ISOLATION AND EXPRESSION OF
cDNA SEQUENCES ENCODING
OVINE FOLLICLE-STIMULATING HORMONE

A thesis submitted for the degree of
Doctor of Philosophy
by
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AMENDMENTS TO THESIS

(i) Page 7, paragraph 3 should read;

"Interestingly, disulphide bridges in the heterodimeric state are less susceptible to reduction by thioredoxin reductase than those of the dissociated subunits, indicating that the disulphide bridges of the free subunits are less accessible in the heterodimeric state (Holmgren and Morgan, 1976)."

(ii) Figure 1.4 (following page 27)

The "splice acceptor site" is incorrectly indicated and should appear as follows;

(iii) Figure 3.1 (following page 70)

The sequence numbers for the amino acid residues upon which the N-terminal degenerate probe were based are in reverse orientation and should read;

Published amino acid sequence*:

\[ +45 \quad \text{Asn} \quad \text{Ile} \quad \text{Gln} \quad \text{Lys} \quad \text{Thr} \quad \text{Cys} \quad +50 \]
Preface

The experiments reported in this thesis were performed by myself except where specified. This thesis complies with the University of Melbourne requirements in being less than 100,000 words in length.

Scientific publications resulting from the studies undertaken in this thesis include:


Acknowledgements

I would especially like to thank my supervisors, Dr. Tim Adams and Dr. Mal Brandon for their support and guidance during the course of these studies and for their advice in the preparation of this thesis. Their enthusiasm, knowledge and friendship has provided the corner-stone of my studies and further developed my love of science.

Thanks also to the many friends within The University of Melbourne Veterinary School who willingly provided technical support and answers to at times a curious novice.

I would also like to acknowledge and thank Ms. Emma James and Ms. Christine Kerr for their excellent typing of this thesis, Mr. Ken Snibson for preparing the photographic work and Mr. Graeme Smith for his computer guidance.

Finally, my special thanks go to my wife Jane-Ellen for her love and companionship, and for providing Millicent and me such a wonderful home.
Abstract

A cDNA library was synthesized from lamb pituitary poly(A)+ RNA for oligonucleotide screening and isolation of cDNA sequences encoding the ovine follicle-stimulating hormone β-subunit. Three cDNA clones were isolated and their nucleotide sequences established by dideoxy sequence analysis. The full length oFSH β-subunit cDNA sequence is 1889 base pairs in length and encodes a 129 amino acid prehormone. Comparison of the predicted amino acid sequence with that determined by amino acid sequence analysis has identified a conservative amino acid substitution Ala for Thr at position +49, a non-conservative substitution Arg for Ser at position +88, and a single C-terminal region amino acid exclusion at position +109. Comparison of the full length ovine follicle-stimulating hormone β-subunit cDNA sequence with the bovine follicle-stimulating hormone β-subunit shows an overall homology of 85% and a coding region homology of 95%.

A mammalian cell expression vector incorporating the SV40 enhancer, origin of replication, small t intron and polyadenylation sequences, and the heavy metal ion inducible human metallothionein IIA promoter, was constructed for the high level expression of the individual ovine follicle-stimulating hormone α- and β-subunits, and the assembly and secretion of ovine follicle-stimulating hormone heterodimers in in vitro cell culture. A variety of α- and β-subunit expression constructs incorporating full length or truncated wild-type cDNA sequences, or mutant β-subunit cDNA sequences, were transfected into the SV40 transformed African green monkey kidney cell line COS-1, and the Chinese hamster ovary cell line CHO-K1. Mammalian cell transfections were examined by Northern blot analysis and metabolic labelling of recombinant α- and β-subunit polypeptides. Expression levels for the recombinant heterodimer were
monitored using a porcine testis plasma membrane radioreceptor assay. This assay is specific for the heterodimeric form of follicle-stimulating hormone.

The results of the mammalian cell expression experiments demonstrated transcription and translation of the individual ovine follicle-stimulating hormone subunit cDNA sequences, and expression and secretion of an ovine follicle-stimulating hormone heterodimer having receptor binding activity in the porcine testis radioreceptor assay. Truncation of the β-subunit cDNA 3' non-coding region sequence resulted in increased levels of β-subunit mRNA and increased heterodimer expression. CHO cell co-transfections incorporating the wild-type α-subunit and the ovine growth hormone/follicle-stimulating hormone β-subunit hybrid cDNA expression constructs, showed the highest levels of expression at approximately 155 ng/10^6 cells/day.

Recombinant α- and β-subunit polypeptides were isolated from stably transfected high producing CHO cell lines for N-terminal amino acid sequence analysis and comparison with pituitary-derived follicle-stimulating hormone subunits by SDS-PAGE and isoelectric focussing. Activities of the recombinant and native follicle-stimulating hormone heterodimers were compared in the porcine testis radioreceptor assay and the in vitro Sertoli cell bioassay. The results of these experiments confirm that co-transfection of mammalian cell cultures with ovine follicle-stimulating hormone α- and β-subunit expression constructs results in the assembly and secretion of a biologically active recombinant heterodimer as determined by in vitro Sertoli cell bioassay. The recombinant ovine follicle-stimulating hormone heterodimer expressed by CHO cells has a higher molecular weight and is more acidic than the pituitary-derived hormone, suggesting a greater degree of sialylation of the recombinant product.
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CHAPTER ONE

LITERATURE REVIEW
1.1 GENERAL INTRODUCTION

Since the isolation of DNA by Miescher in 1869 and its association with inheritability in 1944 (Avery et al., 1944) the development of recombinant DNA techniques has greatly advanced our knowledge in almost every field of experimental biology and medicine.

Major advances including the isolation of restriction endonucleases, nucleic acid hybridisation, DNA sequencing and the engineering of recombinant plasmid and viral vector constructs has enabled the isolation, analysis and modification of genes of interest. DNA fragments and specifically designed DNA expression constructs can be introduced or transfected into appropriate host cell lines thereby enabling investigations into the identity of a gene, the DNA sequences controlling expression of the gene, the cellular processes involved in the biosynthesis and processing of the gene product, and the structural and functional analyses of the gene product.

Promoters and enhancers capable of driving high levels of heterologous gene transcription can be routinely engineered into recombinant expression vector constructs to enable high level expression of a desired gene product. Suitable host cell systems incorporating the expression constructs provide a means by which potentially unlimited supplies of both prokaryotic and eukaryotic recombinant proteins can be synthesised. Proteins previously available in only minute quantities, and often contaminated with other polypeptides, can now be synthesised and isolated from the genetically transformed host cell lines as highly purified bioactive equivalents of the native protein. Recombinant gene expression also provides an alternative source of non-infectious material for the isolation of proteins previously derived from blood or tissue.
Many recombinant proteins have pharmaceutical applications as growth or reproductive regulators, including growth hormone and follicle stimulating hormone, as immunoactive and neuroactive therapeutics, clotting and clot dissolving enzymes such as Factor IX and tissue plasminogen activator, or as immunising antigens. Other recombinant proteins have industrial applications as enzymes in the food and detergent industries and as specific ligands in purification processes.

This chapter reviews the biology and the physicochemical properties of ovine follicle-stimulating hormone, a member of the gonadotrophin family of glycoprotein hormones. The major applications and limitations of a variety of recombinant gene expression systems are then reviewed as possible expression systems for the production of recombinant ovine follicle-stimulating hormone. The focus of the review on expression systems centres on eukaryotic expression vectors and host cell systems suitable for the high level expression of mammalian glycoproteins in tissue culture.
1.2 FOLLICLE-STIMULATING HORMONE: A MEMBER OF THE GLYCOPROTEIN HORMONE FAMILY

1.2.1 Introduction

Follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein consisting of two non-covalently bound subunits, an α-subunit and a β-subunit. The α-subunit is common to all members of the glycoprotein hormone family which includes FSH, luteinizing hormone (LH), thyroid stimulating hormone (TSH) and chorionic gonadotrophin (CG) (for review see Pierce and Parsons, 1981). Each hormone has a unique β-subunit which confers the biological specificity of the heterodimer. FSH, LH and CG have critical roles in the development and control of the reproductive system and are collectively referred to as gonadotrophins, while TSH, which has a critical role in regulating growth and body metabolism, is not considered a reproductive hormone.

The gonadotrophins FSH and LH are ubiquitous to all mammals and are synthesised by a specialised, differentiated subset of cells of the anterior pituitary gland known as the gonadotrophes. There is some conjecture regarding the heterogeneity of hormone expression within individual gonadotrophes. Early immunocytochemical examinations of rat (Childs et al., 1983; Dada et al., 1983) and frog (Gracia-Navarra and Licht, 1987) pituitary gonadotrophes suggested a mixture of hormone specific and dual FSH/LH producing cells. Similar studies of juvenile female rats (Campbell et al., 1987), and more recently bovine gonadotrophes using electronmicroscopy and immunogold staining techniques (Bastings et al., 1990), has identified only LH or FSH containing cells.

CG is a product of the syncytiotrophoblast cells of the placenta (Allen et al., 1973) and has only been described for some members of the primate and equidae families, including humans, and horses. A reported CG-like placental glycoprotein in the rat (Blank and Dufau, 1983) could not be confirmed by Northern blot analysis.
of placental RNA or low stringency Southern blot analysis of genomic DNA with rat LH β-subunit or α-subunit complementary DNA (cDNA) probes (Carr and Chin, 1985). The rat LH β-subunit genomic sequence was selected as a cross hybridising probe as both human and equine CG β-subunits show strong amino acid sequence similarities with the LH β-subunit of each species (see Section 1.2.3). Cross hybridising human and equine CG β-subunit probes were not investigated. Further evidence of pituitary restricted α-subunit expression in mice has been provided in experiments showing that expression and regulation of the pituitary- and placental-specific human α-subunit gene is restricted to the pituitary in transgenic mice (Fox and Solter, 1988). Bovine and ovine pregnancy specific proteins have been isolated from placental tissues (Beckers et al., 1988a; Zoli et al., 1990) although only the bovine glycoprotein described by Beckers and co-workers (1988a) has been described as a CG like glycoprotein on the basis of its parallel dose response curve in the bovine LH radioreceptor assay (RRA, Beckers et al., 1988b). A subunit structure has not been demonstrated for these proteins.

1.2.2 Ontogeny and function
Radioimmunoassays (RIA) of foetal ovine sera have detected FSH and LH as early as day 59 of gestation (Sklar et al., 1981). The normal gestation period for sheep is from 145-155 days. RNAse protection studies have further confirmed oFSH β-subunit gene transcription in the foetal pituitary as early as day 80 of gestation (Bello, unpublished results). Similarly, gonadotrophin expression has been demonstrated at as early as 10 weeks in the foetal human pituitary using a cross-reacting hCG antiserum and immunocytochemical techniques (Baker and Jaffe, 1975).

An initial rise in foetal gonadotrophin levels to a mid-gestational peak stimulates a period of active gonadal growth and development (Dorrington and Armstrong, 1979). The release of pituitary gonadotrophins is then reduced in the third
trimester by negative feedback effects of the developing hypothalamus and central
nervous system inhibitory systems (Sklar et al., 1981). Gonadotrophin levels
remain low in postnatal life until the onset of sexual maturation (Croze and
Franchimont, 1982) and the commencement of cyclic fluctuations associated with
the oestrus cycle and environmental stimuli. Seasonal breeding animals display
periods of anoestrus in response to changes in day length. The changes in day
length result in dramatic changes in the levels of FSH and LH secretion (for review
see Lincoln and Short, 1980).

