leydig tumour (MA10) cells, (designated M5480P), were a gift from Dr M. Ascoli of the University of Iowa, USA. Dulbeccos Modified Eagles Medium, penicillin/streptomycin, Waymouth's MB752/1 medium (with L-Glutamine), Ca^{2+}-Mg^{2+} free phosphate buffered saline and donor horse serum were purchased from Gibco BRL Life Technologies Ltd (Middlesex, UK). Pregnant mares serum gonadotrophin (PMSG) was obtained from Intervet laboratories (Cambridge, UK) and crude hCG from Serono laboratories (Herts. UK). Ovine luteinising hormone (batch oLH-26, potency: 2.3U/mg) and hCG (batch CR-127, potency: 14,900 IU/mg) were donated by NIADDK, (NIH, Bethesda, MD, USA). Cyclic AMP antisera (MS1), was obtained from Dr M Schumacher, Hamburg, Germany and brought thereafter from Calbiochem, Calbiochem-Novachem Corporation (CA 92121, USA). The PKC inhibitor RO 31-8220, was a kind gift from Dr R Abayasekara (Royal Free Hospital School of Medicine, London, UK). The PKC inhibitor GF109203X, was a kind gift from Miss Nickie James (Royal Free Hospital School of Medicine, London, UK). Staurosporine were purchased from Boehringer Mannheim UK (East Sussex, UK). Progesterone antisera was provided by Dr M J Sauer, MAFF, Central Veterinary Laboratory (Weybridge, Surrey, UK). Cholera toxin, dibutyryl-cAMP, forskolin, chloramine T, polyethyleneglycol 6000, glycerol, charcoal and diethylether were purchased from Sigma (Leceistershire, UK).

Molecular biology materials

RNAzol B solution was purchased from AMS Biotechnology UK Ltd (Oxon, UK). dNTPs, IPTG, X-Gal, RNAsin, JM109 high efficiency competent cells, RRL translation system, wheat germ translation system, RRL-coupled TnT system and CytoTox 96 assay kit were purchased from Promega Ltd (Southampton, UK). QIAGEN kits were purchased from QIAGEN Inc (CA 91311, USA). Biotaq polymerase, NH_4 buffer and 50mM $MgCl_2$ were purchased from Bioline, UK Ltd (London, UK). The plasmid pBSLHR/59, containing the full length rat LH/CG receptor cDNA, was a kind gift from Dr P Saunders of the MRC Reproductive Biology Unit, Edinburgh, UK. M-MLV Reverse Transcriptase was purchased from Gibco BRL Life Technologies Ltd (Middlesex, UK). All restriction enzymes, T4 DNA ligase and Random Primed Labelling kit were purchased from Boehringer Mannheim UK (East Sussex, UK). mCAP mRNA capping kit, T3 and T7 RNA polymerase were purchased from Stratagene Ltd (Cambridge, UK). Molecular weight DNA markers (λ Eco RI/Hind III digest and pUC18 Hae III digest) were purchased from Sigma (Leicestershire, UK). Polynucleotide kinase was purchased from Pharmacia Biotech Ltd (Herts. UK). Sequenase Version 2.0 DNA sequencing kit were purchased from Amersham Life Science (Bucks. UK). PCR primers (22167 and 21262) and antisense oligonucleotides 1 and 3 were synthesised by British Bio-technology Products Ltd (Oxford, UK).

Radiochemicals and materials

[^{3}H]-Progesterone (specific activity 103Ci/mmol), carrier free Na-[^{125}I] (specific activity 100Ci/g, [α-^{35}S] dATP (specific activity 600 Ci/mmol), [^{35}S]-methionine (specific activity 400 Ci/mmol), [α-^{32}P] dCTP (specific activity 3000Ci/mmol), Autofluor and Kodak X-ray film were purchased from Amersham Life Science (Bucks. UK). Methyl-[^{14}C] protein markers were purchased from Dupont (UK) Ltd (Herts. UK).

Immunocytochemistry, Western Blotting and protein purification materials

Goat anti-rabbit IgG alkaline phosphatase conjugate and low range molecular weight prestained markers were purchased from Bio-Rad Laboratories Ltd (Herts. UK). Nitrocellulose Hybond-C membranes were purchased from Amersham Life Science (Bucks. UK). BCIP/NBT tablets, Sepharose A and p-Nitrophenyl chloroformate activated

matrix were purchased from Sigma (Leicestershire, UK). Dynabeads M-280 and Dynal MPC-E/E-1 were purchased from Dynal (UK) Ltd (Wirral, UK). Antisense oligonucleotides A and B were synthesised by Dr C Ettelaie (Royal Free Hospital School of Medicine , London UK). Donkey anti-rabbit biotinylated immunoglobulin and streptavidin-fluorescein reagent, originally purchased from Amersham Life Science, were a kind gift from Dr Rachel Helliwell at the Sandoz Institute, London, UK. ABC Vectastain kit was a kind gift from Dr K Teerds (University of Utrecht, Netherlands) and was originally purchased from (Burlingame, CA, USA).

2.2: Methods - DNA amplification

The methods used in DNA and RNA work were as essentially as described by Maniatis and co-workers (Sambrook *et al.* 1982) with some modifications. Plasticware, glassware and media were routinely sterilised by autoclaving for 15-20 minutes at 121°C (15 pounds/inch^2). Chemicals were either autoclaved or filter sterilised and the highest grades available were used. All sources of water used had been double-distilled and then autoclaved.

In addition to the above, it is important when working with nucleic acid, particularly RNA, to establish an RNAse-free environment. This was done by keeping surfaces and equipment dust-free. Benchcote was changed on a regular basis and equipment and glassware autoclaved or baked where possible. Gloves were worn continually. Chemical solutions were autoclaved or filter sterilised and treated with diethylpyrocarbonate (DEPC) where possible (1µl DEPC/ml solution). When DEPC treatment was not possible solutions were dissolved in DEPC-treated water.

2.21: RNA extraction.

Total RNA was extracted using RNAzol B solution. Confluent monolayers of MA10 cells were washed briefly with PBS (0.01M = 10mM sodium phosphate, 140mM NaCl, pH7.4) and then lysed by adding RNAzol B solution at 0.2ml/million cells. The cell suspension was aliquoted into microfuge tubes and chloroform added at 0.1ml/1ml suspension. The tubes were vortexed and left on ice for 5 minutes after which time they were centrifuged at 12000xg at 4°C, for 15 minutes. Centrifugation of the suspension promotes the formation of two phases. The upper phase is aqueous and uncoloured and contains the RNA. This phase was transferred to fresh microfuge tubes and an equal volume of isopropanol was added. The samples were stored at -20°C overnight, after which time they were centrifuged at 12000xg (4°C) for 15 minutes. The RNA precipitate was often not visible but sometimes formed a white/yellow precipitate at the bottom of the tube. The supernatant was removed and the RNA pellet vortexed with 1ml 75% ethanol (DEPC-treated water was used to dilute 100% ethanol) and centrifuged at 7500xg (4°C) for 10 minutes. The pellet was then air dried briefly before either being resuspended in DEPC treated water and measured for purity or stored at -70°C.

2.22: cDNA synthesis using reverse transcriptase

The integrity of total RNA was ascertained by running samples on 1% TBE/agarose gels. The visualisation of ribosomal bands corresponding to the 18S and the 28S ribosomal subunits ensured that the RNA had not been degraded during the extraction procedure.

Approximately 40ng of total RNA was added to a reaction tube containing 1x reaction buffer (50mM Tris-HCL pH8.3, 75mM KCl, 3mM $MgCl_2$), 5mM DTT, 40U RNAsin and 1mM dNTPs. The reaction mix was heated at 70°C for 5 minutes and then cooled rapidly on ice. The 3' gene-specific primer [no. 22617] (5'-CgCgCggCCgCAgATACATTCAgTAATgCAg-3'), (1μM) was added and the reaction mix was incubated at 65°C for 7 minutes before being cooled on ice. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), (200U) was then added and the reaction mix was incubated at 42°C for 50-60 minutes. The samples were stored at -20° overnight before being used in a PCR reaction.

2.23: Polymerase Chain Reaction (PCR)

In order to amplify the C-terminal half of the LH/CG receptor, reverse transcribed DNA was used in a PCR reaction. PCR was carried out in a Minicycler (MJ Research).

Approximately 2-20ng of reverse transcribed DNA was added to a reaction tube containing 1x NH_4 reaction buffer, 1-2mM $MgCl_2$, 1mM dNTPs, 0.5μM 5' gene-specific primer [no. 21262] (5'-CTgCTgCTCATTgCCTCAgTggAC-3') and 0.5μM 3' gene-specific primer [no. 22617] (5'-CgCgCggCCgCAgATACATTCAgTAATgCAg-3'). Mineral oil (20μl), was added to the surface of the reaction mix in order to limit loss of volume by evaporation. Thermal cycling consisted of 94°C for 3 minutes, cooling to 85°C for 2 minutes during which time 5U Biotaq polymerase was added, followed by 30 cycles of 94°C for 1.5 minutes, 55°C for 2 minutes and 72°C for 4 minutes. The final cycle was held at 72°C for a further 7 minutes after which time the reaction temperature dropped to 4°C. The PCR samples were stored at -20°C and the mineral oil aspirated before the samples were thawed for use.

The thermal cycling protocol described here incorporates the use of 'hot start' PCR. Hot start PCR is used to prevent the extension of any primer/DNA complexes which form at low temperatures and would therefore be non-specific. This is achieved by adding the DNA polymerase only after the reaction temperature has reached 85°C.

2.24: Optimisation of PCR - $MgCl_2$

The optimal concentration of magnesium ions required for this PCR system was determined by using buffers with a range of differing concentrations of $MgCl_2$ (between 0.5 and 5mM). The PCR fragments were analysed by 1% TBE/agarose electrophoresis and visualised by UV illumination. The optimisation of PCR had to be reestablished whenever new primers were used. Typically $MgCl_2$ concentrations between 1-2mM were found to be optimal.

2.25: TBE/Agarose gel electrophoresis

Separation of DNA fragments generated by PCR were routinely run on 1% TBE/agarose gels alongside molecular weight markers, using a horizontal submarine gel apparatus (Bio-Rad).

The gels were prepared by dissolving 0.4g agarose in 40ml 0.5x TBE (45mM Tris-borate buffer, 1mM EDTA, pH8.0, ethidium bromide 0.5µg/ml) by microwaving. Cooled molten agarose was poured into a gel former, a comb added and the gel allowed to set for a few minutes at 4°C. Prior to loading onto the gel, DNA samples were mixed with 0.1 volume of 10x loading buffer (0.4% bromophenol blue (w/v) and 67% glycerol in 0.5x TBE buffer (v/v)). Electrophoresis was typically carried out in 0.5x TBE at a constant voltage of 40 volts until the samples were clear of the wells and then at 70 volts thereafter. Molecular weight markers were provided by λ DNA restricted with EcoR I and HIND III (600ng). DNA bands were visualised using a LKB UV transilluminator (2011 Macrovue) and photographed using either a Polaroid hand camera (Polaroid film type 667) or by a scanner.

2.26: Gel extraction of PCR products

A QIAGEN QIAquick kit was used for the extraction of PCR products from agarose gels and the protocol provided was followed. Briefly, the products of interest were excised from the gel using a clean scalpel and placed in a microfuge tube. QX1 buffer was added and the

tube was incubated at 50°C for 10 minutes in order that the gel dissolved. The liquid was then placed in a spin column which was inserted into a clean microfuge tube and this was centrifuged at 13000xg for 60 seconds. The DNA was retained behind the filter and was washed twice with PE buffer. The DNA was eluted into a clean microfuge tube by adding 50µl TE buffer pH8 to the filter and centrifuging at 13000xg for a further 60 seconds. This procedure was repeated, the eluents pooled and the concentration of DNA assessed by TBE/agarose gel electrophoresis.

2.27: Restriction analysis of DNA

Restriction endonucleases type II are DNAses that recognise specific nucleotide sequences. These enzymes cleave double-stranded DNA and produce equal molar fragments of DNA. Samples of both amplified cDNA and cloned DNA fragments underwent digestion with various restriction enzymes in order to ascertain if they were the correct products of PCR or cloning procedures.

The restriction reaction mix was composed of the substrate DNA (between 1-15µg) and the desired restriction enzyme (typically 10U), in its appropriate buffer. Incubation took place at 37°C for between 1-3 hours. The results of the reaction were analysed by electrophoresis on a 1% TBE/agarose gel.

2.3: Methods - Cloning, sequencing and Northern dot blotting

2.31: Preparing amplified DNA for cloning

Blunt ending DNA fragments (Klenow Fill)

This procedure was used to complete the 3' recessed termini of amplified DNA that had been created by the digestion of the DNA with restriction enzymes. The Klenow fragment of *Escherichia coli* DNA polymerase I was used for this purpose since it retains the ability to synthesise DNA but lacks the exonuclease activity sometimes associated with the holoenzyme.

Gel extracted PCR product (7µl) was added to a reaction mix containing 1x Klenow enzyme buffer (50mM Tris-HCl pH7.2, 10mM $MgSO_4$, 0.1mM DTT), 2U Klenow enzyme

and 0.5mM dNTPs. The incubation was at 37°C for 30 minutes and after cooling on ice, the samples underwent a desalting procedure.

Desalting

The desalting protocol from a QIAEX gel extraction kit was used for this purpose. Briefly, the reaction mixture was placed in a spin column which was inserted into a clean microfuge tube. This was centrifuged at 13000xg for 60 seconds. The DNA was retained behind the filter and was washed twice with PE buffer. The DNA was eluted into a clean microfuge tube by adding 25µl TE buffer pH8 to the filter and centrifuging at 13000xg for a further 60 seconds. The eluent was set aside and the eluting procedure repeated. Each eluent sample (4µl) underwent TBE/agarose gel electrophoresis to establish the concentration of DNA in each sample. If the samples were dilute enough then the eluents were pooled, otherwise they were used individually.

Phosphorylation of 5' DNA fragments

This procedure was used to add phosphate groups to the 5' ends of amplified DNA in order that it may ligate efficiently into a dephosphorylated plasmid vector. Desalted PCR product (40µl), was added to a reaction mix containing 5µl 10x ligase buffer + ATP (50mM Tris-HCl pH7.6, 10mM DTT, 500µg/ml BSA) and 3µl polynucleotide kinase (PNK). The reaction mix was incubated at 37°C for 30 minutes, after which time the samples underwent a desalting procedure. Samples from the desalted phosphorylation reactions were run alongside markers on 1% TBE/agarose gel in order to assess the concentration of the DNA.

Ligation

The phosphorylated PCR product (0.86Kb in size) (20ng) was ligated into the dephosphorylated pUC18 sma1/BAP vector (50ng). The reaction consisted of 1.5µl 10x ligase buffer + ATP (50mM Tris-HCl pH7.6, 10mM DTT, 500µg/ml BSA) and 2U T4 DNA ligase. The reaction mix was incubated at 17°C overnight.

2.32: Transformation of competent JM109 cells

E. coli competent JM109 cells were purchased from Promega and were subsequently transformed according to the protocol provided. Briefly, 100µl of cells and 1-50ng cloned DNA were added together in a sterile Falcon (2059) polypropylene tube and placed on ice for 10 minutes. The cells were then heat shocked by placing the tube in a water bath at exactly 42°C for 45-50 seconds and then were immediately placed on ice for 2 minutes. In order to allow for the expression of the antibiotic resistance gene, 900µl of prewarmed SOC medium [see appendix] was then added and the tube incubated in a shaking water bath (225rpm) at 37°C for 60 minutes. Following incubation, 100-200µl of the transformed cells were plated out onto prewarmed LB agar plates containing ampicillin (100µg/ml), IPTG (40µg/ml) and X-Gal (40µg/ml) and incubated at 37°C for 18-24 hours. The presence of IPTG and X-Gal enables the selection of those *E. coli* colonies which contain a recombinant plasmid and are white in colour, over those which contain a wild-type plasmid and are blue in colour. Before selecting white colonies, the plates were first placed at 4°C overnight as this reduced the appearance of 'false' white colonies. White colonies were then picked and inoculated in 10ml of LB broth containing 50µg ampicillin and grown overnight at 37°C with constant shaking.

2.33: Plasmid DNA preparation (Minipreps)

A QIAGEN QIAprep-spin kit was used for this purpose and the protocol provided was followed. Briefly, the 10ml cultures grown as described above were pelleted by centrifugation and resuspended in P1 resuspension buffer. P2 lysis buffer was then added and the tubes inverted to mix. Ice-cold N3 buffer (containing guanidine hydrochloride to precipitate the chromosomal DNA and cell debris) was then added to the tube and mixed by gentle inversion. The tubes were placed on ice for 5 minutes to aid the precipitation procedure. After this time the tubes were microcentrifuged at 10000xg for 10 minutes at room temperature and the supernatant placed in an assembled spin column. The tubes were then centrifuged at 10000xg for 60 seconds and the drainthrough discarded. The spin column was washed twice with PB buffer and then once with PE buffer. The plasmid DNA was then eluted from the column using TE (pH8.5) buffer and centrifuging for 30 seconds. This procedure was repeated, the eluents pooled and the concentration of DNA assessed by TBE/agarose gel electrophoresis.

2.34: Dideoxy-sequencing

Plasmid DNA (3-5μg) was denatured by incubating at room temperature for 15 minutes following the addition of 5μl of NaOH (0.1M) and EDTA (0.5M) in a final volume of 25μl. After incubation, 8.5μl of the denatured plasmid DNA was added to 1μl of 10x TM buffer (100mM $MgCl_2$ and 100mM Tris-HCl pH8.4), along with 0.5pM of forward and reverse universal sequencing primers. The mix was incubated at room temperature for 15 minutes. Sequencing of the plasmid took place using the Sequenase Version 2.0 kit according to the protocol provided. Briefly, 10μl of the primer-annealed template was added to a microfuge tube containing 1μl of DTT (10mM), 2μl of labelling nucleotide mix (1.5μM dGTP, 1.5μM dCTP, 1.5μM dTTP, diluted 1/5), 0.5μl (0.5μCi) [α-^{35}S] dATP and 3 units of Sequenase enzyme (T7 DNA polymerase). The reaction mix was incubated at room temperature for 4-5 minutes after which time the reactions were terminated. This was acheived by adding 3.5μl of labelling reaction into each of 4 microfuge tubes containing 2.5μl of the appropriate dideoxy/deoxynucleotide mixes, (each mix contained 80μM dATP, 80μM dTTP, 80μM dCTP, 80μM dGTP and 50mM NaCl). The relevant dNTP was replaced as follows; 8μM ddATP replaced dATP in the A mix, 8μM ddCTP replaced dCTP in the C mix, 8μM ddGTP replaced dGTP in the G mix and 8μM ddTTP replaced dTTP in the T mix. The reactions were then incubated at 37°C for 5 minutes after which time 4μl of formamide stop mix (98% (v/v) formamide, 10mM EDTA, 0.01% (w/v) bromophenol blue, 0.001% (w/v) xylene cyanol) was added to halt the reactions. The sequencing reactions were heated to 95°C for 5-10 minutes before being loaded onto a sequencing gel.

A 6% (v/v) polyacrylamide sequencing gel was made by mixing 10x filtered TBE buffer with 50% (w/v) urea and 1/7th volume of acrylamide mix (38% (v/v) acrylamide, 2% (v/v) *N, N'*-methylene bisacrylamide). The gel was set with the addition of 1/100th volume of 10% ammonium persulphate and 1/500th volume of TEMED. The sequencing reactions were electrophoresed through the 50ml sequencing gel at 38-40 watts for 2 hours, using 1x TBE as running buffer. The gel was then fixed in 10% (v/v) acetic acid for 30 minutes, blotted onto dry 3MM filter paper and transferred to a slab gel drier. The gel was dried at a temperature of 80°C for 2-3 hours and then exposed to autoradiograph film for a further 18-48 hours. The DNA sequence was read manually from the developed autoradiograph with the aid of a light box.

2.35: Northern dot blotting

Samples of total RNA were prepared as described in section ***2.21***. Using a milliblot system, 1µl and 3µl samples were loaded onto nitrocellulose Hybond C membrane and washed through 3 times with PBS (0.01M pH7). Samples of DEPC-treated water were used to control for background levels of probe hybridisation. Salmon sperm (100µl) was denatured by heating to 100°C for 2 minutes. Prehybridisation buffer was made [see appendix] and the denatured salmon sperm added to this solution. The membrane was placed RNA side up in a hybridisation tube and prewetted with 6x SSC buffer [see appendix]. Excess SSC was removed and the prehybridisation buffer added to the tube. The hybridisation tube was placed in a preheated hybridisation oven at 65°C for 4 hours. After this time the denatured, [α-^{32}P] CTP labelled probe [see section ***2.36***] was added to the prehybridisation solution for 1 hour at 65°C. The radioactive solution was disposed of and the filter removed and placed into a container large enough to ensure adequate washing of both sides of the filter. Cold 2x SSC + 0.1% (w/v) SDS was added and the filter washed for 5 minutes. The filter was then sequentially washed in 1x SSC + 0.1 (w/v) % SDS, 0.5x SSC + 0.1% (w/v) SDS and 0.2x SSC + 0.1% (w/v) SDS, all of which had been preheated to 65°C in a water bath. Each washing step was performed for 30 minutes in a sealed container placed in a shaking water bath at 65°C. After washing the filter was air dried and exposed to autoradiography film for approximately 1 week at -70°C before developing.

2.36: Probe labelling

The C-terminal half of the cloned MA10 LH/CG receptor (200ng) was amplified in a PCR reaction using primers 21262 and 22617 as described in section ***2.23***. The PCR product was electrophoresed on a 1% TBE/agarose gel [see section 2.25], followed by gel extraction. The cDNA was labelled using a Random Primed Labelling kit [Boehringer Mannheim] according to the protocol provided. This method involves the annealing of random hexanucleotides to the template strand of cDNA. These act as primers for the synthesis of complementary cDNA from the 3'OH termini of each primer. Incorporation of dNTPs, one of which has been labelled with a radioactive isotope, in this case [α-^{32}P] dCTP, is carried out by the Klenow fragment of DNA polymerase I. Approximately 25ng of cDNA was denatured by heating to 100°C for 5 minutes, cooled rapidly on ice and added to a reaction mix containing 3µl of dNTP (-dCTP) mix, 2µl of 10x reaction buffer

(containing the hexanucleotide mix), 5µl (or 100µCi) of [α-^{32}P]-CTP (3000 Ci/mmol) and 1µl of Klenow enzyme with sterile water added to a final volume of 20µl. The reaction mix was incubated at 37°C for 30-60 minutes after which time the reaction was stopped by heating to 70°C for 10 minutes thus denaturing the enzyme.

2.4: Methods - Immunopurification

The primary antibodies used in this work have been previously characterised by Pallikaros *et al.* (1995). These antibodies, raised in Dutch half-lop rabbits, were directed against synthetic peptides corresponding to regions of the LH/CG receptor. Peptides 1 and 2 corresponded to two N-terminal regions, whilst peptide 3 corresponded to an intracellular region located on the cytoplasmic tail.

Before an immunopurification protocol could be established in order to purify the LH/CG receptor antigen, it was first necessary to purify antibodies 1, 2 and 3 from rabbit serum stored at -20°C. Immunoaffinity purification columns had already been made in work previously performed in the department (Pallikaros 1995). The column matrices had been cross-linked to either peptide 1, 2 or 3. These peptide-linked columns were then used in the method described below, to immunopurify the respective antibodies from rabbit serum.

2.41: Antibody immunoaffinity purification from rabbit serum

Each antibody serum sample (3-10ml) was repeatedly passed through the relevant peptide column for 2-3h. The column was then washed with sodium phosphate (10mM), NaCl (800mM), (pH7.2) to remove nonspecifically-bound protein. Bound IgG was then eluted with $MgCl_2$ (5M), followed immediately by 10-fold dilution in distilled water as described by Baldwin (1994). Following elution, purified antibodies were dialysed overnight in 4 litres of PBS. All the above steps were carried out at 4°C. Purified antibodies were concentrated to a volume of 1-2ml, depending on the initial serum volume, using the centriprep-30 concentrators and were stored at -70°C. Protein content was later determined by the Bio-Rad method.

2.42: Peptide dot blots to check for antibody recognition

Increasing amounts of peptides 1 and 2 (0-20ng) were immobilised on nitrocellulose Hybond N membranes using a milliblot system. Since the peptides were dissolved in DMF, a dot of this solvent was included (at the same concentration as was present in 20ng of peptide), to act as a background control. The nitocellulose membranes were air dried before undergoing Western blotting [see section ***2.68***] with antibodies 1 and 2 in order to check for antibody-peptide recognition (and to rule out cross-recognition).

Both the antibodies purified during this work and antibodies previously purified by Pallikaros (1995) were used in order to establish an antigen (in this case the LH/CG receptor), immunopurification protocol. There were two main methods used during the immunopurification work; the first method, immunoprecipitation, used magnetic beads linked with anti-rabbit IgG, the second method, immunoaffinity purification, used an activated sepharose-A column linked to the respective anti-peptide antibodies.

2.43: Immunoprecipitation using Dynal beads

This method used magnetic beads, approximately 2.8μm in diameter, which had been coated in sheep anti-rabbit IgG to isolate the antigen of interest via its rabbit antibody. The Dynabeads M-280 were preincubated with either antibody 1 or 2 (20μg antibody/mg beads), at 4°C with gentle agitation, overnight. The beads were then collected using the Dynal MPC (magnetic particle concentrator) and the supernatant discarded. The anti-peptide coated beads were washed in wash buffer (0.01M PBS pH7.4, 0.1% (w/v) BSA, 1% (v/v) FCS) four times for 30 minutes each time at 4°C, collecting the beads and discarding the supernatant before each wash. The beads were then ready to be used to isolate the LH/CG receptor.

LH/CG receptors were solubilised and purified as described in section ***2.62***. The beads were then added at a final concentration of 10^8 beads/ml cell lysate and the mixture was incubated for up to 1 hour at 4°C with gentle agitation. After this time the beads were collected via the MPC, washed as before and the LH/CG receptor eluted.

There were several methods of elution attempted. The first to be tried was elution directly into SDS-PAGE buffer. The pellet of beads was resuspended in 25-50μl of SDS-PAGE

sample buffer and heated to either 100°C or 37°C for 3 minutes or left at room temperature for 15 minutes. The beads were then pelleted via the MPC, and the supernatant collected and loaded onto an SDS-PAGE.

Acetic acid was also used for LH/CG receptor elution. The pellet of beads was resuspended in 100-500µl acetic acid (0.5M) and vortexed for 15 seconds at room temperature. The beads were pelleted via the MPC and the supernatant collected. The supernatant was neutralised using NaOH (1M) solution before being loaded onto SDS-PAGE.

Finally, salt elution was attempted. The pellet of beads were resuspended in 100µl NaCl (2M) and vortexed for 10 seconds at room temperature. The beads were pelleted via the MPC and the procedure repeated twice more. The supernatants were then collected and dialysed for 24 hours at 4°C in PBS (0.01M pH7), before being concentrated and loaded onto an SDS-PAGE.

2.44: **Immunoaffinity purification using activated matrix column chromatography**

For this method Sepharose A, p-Nitrophenyl chloroformate activated matrix was used. The matrix (6ml) was placed in dialysis tubing with a minimum of 2-3ml anti-peptide antibody and transferred to a large beaker containing $NaHCO_3$ (0.1M pH9), for 24-48 hours at 4°C with continuous stirring. After this time the spent buffer was discarded and replaced with ethanolamine (0.1M pH9) for a further 24 hours as before. The solubilised LH/CG receptors were prepared as described in section ***2.63***. The heterogeneous suspension was then loaded onto a 1cm x 20cm chromatography column and circulated for 24-48 hours at 4°C. After loading, the column was washed with Tris-HCl (0.01M pH8.5) to remove unbound protein. Fractions were collected and tested for protein content using the Bio-Rad protein assay. When no further protein was eluted (usually after 1-1.5 hours), the LH/CG receptor was eluted off the column by the addition of NaCl (0.5M in Tris-HCl 0.01M, pH8.5). Fractions (5ml) were collected for 1-2 hours and the LH/CG receptor detected in the fractions by using the Bio-Rad protein assay. (Elution with NaCl (3M) in Tris-HCl 0.01M, pH8.5 was also tried). The column was washed with TrisHCl (0.01M, pH8.5) and stored at 4°C. For long term storage, 0.02% (w/v) NaN_3 was added to the wash buffer to

prevent microbial contamination, but care had to be taken to wash this off thoroughly before the column was next used.

2.5: Methods - Cell-free experiments

2.51: In vitro transcription

In order that enough RNA was available for the *in vitro* translation work, *in vitro* transcription of the full length rat LH/CG receptor (pBSLHR/59) was used for this purpose. The plasmid was digested with the endonuclease NOT1 to produce a linear plasmid which had a T7 RNA polymerase promoter site upstream of the LH/CG receptor gene. An *in vitro* transcription kit [mCAP mRNA capping kit - Stratagene] was used according to the protocol provided. Briefly, a reaction mix was made containing 1μg linearised DNA template, 5μl 5x transcription buffer, 2.5μl cap analogue, 1μl DTT, 1μl RNAsin, 1μl rNTP mix, 10U T7 polymerase and 11.5μl DEPC-treated water. The reaction was incubated at 37°C for 30 minutes. The DNA template was then removed by the addition of 10U of RNAse-free DNAse I and incubated at 37°C for a further 5 minutes. The reaction was stopped by the addition of 100μl of DEPC-treated water and the mixture extracted with 125μl of phenol:chloroform (1:1). The RNA then underwent ethanol precipitation by the addition of 12μl sodium acetate (3M pH5) and 400μl 100% ethanol. The mixture was incubated at -20°C for 30 minutes before being microfuged at 13000xg at 4°C for 20 minutes. The supernatant was removed, the pellet washed in 80% ethanol (20% DEPC-treated water) and allowed to air dry. The capped mRNA was raised in 25μl of RNAse-free TE buffer.

2.52: Coupled *in vitro* transcription/translation (TnT)

This method was adapted from the protocol in the TnT kit (Promega). In an RNAse-free microfuge tube, on ice, a reaction mix was made containing (in the following order), 5μl of rabbit reticulocyte lysate (RRL), 0.8μl [^{35}S]-methionine (400Ci/mmol), 0.2μl amino acids minus methionine, 0.5μl TnT buffer, 2.1μl sterile water, 0.2μl T7 polymerase and 0.2μl RNAsin. To this, 1μl (200ng) of plasmid DNA was added, or replaced by 1μl of sterile water if the reaction was a control. (During this work, the plasmid DNA was added to the TnT reactions in both a linear and a circular form. Linear plasmid DNA was achieved by

endonuclease digestion of circular DNA with NOT1, phenol extraction and ethanol precipitation. Both were found to work well, although circular DNA was frequently used as it saved time). The reaction was incubated in a water bath at 30°C for 1.5 hours, after which time 2µl samples were added to 8µl sterile water and 10µl loading buffer. This mixture was heated to 65°C for 5 minutes in order that good denaturation of the protein was achieved, as were [^{14}C]-protein markers (diluted in loading buffer at a 1:20 dilution). TnT samples then underwent SDS-PAGE alongside the protein markers. After the gel front had reached the bottom of the plate, the gel was placed in fixing buffer (20% (v/v) methanol, 10% (v/v) glacial acetic acid) for 30 minutes with gentle agitation. The gel was then rinsed in distilled water for 10 minutes before being treated with Autofluor for a further 20 minutes. The gel was then blotted onto dry 3MM filter paper and transferred to a slab gel drier. The gel was dried at a temperature of 80°C for 1-3 hours (depending on its size) and then exposed to autoradiograph film for a further 18-48 hours.

Coupled TnT reactions in the presence of antisense oligonucleotides

Each concentrated stock of antisense oligonucleotide underwent a series of 1:10 dilutions to provide a range of different concentrations. The method for the coupled transcription/translation of pBSLHR/59 in the presence of antisense oligonucleotides differed from that described above, only by the inclusion of 1µl of antisense oligonucleotide and 1µl less of sterile water.

2.6: Methods - Cell work

2.61: MA10 cell culture

Stock cultures of MA10 cells were maintained in T75 culture flasks in Waymouth's complete medium (Waymouth's MB752/1 medium with L-Glutamine, supplemented with 15% (v/v) horse serum and 50µg/ml gentamicin) at 37°C in an atmosphere of 5% CO_2 in air. The medium was changed every two days. The stocks were split every 3-4 days depending on the cell density.

The cells were subcultured by washing the cells twice with 5ml Ca^{2+}, Mg^{2+}-free PBS (0.01M pH7) before the addition of 3-4ml trypsin solution (0.5% (w/v) porcine trypsin and

0.2% (w/v) EDTA). The flasks were incubated at 37°C for 2-3 minutes. Waymouth's complete medium was then added to the flasks in order to inhibit the trypsin. The cell suspension was then centrifuged at 1000xg for 10 minutes and the cell pellet was resuspended in fresh medium. Cell numbers were determined using a haemocytometer and viability of the cells was scored by the exclusion of 0.4% (v/v) trypan blue dye. For stimulation/desensitisation experiments like those described in *Chapter Six*, cells were plated at density of 100000 cells/well in 24 well plates, cultured in Waymouth's complete medium and used 24 hours after subculture. For immunocytochemistry experiments cells were cultured on sterile coverslips which had been aseptically placed singularly in 24 well plates, or in pairs in 6 well plates. Cells were then plated at either 75000 cells/well (24 well plates) or 300000 cells/well (6 well plates). For receptor autoradiography experiments, cells were cultured on coverslips and plated at 200000 cells/well (24 well plates) or at 500000 cells/well (6 well plates). For receptor binding studies as described in *Chapter Six*, cells were plated at a minimum of 10^6 cells/well (6 well plates).

2.62: Preparation and solubilisation of gonadal cell membranes

Hyperstimulated ovaries were obtained from 21 day old rats after injection with 50 IU PMSG followed 56 hours later with 25 IU hCG. Thirty day old animals were sacrificed by cervical dislocation (i.e. 7 days after hCG administration), by Dr A Michael or Dr R Abayasekara, both holders of Home Office Licences. Ovarian tissue from these hyperstimulated rats or rat testicular tissue (about 100mg), were homogenised in 1ml PBS (0.01M pH7) containing EDTA (5mM) and *N*-ethylmaleimide (5mM). After centrifugation at 120xg for 5 minutes, the supernatant was aspirated and then centrifuged at 27000xg for 30 minutes. The crude membrane pellet was solubilised in 1ml PBS (0.01M, pH7) containing EDTA (5mM), *N*-ethylmaleimide (5mM), 0.1% (v/v) Triton X-100 and 20% (v/v) glycerol, by stirring on ice for 30 minutes. The suspension was centrifuged at 100000xg (Beckman ultracentrifuge) for 1 hour at 4°C and the resulting supernatant was concentrated using the Amicon centriprep-30 concentrators. The membranes from rat testes and liver were prepared the same way. The solubilised membranes were then used for electrophoresis or stored at -70°C.

The membranes of MA10 cells were prepared as described previously by Hipkin *et al.* (1992). Briefly, culture flasks containing the cells were washed twice with ice-cold buffer A (0.15M NaCl and 20mM HEPES, pH7.4) and subsequently scraped into buffer B (buffer A containing *N*-ethylmalemide (5mM) and protease inhibitors (1mM PMSF, 1μM pepstatin-A, 1μM leupeptin, and 1mM EDTA). The cells were pelleted by centrifugation, lysed by vortexing in buffer C (buffer B containing 0.5% (v/v) Nonidet P-40 and 10% (v/v) glycerol), and centrifuged at 100000xg (Beckman ultracentrifuge) for 30 minutes. The supernatant was concentrated using the centriprep-30 concentrators and the amount of protein was assayed by the Bio-Rad protein assay method.

2.64: Bio-Rad method of protein assay

The stock solution of concentrated Bio-Rad dye was diluted 1/5 in distilled water and filtered. Aliquots of solubilised membrane were diluted 1/10 with distilled water. A standard BSA curve was constructed in the range 0-1mg/ml in distilled water. Standard or sample (20μl) were added to 1ml dye, mixed and incubated at room temperature for 5-10 minutes, after which the extinction was measured at 595nm.

2.65: Diaphorase cytochemistry

Cells were plated in 24 well plates and were washed with PBS (0.01M, pH7) and then incubated with phosphate buffer (0.01M, pH7.4) containing 0.001% (w/v) nitroblue tetrazolium and 0.003% (w/v) NADH) for 30 minutes at 37°C. After this incubation the reagent mixture was removed and the cells were fixed with 50% ethanol/10% formaldehyde (v/v) solution. The cells were then incubated at room temperature for a further 30 minutes.

The percentage of positively stained cells (non viable) was estimated by counting a minimum of 5 fields, each containing about 500 cells, under the light microscope.

2.66: SDS-PAGE

A 40% stock acrylamide/bisacrylamide solution (37:1 v/v) was used to prepare gels for SDS-PAGE.

The 10% separating gel was prepared by mixing 19.35ml distilled water, 10ml 40% acrylamide/bisacrylamide solution, 10ml separating gel buffer (1.5M Tris-HCl pH8.8), 0.2ml (w/v) 20% SDS, 7.5µl TEMED, and 450µl 0.1% (w/v) ammonium persulphate. This solution was then poured into a vertical slab gel (size 16cm x 20cm) and overlaid with butanol and left to set for 1 hour at room temperature.

The 5% stacking gel was prepared by mixing 6.95ml distilled water, 2.5ml 40% acrylamide/bisacrylamide solution, 2.5ml stacking gel buffer (0.5M Tris-HCl pH6.8), 50µl 20% (w/v) SDS, 5µl TEMED, and 100µl 0.1% (w/v) ammonium persulphate.

The butanol was removed from the top of the separating gel and the surface of the gel was rinsed with several changes of distilled water. The stacking gel solution was poured onto the separating gel and a well comb added. The stacking gel was allowed to set for 2-3 hours at room temperature. After setting, the gel was placed in the electrophoresis tank (Protean II xi Vertical Electrophoresis Cells, Bio-Rad) containing 1.5 litres of running buffer (0.025M Tris, 0.19M glycine, 0.1% (w/v) SDS, pH8.3).

Aliquots of 50-100µg protein were mixed with an equal volume of Laemmli's sample buffer [see appendix] and incubated for 15-30 minutes at room temperature before being loaded onto the gel. Prestained low range markers (27.5-106kDa) were used as standards. Proteins were stacked within the gel at 30mA constant current and then separated through the gel at 60mA. After electrophoresis the gel was removed and used for protein staining, Western blotting or ligand blotting.

2.67: Protein Staining

SDS-PAGE gels were stained by immersion in Comassie blue staining solution (10% (v/v) acetic acid, 25% (v/v) propan-2-ol, 0.025% (w/v) Comassie blue R-250), for 1-3 hours with gentle agitation. Destaining was then acheived by immersion in a destaining solution (10% (v/v) acetic acid, 10% (v/v) propan-2-ol) for 5-6 hours (or overnight) with gentle agitation.

2.68: Western blot analysis

Electrophoresed SDS-PAGE gels were placed onto nitrocellulose Hybond-C transfer membranes and soaked in transfer buffer (39mM glycine, 48mM Tris, 0.038% (w/v) SDS, 10% (v/v) methanol). Using an LKB 2117-250 Novablot II unit, the resolved proteins were transferred to the nitrocellulose membrane at a constant current of 150mA for 1-1.5 hours. After transfer, the membrane was washed at room temperature in TBS wash buffer (20mM Tris, 500mM NaCl, pH7.5), for 10 minutes with gentle agitation. To prevent non-specific binding of antibodies, 50ml of TTBS blocking solution (TBS containing 0.2% (v/v) Tween 20 with 5% (w/v) milk powder) was added and incubated for 2 hours with gentle agitation at room temperature. The membranes were then washed with wash buffer (TTBS) twice for 5 minutes. Primary antibody (crude antisera were routinely used at a dilution of 1:500, whereas affinity purified antibodies were used at 2μg/ml) or pre-immune IgG (at the same concentration, or dilution) was added in antibody buffer (TTBS supplemented with 1% (w/v) milk powder) and incubated overnight at room temperature. The membranes were then washed three times for 15 minutes with 100ml TTBS with gentle agitation and then 30ml antibody buffer containing 10μl goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3000) was incubated for 1-2 hours at room temperature. The membranes were again washed 3 times with 100ml TTBS and then further washed twice for 10 minutes with TBS to remove the Tween-20. The binding of antibody to the receptor was detected using BCIP/NBT tablets dissolved in water. The solution was added to the membranes and left at room temperature, with constant agitation for 5-10 minutes or until a balance between protein bands and background was achieved. The membranes were then washed in tap water, photographed and stored in the dark at 4°C.

2.7: Methods - Iodinations and radioimmunoassays

2.71: Iodination of hCG

Highly purified hCG (CR-127) was iodinated with ^{125}I according to the method of Thorell and Johanson (1971) using lactoperoxidase, to a specific activity of 100Ci/g and was purified by Sephadex G-25M chromatography. A G-25M Sephadex chromatography column (PD-10) was washed with 30ml PBS (0.01M, pH7) containing 5% BSA (w/v), to block non-specific binding. HCG (10μg) in 50μl PBS (0.2M, pH7), was added to a tube containing a magnetic microflea situated on top of a magnetic stirrer, together with 15μl

phosphate buffer (0.2M, pH7.5), 10µl Na[^{125}I] (1mCi), and 10µl lactoperoxidase (0.5mg/ml solution in 0.05M PBS, pH7). This was then reacted together for 1 minute by the addition of 0.007% (v/v) H_2O_2. The reaction was stopped by the addition of 1ml PBS (0.01M, pH7) containing 0.1% (w/v) NaN_3 and 10mM KI.

The reaction mixture was added to the column and allowed to enter the PD-10 column before continuing. [^{125}I]-hCG was eluted with PBS (0.01M, pH7). Eight drops/tube were collected in the first four tubes and thereafter four drops/ tube were collected in the remaining 26 tubes. The fractions were then measured for radioactivity in a γ-counter. The fractions that formed the first radioactive peak were pooled. The [^{125}I]-hCG was diluted with PBS (0.01M, pH7) to give a concentration of 1ng [^{125}I]-hCG/µl, stored at -20°C and used within 4 weeks of preparation. Estimation of the specific activity of [^{125}I]-hCG was carried out by competitive binding on crude rat testis Leydig or MA10 cells with known concentrations of purified unlabelled hCG. The results were expressed as a percentage of control binding and the concentration of pure hCG that displaced 50% of the activity was assumed to equal the concentration of the radioactive hCG [see binding studies, section *2.93*].

2.72: Iodination of ScAMP-TME

2'-monosuccinyladenosine-3',5'-cyclic monophosphate tyrosyl methyl ester (ScAMP-TME) was radioiodinated as follows and as described by Brooker *et al.* (1979).

The C_{18} Sep-pak (Waters) column was washed with 20ml methanol, followed by 20ml of distilled water. ScAMP-TME (800ng) (20µl of 40µg/ml succinyl-cAMP tyrosyl methyl ester in sodium acetate; 0.005M, pH4.75) was mixed with 20µl potassium dihydrogen phosphate (0.5M, pH7.0), and 10µl of Na-[^{125}I]. Chloramine T (25µg = 5µl of a 5mg/ml solution, in 0.5M potassium dihydrogen phosphate pH7.0) was then added and the tube was capped and vortexed immediately for 1 minute at room temperature. The reaction was stopped by the addition of 50µl of sodium metabisulphite (5mg/ml sodium metabisulphite solution in distilled water). Sodium acetate buffer (800µl of 0.1M, pH7.4) was then added to the mixture.

The reaction mixture was loaded onto the C_{18} column. Propan-1-ol and sodium acetate buffer (0.1M, pH4.75) was mixed together in the ratio 17.5:82.5 and was used for elution. Fractions (1ml) were collected in borosilicate tubes and the radioactivity of each fraction was measured using a γ-counter. The peak activity fractions containing the eluted [^{125}I]-ScAMP-TME were pooled, and propan-1-ol was added to give a final concentration of 30% (v/v).

Radioimmunoassays (RIAs)

In order to assay for cAMP or progesterone, the cell incubations were stopped by the addition of 40µl of perchloric acid (3M) and the plates stored at -20°C. This effectively lysed the cells so that any measurement of these products was a combination of extracellular and intracellular levels. Before assaying, the plates were thawed at room temperature and the acidified samples neutralised by the addition of 80µl of K_3PO_4 (1M).

2.73: Cyclic AMP RIA

cAMP was determined by the method of Steiner *et al* (1972), with the acetylation modification described by Harper and Brooker (1975). For the cAMP standard curve (range 10-5000fmol cAMP/100µl), cAMP standards in ethanol were aliquoted in triplicate then dried under nitrogen and redissolved in 100µl sodium acetate buffer (50mM, pH6.2). This same sodium acetate buffer (100µl) was aliquoted for totals, non-specific binding (NSB), and zero concentration of cAMP (Bo). Samples were diluted as necessary in this buffer to a final volume of 100µl. Both the standards and the samples were then acetylated by the addition during vortexing of 10µl of the acetylating mixture, (2.7:1 triethylamine:acetic anhydride). The tubes were left to stand for at least 1 hour in a fume hood. Bovine gamma globulin (100µl of 0.3% (w/v) in 0.1M PBS, pH7) was added to the totals and NSB. The cAMP antibody (MS1) was diluted in 0.3% (w/v) bovine gamma globulin at 1:150 dilution and 100µl of antibody solution was added to all tubes except totals and NSB. Iodinated cAMP in PBS (0.1M pH7, 20000cpm/100µl) was then added and the tubes vortexed and incubated overnight at 4°C.

PEG 6000 (16% (w/v) dissolved in tap water) was dispensed into each tube (except the totals) to precipitate the antisera and bound cAMP. The tubes were vortexed and

centrifuged at 3000xg for 30 minutes at 4°C. The supernatant (free cAMP) was then aspirated to waste, the tubes dried and the pellets of antibody-bound cAMP measured in a γ-counter.

2.74: Progesterone RIA

A standard curve for progesterone (range 0.25-31.79pmol/ml) was prepared by aliquoting standards in triplicate. Waymouth's medium supplemented with 0.1% (w/v) BSA (100µl) was aliquoted for totals, non-specific binding (NSB) and zero concentration of progesterone (Bo). Samples were diluted as appropriate in this supplemented Waymouth's medium to a final volume of 100µl. PAS-gelatin buffer (PGB) (100µl) [see appendix] was added to totals and NSB tubes. Progesterone antibody (100µl: diluted 1:4000 in PGB), was added to all tubes except totals and NSB. [^{3}H]-progesterone (specific activity 103Ci/mmol) in PGB was diluted to 10000 cpm/100µl and was added to all tubes. The solution was mixed by vortexing and incubated overnight at 4°C. The following day, ice-cold PGB (500µl) was added to the total tubes. Dextran-coated charcoal suspension (500µl; containing 2.5mg charcoal/ml and 0.25mg dextran/ml in phosphate buffer (0.01M pH7)) was added to the remaining tubes at 4°C in order to precipitate the free [^{3}H]-progesterone. The tubes were then mixed by vortexing and centrifuged at 3000xg for 10 minutes at 4°C. The supernatant was decanted into scintillation vials and 2ml of Ultima-gold scintillation fluid was added. The vials were then vortexed and counted for 5 minutes in a liquid scintillation counter to determine the proportion of antibody bound [^{3}H]-progesterone.

2.8: Methods - Desensitisation and PKC inhibitor protocols

The following will describe the general protocols used in *Chapter Six*. MA10 cells were cultured as described in section ***2.61*** in 24 well plates at 100000 cells/well. The cells were incubated overnight and used 24 hours later.

2.81: PKC inhibitor concentration-dependent studies

Test cells were preincubated for 1 hour in warm Waymouth's experimental medium (Waymouth's medium + 0.1% (w/v) BSA + 0.5mM MIX) supplemented with either of the 3 PKC inhibitors at a range of concentrations. Control cells (no inhibitor), were preincubated for 1 hour in warm Waymouth's experimental medium supplemented with the

equivalent concentration of solvent (DMSO for staurosporine and GF109203X, where a final concentration of 1μM of inhibitor = 1μl DMSO and ethanol for RO 31-8220, where a final concentration of 1μM = 0.1μl ethanol). (Cells for measuring basal levels remained in Waymouth's experimental medium throughout the experiment). After preincubation, the spent medium was replaced with fresh as before but with the addition of LH at a final concentration of 100ng/ml. All cells were incubated for a further 2 hours before the reactions were stopped by the addition of 40μl perchloric acid and the cells placed at -20°C overnight before being assayed as described in sections ***2.73*** and ***2.74***.

2.82: Time course studies in the presence/absence of PKC inhibitors

Test cells were preincubated for 1 hour in Waymouth's experimental medium supplemented with one of the 3 PKC inhibitors at either 1μM or 0.1μM. Control cells (no inhibitor), were preincubated for 1 hour in Waymouth's experimental medium supplemented with DMSO to control for staurosporine and GF109203X (1μl/ml or 0.1μl/ml respectively), or ethanol to control for RO 31-8220 (0.1μl/ml or 0.01μl/ml respectively). (Basal cells were incubated as described above). After the preincubation, the spent medium was replaced with fresh as before but with the addition of LH at a final concentration of 100ng/ml. All cells were incubated for up to 3 hours during which, at various time points, reactions were stopped by the addition of 40μl perchloric acid and the cells placed at -20°C overnight before being assayed as described in sections ***2.73*** and ***2.74***.

2.83: Studies to ascertain if staurosporine had cyclic nucleotide phosphodiesterase activity

Cells were preincubated for 1 hour in Waymouths' experimental medium supplemented with staurosporine (1μM), in the presence or absence of MIX (0.5mM). Medium which did not contain MIX was supplemented with the equivalent concentration of DMSO; the solvent in which MIX was dissolved. After this time, all cells were stimulated with LH (100ng/ml) and the experiment stopped at various time points by the addition of 40μl perchloric acid and the cells were placed overnight at -20°C before being assayed for cAMP as described in section ***2.73***.

2.84: Cholera toxin concentration-dependent studies in the presence of 0.1μM staurosporine

Test cells were preincubated for 1 hour in Waymouth's experimental medium supplemented with staurosporine at 0.1μM. Control cells (no inhibitor), were preincubated for 1 hour in Waymouth's experimental medium supplemented with DMSO (0.1μl/ml). (Basal cells were incubated as described in section ***2.81***). After the preincubation, the spent medium from the test cells was replaced with fresh as before but with the addition of cholera toxin at various concentrations [see *Chapter Six*, section ***6.37***]. All the cells were incubated for a further 2 hours after which time the reactions were stopped by the addition of 40μl perchloric acid and the cells placed at -20°C overnight before being assayed as described in sections ***2.73*** and ***2.74***.

2.85: Forskolin concentration-dependent studies in the presence of 0.1μM staurosporine

This proceeded as described above but with a range of forskolin concentrations in place of cholera toxin [see *Chapter Six*, section ***6.38***].

2.86: Cytotoxicity assay

This assay was based on the protocol provided with the CytoTox 96 assay kit (Promega). This assay measures cellular damage by measuring the amount of the enzyme lactate dehydrogenase (LDH) which is released into the medium surrounding the cells as a result of damage to the cellular membrane. A colour reaction then takes place which is measured using an ELISA plate reader at 492nm. Briefly, cells were plated and cultured as described in section ***2.61***. The cells underwent exposure to the PKC inhibitor for 2-3 hours depending on the duration of the experiment. Control cells were incubated in Waymouth's/0.1% BSA only. After this time 50μl aliquots of media were removed from each well and placed in the wells of a 96 well plate. Reconstituted substrate mix (50μl) was added to each of the wells and the plate covered with foil to protect against the light. The plate was incubated at room temperature for 30 minutes after which time 50μl of stop solution was added to each well. (Cells acting as total controls were first lysed by the addition of lysis buffer and incubated at 37°C for 45 minutes before the addition of the substrate mix. Non-specific background was accounted for by adding the assay components to wells containing experimental media

only.) The absorbence of the wells was measured on an ELISA plate reader at 492nm within one hour of the experiment being stopped.