In the sexually mature animal, FSH and LH interact to play a pivotal role in the
production and secretion of gonadal steroids and polypeptides, and the associated
production and release of gametes (Dorrington and Armstrong, 1979). FSH
stimulates the growth of the ovarian follicles and associated oocyte, and
spermatogenesis in the testis. LH stimulates spermatogenesis in the testis and
ovulation and luteinisation of ovarian follicles. Neither gonadotrophin mediates its
action by direct binding to target germ cells. Instead, both FSH and LH mediate
their actions indirectly via somatic cell binding within the gonads. FSH binds
exclusively to ovarian granulosa cells (Zeleznik et al., 1974) and testicular Sertoli
cells (Dorrington et al., 1975), while LH binds to ovarian thecal (Fortune and
Armstrong, 1977) and interstitial (Savard et al., 1965) cells, and testicular Leydig
cells (Catt et al., 1974). High affinity FSH receptors have been isolated from rat
Sertoli cells (Reichert and Dattatreymury, 1989), and the full length cDNA
sequence coding for the rat FSH receptor has been established (Sprengel et al.,
1990). Similarly, the cDNA sequence for the rat LH receptor has also been
established (McFarland et al., 1989). Nucleotide sequence analyses of both the rat
LH (McFarland et al., 1989) and rat FSH (Sprengel et al., 1990) receptors has
suggested a new mechanism for gonadotrophin-evoked receptor activation in
combining features of the leucine-rich proteoglycan and G-protein-coupled
receptor families.
A variety of steroids, proteins and carbohydrates, including progesterone, oestrogen, inhibin and Mullerian duct inhibiting factor, are secreted by the granulosa and Sertoli cells in response to the various hormonal stimuli (see Table 1.1) to create the environment controlling germ cell differentiation. The complex cellular interactions involved in germ cell differentiation have not been fully established although the folliculogenic activity of FSH in the stimulation or recruitment of small follicles has been demonstrated at the level of DNA synthesis (Roy and Greenwald, 1988). While FSH stimulated [3H] thymidine incorporation in all follicular stages, LH was only active following the appearance of definitive thecal cells.

CG secretion is restricted to the placenta and therefore pregnancy. The predominant LH-like activity of the hCG and eCG is essential for maintenance of the corpus luteum and early pregnancy (Heap, 1972; Ginther, 1979) and is consistent with the strong amino acid sequence similarities each hormone shares with the LH of the same species (see Figure 1.1). Both human and equine CG, and eLH, have strong FSH-like bioactivities in non-related species (Combarnous et al., 1984). While the LH-like activity of hCG is regularly utilised in veterinary and medical practices, it is the FSH-like activity of eCG that has made this hormone the most widely used reproductive stimulant in veterinary medicine.

1.2.3 Structure and evolutionary relationships
The primary structure of the oLH α-subunit (Sairam et al., 1972) is presented in Figure 1.2. The polypeptide sequence is 96 amino acids in length with two asparagine (Asn)-linked oligosaccharide side-chains attached to Asn residues 56 and 82. The oFSH α-subunit sequence was later confirmed as identical to that of the oLH α-subunit (Sairam, 1981). The oFSH β-subunit (see Figure 1.1) is 111 amino acids in length with two Asn-linked oligosaccharide side chains at positions 6 and 23 (Sairam et al., 1981). Molecular weight estimations for the oFSH α- and
Table 1.1
Products secreted by cultured Sertoli cells and granulosa cells under various hormonal stimuli

<table>
<thead>
<tr>
<th>Product</th>
<th>Immature Sertoli cell</th>
<th>Granulosa cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steroids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>-</td>
<td>+ (FSH + Tt)</td>
</tr>
<tr>
<td>Androgens</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estrogens</td>
<td>+ (FSH)</td>
<td>+ (FSH)</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgen-binding protein</td>
<td>+ (FSH, Tt)</td>
<td>-</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>+ (FSH)</td>
<td>+ (FSH, LH)</td>
</tr>
<tr>
<td>Inhibin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>+</td>
<td>+ (FSH)</td>
</tr>
<tr>
<td>Mullerian duct inhibiting factor</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Carbohydrate</strong></td>
<td></td>
<td></td>
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<tr>
<td>Inositol</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- Non detectable.
+ Detectable.
( ) Hormonal requirements.
Tt Testosterone

*[Adapted from Dorrington and Armstrong (1979)]*
**Figure 1.1**
Glycoprotein hormone β-subunit amino acid sequences for rat, human, porcine, bovine and ovine FSH, equine and human CG, and ovine, bovine and porcine LH.
Figure 1.2
Glycoprotein hormone α-subunit amino acid sequences for the ovine, bovine, rat and human polypeptides.

<table>
<thead>
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<th>20</th>
<th>15</th>
<th>10</th>
<th>5</th>
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<th>5</th>
<th>10</th>
<th>15</th>
<th>17</th>
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<tbody>
<tr>
<td>Ovine (1)</td>
<td>Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Leu Ala Ile Leu Ser Leu Phe Leu Gin Ile Leu His Ser Phe Pro Asp Gly Glu Phe Thr Met Gin Gly Cys Pro Glu Cys Lys Leu Lys</td>
<td>Val</td>
<td>Thr</td>
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<td>Bovine (2)</td>
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<tr>
<td>Rat (3)</td>
<td>Cys Arg Val Val Met Met Val His Leu Asp Leu Ile Ile</td>
<td>Ile Phe Val Thr Val His Val Ala Val — — — Asp Thr Gin</td>
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<td>Human (4)</td>
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<td>Ovine</td>
<td>Lys Glu Aan Lys Tyr Phe Ser Lys Pro Asp Ala Pro Ile Tyr Gin Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro Ala Arg Ser Lys Tyr Thr Met Leu Val Pro Lys Aan Ile</td>
<td>Ala</td>
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<tr>
<td>Rat</td>
<td>Leu Gly</td>
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<tr>
<td>Human</td>
<td>Gin Pro Phe Gin Gly Leu</td>
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<th>80</th>
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<th>90</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine</td>
<td>Thr Ser Glu Ala Thr Cys Cys Val Ala Lys Ala Phe Thr Lys Ala Thr Val Met Gly Aan Val Arg Glu Aan His Thr Glu Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser</td>
<td></td>
<td></td>
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<td>Bovine</td>
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<tr>
<td>Rat</td>
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</tr>
<tr>
<td>Human</td>
<td>Ser</td>
<td>Ser Tyr Aan Arg Val</td>
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</tbody>
</table>

- Conserved cysteine residues
- Absence of corresponding amino acid

(1) Bello et al., (1990)
(2) Erwin et al., (1983)
(3) Godine et al., (1982)
(4) Fiddes and Goodman, (1979)
β-subunits, and the native heterodimer, have been established by ultracentrifugation as 15.5 kD (kilodaltons), 18.5 kD and 33 kD respectively (Grimek and McShan, 1974).

Amino acid sequence similarities evident between α- and β-subunits have led to speculation of a common ancestral gene for both subunits (Pierce and Parson, 1981). More recently, differences in the intron-exon configurations of the two subunit genes has discounted this possibility (Fiddes and Talmadge, 1984). Strong evidence does exist, however, for a common FSH, LH, TSH and CG β-subunit ancestral gene, and a separate α-subunit gene. The most striking feature evident in all β-subunit amino acid sequences, irrespective of hormone type or species origin, is the conservation in cysteine residue number and position. Similarly the number and positioning of cysteine residues in all the reported α-subunit sequences is identical. The β-subunits contain 12 cysteine residues while the α-subunits contain 10.

The conservation in number and position of cysteine residues, and the ability of subunits from different species to recombine to form active heterodimers (Pierce and Parsons, 1981), strongly indicates a conservation of intrachain disulphide bridge assignments. The intrachain disulphide assignments reported thus far have been reviewed by Ryan and co-workers (1987), and the assignments α11-35, α14-36, β93-100 and β26-110 were concluded as correct. Interestingly, disulphide bridges in the heterodimeric state are less susceptible to reduction by thioredoxin reductase than those of the dissociated subunits, indicating that the disulphide bridges of the free subunits are in opposition to each other in the heterodimeric state (Holmgren and Morgan, 1976).

The amino acid sequence cysteine-alanine-glycine-tyrosine-cysteine, commonly referred to as the CAGY sequence, is fully conserved in all gonadotrophin β-
subunits except for rat FSH (Maurer, 1987). The rat sequence substitutes alanine for glycine, retaining the hydrophobic nature of the sequence. The CAGY sequence may have a role in receptor recognition (Mullin et al., 1976) or in subunit association (Hayashizaki et al., 1989). Experiments have shown that synthetic peptides homologous to this region can block subunit association in vitro (Ryan et al., 1987) adding support to a possible role in subunit association. Site directed mutagenesis of the hCG ß-subunit CAGY region cDNA sequence has identified the glycine residue as being particularly important in the synthesis of hCG ß-subunits and hCG heterodimers using in vitro transcription, and subsequent in vivo translation in Xenopus laevis oocytes (Azuma, 1990).

The most recent divergence in the gonadotrophin ß-subunit gene family is the emergence of the CG ß-subunit gene from the ancestral LH ß-subunit gene. Fiddes and Goodman (1980) first demonstrated the evolution of the hCG ß-subunit by showing that a single base pair deletion in the LH ß-subunit termination codon enables a read through event in which 3' untranslated LH sequence becomes CG coding sequence. A similar C-terminal amino acid extension is associated with the eCG ß-subunit (Sugino et al., 1987) and a similar evolutionary process has been proposed for the origin of this gene (Bousfield et al., 1987). Interestingly, the eLH (Bousfield et al., 1987) and eCG (Sugino et al., 1987) ß-subunit amino acid sequences are identical. While the likelihood of a single eLH/CG ß-subunit gene has not been disproven, a divergence or duplication in the eLH gene to a separate CG gene may represent an even more recent evolutionary event.

The functional significance, if any, of the CG C-terminal amino acid extension and the four associated O-linked oligosaccharides has not been clearly established. Expression of recombinant hCG in O-glycosylation deficient Chinese hamster ovary (CHO) cells (Matzuk et al., 1987), and chemical cleavage of the C-terminal extension (Bousfield et al., 1989) does not effect subunit association or receptor
binding. Free O-glycosylation deficient hCG β-subunit polypeptides, like the native β-subunit polypeptides, are rapidly secreted from transfected CHO cells. Some evidence has been presented to suggest the C-terminal extension may have a role in reducing the rate of hormone-receptor complex internalisation (Niswender et al., 1985).

1.2.4 Microheterogeneity and the functional significance of gonadotrophin glycosylation

A characteristic of the glycoprotein hormones is their extensive microheterogeneity. Indeed, early biological and physicochemical characterisations of the glycoprotein hormones describe them in terms of a composite, or weighed average, for a heterologous pool of isoforms. The multiple forms or microheterogeneity displayed by each member of the glycoprotein hormone family result from differences in the number and type of associated oligosaccharide side chains (Chappel et al., 1982; Matzuk et al., 1987). More recently, the characterisation of these glycoproteins has focussed on the variety of isoforms, the differences in their biological and physicochemical properties, and the mechanisms by which the non-random distribution of isoforms is generated (for review see Rademacher et al., 1988).

Glycosylation in higher eukaryotes involves a complex array of compartmentalised biochemical pathways (see Figure 1.3). Oligosaccharides are covalently bound to the polypeptide backbone as either N-linked (nitrogen-linked) or O-linked (oxygen-linked) side chains. N-linked or Asn-linked oligosaccharides are synthesised from high mannose precursors which are transferred en bloc from lipid-linked oligosaccharide precursors (Hunt and Summers, 1976; Tabas et al., 1978). The Asn residues within the amino acid sequences Asn-x-threonine (Thr) or Asn-x-serine (Ser), where x can be any amino acid except possibly proline and aspartic acid, are the targets for covalent attachment, although only about one third of the potential Asn-x-Thr/Ser sites in proteins are glycosylated (for review see Kornfeld
Figure 1.3
Pathway of oligosaccharide processing.

Abbreviations:
RER, rough endoplasmic reticulum
(cis), (medial), (trans), compartments of golgi complex
Trans, transferase
[] , class of oligosaccharide side chain
Sugars: ▲, glucose (glic); ◊, mannose (man); ●, N-acetylglucosamine (GlcNAc); ▲, fucose; ●, galactose (gal); ◊, sialic acid (sial)

Sites at which tunicamycin blocks glycosylation, and at which the oligosaccharide side chains become resistant to endoglycosidase H (Endo H) are indicated.

* Adapted from Dormer and Kaufman, (1990)
and Kornfeld, 1985). Following attachment of the high mannose precursor in the endoplasmic reticulum (ER), a series of glycosidases and glycotransferases within the ER and the Golgi complex (GC) modify the structure and composition of the side chain to produce the resultant complex-type oligosaccharides found in association with gonadotrophin hormones. The GC is also the site of O-linked oligosaccharide attachment (Abeijon and Hirschberg, 1987) and oligosaccharide and tyrosine sulphation (Baeuerle and Huttner, 1987). O-linked oligosaccharides are attached to Ser or Thr residues. Consensus sequences for O-linked glycosylation have not been established although secondary structure of the polypeptide backbone is probably important (Sadler, 1984).

The nature and complexity of an oligosaccharide side chain occupying a given glycosylation site depends on the amino acid target sequence, the secondary structure of the polypeptide and resultant accessibility of the target sequence (Green, 1982; Smith and Baenziger, 1988), the cell type expressing the glycoprotein (Rademacher et al., 1988) and the extracellular environment of the cell at the time of synthesis (Goochee and Monica, 1990). For example, differences in accessibility of the bovine LH and FSH α-subunit glycosylation sites, as modulated by the associated β-subunits, result in differential α-subunit glycosylation patterns (Smith and Baenziger, 1988). Cell type specific glycosylation patterns are clearly evident when comparing pituitary-derived eLH and placental-derived eCG. These gonadotrophins share identical amino acid sequences (Bousfield et al., 1987) and yet the carbohydrate contents for eLH and eCG have been established as 24% and 45%, respectively (Landefeld and McShan, 1974; Moore and Ward, 1980).

Gonadotrophin glycosylation has also been shown to vary throughout the human life cycle (Wide, 1987) and the oestrous cycle of the rat (Ulloa-Aguirre et al., 1988). Changes in testosterone levels can affect sialylation of FSH in vitro (Kennedy and
Chappel, 1988) and may explain the reduction in acidic forms of FSH following castration (Robertson et al., 1982). Other steroids, including oestrogen (Weick, 1977), and gonadotrophin-releasing hormone (GnRH, Lui et al., 1976; Miller et al., 1983) may also affect glycosylation.

In reviewing gonadotrophin glycosylation and function, Wilson and co-workers (1990) concluded that, in general, basic isoforms of the gonadotrophins have a greater potency in in vitro assays, but a shorter half-life in vivo, while acidic isoforms have a longer circulatory time and thus are more active in in vivo estimations. In keeping with this observation, two recombinant human FSH products secreted by glycosylation-defective mutant CHO cell lines and deficient in terminal sialic acid residues, have been shown to be as active as wild-type CHO cell recombinant FSH, and pituitary FSH, in the in vitro granulosa cell aromatase assay, but inactive in the in vivo granulosa cell aromatase assay (Galway et al., 1990). The lack of in vivo bioactivity associated with the sialic acid deficient FSH variants correlates with their rapid clearance from serum. The half-life or circulatory clearance rate of glycoproteins is primarily modulated by receptor recognition of specific oligosaccharide side chains, the most well characterised of these receptors being the liver asialoglycoprotein receptor (Ashwell and Harford, 1982). This receptor recognises non-sialylated oligosaccharides and is responsible for the elimination of desialylated glycoproteins from the circulation.

The structure (Green and Baenziger, 1988a) and distribution (Green and Baenziger, 1988b) of oligosaccharide side chains on the ovine, bovine and human pituitary glycoprotein hormones has been reported. Despite very similar amino acid sequences (see Figures 1.1 and 1.2), ovine, bovine and human FSH each display dissimilar relative distributions of sialylated and sulphated oligosaccharide side chains. The ratios of sialylated and sulphated oligosaccharides for ovine,
bovine and human FSHs are 0.95, 4.31 and 12.58 respectively. The range of oligosaccharide side chains established for each FSH preparation also includes at least two species specific sialylated oligosaccharide structures (Green and Baenziger, 1988a; 1988b).

The structure of the oligosaccharides associated with recombinant hFSH expressed in CHO cells (Hard et al., 1990) have also been established. It is interesting to note that this form of recombinant hFSH is more highly sialylated than its native counterpart, and the suggestion has been made that this may lead to a longer circulatory half-life.

As well as affecting clearance rates, oligosaccharides of the glycoprotein hormone family also affect subunit association (Merz, 1988), intracellular degradation (Kaetzel et al., 1989), secretion (Matzuk and Boime, 1988a; 1988b), solubility (Sairam, 1983), receptor binding (Sairam and Bhargavi, 1985), signal transduction (Keene et al., 1989b; Matzuk et al., 1989) and possibly the rate of receptor internalisation (Niswender et al., 1985). Early experiments using chemical and enzymatic deglycosylation procedures established that while N-linked oligosaccharides were not essential for gonadotrophin receptor binding, they were essential for receptor activation and signal transduction (Sairam, 1983). These studies also revealed that deglycosylated gonadotrophins had higher receptor binding affinities than their glycosylated counterparts. By recombining native and deglycosylated oFSH and oLH subunits, Sairam and Bhargavi (1985) were able to identify the α-subunit associated oligosaccharides as essential in receptor activation. More recently, site-directed mutagenesis of individual polypeptide glycosylation sites has established the oligosaccharide moiety attached to Asn-52 of the hCG α-subunit as critical for signal transduction (Matzuk et al., 1989).
Receptor studies examining hCG binding to porcine granulosa cell receptors have revealed different receptor binding site preferences for the native glycosylated, and chemically deglycosylated, forms of hCG (Ji and Ji, 1990). Such differences may provide a structural basis for the previously described native agonist and deglycosylated antagonist forms of gonadotrophin hormones (Sairam, 1983). Considering the range of biological functions associated with the oligosaccharide side chains, it is not surprising that deglycosylated gonadotrophins are biologically inactive, and that on occasions, the polypeptide component of the hormones has been described as "just a platform for presenting different sugars" (cited; Knight, 1989).

1.2.5 FSH assays: applications and limitations

Three assay systems have been described and modified for the quantitation of FSH in vitro and in vivo. These are: (i) the rat ovarian weight hCG augmentation (Steelman and Pohley, 1953) and granulosa cell aromatase (Galway et al., 1990) in vivo bioassays, (ii) the granulosa cell (Van Damme et al., 1979; Jia and Hsueh, 1986) and Sertoli cell (Ritzen et al., 1982; Padmanabhan et al., 1987) in vitro bioassays, and (iii) in vitro radio ligand assays including the porcine testis plasma membrane FSH RRA (Maghuin-Rogister et al., 1978) and a monoclonal antibody based RIA (Benikrane et al., 1988).

The experiments already reviewed in this chapter, comparing various glycosylated and non-glycosylated gonadotrophin hormones in a variety of assay systems, have shown that results established in one assay system will not necessarily be reflected in another assay system. For example, deglycosylated oFSH, which is considerably more active than native glycosylated FSH in the in vitro RRA, is totally inactive in in vitro bioassays (Sairam, 1983; Sairam and Bhargavi, 1985). Partially glycosylated hFSH, synthesised in glycosylation defective mutant CHO cell lines and deficient in terminal sialic acid, is equally as active as wild type recombinant
FSH and native FSH in \textit{in vitro} bioassays and yet inactive in \textit{in vivo} bioassays (Galway \textit{et al.}, 1990). Ultimately, the only suitable assay system for accurately establishing the specific \textit{in vivo} bioactivity of an FSH preparation, which usually will contain a variety of active and less active FSH isoforms, is an \textit{in vivo} bioassay in the target species.

1.2.6 \textbf{Regulation of gonadotrophin subunit expression}

The co-ordinated regulation of pituitary gonadotrophin expression and secretion is essential for the maturation and maintenance of mammalian reproductive capability and therefore the preservation of these species. Hypothalamic GnRH, gonadal steroids including oestrogen, testosterone and progesterone, and gonadal peptides including activin, inhibin and follistatin, each have a role in regulating FSH and LH biosynthesis and secretion.

\textbf{Steroid hormones and GnRH:} The negative feedback effect of oestrogen on both pituitary and serum FSH and LH concentrations was suggested in preliminary experiments showing a rise in gonadotrophin levels following castration (Wise and Ratner, 1980). A similar rise in ovariectomised (OVX) ewes has been normalised by chronic administration of physiological doses of estradiol (E2, Landefeld \textit{et al.}, 1984). Experiments examining gonadotrophin mRNA levels following ovariectomy and subsequent E2 administration in the rat (Gharib \textit{et al.}, 1986; 1987), and following E2 addition to cultured ovine pituitary cells (Hall and Miller, 1986), have confirmed these results.

Considerable evidence exists to suggest the negative feedback effects of oestrogen on gonadotrophin subunit mRNA synthesis are mediated via the hypothalamus. Transcription rates for rat LH and FSH \(\beta\)-subunits, and the common \(\alpha\)-subunit, are negatively regulated by E2 administration \textit{in vivo} (Shupnik \textit{et al.}, 1988), and yet unaffected or even up regulated, as in the case of the LH \(\beta\)-subunit,
by E2 administration to isolated hemipituitaries in vitro (Shupnik et al., 1989). In situ hybridisation experiments examining the effect of gonadectomy and steroid administration on hypothalamic neurone pro-GnRH mRNA levels have provided further evidence for a hypothalamically-mediated negative E2 feedback effect. Castration, and to a lesser extent ovariectomy, increased the number of pro-GnRH mRNA hybridisation signals, while E2 and dihydrotestosterone administration completely blocked this increase (Toranzo et al., 1989). Furthermore, elevated gonadotrophin subunit mRNA levels in OVX ewes have been reduced by hypothalamo-pituitary disconnection (HPD) indicating a stimulatory role for GnRH (Hamernik et al., 1986; Mercer et al., 1989). Pulsatile GnRH administration restored FSH ß- and α-subunit levels in OVX/HPD ewes to those of OVX ewes, while LHβ-subunit levels increased over those of saline controls but were not as high as those observed in OVX ewes (Hamernik and Nett, 1988).