2.9: Methods - LH/CG receptor immunocytochemistry, fluorescent immunohistochemistry, autoradiography and receptor binding assays

2.91: - Receptor immunocytochemistry

This method was adapted for use from the protocol provided in the ABC Vectastain kit. MA10 cells were cultured as described in section *2.61* on plastic coverslips in 24 well plates and washed once in PBS. The cells were fixed in 4% (v/v) formaldehyde in PBS for 30 minutes and were then rinsed six times with PBS. The cells were then dehydrated in 70% ethanol twice, for a minute each time and then in 100% ethanol, twice, for a minute each time. The next step was to treat the cells with 1% (v/v) H_2O_2 in methanol for 30 minutes before washing well with TBS three times and then with glycine (0.1M in TBS) for 30 minutes. The cells were then washed three times with TBS.

As antibody 3 was targeted to an intracellular peptide, when using this antibody, it was first necessary to permeabilise the cells by incubating them in 0.1% (v/v) Triton X-100 in TBS for no longer than 5 minutes. This treatment was not necessary when using antibodies 1 and 2 as they were raised against peptides located on the extracellular domains of the LH/CG receptor. If treated with 0.1% (v/v) Triton X 100, the cells were then washed six times in TBS to removal any residual traces as this detergent is known to contribute to background staining.

A 5% (v/v) solution of normal goat serum was prepared by diluting in TBS. The lid of a 24 well plate was covered in parafilm and 50-75µl drops were placed on this. The coverslips were removed from the wells and placed cell-side downwards onto the goat serum and were left for 30 minutes in an humidified chamber. During this time the antibodies were prepared. Various dilutions were used throughout this work, ranging from 1:100 to 1:500. The dilutions were made in 0.05% (v/v) acetylated BSA in TBS. The drops were placed as before onto a fresh 24 well plate lid, the coverslips placed cell-side downwards and left in an humidified chamber overnight at 4°C. After this time, the coverslips were washed six

times in TBS and the secondary antibody was prepared. Goat anti-rabbit antibody was diluted 1:100 using 0.05% (v/v) acetylated BSA in TBS and the incubation procedure was repeated as previously described for 60 minutes at room temperature. The coverslips were then washed six times in TBS.

The ABC complex of the Vectastain kit was prepared 20 minutes in advance by mixing avidin and biotin at 1:100 dilution using 0.05% (v/v) acetylated BSA in TBS. This was then placed in droplets on the lid of a fresh 24 well plate and the coverslips assembled as before in an humidified chamber for 60 minutes at room temperature. After this time, the coverslips were washed six times in TBS and then once in Tris-HCl (0.05M, pH7.6).

The 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution (0.6mg/ml Tris-HCl (0.05M pH7.6)) was activated by adding 0.03% (v/v) H_2O_2. This was then placed onto the coverslips so as to cover the surface, for 2-5 minutes, or until the cells had turned brown enough. Once adequate colouring had been acheived, the coverslips were washed twice in tap water followed by distilled water. The coverslips were then counterstained by covering the surface with Mayers haematoxylin for 10 minutes. This was then rinsed off under running tap water for 5 minutes, before the coverslips were given a final rinse in distilled water, mounted in aquamount and sealed using clear nail varnish.

2.92: Fluorescent immunohistochemistry

Fresh tissue was frozen in isopentane which had been chilled over dry ice. Frozen 20μm sections were then cut on a cryostat, mounted onto albumin-treated histology slides and stored at -70°C until required.

The frozen sections were fixed in fresh 4% (v/v) paraformaldehyde at room temperature for 30 minutes, after which time they were washed 3 times for 5 minutes in PBS. The slides were then rinsed for 1-2 minutes in PBS + FTAz (PBS + 10% (v/v) horse serum + 0.1% (v/v) Triton X-100 + 0.02% (w/v) sodium azide). Antibodies 1 and 2 were diluted 1:250 in PBS + FTAz. Excess buffer was wiped from the slides to prevent antibody dilution and the sections were covered with 200μl of antibody solution. The slides were loosely covered with parafilm and placed in an humidified chamber at 4°C overnight. The slides were

quickly rinsed in PBS, followed by 3, 5 minute washes in PBS + 0.1% (v/v) Triton X-100. The excess buffer was removed from the slides and the sections were covered with 200µl of donkey anti-rabbit biotinylated immunoglobulin (diluted 1:100 in PBS + FTAz), for 1 hour at room temperature. After this time, the slides were quickly rinsed in PBS, followed by 3, 5 minute washes in PBS + 0.1% Triton X-100. Excess buffer was once again removed and the sections covered with 200µl of streptavidin-fluorescein reagent (diluted 1:100 in PBS + FTAz), for 1 hour at room temperature. The slides were then quickly rinsed in PBS, followed by 3, 5 minute washes in PBS + 0.1% (v/v) Triton X-100. Excess buffer was removed and the slides were mounted in citifluor and visualised on a fluorescent microscope. Photographs were taken using a mounted camera.

2.93: Receptor autoradiography

Two methods of receptor autoradiography were used. Both were essentially the same except that one method used chilled reagents and incubated with [^{125}I]-hCG overnight at 4°C, whilst the second method used room temperature reagents and incubated with [^{125}I]-hCG for 1-1.5 hours at 37°C. The latter method proved more successful.

MA10 cells were cultured as described in section ***2.61*** on plastic coverslips in 24 well plates. The spent medium was aspirated and the coverslips were washed in Waymouth's + 0.1% (w/v) BSA for 5 minutes. [^{125}I]-hCG was diluted in 100ml Waymouth's + 0.1% (w/v) BSA so as to give approximately 100000 cpm/ml. Crude hCG was reconstituted in a small volume of Waymouth's + 0.1% (w/v) BSA and then added to 50ml of [^{125}I]-hCG so as to reach a final concentration of 500 IU/ml. The coverslips were then treated either with 1ml of [^{125}I]-hCG ('hot' hCG), or 1ml of [^{125}I]-hCG + crude hCG ('hot + cold' hCG), and incubated, depending on the method used, as described above. After this time, the spent medium was aspirated. The coverslips were then washed twice for 5 minutes (at 4°C with gentle agitation) with ice-cold Waymouth's + 0.1% (w/v) BSA in order to remove background contamination. The coverslips were blotted dry and exposed to autoradiograph film at -20°C for anything between 1-8 weeks before developing.

2.94: Receptor binding assay

A similar method to the one described above was used to establish whether the PKC inhibitors used in section ***2.83*** had any effect on LH binding to its receptor. Whilst both 4°C and 37°C methods were used, the 37°C method proved more successful. In these experiments cells were plated at a minimum of 10^6/well in 6 well plates, as described in section ***2.61***.

'Hot' and 'hot + cold' solutions were made as described above. Approximately 15ml of the hot medium was supplemented with staurosporine (1μM), whilst the remaining hot medium and the hot + cold medium was supplemented with 0.5μl/ml DMSO. Hot medium supplemented with staurosporine (1μM) was added to the test wells at 2ml/well. Hot medium supplemented with DMSO was added to the 'total' wells and the control wells at 2ml/well. Hot + cold medium supplemented with DMSO was added to the 'NSB' wells at 2ml/well. The cells were then incubated at 37°C for 1.5 hours (or at 4°C for 48 hours). After this time, the medium from all the wells (except that in the 'total' wells which remained), was discarded and the wells washed briefly in ice-cold Waymouth's + 0.1% (w/v) BSA. Fresh, ice-cold Waymouth's + 0.1% (w/v) BSA medium was added to the wells and the cells were scraped from the plate using a rubber policeman and pelleted by centrifugation at 3000xg at 4°C. The supernatant was discarded, fresh ice-cold Waymouth's + 0.1% (w/v) BSA medium was added to wash the cell pellet and the centrifugation step was repeated. This procedure was repeated once more and after the supernatants had been discarded, the cell pellets were counted on a γ counter for 1 minute/tube. The 'total' wells were scraped in the experimental medium and the entire contents of the wells were similarly counted.

This method was also used on cells grown on coverslips as described in section ***2.92***. Test cells were incubated in the presence of hot medium supplemented with staurosporine (1μM) and control cells were incubated in the presence of hot medium supplemented with DMSO (0.5μl/ml). The cells were incubated at either 4°C for 48 hours or at 37°C for 1-1.5 hours. The coverslips were then washed twice for 5 minutes (at 4°C with gentle agitation) with ice-cold Waymouth's + 0.1% (w/v) BSA, blotted dry and exposed to autoradiograph film at -20°C for anything between 1-8 weeks before developing.

2.95: Displacement curve to establish the specific activity of [^{125}I]-hCG

Whilst this method is part of the iodination procedure described in section ***2.71***, it is mentioned here because as a receptor binding assay it is similar in methodology to that described in the previous section. This method was used to determine the specific activity of [^{125}I]-hCG after an iodination procedure. Briefly, 3 x 10^6 cells were added to borosilicate tubes, a concentration range of crude hCG (generally 0-300ng) was added to the tubes and then ice-cold media containing [^{125}I]-hCG at a 1:200 dilution was added at 500µl/tube. The tubes were incubated at 4°C for 48 hours, after which time the cells were pelleted, washed and counted as described above. The results were plotted with counts/minute on the X axis against concentration of crude hCG, allowing the amount of [^{125}I]-hCG that caused 50% displacement of crude hCG to be established. The NSB values were defined as the counts obtained in the presence of 500U of crude hCG, whilst the total values were defined as the counts obtained in the absence of crude hCG or cells.

Chapter Three: The immunopurification of the MA10 LH/CG receptor and immunocytochemistry studies

3.1: Immunopurification

Whilst PCR and cloning techniques provide a powerful way of isolating and analysing the structure of the LH/CG receptor gene, they tell us very little about the functional structure of the LH/CG receptor protein. The *in vitro* transcription and translation work described in *Chapter Five* is involved in the cell-free synthesis of the LH/CG receptor protein, but provides limited information since the protein is in an artificial environment and functionality cannot be assessed. In order to establish what size the mature receptor is, how many, if any, subunits it is composed of, what biochemical modifications contribute to its functional state etc., other techniques are required. The development of techniques such as immunoaffinity purification and immunoprecipitation have proved to be extremely important tools for such research.

Immunoaffinity purification and immunoprecipitation are similar techniques but differ slightly. Immunoaffinity purification is one of the most powerful techniques for the isolation of proteins. Under the proper conditions, purifications of 1000-10000 fold can be achieved routinely in a single step. Immunoaffinity purification protocols take longer than immunoprecipitation protocols as the antibody is bound to a solid support, thus drastically slowing the kinetics of binding. The immunoaffinity purification procedure can be divided into three main steps; the linking of antibody to a solid phase matrix, binding the antigen to the antibody-linked matrix and eluting the antigen from the antibody-linked matrix.

Immunoprecipitation, when linked to SDS-PAGE, can determine important characteristics of the antigen such as its molecular weight, rate of synthesis or degradation, the presence of certain post-translational modifications etc. The immunoprecipitation procedure can be divided into four steps; labelling the antigen of interest (this is not always necessary), lysing cells so as to release the antigen, allowing the formation of antigen-antibody complexes and the purification of the antigen-

antibody. Both types of technique were used in this work and are described more fully in *Chapter Two*, section ***2.4***.

Several investigations have been undertaken to isolate the LH/CG receptor from a variety of species and tissues, including rat testes (Dufau *et al.* 1975), rat ovaries (Kusuda and Dufau 1986, Keinanen *et al.* 1987), bovine ovaries (Saxena *et al.* 1986), porcine ovaries (Wimalasena *et al.* 1986) and murine Leydig tumour cells (Kim *et al.* 1987). These studies have yielded conflicting results indicating the receptor to be made up of 1-4 noncovalently or covalently linked homo- or heteropolypeptide chains. Thus, the reported molecular size of the LH/CG receptor has varied between 12-300kDa. Many of the earlier studies suffered from protocols which resulted in poor yields of relatively impure receptor. The development of immunopurification and affinity purification protocols over the last 10 years has considerably aided this area of research.

Early studies employing chemical or photoaffinity crosslinking techniques by Ascoli and Segaloff (1986) using MA10 cells, Kusuda and Dufau (1986) using rat luteal cells and Minegishi *et al.* (1987) using rat testes, all presented evidence indicating that the LH/CG receptor was present as a single polypeptide chain with a molecular mass of between 73-92kDa. Meanwhile similar studies by Ji *et al.* (1986) using porcine granulosa cells, Hwang and Menon (1984) using rat luteal cells, Bruch *et al.* (1986) using rat luteal cells, all presented evidence that the LH/CG receptor was composed of several subunits with molecular masses ranging from 21kDa-83kDa. One disadvantage of using techniques which involve chemical or photoaffinity crosslinking is that these methods ultimately lead to the analysis of the hormone-receptor complex rather than the free receptor. This is a major drawback when studying the structure of the LH/CG receptor because the hormone is relatively large and has a complex structure (Ascoli and Segaloff (1989). In order to try and circumvent this problem, Kim *et al.* (1987) used biosynthetic labelling to identify the LH/CG receptor of MA10 cells. In this study the workers labelled cells with either [^{35}S]-methionine or [^{35}S]-cysteine in the presence of hCG at 4°C to form the receptor-hormone complex. The hormone-receptor complexes were then extracted using a detergent and immunoprecipitated using an anti-

hCG antibody. The hormone-receptor complex was finally dissociated by exposure to pH3 buffer and analysed on SDS-PAGE. This method resulted in the purification of a 92kDa protein predicted to be the LH/CG receptor. Using this approach the workers were able to demonstrate that the inclusion of two protease inhibitors, EDTA and N-ethylmaleimide, prevented the degradation of the receptor by certain proteases and did not result in the detection of additional bands. However, this approach predicted the molecular mass of the putative LH/CG receptor to be 90-92kDa whereas in a previous study using chemical crosslinking studies the same group had estimated the mass to be 72-83kDa (Ascoli and Segaloff 1986). In a review by Ascoli and Segaloff (1989), the authors suggest that the latter study was the more accurate since the molecular mass of the putative receptor was calculated from the free receptor and not the receptor-hormone complex.

Criteria have been proposed to document the validity of a given proposal for the overall structure of the LH/CG receptor (Ascoli and Segaloff 1989). Firstly, experiments must be done under conditions that are known to prevent receptor degradation. Kellokumpu and Rajaniemi (1985) demonstrated that the LH/CG receptor could be cleaved by proteases found in rat luteal membranes, leading to the detection of a 38kDa or 64kDa fragment in the absence and presence of hCG respectively. These authors showed that whilst compounds such as EDTA and N-ethylmaleimide could prevent such degradation, commonly used protease inhibitors such as leupeptin and pepstatin could only prevent the formation of the 64kDa degradation product. Many of these proteases have been found to contaminate preparations of collagenase and are reported to be capable of degrading the LH/CG receptor (Ascoli and Segaloff 1986). Thus some of the putative receptor subunits previously reported may represent degradation products of the receptor produced by endogenous proteases or by those contaminating collagenase preparations used to disperse gonadal cells (Ascoli and Segaloff 1989). The higher molecular weight forms reported by some workers (Crine *et al.* 1984, Zhang and Menon 1985) have been attributed to products of LH/CG receptor aggregation. The latter has highlighted one of the problems that has considerably hampered the progress of research into the structure/function relationships of the LH/CG receptor [see discussion]. Secondly, the composition of the LH/CG receptor is anticipated to be the

same between sexes and species based on the strong homology found between other GPCRs in different tissues. Thus, in work by Ascoli and co-workers the LH/CG receptor derived from MA10 cells (Ascoli and Segaloff 1986), porcine granulosa cells (Kim *et al.* 1987) and rat luteal cells (Rosemblitt *et al.* 1988) is proposed to consist of a single polypeptide chain. Similar conclusions have been reached by Dufau and co-workers (Kusada and Dufau 1986, Minegishi *et al.* 1987) and by Rebois and co-workers (Rebois *et al.* 1981, Rebois 1982). Finally, the deduced overall structure of the LH/CG receptor should be independent of the methodology used. Thus, Dufau and co-workers (Kusada and Dufau 1986, Minegishi *et al.* 1987), Rajaniemi and co-workers (Kellokumpu and Rajaniemi 1985, Keinanen *et al.* 1987) and Ascoli and co-workers (Ascoli and Segaloff 1986, Kim *et al.* 1987, Rosemblitt *et al.* 1988) have all detected the LH/CG receptor as a single, noncovalently bonded oligomer with a molecular mass of between 73-92kDa, regardless of the methods used (crosslinking, metabolic labelling, immunoprecipitation) (Ascoli and Segloff 1989, Bahl and Sojar 1990).

3.11: Phosphorylation of the LH/CG receptor

The rapid phosphorylation of many G-protein coupled receptors (GPCRs) accompanies stimulus-induced desensitisation, and is therefore postulated to occur in the LH/CG receptor. The role of phosphorylation in desensitisation is discussed in more detail in *Chapter One* and will therefore only be mentioned here briefly.

Desensitisation has been extensively studied in the β_2-adrenergic receptor (β_2AR) (Hausdorff *et al.* 1990), and is achieved by the cAMP dependent protein kinase (PKA) and β_2AR kinase (βARK) mediated phosphorylation of the receptor. The role of β_2AR phosphorylation in agonist-induced desensitisation has been confirmed by site-directed mutagenesis (Dohlman *et al.* 1991). Whether the initial LH/CG-induced desensitisation of the LH/CG receptor is due to its phosphorylation is still unclear, although recent studies have indicated that this would seem increasingly likely. In studies by Rodriguez *et al.* 1992 and Sanchez-Yague *et al.* 1992, experiments using C-terminally truncated rat LH/CG receptors, that therefore lacked potential phosphorylation sites, indicated that these receptors did not demonstrate an hCG-induced desensitisation response. In fact, these mutated receptors actually demonstrated a two-fold increase in the level of

hCG-induced cAMP when compared to cells expressing the full-length LH/CG receptor. Such results support the hypothesis that LH/CG receptor desensitisation is brought about by its phosphorylation. However, conflicting results were obtained in a later study by Zhu *et al.* (1993), whereby the deletion of a similar region of the murine LH/CG receptor did not result in the prevention of LH-induced LH/CG receptor desensitisation. Work by Hipkin *et al.* (1995) demonstrated that hCG and PMA induced phosphorylation of the LH/CG receptor mapped to serines S^{635}, S^{639}, S^{645} and S^{652} in the cytoplasmic tail. Removal of these residues was found to prevent hCG- or 4ß-PMA induced phosphorylation, impair hCG- or 4ß-PMA-induced desensitisation and enhance hCG-induced down-regulation [see section ***1.64***] (Wang *et al.* 1996). Recently, stably transfected cell lines have been made that express a mutated full-length rLHR in which the putative major loci for phosphorylation, S^{635}, T^{638}, S^{639}, S^{649} and S^{653}, have been simultaneously mutated to alanine residues (Wang *et al.* 1997). In response to hCG the mutated receptor demonstrated a level of phosphorylation that, whilst severely blunted compared to the wild-type receptor, was not completely abolished. Likewise, when compared to cells expressing the wild-type receptor, PMA-induced desensitisation was significantly reduced in cells expressing the truncated receptor and hCG-induced desensitisation was delayed, although not abolished. These results suggest that phosphorylation of rLHR is necessary, but not sufficient, for LH/CG receptor desensitisation.

3.12: Immunocytochemistry and the LH/CG receptor

Both immunocytochemical and immunohistochemical techniques play an important role in diagnostics and research. The immunocytochemical localisation of antigens in cellular preparations and tissue sections is a key step in understanding the cellular functions of proteins. Immunocytochemical and histochemical techniques are often used in conjunction with other methods, in studies involved in the localisation of antigens in so-called 'novel' tissues. For example, the LH/CG receptor has recently been demonstrated in many non-gonadal tissues, by these techniques. Some examples of such findings are LH/CG receptor expression in human umbilical cords (Rao *et al.* 1993), human fallopian tubes (Lei *et al.* 1993a), human cytotrophoblasts (Shi *et al.* 1993, Tao *et al.* 1995) and human uterine arteries (Toth *et al.* 1994). Earlier studies

rting the use of LH/CG receptor antibodies (Luborsky and Behrman 1979, sikko and Rajaniemi 1981 and Metsikko and Rajaniemi 1984), were limited by the odies demonstrating species specificity and/or low titres (Pallikaros *et al.* 1995). r work by Pallikaros *et al.* (1995), reported the characterisation of LH/CG receptor directed antibodies that recognised the LH/CG receptor from both rat testis, rat y and MA10 cells at normal levels of LH/CG receptor expression. These antibodies e raised to three different peptide sequences found on the N-terminus and C- inus of the rat ovarian LH/CG receptor. Antibody 1 was raised against a peptide uence found between residues 48-65, antibody 2 was raised against a peptide uence found between residues 187-206 and antibody 3 was raised against a peptide uence found between residues 622-636. The two N-terminal anti-peptide antibodies tibodies 1 and 2), were found to inhibit the binding of [^{125}I]-hCG and the LH- ulated production of cAMP and progesterone (at 10µg/ml). Conversely, the C- minal anti-peptide antibody (antibody 3), had no effect on either [^{125}I]-hCG binding LH-stimulated cAMP and progesterone production.

: Aims

th regards to immunopurification, the aims of this work were two-fold. The first aim s to establish an immunoaffinity purification and/or an immunoprecipitation protocol, ng the LH/CG receptor anti-peptide antibodies that had been previously raised in this partment (Pallikaros *et al.* 1995). With this established, the second aim was to ject MA10 cells to various treatments in the presence of [^{32}P]-ATP and to induce or mic desensitisation. The cells would then be solubilised and the LH/CG receptor munopurified. Analysis of these results would reveal whether the MA10 LH/CG eptor was phosphorylated when desensitised and in addition, whether it was co- munopurified with other proteins. If co-immunopurification with other proteins was monstrated, it would be possible to identify their phosphorylation state.

addition to the above, a third aim of this work was to establish whether the LH/CG eptor anti-peptide antibodies could be successfully used for the munocytochemical detection of the LH/CG receptor. Fixed MA10 cells grown on verslips and frozen rat testicular sections, would be subjected to

immunocytochemical and immunohistochemical staining techniques using these antibodies. It was anticipated that the antibodies would prove to be a useful tool in the immunocytochemical localisation of the LH/CG receptor, particularly when used in conjunction with other techniques such as receptor autoradiography.

3.3: Results

3.31: Anti-peptide antibody purification by affinity chromatography

As described in *Chapter Two*, section ***2.41***, the antipeptide antibodies were affinity purified by loading rabbit serum onto a peptide linked, sepharose column. After elution, dialysis, concentration and Bio-rad protein content determination, the antibodies underwent SDS-PAGE in order to check for purity. ***Figure 3.31a*** shows the results of such a purification procedure of antibodies 1 and 2, after SDS-PAGE and protein staining of the gel. Two different concentrations procedures were used after the antibodies had been dialysed. Antibody 1 was concentrated by centrifugation in Centricon 50 tubes, whilst antibody 2 was concentrated by NH_4SO_4 precipitation. It can be seen that the antibody 1 preparation reached a higher protein concentration than antibody 2, possibly reflecting the differences in effectiveness of each concentration method. The profile of the antibodies appears to differ from what would be expected from the IgG class. This consists of two identical heavy chains each with a molecular mass of 50kDa and two identical light chains each with a molecular mass of 25kDa (Fleischman 1963). The areas of protein staining above and around the largest marker of 106kDa are likely to correspond to either the whole antibody or a combination of heavy and light chains, whilst the major band with a molecular mass of approximately 55kDa corresponds to individual heavy chains. Faint bands with a molecular mass of 25kDa were detected but cannot be seen on this picture, however, if these bands were IgG light chains it would be anticipated that they would be present in similar quantities to that of the heavy chains.

A second method of antibody 1 immunoaffinity purification was also attempted using a sepharose column linked with an anti-IgG antibody. This column would isolate all IgG species and not just antibody 1. However, as antibody 1 would be present in the IgG fraction this mix was also used in Western blotting procedures. ***Figure 3.31b*** shows the results from this purification procedure after SDS-PAGE and protein staining of the gel. As can be seen from the photograph, intense protein staining, ranging in size from approximately 50kDa-<106kDa, indicated that a large amount of IgG had been isolated. The intense protein staining seen between markers 27.5kDa and 32.5kDa in size is likely to represent the light chains of IgG which are approximately 25kDa in size.

3.32: Demonstrating antibody specificity

The newly purified antibodies 1 and 2 were used in a dot blot experiment to confirm their specificity. Peptides 1 and 2 were dotted onto nitrocellulose membrane in increasing concentrations and were then incubated with each of the antibodies. *Figure 3.32* shows the results from such an experiment. It can be seen that antibody 1 has recognised peptide 1 but not peptide 2, whilst the reverse is true for antibody 2. Since the peptides were dissolved in DMF, a control dot of this solvent was also included in the dot blot to act as an indicator of the level of non-specific background.

3.33: Membrane solubilisation

Having successfully immunopurified antipeptide-antibodies from rabbit serum, the next step was to obtain a solubilised membrane preparation containing the LH/CG receptor. The procedures involved in this process are described in *Chapter Two*, section *2.62*. Most of the work described hereafter involved the use of membranes prepared from MA10 cells or rat testes, however, in some instances membranes were prepared from hyper-stimulated rat ovaries or from rat organs such as lung and liver. In each case, the membrane solubilisation procedure followed was the same. *Figure 3.33* shows the results from a solubilisation procedure after SDS-PAGE and protein staining of the gel. Both MA10 cells and hyper-stimulated rat ovaries were used. Although differences can be seen between the two protein profiles, it is impossible to identify one band as corresponding to the LH/CG receptor. An intense area of staining can be seen in the hyper-stimulated ovaries with a molecular mass of approximately 60kDa. Although this banding is less apparent in the MA10 cells, it is unlikely that this corresponds to the LH/CG receptor as previous work in this department (Pallikaros *et al.* 1995) has indicated that the LH/CG receptor detected in this way has a an apparent molecular mass of between 95-100kDa.

3.34: Western blotting

A duplicate gel half from *Figure 3.33*, underwent a Western blotting procedure as described in *Chapter Two*, section *2.68*, using the newly immunopurified antibody 1. As can be seen from *Figure 3.34a*, no distinct bands were obtained that are of the correct size to be the LH/CG receptor. Similar results were obtained in a different experiment using the newly immunopurified antibody 2 (*Figure 3.34b*).

The results seen in *Figures* ***3.34a*** and ***3.34b*** were in contrast to those obtained by Pallikaros (1995),which can be seen in *Figure* ***3.34c***. It was therefore assumed that the newly immunopurified antibodies were different in some way, from those originally purified. In order to clarify this matter, the Western blotting procedure was repeated using the original antibody 1. The results from this Western blot can be seen in *Figure* ***3.34d***. Areas of intense staining can be seen corresponding to proteins with a molecular mass of approximately 100kDa.

3.35: Immunoprecipitation of the MA10 LH/CG receptor

Since the newly immunopurified antibodies 1 and 2 could not demonstrate LH/CG receptor recognition on Western blots, the remaining original stocks of antibody 1 were used for the immunopurification procedures. The first method to be used in this work was that of immunoprecipitation. In order to immunoprecipitate the LH/CG receptor using the antipeptide antibodies, it was first required to incubate the two components together. This method involved the use of magnetic beads that had been precoated with anti-rabbit IgG. The solubilised membrane preparations were incubated with the antibody, after which time the antibody-antigen complexes were then incubated with the anti-rabbit IgG coated beads. The beads linked to the antibody-antigen complexes were retrieved by use of a magnetic particle concentrator and the LH/CG receptor eluted by various methods. The results from this work were disappointing and it was not possible to obtain any results using the anti-rabbit coated magnetic beads in the immunoprecipitation protocol. For this reason the second method of immunoaffinity purification was tried.

3.36: Immunoaffinity purification of the MA10 LH/CG receptor

This method involved the use of an activated sepharose matrix. The antipeptide antibodies were chemically coupled to the sepharose in such a way that ligand attachment occurred via amide groups. After loading the solubilised membrane solution onto the column, the LH/CG receptor was eluted from the column by the addition of salt solution. The eluent was dialysed, concentrated and electrophoresed by SDS-PAGE. Both methods are described more fully in *Chapter Two*, section ***2.4***. *Figure*

3.36a shows the results from an immunoaffinity purification experiment that coupled antibody 1 to an activated sepharose matrix. One half of this SDS-PAGE gel was stained for protein. Distinct protein bands can be seen with a molecular mass of approximately 60kDa. A protein of this size was thought unlikely to be the LH/CG receptor as it was anticipated that LH/CG receptor isolated in this way would have a molecular mass of between 95-100kDa (Pallikaros *et al.* 1995). However, it was thought possible that this band may represent a degraded form of the LH/CG receptor. ***Figure 3.36b*** shows the results obtained when the duplicate half of the gel underwent a Western blotting procedure using antibody 1. It can be seen that the 60kDa protein bands were not detected by Western blotting, whilst a region of intense staining that was absent on the protein stained gel, can be seen on the Western blot, corresponding to a molecular mass of <106kDa.

Because of the large size, it was anticipated that the nature of the protein(s) detected in ***Figure 3.36b,*** was that of unbound antibody 1 that had been eluted from the column during the salt wash. In order to establish whether this was in fact the case, a sample of antibody 1 was electrophoresed by SDS-PAGE and a Western blotting procedure performed using only the secondary antibody. Since the secondary antibody was goat anti-rabbit IgG, it was anticipated that this would detect the protein if it was residual antibody 1, since it was rabbit IgG. ***Figure 3.36c*** shows the result from this experiment. The detection of proteins with a smaller molecular mass than those seen in the previous photograph can be seen in ***Figure 3.36c***. However, it is probable that the detection of these proteins is due to the greater concentration of antibody 1 on this gel, relative to those anticipated to have been detected in ***Figure 3.36b***. Since the profile of staining was otherwise similar to that seen in the previous experiment, it was thought probable that the staining seen in ***Figure 3.36b*** was that of residual antibody 1 which had contaminated the elution step designed to remove any bound antigen. It cannot be ruled out however, that staining in ***Figure 3.36b*** was not that of the immunoprecipitated LH/CG receptor which was at too low a concentration to be detected by protein staining. The nature of the 60kDa protein band was not established. However, it is conceivable that it represents a degraded form of the LH/CG receptor that lacked the epitope to which antibody 1 had been raised, thus eluding detection by Western

blotting. Despite further experiments it was not possible to obtain convincing evidence to suggest that the LH/CG receptor had been isolated via this method.

3.37: Immunocytochemistry using anti-peptide antibodies

Immunocytochemistry studies were carried out using the originally purified stocks of antibodies 1, 2 and 3 on MA10 cells, as described in *Chapter Two*, section ***2.91***. The best pictures were taken when cells were at a low level of confluency, as after this they had a tendency to grow over one another, resulting in a distorted view. *Figure **3.37a*** shows the results of immunostaining on MA10 cells using an antibody termed P1B4. Immunostaining with this antibody was used as a positive control. *Figure **3.37b*** shows the results from a negative control, whereby serum had been substituted for primary antibody. The positive control can be seen to immunostain in and around the cytoplasm of the MA10 cells, as a diffuse brown colour. By contrast, this colouration is largely absent in the negative control.

Antibodies 1 and 2 were both shown to stain MA10 cells in pattern similar manner to the positive control (compare *Figures **3.37c*** and ***3.37d*** with *Figure **3.37a***). As a result the immunostaining observed when using antibodies 1 and 2 was thought to be specific. From these pictures it can be seen that the presence of the LH/CG receptor is not only on the cellular membranes but also inside the cytoplasm, probably attached to intracellular membranes. Evidence for such a distribution has previously been reported (Gulyas *et al.* 1981). The results obtained using antibody 3 (*Figure **3.37e***) however, demonstrates a different pattern of immunostaining from that of the positive control. (Since antibody 3 was directed to an intracellular peptide which would not be accessible unless the cells were permeabilised, it was first necessary to treat the cells with a Triton X-100 (0.1% v/v)).The reason for the differences in the pattern of immunostaining when the results from antibodies P1B4, 1 and 2 are compared to that observed with antibody 3 cannot be explained, but it may be due to this antibody 3 staining non-specific proteins.

3.38*:* Fluorescent immunohistochemistry using anti-peptide antibodies

Fluorescent immunohistochemistry was carried out as described in *Chapter Two*, section ***2.92***. Using this procedure, antibody 2, but *not* antibody 1, was demonstrated to recognise and bind to the Leydig cells of the rat testes. *Figures **3.38a*** and ***3.38b*** show the results from immunohistochemistry on rat testicular tissue, using antibody 2. The seminiferous tubules can be seen as circular structures, inbetween which are located the Leydig cells, seen here fluorescing an intense yellow colour. *Figure* **3.38c** shows the immunohistochemical results of a control section where the primary antibody was omitted. Very little staining of the Leydig cells can be seen when compared to the previous and following figures. The negative results observed when staining was carried with antibody 1 can be seen in *Figure **3.38d***. In light of the positive results observed when using antibody 1 in immunocytochemistry experiments, it was suprising that they were not forthcoming when used in fluorescent immunohistochemistry. However, as is explained in the discussion, the retention of antigenicity is very dependent on the experimental and fixation conditions used. For this reason, different antibodies can generate seemingly conflicting results when different protocols are used.

In this next cross-section (*Figure **3.38e***), in addition to the Leydig cells, it would seem that a blood vessel has also been fluorescently stained. Whilst the staining of the blood vessel cannot be categorically described as specific (no control sections had blood vessels in them), there have been reports of endothelial cells lining the walls of blood vessels in the gonads, containing LH/CG receptors (Toth *et al.* 1994).

The antibodies were also used in non-fluorescent immunohistochemistry on paraffin embedded sections of human ovary, as described in *Chapter Two*, section ***2.91***. Using this method of immunostaining, antibody 3 appeared to specifically stain both granulosa and thecal cells in a human preovulatory follicle, but only after the section had first been treated with Triton X-100 (*Figure **3.38f***). *Figure **3.38g*** demonstrates the negative control where serum was used in place of the primary antibody.

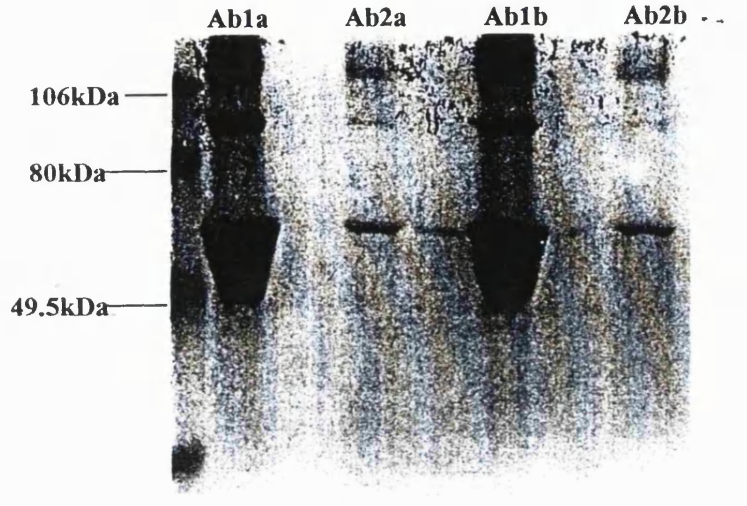


Figure 3.31a: **Anti-peptide antibody purification by affinity chromatography.** Comassie blue protein-stained SDS-PAGE of antibodies 1 and 2 purified by immunoaffinity purification using a peptide-linked sepharose column. The antibody 1 samples were found to be more concentrated than the antibody 2 samples. (Ab1a = 50µl and Ab1b = 40µl of an eluent containing 1µg/µl protein. Ab2a = 50µl and Ab2b = 40 µl of an eluent containing 0.2µg/µl protein. See section ***2.63***)

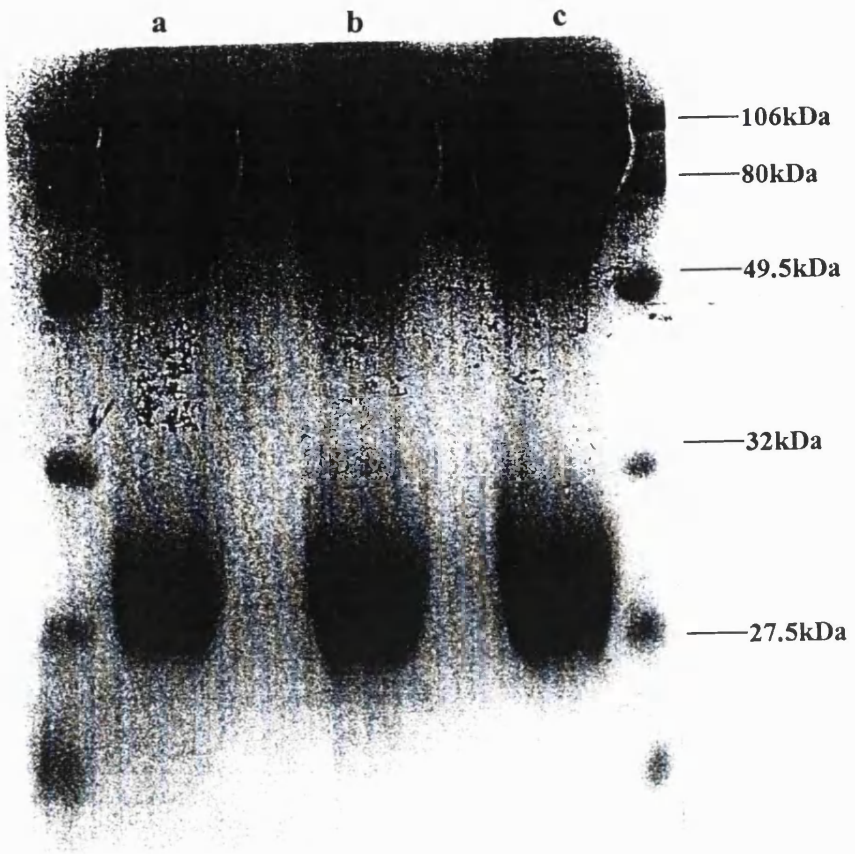
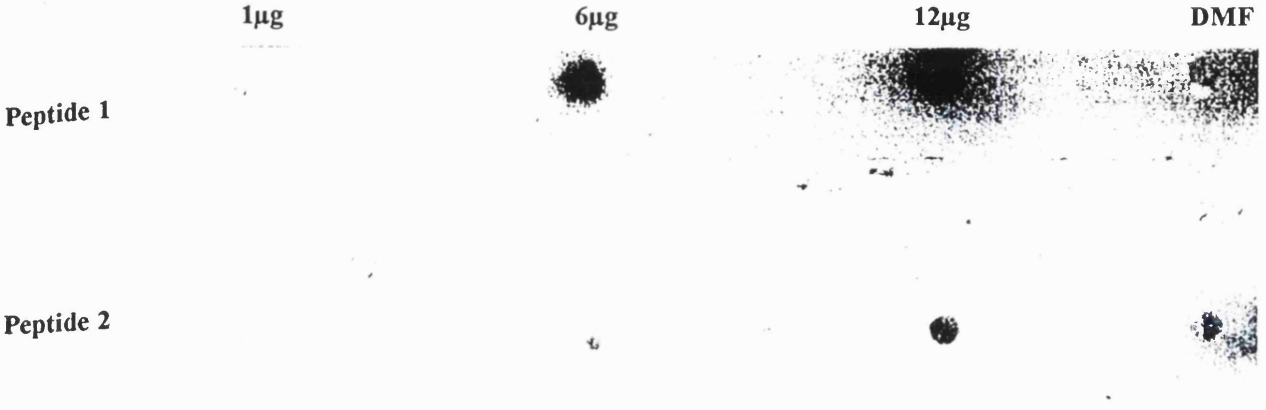
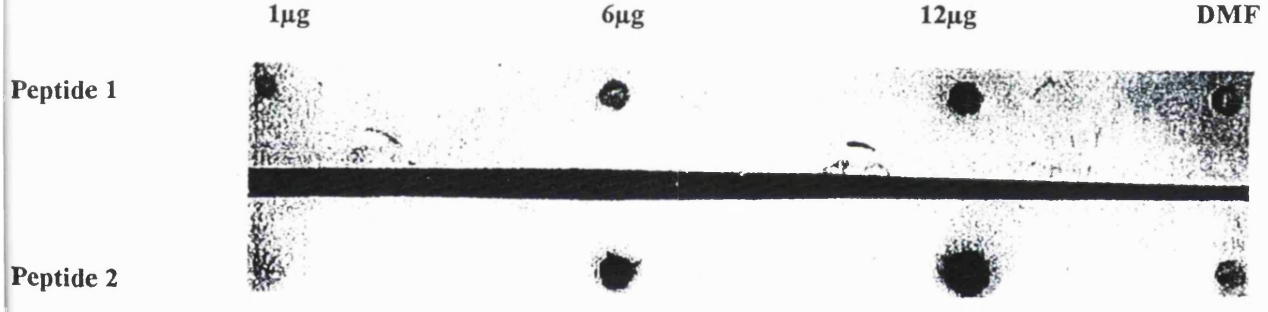


Figure 3.31b: **Anti-IgG antibody purification by affinity chromatography.** Comassie blue protein-stained SDS-PAGE of rabbit IgG purified by immunoaffinity purification using an anti-IgG linked sepharose column. Lanes a, b and c represent 30µl, 40µl and 50µl loadings respectively, of an eluent containing 5µg/µl protein. (See section ***2.63***.)



Dot blot of peptides 1 and 2 incubated with antibody 1



Dot blot of peptides 1 and 2 incubated with antibody 2

Figure 3.32: **Antibody peptide specificity.** The newly purified antibody 1 and antibody 2 were each incubated with peptides 1 and 2 which had been dotted onto nitrocellulose membrane in increasing concentrations, in order to confirm that each antibody specifically recognised its respective peptide.

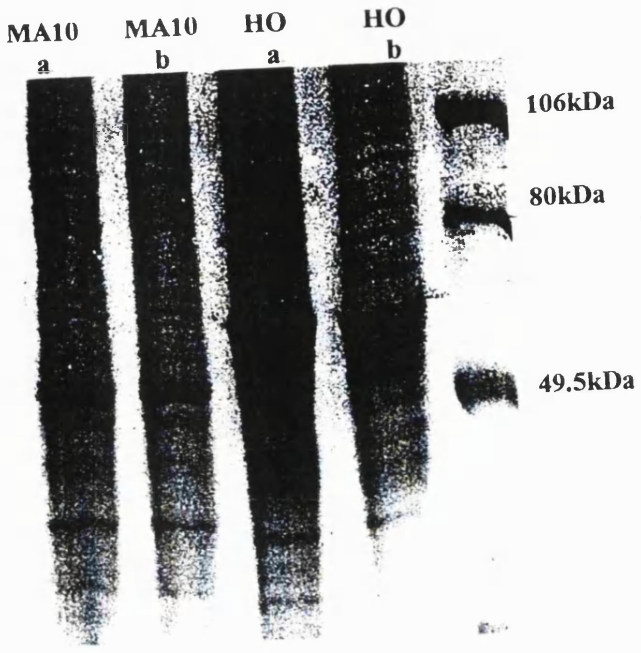
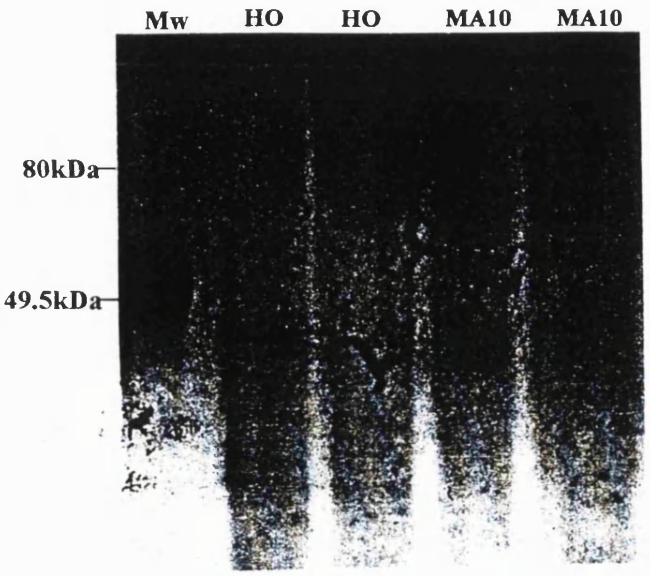


Figure 3.33: **Membrane solubilisation**. A Comassie blue protein-stained SDS-PAGE of solubilised membranes from MA10 cells (a = 40μg protein, b = 25μg protein) and hyperstimulated rat ovaries (HO) (a = 40μg protein, b = 25μg protein).

Figure 3.34a



Figures 3.34a and b: **Western blotting using newly purified antibodies.** These two photographs show the results of a Western blotting procedure performed on the duplicate gel half from *Figure 3.33*. (HO = hyperstimulated rat ovaries). Using both antibody 1 (*Fig. 3.34a*) and 2 (*Fig. 3.34b*) which had been recently immunopurified [see *Figure 3.31*], it was not possible to detect any protein bands with a molecular mass corresponding to the LH/CG receptor.

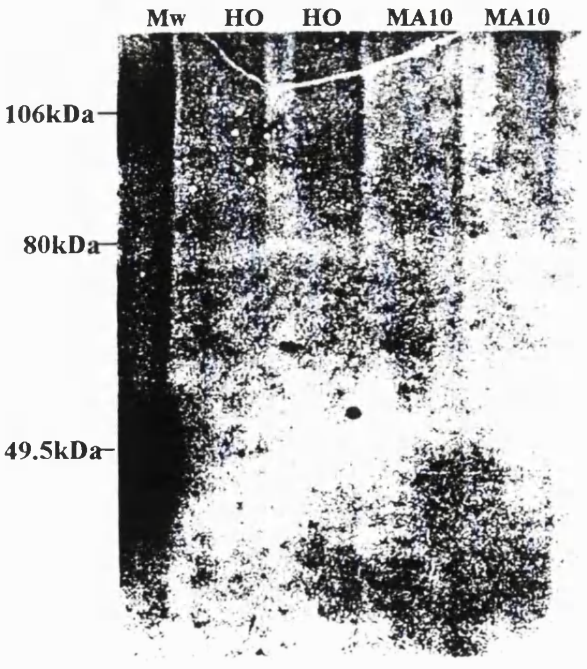
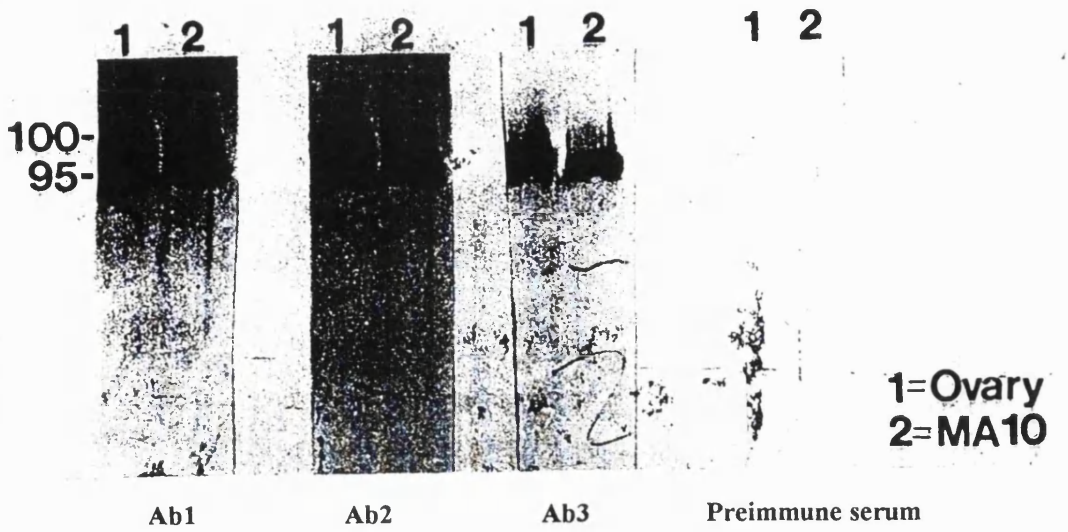


Figure 3.34b

Figure 3.34c



***Figures 3.34c* and *d*: Western blotting using original stocks of antibody 1.** *Figure 3.34c* shows the results previously obtained by Pallikaros (1995) using his immunopurified stock of antibody 1. Bands with a molecular mass of approximately 95-100kDa can be seen in both MA10 and hyperstimulated rat ovary membrane preparations, corresponding to the LH/CG receptor. *Figure 3.34d* shows the results obtained in this work when Western blotting was performed using antibody 1 previously purified by Pallikaros (1995). In this experiment bands of a similar size were also clearly detected in both MA10 and rat hyperstimulated ovary preparations, corresponding to the LH/CG receptor.

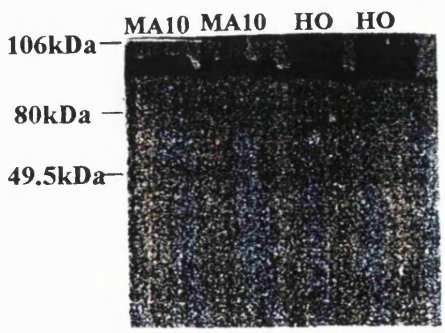


Figure 3.34d

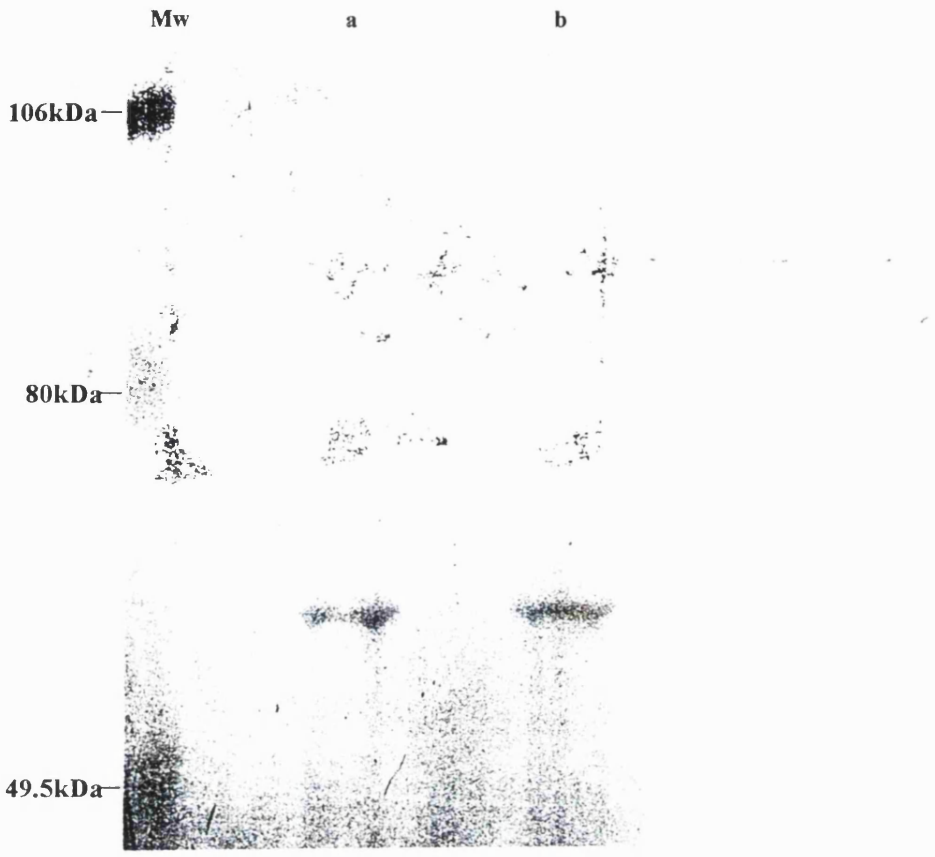


Figure 3.36a: **Immunoaffinity purification.** *Figure 3.36a* shows the results obtained from an immunoaffinity purification of MA10 cell solubilised membranes. The eluent underwent SDS-PAGE electrophoresis. After Comassie blue staining of the gel, distinct protein bands with a molecular mass of approximately 60kDa can be seen, possibly representing a degraded form of the LH/CG receptor. Lane a = 50µl and lane b = 30µl of an eluent containing 0.25µg/µl protein.