It is also interesting to note a positive E2 feedback effect has also been demonstrated in the anoestrous ewe as a three-fold increase in α-subunit mRNA levels during an E2-induced LH surge (Landefeld et al., 1984). This finding is also supported by a further observation that OVX/HPD ewes receiving GnRH and E2 display higher pituitary α-subunit transcript levels than OVX/HPD ewes receiving GnRH alone (Mercer et al., 1989). OVX/HPD ewes receiving only E2 had the lowest levels of α-subunit mRNA indicating α-subunit mRNA is positively regulated by E2, via a direct pituitary action in the presence of GnRH. The previously described E2-induced suppression of pituitary α-subunit mRNA levels in the OVX ewe may have resulted from high, non-physiological levels of E2 administration and a corresponding depletion in GnRH (Nilson et al., 1983). The direct pituitary action of E2 in elevating α-subunit mRNA levels and also suppressing FSH β-subunit mRNA (Mercer et al., 1989) is unique to the ovine and has not been demonstrated for either primates or rodents. These changes in the steady state mRNA levels can result from changes in the rate of mRNA synthesis or mRNA
stability. Nuclear run-off assays described by Shupnik and co-workers (1988) have shown higher rates of gene transcription for LHβ- and α-subunit genes in ovariectomised rats.

Variations in GnRH pulse amplitude and frequency can result in differential regulation of subunit transcription, and α-subunit and heterodimer secretion (Haisenleder et al., 1988; Mercer and Clarke, 1988). A dispersed rat pituitary cell diffusion system, with the advantage of in vitro isolation and controlled signal input, has been used to examine the effects of continuous and pulsatile GnRH infusion on gonadotrophin secretion and mRNA synthesis (Weiss et al., 1990). Results showed that the isolated cell cultures were responsive to each pulse of GnRH but only elicited a single brief release of FSH and LH in response to continuous GnRH infusion. FSH β-subunit mRNA levels in cells under continuous GnRH application were reduced by 52% compared with saline controls, and increased by over 400% in cells receiving pulsatile GnRH application. FSH α-subunit levels increased 160-170% with both regimes while LH β-mRNA levels were unchanged. The divergence in mRNA transcript level responses provides a potential mechanism by which differential regulation of the pituitary gonadotrophins could be achieved.

Progesterone inhibits LH secretion (Hauger et al., 1977) and basal FSH biosynthesis (Batra and Miller, 1985) in the ewe. Rapid sampling techniques utilised in in vivo experiments with rhesus monkeys have provided evidence indicating that the inhibitory effect of progesterone on LH secretion does not affect basal LH secretion but rather reduces LH pulse frequency (Van Vugt et al., 1984). Several studies in rats, and in isolated rat pituitaries, have shown that testosterone has a negative regulatory effect, mediated via the hypothalamus, on LHβ- and α-subunit mRNA synthesis, and a positive regulatory effect on FSH β-subunit mRNA levels at the level of the pituitary (Gharib et al., 1990).
**Gonadal peptides:** Many attempts to purify inhibin, a specific non-steroidal inhibitor of FSH secretion (McCallagh, 1932) were initially unsuccessful. Inhibin-like activity had been reported in various gonadal extracts (Keogh *et al.*, 1976; de Jong and Sharp, 1976) prior to the eventual isolation of two forms of inhibin from porcine follicular fluid (Ling *et al.*, 1985). Both forms of inhibin consist of two dissimilar peptide subunits, the α- and β-subunits, linked by one or more disulphide bridges. Both forms incorporate a common α-subunit of approximately 18 k, and one of two β-subunits, βA and βB, of approximately 14 k. The different β-subunits were identified by N-terminal amino acid sequencing (Ling *et al.*, 1985) and later confirmed by cDNA sequence analysis (Mason *et al.*, 1985).

Early reports described an inhibin-induced suppression in the secretion of FSH, and to a lesser extent LH, both *in vitro* (Steinberger and Steinberger, 1976) and *in vivo* (Findlay *et al.*, 1985). More recent experiments in OVX/HPD ewes have shown that inhibin induces a very rapid and specific lowering of FSH β-subunit mRNA levels by direct action at the level of the pituitary gland (Mercer *et al.*, 1987). No effect was evident for LH β-subunit, α-subunit or prolactin mRNA levels.

Two inhibin-related peptides, activin and FSH releasing protein (FRP), having opposite biological effects to inhibin, have been isolated and characterised (Ling *et al.*, 1986; Vale *et al.*, 1986). Both reports confirmed the somewhat surprising discovery that each of the peptides described is in fact a dimer composed of two inhibin β-subunits. Activin is reported to be a βA/βB heterodimer while FRP is a βA homodimer. Both activin and FRP stimulate FSH β-mRNA synthesis and FSH heterodimer secretion, and have no effect on LH or prolactin mRNA synthesis (Tsonis and Sharp, 1986).

A second unrelated inhibin-like monomeric protein follistatin, which specifically inhibits basal level FSH secretion in rat pituitary cell cultures, has also been
isolated from porcine follicular fluid (Ueno et al., 1987). The 35 kD follistatin amino acid sequence has no sequence homology with the previously described inhibin sequences.

The time course and dose response effects of purified porcine inhibin, recombinant human activin and porcine follistatin in primary rat pituitary cell monolayers have been investigated (Carroll et al., 1989). Inhibin reduced FSH β-subunit mRNA transcripts to below detectable levels within 4 hours of application although FSH secretion was not significantly suppressed until 24 hours. Inhibin did not affect LH β-subunit mRNA levels, although at higher doses it did reduce α-subunit mRNA levels. Activin caused a dose-dependent and parallel increase in FSH β-subunit mRNA levels and FSH secretion, while LH β- and α-subunit mRNA levels remained unchanged. Similarly, no changes were evident in LH β- and α-subunit mRNA levels 24 hours after follistatin administration. FSH β-subunit mRNA transcripts were undetectable 24 hours post follistatin administration.

The mechanism/s by which the gonadal peptides regulate gonadotrophin mRNA levels is not clear. Conceivably, the peptides act directly at the level of the pituitary to influence the rate of gonadotrophin subunit mRNA transcription and/or degradation, thereby regulating the biosynthesis and secretion of the specific polypeptides.
1.3 RECOMBINANT PROTEIN EXPRESSION SYSTEMS

1.3.1 Introduction

The major recombinant expression systems currently being used in research and industrial applications include *Escherichia coli* (*E. coli*), yeast, and *in vitro* insect and mammalian cell culture systems. Each system offers a variety of features which may or may not be advantageous in the production of a recombinant protein. The primary consideration in the selection of an appropriate expression system is the physicochemical nature of the native protein and the corresponding degree of similarity required for the recombinant product for its intended application. This section reviews the systems currently available for the production of recombinant eukaryotic proteins. The main features of each system are summarised in Table 1.2.

1.3.2 Prokaryotic expression systems

**Bacteria:** The major advantages of the bacterial expression systems include cheap and simple culture media, high product yields and simplified scale up. These advantages are often offset in the production of eukaryotic proteins by the limited co- and post-translational processes *E. coli* can undertake, and the complexity of product purification and renaturation. Eukaryotic co-translational and post-translational modifications such as glycosylation, sulphation, disulphide bridge formation, proteolytic cleavage and the assembly of multimeric forms, many of which are essential processes in the assembly of biologically active eukaryotic proteins, require interprotein interactions and special compartmentalised processes unique to eukaryotic organelles such as the rough endoplasmic reticulum (RER) and GC (see Figure 1.3).

While heterologous proteins expressed in *E. coli* do form disulphide bonds, the reducing environment of the bacterial cytoplasm does not result in accurate eukaryotic disulphide bridge formation of cysteine rich heterologous proteins, and
Table 1.2
Recombinant protein expression systems; major features for consideration in the \textit{in vitro} synthesis of recombinant proteins.

<table>
<thead>
<tr>
<th>EXPRESSION SYSTEM</th>
<th>Prokaryotic</th>
<th>Eukaryotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli</td>
<td>Yeast</td>
</tr>
<tr>
<td>\textit{Recombinant Product Processing:}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycosylation</td>
<td>No</td>
<td>N- and O-linked (can hyperglycosylate)</td>
</tr>
<tr>
<td>Folding</td>
<td>Poor</td>
<td>Yes</td>
</tr>
<tr>
<td>Secretion</td>
<td>Occasionally</td>
<td>Variable</td>
</tr>
<tr>
<td>Proteases/ Degradation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Multimeric Assembly</td>
<td>Possible</td>
<td>Yes</td>
</tr>
<tr>
<td>\textit{Production Considerations}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient Requirements</td>
<td>Simple</td>
<td>Simple</td>
</tr>
<tr>
<td>Media Cost</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Scale Up</td>
<td>Simple</td>
<td>Simple</td>
</tr>
<tr>
<td>Maximum Productivity</td>
<td>(&gt;1\text{ g/litre}) (^{(1)})</td>
<td>0.4 g/litre (^{(2)})</td>
</tr>
</tbody>
</table>

(1) Gold (1990)
(2) Cregg \textit{et al.}, (1987)
(3) Marumoto \textit{et al.}, (1987)
(4) Friedman \textit{et al.}, (1989)
often assists in precipitation or aggregation of the recombinant product (Kohno et al., 1990). The formation of electron-dense cytoplasmic aggregates, otherwise called inclusion bodies, which consist primarily of the recombinant protein may be an advantage in that they provide a simplified approach to product isolation. Processes involved in the solublisation and refolding of the proteins contained in inclusion bodies use strong denaturants such as 6M urea or 8M guanidium hydrochloride, and strong reducing agents such as 2-mercaptoethanol or dithiothreitol (Marston, 1986). In some cases these processes result in irreversible denaturation, or difficulties in refolding the recombinant product and hence approaches which avoid the production of inclusion bodies have been developed.

One approach which avoids the need for product refolding utilises prokaryotic/eukaryotic gene fusions to direct the hybrid polypeptide product to processing pathways within *E. coli* which secrete the protein into the more oxidising environments of the periplasmic space or extracellular medium (Uhlen and Moks, 1990). This system has been successfully applied in the secretion of complex eukaryotic proteins such as active human-mouse chimaeric antibody fragments (Better et al., 1988), and immunoactive antibody heavy chain variable regions (Ward et al., 1989). Several other possible approaches for the production of soluble eukaryotic polypeptides in *E. coli*, including cloning mammalian foldases and mutation of the post-translational processes of the host bacteria, have been proposed (Schein, 1989).

Another bacterial species, *Bacillus subtilis*, has an added advantage in its ability to secrete large amounts of protein. It is also interesting to note that *Bacilli* do not produce endotoxins and several strains, including *Bacillus subtilis*, have been approved for the production of proteins for human use. Recombinant expression levels for bacterial α-amylase and human α-interferon of 250-400 mg/l and 1-2 mg/l, respectively, have been reported (Schiøn et al., 1986). Hybrid gene fusions
which result in protein secretion or protective conformational changes in the hybrid product, are also useful in avoiding proteolysis of heterologous proteins in *E. coli* (Uhlen and Moks, 1990).