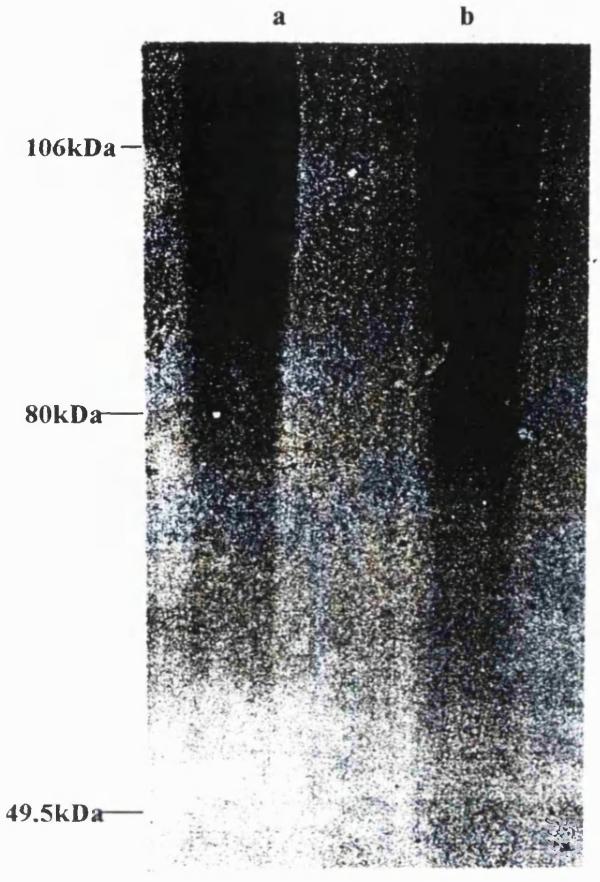


Figure 3.36b shows the results after the duplicate half of the gel seen in *Figure 3.36a* was Western blotted using antibody 1. As can be seen an area with a large molecular mass stained intensely on a part of the gel were no protein staining could be detected. Conversely, the protein bands seen on the gel have not immunostained on the Western blot. Lane a = 30µl and lane b = 50µl of an eluent containing 0.25µg/µl protein.

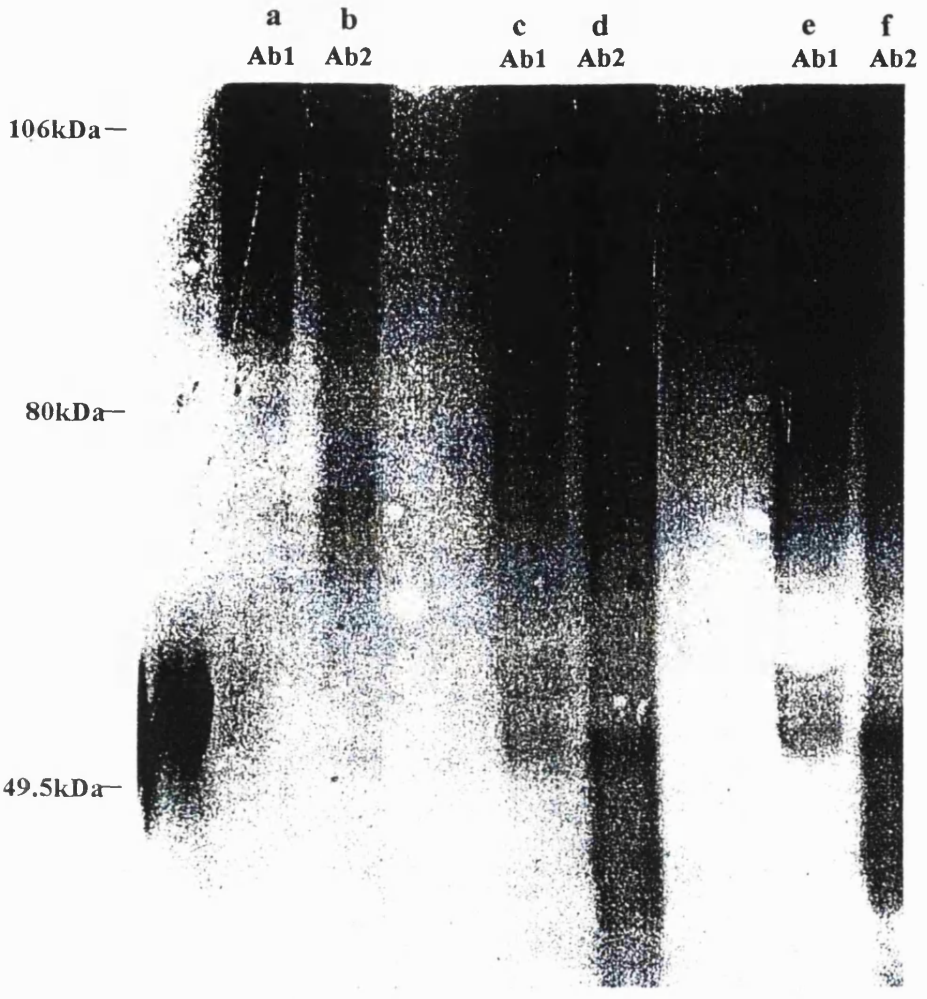
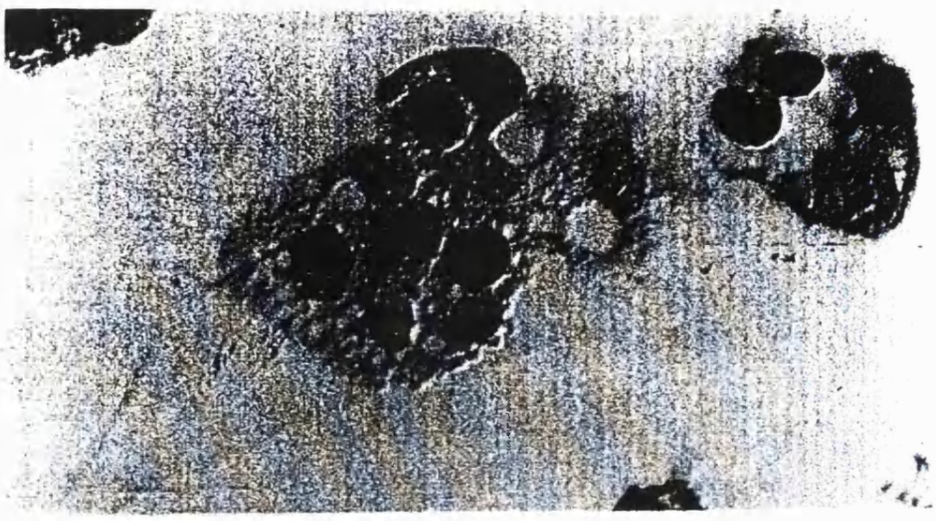


Figure 3.36c: **Immunoaffinity purification.** This photograph shows the results from an experiment to determine whether the protein immunostained in *Figure* ***3.36b*** was that of antibody 1. Samples of antibodies 1 and 2 were electrophoresed and transferred to membrane. The membrane then underwent a Western blotting protocol using only the secondary anti-rabbit antibody. The results after colour development can be seen. The large areas of staining are similar to that seen in *Figure* ***3.36b*** suggesting that the protein eluted after immunoprecipitation was indeed residual antibody 1 that had been recognised by the secondary antibody resulting in immunostaining. Lanes a, c and e = 25μg. 40μg and 50μg of antibody 1 respectively, whilst lanes b, d and f = 25μg, 50μg and 60μg of antibody 2 respectively.

Figure 3.37a **x400**



***Figures 3.37a* and *b:* Immunocytochemistry**.(Magnification x400). *Figure 3.37a* demonstrates MA10 cells immunostained with the LH/CG receptor antibody P1B4, used in these experiments as a positive control. Positive staining can be seen as a brown colouration around the blue stained nuclei. *Figure 3.37b* demonstrates the results of a negative control whereby the primary antibody was replaced with serum.

Figure 3.37b **x400**

Figure 3.37c **x400**

Figures 3.37c* and *d:* Immunocytochemistry.** (Magnification x400). These figures demonstrate MA10 cells immunostained with either antibody 1 (*Figure **3.37c) or antibody 2 *(Figure **3.37d***). Positive staining can be seen as a brown colouration. This staining is thought to be specific as it is very similar to the pattern of staining seen in the positive control (*Figure **3.37a***).

Figure 3.37d **x400**

x400

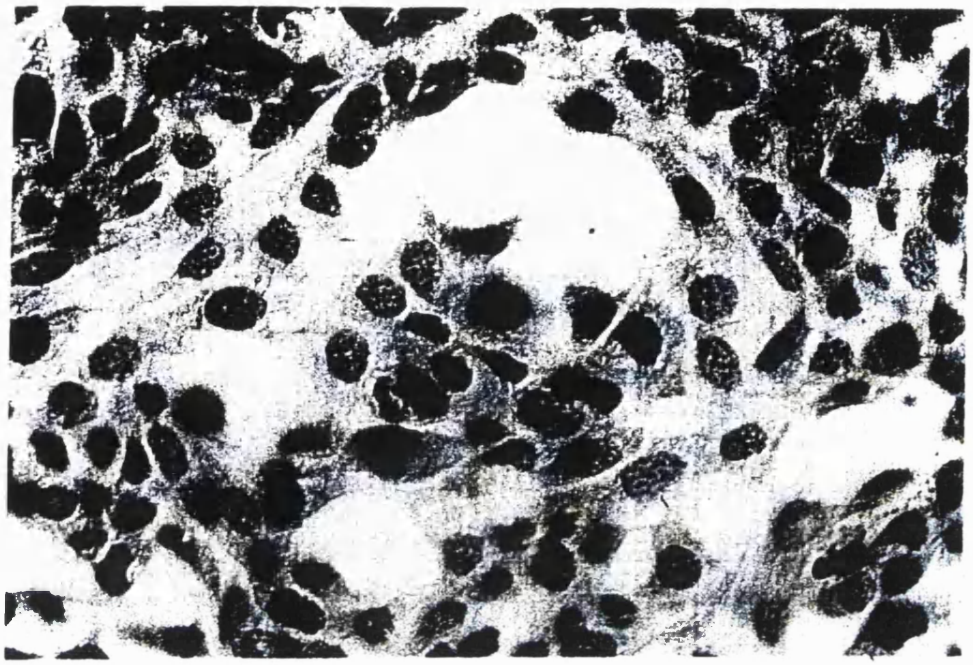
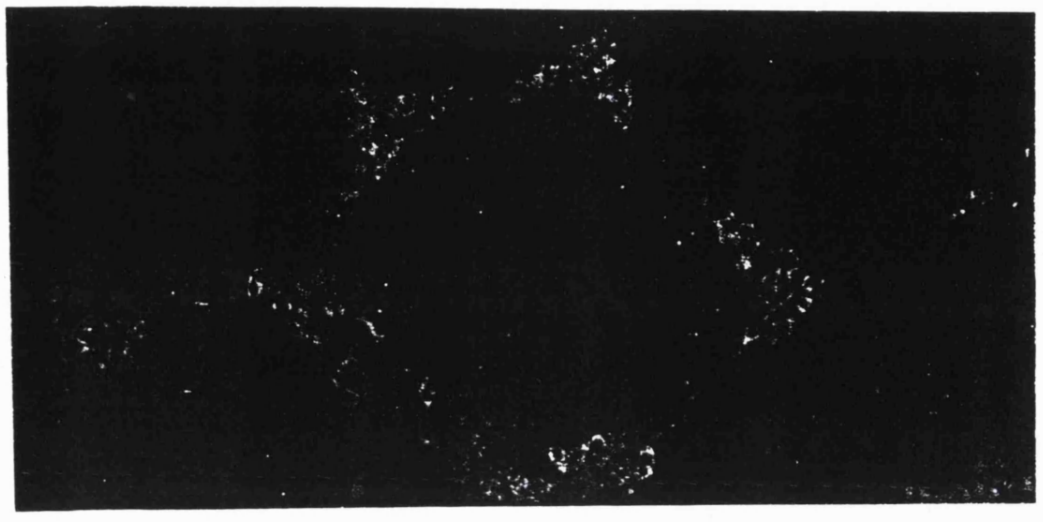


Figure 3.37e: **Immunocytochemistry.** (Magnification x400). This figure demonstrates the results obtained after MA10 cells had been pretreated with Triton X-100 and then immunostained with antibody 3. Any brown staining seen is thought to be non-specific as the pattern of staining is not as is seen in the positive control (***Figure 3.37a***), but is more concentrated in the nuclei.

Figure 3.38a **x400**



***Figures 3.38a* and b: Fluorescent immunohistochemistry**. (Magnification x400 and x200 respectively). These figures demonstrate the positive results obtained when antibody 2 was used in immunohistochemistry experiments using frozen rat testicular tissue. Fluorescent yellow immunostaining can be seen on the Leydig cells surrounding the seminiferous tubules.

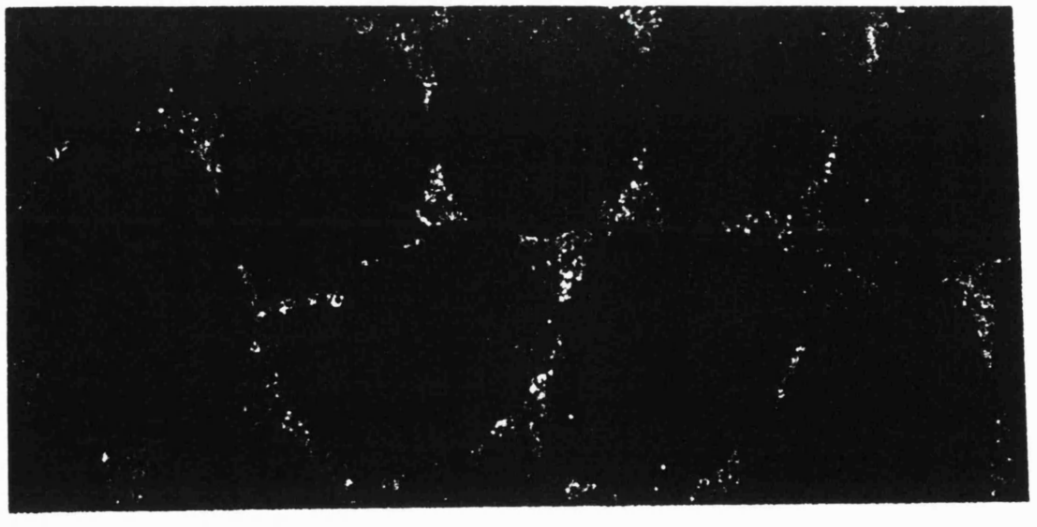
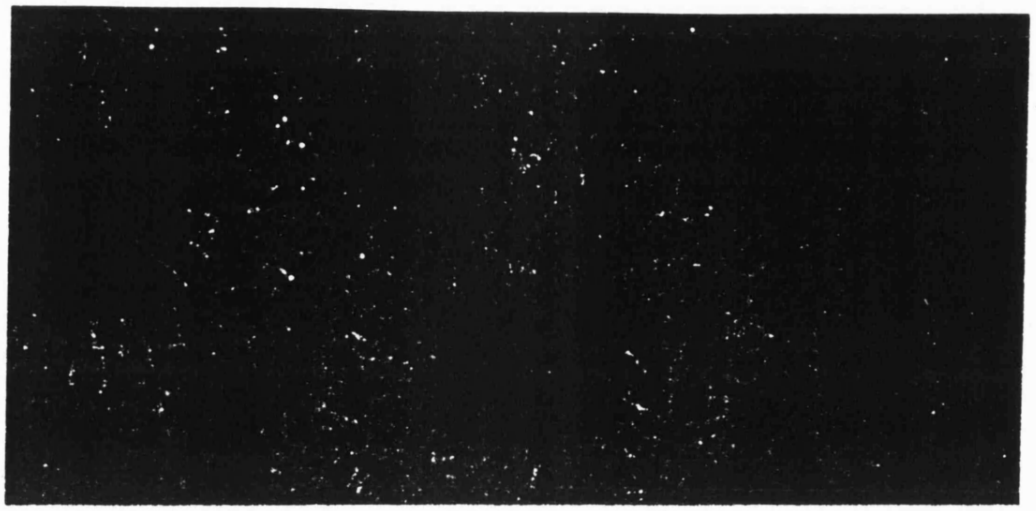


Figure 3.38b **x200**

Figure 3.38c **x400**



Figures 3.38c **and d*:* Fluorescent immunohistochemistry.** (Magnification x400). *Figure* ***3.38c*** demonstrates the results obtained from a negative control whereby the primary antibody had been omitted. Similar results to the negative control were obtained when antibody 1 was used as the primary antibody (*Figure* ***3.38d***). These results indicated that antibody 1 did not recognise its specific antigen under these conditions of tissue fixation.

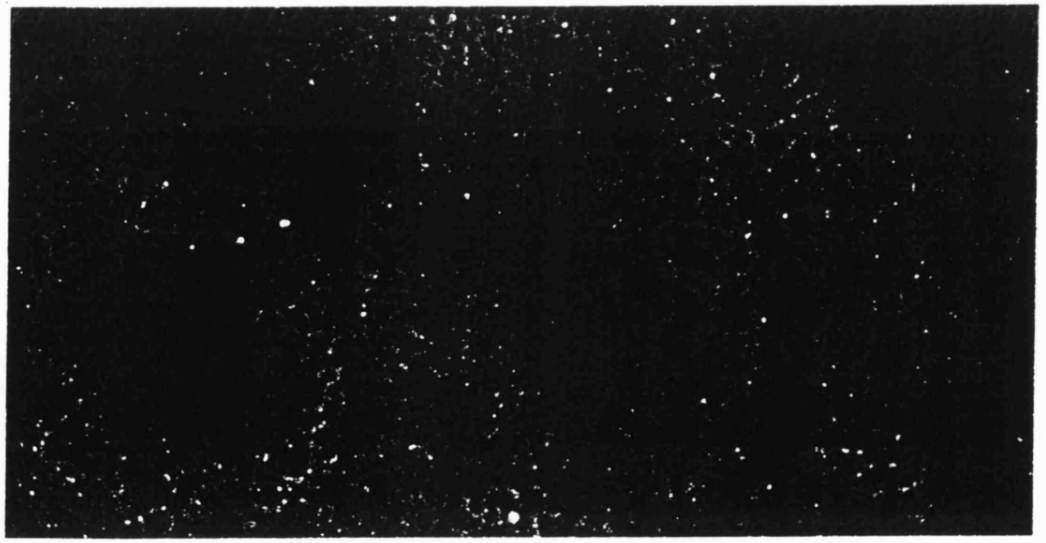


Figure 3.38d **x400**

x400

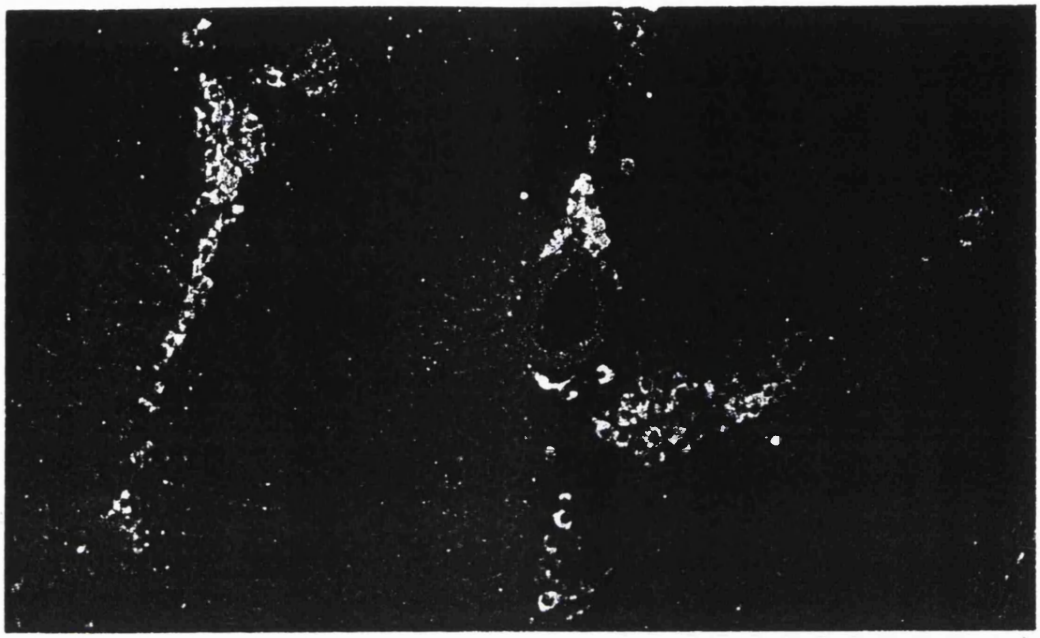
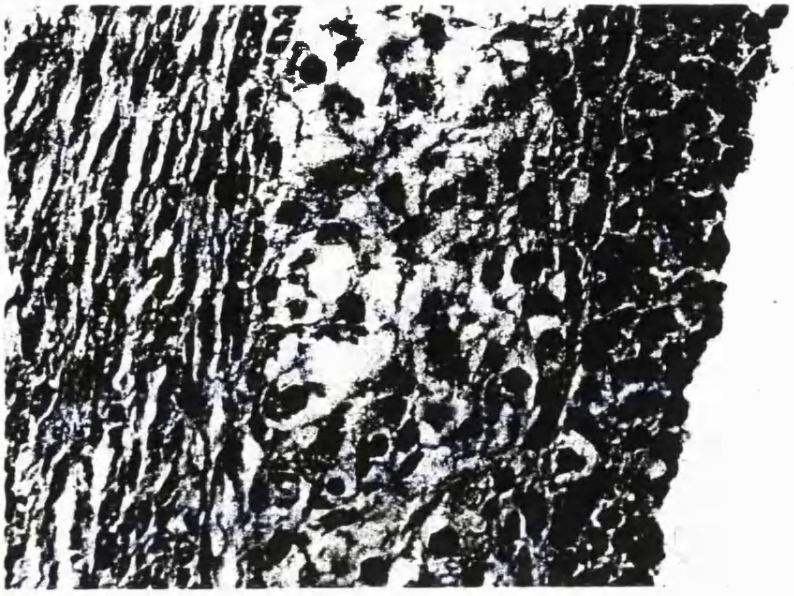


Figure 3.38e: **Fluorescent immunohistochemistry.** (Magnification x400). In addition to the positive immunostaining of Leydig cells using antibody 2, this figure also demonstrates the probable positive immunostaining of a blood vessel.

Figure 3.38f **x400**



Figures 3.38f **and** ***g:*** **Immunohistochemistry.** (Magnification x400 and x200 respectively). ***Figure 3.38f*** demonstrates a paraffin embedded section of a human preovulatory follicle which has been immunostained with antibody 3 after pretreatment with Triton X-100. Specific immunostaining of the granulosa and thecal cells can be seen as a brown colouration when compared to the negative control section (***Figure 3.38g***), where the primary antibody was replaced with serum.

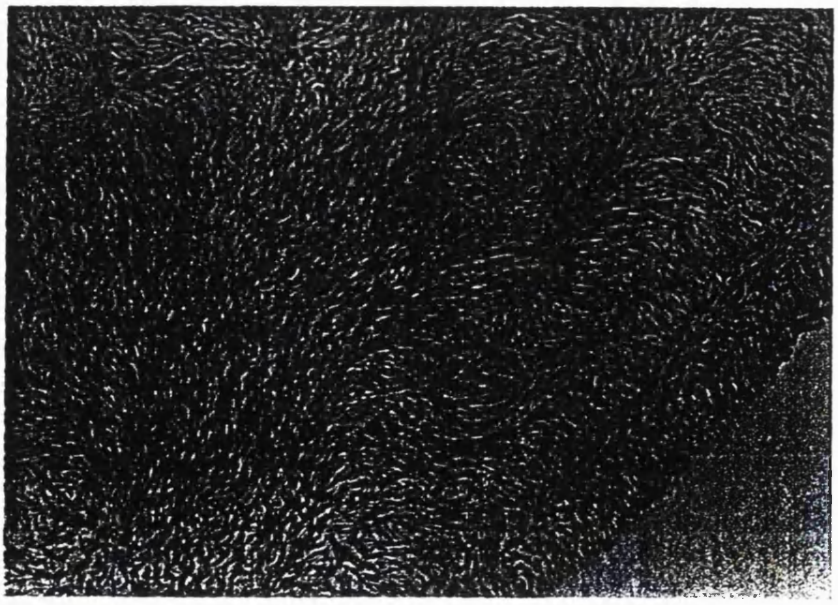


Figure 3.38g **x200**

3.4: Discussion

One of the aims of the work described in this chapter was to establish an immunopurification protocol using existing anti-peptide LH/CG receptor antibodies, in order that this receptor may be immunopurified. Despite the use of two different methods, the establishment of such a protocol proved unsuccessful. The reasons for this are unclear, but could have been due to the quality of the antibodies, or the quality of the antigen-containing membrane preparation.

3.41: Quality of antibodies?

The LH/CG receptor antibodies purified in this work were demonstrated to recognise the peptides against which they had been raised. However, these antibodies could not recognise the LH/CG receptor on Western blots of MA10 cells or hyper-stimulated ovary preparations. It was initially thought that this lack of recognition was due to a problem in the Western blotting procedure, however, in view of the fact that the original antibody 1 could clearly recognise a Western blotted MA10 preparation, this would appear unlikely.

The antibodies that were immunopurified in this work were obtained from batches of serum that had been stored for up to 4 years at -20°C. In addition, much of this serum had been obtained from bleeds taken either early on in the immunisation procedure or towards the end. This could have resulted in the purification of antibodies that had a lower level of affinity for the LH/CG receptor antigen, compared to those purified originally (Pallikaros 1995).

3.42: Quality of antigen-containing membrane preparation?

The originally purified stocks of antibodies 1 and 2, which had been previously demonstrated to recognise the LH/CG receptor on Western blots (Pallikaros 1995), could not demonstrate the ability to immunoprecipitate or immunoaffinity purify the LH/CG receptor antigen. This could indicate that the failure to establish such a protocol was the result of either low levels of antigen present in the membrane preparation or the formation of antigen aggregates which prevented antibody recognition (Harlow and Lane 1988). The work described here used either tissue from

hyper-stimulated rat ovaries or MA10 cells. Both of these are known to express the LH/CG receptor to high levels (although hyper-stimulated rat ovaries have been reported to have a specific binding capacity of 10-50 times higher than homogenates prepared from rat testis or MA10 cells (Ascoli and Segaloff 1989)) and therefore scarcity of the starting antigen is not considered a likely reason for the failure to establish an immunopurification protocol. In addition, the concentration of the solubilised LH/CG receptor was not thought to be a causal factor as each solubilisation procedure incorporated a concentration step so as to obtain as concentrated a protein solution as possible. The presence of other solubilised proteins in this concentrated protein fraction may have, however, interfered with the avidity of antibody-antigen complexes. Avidity is a measure of the overall stability of the complex between antibodies and antigens and can be affected by the geometric arrangement assumed by antibodies and antigens in the presence of other protein components (Harlow and Lane 1988). Another factor that may well have affected the avidity of antibody-antigen complexes is that of antigen aggregation.

3.43: LH/CG receptor self-aggregation and lability

The LH/CG receptor appears to form aggregates with itself very easily. Within the cysteine residues of the LH/CG receptor are free thiol groups which are thought to undergo rapid oxidation resulting in the formation of intermolecular disulphide bonds and thus LH/CG receptor aggregation (Dufau *et al.* 1974, Bahl and Sojar 1990). In addition, due to the presence of seven hydrophobic domains, the LH/CG receptor contains many hydrophobic residues. These are thought to contribute to receptor aggregation during membrane solubilisation, by 'sticking together' in an attempt to keep the hydrophobic residues away from water (Bahl and Sojar 1990). Despite the presence of a detergent in the membrane solubilisation procedure used in this work, (0.5% (v/v) Nonidet P-40) such hydrophobic interactions can still occur, particularly if the receptor is expressed abundantly. Such aggregation would be predicted to prevent recognition by antibodies, or at the very least, considerably lower the avidity of the antibody-antigen complex. In order that LH/CG receptor aggregation is kept to a minimum, immunopurification protocols need to include detergents and, more importantly, compounds that act to protect the thiol groups from oxidation (Bahl and

Sojar 1990). These compounds include S-alkylating agents or low levels of mercaptoethanol, DTT, cysteine and sodium sulphite. The preparation and solubilisation of MA10 and rat hyper-stimulated ovary membranes were carried out in the presence of N-ethylmalemide (5mM), an S-alkylating agent. However, it is probable that this was too low a concentration or that other compounds may have worked better.

Another problem encountered in the isolation of the LH/CG receptor has been its extreme lability. The binding activity of the free receptor was found to decline dramatically within a few hours of solubilisation (Dufau *et al.* 1973, Charreau *et al.* 1974). However, the addition of glycerol to the buffers during the solubilisation and purification procedures has reduced this considerably (Bahl and Sojar 1990). Glycerol ((v/v) 10%) and a range of protease inhibitors were included during the membrane solubilisation and purification procedures described in this work, in an attempt to decrease the levels of LH/CG receptor degradation [see *Chapter Two*, section ***2.63***].

3.44: The use of anti-peptide antibodies in immunocytochemistry

The problem of LH/CG receptor aggregation and/or proteolytic degradation would seem to be the most likely area contributing to the difficulties experienced in establishing an immunopurification protocol. Adding substance to this theory are the results which were obtained in later imunocyto- and immunohistochemical experiments using the original stocks of antibodies as purified by Pallikaros (1995). During the immunocytochemical studies on MA10 cells, antibody 1 was found to demonstrate LH/CG receptor-specific immunostaining on MA10 cells. In addition, during immunohistochemistry on human ovarian paraffin-embedded sections, antibody 3, (raised against a cytoplasmically located antigen) was shown to demonstrate granulosa and luteal cell-specific immunostaining, although only after the sections had been treated with a detergent. (This treatment had previously been thought to be unnecessary as the act of sectioning was anticipated to provide sufficient access to the cytoplasm). Later work found that antibody 2 could demonstrate Leydig cell-specific fluorescent immunostaining on rat testicular cryostat sections. Thus, all three

antibodies were demonstrated to give positive results when used in immunostaining experiments, indicating that they were still able to bind to their respective antigens.

The reasons for the differences in cell-specific staining between the three antibodies is likely to be due to the differences in cell and tissue preparation. The failure of antibodies to immunostain cells or tissue sections does not necessarily reflect the absence of the antigen, but may be due to the inability of the antibody to recognise the antigen under the conditions used to prepare and incubate the material of interest (Brown *et al.* 1996). Paraffin-embedded sections are subjected to several harsh treatments prior to immunostaining, which is thought on occasions, to be responsible for the loss of antigenicity (Polak and Van Noorden 1988). Cryostat sections are generally regarded as better for retaining antigenicity, particularly of surface antigens, however cellular morphology can often be lost using this form of tissue fixation and high levels of background can mask some results (Polak and Van Noorden 1988).

3.5: Summary

One of the aims of this work was to establish an immunoprecipitation protocol in order that the LH/CG receptor could be isolated, so that it could be established if, when in a desensitised state, the LH/CG receptor of MA10 cells underwent phosphorylation. Despite using two methods of immunopurification and various antibodies, this proved unsuccessful. It is anticipated that this was likely to be due to LH/CG receptor aggregation rendering the antigenic sites of the protein inaccessible to the antibodies. This argument is enhanced by the fact that these antibodies were successfully used in immunocytochemistry experiments, (another aim of this work). Thus, it was possible to demonstrate that anti-peptide antibodies, previously only used in Western blotting experiments, could, (depending on the method of fixation) be used for the immunocyto- and immunohistochemical detection of the LH/CG receptor in MA10 cells and human ovarian and rat testicular tissue.

Chapter Four: The amplification, cloning and sequencing of the C-terminal half of the MA10 LH/CG receptor.

4.1: Cloning and sequencing of the LH/CG receptor gene

In recent years, the LH/CG receptor gene has been cloned and sequenced from several species. In the late 1980s, sequence analysis of the ovarian LH/CG receptor gene was performed from ovarian tissue taken from the rat (McFarland *et al.* 1989), mouse (Gudermann *et al.* 1992a), and human (Minegishi *et al.* 1990). Similarly, the testicular LH/CG receptor gene sequence was established from porcine Leydig cells, (Loosfelt *et al.* 1989), from rat Leydig cells (Tsai-Morris *et al.* 1991) and more recently from marmoset monkey (Zhang *et al.* 1997). Sequence alignment of the LH/CG receptor genes from different species has revealed a high degree of homology. For instance, the human ovarian LH/CG receptor gene has been shown to share, overall, 85% sequence homology with the rat ovarian LH/CG receptor gene and 87% sequence homology with the porcine ovarian LH/CG receptor gene (Minegishi *et al.* 1990).

Elucidation of the sequence of the LH/CG receptor genes allowed comparisons to be made with the genes of other proteins. Sequence alignments revealed considerable homology to the G-protein coupled receptor (GPCR) family. The sequence homology was particularly evident in the regions thought to comprise the seven transmembrane domains that typifies this family of proteins. The most highly conserved regions show over 90% homology when the human, pig and rat LH/CG receptor genes are compared (Minegishi *et al.* 1990). Hydropathy plots from the sequence data of the respective LH/CG receptor clones suggested a possible model for the organisation of the LH/CG receptor protein. The protein model consisted of a large NH_2-terminal part, approximately 333 amino-acids long, that was thought to be the extracellular ligand-binding domain of the receptor (Loosfelt *et al.* 1989, Minegishi *et al.* 1990, McFarland *et al.* 1989). This putative extracellular domain contained six potential, *N*-linked glycosylation sites and was followed by a region of approximately 266 amino-acids that suggested seven possible transmembrane segments (Loosfelt *et al.* 1989). The LH/CG receptor protein model ended in an intracellular COOH-terminal region of approximately 70 amino-acids, that was found to contain potential serine, threonine and tyrosine phosphorylation sites (Loosfelt *et al.* 1989, Minegishi *et al.* 1990). The structure and

functional relationship of the LH/CG receptor is discussed in more detail elsewhere in this thesis.

4.12: Differences in LH/CG receptor function between closely related species

Despite the high level of structural homology of the LH/CG receptor across a range of species, differences in the amino-acid sequence nevertheless exist. In work reviewed by Rommerts and Cooke (1988), it had been demonstrated that rat Leydig cells could be desensitised, with regards to cAMP production, by activators of PKC but not by activators of PKA. However, previous work in the department (West and Cooke 1991a, West *et al.* 1991) indicated that under the same experimental conditions, mouse Leydig cells could undergo desensitisation in response to stimulation with activators of both PKC and PKA. This desensitisation was complete, affecting both cAMP levels and steroid production. Thus, there obvious differences in the way in which different species undergo the desensitisation process and these may be related to the differences found in amino-acid sequence between species.

4.2: Aims

The aim of the work described in this chapter was to isolate, sequence and clone the MA10 LH/CG receptor. This was carried out for the following reasons. Firstly, although the mouse ovarian sequence had been published, the MA10 LH/CG receptor sequence had not been established. Due to its neoplastic nature, it was important to determine if there were any nucleotide differences, especially with respect to the putative phosphorylation consensus sequences. By carrying out sequencing analysis of the MA10 LH/CG receptor we could ascertain whether the MA10 cells contained these same base changes found in the mouse ovarian sequence and that were different in the rat ovarian sequence. This would confirm that results obtained using MA10 cells as a model for desensitisation studies would be the same as those obtained if normal Leydig cells were used.

Secondly, the cloning of the LH/CG receptor would provide a way in which to confirm any effects observed resulting from the use of an antisense strategy [see *Chapter Five*]. Previous work in the department had developed an antisense strategy which had been

reported to produce C-terminally truncated receptors because of differences found in desensitisation responses (West and Cooke 1991b). In order to confirm that incubation with these antisense oligonucleotides had resulted in C-terminal truncations and therefore that these truncations were responsible for changes in the desensitisation responses reported, the cloned MA10 LH/CG receptor would be similarly truncated by the use of restriction enzymes. Expression of the truncated LH/CG receptor cDNA in a mammalian expression system, would be anticipated to mimic the desensitisation responses previously reported if the antisense oligonucleotides had worked as was hypothesised.

4.3: Results

4.31: Extraction of RNA using RNAzol

In order to obtain MA10 LH/CG receptor cDNA for cloning and sequencing, it was first necessary to obtain total RNA from MA10 cells. In cells where the gene of interest is being transcribed and translated to a high level, such as a house keeping gene, it is usually possible to first extract the total RNA and then to follow this with a procedure that isolates only the messenger RNA. This allows for a cleaner and more efficient first strand synthesis procedure. In this work however, because of the low level of LH/CG receptor mRNA present in the MA10 cells, it was found that more LH/CG receptor cDNA could be synthesised if total RNA was used in the first strand reaction.

As is discussed in more detail in *Chapter Two*, MA10 cells were grown to confluency in culture flasks and RNAzol solution was added to extract the RNA. After agarose gel electrophoresis the clear identification of ribosomal bands indicated that RNA had been successfully extracted. *Figure 4.31* shows RNA that had been extracted from flasks of MA10 cells of different passage numbers. The ribosomal bands corresponding to the 18S and the 28S subunits of ribosomal RNA can be clearly seen. The background smearing is residual DNA that was not removed by the RNAzol.

4.32: cDNA synthesis

RNA (or mRNA) cannot itself be ligated into a cloning vector. It first has to be converted into DNA by complementary DNA (cDNA) synthesis. This is done by using a reverse trancriptase enzyme which synthesises a DNA polynucleotide strand complementary to an existing RNA strand. The reverse transcriptase enzyme used in this work was Murine Mammary Leukaemia Virus Reverse Transcriptase (MMLV-RT), so-called because it was first isolated from a virus that infected mouse mammary glands. Total RNA was added to a cDNA reaction containing a 3'gene-specific primer. The primer annealed to the 3' end of the RNA and MMLV-RT then transcribed DNA in a 5'-3' direction. This reaction resulted in a double-stranded hybrid consisting of one strand of DNA and one strand of RNA. The RNA strand could be removed by treating with a

mild alkali but was often left as it appeared to have no adverse effect on the PCR procedure.

4.33: Primer design

The primers, more than anything else, can determine the success or failure of a PCR. The sequence of the primers used in this work were based on the published mouse sequence (Guderman *et al.* 1992a). Areas flanking the LH/CG receptor gene were chosen that had a random base distribution and were free from unusual sequences such as stretches of polypurines or polypyrimidines. Unusual sequence runs such as these can generate secondary structures, such as hairpin loops, which can hinder primer hybridisation. The 3' terminus of the primer is the end from which extension takes place and therefore good hybridisation is required at this point in order that mispriming events are kept to a minimum. For this reason, care was taken to ensure that this area was also free from regions containing significant secondary structures. The primers used in PCRs are incorporated into the final amplification product and PCR is often used in order to introduce new sequences into genes of interest. These regions would not be involved in the initial hybridisation to the template, but would overhang at the 5' ends and become incorporated during subsequent extension cycles In this work restriction sites for the endonucleases NOT1 and SAL1 were introduced into the original primer pair in order to aid in the cloning procedure.

The original primer pair was designed to amplify the whole of the MA10 LH/CG receptor gene (approximately 2.1kb) in one reaction. This proved unsuccessful and the primer design strategy was re-evaluated. It was then decided to amplify the MA10 LH/CG receptor gene in two halves; the C-terminal half which was of greater interest as it contained the region of the LH/CG receptor thought to be involved in intracellular signalling, and the N-terminal half. These new primer pairs were not designed to contain restriction sites and they were the primers used routinely in the work described hereafter. After each primer had been designed, its sequence was checked on a DNASTAR database for complementarity to each other, (in order to prevent primer dimerisation), and to other sequences in the rat genome.

The primers used in this work were synthesised to order by British Biotech. The diagram in *Figure 4.33a*, depicts the cDNA sequence and the sites of primer hybridisation.

Before the primers were used in the MA10 LH/CG receptor PCR, they were tested using the rat LH/CG receptor as a template. This was done to check that the primers were of the correct sequence and also to enable the optimal concentrations of the primer pair and $MgCl_2$ to be established.

Figure 4.33b shows the results from two rat LH/CG receptor PCRs using the two different primer pairs. In the first PCR the primers had been designed to amplify the N-terminal half of the rat LH/CG receptor. In the second PCR the primers had been designed to amplify the C-terminal half of the rat LH/CG receptor. From the sequence data it was deduced that the N-terminal PCR product should be 1322 bp in size and that the C-terminal half should be 868 bp in size. From the photograph it can be seen that the N-terminal product lies between markers of 947-1375bp and that the C-terminal product lies between markers of 831-947bp in size. Restriction digest analysis of the two halves gave the expected profile, (results not shown). From these results it was concluded that the primers had been designed accurately. This figure also demonstrates the results of a $MgCl_2$ titration [see next section].

4.34: $MgCl_2$ concentration optimisation

The concentration of Mg^{2+} can have a profound effect on the yield and specificity of a PCR by affecting the activity of the *Taq* polymerase. Supplied in the form of $MgCl_2$, each PCR has an optimal Mg^{2+} concentration which needs to be established beforehand by titration experiments. Generally concentrations of approximately 1.5mM are optimal but this can vary between different PCRs. Excess Mg^{2+} often results in an increase in non-specific amplification products, whereas insufficient Mg^{2+} will reduce the yield.

In addition to demonstrating the PCR products obtained using the two different primer pairs, *Figure 4.33b* also shows the results of a $MgCl_2$ titration. Using the N terminal pair of primers, the optimal $MgCl_2$ concentration was between 0.5-1mM. Using the C

terminal pair of primers however, the optimal $MgCl_2$ concentration was between 1-3mM. (1mM was routinely used in later PCRs).

4.35: The amplification of the C-terminal half of the MA10 LH/CG receptor

The total RNA extracted from MA10 cells was reverse transcribed using a gene-specific primer. This cDNA then underwent amplification using the two gene-specific primers that had been previously demonstrated to amplify the C-terminal half of the rat LH/CG receptor gene. Previous experiments had already established the optimal $MgCl_2$ and primer concentrations and had also allowed a high primer hybridisation temperature (62°C), to be achieved. (This was advantageous as the higher the primer hybridisation temperature, the lower the number of mispriming events). *Figure **4.35*** shows the results from this PCR. A single, well defined band with a molecular weight of between 947 and 831 base pairs can clearly be seen.

4.36: The restriction digest analysis of the amplified PCR product

Using the published mouse sequence as a guide, various endonucleases were selected and used to digest the PCR product in separate enzyme reactions. This was done in order to confirm, as so far as was possible, the identity of this PCR product before the cloning procedure was carried out. Table ***4.36*** shows the endonucleases selected and the fragment sizes expected if the amplified product was the C-terminal half of the MA10 LH/CG receptor. Figure ***4.36*** shows the results from the restriction digest of the extracted PCR product. The sizes of the digested fragments corresponded closely to those anticipated, indicating that the identity of this PCR product was likely to be the C-terminus of the MA10 LH/CG receptor.

4.37: Cloning

The extracted and desalted PCR product underwent a number of further reactions, as described in *Chapter Two*, before it was ready to be ligated into the plasmid vector pUC18. The recombinant plasmid was used to transform *E.coli* derived cells and these were then streaked out onto agar plates in order that single recombinant colonies could be identified. Recombinant colonies were picked, grown up in broth in order that a

plasmid extraction/purification procedure, and subsequent restriction digest analysis could be performed.

4.38: Restriction analysis of clones

In *Figure 4.38a* it can be seen that the multiple cloning region of pUC18 is bordered by a Hind III restriction site on one side and an EcoR 1 site on the other. Using both EcoR I and Hind III, it was possible to remove the region containing the DNA insert, from the purified plasmid preparations of each of the clones picked. If the C-terminal fragment had been cloned the size of the insert was anticipated to be approximately 800-900bp. *Figure 4.38b* shows the results from this digest. It can be seen that of the 12 recombinant colonies picked by blue/white selection, colonies 1, 2 and 3 each have an insert of the right size to be the C-terminal half of the MA10 LH/CG receptor.

Before sequence analysis was attempted however, further restriction digests were performed so as to ascertain with more certainty the nature of the inserts [*see Figure 4.38c*]. As described previously, the restriction endonucleases NCO 1 and NSI 1 cut the C-terminal rat LH/CG receptor at 1564bp and 1761bp respectively. Each clone underwent two different digestions. EcoR 1 + NSI 1 was anticipated to generate a cleaved fragment of either 521bp or 368bp depending on the orientation of the insert. EcoR 1 + NCO 1 was anticipated to generate a cleaved fragment of either 565bp or 324bp, depending on the orientation of the insert. (The plasmid pUC18 also underwent these digestion reactions, including the EcoR 1 and Hind III, to ensure that none of 4 restriction sites occurred anywhere else in the plasmid which would generate extra fragments).

From the results of the digest shown in *Figure 4.38c*, it can be seen that in all three clones, as anticipated, a fragment of 800bp has been generated by digesting with Hind III and EcoR 1. Both clones 1 and 2 generate fragments of approximately 500bp on digestion with EcoR 1 and NSI 1. Similarly, both clones 1 and 2 generate a fragment of less than 500bp on digestion with EcoR 1 and NCO 1. Clone 3, despite containing an insert of the right size, did not generate a similar band profile on digestion with the same enzymes and did not, therefore, undergo further analysis. There were no extra bands

seen, other than that of the plasmid itself, when pUC18 underwent digestion with the enzymes used, confirming that those generated from the inserts were specific.

From the band profile obtained, it was possible to ascertain the orientation of the insert in the plasmid. Both clones 1 and 2 were orientated in the same way, as can be seen in *Figure **4.38d**.*

4.39: Sequence analysis of cloned cDNA

From the results of dideoxy-sequencing, approximately the first 500bp and the last 500bp could be read of the C-terminal half of the MA10 LH/CG receptor gene. The sequence of bases was identical to that of the analogous region in the published mouse ovarian LH/CG receptor gene. This preliminary sequence data was enough to confirm that the MA10 LH/CG receptor gene had been successfully cloned. *Figure **4.39*** shows part of one such sequencing reaction. In order to save time, further sequence analysis was performed by the Babraham Institute. Analysis of the resulting data confirmed that there were no significant sequence differences from that of the published mouse ovarian sequence (Gudermann et al 1992a).

*Figure **4.4a*** shows the results obtained when LH/CG receptor total RNA was isolated from rat testes, rat liver, rat brain and MA10 cells. Whilst well-defined bands corresponding to the ribosomal subunits can be seen in the RNA isolated from MA10 cells, rat testes and rat brain, these bands are less well-defined in the total RNA obtained from liver. As a result this sample was thought to be degraded.

The autoradiograph in F*igure **4.4b*** shows the results obtained when samples from the total RNA extraction described above were used in a Northern dot blot experiment. The blot was probed with the cloned C-terminal fragment of the MA10 LH/CG receptor which had been labelled with [^{32}P]-dATP using the enzyme terminal transferase [see *Chapter Two*, section ***2.36***]. The degraded liver RNA was used as a control for this experiment. After several weeks at -20°C the autoradiograph was developed. Unsurprisingly, areas of probe hybridisation can be seen in the 3µl sample from MA10 cells and in both the 1µl and 3µl samples from rat testes. However, probe

hybridisation can *also* be seen in the 1µl sample (faint) and 3µl sample from rat brain indicating the presence of LH/CG receptor mRNA in this sample. Probe hybridisation was not seen in the degraded liver sample.

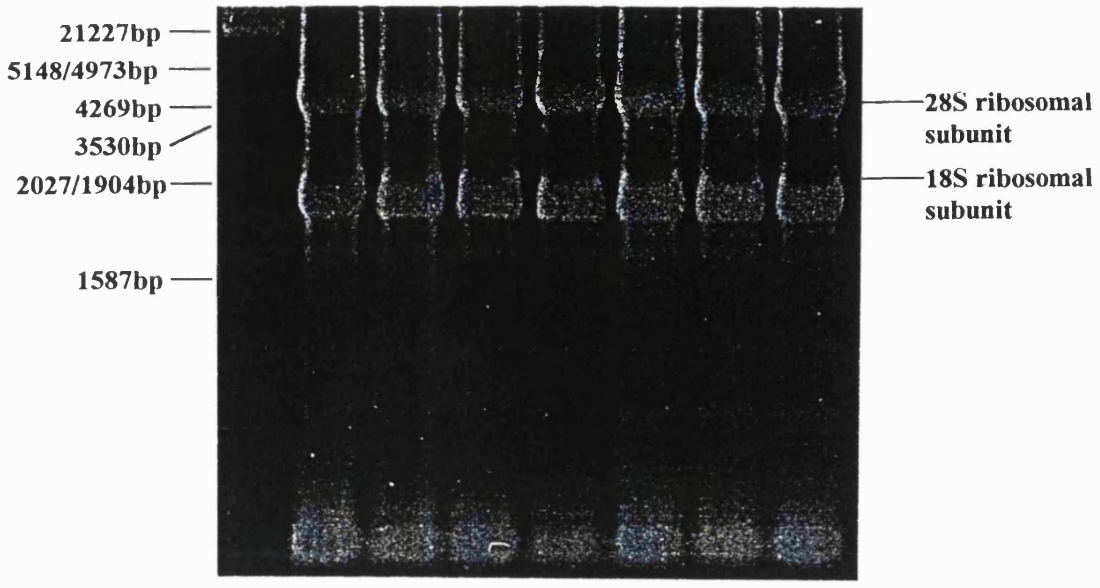


Figure 4.31: **Extraction of total RNA using RNAzol examined by agarose gel electrophoresis**. Total RNA was extracted from MA10 cells of different passages using the RNAzol protocol described in *Chapter Two*, section ***2.21***. The 28S and 18S ribosomal subunits can clearly be seen indicating that the RNA is not degraded.

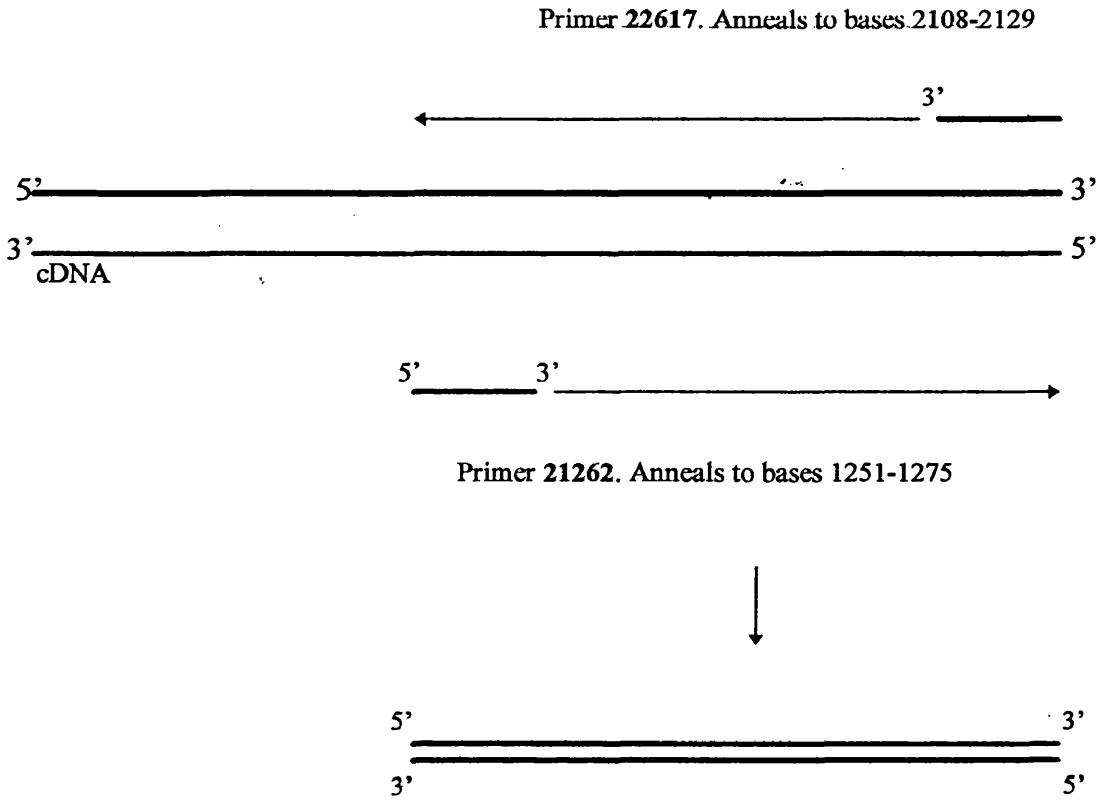
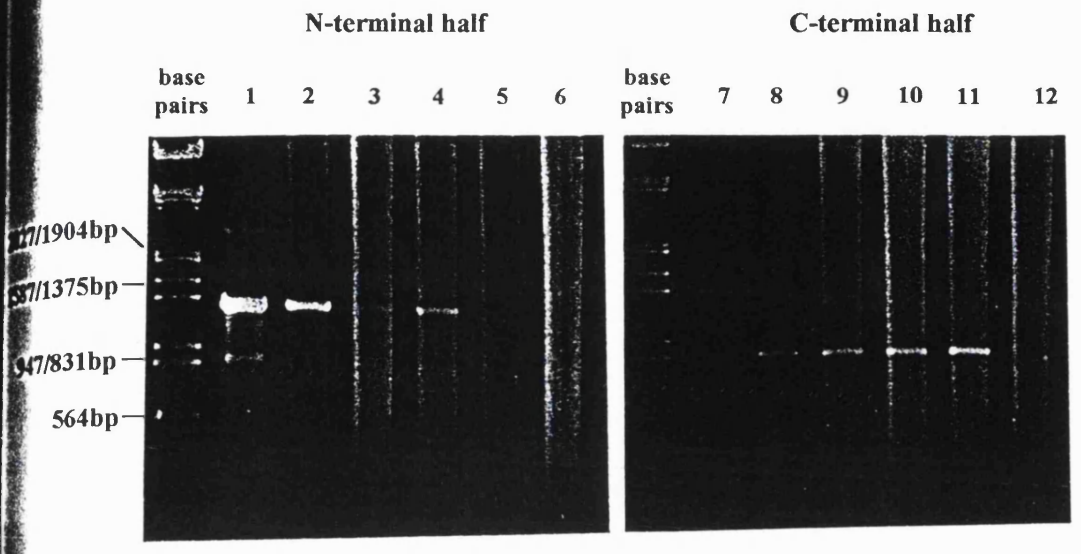


Figure 4.33a: This diagram depicts the cDNA hybridisation sites of the two gene-specific primers designed and used in this work, resulting in the predicted amplification of the C-terminal portion of the LH/CG receptor.



N-terminal amplification reaction of cloned rat LH/CG receptor and $MgCl_2$ titration
Lane 1 = 0.5mM $MgCl_2$, **Lane 2** = 1mM $MgCl_2$, **Lane 3** = 1.5mM $MgCl_2$, **Lane 4** = 2mM $MgCl_2$, **Lane 5** = 3mM $MgCl_2$, **Lane 6** = control (no cDNA).

C-terminal amplification reaction of cloned rat LH/CG receptor and $MgCl_2$ titration
Lane 7 = 0.5mM $MgCl_2$, **Lane 8** = 1mM $MgCl_2$, **Lane 9** = 1.5mM $MgCl_2$, **Lane 10** = 2mM $MgCl_2$ **Lane 11** = 3mM $MgCl_2$, **Lane 12** = control (no cDNA).

Figure 4.33b: **Testing of PCR primers on rat LH/CG receptor cDNA and demonstration of $MgCl_2$ titration as examined by agarose gel electrophoresis.** This figure demonstrates the results obtained from testing the two primer pairs using a cloned rat LH/CG receptor. This experiment also included a $MgCl_2$ titration to ascertain the optimal concentration of Mg^{2+} for each primer pair.

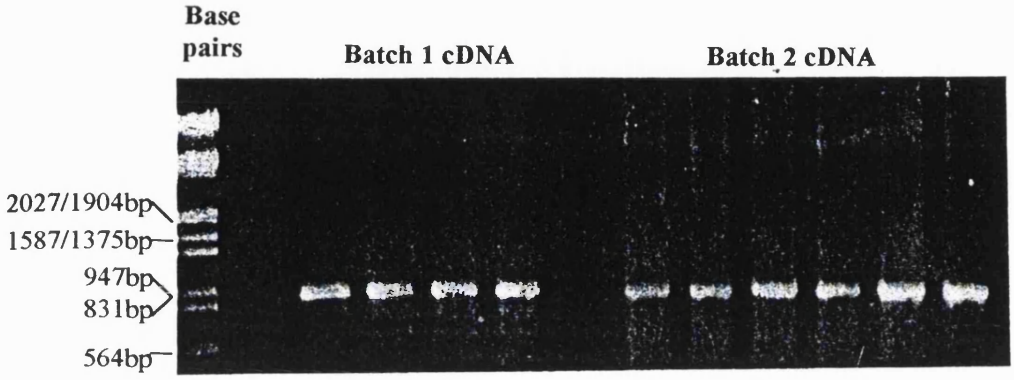
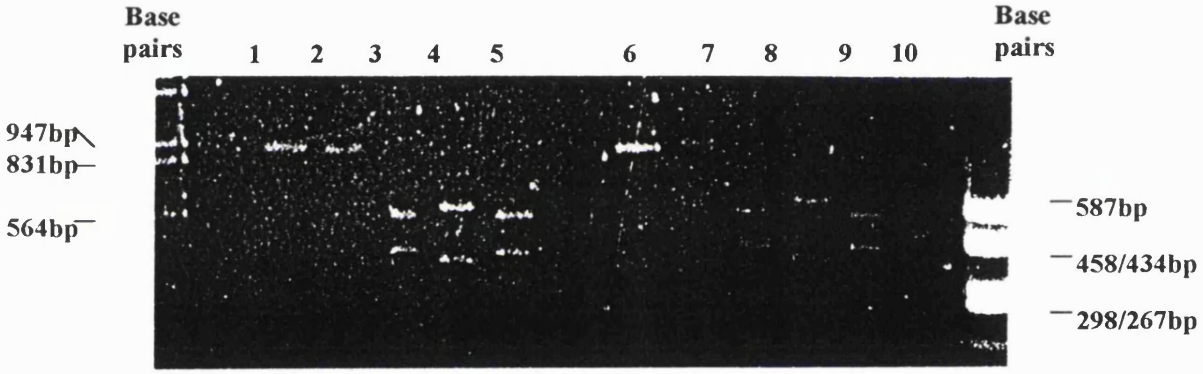


Figure 4.35: **The amplification of the C-terminal half of the MA10 LH/CG receptor examined by agarose gel electrophoresis.** This photograph shows the results of two identical PCR reactions carried out on two different batches of cDNA. A single PCR product anticipated to be the C-terminal half of the MA10 LH/CG receptor, can clearly be seen approximately 900bp in size.

Restriction enzyme	Location on mouse sequence	Anticipated fragment sizes
BAN I	Nucleotide 1763	523 and 366bp
NCO I	Nucleotide 1564	324 and 565bp
NSI I	Nucleotide 1761	521 and 368bp

Table 4.36: The predicted fragment sizes of the PCR product after digestion with each respective endonuclease.

Figure 4.36: **Restriction digest analysis of the amplified PCR product**



Lane 1 = PCR fragment, **Lane 2** = PCR product incubated with buffer only, **Lane 3** = PCR product digested with BAN 1, **Lane 4** = PCR product digested with NCO 1, **Lane 5** = PCR product digested with NSI 1. (Lanes 6, 7, 8, 9 and 10 are as Lanes 1, 2, 3, 4 and 5 respectively).

Figure ***4.36*** shows the results obtained after the PCR product had been digested with each of the three restriction enzymes listed in *Table* ***4.36.*** Samples of the digest reactions were electrophoresed on an 1%/TBE agarose gel. From the photograph it can be seen that the predicted fragment sizes listed in *Table* ***4.36*** were actually obtained in this digestion reaction. Thus, these results suggested that the PCR product was the C-terminal half of the MA10 LH/CG receptor.

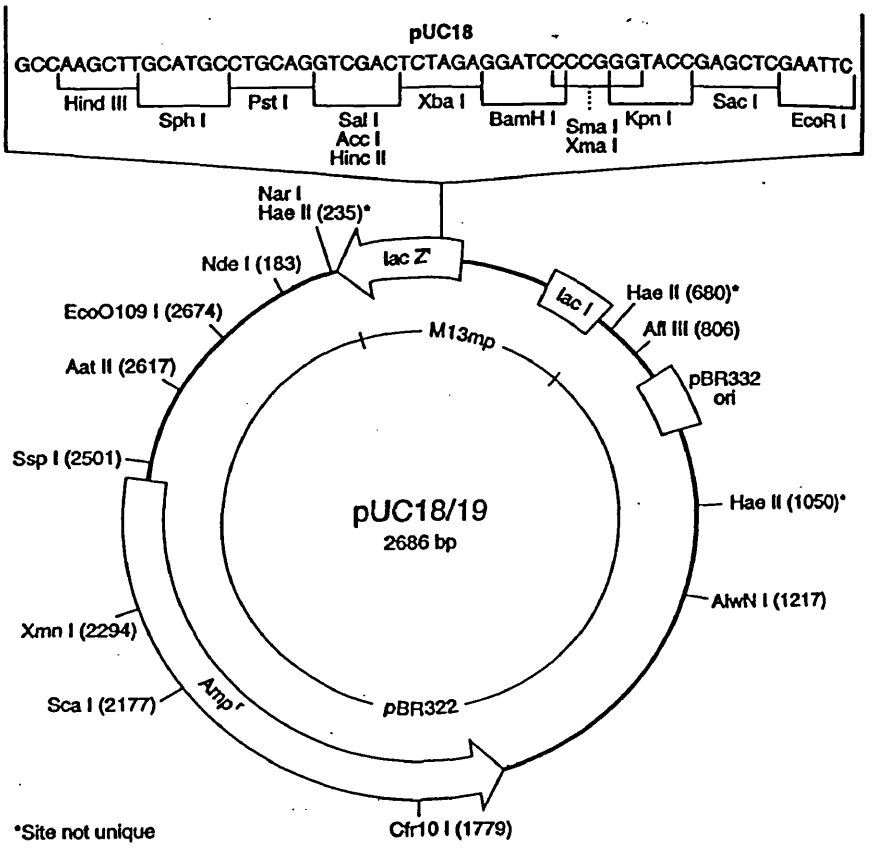
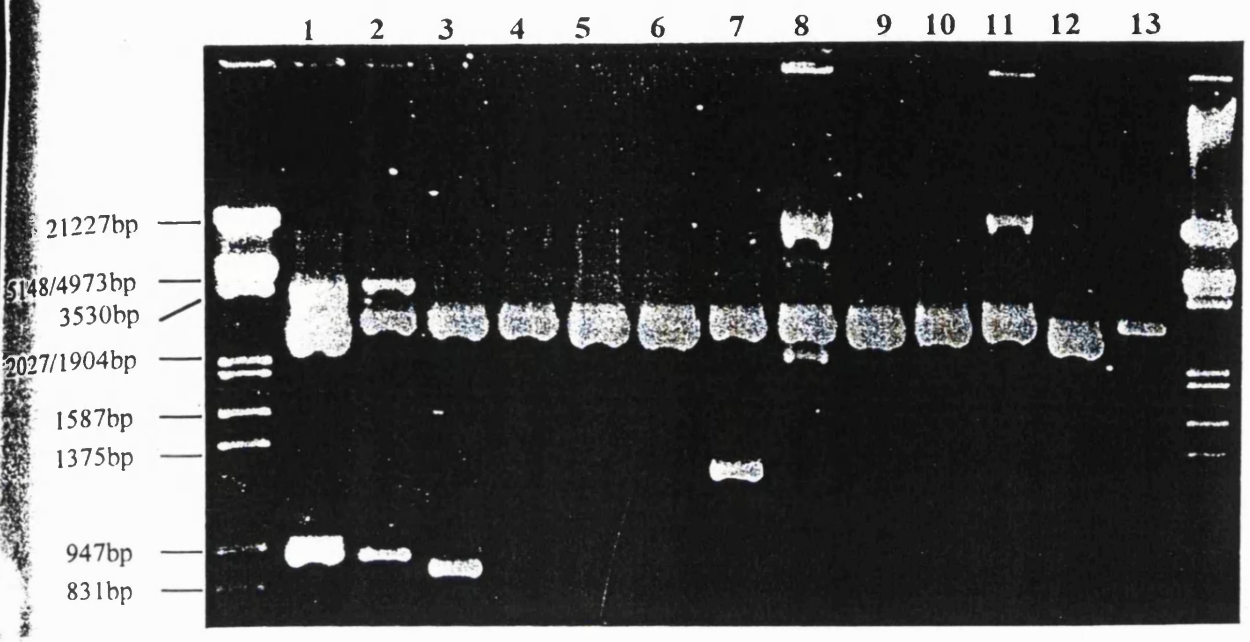
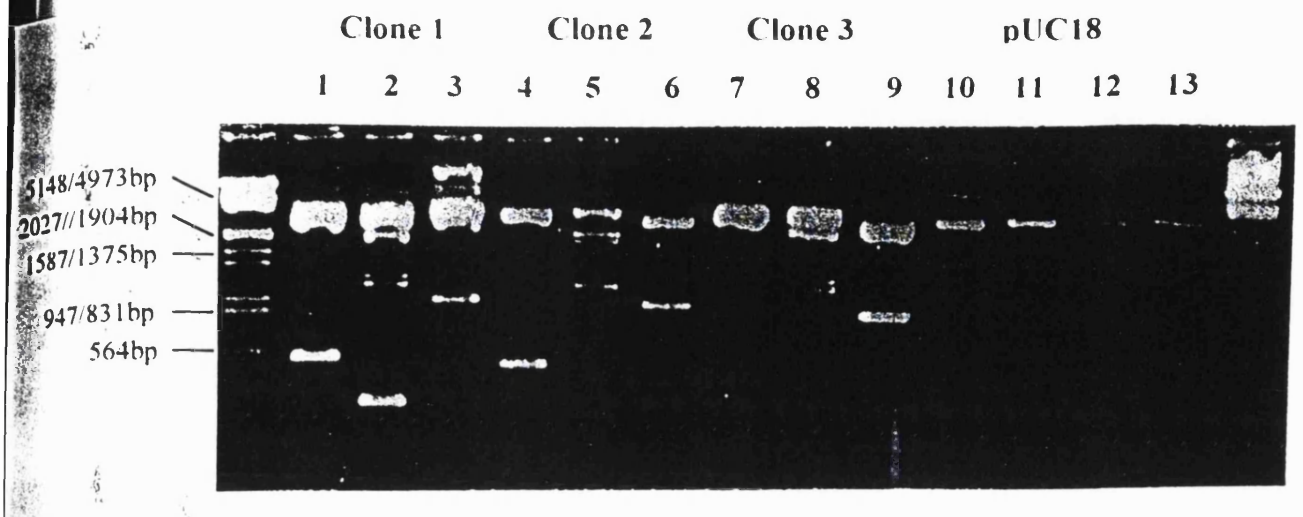


Figure 4.38a: **Diagram demonstrating the restriction endonuclease map of the plasmid pUC 18.** The multiple cloning site within the lac Z gene is depicted. *This figure was taken from the Pharmacia Biotech catalogue 1996.*



All clones were digested with EcoR I/HIND III;
Lane 1 = clone 1, **Lane 2** = clone 2; **Lane 3** = clone3; **Lane 4** = clone 4; **Lane 5** = clone 5;
Lane 6 = clone 6; **Lane 7** = clone 7; **Lane 8** = clone 8; **Lane 9** = clone 9; **Lane 10** = clone 10;
Lane 11 = clone 11, **Lane 12** = clone 12; **Lane 13** = clone 13.

Figure 4.38b: **Removal of the cloned insert by restriction digest as examined by agarose gel electrophoresis**. Each of the twelve clones which had been picked from a blue/white selection plate were grown up in broth and recombinant plasmid isolated. Each plasmid preparation underwent digestion with EcoR 1 and HIND III which removed the cloned insert from the multiple cloning site. The size of the cloned insert was predicted to be 800-900bp in size. From this photograph it can be seen that clones 1, 2 and 3 contained an insert of the correct size.



Lane 1 = clone 1 digested with EcoR I/NSI I; **Lane 2** = clone 1 digested with EcoR I/NCO I; **Lane 3** = clone 1 digested with EcoR 1/HIND III

Lane 4 = clone 2 digested with EcoR I/NSI I; **Lane 5** = clone 2 digested with EcoR I/NCO I; **Lane 6** = clone 2 digested with EcoR 1/HIND III

Lane 7 = clone 3 digested with EcoR I/NSI I; **Lane 8** = clone 3 digested with EcoR I/NCO I; **Lane 9** = clone 3 digested with EcoR 1/HIND III

Lane 10 = pUC18 undigested; **Lane 11** = pUC18 digested with EcoR I/NSI I; **Lane 12** = pUC18 digested with EcoR I/NCO I; **Lane 13** = pUC18 digested with EcoR 1/HIND III

Figure 4.38c: **Restriction analysis of cloned inserts 1, 2 and 3 as examined by agarose gel electrophoresis**. Further analysis of these cloned inserts was required before sequencing analysis was performed. Digestion of the cloned inserts with the restriction enzymes EcoR I/NSI was anticipated to result in band sizes of 521bp or 1608bp, whilst digestion with EcoR I/NCO I was anticipated to result in band sizes of 324bp or 1805bp. (Digestion EcoR I/HIND III would simply remove the cloned insert from the multiple cloning site). As can be seen from the photograph, only clones 1 and 2 gave the anticipated band profile. The results from the digestion of clone 3 would indicate that this insert cannot be the amplified C-terminal half of the MA10 LH/CG receptor. As expected, digestion of the empty plasmid did not result in the production of any bands.

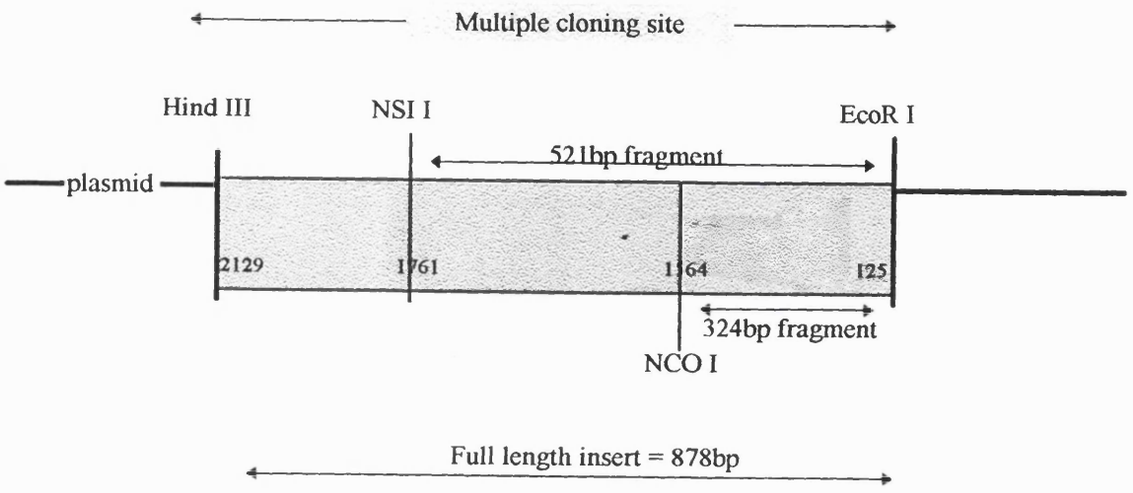


Figure 4.38d: **Orientation of cloned insert**. Diagram depicting the orientation of the cloned insert in relation to the multiple cloning site of the plasmid.

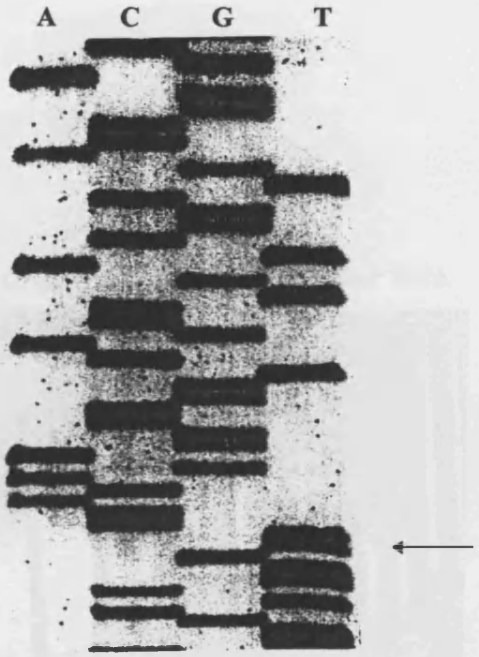


Figure 4.39: **Sequence analysis data of cloned cDNA.** This photograph shows a portion of the sequencing gel that was initially run to determine the identity of the two clones. The arrow indicates the beginning of the PCR primer sequence on one of these sequencing gels. This sequencing analysis confirmed the identity of both clones to be the C-terminal half of the MA10 LH/CG receptor. Further sequencing analysis of both clones was performed by the Babraham Institute, Cambridge. The automated sequencing data from these analyses can be seen in *Appendix 2*.

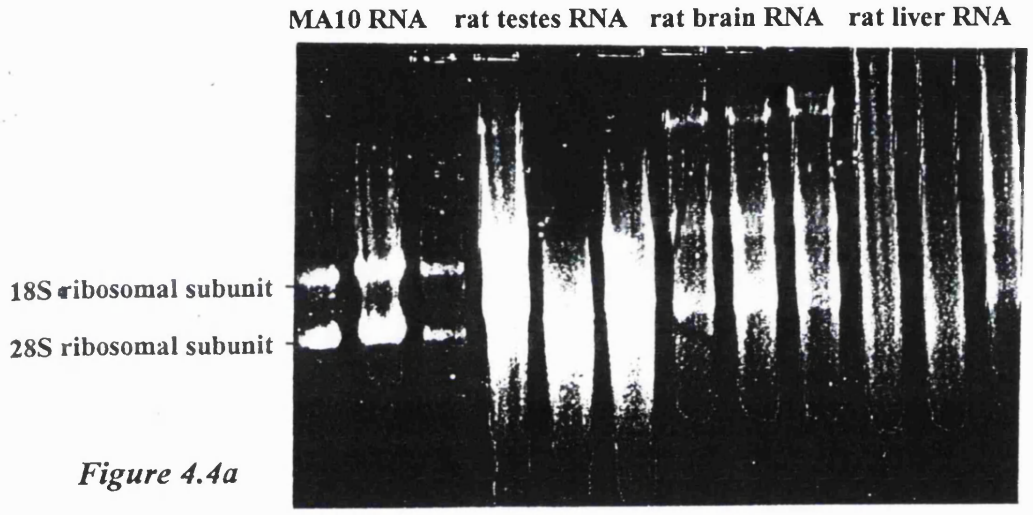


Figure 4.4a

Figures 4.4a and b (next page): **Northern dot blot.** ***Figure 4.4a*** shows the results from a total RNA extraction from different tissues. Whilst the ribosomal bands can be seen in the samples taken from MA10 cells, rat testes and rat brain, no such bands can be seen in the samples from rat liver which appears to be degraded.

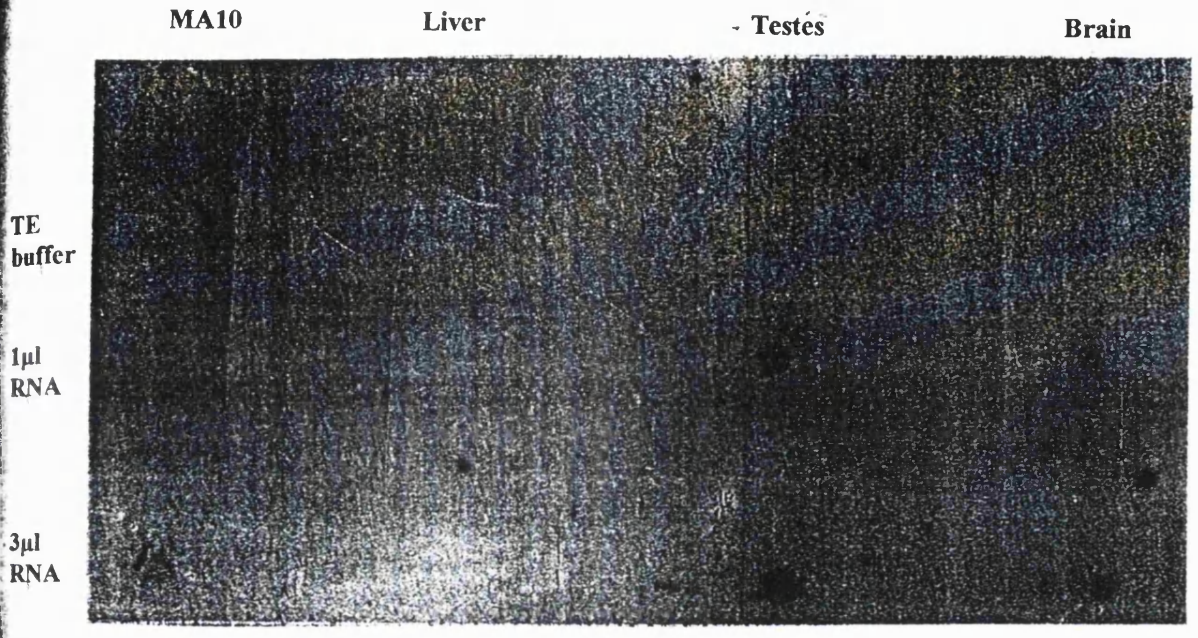


Figure 4.4b: This figure demonstrates the results from a Northern dot blot of various tissues which had been probed with the labelled C-terminal half of the MA10 LH/CG receptor. Hybridisation has occurred on total RNA isolated from MA10 cells, testes and brain. No hybridisation occurred on total RNA isolated from rat liver, however as this sample was degraded the possibility of LH/CG receptor mRNA in liver cannot be excluded from this experiment.

4.4: Discussion

In this work it was originally intended to amplify and clone the whole of the MA10 LH/CG receptor in order to elucidate the sequence of this protein and to provide a clone for use in mutation studies. The first set of primers that were synthesised were designed to anneal from nucleotides 6-29 and 2085-2108, based on the published sequence of the mouse LH/CG receptor (Gudermann *et al.* 1992a, thus encompassing the AUG start codon and the TAA stop codon. Despite extensive optimisation procedures it proved impossible to amplify the MA10 LH/CG receptor in one reaction.

Large pieces of DNA are generally harder to amplify because of the difficulty in supplying enough reaction components to prevent their exhaustion and because of the greater likelihood of incomplete strand synthesis by *Taq* polymerase. The full length LH/CG receptor was calculated to be 2102 bp which was anticipated to be well within the working range for PCR. In view of the difficulties experienced in the amplification of the LH/CG receptor gene in its entirety however, it was decided to design further primers which would enable the amplification reactions to proceed in two halves.

The new N-terminal primers were designed to amplify bases 6-1328 and the new C-terminal primers were designed to amplify bases 1251-2129. After extensive optimisation of the PCR conditions, the C-terminal half of the MA10 LH/CG receptor was successfully amplified. The N-terminal half however, was not. The reasons for this were not clear. The size of the amplification product was small enough to amplify easily. The primers were obviously working as intended since they amplified the correct fragment from the cloned rat LH/CG receptor. It was possible however, that due to mRNA secondary structure, the 5' primer was not annealing adequately to MA10 LH/CG receptor mRNA in the PCR proper. To investigate this further, the first 450 bases of the rat LH/CG receptor were analysed for secondary structure formation. Using the SQUIGGLES program, bases 1-30 were found to anneal with bases 300-350, forming considerable hairpin formation, as can be seen in *Figure 4.5*.

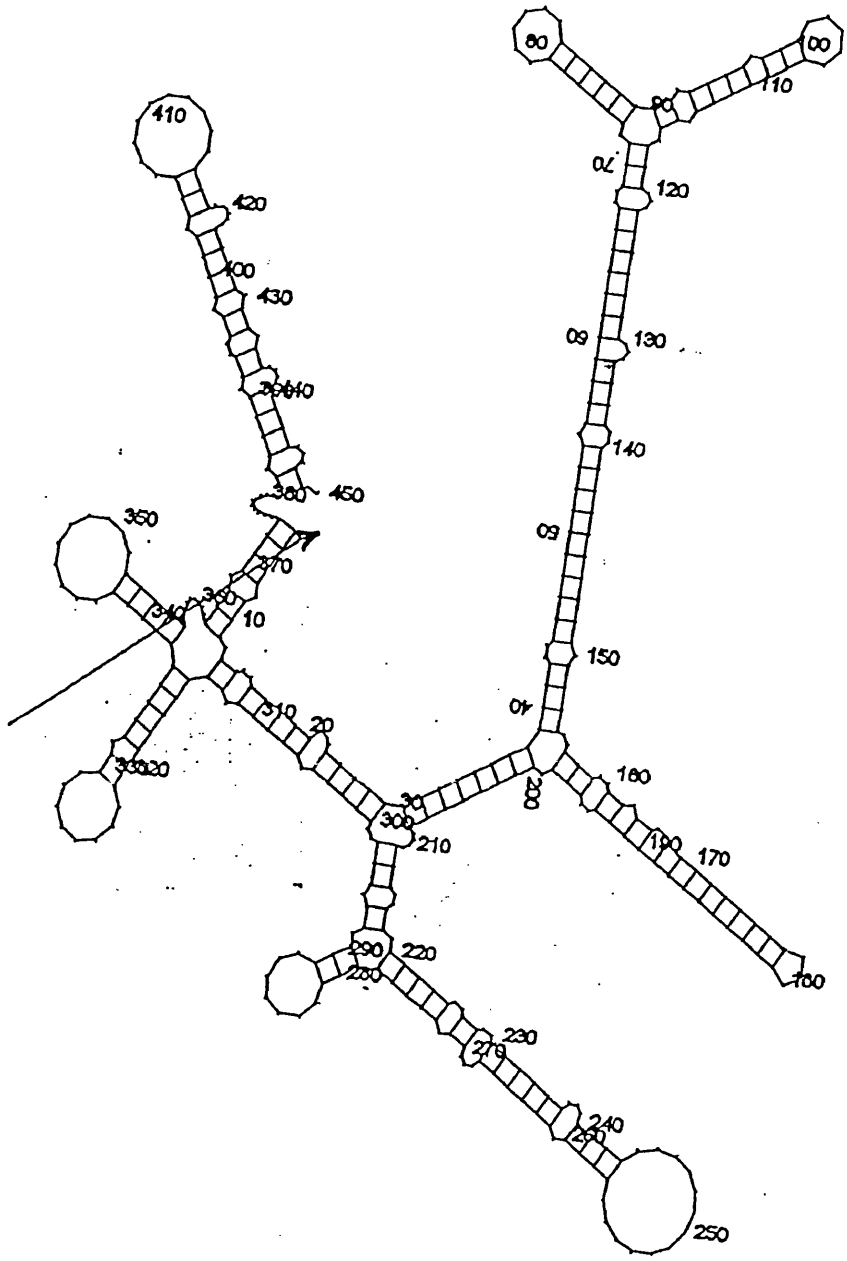


Figure 4.5: **Diagram depicting the potential secondary structure of the first 450bp of the MA10 LH/CG receptor mRNA** (as predicted by SQIGGLES program). The arrow indicates the first base pair of the mRNA sequence.

This then, may explain why the N-terminal primers could amplify the rat LH/CG receptor that had been cloned into a plasmid and therefore did not possess significant secondary structure, and those same primers failing to amplify the MA10 LH/CG receptor from mRNA that may possess considerable secondary structure, particularly around the 5'-primer annealing region.

The C-terminal half (878bp) of the MA10 LH/CG receptor was cloned and sequenced. Analysis of this sequence revealed that there were no major base differences when compared to that of the published mouse ovarian sequence (Guderman *et al.* 1992a). One nucleotide difference was found in clone 1, where at nucleotide 1656 there was a base change from guanine to adenosine. This base change would potentially result in an amino-acid change from threonine to methionine. However, this substitution was not repeated in clone 2, which had an identical sequence to that of the murine ovarian LH/CG receptor, so it is anticipated that this base change was due to an error in the sequence analysis. The phosphorylation consensus sequences were therefore demonstrated to be identical to those found in the mouse ovarian LH/CG receptor [see *Chapter One*]. There are many base changes between the C-terminal regions of the rat and mouse LH/CG receptor, some of which are located in the putative phosphorylation consensus sequences. The cytoplasmic regions of the rat LH/CG receptor were examined for potential phosphorylation consensus sequences [see Segaloff and Ascoli 1993]. Based on the amino-acid sequence and using the criteria for protein kinase phosphorylation consensus sequences as reported by Kennelly and Krebs (1991), it would seem that there are two weak PKA sites, nine weak PKC sites and two strong PKC sites in this area of the rat LH/CG receptor. When the mouse sequence (and MA10 sequence), is examined in this way and compared to the rat sequence, one identical weak PKA site, six identical weak PKC sites and two identical strong PKC sites are found. However, in the mouse sequence an additional strong PKC site is found. Comparison of the rat and mouse sequences also reveals three weak PKC sites and one weak PKA site that are not shared. Thus, examining the amino-acid sequences of the LH/CG receptor from these two species in this way, would indicate that there are differences in phosphorylation consensus sequences between rat and mouse, and

this may explain why the two species respond to activators of PKC and/or PKA differently.

4.5: Summary

The aim of this work was to PCR and clone the MA10 LH/CG receptor gene in order to confirm the nucleotide sequence of this gene. We were particularly interested in the cytoplasmic tail region with respect to the phosphorylation consensus sequences that were present. To this end we were successful in cloning and sequencing approximately the latter 878 bases and on sequencing analysis it was found that there were no significant base variations from the published murine ovarian sequence (Guderman *et al.* 1992a). Thus despite the neoplastic origin of this cell type, the MA10 cell would be anticipated to undergo signal transduction and desensitisation responses like that of normal, non-neoplastic Leydig cells.

Chapter Five: Antisense technology and cell-free translation

5.1: Introduction

Over the last 14 years there has been a wealth of research conducted demonstrating the use of antisense oligonucleotides in the study of cellular functions associated with particular DNA sequences. Antisense oligonucleotides exert their effects by a process known as translational arrest. There are two main ways in which translational arrest occurs. Firstly, if oligonucleotides are targeted to the region around the AUG initiation site of translation then it is anticipated that antisense oligonucleotides may prevent the binding and/or assembly of the 80S ribosome in an RNAse H-independent manner (Liebhaber *et al.* 1992, Persaud and Jones 1994). Secondly, particular oligonucleotides targeted to the coding region of the mRNA can induce mRNA cleavage if they activate RNAse H activity, (Minshall and Hunt 1986, Helene and Toulme 1990, Probst and Skutella 1996).

5.11: Antisense oligonucleotide binding to the AUG start site of translation

The mature 80S ribosome consists of two subunits, the 30S and the 50S subunits. All proteins are synthesised from the initiation codon, AUG, which encodes for the amino-acid methionine. Approximately 10 base pairs upstream from the initiation codon, in the 5' UTR, lies a region rich in purines. This sequence is called the Shine-Dalgarno sequence. During ribosome assembly, the 30S ribosomal subunit associates with the Shine-Dalgarno sequence and forms a complex with initiation factors, GTP and the initiator tRNA encoding for methionine. With all these factors present, the 50S ribosomal subunit joins the complex and the mature 80S ribosome is complete. The initiator tRNA allows the 80S ribosome to scan forward until the AUG codon is reached. At this point protein synthesis begins and peptide bonds form between adjacent amino-acids. It is thought that if antisense oligonucleotides hybridise to either the AUG initiation codon or to the Shine-Dalgarno sequence, the process of ribosome assembly and/or binding will be prevented. Although the ribosomal complex contains unwindase activity it is not thought to be active during the initiation phase of translation (Helene and Toulme 1990, Liebhaber *et al.* 1992). Many studies have demonstrated the effectiveness of antisense oligonucleotides targeted to the AUG

initiation codon in preventing protein translation in cell-free systems (Mizutani *et al.* 1995, Wakita and Wands 1994, Blake *et al.* 1995).

5.12: Antisense oligonucleotides targeted to the coding region of the mRNA

There have been several studies where antisense oligonucleotides targeted to the coding region of mRNA have been found to prevent translation. However, most of these studies have taken place in cell culture or in cell-free wheat germ systems. The enzyme RNAse H is present in each of these experimental systems and is thought to be essential for the effects of the antisense oligonucleotide in each case. RNAse H is present mainly in the nucleus of cells and is involved in the processes of DNA repair and replication. The function of RNAse H is to degrade any RNA/DNA duplexes that might arise during these cellular processes. Since cleaved RNA are generally degraded by cellular enzymes, their half-life is short. Cleaved mRNAs can therefore no longer support translation (Cazenave *et al.* 1987, Helene and Toulme 1990). Despite this there are rare reports of truncated proteins being synthesised, (Haeuptle *et al.* 1986, West and Cooke 1991b).

The effectiveness of antisense oligonucleotides in preventing translation then, would appear to be partly dependent on which experimental system is used and whether or not it contains RNAse H. It was generally agreed that cell-free systems derived from reticulocytes (non-nucleated cells) contained lower levels of RNAse H activity than were normally found in cell-free systems derived from wheat germ or in whole cells. These lower levels of RNAse H were not thought sufficient to support hybrid-arrested translation (Walder and Walder 1988, Boiziau *et al.* 1991). However, characterisation of the RNAse H activities present in cell-free systems derived from rabbit reticulocytes and wheat germ have demonstrated that RNAse H activity is similar in both systems (Cazenave *et al.* 1993). Moreover, the predominant RNAse H of the rabbit reticulocyte lysate (RRL) is reported to be a class 1 RNAse H, whereas the predominant RNAse H from the wheat germ extract is reported to be of class 2. The physiological state of the cell appears to govern which class of RNAse H predominates (Crouch and Dirksen 1982). For example, class 1 RNAse H activity seems to correlate

with the process of DNA replication whilst class 2 RNAse H correlates with RNA transcription (Busen *et al.* 1977).

5.13: Problems encountered using antisense oligonucleotides

One of the major problems encountered by researchers using antisense strategies in cells has been how to avoid antisense oligonucleotide degradation (Malcom 1992, Tidd 1992, Stein and Cheng 1993). Before an antisense oligonucleotide can exert its desired effect on gene expression, it must first escape nuclease attack in the extracellular medium and after crossing the cell membrane, must escape the attack of intracellular nucleases. Unmodified oligonucleotides are very susceptible to nuclease attack and although deoxyribonucleotides are more resistant than their ribonucleotide counterparts, the half-life of even these is approximately 10 minutes in cell culture medium containing 10% heat inactivated fetal calf serum at 37°C (Akhtar *et al.* 1991, Malcom 1992, Stein and Cheng 1993).

Independent studies by Loke *et al.* (1989) and Yakubov *et al.* (1989) reported the existence of an 80kDa surface protein which was suggested to mediate the transport of oligonucleotides into the cell. Despite this observation, some researchers have experienced difficulties in introducing large, negatively charged molecules across the cell membrane (Tidd 1992, Krieg 1993, Stein and Cheng 1993). A further problem has been how to ensure hybridisation specificity. Once inside the cell the antisense oligonucleotide must hybridise in a completely specific fashion with the intended target sequence (Crooke 1993, Giles *et al.* 1993). Whilst it is relatively easy to measure the stability, stringency and melting temperature of a particular heteroduplex *in vitro*, in many cases intracellular conditions of temperature, pH, ionic strength and counter ions are either unknown or certainly uncontrollable, and may therefore not provide ideal hybridisation conditions (Malcom 1992). The restrictions imposed by using unmodified oligonucleotides has led to the development of methodology which allows these problems to be largely overcome or circumvented.

5.14: Design and modifications of antisense oligonucleotides

With the rise in popularity of antisense technology, a large amount of recent work has been aimed at developing oligonucleotides that display an increased resistance to DNAses, an enhanced affinity for their target and improved uptake by intact cells. Some of the more common modifications are discussed below.

In work by Ts'o (1991) it became apparent that substitution of the charged oxygen on the oligonucleotide backbone with a methyl group rendered antisense oligonucleotides virtually resistant to degradation by nucleases. The resulting oligonucleotides, called methylphosphonates, were found to penetrate the cell membrane easily (possibly by passive diffusion), form stable duplexes with specific sequences and suppress gene expression in culture in a sequence-specific fashion (Ts'o 1991). However, the methylphosphonates were not without problems themselves. They have been expensive to make and are poorly water soluble requiring large concentrations (approximately 100μM) to be used. In addition methylphosphonates do not activate RNAse H activity.

An alternative to the above was found in the development of phosphorothioate oligonucleotides. In these oligonucleotides the charged oxygen atom is replaced by a charged sulphur atom with the result that these derivatives are water soluble and far more potent, often requiring only nanomolar concentrations (Stein *et al.* 1988). These modified oligonucleotides were reported to be efficiently taken up by cells via an 80kDa membrane receptor and were able to activate RNAse H (Stein *et al.* 1988). The main problem encountered using the phosphorothioate oligonucleotides is that they are far less sequence-specific, particularly when they are used at high concentrations and/or are longer than average in length (approximately 20bp) (Cazenave *et al.* 1989, Brown *et al.* 1994).

In another class of nuclease resistant oligonucleotides, called α-oligomers (Morvan *et al.* 1986), the normal ß-configuration of sugar and base is replaced by an α-configuration. As a consequence of the inverted sugar, α-oligomers bind in a parallel fashion to their target nucleic acid. The affinity of the α-oligomers is much higher that is the case for the ß-oligomers, but α-oligomers do not activate RNAse H and are poor

inhibitors of *in vitro* translation when directed to the translation initiation site or coding region of mRNA (Cazenave *et al.* 1989). In contrast, α-oligomers directed to the CAP site specifically inhibited ß-globin synthesis *in vitro* at micromolar concentrations (Boiziau *et al.* 1991).

It is evident from the work described above that despite different modifications contributing positive functions to the oligonucleotides in question, no one modification is ideal. The solution appears therefore, to combine the positive attributes from unmodified and modified oligonucleotides into a chimeric molecule. Chimeric oligonucleotides generally consist of a region of consecutive unmodified nucleotides flanked on the 3' and 5' ends by consecutive methylphosphonate nucleotides. This strategy was shown to markedly improve the specificity with which RNAse H cleaved its target mRNA. It has since been possible to decrease the stretch of unmodified nucleotides further to two or three residues (Giles and Tidd 1992, Giles *et al.* 1993), thus increasing the specificity of the target region. The chimeric oligonucleotides were found to combine the advantages of the methylphosphonates (improved uptake, nuclease resistance) with those of the unmodified nucleotides (RNAse H activation, improved water solubility), whilst minimising the non-specific cleavage of mRNA by RNAse H.

Another oligonucleotide modification designed to improve cell uptake, nuclease resistance and, in some cases, RNAse H cleavage has been to conjugate the 5' and/or the 3' end of the oligonucleotide with groups possessing these properties such as lipophillic groups like cholesterol. In work by Boutorine *et al.* (1992), the presence of cholesterol conjugated to the 5' end of an antisense oligonucleotide displayed increased resistance to nuclease attack and a 30-100 fold increase in uptake by human cancer cells. The increase in uptake was thought to be due in part, to the cholesterol-conjugated oligonucleotide binding to low density lipoprotein (LDL) and uptake via LDL-receptor mediated endocytosis (Krieg *et al.* 1993). There have however, been reservations about how effective these cholesterol-conjugated oligonucleotides are at binding to target RNA (Boiziau and Toulme 1991).

Finally, other modifications that have been reported have included the encapsulation of either modified or unmodified oligonucleotides in liposomes (Iversen 1991). This has been demonstrated to enhance cellular uptake and to provide resistance from nuclease attack. Similarly, the addition of cationic lipids to the incubation medium of cultured cells has been shown to enhance the uptake of antisense oligonucleotides (Capaccioli *et al.* 1993). Altering the concentration of extracellular ATP in culture has been shown to increase the permeability of cells to macromolecules of up to 20kDa (Saribas *et al.* 1993). It is also possible that oligonucleotides could be introduced into cells by a method of electropermeabilisation reported by Gabriel and Teissie (1994).

5.15: The application of antisense technology to endocrine systems

So far antisense technology has not been widely used in endocrine cells but may well become a standard approach to determining the role played by particular proteins in stimulus-response coupling in endocrine cell systems. Rather than presenting an exhaustive list of publications, three representative cases where antisense technology has been applied to endocrine systems will be described.

The LH/CG receptor

Previous work in this laboratory incubated growing MA10 cells with unmodified antisense oligonucleotides (West and Cooke 1991b). The oligonucleotides had been designed to hybridise to different regions of the rat LH/CG receptor mRNA. The work was designed to establish which regions of the LH/CG receptor were involved in the desensitisation response. MA10 cells were preincubated for up to 48 hours with each antisense oligonucleotide at a concentration of 2.5μM. The cells were then incubated with desensitising doses of LH, 4ß-PMA and dibutyryl-cAMP and the incubation medium was assayed for the presence of cAMP and the steroid pregnenelone. Antisense oligonucleotide 1 was targeted to the extracellular domain. Cells preincubated with antisense oligonucleotide 1 for 24 hours demonstrated a 75% loss in [^{125}I]-hCG, binding but LH-stimulated cAMP and pregnenolone production were not affected. When the cells were incubated with antisense oligonucleotide 1 for 48 hours however, there was a 98% loss in [^{125}I]-hCG binding and a complete loss of LH-stimulated cAMP and pregnenolone production.

A second antisense oligonucleotide was targeted to the third intracellular loop in an attempt to delete the 7th transmembrane domain and cytoplasmic tail. Cells pretreated with this antisense oligonucleotide did not demonstrate LH or 4ß-PMA-induced desensitisation as measured by the loss of cAMP production. Cells pretreated with this antisense oligonucleotide did demonstrate LH-induced but not dibutyryl cAMP or 4ß-PMA-induced desensitisation as measured by pregnenolone production, indicating that LH action involves additional mechanisms compared to dibutyryl cAMP and 4ß-PMA. It was also found that loss of binding sites caused by the incubation of cells with dibutyryl cAMP and LH was prevented by preincubation with this antisense oligonucleotide (West *et al.* 1991).

A third antisense oligonucleotide targeted to the last 7 amino-acids of the cytoplasmic tail did not prevent LH, dibutyryl cAMP or 4ß-PMA-induced desensitisation as measured by pregnenolone production. However the loss of binding sites caused by the incubation of cells with dibutyryl cAMP and LH was prevented by preincubation with this antisense oligonucleotide (West *et al.* 1991). This indicated that the loss of [^{125}I]-hCG binding sites could be discriminated from LH-induced desensitisation.

Diazepam-binding inhibitor

MA10 cells were also used in another antisense study investigating the role of the diazepam-binding inhibitor (DBI) (Boujrad *et al.* 1993). Cholesterol-linked, phosphorothioate antisense oligonucleotides were incubated with MA10 cells in culture in order to assess whether DBI was implicated in hormone-stimulated steroidogenesis. The results of this work showed that when MA10 cells were incubated with the antisense oligonucleotides, there was a dose dependent loss in their ability to respond to hCG by producing progesterone. The researchers concluded that DBI was involved in the acute stimulation of steroidogenesis by trophic hormones.

PKC isoforms

Another study examining the role of protein kinases in signal transduction was performed by Godson *et al.* (1993). In this work, canine kidney cells were transfected with antisense cDNAs to either the PKCα or PKCß isoforms, or both. In cells

transfected with antisense PKCα or both PKCα and PKCß isoforms, phorbol ester-stimulated release of arachidonate and its metabolites was inhibited. In cells that had been transfected with only cDNA antisense to the PKCß isoform, phorbol ester-stimulated arachidonate release was not significantly different from control cells. This work clearly demonstrated the use of an antisense approach in defining the functions of particular isoforms of PKC.

5.16: The therapeutic potential of antisense technology

From the work described above, it is clear that the antisense oligonucleotide approach, in defining the roles of particular proteins in cell signalling events, is a powerful tool for endocrinologists. Furthermore, the therapeutic potential of these agents is great, given their ability to act specifically on a particular cellular target. In athymic mice, continuous administration of oligonucleotides antisense to N-myc, a DNA-binding phosphoprotein, via a subcutaneously implanted micro-osmotic pump, resulted in a reduction in the growth of a neuroendocrine tumour, concomitant with reduced N-myc expression in the tumour (Whitesell *et al.* 1991). At present, the applicability of administration of antisense oligonucleotides to humans for the treatment of some cancers, herpes simplex virus II and the inhibition of the replication of the human immunodeficiency virus is being evaluated. The potential use of antisense oligonucleotides as inhibitors of autoantibody production may be therapeutically applicable to autoimmune endocrine diseases, such as Grave's disease and some types of insulin-dependent diabetes mellitus (Crooke 1993, Persaud and Jones 1994).

5.2: Aims

The aim at the start of this work was to continue to use the antisense strategy previously designed in the laboratory to generate truncated LH/CG receptors in MA10 cells as was reported to have been demonstrated by West and Cooke (1991b and 1992). The generation of these mutated receptors would provide information about which parts of the LH/CG receptor were important for functions such as G protein coupling, membrane insertion, desensitisation and/or phosphorylation. The mutations that were predicted to result from the use of an antisense strategy would be mimicked by the transfection of truncated MA10 LH/CG receptor cDNA into a mammalian

steroidogenic cell line. In this way then, it would be possible to confirm that incubation with the antisense oligonucleotide had generated the truncation predicted, and that the effects of cell signalling observed when incubating cells with antisense oligonucleotides were as a result of the production of truncated receptors and not because of another non-specific mechanism. Experiments such as these would provide valuable information as to how much of a viable alternative the use of an antisense strategy is compared to the more traditionally used technique of site-directed mutagenesis.

Soon after this work began, major problems were encountered during experiments designed to confirm the results of the previous work. It was soon realised that another experimental system would have to be designed before the work could progress further. To this end, the aim of the work evolved to include the establishment of cell-free methods in which to first test antisense oligonucleotides for effectiveness. Once suitable antisense oligonucleotides had been established as effective in the cell-free systems, it was intended to return to using the cultured MA10 cells to ascertain whether the results could be repeated.

5.3: Results

The first antisense experiment to be performed during this work used antisense oligonucleotide 1, one of the five original unmodified antisense oligonucleotides designed and used in the previous work reported in West and Cooke (1991b). During preliminary experiments designed to repeat the methodology of the aforementioned work, contrary to what had been previously demonstrated, it was found that MA10 cells that had been incubated for 24 hours with antisense oligonucleotide 1, did respond to LH. LH-stimulated progesterone production was however, decreased (approximately 3-fold) when compared to control cells. LH/CG receptor binding assays performed at 37°C, found that cells incubated with antisense oligonucleotide 1 demonstrated no difference in the specific binding of [^{125}I]-hCG when compared to control cells. However, repeating this work was fraught with difficulties as will be discussed later and it is impossible to say whether these early results provided a representative picture of what was happening during the incubation of cells with antisense oligonucleotide 1.