While many folding and proteolytic problems associated with recombinant eukaryotic protein expression in *E. coli* have been resolved, and biologically active eukaryotic polypeptides including human growth hormone (Hsiung *et al.*, 1989) are being expressed and secreted at high levels, the transfer of eukaryotic genes into the prokaryotic systems to provide more complex "eukaryotic" processes such as glycosylation appears impossible. Eukaryotic glycoproteins such as the gonadotrophin hormones, whose biological activity is dependent on associated carbohydrate side chains, cannot be expressed in biologically active conformations in *E. coli* or other bacterial systems. Other glycoproteins including murine and human leukaemia inhibitory factors (LIF, Gearing *et al.*, 1987; Gough *et al.*, 1988), are not dependent on glycosylation for bioactivity and have been successfully expressed in *E. coli* (Gearing *et al.*, 1989).

Another limitation of the bacterial expression systems is their inability to process intron-containing genomic DNA sequences. While not a major limitation, this feature does restrict the source of heterologous nucleotide sequences which can be expressed in *E. coli* to intron-free genomic and cDNA sequences.

1.3.3 Eukaryotic expression systems

**Yeast:** Heterologous gene expression in *Saccharomyces cerevisiae* combines many of the higher eukaryotic cellular processes with many of the features of bacterial systems, including rapid growth to high cell densities in simple defined media (Emr, 1990). Many yeast expression systems, like bacterial systems, use hybrid gene fusions to direct hybrid gene products to secretory pathways and/or reduce proteolytic degradation (Brake, 1990). Other recombinant proteins are
automatically secreted. Unlike *E. coli*, yeast are capable of efficient glycosylation. Unfortunately, *Saccharomyces* specific glycosylation processes result in yeast-specific oligosaccharide side chains, and often hyperglycosylation of heterologous glycoproteins. For example, the molecular weight of human immunodeficiency virus (HIV) glycoprotein 120, the viral glycoprotein that mediates HIV infection by binding to T lymphocyte CD4 cell surface receptors, is 120 kD when expressed in mammalian cells, and up to 600 kD when expressed in yeast. Unfortunately, as the 600 kD yeast glycoprotein fails to bind to the CD4 receptor (Hitzeman *et al.*, 1990), this form of gp120 has limited application in HIV research. Another yeast strain, *Pichia pastoris*, reportedly restricts N-linked glycosylation to high mannose core structures and does not hyperglycosylate proteins (for review see Van Brunt, 1986).

**Insect Cells:** Insect cell culture techniques for recombinant protein expression centre on the use of recombinant baculovirus expression vector systems (Summers and Smith, 1987). These viral expression vectors predominantly utilise the viral polyhedrin gene promoter to drive high level expression of associated heterologous gene products. The polyhedrin gene product is the major structural component of viral occlusions accumulating to levels as high as 1 mg/ml in infected insect cell cultures (Summers and Smith, 1987). While the great majority of mammalian cell post-translational processes, including proteolytic cleavage, disulphide bridge formation, secretion, assembly of multimeric polypeptides and membrane targeting, are also found in insect cells (Luckow and Summers, 1988), a divergence in insect and vertebrate N-linked glycosylation patterns results in differential glycosylation. Insect cell oligosaccharide processing appears to be restricted to the addition of a high mannose/N-acetyl glucosamine core structure and terminal sugar trimming (Butters and Hughes, 1981; Hsieh and Robbins, 1984). The lack of sialic acid, galactose and fucose present in mosquito cell oligosaccharides is consistent with the low level of active glucosaminyl-, galactosyl- and sialyltransferases evident in these cells (Butters and Hughes,
O-linked oligosaccharides have been reported in association with recombinant insect cell culture products although the structure of the side chains has not been determined (Luckow and Summers, 1988).

Despite differences in the nature of insect cell glycosylation, the expression of heterologous eukaryotic gene products in insect cells is becoming increasingly popular. Expression levels as high as 300 µg/ml culture media and up to 7.2 mg/ml in insect larvae haemolymph have been reported for hybrid proteins such as the baculovirus polyhedrin-insulin-like growth factor II (Marumoto et al., 1987). Non-hybrid gene expression levels as high as 75 µg/ml have been reported for human α-interferon in haemolymph (Maeda et al., 1985). In contrast, expression levels for individual influenza viral polymerase proteins in in vitro cell culture, ranging between 3-5 µg/ml, have been less impressive (St. Angelo et al., 1987).

**In vitro translation:** In vitro translation systems, such as the wheat germ extract (Roberts and Paterson, 1973) and rabbit reticulocyte lysate (Pelham and Jackson, 1976) systems, provide cell free systems for the translation of mRNA isolated from tissues, or synthesised in vitro. Specific template mRNA preparations can be generated in vitro as biologically active, unprocessed mRNA from plasmid vector constructs containing the highly specific bacteriophage T7 (McAllister et al., 1981) or SP6 (Melton et al., 1984) promoters and a DNA sequence of interest. Exclusive transcription is initiated from these promoters by the promoter-specific T7 and SP6 RNA polymerases, respectively. Signal peptide cleavage, membrane insertion and limited N-linked core glycosylation of polypeptide products can be achieved via co-translational processing in the presence of canine pancreatic microsomal membranes (for review see Walter and Blobel, 1983).

This system has application in studying mRNA structure and function and in polypeptide analysis. In vitro transcription/translation remains an analytical
expression technology (for review see Chaimberlin et al., 1983) although maximum yields in the order of 100 mg/litre have been reported and production systems have been proposed (Riordan, 1987).

In vitro mammalian cell culture: The most commonly selected expression system for the production of biologically active mammalian glycoproteins and complex polypeptides is in vitro mammalian cell culture. Cell lines continuously maintained in tissue culture are transfected with specifically designed expression constructs capable of mediating high level expression of the associated DNA-encoded recombinant gene product. The full complement of mammalian-specific co- and post-translational processes for biosynthesis, folding and secretion of polypeptides are maintained in most immortalised host cell lineages, although cell type specific and mutant cell line specificities need to be considered. The components and design of the expression vector construct depends on the level and duration of recombinant expression required, the method of cell transfection and the cell line selected for transfection.

As shown in Table 1.2, the advantages offered by mammalian cell expression systems need to be weighed against the disadvantages of cost and difficulty in scale up. The high cost and limited productivity of mammalian cell culture systems generally limit their use to products of high value needed in low quantity. In vitro mammalian cell culture systems for recombinant protein expression are discussed in detail later in this chapter.

Transgenic animals: The introduction of new genes and the modification of existing genes through the production of transgenic animals has advanced our understanding of gene regulation, oncogenesis, the immune system, mammalian development and other areas of biology (Jaenisch, 1988). By extending these techniques to domestic animals, research now includes the design of transgenic,
disease resistant and feed efficient livestock (Pursel et al., 1989), and transgenic animals as producers of harvestable recombinant proteins (Van Brunt, 1988). In particular, attention has now focused on the utilisation of mammary-specific gene regulatory elements to direct expression of heterologous gene products to the mammary glands of transgenic animals.

Transgenic mice carrying the sheep β-lactoglobulin (BLG) gene (Simons et al., 1987), or a heterologous gene construct incorporating the murine whey acidic protein promoter and coding region sequences of the human tissue plasminogen activator (tPA) gene (Simons et al., 1987), show recombinant protein expression levels in their milk of 23.2 mg/ml and 50 µg/ml respectively. However, similar levels of expression have not been achieved in the milk of transgenic animals of other species, for example, transgenic sheep carrying heterologous gene constructs which incorporate the BGL promoter sequence show mammary gland specific expression levels ranging between 20-25 ng/ml for human factor IX, and 3-18 µg/ml for human α-1 antitrypsin (Simons, 1989).

Although technical difficulties associated with the production of transgenic animals remain considerable, major advances in gene transfer techniques including the development of embryo-derived stem (ES) cells (Evans and Kaufman 1981; Martin 1981) promise improved efficiencies. The advantages of ES cell techniques have so far been restricted to the mouse although in vitro pluripotent embryonic lineages have been described for pigs (Evans et al., 1990) and hamsters (Doetschman et al., 1988), and their ability to contribute to germ line chimaeras is currently being investigated. Early excitement over a recent report describing the production of transgenic animals by using sperm cells as vectors (Lavitrano et al., 1989) has proven unfounded. Methods for the production of transgenic livestock are therefore currently restricted to microinjection and electroporation of single cell embryos (for review see Wilmut et al., 1990).
Another limitation in developing transgenic animals for recombinant protein expression is the possible deleterious effects of recombinant products on the physiological well-being of the host. For example, transgenic mice expressing the ovine growth hormone gene under the control of the mouse metallothionein promoter develop pathogenic lesions in the liver (Orian et al., 1989). While targeting recombinant protein expression to the mammary gland essentially isolates the recombinant protein in this tissue, expression of murine whey acidic protein in the mammary glands of transgenic pigs has resulted in decreased periods of lactation (Wall et al., 1991).
1.4 OPTIMISING HETEROLOGOUS GENE EXPRESSION IN MAMMALIAN CELL CULTURE SYSTEMS

1.4.1 An overview of gene expression

The expression of all genes, whether they be homologous genes in their native state or transfected gene constructs, is critically dependent on the arrangement in cis of several DNA sequence elements. These can be fundamentally divided into the DNA sequences controlling gene transcription, and the gene sequences which are transcribed (see Figure 1.4). While the transcriptional control elements, including promoters, suppressors and enhancers, regulate gene expression at the level of mRNA synthesis, a variety of transcribed sequences including the mRNA polyadenylation signals (for review see Birnstiel et al., 1985), translation initiation sequences (Kozak, 1981; 1984b; 1986) and sequences controlling the stability of the mRNA transcript (Shaw and Kamen, 1986; Cleveland and Yen, 1989), also regulate gene expression at the level of translation. Ultimately the gene product itself can further influence the level of gene expression by directly affecting the host cell or tissue (Heyman et al., 1989). This section reviews the major genetic elements regulating gene expression as components which maybe useful for optimising the over-expression of heterologous genes in vitro.

1.4.2 Promoters and enhancers

Promoters and enhancers are structurally and functionally related in that they each contain discrete DNA sequence elements which interact with proteins known as transcription factors, to regulate the rate of gene transcription (for review see McKnight and Tjian, 1986). Promoters differ from enhancers in that they are always located upstream, usually within a few hundred base pairs, of the transcription start site, and often contain AT-rich or so called "TATA box" sequences which ensure the accurate initiation of transcription further downstream (Breathnach and Chambon, 1981). Enhancers, on the other hand, act in an orientation and distance independent manner, whether located upstream or downstream of the
Figure 1.4
Schematic presentation showing the expression of a typical eukaryotic gene.
transcriptional start site to increase the rate of promoter-mediated transcription as much as 10- to 100-fold (Serfling et al., 1985; Kaufman, 1990a). The enhancer for the human T cell receptor β-chain gene, for example, activates its promoter from a position at least 27,000 bp downstream of the promoter site (Kripenfort et al., 1988). Despite these differences, the delineation of regulatory sequences into promoter or enhancer elements is often difficult, with many sharing common DNA-binding sequence motifs such as the GGGCGG sequence (Boshart et al., 1985; Myers et al., 1986) and the GC-rich β-globin sequences (Myers et al., 1986). This is especially true for the small mammalian viruses such as simian virus 40 (SV40), which have evolved compact regulatory regions in which promoter and enhancer regions overlap (Hansen and Sharp, 1983; Rio and Tjian, 1984).