Because of these initial difficulties and also in order to investigate the mechanisms of the antisense strategy in more detail, it was decided to establish and carry out this work in a cell-free system. As reported in *Chapter Three*, section ***3.4***, the rat LH/CG receptor mRNA contains large amounts of secondary structure, particularly around the N-terminus. It was therefore decided to use a coupled transcription/translation system in order to try and minimise the effects of mRNA secondary structure. Since this system begins to translate mRNA as soon as it has been transcribed, mRNA secondary structure is minimised.

5.31: The coupled transcription/translation of the LH/CG receptor in a cell-free system

Figure ***5.31*** shows the results from a coupled transcription/translation experiments. Plasmids containing the LH/CG receptor, the FSHR, the Kallmann protein and ß-galactosidase were transcribed and translated in a coupled reticulocyte reaction (TnT reaction). Although faint due to underexposure, bands can be seen that correspond in size to the respective proteins. A single band, corresponding in size to the

unglycosylated LH/CG receptor (approximately 65kDa) is absent in a control reaction that contained water in place of a plasmid. The results from this work would indicate that the synthesis of the LH/CG receptor (and other proteins) had been successfully established in a coupled cell-free system. Since the water control is devoid of any protein bands, it indicates that those present are specific.

Having established a cell-free system where the LH/CG receptor appeared to be synthesised, it was now possible to add antisense oligonucleotide 1 in order to see if LH/CG receptor could be prevented. A new stock of antisense oligonucleotide 1 had been made that was an exact copy of the one used by West and Cooke (1991b). Because the oligonucleotide was designed to be used in a cell-free system it was not thought that nuclease degradation would pose a problem. For this reason, the oligonucleotide did not contain modifications and was synthesised from unmodified bases.

5.32: The use of antisense oligonucleotide 1 in the cell-free system

Figure ***5.32a*** demonstrates the regions of the LH/CG receptor which correspond to the mRNA sites to which the original five antisense oligonucleotides were targeted. Antisense oligonucleotides 1 and 3 were used in the work reported here.

Figure ***5.32b*** shows the results from a TnT experiment using antisense oligonucleotide 1. A plasmid containing the ß-galactosidase gene (ß-gal) was used as a control for antisense oligonucleotide sequence-specificity. Both the control plasmid and the plasmid containing the LH/CG receptor gene were transcribed and translated in increasing concentrations of antisense oligonucleotide 1. In the lanes where antisense oligonucleotide 1 was absent, bands can be seen that correspond in size to both the LH/CG receptor and the ß-gal proteins. As the concentration of antisense oligonucleotide 1 increases, the level of synthesis of the ß-gal control plasmid is not altered. In the presence of increasing concentrations of antisense oligonucleotide 1, the intensity of the putative LH/CG receptor band becomes less prominent but the size of the protein remains the same. If this antisense oligonucleotide were to block the translation of the LH/CG receptor, as is suggested in West and Cooke (1991b), then it

would be anticipated that this band would disappear when the concentration of antisense oligonucleotide was obtained that was capable of completely blocking the translation of the LH/CG receptor mRNA. Since the putative LH/CG receptor band becomes less prominent in increasing concentrations of the antisense oligonucleotide, but does not disappear altogether, it must be concluded that the highest concentration of the antisense oligonucleotide 1 (32μM) was not high enough to prevent complete translation of the LH/CG receptor mRNA.

It is not certain as to the nature of the second prominent band that can be seen on the autoradiograph. However, since this band is also reduced in intensity as the concentration of antisense oligonucleotide 1 increases and it is possible that it represents either an alternative mRNA transcript that has been translated into protein, or an alternatively translated form of the LH/CG receptor. From analysis of the rat sequence it was found that in addition to the first AUG start site of translation, there were 11 other AUG codons in phase. It is possible that one or more of these AUG codons was used as an alternative or 'mistaken' start site of translation.

The results from further experiments using antisense oligonucleotide 1 proved to be inconclusive, however. *Figure 5.32c* shows the results from another TnT experiment using antisense oligonucleotide 1 at a range of concentrations. Although faint, a band of approximately 65kDa can be seen in the lanes containing the LH/CG receptor plasmid, the addition of antisense oligonucleotide 1 does not appear to have an effect on the intensity of this band until the highest concentration (32μM) is reached. At this point the protein band corresponding to the LH/CG receptor disappears indicating that the oligonucleotide has prevented LH/CG receptor synthesis. This effect however, is not specific as at the same concentration, the protein bands from the ß-gal control plasmid also disappear.

5.33: The use of antisense oligonucleotide 3 in the cell-free system

Antisense oligonucleotide 3, previously used in work by West and Cooke (1991b), was also tested in the cell-free system. This oligonucleotide was targeted to the mRNA in a coding region located on the cytoplasmic tail. Antisense oligonucleotide 3 was thought

to cause the production of truncated LH/CG receptors since treatment with this oligonucleotide was reported to prevent LH-stimulated and 4ß-PMA-induced cAMP desensitisation. 4ß-PMA-induced and dibutyryl cAMP-induced pregnenolone desensitisation was also prevented, but binding of [^{125}I]-hCG was not affected by this oligonucleotide. Conversely, treatment with antisense oligonucleotide 3 was found to have no affect on LH-stimulated cAMP or pregnenolone production or binding of [^{125}I]-hCG (West and Cooke 1991b and 1992). *Figure **5.33*** shows the results obtained when antisense oligonucleotide 3 was added to a TnT reaction of the LH/CG receptor at varying concentrations. At the highest concentration of antisense oligonucleotide 3 (35µM), the intensity of the LH/CG receptor band begins to fade, however, this is not due to a specific effect of the antisense oligonucleotide, as the intensity of the ß-Gal band is also fainter at this concentration.

5.34: Can antisense oligonucleotides 1 and 3 prevent transcription?

mRNA is transcribed from the sense strand of DNA and is therefore also described as "sense". Oligonucleotides complementary to the mRNA will therefore be "antisense". Thus, such antisense oligonucleotides will possess the same sequence of bases as the coding strand of DNA and will be complementary to the non-coding strand of DNA. If antisense oligonucleotides entered the transcription bubble, they could theoretically anneal with the non-coding DNA strand and interfere with the process of transcription by steric hindrance (triplex formation). Whilst the above experiments were being performed, it was also decided to investigate whether the antisense oligonucleotides used in this work could have an effect on the level of LH/CG receptor gene transcription. *Figures **5.34a*** show the results from such an experiment using antisense oligonucleotide 1. Bands can be seen that correspond to the size of the rat LH/CG receptor mRNA (approximately 2.1kb). The presence of the antisense oligonucleotide had no effect until the top concentration of 32µM is reached, at which point the LH/CG receptor mRNA band disappears. *Figure **5.34b*** shows the same experiment using antisense oligonucleotide 3. This oligonucleotide did not appear to effect LH/CG receptor transcription at its top concentration (35µM). However, due to the low level of synthesis of the control plasmid however, it is not possible to ascertain whether these effects on LH/CG receptor gene transcription were specific or not. It is possible

that when triplex formation occurs near the beginning of gene transcription, that the ribosomal complex may be inhibited sterically or otherwise. When the triplex formation occurs further downstream the ribsomal complex may be less inhibited by its presence.

5.35: The synthesis of new antisense oligonucleotides to around the start site of translation

Antisense oligonucleotide 1 was designed in previous work, to hybridise to bases 105-125 of the MA10 LH/CG receptor mRNA. This area of the mRNA corresponded to a region starting 33 amino-acids downstream of the start site of translation. As cited in much of the literature described above, oligonucleotides that hybridise to regions of the mRNA upstream or covering the start site of translation were found to generally be more effective at preventing the translation of mRNA into protein. For this reason it was decided to synthesise two further antisense oligonucleotides that would hybridise to these regions. *Figure 5.35a* shows the sites to which the new antisense oligonucleotides were designed to hybridise. Antisense oligonucleotide A was designed to anneal to bases -10 to 12 and antisense oligonucleotide B was designed to hybridise to bases -1 to 18. Both antisense sequences were designed to cover the start site of translation with antisense oligonucleotide A extending further upstream into the 5'UTR than antisense oligonucleotide B.

Figure 5.35b shows the results from a TnT experiment using antisense oligonucleotide A. It can be see that when this oligonucleotide is present at the highest concentration (24μM), the intensity of the LH/CG receptor band decreases, however, once again this effect is not specific because the same phenomenon can be seen in the ß-gal control. As other similar results were obtained when using this antisense oligonucleotide, it would appear that antisense oligonucleotide A is not able to prevent translation of the LH/CG receptor despite being targeted to a region generally thought to be effective in preventing ribosome assembly and/or binding (Blake *et al.* 1985, Mizutani *et al.* 1995, Wakita and Wands 1994).

Figure 5.35c shows the results from a similar TnT experiment using antisense oligonucleotide B. The intensity of the LH/CG receptor band does appear to be fainter

at the highest concentration of antisense oligonucleotide B (2.6μM) when compared to the control lane, however, this could be due to differences in sample loading. The intensity of the ß-gal protein does not alter with an increase in antisense oligonucleotide B concentration, suggesting that this antisense oligonucleotide could be effective in specifically preventing the translation of LH/CG receptor mRNA, providing it could be synthesised at a higher concentration.

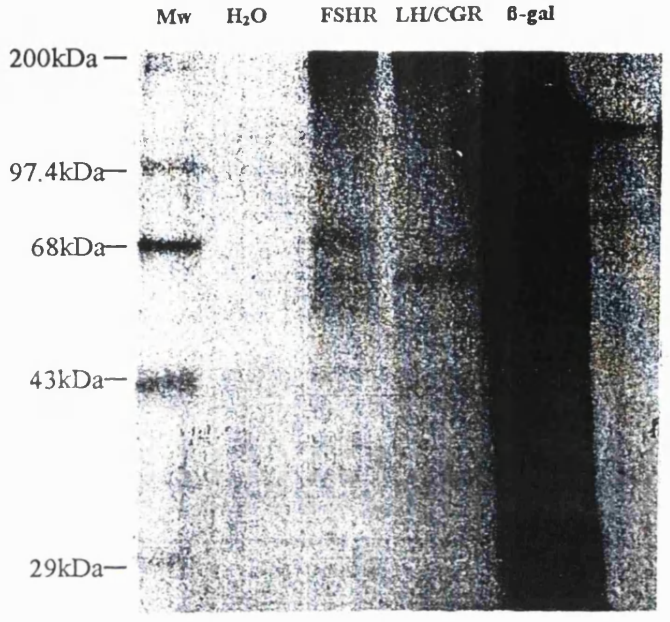


Figure 5.31: **Cell-free synthesis of the LH/CG receptor.** This photograph shows the results obtained when the plasmid containing the full-length rat LH/CG receptor (pBSLHR/59) was transcribed and translated using a coupled RRL cell-free system. A band of approximately 65kDa can be seen corresponding to the unglycosylated LH/CG receptor, whilst a band of approximately 70kDa can be seen corresponding to the unglycosylated FSHR. No bands can be seen in the H_20 control, whilst the ß-gal control can clearly be seen and is present at too high a concentration.

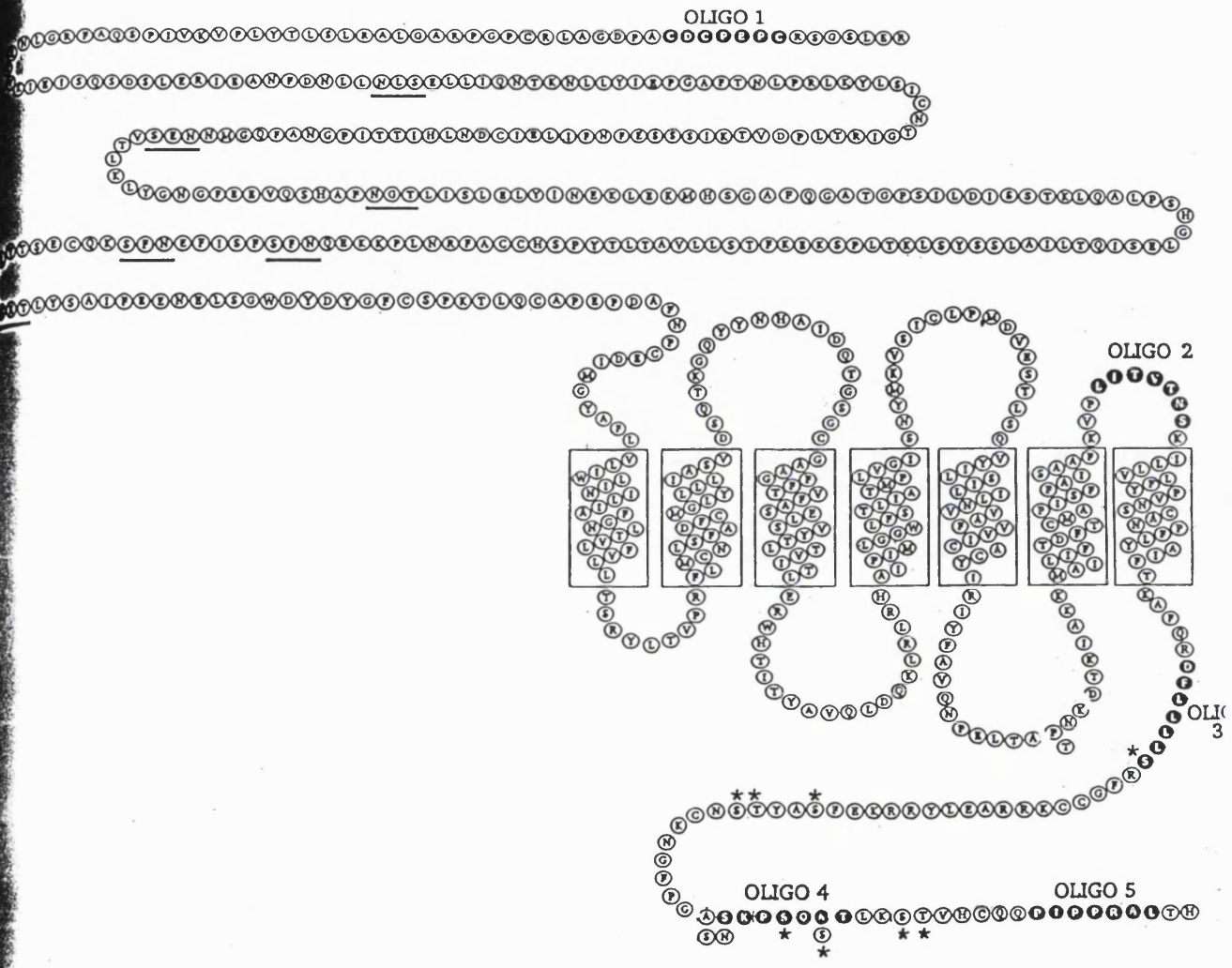


Figure 5.32a: **Diagram showing the amino-acid sequence of the rat LH/CG receptor and the regions to which the original antisense oligonucleotides were targeted (West and Cooke 1991b).** Not shown on this diagram are the regions to which antisense oligonucleotides A and B were targeted as they were directed to the 5'UTR.

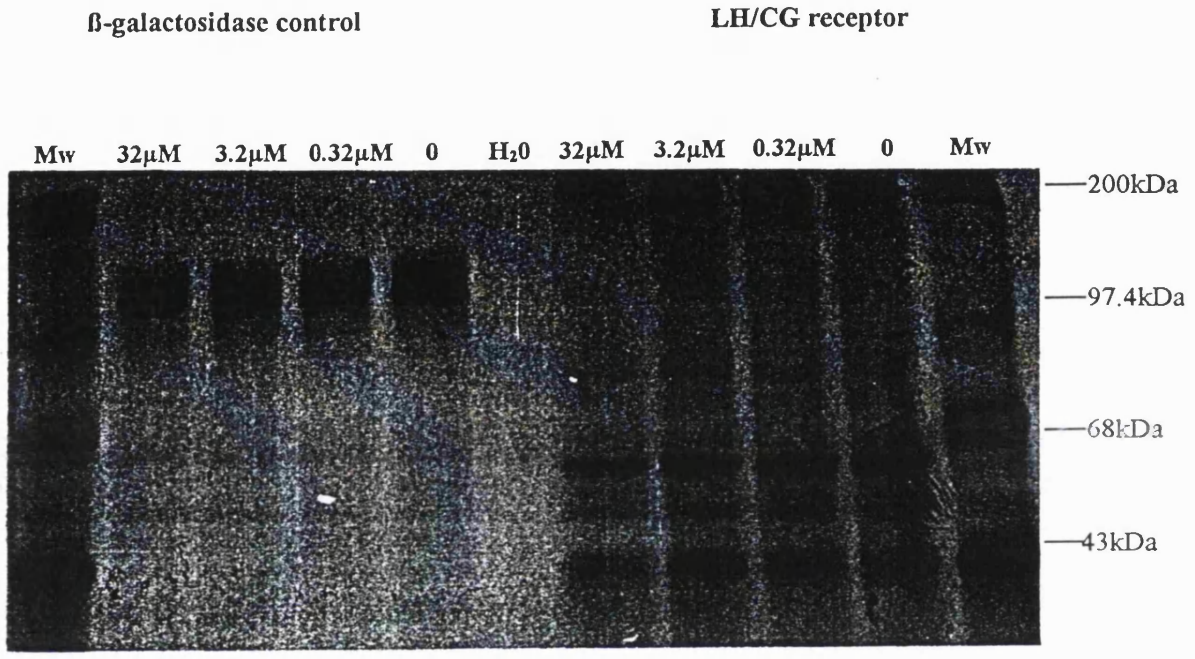


Figure 5.32b: **The effect of antisense oligonucleotide 1 on LH/CG receptor synthesis.** Bands corresponding to the unglycosylated LH/CG receptor, with an approximate molecular mass of 65kDa can be seen. This band seems to fade in intensity with the addition of increasing concentrations of antisense oligonucleotide 1, although no change in the size of the LH/CG receptor can be observed (compare to the lanes where antisense oligonucleotide 1 is absent). The decrease in band intensity could be due to the specific interaction of antisense oligonucleotide 1 with LH/CG receptor synthesis, as no similar decrease is observed in the lanes where ß-gal is present as a control.

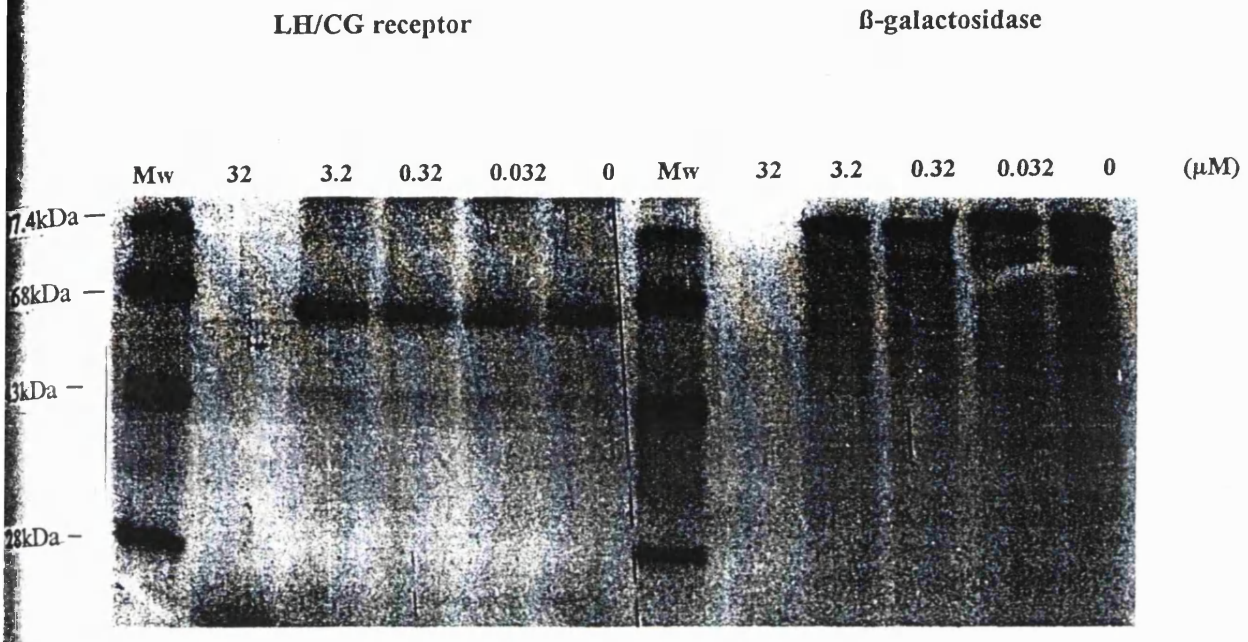


Figure 5.32c: **Another TnT reaction with antisense oligonucleotide 1.** This photograph shows the results from a similar experiment to that seen in ***Figure 5.32b***, except the highest concentration of antisense oligonucleotide is now 32µM. However, in this photograph, increasing concentrations of antisense oligonucleotide 1 do not appear to have any effect on the level of LH/CG receptor synthesis until this highest concentration is reached. At this point, both LH/CG receptor and ß-gal synthesis are prevented, indicating that this is a non-specific effect of the antisense oligonucleotide.

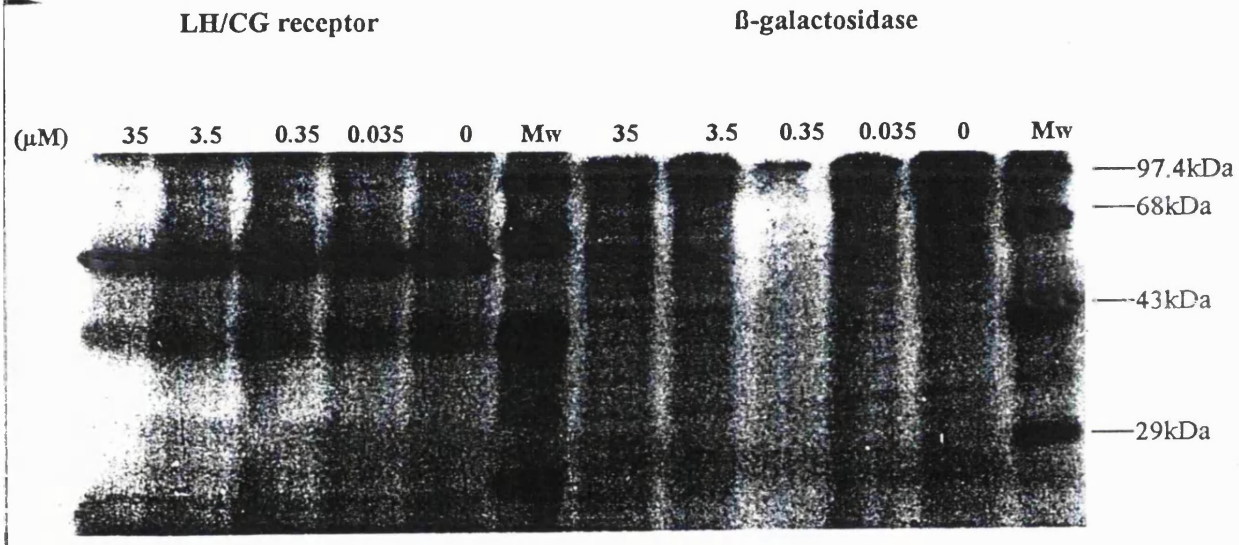


Figure 5.33: **The effect of antisense oligonucleotide 3 in LH/CG cell-free synthesis.** The band of approximately 65kDa corresponding to the unglycosylated LH/CG receptor can be seen . The addition of increasing concentrations of antisense oligonucleotide 3 has no effect on the size of the putative LH/CG receptor. However, LH/CG receptor synthesis does begin to decrease in intensity in the presence of the highest concentration of antisense oligonucleotide 3 (35µM). This would seem to be a specific effect as a similar decrease in the intensity of the ß-gal control is not seen.

Figure 5.34a: Antisense oligonucleotide 1

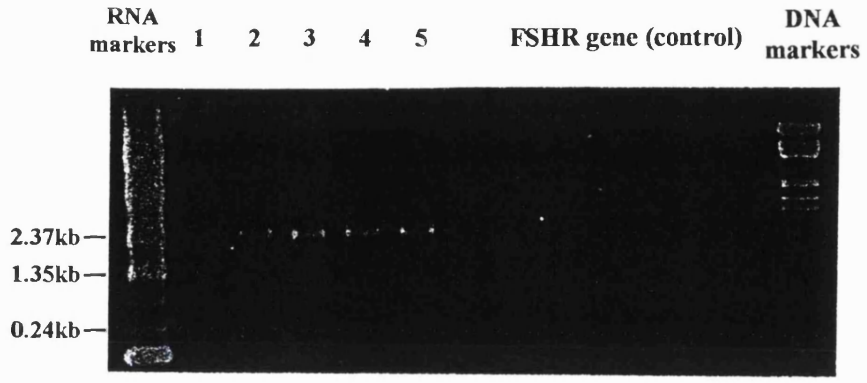


Figure 5.34b: Antisense oligonucleotide 3

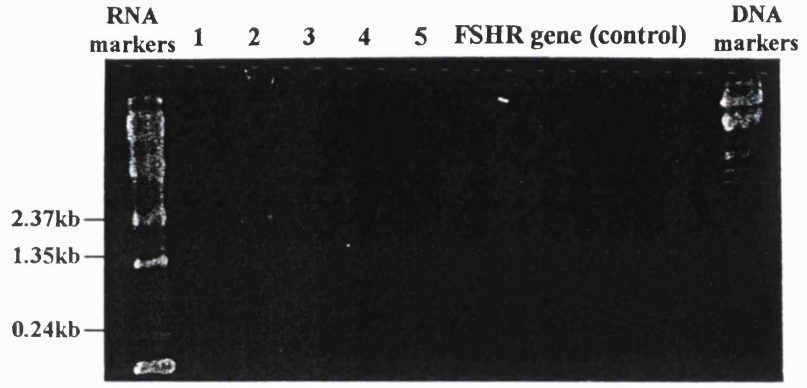


Figure 5.34a: Lane 1 = 32μM, Lane 2 = 3.2 μM, Lane 3 = 0.32μM, Lane 4 = 0.032μM, Lane 5 = 0μM.
Figure 5.34b: Lane 1 = 35μM, Lane 2 = 3.5μM, Lane 3 = 0.35μM, Lane 4 = 0.035μM, Lane 5 = 0μM

Figures 5.34a* and *b:* The effect of antisense oligonucleotides 1 and 3 on LH/CG receptor gene transcription.** (Figures are photographs of RNA samples electrophoresed on 0.1% TBE/agarose gels). *Figure* ***5.34a demonstrates the results obtained when antisense oligonucleotide 1 was used in a LH/CG receptor gene transcription reaction. RNA bands of the transcribed gene can be seen corresponding to a size of approximately 2kb. Only when the top concentration of oligonucleotide was reached did transcription appear to be inhibited. Conversely antisense oligonucleotide 3 did not appear to have an effect on gene transcription. The transcription of the FSHR gene was used as a control in these experiments but can only be seen faintly in these pictures.

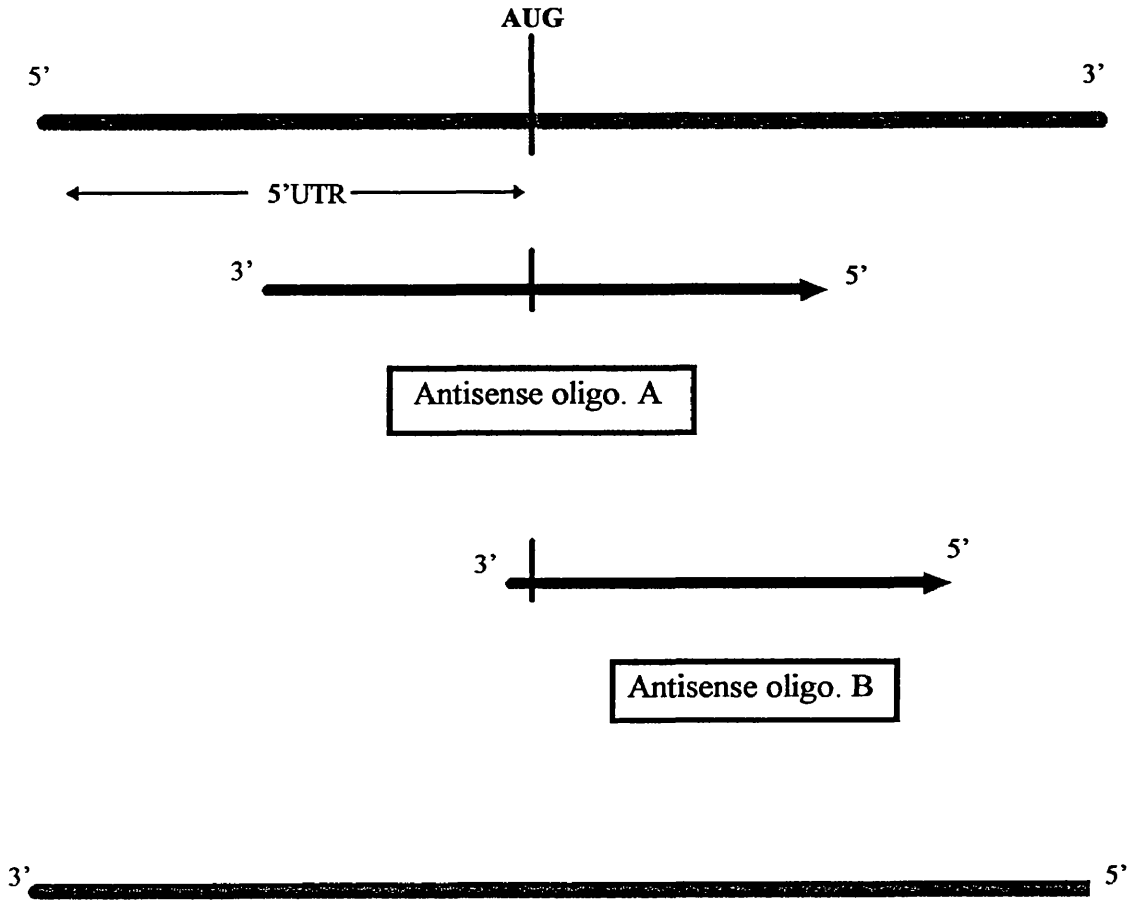


Figure 5.35a: Diagram of the LH/CG receptor mRNA demonstrating the target sites of antisense oligonucleotides A and B

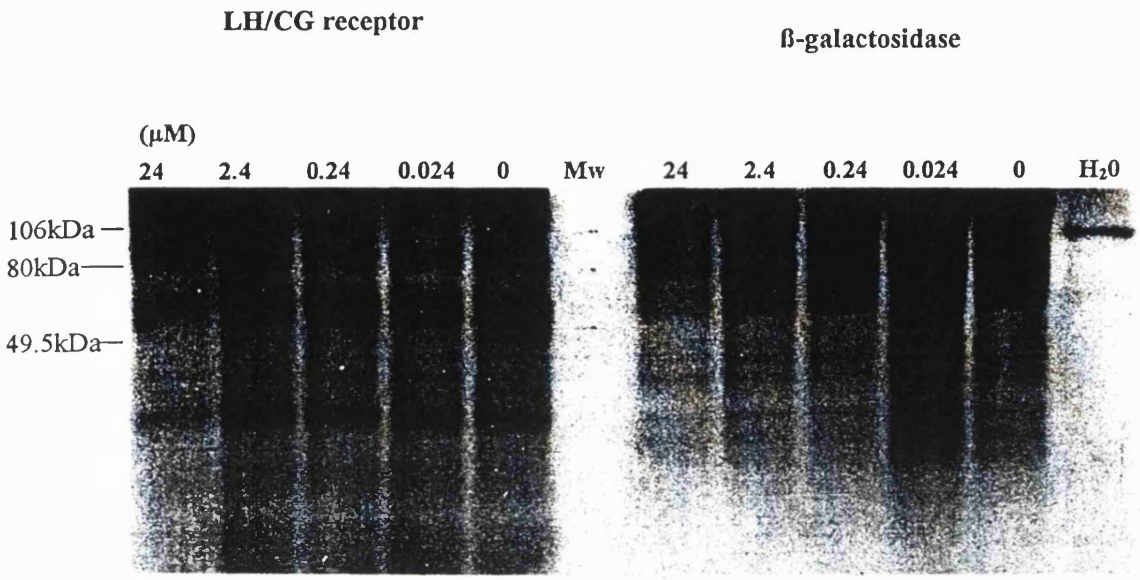


Figure 5.35b: **The effect of antisense oligonucleotide A on LH/CG receptor cell-free synthesis.** The band of approximately 65kDa corresponding to the unglycosylated LH/CG receptor can be seen. (It is not known what the previously unseen band with a molecular mass of approximately 80kDa represents, although it is probably an experimental artefact). The addition of increasing concentrations of antisense oligonucleotide A does not appear to alter the level of LH/CG receptor synthesis.

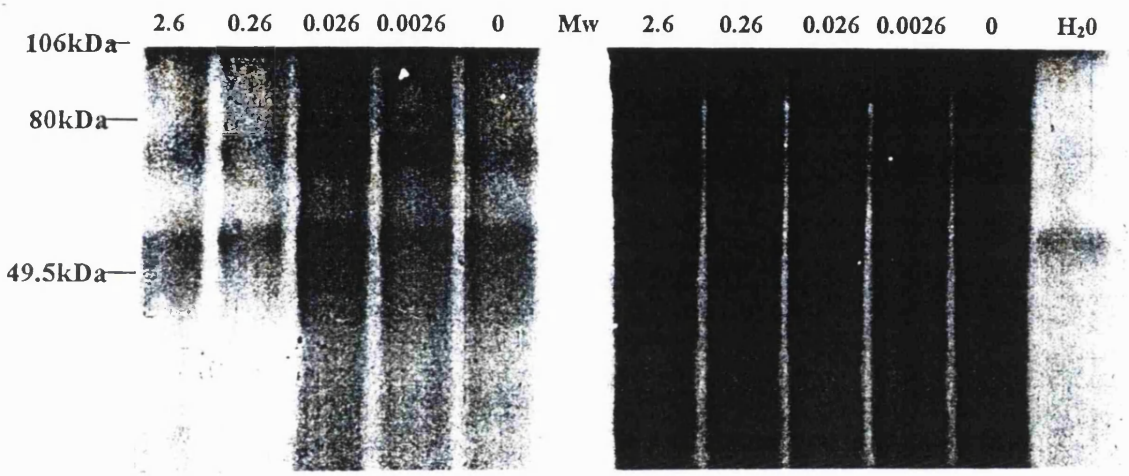


Figure 5.35c: **The effect of antisense oligonucleotide B on LH/CG receptor cell-free synthesis.** The band of approximately 65kDa corresponding to the unglycosylated LH/CG receptor can be seen to decrease in intensity as the concentration of antisense oligonucleotide B increases. This effect would appear to be specific as the intensity of the ß-galactosidase controls remain unaffected by the increase in the antisense oligonucleotide B concentration.

5.4: Discussion

The aim at the start of this work was to continue to use the antisense strategy previously designed in the laboratory to generate truncated LH/CG receptors in MA10 cells reported by West and Cooke (1991b). The generation of these mutated receptors would provide information about which parts of the LH/CG receptor were important for functions such as G protein coupling, membrane insertion, desensitisation and/or phosphorylation. However, during preliminary experiments, major problems were encountered in trying to reproduce the methodology and results previously reported.

5.41: Problems encountered in trying to repeat previous methodology

Two major problems were encountered whilst trying to repeat previous work (West 1992- PhD thesis). In brief; firstly it was found impossible to obtain adequate specific binding during [^{125}I]-hCG binding studies incubated at 4°C overnight, using 200000 cells as previously documented. When further investigated it was found that adequate specific binding could only be obtained at 4°C if a minimum of 2 million cells were used and incubated at 4°C for 48 hours.

Secondly, it was found impossible to reproduce the results of studies that had identified trypsinisation as being efficient at removing existing LH/CG receptors from the cell surface. This was important because if the effects of antisense oligonucleotides were to be assessed, existing unmodified LH/CG receptors had to be first be removed from the cell surface. If this was not done efficiently these existing LH/CG receptors would mask the results from any antisense modified LH/CG receptors, during [^{125}I]-hCG binding studies. When the methodology was repeated it was found that trypsinisation of MA10 cells had little effect on decreasing [^{125}I]-hCG binding.

The inability to repeat these basic techniques had far reaching consequences for the progression of this work. As a result of the above, it was impossible to establish whether the results from the two preliminary studies described at the beginning of this results chapter, were representative and/or valid. To reiterate, in this study cultured MA10 cells were incubated for 24 and 48 hours with antisense oligonucleotide 1. The results from the first study found that after incubation with antisense oligonucleotide 1,

LH-stimulated progesterone production was not prevented, although it was decreased by approximately 3-fold when compared to control cells. The results from the second study found that cells were able to bind [^{125}I] hCG (at 37°C) to the same extent as the control cells that had not been pretreated with antisense oligonucleotide 1. These results conflicted with those previously documented by West and Cooke (1991b) which found the cells pretreated with antisense oligonucleotide 1 to be unresponsive to LH as measured by pregnenolone assay, with the cells failing to demonstrate [^{125}I] hCG binding (at 4°C) in receptor binding assays.

5.42: Other problems

As discussed earlier in this chapter, the successful use of an antisense strategy is dependent on many parameters. In cell culture, antisense oligonucleotides need to be resistant to degradation both in the culture medium and inside the cell. They need to be readily taken up by the cell so as to reach the desired concentration inside the cell. They have to hybridise with their target in a specific manner. Despite not being necessarily representative, our preliminary results indicated that antisense oligonucleotide 1 was not having an effect on LH/CG receptor synthesis. We therefore had to elucidate where else in the experimental protocol we might be encountering problems.

Firstly, unmodified oligonucleotides are not generally stable in culture medium, especially if serum is present, for long periods of time at 37°C. We therefore thought it unlikely that antisense oligonucleotide 1 would remain undegraded for up to periods of 24 hours.

Secondly, despite the reported existence of a membrane protein potentially involved in oligonucleotide uptake (Loke *et al.* 1989, Yakubov *et al.* 1989), some antisense studies have still had problems with the uptake of unmodified oligonucleotides by the cell (Tidd 1992, Krieg 1993, Stein and Cheng 1993). Thus, our seemingly negative results could have been as a result of little or no oligonucleotide getting into the cell.

Because the binding studies were not working using the previously established methodology, it was impossible to detect the effects of any changes made to the experimental conditions in an attempt to reduce some of these problems. It was therefore decided to establish a cell-free system of monitoring LH/CG receptor synthesis.

It was found that the reticulocyte derived, coupled TnT system could be used successfully to synthesise the LH/CG receptor protein. Experiments containing the LH/CG receptor plasmid were found to produce a reaction product of approximately 65kDa. Since the synthesis of the LH/CG receptor was taking place in cell-free conditions, glycosylation modifications usually found in the mature LH/CG receptor (70-92kDa) were not anticipated. Thus, the size of the putative LH/CG receptor was thought to be accurate. After we had demonstrated that the LH/CG receptor could be synthesised in a cell-free system, antisense oligonucleotide 1 was added in order to assess its effectiveness in preventing LH/CG receptor synthesis.

It had been documented (West and Cooke 1991b) that antisense oligonucleotide 1 had prevented the synthesis of the whole of the LH/CG receptor synthesis when it had been added to cell culture. It was therefore anticipated that in the cell-free system in the presence of antisense oligonucleotide 1, LH/CG receptor synthesis would not occur and thus no putative LH/CG receptor protein bands would be detected. However, apart from a slight decrease in the level of LH/CG receptor synthesis in one experiment (***Figure 5.32b***), antisense oligonucleotide 1 could not be found to demonstrate any specific effect on the level of LH/CG receptor synthesis.

Similar results were demonstrated when antisense oligonucleotide 3 was added to the cell-free system. Previously, it had been suggested that incubation of MA10 cells with antisense oligonucleotide 3 had resulted in LH/CG receptors that were C-terminally truncated. Whilst these cells bound [^{125}I]-hCG at comparable levels to that of the controls, they did not undergo cAMP desensitisation when stimulated with LH or 4ß-PMA, nor pregneneolone desensitisation when stimulated with 4ß-PMA or dibutyryl cAMP. However, LH-stimulated levels of cAMP and pregnenolone were unaffected by

incubation with this oligonucleotide. Antisense oligonucleotide 3 was targeted to nucleotides corresponding to amino-acids 269-306. It was anticipated from results obtained by West and Cooke (1992), that mRNA cleavage may occur at this site presumably via an RNAse H directed mechanism. This would result either in the synthesis of a truncated LH/CG receptor, which would be detected either as a decrease in the molecular mass of the LH/CG receptor of approximately 0.8kDa or, as is more likely, the cleaved mRNA would be degraded by enzymes resulting in the complete ablation of LH/CG receptor synthesis. Whilst a decrease in molecular mass of 0.8kDa would be difficult to detect on SDS-PAGE, no decrease in the overall level of LH/CG receptor synthesis or molecular mass was seen on SDS-PAGE when compared to the control (no antisense oligonucleotide 3). At high concentrations of oligonucleotide (35μM) the LH/CG receptor band intensity was found to decrease. However, this was not a specific effect of the oligonucleotide as the ß-Galactosidase control band also began to decrease in intensity.

There are a number of possible reasons as to why antisense oligonucleotides 1 and 3 were not observed to have an effect on LH/CG receptor synthesis. Firstly, the RNAse H content of the reticulocyte derived cell-free system may not have been high enough to direct mRNA cleavage at the site of oligonucleotide hybridisation. Unfortunately, due to RNAse H requiring very stringent buffer conditions, the obvious experiment whereby RNAse H was added to the cell-free translation reaction proved inconclusive and has not been included in this thesis. If finances had been permitting, these experiments could have been repeated in a cell-free wheat germ coupled TnT system. Despite reports suggesting that the level of RNAse H activity does not differ much between RRL systems and wheat germ extracts (Cazenave *et al.* 1993), it is possible that in the wheat germ system RNAse H was marginally greater or of a class that permitted the cleavage of an mRNA-DNA hybrid.

Because antisense oligonucleotides 1 and 3 had been aliquoted and stored at -70°C on arrival, degradation had not been considered a likely explanation for the lack of an effect on LH/CG receptor synthesis. However, the

integrity of antisense oligonucleotides 1 and 3 had not been confirmed and degradation cannot, therefore, be ruled out. Finally, it is possible that antisense oligonucleotides 1 and 3 simply do not prevent or alter the synthesis of the LH/CG receptor under the cell-free conditions described here but are able to do so within intact cells. However, as was discussed earlier, even if mRNA cleavage had been obtained in the cell experiments performed by West and Cooke (1991b), it is unlikely that such mRNAs would have existed in the cell long enough to be translated into proteins. In the event that a cleaved mRNA were translated into protein, it is even more unlikely that it would insert in the membrane in the correct orientation for ligand binding, since many tranfection studies have demonstrated that mutated proteins are often trapped intracellularly (Xie *et al.* 1990, Moyle *et al.* 1991, Braun *et al.* 1991, Ji and Ji 1991, Zhu *et al.* 1995).

The levels of cellular processing which antisense oligonucleotides could affect are numerous and include the prevention of transcription and post-transcriptional processing in addition to preventing translation (Helene and Toulme 1990, Ghosh and Cohen 1992, Hogan 1993). It is possible that antisense oligonucleotides present in the cytoplasm of a cell could enter the nucleus and interfere with gene expression at the level of transcription. Whilst some antisense oligonucleotides are designed specifically for the purpose of hybridising to specific genes in order to prevent their transcription, antisense oligonucleotides targeted to mRNA would only be complementary to the non-coding strand of DNA. It is possible however, that the subsequent formation of a DNA triplex within the transcription bubble could prevent transcription of the gene by steric hindrance. This theory was tested by preannealing antisense oligonucleotides 1 and 3 to a denatured plasmid containing the LH/CG receptor gene and then allowing transcription to take place. Neither antisense oligonucleotide was found to demonstrate a specific effect on the level of LH/CG receptor transcription. These results suggest that in cell-free studies such as these, antisense oligonucleotides designed to target specific mRNA sequences are unlikely to have an effect on gene transcription. Therefore, any effects seen by using antisense oligonucleotides in cell-free studies would be anticipated to occur after transcription has taken place.

In light of the many reports of the successful prevention of translation by antisense oligonucleotides designed to regions around the AUG start codon, two new antisense oligonucleotides were synthesised. These were subsequently used in the cell-free system in order to ascertain whether the translation of the LH/CG receptor could be prevented. Both oligonucleotides were designed to cover the AUG initiation codon, whilst antisense oligonucleotide A extended into the 5' UTR. Suprisingly, neither of these new antisense oligonucleotides were particularly effective in preventing LH/CG receptor translation. These results could be due to the antisense oligonucleotides not being present at high enough concentrations for a clear effect on LH/CG receptor synthesis to be determined. After its initial synthesis, the concentration of antisense oligonucleotide B was found to be much lower than that of A. Had it been possible to achieve higher concentrations of antisense oligonucleotide B in the cell-free reactions, alterations in the level or size of the LH/CG receptor protein may have been demonstrated less ambiguously. In addition, other factors can also influence the efficiency of antisense oligonucleotide hybridisation to the target mRNA. Examples are, the position of the target site on the mRNA, the chain length of the oligonucleotide, the presence or absence of secondary structure at the binding site, the salt content and/or temperature of the reaction, the G-C content of the oligonucleotides and/or target regions, (Ghosh and Cohen 1992).

5.5: Summary

The aim of this work had been to continue a previous study started by West and Cooke (1991b and 1992). However, it was found impossible to reproduce the original results and it was subsequently decided to establish a cell-free method of assessing the potential affects of antisense oligonucleotides on LH/CG receptor synthesis. The reticulocyte lysate derived, coupled transcription/translation system was successfully used to synthesis the putative LH/CG receptor protein. With a cell-free system established, it was possible to test two of the original antisense oligonucleotides used in work by West and Cooke (1991b and 1992). It was found that neither antisense oligonucleotide 1 or 3 had any effect on the level of LH/CG receptor synthesis in this cell-free system. Neither was it possible to demonstrate any effect at the level of transcription. Since both of these oligonucleotides had been targeted to the coding

region of the LH/CG receptor mRNA, (an unreliable site of hybridisation unless RNAse H is present), new antisense oligonucleotides were subsequently designed that were targeted to the region around the start site of translation. Antisense oligonucleotide A, (targeted to cover the initiation codon and extend into the 5' UTR), did not prevent the translation of the LH/CG receptor. Ambiguous results obtained with antisense oligonucleotide B, (targeted to cover the initiation codon and extend upstream), suggested that at higher concentrations, this antisense oligonucleotide may be able to specifically prevent LH/CG receptor mRNA translation [see *Chapter Eight*].

Chapter Six: PKC inhibitor studies on LH/CG receptor function

6.1: Introduction

It is generally accepted that cAMP is the major second messenger in LH/CG receptor signal transduction, primarily because it satisfies most of the criteria devised by Sutherland (1972). It would seem logical to suggest then, that the adenylyl cyclase/cAMP pathway of LH/CG receptor signal transduction may also be involved in mediating LH/CG receptor desensitisation, by the activation of PKA for example. In addition, it is well established that PKA plays an important role in the desensitisation of other GPCRs such as the $ß_2$-adrenergic receptor ($ß_2$AR) (Hausdorff *et al.* 1990), and rhodopsin (Stryer 1991) [see *Chapter One*, section ***1.53***].

Whilst it is possible that PKA is involved in LH/CG receptor desensitisation, recent research suggests that other signal transduction pathways are also involved in mediating the cellular actions of LH and hCG. Reports of other second messenger systems involved in LH signal transduction include inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG) (Davis *et al.* 1986, Davis *et al.* 1987), arachidonic acid (Cooke *et al.* 1989, Lopez-Ruiz *et al.* 1992) and Ca^{2+}/calmodulin (Cooke and Sullivan 1985).

6.12: IP_3 and DAG

IP_3 and DAG are generated by the action of phospholipase C (PLC) on phosphatidylinositol 4, 5-bisphosphate and can act either independently or together, leading to the production or mobilisation of other messengers and modulators (Nishizuka 1984). For example, IP_3 can stimulate the release of Ca^{2+} from the endoplasmic reticulum (Rana and Hokin 1990). This resulting rise in intracellular Ca^{2+} concentration, coupled with the generation of DAG, results in the synergistic activation of the calcium-dependent protein kinase (PKC) which translocates to the cell membrane (Rana and Hokin 1990). Several GPCRs have been found to stimulate PLC, including the calcitonin receptor (Chabre *et al.* 1992), the gonadotropin-releasing hormone receptor (GnRHR) (Hsieh and Martin 1992) and the thyroid stimulating hormone receptor (TSHR) (Van Sande *et al.* 1990). There is also evidence to suggest that the LH/CG receptor may be coupled to PLC and that LH activation of the

receptor can generate the production of IP_3 and DAG and/or calcium mobilisation. For example, studies by Gudermann *et al.* (1992a and 1992b) have demonstrated that LH and hCG can stimulate both adenylyl cyclase activity and phosphatidylinositol 4, 5-bisphosphate hydrolysis, whilst work by Hipkin *et al.* (1993), demonstrated that an increase in LH/CG receptor phosphorylation by exposure to hCG could be mimicked by the PKC activator, 4ß-PMA [see *Chapter One*, section ***1.53***].

Among the 21 different G-protein $_{\alpha}$-subunits known to exist (Hepler and Gilman 1992) only $G_{\alpha s}$ and olfactory-specific $G_{\alpha olf}$ subunits stimulate adenylyl cyclase. Similarly subunits of the $G_{\alpha q}$ and $G_{\alpha o}$ classes strongly promote activity of PLC (Cockcroft and Thomas 1992, Exton 1993). The mechanisms by which G_s activating receptors activate two effectors is not known. In a report by Rosenthal *et al.* (1993), two models are proposed. In the first the activated receptor interacts with two G-proteins, $G_{\alpha s}$ and $G_{\alpha q}$. $G_{\alpha s}$ activates adenylyl cyclase and $G_{\alpha q}$ activates PLC. In the second model activation of a single G-protein, G_s, induces its dissociation into $G_{\alpha s}$ and $_{\beta\gamma}$ complexes. The former activates adenylyl cyclase while the later activates PLC.

6.13: PKC

PKC is a key regulatory element in signal transduction and exerts its effects by catalysing the phosphorylation of specific substrates. PKC is not a single enzyme but consists of a structurally homologous family of isoenzymes (Nishizuka 1988). To date there are 11 known isoenzymes of PKC [for reviews see Hug and Sarre 1993, Liu 1996]. Each isoform consists of a regulatory domain joined by a hinge region to a catalytic domain. The catalytic domain contains the active site of the enzyme and the regulatory domain binds the activators and regulators of PKC (Hug and Sarre 1993, Liu 1996). Each of the 11 isoforms have been cloned and characterised. Structural differences in the regulatory domain and different activation conditions have been the basis for the division of the isoforms into four major groups (Liu 1996) [see table below].

PKC family	Isoforms	Characteristics
cPKC (classical)	α, ß1, ßII, γ	Ca^{2+}-dependent
nPKC (novel)	δ, ε, η, θ	Ca^{2+}-independent
aPKC (atypical)	ζ, λ	Do not respond to phorbol esters and are Ca^{2+}-independent
PKCμ	-	Intermediate between aPKC and nPKC

Table 6: Eleven PKC isoforms of the PKC family can be divided into 4 groups according to structural differences in the regulatory domain and different activation conditions.

The regulatory domain of all PKC isoforms consists of variable regions interspersed by conserved regions. All PKC isoforms possess at least one conserved region (c1), which contains two tandem-repeated cysteine-rich sequences, resembling the consensus sequences for 'zinc-fingers' (Hug and Sarre 1993, Liu 1996). These tandem repeats have been found to be essential for DAG and phorbol ester binding/activation of PKC (Hubbard *et al.* 1991), hence many isoforms of PKC can be activated by phorbol esters. A second conserved region (c2) is found in the cPKC isoforms, but not the nPKC or aPKC isoforms. Since neither the nPKC or aPKC isoforms have an absolute requirement for Ca^{2+} for activity, the c2 region is thought to be involved in Ca^{2+} binding in cPKC isoforms (Liu 1996).

The cPKC group of isoforms are the most well-studied groups of the PKC family. Activation of these kinases involves complex interactions with their activators. Firstly, in the presence of phospholipids, cPKC binds to phosphatidylserine in the plasma membrane. This binding is highly co-operative and is greatly enhanced in the presence of Ca^{2+} in a concentration-dependent manner (Liu *et al.* 1994). cPKC binds at least 8 Ca^{2+} ions/protein at the interface between the protein and the plasma membrane, thus forming a PKC, phospholipid, Ca^{2+} complex (Liu 1996). This complex becomes

activated via its association with DAG. Binding of DAG to the complex is thought to cause a conformational change which results in the dissociation of the regulatory region from the substrate binding site located in the catalytic domain. This allows the substrate access to the enzyme for phosphorylation (Orr *et al.* 1992). Recent evidence has indicated that PKC may require the phosphorylation of its own catalytic domain before it is itself fully activated (Hug and Sarre 1993, Lui 1996). Such phosphorylation may induce a conformational change allowing the substrate access to the catalytic site. It has been suggested that each PKC isoform is rendered catalytically active as a result of its phosphorylation by another specific PKC isoform, thus resulting in a phosphorylation cascade within the PKC family (Newton and Taylor 1995). Activated PKC isoenzymes undergo selective translocation from the cytosol to the cell membrane, via mechanisms that are not yet fully understood. Once at the cell membrane, PKC becomes tightly associated by binding directly to phospholipids (Liu 1996). PKC is a serine/threonine kinase and thus catalyses the transfer of phosphate from ATP to the free hydroxyl group of these residues on substrate proteins (Nishizuka 1980).

In the presence of phosphotidylserine and Ca^{2+}, PKC can also be activated by a number of phosphoinositides such as phosphotidylinositol 3, 4, 5-trisphosphate (PIP_3) (Liu 1996). Cis-unsaturated fatty acids, which are derived from the cell membrane via the action of phospholipase A_2, are also activators of PKC (Nishizuka 1992). However, activation by fatty acids is independent of the presence of phosphotidylserine which may suggest that fatty acids activate PKC to phosphorylate different sets of substrates (Liu 1996).

6.14: Arachidonic acid

LH-stimulation of rat Leydig cells has been reported to cause the rapid release of arachidonic acid (Cooke *et al.* 1991), and in turn, arachidonic acid and its metabolites have been demonstrated to be involved in LH-induced steroidogenesis (Dix *et al.* 1984). The fatty acid, arachidonic acid, in addition to DAG, has also been shown to be a physiological regulator of cPKC in various tissues (McPhail 1984, Nishizuka 1992, Wakelam *et al.* 1993). As cPKC isotypes exist in Leydig cells (Pelosin *et al.* 1991), it

is possible that they could be activated by arachidonic acid and may thus mediate the effects of arachidonic acid in LH-induced steroidogenesis (Lopez-Ruiz *et al.* 1992). In a study by Lopez-Ruiz *et al.* (1992), pretreatment with either arachidonic acid or activators of PKC was found to have an inhibitory effect on rat Leydig cell steroidogenesis. In addition, when PKC was downregulated by pretreating rat Leydig cells with arachidonic acid or PMA, steroidogenesis was found to be enhanced. These results suggest that steroidogenesis in rat Leydig cells is normally under tonic inhibitory control by PKC (Lopez-Ruiz *et al.* 1992). Arachidonic acid is probably released to the cytosol via the activation of PLA_2, however, arachidonic acid release can also occur via PLD activation and/or PLC activation followed by the hydrolysis of DAG by DAG lipase (Rana and Hokin 1990, Lopez-Ruiz *et al.* 1992). Since both PLA_2 and PLC can be coupled to a common membrane receptor (Lapetina 1982), they may be activated by the same hormone (Lopez-Ruiz *et al.* 1992).

6.15: Ca2+/calmodulin and LH/CG receptor signal transduction

The involvement of Ca^{2+} in gonadal steroidogenesis has been suggested by findings which demonstrate that removal of extracellular Ca^{2+}, or the addition of Ca^{2+} channel blockers or calmodulin antagonists, results in the blunting of the steroidogenic response in Leydig cells (Janzen *et al.* 1976), granulosa cells (Veldhuis *et al.* 1984) and luteal cells (Dorflinger *et al.* 1984). In addition, work by Hall *et al.* (1981) demonstrated that the transport of cholesterol to the mitochondria and steroid biosynthesis was enhanced in rat Leydig cells that had been treated with liposomes containing Ca^{2+} and calmodulin. Calmodulin is a ubiquitously expressed Ca^{2+} binding protein (Sacks *et al.* 1995). Calmodulin becomes activated on binding Ca^{2+} and so serves as a transducer of Ca^{2+} signals by subsequently binding to and activating essential regulatory enzymes such as Ca^{2+}/calmodulin kinase II (CaM-kinase II) (Sacks *et al* 1995). It is clear then, that in many cell types, Ca^{2+} can regulate the intracellular concentration of cAMP. In addition, some cell types have been demonstrated to contain an adenylyl cyclase that is stimulated by calmodulin [for review see MacNeil *et al.* 1985]. Thus in these cells, Ca^{2+} has a stimulatory effect on the levels of intracellular cAMP, however, this is not the case in all cells.

In response to reports that LH/CG receptor activation could lead to an increase in IP3 (Davis *et al.* 1986, Davis *et al.* 1987) and cytosolic Ca^{2+} (Sullivan and Cooke 1986, Davis *et al.* 1987), the effects of Ca^{2+} on hCG-stimulated cAMP accumulation and steroid biosynthesis in MA10 cells, were studied by Pereira *et al.* (1988). In these studies, the addition of the Ca^{2+} ionophore A23187 resulted in a concentration-dependent decrease in hCG-stimulated cAMP accumulation, whilst removal of extracellular Ca^{2+} completely prevented this effect of A23187. The authors therefore concluded that Ca^{2+} is an inhibitor of hCG-stimulated adenylyl cyclase activity in MA10 cells. As a result, this inhibition was found to impose a limitation on the ability of hCG to activate steroidogenesis. Ca^{2+} has also been demonstrated to have an inhibitory effect on adenylyl cyclase activity in rat luteal cells (Dorflinger *et al.* 1984), porcine parathyroid cells (Oldham *et al.* 1984) and rat anterior pituitary gland (Giannattasio *et al.* 1987).

6.16: PKC inhibitors

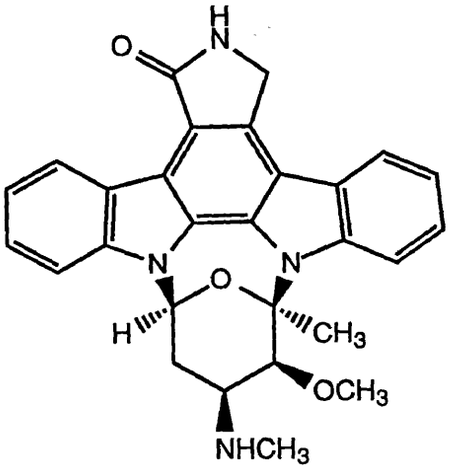
The use of PKC inhibitors has helped to clarify the role of PKC in cellular functions, but many PKC inhibitors fail to show potent PKC selectivity, thus hampering this area of research (Davis *et al.* 1992).

The indolocarbazole, staurosporine, was first discovered as a potent inhibitor of PKC by Tamaoki *et al.* (1986), and has been widely used since in many studies to determine the role of PKC in cellular processes. Staurosporine is a microbial alkaloid which acts at the ATP-binding site on protein kinase catalytic domains (Persaud *et al.* 1993). Whilst the use of staurosporine as a PKC inhibitor resulted from the realisation that earlier PKC inhibitors failed to show selectivity for PKC, staurosporine has in turn been discovered to inhibit a variety of other protein kinases (Ruegg and Burgess 1989). In particular, it has been reported that staurosporine is an effective inhibitor of rat brain Ca^{2+}/calmodulin-dependent protein kinase II (Persaud *et al.* 1993), human liver tyrosine protein kinase (Fallon 1990) and bovine heart PKA (Tamaoki *et al.* 1986). As a result of this lack of PKC selectivity, many studies have focused on developing derivatives of staurosporine that demonstrate a greater specificity for PKC inhibition. Compounds were synthesised which were based on the chemical structure of

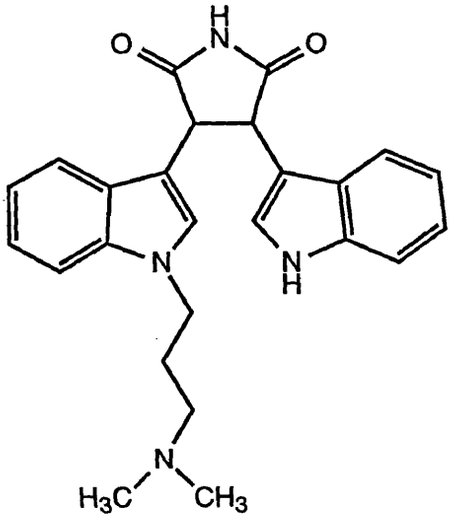
staurosporine, but which contained modifications (Toullec *et al.* 1991, Wilkinson *et al.* 1993). The resulting bisindolylmalemides were demonstrated to compete with ATP for binding to the enzyme (Wilkinson *et al.* 1993), and to display a potent inhibition of PKC with a relatively high degree of selectivity (Toullec *et al.* 1991) [see *Figure 6.1*].

The bisindolylmalemide, GF109203X, is reported to be a potent and selective inhibitor of PKC (Toullec *et al.* 1991). In this work, the authors compared the actions of staurosporine and GF109203X in two cellular models; human platelets and Swiss 3T3 fibroblasts. GF109203X was found to be as efficient as staurosporine in inhibiting PKC activity in these systems (IC_{50}=10nM), but much less active against PKA and tyrosine kinases.

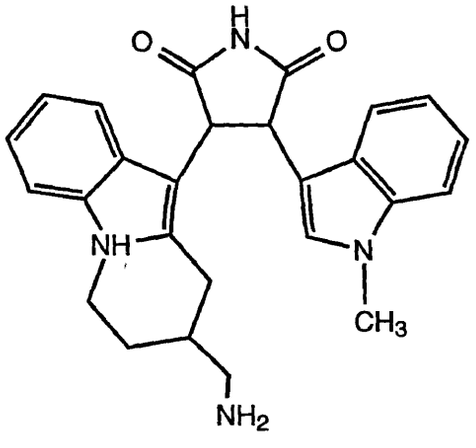
Similarly, in a report by Dieter and Fitzke (1991), two other bisindolylmalemide compounds, RO 31-8220 and RO 31-7549, were examined as potential PKC inhibitors and compared against staurosporine in cultured liver macrophages. Staurosporine and RO 31-8220 were found to be equally active against PKC *in vitro*, but had a 10-100-fold lower potency against PKA or Ca^{2+}/calmodulin-dependent protein kinase as staurosporine. *Figure 6.1* shows the chemical structures of staurosporine, GF109203X and RO 31-8220.



Staurosporine



GF109203X



RO 31-8220

Figure 6.1: Diagram depicting the chemical structure of staurosporine, an indolocarbazole, and its bis-indolylmaleimide derivatives, GF109203X and RO 31-8220.

6.2: Aims

In light of the mounting evidence of dual or multiple signal transduction pathways existing in some GPCR systems, the aim of this part of the project was to establish if PKC was involved in LH/CG receptor desensitisation in MA10 cells. If PKC involvement could be demonstrated in this system, it would indicate that signal transduction pathways other than the adenylyl cyclase/cAMP pathway are coupled to the LH/CG receptor in these cells. It was decided to stimulate growing MA10 cells in the presence and absence of various PKC inhibitors in order to see what effects, if any, this treatment had on the process of LH-stimulated, cholera toxin-stimulated and forskolin-stimulated desensitisation. This work has focused on the use of three main PKC inhibitors; staurosporine, GF109203X and RO 31-8220.

6.3: Results

All the experiments described here were performed in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) (0.5mM), unless otherwise stated. In addition, in the absence of PKC inhibitors, basal and control wells were treated with solvent to a final concentration as was present in the experimental wells, (DMSO for staurosporine and GF109203X, ethanol for RO 31-8220). All the PKC inhibitors were tested in the absence of LH/CG receptor stimulation and were found to have no effect on the basal levels of cAMP.

6.31: The concentration-dependent effects of PKC inhibitors on LH-stimulated cAMP production

These experiments were designed in order to establish the effects that a range of PKC inhibitor concentrations had on LH-stimulated cAMP production. The experiments were carried out as described in *Chapter Two*, section ***2.81***, over a total of 3 hours. *Figure **6.31a*** is a representative graph showing the concentration-dependent effects of staurosporine on LH-stimulated cAMP production. From this graph it can be seen that there was a staurosporine concentration-dependent increase in LH-stimulated cAMP production, with maximal cAMP levels being attained at the highest concentration of staurosporine (1μM).

*Figure **6.31b*** represents the concentration-dependent effects of GF109203X on LH-stimulated cAMP production. This graph demonstrates, in contrast to the results obtained with staurosporine, that there was a GF109203X concentration-dependent decrease in LH-stimulated cAMP production. From this graph it can be seen that GF109203X present at concentrations of 1μM or more, resulted in a 2-3 fold decrease in cAMP levels.

*Figure **6.31c*** represents the concentration-dependent effects of RO 31-8220 on LH-stimulated cAMP production. This graph demonstrates, as was seen in the results obtained with GF109203X, that there was a RO 31-8220 concentration-dependent decrease in LH-stimulated cAMP production. From this graph it can be seen that RO

31-8220 present at concentrations of 1μM or more, resulted in a 2-5 fold decrease in cAMP levels.

6.32: The concentration-dependent effect of PKC inhibitors on LH-stimulated steroid production

This graph represents the results of two progesterone assays performed on previous experiments described in section ***6.31***. Progesterone assays were performed in order to establish how the PKC inhibitors affected LH-stimulated steroid production. *Figure **6.32*** represents the mean results from these assays. (Note: the values for RO 31-8220 are not shown here for the sake of clarity, however they were very similar to the values obtained with GF109203X.)

6.33: Time course experiments measuring LH-stimulated cAMP production in the presence and absence of PKC inhibitors

These experiments were designed in order to establish if the presence of the PKC inhibitors could prevent or delay the process of LH-stimulated desensitisation as measured by the production of cAMP over time. These experiments were performed as described in *Chapter Two*, section ***2.82***. The time course experiments took place over 2/2.5 hours, with reactions stopped at intervals of 0, 30, 60 and 120/150 minutes.

The results from time course experiments using staurosporine (1μM) are represented in *Figure **6.33a***. The addition of 1μM staurosporine was seen to enhance the production of LH-stimulated cAMP at all time points after 0 minutes. By the end of the time course, in four identical experiments, the amount of cAMP produced by cells stimulated with LH in the presence of 1μM staurosporine was between 1.5-3.5 times greater when compared to cells where staurosporine had been absent. The results from similar time course experiments, using staurosporine at a lower concentration (0.1μM) can be seen represented in *Figure **6.33b***. The lower concentration of staurosporine had no significant effect on the level of LH-stimulated cAMP.

The results from time course experiments using GF109302X (1μM) are represented in *Figure **6.33c***. The addition of 1μM GF109203X was seen to decrease the production

of LH-stimulated cAMP at all time points after approximately 15 minutes. After two hours incubation, in three identical experiments, the amount of cAMP produced by cells stimulated with LH in the presence of 1μM GF109203X was between 2-2.5-fold less when compared to cells where GF109203X had been absent. The results from similar time course experiments, using GF109203X at a lower concentration (0.1μM) can be seen represented in ***Figure 6.33d***. The lower concentration of GF109203X had no significant effect on the level of LH-stimulated cAMP.

The results from time course experiments using RO 31-8220 (1μM) are represented in ***Figure 6.33e***. As was demonstrated using GF109203X (1μM), the addition of 1μM RO 31-8220 was seen to decrease the production of LH-stimulated cAMP at all time points after 0 minutes. After two hours incubation, in three identical experiments, the amount of cAMP produced by cells stimulated with LH in the presence of 1μM RO 31-8220 was approximately 2-fold less when compared to cells where RO 31-8220 had been absent. The results from similar time course experiments, using RO 31-8220 at a lower concentration (0.1μM) can be seen represented in ***Figure 6.33f***. The lower concentration of RO 31-8220 had no significant effect on the level of LH-stimulated cAMP.

6.34: Is staurosporine acting as an inhibitor of phosphodiesterase activity?

In order to determine if the effects of staurosporine could be explained by inhibition of phosphodiesterases, experiments were performed as described in *Chapter Two*, section ***2.85***. These experiments measured the level of LH-stimulated cAMP production in the presence of staurosporine, but in the presence or absence of the phosphodiesterase inhibitor, MIX (0.5mM). The results of these experiments are represented in ***Figure 6.34***. From these results it can be seen that in the absence of MIX but in the presence of staurosporine (1μM), the levels of LH-stimulated cAMP are significantly lower when compared to levels obtained in the presence of MIX. Thus, these results suggest that the enhancement of LH-stimulated cAMP cannot be attributed to staurosporine acting as a phosphodiesterase inhibitor.

6.35: Cytotoxicity studies on staurosporine

In order to assess whether staurosporine had any cellular cytotoxic effects which may account for the enhancement of LH-stimulated cAMP production, experiments were performed in the presence and absence of staurosporine (0.1-1μM) and LH (100ng/ml). Cellular damage was measured using the Cytotox 96 assay kit, as described in *Chapter Two* section ***2.86***. This assay measures the activity of the enzyme lactate dehydrogenase (LDH), which is released into the medium surrounding the cells as a result of damage to the plasma membrane. A colour reaction then takes place which is measured using an ELISA plate reader at 492nm. *Figure **6.35*** represents the results from preliminary experiments. (The total values are obtained by lysing untreated cells at the end of the experiment). These results suggest that staurosporine, at concentrations of 1μM or less, has a minimal cytotoxic effect on MA10 cells.

6.36: The effect of staurosporine on LH binding to its receptor

In order to rule out the possibility that staurosporine may enhance LH-stimulated cAMP production via an interaction with the LH/CG receptor, receptor binding assays were carried out in the presence of [^{125}I]hCG and the presence and absence of staurosporine (1μM) as described in *Chapter Two*, section ***2.93***. *Figure **6.36a*** represents the results from these experiments. Very little difference in [^{125}I]hCG binding to the LH/CG receptor can be seen between the cells preincubated in the presence of staurosporine (1μM) and those not. Thus, the results from these experiments would suggest that staurosporine at concentrations of 1μM or less, do not have any effects on ligand-receptor binding.

Figure ***6.36b*** shows the results from a receptor autoradiography experiment performed simultaneously with one of the previous binding studies. Cells were grown on coverslips and then incubated in the presence of [^{125}I]hCG and in the presence or absence of 1μM staurosporine, as described in *Chapter Two*, section ***2.94***. The cells were incubated either at 37°C for 1.5 hours or at 4°C for 48 hours. From the photograph, it can be seen that when the binding was carried out at 4°C, the level of [^{125}I]hCG binding does not exceed that observed in the non-specific control. As a result, it is impossible to ascertain whether the presence of staurosporine has altered the binding of [^{125}I]hCG to the LH/CG receptor. In contrast however, when the

binding was carried out at 37°C, very little [^{125}I]hCG binding was seen in the non-specific controls compared to that seen on the test coverslips. This would indicate that specific binding had occurred. Visual comparison of the level of [^{125}I]hCG binding obtained in the presence of staurosporine compared with in its absence, would agree with the results observed in the binding studies, i.e. that at 37°C, the presence of 1μM staurosporine had no effect on ligand binding.

6.37: The effect of staurosporine on cholera toxin-stimulated cAMP production

In addition to using LH to stimulate MA10 cells, cholera toxin was also used. Cholera toxin works by inhibiting the GTPase activity of the $G_{s\alpha}$ subunit, thus rendering it constituitively active, resulting in the constant production of cAMP from adenylyl cyclase in the absence of LH. It was of interest therefore, to see whether a PKC inhibitor such as staurosporine had any effect on this receptor-independent activator of adenylyl cyclase. These experiments were performed as described in *Chapter Two*, section ***2.83***, over a total period of 3 hours. As is explained in the discussion, preliminary experiments established that at a concentration of 1μM, staurosporine potentiated cholera toxin-stimulated (and forskolin-stimulated, see next section) cAMP production. Following a change in the supplier of staurosporine it was found that the new batch of staurosporine could duplicate the results seen previously, not only at 1μM but also at the lower concentrations of 0.5μM and 0.1μM. Hence, the experiments described here were all performed using staurosporine at 0.1μM. The results from experiments using a range of cholera toxin concentrations in the presence and absence of staurosporine (0.1μM) can be seen represented in *Figure* ***6.37***. Staurosporine (0.1μM) was found to potentiate cAMP production across a concentration range of cholera toxin.

6.38: The effect of staurosporine on forskolin-stimulated cAMP production

The effect of staurosporine (0.1μM) was also studied on forskolin-stimulated cAMP production. Forskolin works by the direct activation of adenylyl cyclase, again resulting in the constant production of cAMP in the absence of LH. As was the case with cholera toxin, it was of interest to see whether staurosporine had any effect on this receptor-independent activator of adenylyl cyclase. These experiments were

performed as described in *Chapter Two*, section ***2.84***, over a total period of 3 hours. The results from experiments using a range of forskolin concentrations in the presence and absence of staurosporine (0.1μM) can be seen represented in *Figure **6.38***. As was seen in the cholera toxin experiments, the presence of staurosporine (0.1μM) was found to potentiate cAMP production across a concentration range of forskolin.

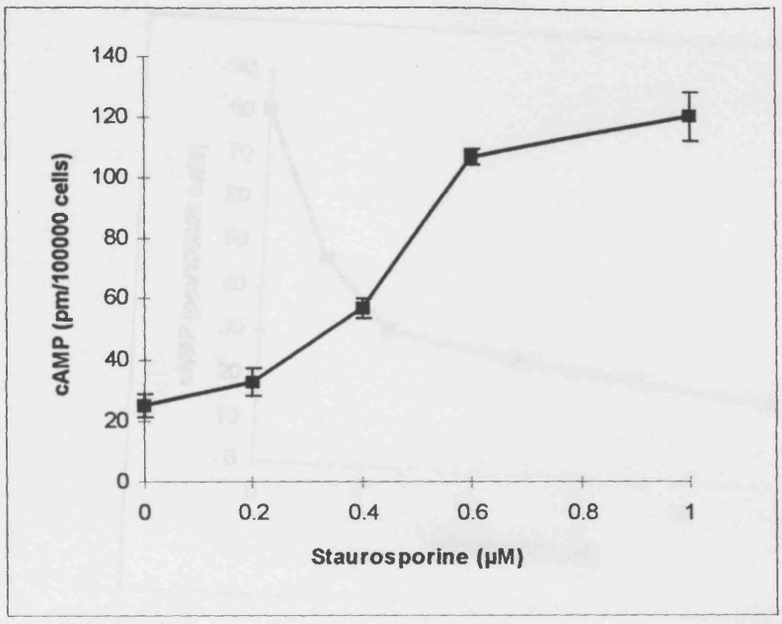


Figure 6.31a: **The concentration-dependent of staurosporine on LH-stimulated cAMP production.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence of varying concentrations of staurosporine (0-1µM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). After 2 hours the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (1 way without replication). The individual Anova values were 1.07×10^{-10}, 7.8×10^{-3} and 1.79×10^{-11}. Mean basal values after a total of 3 hours = 2.95 ± 2 cAMP (pm/100000 cells).

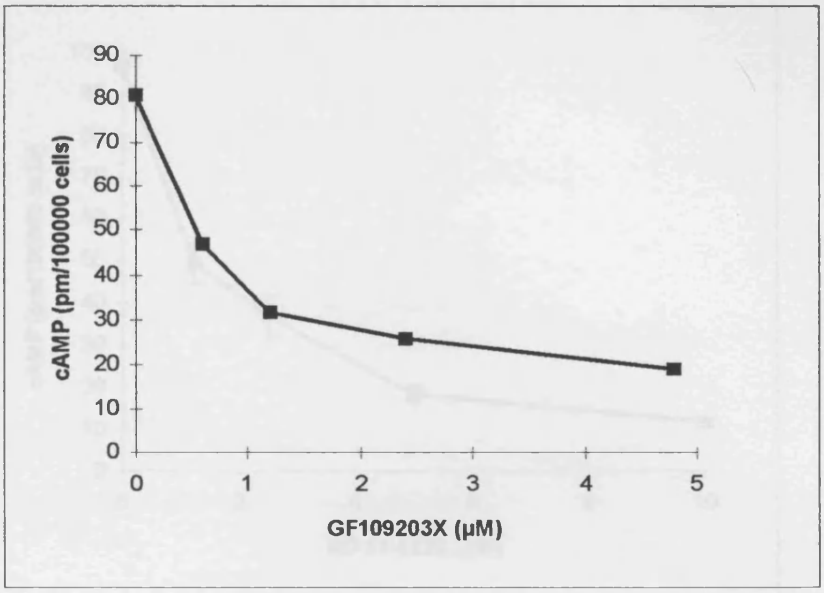


Figure 6.31b: **The concentration-dependent effect of GF109203X on LH-stimulated cAMP production.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence of varying concentrations of GF109203X (0-1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). After 2 hours the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

The values given are the mean +/- SEM of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (1 way without replication). The individual Anova values were 4×10^{-13}, 3.55×10^{-7} and 2.69×10^{-3}. Mean basal values after a total of 3 hours = 9.4 ± 2.2 cAMP (pm/100000 cells).

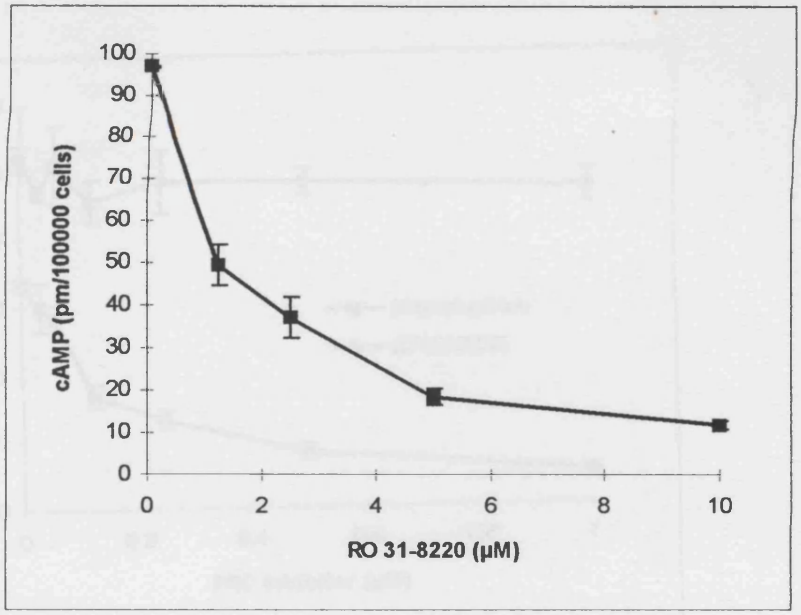


Figure 6.31c: **The concentration-dependent effect of RO 31-8220 on LH-stimulated cAMP production.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence of varying concentrations of RO 31-8220 (0-10μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). After 2 hours the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (1 way without replication). The individual Anova values were 4.32×10^{-5}, 1.84×10^{-6} and 2.1×10^{-10}. The Mean basal values after a total of 3 hours = 10.7 ± 2.1 cAMP (pm/100000 cells) .

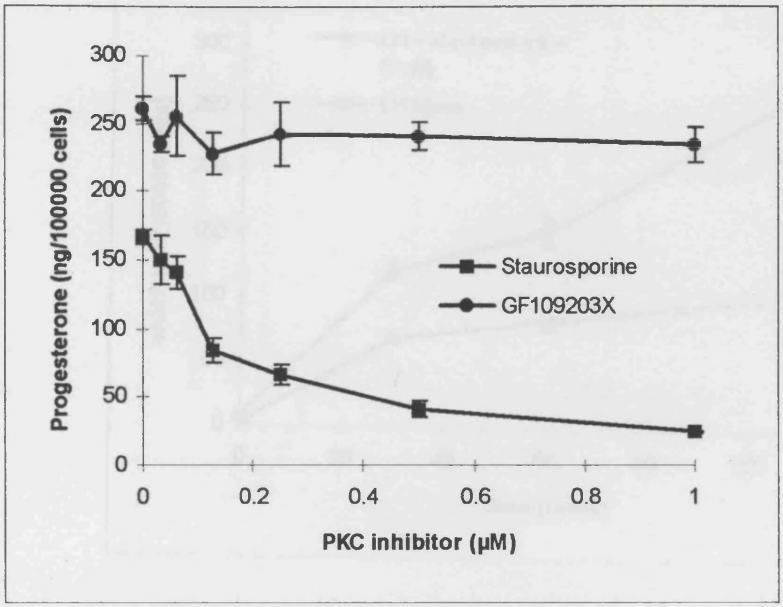


Figure 6.32: **The concentration-dependent effect of PKC inhibitors on LH-stimulated steroid production.** These assays were performed on experiments described in sections ***6.31a, b*** and ***c***, in accordance with the protocol detailed in *Chapter Two*, section ***2.74***.

As this graph represents only two assays, it was not possible to perform statistical analysis. Mean basal values after a total of 3 hours = 8.6 ± 2.3 progesterone (ng/100000 cells).

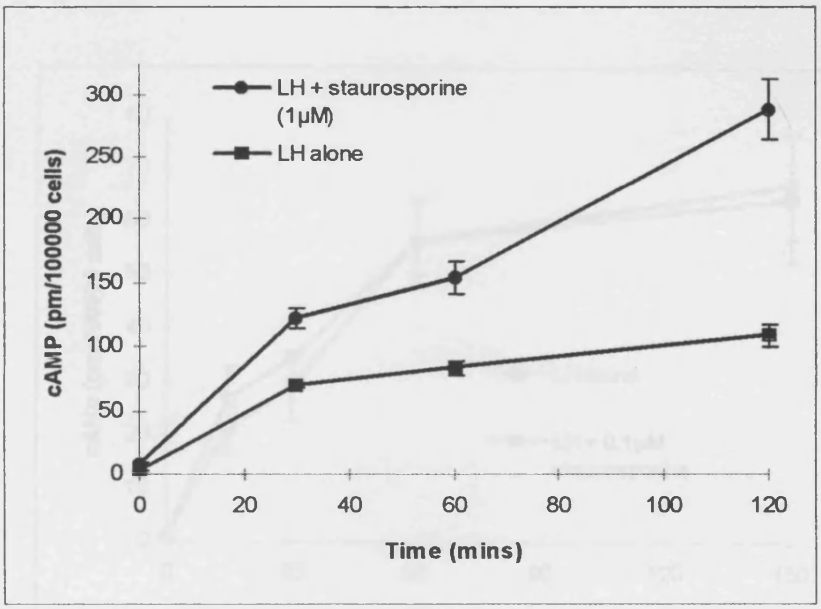


Figure 6.33a*: The effects of staurosporine (1μM) over time, on LH-stimulated cAMP production by MA10 cells.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of staurosporine (1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 120 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73.

The values shown are the mean +/- SEM of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 3.52×10^{-6}, 1.03×10^{-3} and 1.27×10^{-4}. Individual experiments were also analysed by paired *t* tests. Mean basal values after a total of 3 hours = 2.7 ± 0.17 cAMP (pm/100000 cells).

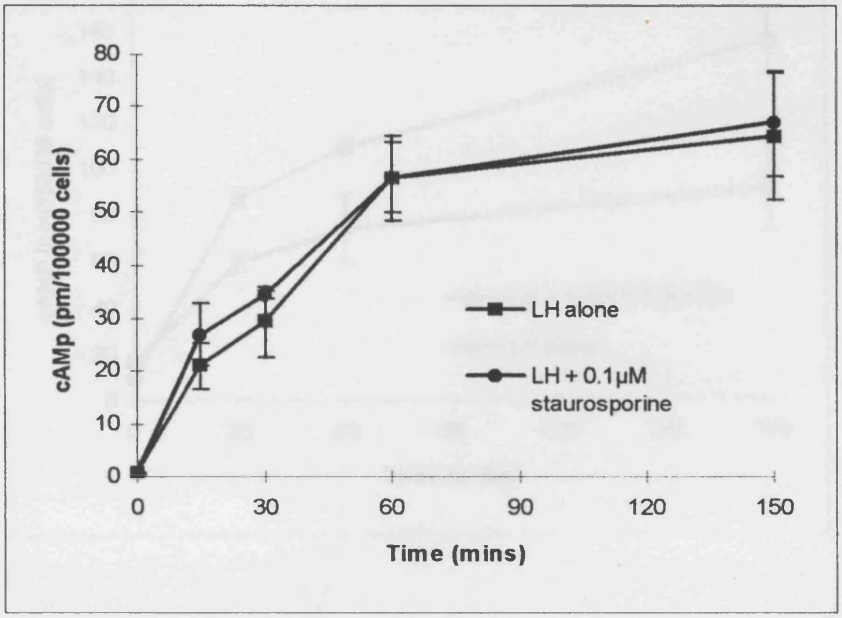


Figure 6.33b: **The effects of staurosporine (0.1µM) over time, on LH-stimulated cAMP production by MA10 cells.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of staurosporine (0.1µM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 150 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. When analysed by ANOVA (2 way without replication), none of the three experiments showed significant effects of staurosporine (0.1µM) (P was always greater than 0.05). The individual Anova values were 0.26, 0.732 and 0.58. Mean basal values after a total of 3.5 hours = 1.34 ± 0.61 cAMP (pm/100000 cells).

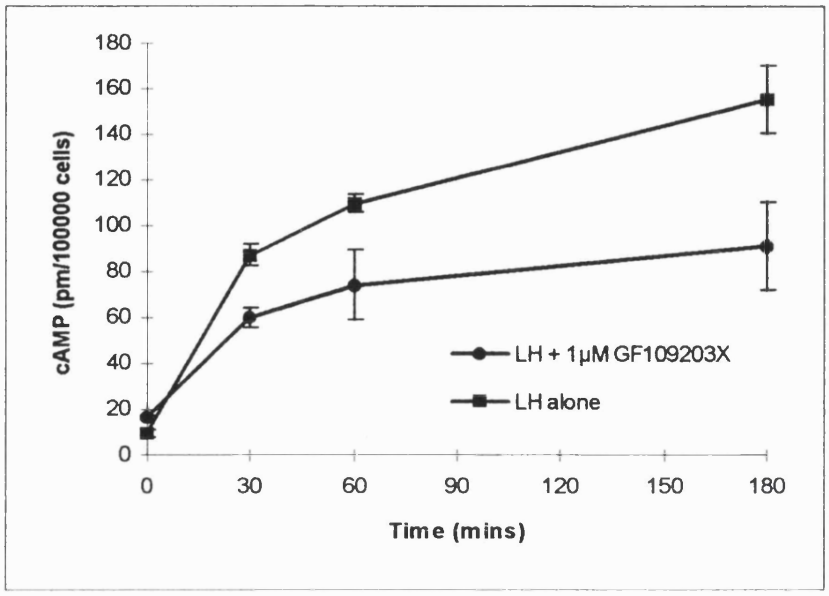


Figure 6.33c: **The effects of GF109203X (1μM) over time, on LH-stimulated cAMP production by MA10 cells.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of GF109203X (1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 120/180 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

The values given are the mean +/- SEM of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 5.09×10^{-5}, 2.36×10^{-3} and 1.4×10^{-4}. Individual experiments were also analysed by paired *t* tests. Mean basal values after a total of 4 hours = 7.9 ± 0.3 cAMP (pm/100000 cells).

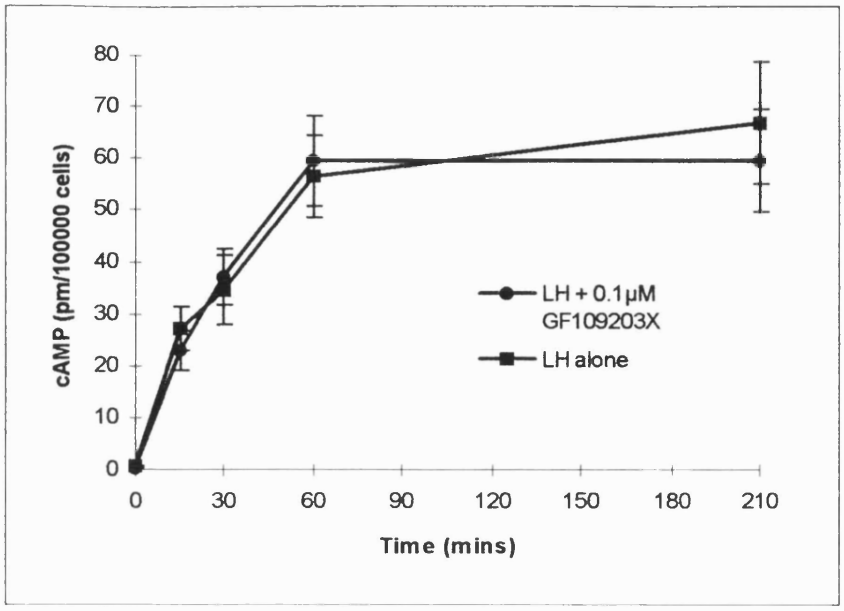


Figure 6.33d: **The effects of GF109203X (0.1μM) over time, on LH-stimulated cAMP production by MA10 cells**. MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of GF109203X (0.1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 150/210 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. When analysed by ANOVA (2 way without replication), none of the three experiments showed significant effects of GF109203X (P was always greater than 0.05). The individual Anova values were 0.36, 0.19 and 0.22. Mean basal values after a total of 3.5-4.5 hours = 0.89 ± 0.08 cAMP (pm/100000 cells).

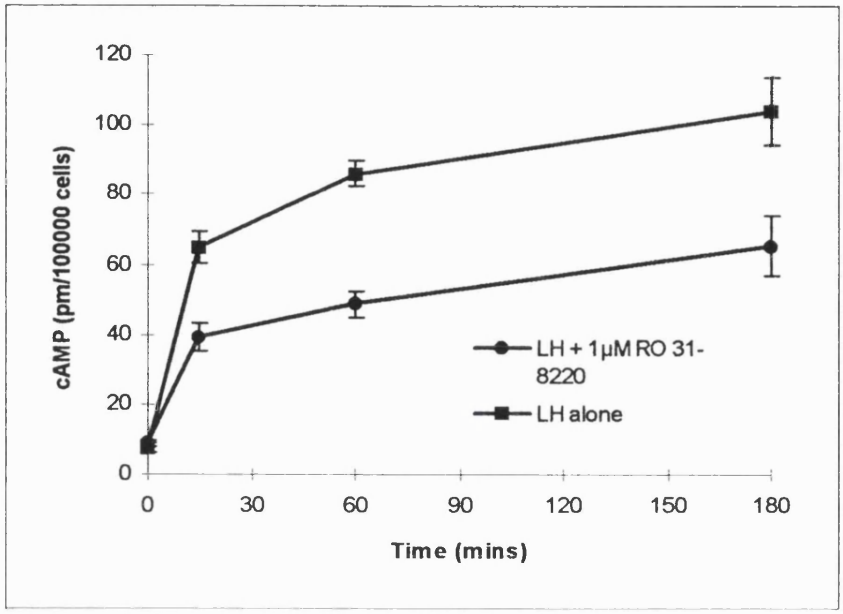


Figure 6.33e: **The effects of RO 31-8220 (3μM) over time, on LH-stimulated cAMP production by MA10 cells**. MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of RO 31-8220 (1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 120/210 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

The values given are the mean +/- SEM of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 8.38×10^{-4}, 3.59×10^{-3} and 2.7×10^{-3}. Individual experiments were also analysed by paired *t* tests. Mean basal values after a total of 4 hours = 4.3 ± 0.91 cAMP (pm/100000 cells).

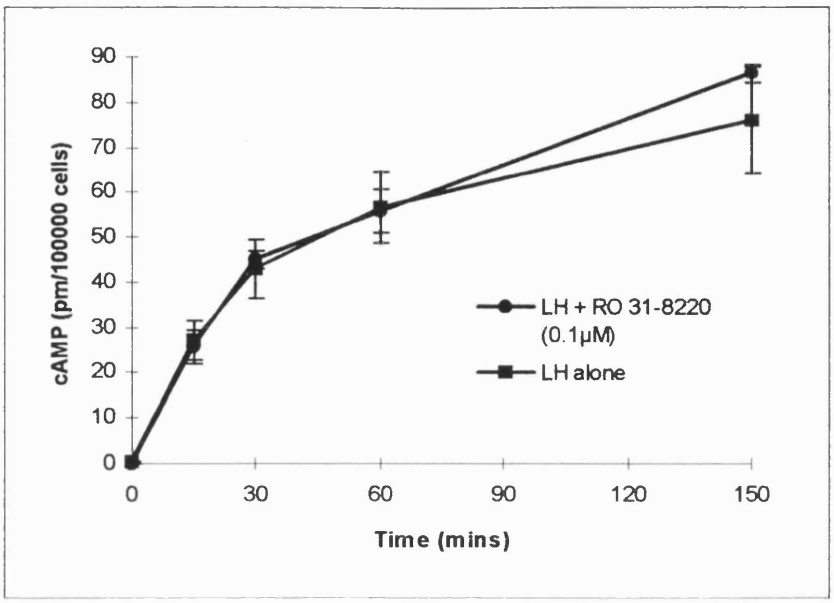


Figure 6.33f: **The effects of RO 31-8220 (0.1μM) over time, on LH-stimulated cAMP production by MA10 cells**. MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of RO 31-8220 (0.1μM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). At time points of 0, 30, 60 and 150 minutes the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. When analysed by ANOVA (2 way without replication), none of the three experiments showed a significant effect of RO 31-8220 (P was always greater than 0.05). The individual Anova values were 0.38, 0.26 and 0.45. Mean basal values after a total of 3.5 hours = 0.76 ± 0.5 cAMP (pm/100000 cells).

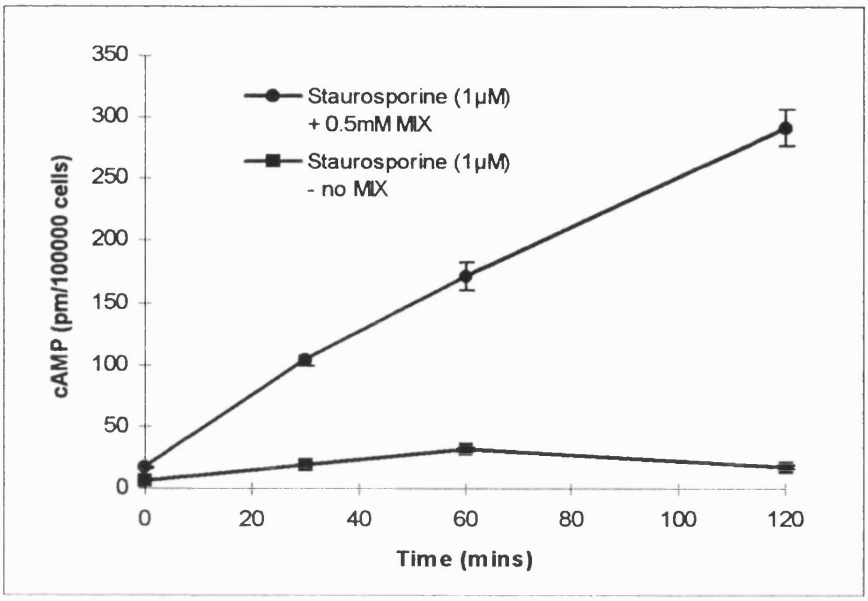


Figure 6.34: **The effects of staurosporine (1μM) on LH-stimulated cAMP production in the presence and absence of the phosphodiesterase inhibitor, MIX (0.5mM).** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence of staurosporine (1μM), and in the presence or absence of MIX (0.5mM) for 1 hour. Fresh media was then added as before with the addition of LH (100ng/ml). After 2 hours at 37°C the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 6.87×10^{-6}, 1.03×10^{-3} and 1.46×10^{-4}. Individual experiments were also analysed by paired *t* tests. Mean basal values after a total of 3 hours = 4.8 ± 1.1 cAMP (pm/100000 cells).

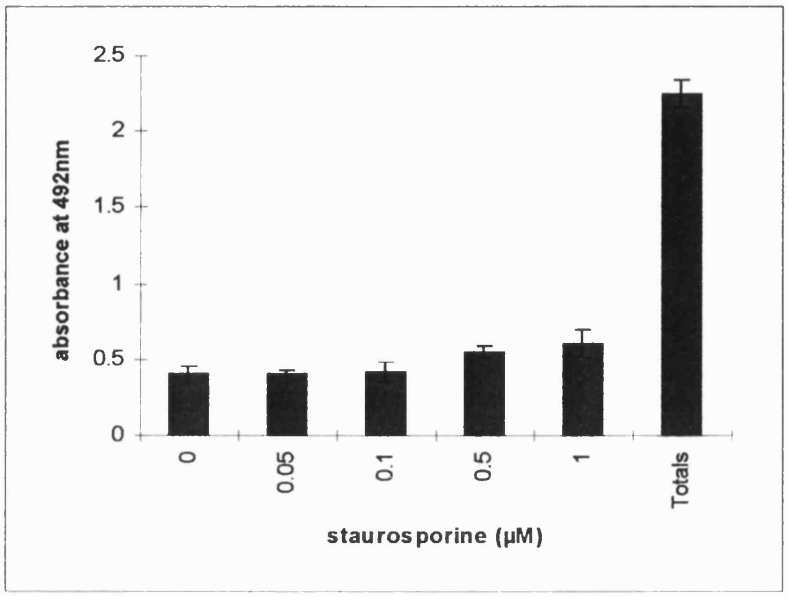


Figure 6.35: **The cytotoxic effects of staurosporine in the presence of LH (100ng/ml), over 2 hours.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of staurosporine (1μM) for 1 hour. LH (100ng/ml) was then added to all wells. After 2 hours, aliquots of media were removed from each well so that a cytotoxicity assay could be carried as described in *Chapter Two*, section ***2.85***. As this graph is representative of two assays, it was not possible to perform statistical analysis.

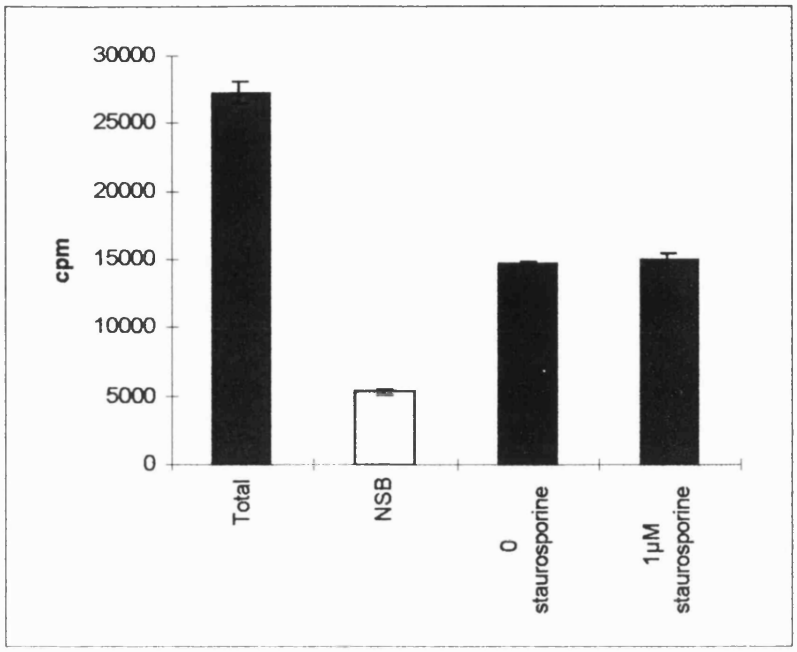


Figure 6.36a: **The effect of staurosporine on the binding of [^{125}I]-hCG to the LH/CG receptor.** MA10 cells (a minimum of 10^6 cells/well in 6 well plates), were preincubated in the presence of or absence of staurosporine (1μM). The wells were then treated with a combination of [^{125}I]-hCG and crude hCG, and incubated for a further 1.5 hours at 37°C. The cells were scraped, pelleted and counted as described in *Chapter Two*, section ***2.93***.

This experiment is representative of 3 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication).The individual Anova values were 5.6×10^{-4}, 8.23×10^{-7} and 1.32×10^{-4}. Individual experiments were also analysed by paired *t* tests.

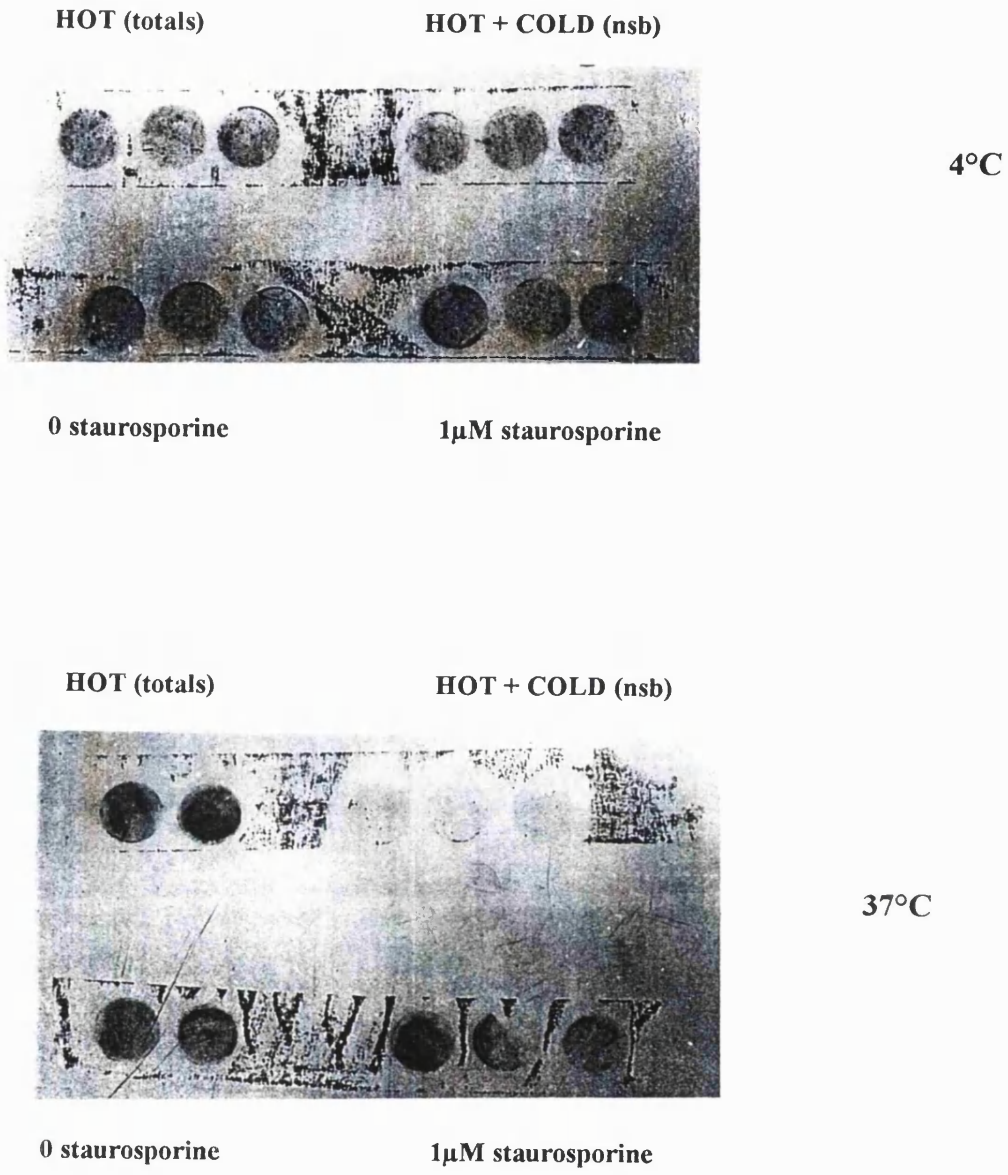


Figure 6.36b: **The effect of staurosporine on the binding of [^{125}I]hCG to the LH/CG receptor.** Receptor autoradiography was performed simultaneously alongside the binding studies just described. These experiments were carried out as detailed in *Chapter Two*, section ***2.94***. The top photograph shows the lack of specific binding acheived when the incubation temperature was 4°C (for 48 hours). In contrast the lower photograph shows the specific binding obtained when the incubation temperature was 37°C (for 1.5 hours). The demonstration of similar levels of [^{125}I]hCG binding between cells incubated in the presence of 1μM staurosporine and those that were not, would suggest that at 37°C, 1μM staurosporine has no effect on ligand binding to the LH/CG receptor.