Transcription factors, or trans-acting DNA-binding proteins, bind short modular DNA sequence arrays within the promoter, enhancer and suppressor regions to activate or repress RNA polymerase II-mediated gene transcription (for review see Mitchell and Tjian, 1989). Many transcription factors share related DNA sequence specificities and multiple DNA-binding domains, and may compete for DNA-binding sites to control the rate of transcription. The proteins binding to common regulatory sequences such as the TATA box and GGGCGG binding domains, including the human TATA box binding protein TFIID, and the human GGGCGG binding protein Sp1, have been isolated and characterised (Peterson et al., 1990; Dynan and Tjian, 1983). The presence of TFIID maintains unregulated transcription of some constitutively expressed genes while other signal-dependent transcription factors require activation. For example, the regulatory protein which binds to the human heat shock protein 70 (hsp70) promoter, can only bind to the DNA following activation by heat shock stimuli (Kingston et al., 1987). The control mechanisms which activate and inactivate transcription factors vary considerably. For example, the yeast heat shock transcription factors, unlike the human example, bind to target sequences prior to being activated by phosphorylation (Sorger and Pehlem, 1988).
Different promoters and enhancers display a variety of tissue specificities, modes of induction and levels of associated gene transcription. The most commonly utilised regulatory sequences for high level expression are the mammalian viral sequences which constitutively transcribe high levels of mRNA in a wide variety of host cell systems. These include elements from the SV40 (McKnight and Tjian, 1986) and human cytomegalovirus [HCMV, (Boshart et al., 1985)] genomes. The avian Rous sarcoma virus (RSV) long terminal repeat (LTR) sequence also shows strong promoter activity in a broad host cell range (Gorman et al., 1982). Other viral elements show strong host cell preference, for example promoters isolated from the Moloney murine sarcoma virus are more active in mouse cells than monkey cells (Laimins et al., 1982). Stricter cell type specificity has been described for many cellular genes including the regulatory elements controlling immunoglobulin gene expression (Banerji et al., 1983; Grosschedl and Baltimore, 1985). Interestingly, the regulatory sequences controlling immunoglobulin expression have been utilised in transgene expression constructs for lymphocyte specific expression in vivo and in subsequent transgene hybridomas in vitro (Pavirani et al., 1989). Other strong eukaryotic cellular promoters include the human metallothionein (Karin and Holtgreve, 1984) and mouse β-globin (Myers et al., 1986) promoters.

While there has been no systematic comparison of the efficiencies of these strong promoters and enhancers some comparisons have been made. The RSV-LTR has been shown to be 3-10 times more active in expressing chloramphenicol acetyltransferase (CAT) reporter gene constructs than similar SV40 early region promoter constructs in the African green monkey kidney cell line CV-1 and murine NIH-3T3 cells respectively, but 50% less active than the same promoter in CHO cells (Gorman et al., 1982). The HCMV enhancer has been shown to be 3-5 times more active in initiating transcription of an associated β-globin gene than the SV40 enhancer in similar constructs in CV-1 cells (Boshart et al., 1985).
A particularly well characterised cellular promoter is the human metallothionein IIA (hMTIIA) promoter which is active in many cell types including hepatic, renal, neuronal, lymphocytic, fibroblastic and epithelial cells (Haslinger and Karin, 1985). Proteins encoded for by the metallothionein gene family play an important role in heavy metal homeostasis and heavy metal detoxification (for review see Karin, 1985). Putative 5' regulatory sequences common to the hMTIIA and mouse MTI genes were first identified by Karin and Richards (1982). Further experiments utilising 5' deletion and mutational analyses identified hMTIIA promoter sequence elements responsible for basal, and cadmium or glucocorticoid-induced expression of hMTIIA-thymidine kinase fusion genes in transient NIH-3T3 cell transfections (Karin and Holtgreve 1984; Karin et al., 1984a). An observation that the hMTIIA promoter was capable of activating the herpes simplex thymidine kinase gene promoter from a distance (Karin et al., 1984b) lead to the identification of two enhancer element repeat sequences with strikingly similar structural and functional organisation to that of the SV40 enhancer element (Haslinger and Karin, 1985).

The hMTIIA enhancer sequences were subsequently found to contain both basal level and metal response elements (BLE and MRE respectively), with the MRE acting as positive regulators of BLE activity in the presence of heavy metal ions. Two separate sequence elements including a GC box, recognised by the transcription factor Sp1 and necessary for basal level expression, and a glucocorticoid response element (GRE) acting as a hormone-dependent enhancer element were also described (Karin et al., 1987). The GRE enhancer element was found to be independently capable of mediating steroid-induced transcription, while the MRE elements required an accompanying BLE to mediate heavy-metal ion induced transcription. By changing the ratios of MRE to BLE, McNeall and co-workers (1989) have been able to increase the metal ion inducability of the promoter.
One novel eukaryotic expression system which utilises a prokaryotic promoter sequence has been described (Fuerst et al., 1986). The bacteriophage T7 promoter used in this system displays strong specificity for the prokaryotic RNA polymerase T7 and is therefore reliant upon the co-expression of this heterologous enzyme. The high transcriptase activity and stringent promoter specificity offered by the T7 system is, in many cases, offset by the prokaryotic-like mRNA transcribed by T7 polymerase in the cytoplasm of the host cell (Moss et al., 1990). The low levels of 5' mRNA capping associated with cytoplasmic transcription have been partially addressed by infecting host cells with vaccinia virus. Infecting the cells with vaccinia virus, which is believed to supply the necessary mRNA capping and methylation enzymes required for efficient eukaryotic mRNA translation, resulted in capping and methylation of only 5% of the mRNA transcribed in the cytoplasm (Fuerst and Moss, 1989). Another approach, which avoids the need for mRNA capping, is to include 5' cap-independent mRNA untranslated sequences from encephalomyocarditis virus, which facilitate cap-independent ribosome binding, immediately upstream of the coding sequence (Jang et al., 1989). The incorporation of these sequences has resulted in a four- to seven-fold increase in CAT reporter gene expression in vaccinia virus infected cells (Elroy-Stein et al., 1989).

1.4.3 mRNA processing
Eukaryotic RNA transcripts undergo a variety of post-transcriptional modifications or RNA processing events within the cell nucleus prior to being transported to the cytoplasm for translation (see Figures 1.4 and 1.5). The primary RNA processing event for RNA molecules destined for translation is the addition of a 5' terminal capping nucleotide, usually 7-methylguanosine. The addition of the capping nucleotide is considered essential for the initiation of translation, the first step in mRNA translation being the binding of translation initiation factors at the 5' cap site (for review see Pain, 1986), and stabilisation of mRNA transcripts (Furuichi et al.,
Figure 1.5
Major levels of eukaryotic gene regulation.
While further optimisation of the endogenous process for the over-expression of heterologous genes has not been attempted, several cap-independent 5' viral mRNA sequences have been described (Jackson, 1988; Jang et al., 1989) and utilised to increase the translational efficiency of cytoplasmically transcribed heterologous gene transcripts.

A second event in eukaryotic RNA processing is the addition of the 3' poly-adenylic acid [poly(A)] tail. Like 5' capping, the polyadenylation of RNA plays an important role in protecting mRNA from nuclease digestion (Zeevi et al., 1982) and is therefore an essential element for the over-expression of heterologous genes. In examining the importance of polyadenylation, Kaufman and Sharp (1983) reported a 10-fold reduction in dihydrofolate reductase (DHFR) expression levels following the removal of the polyadenylation signal. Similarly, a single point mutation in the human α2-globin polyadenylation signal, changing the consensus AAUAAA signal to AAUAAG, reduces endogenous α2-globin expression to 5% of normal levels and results in the clinical condition known as α-thalassemia (Higgs et al., 1983). A variety of potential polyadenylation signal sequences other than the consensus AAUAAA sequence have been identified including AAUAAA, AUUAAA, AAUACA and AAUAAAU (for review see Birnstiel et al., 1985). The length of the poly(A) tail has also been shown to correlate with the stability of several mRNA species, including human growth hormone, which can be stabilised by a glucocorticoid-induced increase in the length of the poly(A) tail (Peck and Alex, 1987), insulin (Muschel et al., 1986) and vasopressin (Carrazana et al., 1988).

To ensure the polyadenylation of heterologous mRNAs encoded by DNA sequences which may or may not contain active polyadenylation sites, most expression vector constructs incorporate a strong heterologous polyadenylation signal. Two of the most commonly utilised signals are the SV40 and β-globin sequences. The inclusion of these sequences, downstream of protein encoding
nucleotide sequence, ensures the transcripts are polyadenylated. The intron sequences, generally beginning with the nucleotides 5'-GU-3' and ending with 5'-AG-3' (Breathnach and Chambon, 1981), are removed following polyadenylation by a process known as RNA splicing. While some controversy exists as to the role intron sequences play in gene expression, the inclusion of such sequences in cDNA expression vectors has been broadly accepted as beneficial. Some reports have also suggested that the requirement for a functional intron may be promoter specific (Kriegler, 1990). Interestingly, while the benefit of intron sequences in in vitro expression has not been clearly established, introns have been shown to play an important role in the levels of transgene expression in mice (Brinster et al., 1988).

1.4.4 RNA sequence components and secondary structure

Nucleotide sequences other than the 5' cap and poly(A) tail of mRNA can greatly affect both mRNA stability (Cosman, 1987; Cleveland and Yen, 1989) and translational efficiency (Kozak 1980; 1984b; 1986). While both 5' and 3' mRNA destabilising elements have been described, most destabilising sequence motifs have been associated with the 3' non-translated region (Brawerman, 1989). One particular sequence motif, AUUUA, has been identified in association with a variety of oncogenes, cytokines and transcriptional activators (for review see Cosman, 1987), and an AUUUA-specific mRNA binding protein has been identified (Malter, 1989). Deletion or replacement of these 3' sequence motifs has significantly stabilised c-fos mRNA in NIH-3T3 cells and dramatically slows poly(A) tail shortening (Wilson and Treisman, 1988). Similarly, the deletion or modification of such sequences in genes for high level expression must be considered if optimal expression levels are to be achieved.

Secondary structure of the mRNA transcript also modulates translational accuracy and efficiency (Kozak, 1980). While some secondary structure appears necessary
to facilitate correct initiation of translation by impeding ribosomal migration beyond the translation initiation site (Kozak, 1980), excessive mRNA hairpin formation can also reduce translational efficiency (Pelletier and Sonenberg, 1985). A 30-40% difference in the rate of mouse and rabbit α- and βmaj-globin mRNA translation initiation has been partly explained on the basis of AUG initiation codon accessibility as determined by their susceptibility to nucleases (Pavlakis et al., 1980).