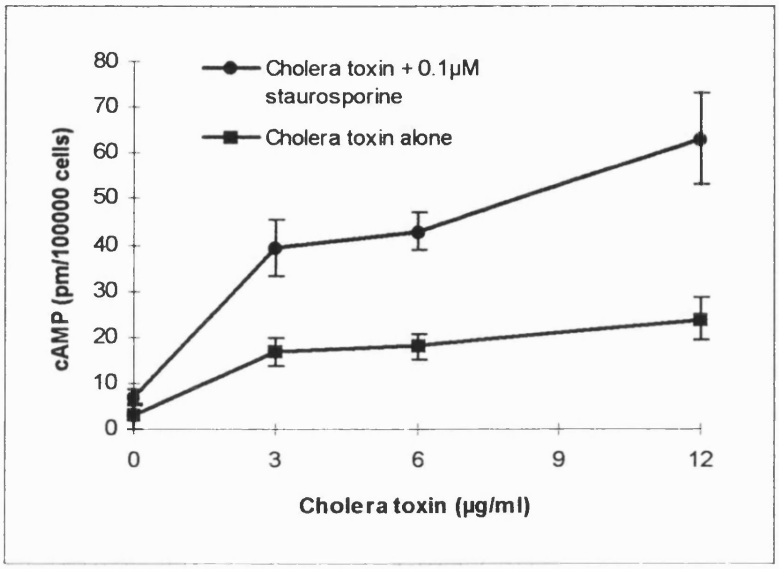


Figure 6.37: **The effects of staurosporine (0.1μM) on cholera toxin dose response experiments.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of staurosporine (0.1μM) for 1 hour. Fresh media was then added as before with the addition of cholera toxin at 0, 3, 6 and 12/25mg/ml. After 2 hours at 37°C the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 4 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 5.88×10^{-4}, 2.7×10^{-5}, 4.19×10^{-5} and 8.59×10^{-3}.Individual experiments were also analysed by paired *t* tests. Mean basal values after at total of 3 hours = 3.8 ± 0.5 cAMP (pm/100000 cells).

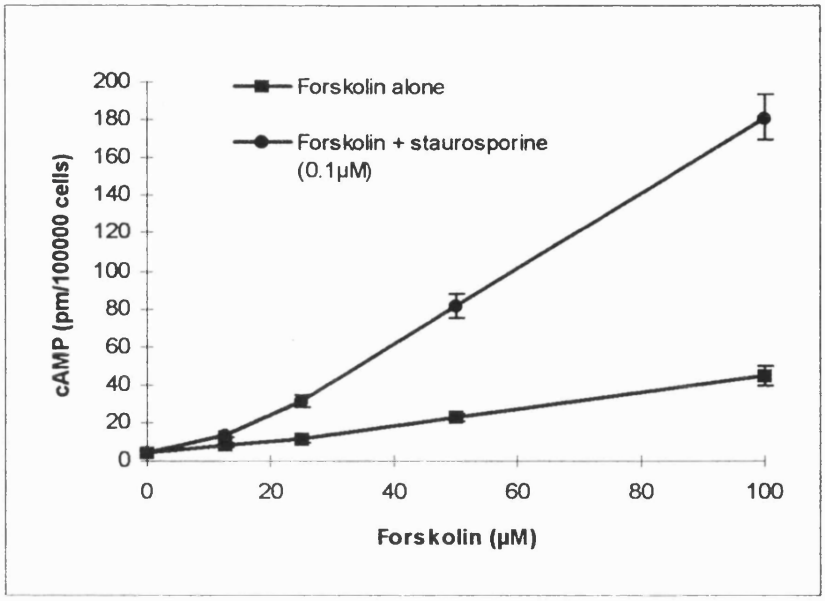


Figure 6.38: **The effects of staurosporine (0.1μM) on forskolin dose response experiments.** MA10 cells (100000 cells/well in 24 well plates), were preincubated in the presence or absence of staurosporine (0.1μM) for 1 hour. Fresh media was then added as before with the addition of forskolin at 0, 12.5, 25, 50 and 100μM. After 2 hours at 37°C the reactions were stopped and assayed for cAMP by RIA as described in *Chapter Two*, section ***2.73***.

This experiment is representative of 4 independent experiments. Each experiment showed a significance of $P<0.05$ when analysed by ANOVA (2 way without replication). The individual Anova values were 1.18×10^{-3}, 1.04×10^{-3}, 3.14×10^{-3} and 1.03×10^{-3}. Individual experiments were also analysed by paired *t* tests. Mean basal values after at total of 3 hours = 3.3 ± 1.2 cAMP (pm/100000 cells).

6.4: Discussion

In recent years it has been suggested that a number of GPCRs, including the LH/CG receptor, are coupled to more than one second messenger pathway. It is generally accepted that the enzyme adenylyl cyclase is involved in LH/CG receptor signal transduction via the production of cAMP. Recent reports have also suggested that the LH/CG receptor may also be coupled to the enzyme PLC (Gudermann *et al.* 1992a and 1992b, Hipkin *et al.* 1993). If the PLC activation were involved in LH/CG receptor signal transduction, it would be reasonable to suggest that the Ca^{2+}-dependent protein kinase (PKC), could be involved in the phosphorylation of other proteins, or maybe even the receptor itself. These studies were therefore designed to establish if PKC was involved in MA10 LH/CG receptor signal transduction. Using inhibitors of PKC, it was anticipated that if PKC was involved, LH-stimulated cAMP production may be enhanced when the inhibitor was present (preventing desensitisation), and unaffected (undergoing desensitisation), when the inhibitor was absent.

During the course of this work, the standard working concentration of staurosporine and its derivatives, GF109203X and RO 31-8220 was set at 1μM. The results from a number of dose response studies, using a range of staurosporine concentrations, indicated that LH-stimulated cAMP production could be enhanced 2-3-fold in the presence of staurosporine (1μM). In contrast however, GF109203X and RO 31-8220 were demonstrated to inhibit LH-stimulated cAMP production (*Figures **6.31a** , **b** and **c***). This would indicate that the staurosporine-dependent effects on LH-mediated cAMP are unlikely to be as a result of the inhibition of PKC. In addition, preliminary experiments indicated that MA10 progesterone production could be inhibited in a dose-dependent manner by staurosporine, whilst GF109203X and RO 31-8820 were found to have no effect on steroidogenesis (*Figure **6.32***). Since staurosporine can be considered an inhibitor of more than one type of protein kinase depending on the concentration used (Tamakoi *et al.* 1986, Ruegg and Burgess 1989, Gadbois *et al.* 1992), it is possible that the decrease in steroidogenesis was as a result of such inhibition downstream to cAMP accumulation, thus resulting in the interruption of steroidogenesis. These results are in agreement with those obtained in other studies whereby the effects of staurosporine were studied on LH-stimulated cAMP production

and steroidogenesis (Jamaluddin *et al.* 1994) and FSH-stimulated cAMP production and steroidogenesis (Keren-Tal *et al.* 1996). In the former study, staurosporine was found to dose-dependently potentiate LH-stimulated cAMP production but inhibit progesterone production in avian granulosa cells. In the latter study, the presence of 0.05μM staurosporine resulted in a 2-3-fold increase in FSH-stimulated cAMP production in FSH receptor transfected-granulosa cells, whilst FSH-stimulated steroidogenesis was completely blocked by staurosporine at this concentration.

In addition to the results obtained in the concentration-dependent experiments, it was of interest to establish whether staurosporine could alter the production of LH-stimulated cAMP over time. If so, this may indicate that staurosporine was having an effect on the process of desensitisation, rather than simply enhancing cAMP production in some way. The results from the time course studies on LH-stimulated cAMP production in the presence of staurosporine (1μM) (***Figure 6.33a***), suggests that staurosporine acts in part, by delaying the onset of desensitisation. This is because the production of LH-stimulated cAMP continues to rise long after a plateau has been reached in the absence of staurosporine. However, once again, the effects of staurosporine cannot be mimicked by GF109203X and RO 31-8220, (***Figures 6.33c*** and ***6.33e*** respectively). The use of these specific PKC inhibitors (at 1μM) has an inhibitory effect on LH-stimulated cAMP production. In time course studies where the inhibitors were present at a concentration of 0.1μM, no significant effects were seen on the levels of LH-stimulated cAMP production (***Figures 6.33b***, ***d*** and ***f***).

The possibility that the effects seen with staurosporine (1μM) were due to its acting as a phosphodiesterase (PDE) inhibitor were ruled out by experiments which were performed in the presence of staurosporine, but in the absence of MIX. If staurosporine were acting as a PDE inhibitor, the profiles of +/- MIX would be expected to be similar. However, as can be seen in ***Figure 6.34***, when MIX is absent but staurosporine present, the level of LH-stimulated cAMP production is much lower than when MIX is present. This would indicate that the effects of staurosporine are not due to the possession of PDE inhibitor activity.

Staurosporine has been reported to have a cytotoxic effect in some cellular systems. For example, in a technical report by Hengerer and Mathe (1996) staurosporine was used at a concentration of 1μM to induce apoptosis in PC12 cells, (although this was only fully acheived after 3-16 hours incubation). However, it is also suggested in a report by Persaud *et al.* (1993), that concentrations of up to 10μM staurosporine can be used without adverse effects, although at these concentrations, the authors concede that non-specific PKC effects would be expected. To establish if staurosporine was capable of causing damage to the MA10 plasma membrane, preliminary experiments were performed in the presence and absence of varying concentrations of staurosporine. The experimental media was then assayed for the presence of LDH, an enzyme that is released from damaged cells. The results from these preliminary studies indicated that at concentrations of 1μM or less, staurosporine did not appear to damage the plasma membrane of MA10 cells (*Figure 6.35*).

Experiments were also performed to rule out the possibility that staurosporine at 1μM may alter the binding of LH to its receptor. From the results represented in *Figure 6.36a* and *b*, it can be seen that there was no difference in LH binding to its receptor in the presence or absence of staurosporine (1μM). Similar results were obtained in experiments that were carried out using GF109203X (1μM). However, due to the low levels of specific binding in the latter experiments, it was impossible to be certain that [^{125}I]-hCG binding had not been affected by GF109203X and these results have therefore not been included in this thesis.

Staurosporine at a concentration of 1μM then, was not found to increase LH-stimulated cAMP production by acting as a phosphodiesterase inhibitor, nor was it found to be cytotoxic to MA10 cells or alter ligand-receptor binding. Since GF109203X and RO 31-8220, PKC inhibitors with a greater specificity than staurosporine, it would appear likely then, that the enhancement of LH-stimulated cAMP production seen in the presence of staurosporine (1μM), is due to the non-specific inhibition of enzymes other than PKC. It is known that staurosporine can inhibit other kinases such as CaM-kinase II (Persuad *et al.* 1993). CaM-kinase II has been demonstrated *in vitro* to phosphorylate a large number of proteins (Colbran *et al.*

1989). In the presence of Ca^{2+}/calmodulin, CaM-kinase II undergoes autophosphorylation on multiple serine and threonine residues, an event which appears to precede the phosphorylation of substrate proteins (Kwiatkowski *et al.* 1988). In light of the research described earlier, whereby Ca^{2+} has been demonstrated to have an inhibitory effect on adenylyl cyclase (Pereira *et al.* 1988, Dorflinger *et al.* 1984, Oldham *et al.* 1984, Giannattasio *et al.* 1987), it is suggested that the enhancement of LH-stimulated cAMP in the presence of staurosporine that has been demonstrated in this work, is due to the non-PKC-specific inhibition of CaM-kinase II by staurosporine.

Adenylyl cyclases exhibit diverse regulatory properties that provide interesting mechanisms for regulation of cAMP by extracellular and intracellular signals (Tang and Gilman 1992). Thus, regulation of adenylyl cyclases by various protein kinases generates cross-talk between the cAMP regulatory system and other signal transduction systems, as well as mechanisms for feedback inhibition or indeed amplification of cAMP signals (Wei *et al.* 1996). The latter may help to explain the decrease in cAMP production seen in the presence of GF109203X and RO 31-8220.

If we can assume that the PKC inhibitors GF109203X and RO 31-8220, are specific for PKC, why then would inhibiting PKC result in a decrease in LH-stimulated cAMP production? A criticism of this work would be that the cytotoxic effects of these two inhibitors were not established at 1μM, however, it must be noted that at the same concentration the parent compound, staurosporine, did not appear to have a cytotoxic effect on MA10 cells. In addition, other studies (Keren-Tal *et al.* 1996), have used GF109203X at concentrations up to 3-fold higher than were used in this work, with no adverse cytotoxic effects. An alternative explanation for the fall in LH-stimulated cAMP production in the presence of GF109203X and RO 31-8220, is that these inhibitors somehow affect the binding of LH to the receptor. (Staurosporine at 1.5μM has been reported to block the binding of [^{125}I]-thrombin to platelets (Puri and Colman 1993). Whilst it was not possible to establish whether this was indeed the case, it was demonstrated that the structurally similar compound staurosporine, did not alter the binding of LH to its receptor and it is therefore thought unlikely that GF109203X and RO 31-8220 would act differently. A more substantial argument for why PKC

inhibition may affect cAMP levels is provided by studies which report that PKC can directly interact with adenylyl cyclase resulting in a stimulation of its activity (Yoshimasa *et al.* 1987, Choi *et al.* 1993, Jacobowitz and Iyengar 1994, Kawabe *et al.* 1994, Lai *et al.* 1997). For example, the results from studies by Yoshimasa *et al.* (1987) suggest that PKC may function as an activator of adenylyl cyclase, since a phorbol ester that activates PKC results in an increase in adenylyl cyclase activity. In a report by Lai *et al.* (1997), it is suggested that a Ca^{2+}-independent PKC inhibits adenylyl cyclase activity by phosphorylation. Thus, it is suggested that the decrease in cAMP production that is observed in the presence of 1μM GF109203X and RO 31-8220, is the result of PKC inhibition which in turn results in the subsequent decrease in adenylyl cyclase activity.

During this work, it has been demonstrated that the presence of staurosporine at the lower concentration of 0.1μM, could significantly increase the level of cholera toxin-stimulated cAMP over a concentration range of the G-protein agonist, compared to when the inhibitor was absent (***Figure 6.37***). These results reflect those obtained by Keren-Tal *et al.* (1996) when studying their FSH-responsive cell line in the presence of staurosporine at a concentration of 0.05μM. However, unlike the results reported by this latter study and by Jamaluddin *et al.* (1994), during the work described herein, forskolin-stimulated cAMP was *also* found to be potentiated by the presence of staurosporine at 0.1μM (***Figure 6.38***). There are two possible explanations for the staurosporine enhancement of cholera toxin- and forskolin-mediated cAMP levels. Firstly, it is possible that like GF109203X and RO 31-8220, staurosporine is having a direct effect on the activity of adenylyl cyclase itself. In this case however, staurosporine would be acting to enhance adenylyl cyclase activity as opposed to lowering it as is proposed for GF109203X and RO 31-8220. Secondly, as is discussed above, it is possible that staurosporine is acting non-specifically to inhibit another kinase such as CaM-kinase II. Such inhibition may in turn prevent the inhibition of adenylyl cyclase by Ca^{2+}, resulting in an increase in both cholera toxin- and forskolin-mediated cAMP production.

The effects of this lower concentration of staurosporine on cholera toxin-stimulated and forskolin-stimulated cAMP are harder to explain, as in LH-stimulated experiments 0.1μM staurosporine was found to have no significant effect on cAMP levels. To reiterate, it is anticipated that these observations reflect a change in the quality of staurosporine as a result of changing suppliers during this work. Whilst the new batch of staurosporine was observed to potentiate both cholera toxin-stimulated and forskolin-stimulated cAMP production at 1μM, the lower concentration of 0.1μM staurosporine was used routinely in subsequent experiments so as to conserve resources.

6.5: Summary

In this work then, we have examined the effects of three PKC inhibitors on LH-, cholera toxin- and forskolin-stimulated cAMP production in MA10 cells. Staurosporine was found to increase cAMP production in the presence of LH, cholera toxin and forskolin. Whilst the effect of staurosporine was not as a result of possessing PDE activity or altering LH binding to its receptor, it is likely that it was not via PKC inhibition, since inhibitors known to possess a greater specificity for PKC did not produce similar results. Some reports have suggested that Ca^{2+} can act as an inhibitor of adenylyl cyclase activity. Since staurosporine is known to inhibit CaM-kinase II, it is suggested that the increase in cAMP levels in the presence of this kinase inhibitor, is due to the inhibition of CaM-kinase II, resulting in the removal of the inhibitory effects of Ca^{2+} on adenylyl cyclase and a subsequent increase in cAMP. It is suggested that the decrease in cAMP levels observed in the presence of specific PKC inhibitors, GF109203X and RO 31-8820, are due to the inhibition of PKC isotypes that are involved in the direct stimulation of adenylyl cyclase.

Since neither GF109203X or RO 31-8220 enhanced LH-stimulated cAMP production, it would seem unlikely that they are directly involved with LH/CG receptor desensitisation. However, the results from this work suggests that PKC isoforms may be involved in LH/CG receptor signal transduction, acting at the level of adenylyl cyclase. The results from studies using staurosporine indicate that staurosporine itself may have been directly activating adenylyl cyclase activity. Alternatively, since

staurosporine is known to inhibit CaM-kinase II, these results could suggest a putative role for this enzyme in maintaining Ca^{2+} inhibition of adenylyl cyclase.

Chapter Seven: General Discussion

The overall aim of this project was to learn more about the structure/function relationships of the MA10 LH/CG receptor with a particular emphasis on understanding more fully the process of desensitisation. It was intended to approach this project from several different angles, using a number of methods. Some of these techniques were not routinely used in the department prior to this project and had first to be established.

At the time this work began the concept of LH/CG receptor phosphorylation being the causal factor in the desensitisation response was a fiercely debated issue. In light of what was known regarding receptor phosphorylation and desensitisation of the cAMP response in the related β_2AR, many workers believed that an analogous system of signal transduction existed in the LH/CG receptor. Thus, much recent research has focused on investigating whether LH/CG receptor phosphorylation and ligand-induced adenylyl cyclase desensitisation were linked. The existence of LH/CG receptor antibodies and development of immunoprecipitation techniques greatly aided this work. Results demonstrating that ligand binding increased LH/CG receptor phosphorylation (Hipkin *et al.* 1993) and decreased cAMP production (Wang *et al.* 1997) suggests that LH/CG receptor phosphorylation and desensitisation may indeed be linked. However, research by others (Lamm and Hunzicker-Dunn 1994, Lamm *et al.* 1994) has proposed that phosphorylation of the LH/CG receptor was not responsible for ligand-induced cAMP desensitisation in isolated ovarian membranes.

Whilst the results from the studies mentioned here and in *Chapter Four* are not disputed, it must be noted that all these studies were either performed using LH/CG receptors which had been transfected into non-steroidogenic cell lines, or in cell membranes lacking an intracellular environment. Thus, the production and characterisation of LH/CG receptor anti-peptide antibodies in our laboratory (Pallikaros *et al.* 1995) provided an ideal opportunity of establishing whether the LH/CG receptor of MA10 cells became

phosphorylated when treated with desensitising concentrations of ligand in a native steroidogenic environment.

For the work described in *Chapter Four*, it was anticipated that MA10 cells or membranes would be treated with desensitising concentrations of hCG or LH in the presence of [^{32}P] or [^{32}P]-ATP respectively, proteins would be obtained by membrane solubilisation and the LH/CG receptors isolated via immunopurification with specific LH/CG receptor antibodies. During the course of this work, both immunoaffinity purification and immunoprecipitation purification were attempted. The anti-peptide antibodies were demonstrated to recognise the LH/CG receptor from MA10 cells and hyper-stimulated rat ovaries on Western blots, but despite this it was impossible to detect immunopurified LH/CG receptor by either method of purification. Since the LH/CG receptor is highly labile and contains many hydrophobic and cysteine residues, it would seem likely that the LH/CG receptor became degraded or formed aggregates, thus rendering the proteins unrecognisable to the antibodies.

The latter half of *Chapter Four* describes the results obtained when the same antibodies that were used in the immunopurification protocols were used in immunocyto- and immunohistochemistry techniques. These experiments were performed in order to assess the suitability of these antibodies as tools with which to study the intracellular trafficking of the LH/CG receptor in later work [see *Chapter Eight*]. The results from these experiments indicated that each of the three antibodies differed in their ability to recognise the respective antigens depending on the method of cell/tissue fixation used. However, the fact that these antibodies were found to recognise anti-peptide sequences of the LH/CG receptor after they had seemingly failed to immunoprecipitate the receptor, adds credence to the suggestion that the failure to establish an immunopurification protocol was likely to be as a result of problems with the solubilised membrane preparation and not the antibodies themselves. Antibody 2 demonstrated specific binding to Leydig cells in frozen sections of rat testes. Since this method of fixation is thought to cause the least alteration

in antigen conformation, antibody 2 proves to a be a good candidate for use in further studies using living cells [see *Chapter Eight*].

Whilst it is known that particular intracellular regions of the $ß_2$AR are involved in coupling to adenylyl cyclase and that others are phosphorylated by kinases during receptor desensitisation, similar regions had not been fully elucidated in the LH/CG receptor. Therefore, another approach to investigating the process of desensitisation in this receptor would have been to mutate nucleotides in specific regions or generate receptors that lacked parts of the native protein. Some researchers had been successful in generating and expressing C-terminally truncated LH/CG receptors in transfected cell lines (Sanchez-Yague *et al.* 1992, Zhu *et al.* 1993) leading to conflicting results regarding the putative roles of the missing regions in receptor/effector coupling, phosphorylation and ligand-induced desensitisation. Previous work in this department had reported the establishment of a novel method for producing truncated LH/CG receptors in rat Leydig cells. This method employed an antisense strategy to prevent the translation of specific regions of the LH/CG receptor and subsequently demonstrated truncated mutant receptors to be deficient at undergoing desensitisation with regards to cAMP and/or steroidogenesis, in response to stimulation with LH and 4ß-PMA. Thus, the initial aim of the work described in *Chapter Five* was to continue using antisense oligonucleotides to elucidate the regions of the LH/CG receptor that were important to the process of desensitisation. Because these experiments could be performed in native MA10 cells, the effects of particular truncations on the capability of the receptor to undergo cAMP production and steroidogenesis would be examined by stimulation with LH, followed by radioimmunoassay detection of cAMP and progesterone levels. Similarly, the effects of truncations on membrane insertion and hormone binding could also have been examined by techniques such as receptor binding assays and Western blotting experiments.

It was anticipated that the generation of LH/CG receptors which contained relatively large truncations would be detected on Western or ligand blots as having a lower molecular mass than that of the wild-type receptor. However, using these methods of detection it

was predicted that the molecular mass of LH/CG receptors which had only undergone small truncations would be difficult to assess. Thus, in order to verify the results obtained from the antisense studies and also to establish that incubation of cells with antisense oligonucleotides did generate truncated receptors as previously reported (West and Cooke 1991b and 1992), it was decided to duplicate the predicted LH/CG receptor truncations by another method. This would involve the generation of MA10 LH/CG receptor cDNA, its truncation by the use of restriction enzymes, followed by the transfection of this cDNA into a steroidogenic cell line that does not express native LH/CG receptors. The transfected cells containing the restriction enzyme-generated truncated LH/CG receptors would have been anticipated to respond to ligand or 4ß-PMA in a similar manner to MA10 cells containing truncated LH/CG receptors generated by antisense oligonucleotides, if the antisense strategy had worked as was previously predicted. Hence, the work described in *Chapter Three* was performed in part for these reasons and is referred to again later in this discussion.

Preliminary investigations using the antisense oligonucleotides in cultured MA10 cell experiments encountered difficulties in duplicating the previously reported results [see *Chapter Five*]. As a result, the aims of this part of the work were extended to first include the establishment of a cell-free system in which the LH/CG receptor could be synthesised. In this cell-free environment many of the problems encountered when trying to perform experiments in cells are avoided. Thus, such a system was anticipated to provide an ideal environment in which to study the efficacy of different antisense oligonucleotides in preventing or altering the synthesis of the LH/CG receptor. The demonstration of a distinct band with an approximate molecular mass of 65kDa corresponded to the anticipated molecular mass of the unglycosylated LH/CG receptor. Contrary to the previous results reported by West and Cooke (1991b), the addition of either antisense oligonucleotide 1 (targeted to near the N-terminus of the LH/CG receptor), or antisense oligonucleotide 3 (targeted to the third intracellular loop), had no detectable effect on the level of synthesis or size of the putative LH/CG receptor. It is possible that an increase in

the RNAse H content of this cell-free system may have generated different results, but as is explained in *Chapter Five*, it was not possible to investigate this.

In an attempt to establish an antisense strategy that *would* work using the rabbit reticulocyte cell-free system, to either prevent or alter the level of LH/CG receptor synthesis, two new antisense oligonucleotides were developed. In light of recent literature (Wakita and Wands 1994, Mizutari *et al.* 1995) suggesting that not all regions on the mRNA were ideal as target sites for antisense oligonucelotides, the new oligonucleotides were targeted to a region covering the start site of translation with antisense oligonucleotide A extending downstream into the 5'UTR. Suprisingly, neither of these antisense oligonucleotides had any discernible effect on the level of LH/CG receptor synthesis either.

Thus, the results from the work described in *Chapter Three* have demonstrated the novel, cell-free synthesis of the LH/CG receptor, providing a system in which to test the effects of antisense oligonucleotides on protein synthesis. Antisense oligonucleotides which had been previously reported as causing truncated LH/CG receptors were found to have no detectable effect on the level or size of LH/CG receptor synthesis in this system.

The aim of the work described in *Chapter Four* was to amplify and clone the LH/CG receptor from a murine Leydig tumour cell line (MA10) for reasons pertaining to the

antisense work described earlier and to establish if the LH/CG receptor from this neoplastic testicular cell contained sequence discrepancies compared to the published mouse ovarian sequence. Areas of the MA10 LH/CG receptor which were of particular interest were the intracellular regions which incorporated the putative phosphorylation consensus sequences sites and which were therefore anticipated to be involved in receptor desensitisation. The results from studies by West *et al.* (1991) and those reviewed in Rommerts and Cooke (1988), suggested that amino-acid or nucleotide differences within these regions might explain why different species respond to activators of protein kinases by undergoing desensitisation differently. One way in which to examine the putative phosphorylation consensus sequences for differences between species, would have been to perform amino-acid sequencing analysis as opposed to nucleotide sequence analysis. Because of the degenerate nature of the amino-acid code, not every base change will translate into an amino-acid difference. Examining the nucleotide sequence then is not necessarily the most convenient way of establishing whether amino-acid differences exist between related proteins. However, nucleotide sequence analysis was used in this instance as it was anticipated that truncations would then be made to the DNA in order to confirm the effects (if any) on LH/CG receptor function produced by the use of an antisense strategy.

Because the initial amplification of the full length MA10 LH/CG receptor proved difficult, it was decided to concentrate on the amplification of the C-terminus as this is where the putative phosphorylation consensus sequences are located. It was anticipated the N-terminal half of the LH/CG receptor would be obtained at a later date and that the two halves could be ligated together during cloning. However, due to time and financial constraints, further amplification of the N-terminal half of the MA10 LH/CG receptor was not attempted. As a result it was not possible to generate truncated receptors for use in functional studies alongside the antisense work described in *Chapter Five* and the potential use of the C-terminal region of the MA10 LH/CG receptor is described further in *Chapter Eight*. The amplification and cloning of MA10 cDNA produced two individual clones which on sequence analysis and comparison with the published mouse ovarian sequence,

proved to be the MA10 LH/CG receptor. A single base change in one of the clones was thought to be a misread base as it was not duplicated in the second clone. Thus, this work established that the C-terminal half of the MA10 LH/CG receptor was identical to the mouse ovarian sequence with no base differences found in the putative phosphorylation consensus sites.

Additional preliminary work using the cloned C-terminal half of the MA10 LH/CG receptor labeled with [^{32}P]-dCTP to probe Northern dot blots of total RNA extracted from rat testicular, liver and brain tissue and MA10 cells, indicated the presence of LH/CG receptor mRNA in rat brain. This result agrees with those by others (Lei *et al.* 1993, Al-Hader *et al.* 1997a, Al-Hader *et al.* 1997b) who have detected LH/CG receptor mRNA in a number of other tissues including the brain. This area of research is discussed further in *Chapter Eight*.

Research into the process of desensitisation in the $ß_2$AR has indicated that both PKA and ßARK are involved in the phosphorylation/desensitisation response (Hausdorff *et al.* 1989). A recent study by Chuang *et al.* (1995) also implicated PKC as having a potential role in $ß_2$AR desensitisation. The putative kinases involved in LH/CG receptor phosphorylation have not, as yet, been identified. The potential role of PKA in the desensitisation response of the LH/CG receptor has been the subject of much research in the last decade, resulting in conflicting points of view. Reports by researchers such as Minegishi *et al.* (1989), Wang and Ascoli (1990), Hipkin *et al.* (1993), suggest the involvement of PKA in LH/CG receptor ligand-mediated phosphorylation, whilst conflicting evidence has been reported by researchers such as Hunzicker-Dunn and Birnbaumer (1981), Rebois and Fishman (1986), McFarland *et al.* 1989, Lamm and Hunzicker-Dunn (1994). Many other GPCRs have been demonstrated to couple to the PLC signal tranducing pathway and some studies, (Hipkin *et al.* 1993), have suggested that PKC may be involved in LH/CG receptor phosphorylation. Since PKC is activated in PLC signal transduction, the primary aim of the work described in *Chapter Six* was to investigate whether inhibition of PKC had an effect on LH/CG receptor desensitisation in

MA10 cells. The results from this work demonstrated that the non-specific PKC inhibitor staurosporine, augmented LH-stimulated cAMP levels over periods of 2-3 hours. PKC inhibitors reported to demonstrate a greater specificity for PKC (GF109203X and RO 31-8220) did not mimic this effect and consistently decreased the level of LH-stimulated cAMP when compared to the controls. In addition, staurosporine was found to augment cholera toxin- and forskolin-stimulated cAMP production, although due to time constraints it was not possible to test the effects of the specific PKC inhibitors on cholera toxin- and forskolin-stimulated cAMP production. Staurosporine was found to inhibit steroidogenesis however, the specific PKC inhibitors did not appear to have any effect.

Staurosporine had been previously reported to augment ligand-stimulated cAMP levels (Jamaluddin *et al.* 1994, Keren-Tal *et al.* 1996) and cholera toxin-stimulated cAMP levels (Keren-Tal *et al.*) in other cell systems. However, the results presented in *Chapter Six* also show a significant increase in forskolin-stimulated cAMP levels in the presence of staurosporine which was not demonstrated in these previous studies. Since neither GF109203X or RO 31-8220 demonstrated an augmentation of LH-stimulated cAMP levels, the increase in cAMP levels observed was concluded to be via a non-PKC specific action of staurosporine. Staurosporine has acquired a reputation as a 'dirty' compound and is known to inhibit other protein kinases in addition to PKC, particularly at higher concentrations (Tamakoi *et al.* 1986, Ruegg and Burgess 1989, Gadbois *et al.* 1992, Persuad *et al.* 1993). The decrease in LH-stimulated steroidogenesis that was observed in the presence of staurosporine could therefore, be explained by this compound inhibiting the activity of kinases involved in steroidogenesis, at a point after cAMP accumulation. The increase in LH-stimulated cAMP levels that was observed in the presence of staurosporine, but not in the presence of the specific PKC inhibitors, may be explained by staurosporine having an inhibitory effect on CaM-kinase II. Since Ca^{2+} has been shown to have an inhibitory effect on adenylyl cyclase (Pereira *et al.* 1988, Dorflinger *et al.* 1984, Oldham *et al.* 1984 Giannattasio *et al.* 1987) it is conceivable that by inhibiting the activity of CaM-kinase II, the effects of Ca^{2+} are not conveyed to adenylyl cyclase. Thus, in the presence of staurosporine cAMP levels would be expected to increase. The results from

this work then, suggest that PKC is not involved in LH/CG receptor desensitisation. This is in agreement with more recent reports by Gudermann *et al.* (1995) and Hipkin *et al.* (1995), which taken together, suggest that whilst phorbol ester activators of PKC could induce LH/CG receptor phosphorylation, they did not cause LH/CG receptor desensitisation. However, PKC is not a single protein but instead represents a family of distinct isoenzymes. These isoenzymes are expressed in different tissues and have different activation requirements. Since no one PKC inhibitor has been demonstrated to inhibit all known isoforms of PKC, it is premature to completely exclude the involvement of 'PKC' from the process of LH/CG receptor desensitisation. Further investigations into the putative role of CaM-kinase II in LH/CG receptor desensitisation are required [see *Chapter Eight*].

Chapter Eight: Future Work

Many recent studies have demonstrated that several nongonadal reproductive tissues including their blood vessels contain LH/CG receptor mRNA and receptor protein (Lei *et al.* 1992, Toth *et al.* 1994, Tao *et al.* 1995). In addition, LH/CG receptor mRNA has also been demonstrated in rat brain (Lei *et al.* 1993, Al-Hader *et al.* 1997a, Al-Hader *et al.* 1997b), in lactating rat mammary glands (Tao *et al.* 1997), human adrenal glands (Pabon *et al.* 1996a) and human skin (Pabon *et al.* 1996b). Obviously the presence of LH/CG receptor mRNA does not necessarily mean that functional LH/CG receptors are expressed in these tissues, however the use of antibodies which are capable of recognising the LH/CG receptor in different tissues would aid in the identification of those tissues which express the LH/CG receptor protein. Previous work in the department (Pallikaros *et al.* 1995) had been successful in raising antibodies that could recognise the LH/CG receptor from hyper-stimulated rat ovaries, rat Leydig cells and MA10 cells. In the work described in this thesis, these antibodies have been successfully used to identify the LH/CG receptor in immunocytochemistry experiments using MA10 cells, and immunohistochemistry experiments using paraffin and frozen sections of ovarian and testicular tissues. In the immunohistochemistry experiments, antibody 2 detected expression of the LH/CG receptor in Leydig cells, but also appeared to immunostain the blood vessels of a testicular section. These results are in agreement with those by Toth *et al.* (1994), described above. These antibodies then, are excellent candidates for the study and demonstration of LH/CG receptor expression in non-gonadal tissues by immunohistochemical methods. In addition, LH/CG receptor antibodies directly labelled with a fluorescent tag could be used in confocal microscopy studies. This technique is used to follow the intracellular passage of proteins through the cell and could be used to establish if the processes of LH/CG receptor internalisation/degradation etc. occurs in a similar manner to those in gonadal cells. Double immunofluorescent studies using the labelled LH/CG receptor antibodies in conjuction with another antibody labelled with a different immunofluorescent tag, would allow the study of how the LH/CG receptor interacts with cytoskeletal components of the cell when internalised, or shed light on the specific factors that are involved in the internalisation of glycoprotein hormone receptors.

During the work described in this thesis it was possible to clone the latter 878bp of the MA10 LH/CG receptor. Whilst this fragment has been termed the 'C-terminal half' of the LH/CG receptor, it actually encompasses most of the transmembrane domain region, beginning at the end of the first transmembrane region. Thus, this clone contains all the intracellular regions of the LH/CG receptor and could prove to be an ideal candidate for use in the study of chimeric receptors. Such chimeric receptors involving the use of the LH/CG receptor have previously been used to study the functions of the TSHR (Nagayama et al. 1991) and the $ß_2$AR (Moyle et al. 1991). In addition, chimeric receptors formed between the FSHR and the LH/CG receptor have been used to study IP formation in the presence of either stimulating doses of FSH or LH (Hirsch et al. 1996). In this study, the region of the LH/CG receptor encompassing the 5th transmembrane domain to the end of the cytoplasmic tail was found to be important in IP release. Studies by Kobilka et al. (1988), have attempted to delineate the regions of the human $ß_2$AR that are responsible for G_s activation by the use of chimeric receptors consisting of the human $ß_2$AR and the human α_2-AR. The results from this work found that chimeric receptors that contained only a portion of the 3rd intracellular loop of the $ß_2$AR lost the ability to stimulate adenylate cyclase. Thus, similar studies to these could be performed using the cloned C-terminal half of the MA10 LH/CG receptor. In addition, one of the major problems of LH/CG receptor research has been the lack of steroidogenic cells in which to transfect mutated, truncated or chimeric receptors for the study of receptor signalling. Many studies have used COS or HEK cells for transfection studies, but these cells provide a relatively alien environment in which to study the structure/function relationships with regards to downstream events such as the intramitochondrial transfer of cholesterol or the synthesis of androgens and oestrogens. The formation of a chimeric receptor involving the intracellular regions of the MA10 LH/CG receptor and the extracellular regions of another GPCR would mean that LH/CG receptor cell signalling and steroidogenesis could be studied *in situ* in Leydig cells. The chimeric receptor would be activated not by LH, but by a ligand which would not have been previously recognised such as thrombin or TSH.

In light of the inability to repeat the results obtained by West and Cooke (1991b) by the use of an antisense strategy, it would be of interest to generate the putative truncations and use a mammalian expression system to establish how the truncations affect events such as membrane insertion, G-protein coupling/uncoupling, receptor internalisation etc. As it was not possible to amplify the N-terminal half of the MA10 LH/CG receptor during this work, it would be proposed to obtain a full length clone of the murine LH/CG receptor in which to perform these studies.

During the work described in this thesis it was possible to establish a cell-free system for generating LH/CG receptor protein from cDNA clones. Future work using this system would involve the identification of the LH/CG receptor by the use of LH/CG receptor antibodies or [^{125}I]-hCG on dot blots. This is not as simple as it first appears because the amount of protein generated in cell-free systems is generally quite low (150-500ng) and is not always detectable by these types of techniques. In addition, cell-free systems do not allow for co- or posttranslational processing events to occur. Thus, the LH/CG receptor protein will not be glycosylated, nor will it necessarily assume its native conformation in the absence of membranes. For these reasons the protein may not be recognised by antibodies or ligand. However, the rabbit reticulocyte lysate cell-free system can be modified by the addition of microsomal vesicles derived from mammalian pancreas. These vesicles permit processing events such as signal peptide cleavage, membrane insertion, translocation core glycosylation to occur during or after protein translation. Future work would include the addition of these membranes to the TnT LH/CG receptor reaction, followed by the detection of the receptor by Western or ligand blotting techniques. It is also possible to incorporate a histidine tag into the recombinant plasmid so that when the cDNA has been translated into protein, the protein of interest can be identified by Western blotting with anti histidine antibodies. If it proved possible to detect the LH/CG receptor in this way, cell-free synthesis may provide an easier alternative to mammalian cell expression. For example, truncations or mutations could be made in the LH/CG receptor cDNA which could then be translated into protein using the cell-free system in the presence of microsomal vesicles. In this way it would be possible to detect whether specific truncations or mutations of the LH/CG receptor effect ligand binding. In addition it

should be possible to clone and translate the multiple mRNA transcripts of LH/CG receptor that have been reported. Using ligand binding experiments and/or Western blotting techniques, it would be possible to ascertain whether these transcripts could be translated into putative proteins.

In view of reports by Hipkin et al. (1993) it would seem that ligand activation of the LH/CG receptor does result in receptor phosphorylation. Whether receptor phosphorylation is responsible for the desensitisation response has yet to be categorically established. However, there is a large body of evidence that suggests that LH/CG receptor desensitisation is related to phosphorylation of the receptor. In order to induce receptor desensitisation, it would appear that specific regions of the LH/CG receptor must undergo phosphorylation, as the demonstration of receptor phosphorylation does not necessarily result in LH/CG receptor desensitisation (Hipkin et al. 1993). Thus it would seem probable that phosphorylation of specific consensus sequences on the LH/CG receptor is mediated by a protein which has yet to be identified. Much research has focused on PKA as having a role in LH/CG receptor phosphorylation, however, it would seem from the literature reviewed here that if PKA is involved, it is not likely to be the only kinase mediating LH/CG receptor desensitisation events. In addition, PKA represents a family of isoenzymes which are expressed in a tissue specific manner. Since much of the research examined here has utilised different cell lines or cell types in which to perform desensitisation studies, discrepant results could be explained by encountering different isoforms of PKA. Further studies examining the role of PKA in LH/CG receptor desensitisation should make use of the recent development of antibodies specific for each PKA isoform. In this way then, the presence or absence of a particular PKA isoenzyme could first be established. Studies to ascertain whether this particular PKA isoenzyme was involved in LH/CG receptor desensitisation could then be examined by the use of inhibitors or activators.

The results from work presented in this thesis agrees with several other studies which indicate that a role for PKC in LH/CG receptor desensitisation would seem increasingly unlikely. However, as with PKA, this issue is complicated by the fact that

PKC consists of many isoenzymes which are expressed in a tissue specific manner. Once again, the use of PKC antibodies specific for each isoenzyme would enable the presence of PKC isoenzymes to be identified in particular cells and desensitisation studies could then make use of PKC inhibitors to assess the role that PKC may play in this process.

Since many studies cited here have indicated that neither PKA or PKC play a definitive role in LH/CG receptor desensitisation, it may be prudent to look towards other kinases as participants in this process. The results from the work described in this thesis suggests that CaM-kinase II may be involved in LH/CG receptor desensitisation in MA10 cells. Antibodies to CaM-kinase II and inhibitor compounds are available and future work should make use of these to investigate the role of this kinase further.

In light of what is known about the process of desensitisation in the $ß_2$AR it is highly possible that a second messenger-independent kinase analogous to ßARK, is responsible for the agonist-induced desensitisation of the LH/CG receptor. The role of ßARK in $ß_2$AR desensitisation was elucidated by the use of S49 lymphoma cells which were defective in either PKA or G_s (Benovic *et al.* 1986 and 1987). Direct evidence that ßARK mediated $ß_2$AR phosphorylation was provided by the use of inhibitors of ßARK and PKA whereby heparin and a heat stable inhibitor peptide of PKA (PKI) were found to completely block agonist-induced phosphorylation of the ßARs (Lohse *et al.* 1989, Lohse *et al.* 1990). The discovery of a second form of ßARK (ßARK2) (Benovic *et al.* 1991) and the subsequent identification of a number of other socalled G-protein coupled receptor kinases (GPKs) [for review see Premont *et al.* 1995], has indicated that these proteins are members of a multigene family. In a recent study by Iacovelli *et al.* (1996), cotransfection studies of COS7 cells with the TSHR, GRK2 and ß-arrestin1, demonstrated that TSHR-stimulated cAMP accumulation could be controlled by a GRK2/ß-arrestin1 mechanism. In addition, Nagayama *et al.* (1996), have recently demonstrated the involvement of GRK5 in TSHR homologous desensitisation. Thus, it would seem highly possible that in cells expressing the LH/CG receptor, similar mechanisms could be involved in LH/CG receptor desensitisation. Future work should involve the examination of Leydig or granulosa-luteal cells for the

presence or absence of GRKs using molecular biological techniques such as PCR or Northern blotting. In addition, the effect(s) of ßARK inhibitors and/or PKA/PKC on LH/CG receptor desensitisation could be examined in MA10 cells. In addition to these studies, it would be of interest to examine the role of phosphatases in LH/CG receptor desensitisation by the use of specific inhibitors and antibodies.

Lammelli's sample buffer: 0.0625M Tris-HCl pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, 0.05% (w/v) bromophenol blue. Aliquot and store at -20°C.

Pas Gelatin Buffer (PGB): 4.42g KH_2PO_4, 124.16g $Na_2HPO_4.12H_20$, 9g NaCl, 1g NaN_3, 1g Gelatin, add distilled water to 1 litre and dissolve stirring over low heat.

Prehybridisation buffer: 3ml 20x SSC buffer [see below], 1ml 50x Denhardts solution, 100µl 10mg/ml salmon sperm DNA, 20% (w/v) SDS. Measure the SSC buffer in a 10ml measuring cylinder, add the Denhardts solution and add distilled water to approximately 9ml. Then add SDS (adding the SDS before the water will cause the SDS to precipitate). Add water to 10ml.

SOC medium: 2g Bacto-Tryptone, 0.5g Bacto-Yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M glucose (filter sterilised), 1ml Mg^{2+} stock (1M $MgCl_2.6H_2O$,1M $MgSO_4.7H_2O$ - filter sterilised). Add distilled H_2O to 100ml and filter sterilise the complete solution.

SSC buffer (20x solution): Add 87.7g NaCl and 44.1g sodium citrate to 400ml DEPC treated water. pH to 7.2 with NaOH and add DEPC treated water to 500ml. Sterilise by autoclaving.

Appendix 2

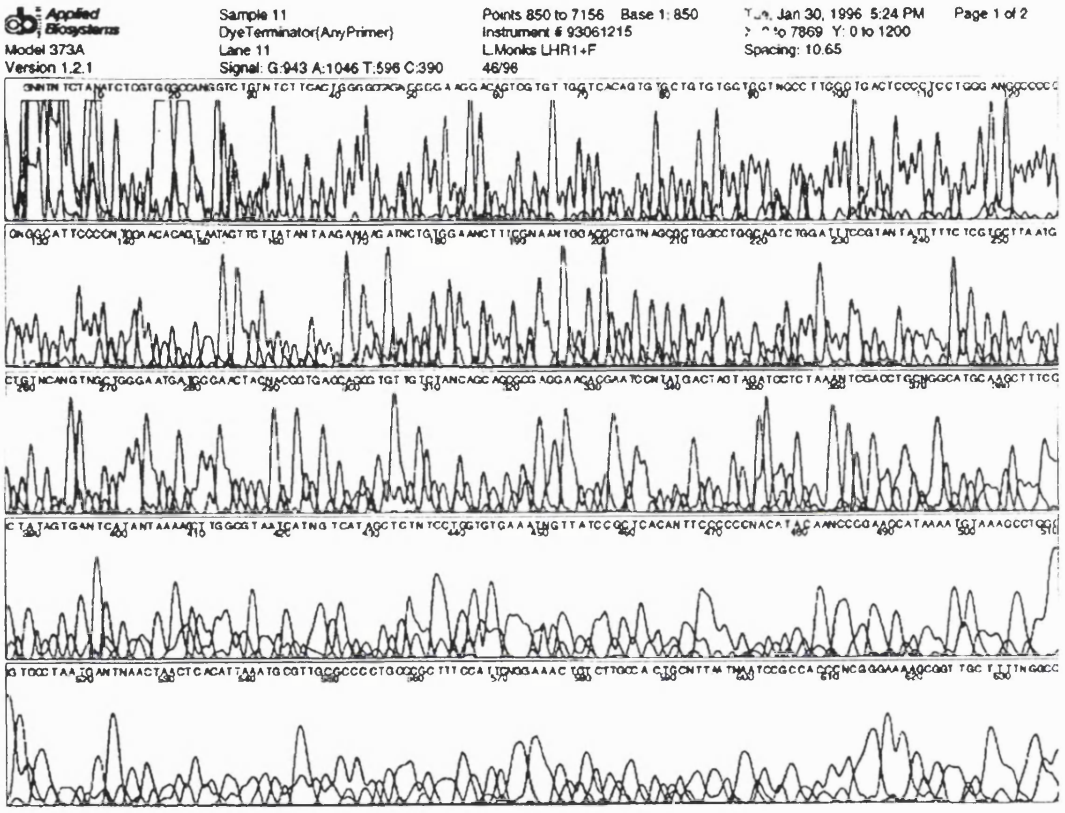


Figure A1: Sequence data provided by the Babraham Institute, of cloned insert number 1 using the forward universal primer.

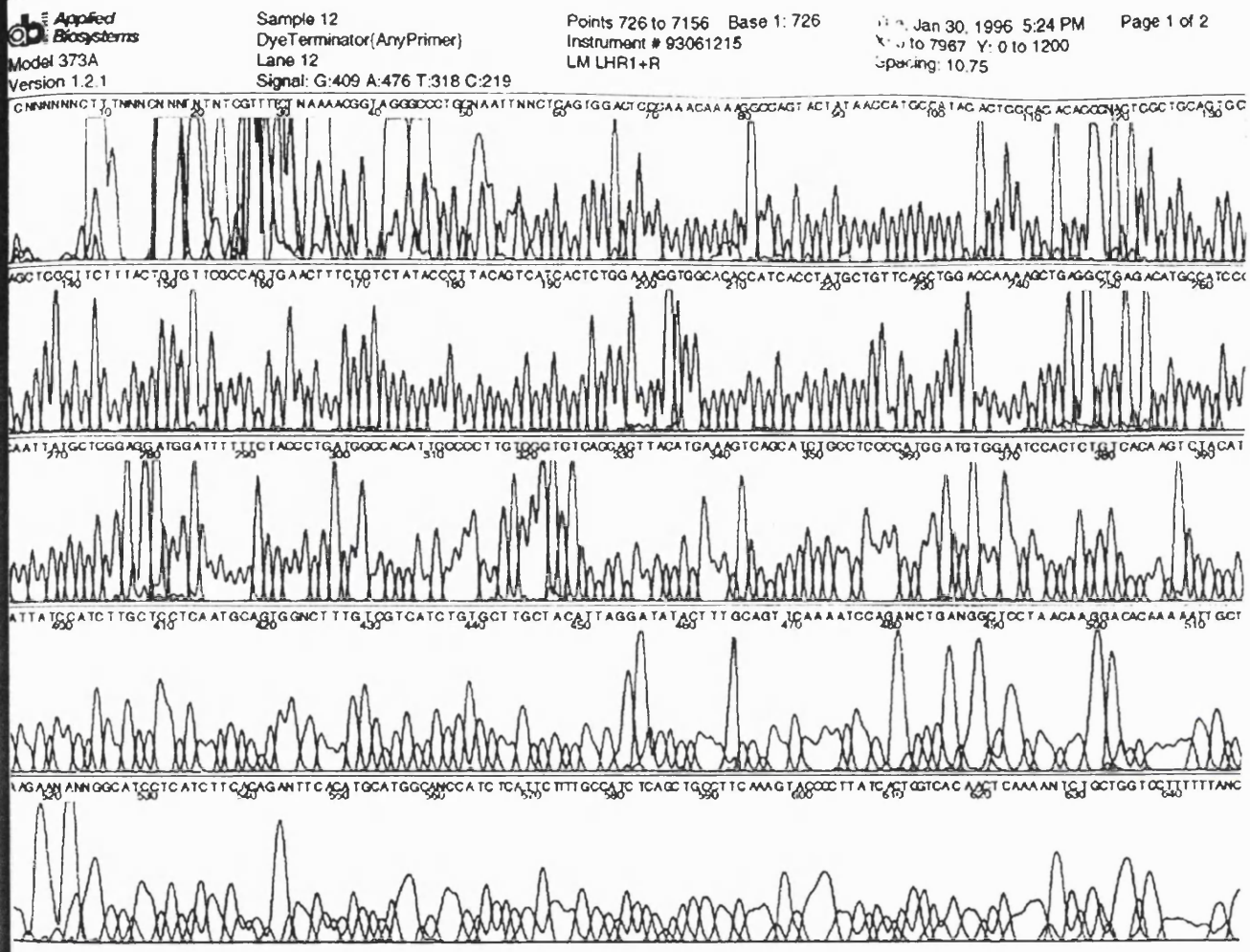


Figure A2: Sequence data provided by the Babraham Institute, of cloned insert number 1 using the reverse universal primer.