The nucleotide sequence of the mRNA coding region can also influence gene expression levels independently of mRNA secondary structure. Highly expressed genes in *E. coli* and some lower eukaryotes show a bias for certain codon usage, and the amounts of accepting tRNAs have been shown to correlate with codon bias (Ernst, 1988). In higher eukaryotes the sequence surrounding the AUG translation initiation codon has been shown to be particularly important in initiating translation and an optimised consensus sequence, ACCAUGG, has been described (Kozak 1984b, 1986). The possibility that the consensus ACCAUGG sequence is preferentially recognised by a complementary sequence within the 18S ribosomal RNA has been suggested (Kozak, 1986).

### 1.4.5 DNA transfer techniques

The two major methods used for transferring foreign gene sequences into eukaryotic host cells include DNA transfer by viral infection and direct DNA transfer by chemically-mediated or physical microinjection and electroporation techniques. Viral-mediated DNA transfer is reliant on the replacement of viral gene sequences with DNA coding the protein of interest, and the subsequent transfection of the recombinant viral genome into permissive cell lines which enable packaging of the DNA into infectious viral particles (for review see Sambrook and Gething, 1988). Susceptible cell lines are then infected with the recombinant virus.

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The major advantage of viral-mediated DNA transfer is the high efficiency with which foreign DNA is transferred. The infective nature of viruses and the ability to produce high-titre stocks theoretically ensures that the DNA is transferred to every cell in the transfection culture. Plasmid-based vectors and isolated gene sequences are non-infective and therefore rely on a variety of DNA transfer techniques which provide up to a maximum of about 40% transfected cells (Sambrook et al., 1989, Keown et al., 1990).

Viral vectors differ considerably in their molecular structure and display a variety of features. Most mammalian viruses, including SV40 and vaccinia virus, are lytic in host cell systems and as such often provide only short term or transient expression of wild-type and transferred heterologous genes of the recombinant viral genome. The strong immunogenic responses generated as a result of vaccinia virus infection have identified this viral expression vector as having particular application in the production of recombinant vaccines. For example, mice vaccinated with a recombinant vaccinia virus incorporating the rabies virus envelope glycoprotein G cDNA have shown 100% protective immunity against challenge with street rabies virus (Kieny et al., 1984). This success has been followed by the successful oral vaccination of foxes (Blancou et al., 1986). Other viral vectors based on the RNA retroviruses can integrate DNA into the host cell genome and may have particular clinical application as vehicles for the introduction of foreign genes in gene therapy programs (Friedman, 1989). These systems are reliant on the in vitro production of helper-free replication-defective recombinant viruses which can infect but not multiply within the host.

While the strong promoters, infective nature and ability of many viral vectors to replicate extra-chromosomally has attracted widespread investigation into their use as high-level expression vectors, other features of the viral systems have limited their use. For example, the limited biological understanding and oncogenic nature
of many mammalian viral vectors has demanded specialised handling techniques which can make them difficult to utilise. Also, many viral vectors have limited host cell ranges and/or foreign DNA capacities which further restrict their use. Despite these restrictions, many mammalian viral expression systems have been successfully utilised for the over-expression of heterologous gene products. For example, monkey CV-1 cells, infected with a single SV40 viral vector incorporating both hCG α- and β-subunit cDNA sequences in place of the viral protein (VP) 2 and VP1 coding regions respectively, express 1.1 mg hCG/litre/24 hours (Reddy et al., 1985). Similarly, co-infection of CV-1 cells with recombinant vaccinia virus expression vectors incorporating either the hCG α- or β-subunit gene coding region sequences, has resulted in hCG expression levels of 0.33 μg/10^6 cells/24 hours (Chakrabarti et al., 1989).

A variety of techniques are available for DNA-mediated gene transfer, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfer, liposome-mediated transfer, electroporation and microinjection. Many variations of each technique have been reported and several review articles provide good examples of each (Sambrook et al., 1989; Keown et al., 1990). The calcium phosphate co-precipitation transfection technique (Wigler et al., 1979) relies on the mixing of purified DNA with buffers containing phosphate and calcium chloride and the subsequent exposure of the cells for transfection to the fine, DNA-containing, calcium phosphate precipitate. The formation of the calcium phosphate/DNA precipitate may also facilitate DNA transfer by concentrating the DNA at the cell surface and protecting the DNA from extra- and intra-cellular nucleases (Loyter et al., 1982). The DEAE-dextran-mediated DNA transfection technique (Warden and Thorne, 1968) results in the formation of a soluble DEAE-dextran/DNA complex. The mechanism/s by which the cells take up the DNA complexes have not been established although it is believed to involve endocytosis. Liposome-mediated DNA transfection relies on the encapsulation of DNA within a liposomal membrane.
and the subsequent fusion of the liposome with the cell membrane (Felgner et al., 1987). Electroporation, as the name suggests, enables the transfer of DNA across cell membranes by momentarily porating the membrane through the application of an electric shock (Potter et al., 1984). Similarly, microinjection uses micromanipulation techniques for the direct nuclear injection of solutions containing DNA (Capecchi, 1980).

The technique selected will depend primarily on the nature of the expression system, whether it be transient or constitutive, and the host cell lineage. Calcium phosphate co-precipitation is well suited for the establishment of both transient and constitutive expression in many host cell lines while DEAE-dextran-mediated transfer is suitable for establishing only transient expression systems (McCutchan and Pagano, 1968). Liposome-mediated DNA transfer is reportedly 6-10 times more efficient than DEAE-dextran transfection and 6-80 times more efficient than calcium phosphate co-precipitation in the establishment of both transient and constitutive expression systems (Felgner and Holm, 1989).

1.4.6 Selectable markers
The transfection efficiencies associated with DNA-mediated transfer techniques necessitate the incorporation of a selectable marker gene so as to enable the identification and isolation of clonal cell lines carrying and expressing the integrated DNA sequences. In most instances, selectable markers do not directly affect the level of heterologous gene expression. Some selectable markers (see Section 1.4.7), however, have further application in providing a means by which the copy number of the transfected gene construct can be amplified.

Drug resistance markers can be classed as either dominant or recessive. Recessive drug resistance markers utilise host cell systems deficient in the biochemical activity being selected for, usually the purine and pyrimidine salvage pathway (see Figure 1.6). For example, cells with a non-functional salvage
Figure 1.6

*De novo* and salvage biosynthetic pathways for purines and pyrimidines involving available selectable markers.

*De novo* enzymes: DHFR, dihydrofolate reductase; CAD, carbamoyl-P synthase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; IMPDH, inosine-monophosphate dehydrogenase. Salvage enzymes: TK, thymidine kinase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; AK, adenosine kinase; APRT, adenine phosphoribosyltransferase. Solid arrows indicate single reactions, dashed arrows indicate multiple reactions. Solid squares indicate reactions that are inhibited by folate analogs such as MTX and aminopterin. Hatched squares indicate the principle reactions inhibited by azaserine while the open square indicates the reaction inhibited by mycophenolic acid.

[Adapted from Kaufman (1990b)]
pathway do not grow when the \textit{de novo} biosynthesis of purines and pyrimidines is blocked unless they express a transferred marker gene which rescues the salvage pathway. Selectable markers which utilise this strategy include hypoxanthine-guanine phosphoribosyl transferase (HGPRT, Szybalska and Szybalska, 1962), thymidine kinase (TK, Littlefield, 1966) and adenine phosphoribosyl transferase (APRT, Wigler \textit{et al.}, 1979).

Dominant selectable markers are not dependent on the availability of cell lines deficient in a particular biochemical pathway, but rather confer resistance to toxic selective agents such as the neomycin analog G-418 sulphate which blocks translation in mammalian cells (Southern and Berg, 1982). Resistance to G-418 sulphate is conferred in transfected cells by the expression of the bacterial transposon Tn5 aminoglycoside phosphotransferase gene ($\text{Neo}^+$), under the transcription control of a eukaryotic promoter such as the SV40 early promoter (Southern and Berg, 1982) or the Herpes simplex TK promoter (Thomas and Capecchi, 1987). The $\text{Neo}^+$ gene product inactivates the G-418 sulphate permitting selection of the transfected cell population.

Another dominant selection system utilises the bacterial xanthine-guanine phosphoribosyl transferase (XGPRT or gpt) gene (Mulligan and Berg, 1981; see Figure 1.6). In this system the \textit{de novo} conversion of inosinic acid (IMP) to xanthine-monophosphate (XMP) by inosine-monophosphate dehydrogenase is blocked by the addition of mycophenolic acid. Cells transfected with XGPRT gene express the prokaryotic XGPRT enzyme which is not inhibited by mycophenolic acid. Cells expressing XGPRT covert xanthine to xanthine-monophosphate, thereby maintaining the xanthine-guanine monophosphate pathway (see Figure 1.6).
One commonly used recessive selectable marker, which also has application as a dominant marker is the dihydrofolate reductase (DHFR) gene (Murray et al., 1983). DHFR catalyses the conversion of folate to tetrahydrofolate (FH₄) which is necessary for the biosynthesis of glycine from serine, and also for purine biosynthesis (Kaufman, 1990b; see Figure 1.6). CHO cell lines deficient in DHFR have been described (Urlaub and Chasin, 1980) and utilised in the selection of transfected cell lines expressing bLH (Kaetzel et al., 1985). DHFR-deficient cells transfected with the DHFR gene will grow in medium lacking added nucleosides while non-transfected cells do not. Further selective pressure for cell lines expressing high levels of DHFR can be applied by incorporating a folic acid analogue, methotrexate (MTX), which binds to and inhibits DHFR, leading to cell death. By titrating media MTX concentrations, the highest DHFR producing cell lines can be selected. More importantly, step-wise increases in the level of MTX in selection media can lead to DHFR gene amplification (Alt et al., 1978) and co-amplification of associated heterologous genes (see Section 1.4.7).

1.4.7 Amplifiable markers
The co-amplification of heterologous genes in conjunction with the DHFR gene has particular application in the over-expression of recombinant gene products. For example, recombinant human factor IX expression levels for CHO cells co-transfected with both DHFR and factor IX expression constructs, have been increased 3000-fold to levels as high as 100 µg/ml by stepwise increases in the concentration of MTX, from 0 to 200 µM, in the selection medium (Kaufman et al., 1986). Similarly, MTX-induced amplification of the bovine glycoprotein α-subunit gene, in CHO cells co-transfected with bovine α- and LH β-subunit expression constructs, has resulted in an 11-fold increase in bLH expression (Kaetzel and Nilson, 1988). Surprisingly, this result was achieved without β-subunit gene amplification.
While heterologous DHFR gene amplification in transfected DHFR deficient CHO cell lines has achieved DHFR gene copy numbers in excess of 2000 copies/cell (Crouse et al., 1983), similar levels of amplification have not been achieved in non-DHFR deficient cell lines. Another amplifiable marker, glutamine synthetase (GS) has recently been compared with the DHFR marker in non-DHFR-deficient CHO cells (Cockett, 1990). Reporter gene expression levels were found to be consistently higher following GS-mediated amplification than following DHFR amplification. Differences in transfection colony numbers following the GS-based selection procedure were found to be ten-fold lower than those following the DHFR selection, suggesting a 10-fold higher stringency of the GS selection system which may have resulted in the selection of higher producing cell lines. Overall, the GS-selected cell lines had higher reporter gene copy numbers. Selection for GS is applied through the incorporation of the enzyme substrate methionine sulfoximine. Modification of the methionine sulfoximine results in irreversibly binding of the resultant methionine sulfoximine phosphate, and inactivation of the enzyme (Young and Ringold, 1983).