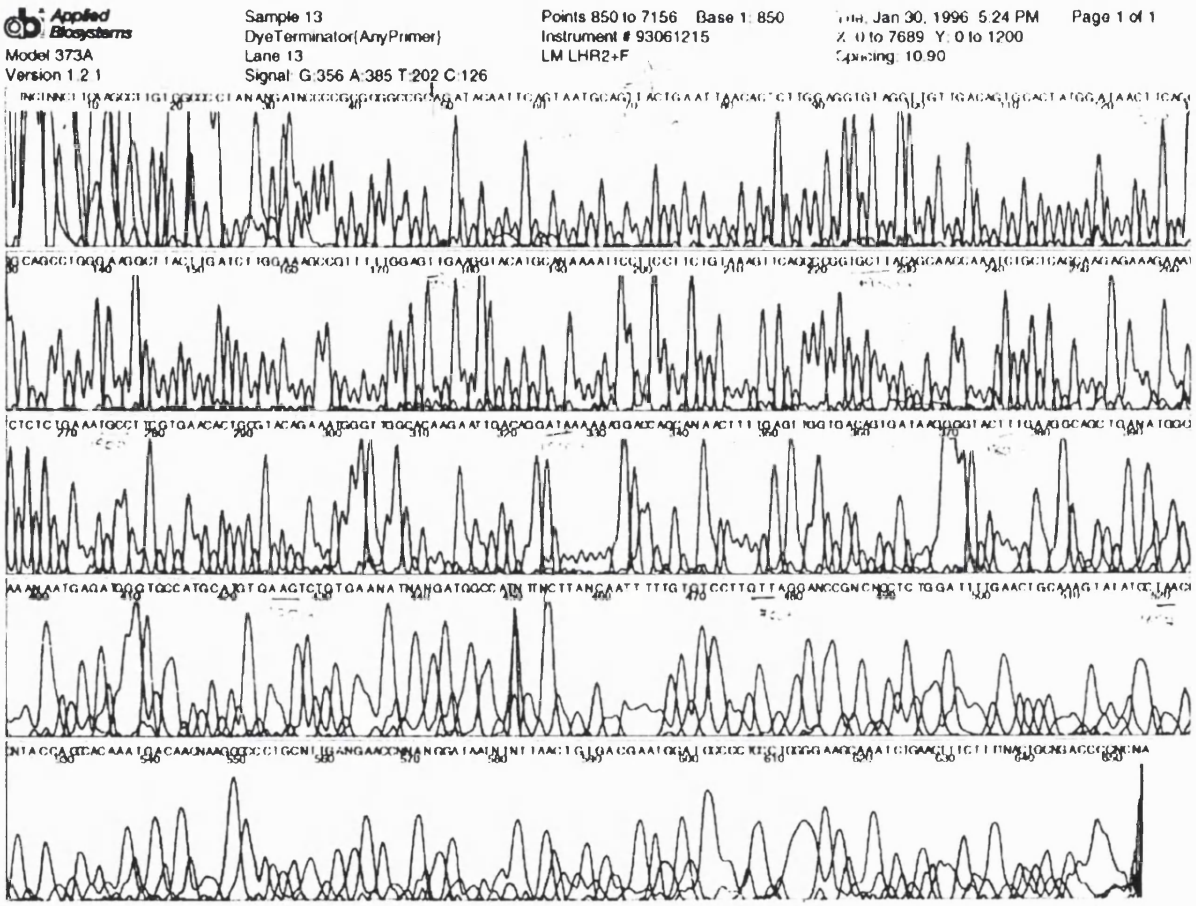


Figure A3: Sequence data provided by the Babraham Institute, of cloned insert number 2 using the forward universal primer.

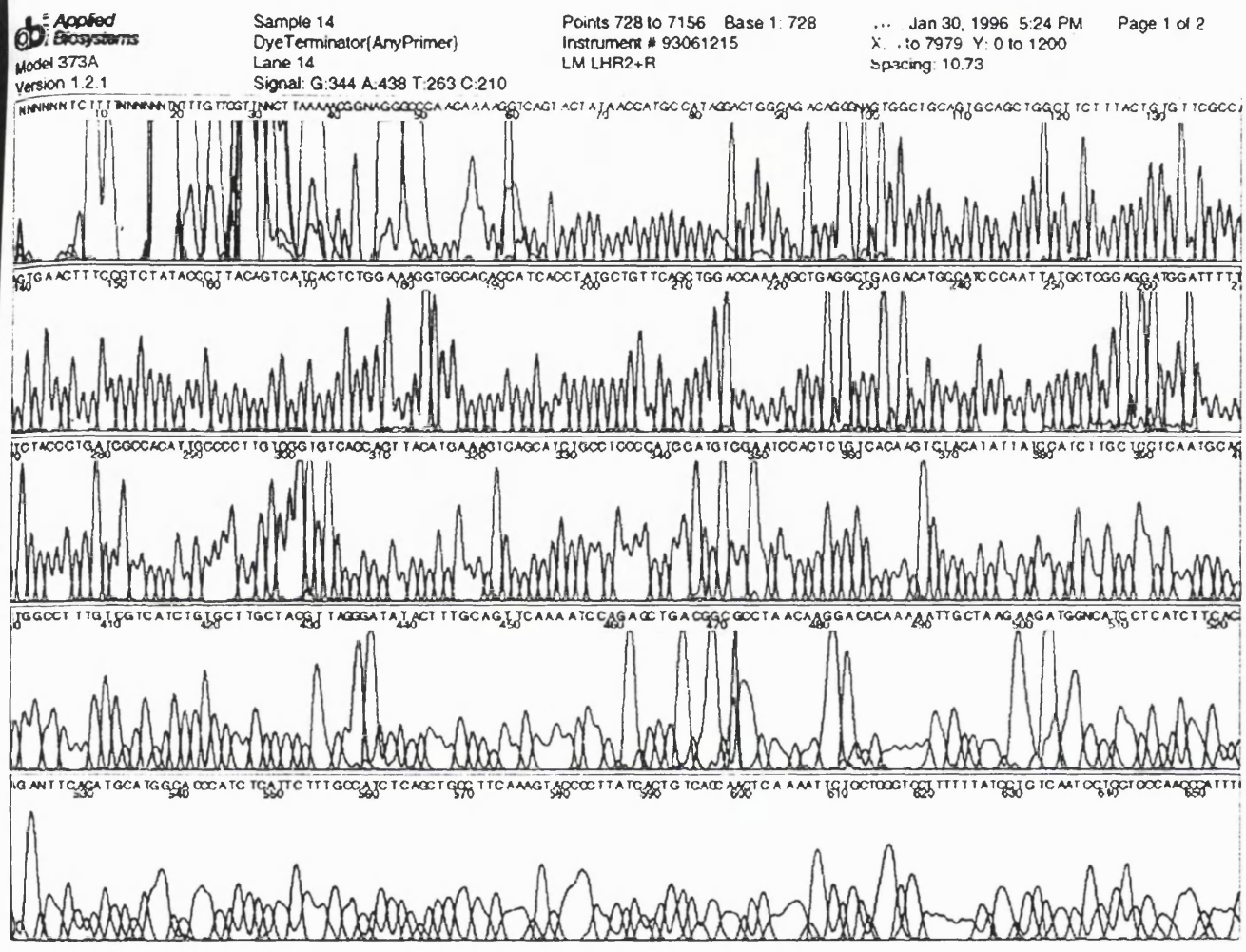


Figure A4: Sequence data provided by the Babraham Institute, of cloned insert number 2 using the reverse universal primer.

Chapter Nine: References

Agrawal S, Rustagi P, Shaw D. 1995. Toxicology Letters. 82/83:431-434.

Akhtar S and Agrawal S. 1997. Trends in Pharmocological Sciences. 18: 12-18.

Akhtar S, Kole R, Juliano R. 1991. Life Sciences. 49: 1793-1801.

Al-Hader A, Lei Z, Rao C. 1997a. Biology of Reproduction. 56: 1071-1076.

Al-Hader A, Lei Z, Rao C. 1997b. Biology of Reproduction. 56: 501-507.

Ascoli M. 1981. Endocrinology. 108: 88-95.

Ascoli M, Pignataro O, Segaloff D. 1989. Journal of Biological Chemistry. 264: 6674-6681.

Ascoli M and Segaloff D. 1986. Journal of Biological Chemistry. 261: 3807-3815.

Ascoli M and Segaloff D. 1989. Endocrine Reviews. 10: 27-44.

Bacich D, Rohan R, Norman R, Rodgers R. 1994. Endocrinology. 135:735-744.

Baertschi A. 1994. Molecular and Cellular Endocrinology. 101: R15-24.

Bagshawe K and Harland S. 1976. Cancer. 38:112-118.

Bahl O, Sojar H. 1990. Receptor Purification. Vol.1: 173-190. Published by the Humana Press.

Baldwin J. 1993. The EMBO Journal. 12: 1693-1703.

Baldwin S. 1994. In: Methods in Molecular Biology. Edited by Graham J. Published by the Humanan Press. Towata NJ.

Benovic J, Strasser R, Caron M, Lefkowitz R. 1986. PNAS USA. 83: 2797-2801.

Benovic J, Regan J, Matsui H, Mayor F, Cotecchia S, Leeb-Lundberg L, Caron M, Lefkowitz R. 1987. Journal of Biological Chemistry. 262:17251-17253.

Benovic J, Onorato J, Arriza J, Stone W, Lohse M, Jenkins N, Gilbert D, Copeland N, Caron M, Leftkowitz R. 1991. Journal of Biological Chemistry. 266: 14939-14946.

Birnbaumer L. 1987. Trends in Pharmocological Sciences. 8: 209-217.

Birnbaumer L. 1990. FASEB Journal. 4: 3178-3188.

Birnbaumer L. 1992. Cell. 71: 1069-1072.

Bhowmick N, Huang J, Puett D, Issacs N, Lapthorn A. 1996. Molecular Endocrinology. 10: 1147-59

Blake K, Murakami A, Miller P. 1985. Biochemistry. 24: 6132-6138.

Bluml K, Mutschler E, Wess J. 1994. PNAS USA. 91: 7980-7984.

Bockaert J, Hunzicker-Dunn M, Birnbaumer L. 1976. Journal of Biological Chemistry. 251: 2653-63.

Boiziau C and Toulme J. 1991. Biochimie. 73: 1403-1408.

Boiziau C, Kurfurst R, Cazenave C, Roig V, Thuong N, Toulme J. 1991. Nucleic Acids Research 19: 1113-1119.

Boujrad N, Hudson J, Papadopoulos V. 1993. PNAS USA. 90: 5728-5731.

Boutorine A, Boiziau C, Le Doan T, Toulme J, Helen C. 1992. 74: 485-489.

Bouvier M, Hausdorff W, De Blasi A, O'Dowd B, Kobilka B, Caron M, Lefkowitz R. 1988. Nature. 333: 370-373.

Braun T, Schofield P, Sprengel R. 1991. EMBO Journal. 10: 1885-1890.

Braunstein G. 1981. Fertility and Sterility. 36: 164-172.

Brooker G. 1979. Advances in Cyclic Nucleotide Research. 10: 1-33.

Brown D, Kang S-H, Gryaznov S, DeDionisio L, Heidenreich O, Sullivan S, Xu X, Nerenberg M. 1994. Journal of Biological Chemistry. 269: 26801-26805.

Brown D, Lydon J, McLaughlin M, Stuart-Tilley A, Tyszkowski R, Alper S, 1996. Histology and Cell Biology. 105: 261-267.

Bruch B, Thotakura, Bahl O. 1986. Journal of Biological Chemistry. 261: 9450-9460.

Budnik L and Mukhopadhyay A. 1987. Molecular and Cellular Endocrinology. 54: 51-61.

Burstein S and Gut M. 1976. Steroids. 28: 115-131.

Busen W, Peters J, Hausen P. 1977. European Journal of Biochemistry. 74: 203-208.

Capaccioli S, Di Pasquale G, Mini E, Mazzei T, Quattrone A. 1993. Biochemical and Biophysical Research Communications. 197: 818-825.

Catt K, Baukal A, Davies T, Dufau M. 1979a. Endocrinology. 104: 17-25.

Catt K, Harwood J, Aguilera G, Dufau M.1979b. Nature. 280: 109-116.

Cazenave C, Loreau N, Thuong N, Toulme J-J, Helene C. 1987. Nucleic Acids Research. 17: 4255-4273.

Cazenave C, Stein C, Loreau N, Thuong N, Neckers L, Subasinghe C, Helene C, Cohen J, Toulme J. 1989. Nucleic Acids Research 17: 4255-4273.

Cazenave C, Frank P, Busen W. 1993. Biochimie. 75: 113-122

Chabre O, Conklin B, Lin H, Lodish H, Wilson E, Ives H, Catanzariti L, Hemmings B, Bourne H. 1992. Molecular Endocrinology. 6: 551-556. et al 1992

Charreau E, Dufau M, Catt K. 1974. Journal of Biological Chemistry. 249: 4189-4195.

Cheung A, Huang R, Grazino M, Strader C. 1991. FEBS Letters. 279: 277-280.

Choi E, Wong S, Dittman, Storm D. 1993. Biochemistry. 32: 1891-1894.

Chuang T, LeVine H, De Blasi A. 1995. Journal of Biological Chemistry. 270: 18660-18665.

Clark B, Wells J, King S, Stocco D. 1994. Journal of Biological Chemistry. 269: 28314-22.

Clayton R. 1982. Endocrinology. 111: 152-161.

Clayton R. 1989. Journal of Endocrinology. 120: 11-19.

Cockcroft S and Thomas G. 1992. Biochemical Journal. 288: 1-14.

Colbran R, Schworer C, Hashimoto Y, Fong Y, Rich D, Smith M, Soderling T. 1989. Biochemical Journal. 258: 313-325.

Cooke B, Dix C, Habberfield A, Sullivan M. 1986. In: Hormone and Cell Regulation (Proceedings of the 10th European Symposium), 139: 111-128. Edited by Nunez J, Dumont J, King J. Published by John Libby and Co.

Cooke B, Platts E, Abayasekara R, Kurlak L, Schulster D, Sullivan M. 1989. Journal of Fertility. Supplement. 37: 139-145.

Cooke B and Sullivan M. 1985. Molecular and Cellular Endocrinology. 41: 115-122.

Cooke B, Lindh L, Janszen F. 1977. FEBS Letters. 73: 67-71.

Cooke B. 1996. Chapter 17. In: The Leydig Cell. Edited by Payne A, Hardy M, Russell L. Published by Cache River Press.

Crine P, Aubry M, Potier M. 1984. Annual New York Academy of Science. 438: 224-226.

Crooke S. 1993. FASEB Journal. 7: 533-539.

Crouch R and Dirksen M-L. 1985. In: Nucleases. Edited by Linn S and Roberts R. Published by Cold Spring Harbour Laboratory Press. USA.

Cunha G, Donjacour A, Cooke P, Mee S, Bigsby R, Higgins S. 1987. Endocrine Reviews. 8: 338-362.

Davis J, Weakland L, West L, Farese R. 1986. Biochemical Journal. 238: 597-604.

Davis J, Weakland L, Farese R, West L. 1987. Journal of Biological Chemistry. 262: 8515-8521.

Davis P, Elliot L, Harris W, Hill C, Hurst S, Keech E, Kumar M, Lawton G, Nixon J, Wilkinson S. 1992. Journal of Medicinal Chemistry. 35: 994-1001.

Davis D, Rozell T, Liu X, Segaloff D. 1997. Molecular Endocrinology. 11: 550-562.

Davidson J, van der Merwe, Wakefield I, Millar P. 1991. Molecular and Cellular Endocrinology. 76: C33-C38.

Desjardins C. 1981. Biology of Reproduction. 24: 1-21.

Dieter P and Fitzke E. 1991. Biochemical and Biophysical Research Communications. 181: 396-401.

Dix C, Schumacher M, Cooke B. 1982. Biochemical Journal. 202: 739-45.

Dix C, Habberfield A, Sullivan M, Cooke B. 1984. Biochemical Journal. 219: 529-537.

Dix C, Habberfield A, Cooke B. 1987. Biochemical Journal. 243: 373-77.

Dixon R, Furutachi T, Leiberman S. 1970. Biochemical and Biophysical Research Communications. 40: 161-169.

Dohlman H, Thorner J, Caron M, Lefkowitz R. 1991. Annual Review of Biochemistry. 60: 653-88.

Dorflinger L, Albert P, Williams A, Behrman H. 1984. Endocrinology. 114: 1208-1215.

Dufau M, Charreau E, Ryan D, Catt K. 1974. FEBS Letters. 39: 149-53.

Dufau M, Ryan D, Baukat A, Catt K. 1975. Journal of Biological Chemistry. 250: 4822-4825.

Dufau M, Horner K, Hayashi K, Tsuruhara T, Conn P, Catt K. 1978. Journal of Biological Chemistry. 3721-3729.

Dufau M, Cigorraga S, Baukal A, Sorrell S, Batar J, Neubauer J. 1979. Endocrinology. 105: 1314-1321.

Eason M, Jacinto M, Theiss C, Liggett S. 1994. PNAS USA. 91: 11178-11182.

Ekstrom R and Hunzicker-Dunn M. 1989a. Endocrinology. 124: 956-963.

Ekstrom R and Hunzicker-Dunn M. 1989b. Endocrinology. 125: 2470-2474.

Emanuele N, Oslapas R, Connick E, Kirsteins L, Lawrence A. 1981. Neuroendocrinology. 33: 12-17.

Emanuele N, Anderson J, Andersen E, Connick E, Baker G, Kirsteins L. 1983. Neuroendocrinology. 36: 254-260.

Exton J. 1993. Advances in Second Messenger and Phosphoprotein Research. 28: 65-71. Published by Raven Press, Ltd., New York.

Ezra E and Salomon Y. 1980. Journal of Biological Chemistry. 255: 653-658.

Ezra E and Salomon Y. 1981. Journal of Biological Chemistry. 256: 5377-5382.

Fallon R. 1990. Biochemical and Biophysical Research Communications. 170: 1191-1196.

Feuilloley M and Vaudry H. 1996. Endocrine Reviews. 17: 269-288.

Findlay D, Houssami S, Lin H, Myers D, Brady C, Darcy P, Ikeda K, Martin J, Sexton P. 1994. Molecular Endocrinology. 8: 1691-1700.

Fleischman J, Porter R, Press E. 1963. Biochemical Journal. 88: 220-228.

Freeman D. 1989. Endocrinology. 124: 2527-2534.

Gabriel B and Teissie J. 1994. European Journal of Biochemistry. 223: 25-33.

Gadbois D, Crissman H, Tobey R, Bradbury E. 1992. PNAS USA. 89: 8626-8630.

Garnier M, Boujrad N, Oke B, Brown A, Riond J, Ferrara P, Shoyab M, Suarez-Quian C, Papadopoulos V. 1993. Endocrinology. 132: 444-458.

Genty N, Salesse R, Garnier J. 1987. Biology of the Cell. 59: 129-135.
Ghosh M and Cohen J. 1992. Progress in Nucleic Acid Research and Molecular Biology. 42: 79-126.

Giannattasio G, Bianchi R, Spada A, Vallar L. 1987. Endocrinology. 120: 2611-2619.

Gilchrist R, Ryu K-S, Ji I, Ji T. 1996. Journal of Biological Chemistry. 271: 19283-19287.

Giles R, Spiller D, Tidd D. 1993. Anti-Cancer Drug Design. 8: 33-51.

Giles R and Tidd D. 1992. Nucleic Acids Research. 20: 763-770.

Godson C, Bell K, Insel P. 1993. Journal of Biological Chemistry. 268: 11946-11950.

Gudermann T, Birnbaumer M, Birnbaumer L. 1992a. Journal of Biological Chemistry. 267: 4479-4488.

Gudermann T, Nichols C, Levy F, Birnbaumer M, Birnbaumer L. 1992b. Molecular Endocrinology. 6: 272-278.

Gudermann T, Birnbaumer M, Birnbaumer L. 1995. Molecular and Cellular Endocrinology. 110: 125-135.

Gulyas B, Matsuura S, Chen H, Yuan L, Hodgen G. 1981. Biology of Reproduction. 25: 609-620.

Gura T. 1995. Science. 270: 575-577.

Gutowski S, Smerka A, Nowak L, Wu D, Simon M, Sternweis P. 1991. Journal of Biological Chemistry. 266: 20519-20524.

Habberfield A, Dix C, Cooke B. 1986. Biochemical Journal. 233: 369-376.

Haeuptle M-T, Frank R, Dobberstein B. 1986. Nucleic Acids Research. 14: 1427-1444.

Hadley M. 1992. In: Endocrinology. Edited by Hadley M. 3rd edition. Published by Prentice Hall Inc.

Haisenleder D, Dalkin A, Ortolano G, Marshall J, Shupnik M. 1991. Endocrinology. 128: 509-517.

Hall P, Osawa S, Mrotek J. 1981. Endocrinology. 109: 1677-1682.

Harlow E and Lane D. 1988. In: Antibodies: a laboratory manual. Published by Cold Spring Harbour Laboratory Press. USA.

Harris P, Perry L, Chard T, Chaudry L, Cooke B, Touzel R, Croates P, Lowe D, Afshar F, Wass J, Besser G. 1988. Clinical Endocrinology. 29:503-508.

Haseloff J and Gerlach W. 1988. Nature 334: 585-591.

Hausdorff W, Bouvier M, O'Dowd, Irons G, Caron M, Lefkowitz R. 1989. Journal of Biological Chemistry. 264: 12657-12665.

Hausdorff W, Caron M, Lefkowitz R. 1990. FASEB. J. 4: 2881-2889.

Hedin K, Duerson K, Clapham D. 1993. Cellular Signalling. 5: 505-518.

Helene C and Toulme J-J. 1990. Biochimica and Biophysica Acta. 99-125.

Hengerer B and Mathe D. 1996. Promega Notes. 58: 40-42.

Hertel C, Munnally M, Wong S, Murphy E, Ross E, Perkins J. 1990. Journal of Biological Chemistry. 265: 17988-17994.

Hepler J and Gilman A. 1992. Trends in Biological Science. 17: 383-387.

Herrlich A, Kuhn B, Grosse R, Schmid A, Schultz G, Gudermann T. 1996. Journal of Biological Chemistry. 271: 16764-16772.

Hipkin R, Sanchez-Yague J, Ascoli M. 1993. Molecular Endocrinology. 7: 823-832.

Hipkin R, Wang Z, Ascoli M. 1995. Molecular Endocrinology. 9: 151-158.

Hirsch B, Kudo M, Naro F, Conti M, Hsueh A. 1996. Molecular Endocrinology. 10: 1127-1137.

Hogan M. 1993. Clinical Chemistry. 39: 712-713.

Hoshina M, Boothby M, Hussa R, Patillo R, Camel H, Boime I. 1985. Placenta. 6: 163-172.

Hostetter G, Gallo R, Brownfield M. 1981. Neuroendocrinology. 33: 241-245.

Hsieh K and Martin T. 1992. Molecular Endocrinology. 6: 1673-1681.

Hsueh A, Dufau M, Catt K. 1976. Biochemical and Biophysical Research Communications. 72: 1145-1152.

Hsueh A, Dufau M, Catt K. 1977. PNAS USA. 74: 592-595.

Hubbard S, Bishop W, Kirschmeier P, George S, Cramer S, Hendrickson W. 1991. Science. 254: 1776-1779.

Hug H and Sarre T. 1993. Biochemical Journal. 291: 329-343.

Huhtaniemi I, Katikineni M, Chan V, Catt K. 1981. Endocrinology. 108: 58-65.

Hunzicker-Dunn M and Birnbaumer L. 1981. Endocrinology. 109: 345-351.

Hwang J and Menon K. 1984. Journal of Biological Chemistry. 259: 1978-1985.

Iacovelli L, Franchetti R, Masini M, De Blasi A. 1996. Molecular Endocrinology. 10: 1138-1146.

Ikezu T, Okamoto T, Ogata E, Nishimoto I. 1992. FEBS Letters. 311: 29-32.

Inoue Y and Rebois R. 1989. Journal of Biological Chemistry. 264: 8504-8508.

Iversen P. 1991. Anticancer Drug Design. 6: 531-538.

Jacobowitz O and Iyengar R. 1994. PNAS USA. 91: 10630-10634.

Jamaluddin M, Molnar M, Marrone B, Hertelendy F. 1994. General and Comparative Endocrinology. 93:471-479.

Janszen F, Cooke B, Van Driel M. Van Der Molen H. 1976. Biochemical Journal. 160: 433-437.

Ji I and Ji T. 1991. Endocrinology. 128: 2648-50.

Kandel E. 1991. In: Principles of Neural Science. Edited by Kandel E, Schwartz J, Jessell J. 3rd edition. Published by Elsevier Scince Publishing Company Inc.

Karnik S, Ridge K, Bhattacharya S, Khorana G. 1993. PNAS USA 90: 40-44.

Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy C, Ishikawa Y. 1994. Journal of Biological Chemistry. 269: 16554-16558.

Kawate N and Menon K. 1994. Journal of Biological Chemistry. 269: 30651-30658.

Kawate N, Peegel H, Menon K. 1997. Molecular and Cellular Endocrinology. 127: 211-219.

Keinanen K, Kellokumpu S, Metsikko M, Rajaniemi H. 1987. Journal of Biological Chemistry. 262: 7920-7926.

Keinanen K and Rajaniemi H. 1986. Biochemical Journal. 239: 83-87.

Kellokumpu S and Rajaniemi H. 1985. Endocrinology. 116: 707-714.
Kennedy M and Limbird L. 1993. Journal of Biological Chemistry. 268: 8003-8011.

Kennelly P and Krebs E. 1991. Journal of Biological Chemistry. 266: 15555-15558.

Keren-Tal I, Dantes A, Amsterdam A, 1996. Molecular and Cellular Endocrinology. 116: 39-48.

Keutmann H. 1992. Molecular and Cellular Endocrinology. 86: C1-C6.

Keutmann H, McIlroy P, Berget E, Ryan R. 1983. Biochemistry. 22: 3067-3072.

Kim I, Ascoli M, Segaloff D. 1987. Journal of Biological Chemistry. 262: 470-477.

Kirchick H and Birnbaumer L. 1983. Endocrinology. 113: 1629-1637.

Kirchick H, Iyengar R, Birnbaumer L. 1983. Endocrinology. 113: 1638-1646.

Kliman H, Nestler J, Sermasi E, Sanger J, Strauss J. 1986. Endocrinology. 118: 1567-1582.

Kobilka B, Kobilka T, Daniel K, Regan J, Caron M, Lefkowitz R. 1988. Science. 240: 1310-1316.

Koo Y, Ji I, Slaughter R, Ji T. 1991. Endocrinology. 128: 2297-2308.

Koo Y, Ji I, Ji T. 1994. Endocrinology. 134: 19-26.

Kosugi S, Shenkar A, Mori T. 1994. FEBS Letters. 356: 291-294.

Kosugi S, Mori T, Shenkar A. 1996. Journal of Biological Chemistry. 31813-31817.

Krieg A. 1993. Clinical Chemistry. 39: 710-712.

Krieg A, Tonkinson J, Matson, Zho Q, Saxon M, Zhang L, Bhanja U, Yakubov L, Stein C. 1993. PNAS USA. 90: 1048-1052.

Kusuda S and Dufau M. 1986. Journal of Biological Chemistry. 261: 16161-16168.

Kwiatkowski A, Shell D, King M. 1988. Journal of Biological Chemistry. 263: 6484-6486.

Lai H, Yang T-H, Messing R, Ching Y-H, Lin S-C, Chern Y. 1997. Journal of Biological Chemistry. 272: 4970-4977.

Lamm M and Hunzicker-Dunn M. 1994. Molecular Endocrinology. 8: 1537-1546.

Lamm M, Ekstrom R, Maizels E, Rajagopalan R, Hunzicker-Dunn M. 1994. Endocrinology. 134: 1745-1754.

Lapetina E, Billah M, Cuatrecasas P. 1982. Nature. 292: 367-369.

Laue L, Wu S, Kuido M, Hseuh A, Cutler G, Griffin J, Wilson J, Brain C, Berry A, Grant D, Chan W. 1995. Human Molecular Genetics. 4. no. 8: 1429-1433.

Laue L, Wu S, Kuido M, Bourdony C, Cutler G, Hsuer A, Chan W. 1996a. Molecular Endocrinology. 10. no.8: 987-97.

Laue L, Wu S, Kuido M, Hseuh A, Cutler G, Jelly D, Diamond F, Chan W. 1996b. Biochemical and Molecular Medicine. 58: 192-198.

Laurenza A, Sutkowski E, Seamon K. 1989. Trends in Pharmacologcal Sciences. 10:442-447.

Lechan R. 1987. Endocrinol. Metab. Clin. North America. 16: 475-501.

Lefkowitz R and Caron M. 1987. Recent Progress In Hormone Research. 43: 469-497.

Lefkowitz R, Hausdorff W, Caron M. 1990. Trends in Pharmacological Sciences. 11: 190-194.

Lei Z and Rao C. 1992. Trophoblast Research. 6: 213-224.

Lei Z, Rao C, Kornyei J, Licht P, Hiatt E. 1993. Endocrinology. 132: 2262-2270.

Lei Z, Resef E, Rao V. 1992. Journal of Clinical Endocrinology and Metabolism. 75: 651-659.

Lei Z, Toth P, Rao Ch, Pridham D. 1993. Journal of Clinical Endocrinology and Metabolism. 77: 863-872.

Liebhaber S, Russell J, Cash F, Eshleman S. 1992. In: Gene Regulation: Biology of Antisense RNA and DNA. Edited by Ecrikson R and Izant J. Published by Raven Press Ltd. New York.

Liu T, Pu H, Jackson G. 1992. Endocrinology. 131: 2711-2716

Liu J. 1996. Molecular and Cellular Endocrinology. 116: 1-29.

Liu J, Powell K, Sudhof T, Robinson P. 1994. Journal of Biological Chemistry. 269: 21043-21050.

Lloyd C and Ascoli M. 1983. Journal of Cell Biology. 96: 521-526.

Loke S, Stein C, Zhang X, Mori K, Nakanishi M, Subasinghe C, Cohen J, Neckers L. 1989. PNAS USA. 86: 3474-3478.

Lohse M, Lefkowitz R, Caron M, Benovic J. 1989. PNAS USA. 86: 3011-3015.

Lohse M. 1993. Biochimica and Biophysica Acta. 1179: 171-188.

Lohse M, Benovic J, Caron M, Lefkowitz, R. 1990. Journal of Biological Chemistry. 265: 3202-3209.

Loosfelt H, Misrahi M, Atger M, Salesse R, Tu Vu M, Hai-Luu Thi, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J, Milgrom M. 1989. Science. 245: 525-528.

Lopez-Ruiz M, Choi M, Rose M, West A, Cooke B. 1992. Endocrinology. 130: 1122-1130.

Lorenz W, Inglese J, Palczewski K, Onorato J, Caron M, Leftkowitz R. 1991. PNAS USA. 88: 8715-8719.

Lu D and Menon M. 1994. European Journal of Biochemistry. 222: 753-60.

Luborsky J and Behrman H. 1979. Biochemical and Biophysical Research Communications 90: 1407-1413.

Lustig K, Conklin B, Herzmark P, Taussig R, Bourne H. 1993. Journal of Biological Chemistry. 268: 13900-13905.

Malcolm D. 1992. Biochemical Society Transactions. 20: 745-746.

Malek D, Munch G, Palm D. 1993. FEBS Letters. 325: 215-219.

Marshall J and Kelsh R. 1986. New England Journal of Medicine. 315: 1459-1468.

Matsumoto M and Bremner W. 1989. Journal of Steroid Biochemistry. 33: 789-790.

Matzuk M, Kornmeier C, Whitfield G, Kourides I, Boime I. 1988. Molecular Endocrinology. 2: 95-100.

McFarland K, Sprengel R, Phillips H, Kohler M, Rosemblit N, Nikolics K, Segaloff D, Seeburg P. 1989. Science. 245: 494-499.

McIntosh R and McIntosh J. 1985. Endocrinology. 117: 169-179.

McNeil S, Lakey T, Tomlinson S. 1985. Cell Calcium. 6: 213-226

McPhail L. 1984. Science. 244: 622-625.

Meduri G, Vuhai-luuthi M, Jolivet A, Milgrom E. 1992. Endocrinology. 131: 366-373

Metsikko K and Rajaniemi H. 1981. Endocrinology. 109: 1399-1403.
Metsikko K and Rajaniemi H. 1984. Biochemical Journal. 224: 467-471.

Minegishi T, Nakamura K, Takakura Y, Miyamoto K, Hasegawa Y, Ibuki Y, Igarashi M. 1990. Biochemical and Biophysical Research Communications. 172. no.3: 1049-1054.

Minegishi T, Kusuda S, Dufau M. 1987. Journal of Biological Chemistry. 262: 17138-17143.

Minegishi T, Delgado C, Dufau M. 1989. PNAS USA. 86: 1470-1474.

Minshall J and Hunt T. 1986. Nucleic Acids Research. 14: 6433-6451.

Misrahi M, Hai M, Ghinea N, Loosfelt H, Meduri G, Atger M, Jolivet A, Gross B, Savouret J, Dessen P, Milgrom E. 1993. In: The Ovary. Edited by Adashi E, Leung P. Published by Raven Press Ltd. New York.

Mizutani T, Kato N, Hirota M, Sugiyama K, Murakami A, Shimotohno K. 1995. Biochemical and Biophysical Research Communications. 212: 906-911.

Moffett S, Mouillac B, Bonin H, Bouvier M. 1993. The EMBO Journal. 12: 349-356.

Morgan N. 1989. In : Cell Signalling. Published by the Open University Press. Milton Keynes, UK.

Mori H and Christensen A. 1980. Journal of Biological Chemistry. 84: 340-354.

Morrison D, O'Brien P, Pepperberg D. 1991. Journal of Biological Chemistry. 266: 20118-20123.

Morvan F, Rayner E, Imbach J, Chang D, Lown J. 1986. Nucleic Acids Research. 14: 5019-5035.

Moss J and Vaughan M, 1994. Current Topics in Cellular Regulation. 32: 49-72.

Mouillac B, Caron M, Bonin H, Dennis M, Bouvier M. 1992. Journal of Biological Chemistry. 267: 21733-21737.

Moyle W, Bernard M, Myers R, Marko O, Strader C. 1991. Journal of Biological Chemistry. 266: 10807-10812.

Muid R, Dale M, Davis P, Elliott L, Hill C, Kumar H, Lawton G, Twomey B, Wadsworth J, Wilkinson S, Nixon J. 1991 FEBS Letters. 293: 169-172.

Nagayama Y, Wadsworth H, Chazenbalk G, Russo D, Seto P, Rapoport B. 1991. PNAS USA. 88: 902-905.

Nagayama Y, Tanaka K, Hara T, Namba H, Yamashita S, Taniyama K, Niwa M. 1996. Journal of Biological Chemistry. 271: 10143-10148.

Newton A and Taylor S. 1995. FEBS Letters 1: 2-3

Nishizuka Y. 1980. Molecular Biology, Biochemistry and Biophysics. 32: 113-115.

Nishizuka Y. 1984. Nature. 308: 693-698.

Nishizuka Y. 1988. Nature 334: 661-665.

Nishizuka Y. 1992 Science. 258: 607-614.

Nussenveig D, Heinflink M, Gershengorn M. 1993. Journal of Biological Chemistry. 268: 2389-2392.

O'Dowd B, Hnatowich M, Caron M, Lefkowitz R, Bouvier M. 1989. Journal of Biological Chemistry. 264: 7564-7569.

Oldham S, Molloy C, Lipson L. 1984. Endocrinology. 114: 207-214.

Orr J, Keranen L, Newton A. 1992. Journal of Biological Chemistry. 267: 15263-15266.

Pabon J, Li X, Lei Z, Sanfilippo J, Yussman M, Rao C. 1996a. Journal of Clinical Endocrinology and Metabolism. 81: 2739-2400.

Pabon J, Bird J, Li X, Huang Z, Lei Z, Sanfilippo J, Yussman M, Rao C. 1996b. Journal of Clinical Endocrinology and Metabolism. 81: 2738-2741.

Palczewski K and Benovic J. 1991. Trends in Biochemical Science. 16: 387-391.

Pallikaros Z. 1995. PhD thesis

Pallikaros Z, Schulster D, Baldwin S, Helliwell R, Michael A, Cooke B. 1995. Molecular and Cellular Endocrinology. 114: 57-68.

Papadopoulos V, Berkovich A, Kreuger K, Costa E, Guidotti A. 1991. Endocrinology. 129: 1481-1488.

Papadopoulos V, Guarneri P, Kreuger K, Guidotti A, Costa E. 1992. PNAS USA. 89: 5113-5117.

Papadopoulos V, Mukhin A, Costa E, Kreuger K. 1990. Journal of Biological Chemistry. 265: 3772-3779.

Papac D, Thornburg K, Bullesbach E, Crouch R, Knapp D. 1992. Journal of Biological Chemistry. 267: 16889-16894.

Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mochel J, Dumont J, Vassart G. 1993. Nature. 365: 649-651.

Patthy L. 1987. FEBS Letters. 214: 1-7.

Payne A, Kelch R, Musich S, Halpern M. 1976. Journal of Clinical Endocrinology and Metabolism. 42: 1081-1087.

Payne A and Shaughnessy P. 1996. Chapter 12 In: The Leydig Cell. Edited by Payne A, Hardy M, Russell L. Published by Cache River Press.

Pedersen R and Brownie A. 1983. PNAS USA. 80:1882-1886.

Pedersen R and Brownie A. 1987. Science. 236: 188-190.

Pelosin F, Ricouart A, Sergheraert C, Benahmed M, Chambaz E. 1991. Molecular and Cellular Endocrinology. 75:149-155.

Pereira M, Segaloff D, Ascoli M. 1988. Endocrinology. 122: 2232-2239.

Persaud S, Jones P, Howell S. 1993. Molecular and Cellular Endocrinology. 94: 55-60.

Persaud S and Jones P. 1994. Journal of Molecular Endocrinology. 12: 127-130.

Pierce J and Parsons T. 1981. Annual Review of Biochemistry. 50: 465-495.

Platts E, Schulster D, Cooke B. 1988. Biochemical Journal. 253: 895-899.

Polark J and Van Noorden S. 1988. In: An introduction to immunocytochemistry: current techniques and problems. Published by the Oxford University Press.

Premont R, Inglese J, Lefkowitz R. 1995. FASEB Journal. 9: 175-182.

Probst W, Snyder L, Schulster D, Brosius J, Sealfon S. 1992. DNA Cell Biology. 11: 1-20.

Probst J and Skutella T. 1996. Biochemical and Biophysical Research Communications. 225: 861-868.

Puri R and Colman R. 1993. Analytical Biochemistry. 210: 50-57.

Quintana J, Hipkin W, Sanchez-Yague J, Ascoli M. 1994. Journal of Biological Chemistry. 269: 8772-8779.

Raeside J and Lobb D. 1984. Journal of Steroid Biochemistry. 20: 1267-1272.

Rajkumar K, Chedrese P, Ly H, Murphy B. 1991. Journal of Endocrinology. 130: 273-280.

Rana R and Hokin L. 1990. Physiological Reviews. 70: 115-164.

Ransnas L and Insel P. 1988. Journal of Biological Chemistry. 263: 17239-17242.

Rao Ch, Li X, Toth P, Lei Z, Cook V. 1993. Journal of Clinical Endocrinology and Metabolism. 77: 1706-1714.

Rebois R. 1982. Journal of Cell Biology. 94:70-76.

Rebois R, Omedeo-Sale F, Brady R, Fishman P. 1981. PNAS USA. 78: 2086-2089.

Rebois R and Fishman P. 1986. Endocrinology. 118: 2340-2348.

Redding T, Schally A, Arimura A, Matsuo H. 1972. Endocrinology. 90: 764-770.

Reshef E, Lei Z, Rao C, Pridham D, Chegini N, Luborsky J. 1990. Journal of Clinical Endocrinology and Metabolism. 70: 421-430.

Rheaume E, Tonon M, Smih F, Simard J, Desy L, Vaudry H, Pelletier G. 1990. Endocrinology. 127: 1986-1994.

Rhoades R and Pflanzer R. 1989. In: Human Physiology. Published by Sanders College Publishing.

Richards J. 1994. Endocrine Reviews. 15: 725-751.

Rodriguez M, Xie Y-B, Wang H, Collison K, Segaloff D. 1992. Molecular Endocrinology. 6: 327-336.

Rodriguez M and Segaloff D. 1990. Endocrinology. 127: 674-681.

Rommerts F and Cooke B. 1988. In: New Comprehensive Biochemistry: Hormones and their Actions, volume II. 163-180. Edited by Cooke B, King R, van der Molen H. Published by Elsevier, Amsterdam.

Rosemblitt N, Ascoli M, Segaloff D. 1988. Endocrinology. 123:2284-2290.

Rosenthal W, Gudermann T, Antaramian A, Seibold A, Lonergan M, Arthus M, Hendy G, Birnbaumer L, Bichet D, Birnbaumer M. 1993. Advances in Second Messenger and Phosphoprotein Research. 28: 47-55. Published by Raven Press Ltd.

Ruegg U and Burgess G. 1989. Trends in Pharmacological Sciences. 10: 218-220.

Ruiz-Gomez A and Mayor F. 1997. Journal of Biological Chemistry. 272:9601-9604.

Sacks D, Mazus B, Joyal J. 1995. Biochemical Journal. 312: 197-204.

Saez J. 1994. Endocrine Reviews. 15: 574-626.

Sairam M. 1983. In: Hormonal Proteins and Peptides. Edited by Li C. Published by Academic New York Press.

Sairam M and Bhargavi G. 1985. Science. 229: 65-67.

Salesse R, Dacheux F, Genty N, Garnier J. 1989. Biology of the Cell. 66: 297-306.

Salustri A, Yanagishita M, Underhill C, Laurent T, Hascall V. 1992. Developmental Biology. 151: 541-551.

Sambrook J, Fritsch E, Maniatis T. 1989. In: Molecular cloning: a laboratory manual. Published by Cold Sring Harbour Laboratory Press. USA.

Sanchez-Yague J, Rodriguez M, Segaloff D, Ascoli M. 1992. Journal of Biological Chemistry. 11: 7217-7220.

Sanchez-Yague J, Hipkin R, Ascoli M. 1993. Endocrinology. 132: 1007-1016.

Saribas A, Lustig K, Zhang X, Weisman G. 1993. Analytical Biochemistry. 209: 45-52.

Saxena B, Dattatreyamurty B, Ota H, Milkov V, Rathnam P. 1986. Biochemistry. 25: 7943-7950.

Seamon K, Padgett W, Daly J. 1981. PNAS USA. 78: 3363-3367.

Seamon K and Daly J. 1986. Advances in Cyclic Nucleotide Research. 20: 1-150.

Segaloff D and Ascoli M. 1993. Endocrine Reviews. 14. no. 3: 324-47.

Segaloff D, Sprengel R, Nikolics K, Ascoli M. 1990. Recent Progress in Hormone Research. 46: 261-301.

Sharpe R. 1976. Nature. 264: 644-646.

Sharpe R. 1977. Biochemical and Biophysical Research Communications. 76: 957-967.

Shenkar A, Laue L, Kosugi S, Merendino J, Minegishi T, Cutler G. 1993. Nature. 365: 652-654.

Shi Q, Lei Z, Rao Ch, Lin J. 1993. Endocrinology. 132: 1387-1395.

Shih M and Malbon C. 1994. PNAS USA. 91: 12193-12197.
Simon M, Strathmann M, Gautam N. 1991. Science. 252: 802-808.

Skinner M. 1991. Endocrine Reviews. 12: 45-77.

Sokka T, Hamalainen T, Huhtaniemi I. 1992. Endocrinology. 130: 1738-1740.

Spampinato S, Canossa M, Carboni L, Campana G, Leanza G, Ferri S. 1994. PNAS USA. 91: 8072-8076.

Stefan C and Blumer K. 1994. Molecular and Cellular Biology. 14: 3339-3349.

Stein C, Subasinghe C, Shinokuza K, Cohen J. 1988. Nucleic Acids Research. 16: 3209-3221.

Stein C and Cheng Y-C. 1993. Science 261: 1004-1012.

Steiner A, Parker C, Kipnis D. 1972. Journal of Biological Chemistry. 247: 1106-1113.

Stocco D. 1996. Chapter 11. In: The Leydig Cell. Edited by Payne A, Hardy M, Russell L. Published by Cache River Press.

Stocco D and Clark B. 1996. Biochem. Pharmacol. 51: 197-205.

Stocco D and Khan S. 1992. Molecular and Cellular Endocrinology. 84: 185-194.

Stojilkovic S and Catt K. 1992. Endocrine Reviews. 13: 256-280.

Strader C, Sigal I, Dixon R. 1989. FASEB Journal. 31825-31832.

Strott C. 1977. Journal of Biological Chemistry. 252: 464-470.

Stryer L. 1991. Journal of Biological Chemistry 266: 10711-10714.

Sullivan M and Cooke B. 1986. Biochemical Journal. 236: 45-51.

Sutherland E. 1972. Science. 177: 401-408.

Talmadge K, Vamvakopoulos N, Fiddes J. 1984. Nature. 307: 37-40.

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F. 1986. Biochemical and Biophysical Research Communications. 135: 397-402.

Tang W and Gilman A. 1992. Cell.70: 869-872.

Tao Y-X, Lei Z, Hofmann G, Rao C. 1995. Biology of Reproduction. 53:899-904.

Tao Y-X, Lei Z, Rao C. 1997. Life Sciences. 60:1297-1303.

Tapanainen J, Lapolt P, Perlas E, Hseueh A. 1993. Endocrinology. 133: 2875-2880.

Tapanainen J, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi I. 1997. Nature Genetics. 15: 205-206.

Tena-Sempere M, Chang F-P, Huhtaniemi I. 1994. Endocrinology. 135: 1018-1024.

Thorell J and Johanson B. 1971. Biochimica and Biophysica Acta. 251: 363-369.

Tidd D. 1992. Biochemical Society Transactions. 20: 746-749.

Toth P, Li X, Rao Ch, Lincoln S, Sanfilippo J, Spinnato II J, Yussman M. 1994. Journal of Clinical Endocrinology and Metabolism. 79: 307-315.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. 1991. Journal of Biological Chemistry. 266: 15771-15781.

Tsai-Morris C, Buczko E, Wang W, Xie X, Dufau M. 1991. Journal of Biological Chemistry. 266: 11355-11359.

Ts'o P. 1991. Antisense Research and Development. 1: 273-76.

Tsuruhara T, Dufau M, Cigorraga S, Catt K. 1977. Journal of Biological Chemistry. 252: 9002-9009.

Vahouny G, Chanderahan R, Noland B, ScallenT. 1985. Endocrine Research. 10: 473-505.

Van Koppen C and Nathanson N. 1991. Journal of Neurochemistry. 57: 1873-1877.

Van Sande J, Raspe E, perret J, Lejeune C, Maenhaut C, Vassart G Dumont J. 1990. Molecular and Cellular Endocrinology. 74: R1-R6.

Van Sande J, Allgeier A, Massart C, Czernilofsky A, Vassart G, Dumont J, Maenaut C. 1993. European Journal of Pharmacology. 247: 177-184.

Veldhuis J, Klase P, Demers L, Chafouleas J. 1984. Endocrinology. 114: 441-449.

Vuhai-Luuthi M, Misrahi, Houllier A, Jolivet A, Milgrom E. 1992. Biochemistry. 31: 8377-8383.

Wakelam M, Pettitt T, Kaur P, Briscoe C, Stewart A, Paul A, Paterson A, Cross M, Gardner S, Currie S, McNulty E, Plevin R, Cook S. 1993. Advances in Second Messenger and Phosphoprotein Research. 28: 73-79. Published by Raven Press, Ltd.

Wakita T and Wands J. 1994. Journal of Biological Chemistry. 269: 14205-14210.

Walder R and Walder J. 1988. PNAS USA. 85: 5011-5015.

Wang H and Ascoli M. 1990. Molecular Endocrinology. 4: 80-90.

Wang H, Ascoli M, Segaloff D. 1991. Endocrinology. 129: 133-138.

Wang H, Nelson S, Ascoli M, Segaloff D. 1992. Molecular Endocrinology. 6: 320-326.

Wang Z, Hipkin W, Ascoli M. 1996. Molecular Endocrinology. 10: 748-759.

Wang Z, Liu X, Ascoli M. 1997. Molecular Endocrinology. 11: 183-192.

Wei J, Wayman G, Storm D. 1996. Journal of Biological Chemistry. 271: 24231-24235.

Weinstein R, Kelch R, Jenner M, Kaplan S, Grumbach M. 1974. Journal Clinical Investigation. 53: 1-6.

West A. 1992. PhD thesis.

West A, Cooke B. 1991a. Endocrinology. 128: 363-370.

West A and Cooke B. 1991b. Molecular and Cellular Endocrinology. 79: R9-R14.

West A and Cooke B. 1992. Biochemical Society Transactions. 20: 320S.

West A, Lopez-Ruiz M, Cooke B. 1991. Molecular and Cellular Endocrinology. 77: R7-R11.

Weuesten J, Smals A, Hofman J, Kloppenborg P, Benraad T. 1987. Endocrinology. 120: 1913-1919.

Whitesell L, Rosolen A, Neckers L. 1991. Antisense Research and Development. 1: 343-350.

Whitnall M, Lee Y, Driscoll W, Strott C. 1990. Journal of Histochemistry and Cytochemistry. 38: 1607-1614.

Wilden U, Hall S, Kuhn H. 1986. PNAS USA. 83:1174-1178.

Wilkinson S, Parker P, Nixon J. 1993. Biochemical Journal. 294: 335-337.

Wimalasena J, Abel J, Wiebe J, Chen T. 1986. Journal of Biological Chemistry. 261: 9416-9420.

Woodruff T, Besecke L, Groome N, Draper L, Schwarz N, Weiss J. 1996. Endocrinology. 137: 5463-5467.

Woolf T. 1995. Antisense Research and Development. 5: 227-232.

Xie Y-B, Wang H, Segaloff D. 1990. Journal of Biological Chemistry. 265: 21411-21414.

Yakubov L, Deeva E, Zarytova V, Ivanova E, Ryte A, Yurchenko L, Vlassov V. 1989. PNAS USA. 86: 6454-6458.

Yanaihara T and Troen P. 1972. Journal of Clinical Endocrinology and Metabolism. 34: 783-792.

Yoshimasa T, Sibley D, Bouvier M, Lefkowitz R, Caron M. 1987. Nature. 327: 67-70.

Zhang F-P, Hamalainen T, Kaipia A, Pakarinen P, Huhtaniemi I. 1994. Endocrinology. 134: 2206-2213.

Zhang F-P, Rannikko A, Manna P, Fraser H, Huhtaniemi I. 1997. Endocrinology. 138: 2481-2490.

Zhao Q, Matson S, Herrera C, Fisher E, Yu H, Krieg A. 1993. Antisense Research and Development. 3: 53-66.

Zhu X, Gudermann T, Birnbaumer M, Birnbaumer L. 1993. Journal of Biological Chemistry. 268: 1723-1728.

Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L. 1994. Molecular Pharmacology. 46: 460-469.

Zhu H, Wang H, Ascoli M. 1995. Molecular Endocrinology. 9: 141-150.

Ziecik A, Stanchev P, Tilton J. 1986. Endocrinology. 119: 1159-1163.

Zirkin B, Awoniyl C, Griswold M, Russel L, Sharpe R. 1994. Journal of Andrology. 15: 273-276.