The replicative nature of viral vectors also provides a mechanism for heterologous gene amplification. Trans-acting factors provided by genes of the viral vector, helper virus or the host cell genome, bind to viral DNA sequences known as viral replicons and initiate episomal replication (Sambrook and Gething, 1988). The level of replication is primarily dependent on the type of virus. For example, the SV40 virus can generate up to $10^5$ copies per cell (Mellon et al., 1981) while the BPV virus generates between 30 and 200 copies per cell (Sambrook et al., 1985). A fragment of the BPV genome known as the 69% transforming fragment (Sarver et al., 1981) has been included in several plasmid-based expression vectors to ensure episomal replication and resulted in moderate expression of associated heterologous transcription units (Campo, 1985). While BPV-mediated cell transformation is restricted to murine C-127 cells, and provides a morphologically
based selection system for the identification of transfected cell lines, the BPV-mediated replication of transfected heterologous gene constructs incorporating the 69% transforming fragment is not. Similarly, a 300 bp fragment of the SV40 genome, which includes the SV40 origin of replication and several promoter/enhancer sequences (McKnight and Tjian, 1986), has been included in many plasmid-based transient expression vectors. Replication of these vectors is dependent on the co-expression of SV40 large T antigen which binds to the SV40 origin of replication and initiates SV40 DNA synthesis (Borowiec et al., 1990; Prives, 1990). While some SV40 based plasmid vectors include large T antigen transcription units, most rely on large T antigen expression by SV40 transformed host cell lineages such as COS (Gluzman, 1981).

1.4.8 Cell lineages for heterologous gene expression

The most commonly utilised cell line for high level transient expression is the SV40 transformed, African green monkey kidney cell line COS (Gluzman, 1981). This cell line was generated by transforming the kidney cell line CV-1, with origin defective SV40 DNA to enable constitutive expression of SV40 large T antigen. In an attempt to modulate T antigen expression and therefore plasmid replication, Rio and co-workers (1985) developed a COS cell line expressing a temperature sensitive large T antigen which is active at 33°C but inactive in cells cultured at 40°C.

Cell lines suitable for both transient and constitutive expression of heterologous gene constructs include the CHO (for review see Gottesman, 1987), and mouse NIH-3T3 (Jainchill et al., 1969) and C-127 (Lowy et al., 1978) cell lines. CHO cells are particularly well suited for a number of reasons. A comparative study examining human interferon-β1 glycosylation patterns for three recombinant products expressed in CHO, C-127 and human lung adenocarcinoma cells has shown that only the human interferon-β1 expressed by CHO cells closely
resembles the native form (Kagawa et al., 1988). This is of particular importance in selecting a cell line for the expression of recombinant FSH and other glycoproteins whose biological activity is critically dependent of the associated oligosaccharide side-chains (Galway et al., 1990). Furthermore, introduced genes can be routinely amplified and stably integrated chromosomally in CHO cells (Kaufman et al., 1985). CHO cells also secrete efficiently and are suitable for large scale cell culture in defined low protein media (Arathoon and Birch, 1986). As many as 80 classes of CHO cell lines (Gottesman, 1985) including a variety of glycosylation-defective mutant cell lines (Gottlieb et al., 1974; Briles et al., 1977) and a DHFR deficient CHO cell line (Urlaub and Chasin, 1980) have been described.

The cell lines and expression systems described in this chapter provide a wide range of options for establishing in vitro mammalian cell expression systems to synthesize and secrete recombinant oFSH. The following chapters describe a number of experimental approaches which have been undertaken to achieve this goal.
2.1 General laboratory reagents

Unless otherwise stated, all laboratory reagents were analytical grade and supplied by Ajax Chemicals, BDH, Boehringer Mannheim or Sigma Chemicals.

Solvents for DNA and RNA extraction including phenol, phenol/chloroform/isoamyl alcohol, chloroform/isoamyl alcohol and others were prepared (see Appendix 1) as described by Sambrook and co-workers (1989). Analytical grade phenol and isoamyl alcohol were supplied by BDH Chemicals.

Particular attention and care was taken in the preparation and handling of all RNA reagents. Glassware was baked overnight at 270°C and where appropriate solutions were treated with diethyl pyrocarbonate to inactivate contaminating RNAses (DEPC, for details see Sambrook et al., 1989).

2.2 DNA cloning and manipulation techniques

2.2.1 DNA restriction enzyme digests

Restriction enzymes were supplied by Pharmacia and New England Biolabs and used as recommended by the manufacturers. Concentrated (10x) restriction enzyme buffers were prepared as described by Maniatis and co-workers (1982).

2.2.2 Agarose gel electrophoresis

DNA samples were analysed on 0.8 - 1.5% agarose (Agarose NA, Pharmacia) TAE (Appendix 1) gels containing 0.5 µg/ml ethidium bromide (Appendix 1) using standard techniques. DNA samples were visualised by UV illumination on a horizontal UV transilluminator (Chromato-vue model TM40, UVP, USA). Low melting temperature agarose (LMT, Sea Plaque FMC Bioproducts) was
substituted for routine agarose when DNA fragments were to be recovered following electrophoresis. The DNA band of interest was excised from the gel using a sterile scalpel blade and trimmed of excess agarose. The agarose block then was dissolved in 3 volumes of 40 mM Tris HCl (pH 8.0), 1 mM EDTA (Appendix 1) by heating the agarose containing solution to 65°C for 10-15 minutes. Samples were extracted twice with an equal volume of phenol, then once with an equal volume of phenol/chloroform and once with an equal volume of chloroform alone. DNA was then precipitated by adding an equal volume of 3M sodium acetate pH 5.2, (Appendix 1), and 2 final volumes (RNA plus sodium acetate) of ethanol. If small quantities of DNA were being recovered, 5-10 µg of transfer RNA (tRNA, Boehringer) was added as carrier to assist precipitation and visualisation of the DNA pellet.

2.2.3 Restriction enzyme digestion, dephosphorylation and insert ligation of cloning vectors

Restriction enzyme digests of cloning vectors were monitored by agarose gel electrophoresis and routinely subjected to a secondary reaction with fresh enzyme and buffer to ensure complete digestion. Vectors were dephosphorylated by adding 6-8 units calf intestine alkaline phosphatase (cat. # 713023, Boehringer) to the completed secondary reaction according to the one-step method described by the manufacturer. Vector DNA was precipitated with or without carrier tRNA by adding an equal volume 4M ammonium acetate, and 2 final volumes (DNA plus ammonium acetate) of ethanol following 2 phenol/chloroform extractions and a single chloroform extraction.

Inserts were ligated into vectors at molar ratios of insert to vector ranging from 0.5:1 to 50:1 as described by Maniatis and co-workers (1982).
2.3 Design, synthesis and radiolabelling of synthetic oligonucleotide probes for cDNA library screening

2.3.1 Design and synthesis of oligonucleotides

Two pools of degenerate oligonucleotides were designed and synthesized (Baker Institute, Melbourne) on the basis of the published amino acid sequence of the oFSH β-subunit (Sairam et al., 1981). A third non-degenerate oligonucleotide was designed and synthesized (Bresatec, Adelaide) on the basis of a common region of the ovine and bovine FSH β-subunit amino acid sequences (Pierce and Parsons, 1981), and the bovine FSH (bFSH) β-subunit cDNA sequence (Maurer and Beck, 1986). The degenerate oligonucleotide pools were designed to target cDNA coding regions encoding both N- and C-terminal amino acid sequences with limited codon degeneracy. The sequence and estimated melting temperatures (T_M) for the preparations of oligonucleotides are detailed in Chapter 3.

2.3.2 T4 polynucleotide kinase (PNK) radiolabelling of synthetic oligonucleotide probes

Oligonucleotide probes were prepared by T4 PNK end labelling with γ^32P-dATP (≥ 5000 Ci/mmol, code 10218, Amersham) according to the method described by Maniatis and co-workers (1982). Routinely, 100 ng of oligonucleotide was labelled with 50 μCi γ^32P-dATP to a specific activity of 3.6-4.8 x 10^8 cpm/μg.

2.4 Preparation of ovine pituitary poly(A)^+ RNA

2.4.1 Tissue collection

Ovine pituitaries were collected from 8-12 month old lambs of either sex at a local abattoir (Gilbertson's Abattoir, Brooklyn) within 10 minutes of slaughter and frozen immediately in liquid nitrogen. Pituitaries were then stored at -70°C prior to use.
2.4.2 RNA extraction

Total pituitary RNA was extracted according to the method described by Chirgwin and co-workers (1979) with the following modifications. Homogenising buffer was prepared as 5 M guanidinium thiocyanate (Fluka), 10 mM Tris HCl (pH 7.5), 1% β-mercaptoethanol (Sigma). Frozen tissues (1 gram/10 ml homogenising buffer) were homogenised for 2-3 minutes at high speed using a Polytron PT10/35 homogeniser (Polytron, Switzerland). Sodium N-lauroylsarcosine (sarkosyl, Fluka) was then added to a final concentration of 1% and homogenised for a further 10-15 seconds. Particulate material was removed by centrifuging homogenates at 16,000 g for 10 minutes at 4°C. Supernatants were layered over a quarter volume of 5.7 M cesium chloride (CsCl) and centrifuged at 120,000 g for 20 hours at 18°C. Supernatants were aspirated prior to inverting the tubes for air drying. RNA pellets were resuspended in DEPC treated water, extracted twice with phenol/chloroform and twice with chloroform. Total RNA was precipitated by sodium acetate/ethanol precipitation.

2.4.3 Selection of poly (A)+ RNA

Poly (A)+ RNA was isolated from total cellular RNA by twice repeating the oligo (dT)-cellulose (Pharmacia) affinity chromatography procedure described by Maniatis and co-workers (1982). Samples were quantitated by OD260 spectrophotometric determination and analysed by 0.66 M formaldehyde, 1.0-1.2% agarose gel electrophoresis (Fourney et al., 1988). RNA was transferred to nylon membranes (Hybond-N, Amersham) by capillary transfer using 20x SSC (3 M NaCl, 0.3 M Tri-sodium citrate). Filters were air dried prior to 2 minute UV illumination to cross-link RNA to the membrane.
2.5 Construction and screening of cDNA libraries

2.5.1 Synthesis and cloning of two ovine lamb pituitary λgt 10 cDNA libraries

The first cDNA library was constructed to be fully representative while the second smaller library was enriched for cDNA transcripts >500 base pairs (bp) in length by size selection chromatography. cDNA was synthesized using the Amersham cDNA synthesis system (code 1256, Amersham) and packaged into the bacteriophage vector λgt10 using the Amersham cDNA cloning system λgt10 (code 1257, Amersham) according to the manufacturers instructions. Reactions were monitored through the incorporation of $^{32}\text{P}$-labelled dATP (3000 Ci/mmol, code PB10204, Amersham) rather than the recommended $^{32}\text{P}$-dTTP which was not routinely available in our laboratory. A control cDNA synthesis reaction incorporating 0.5 μg RNA [0.24-9.5 kilobase (Kb) RNA ladder, catalogue # 5620SA, BRL] was run simultaneously with each pituitary RNA cDNA reaction to monitor the efficiency of the system.

The Amersham cDNA synthesis kit is based on the method described by Gubler and Hoffman (1983). This method is an improved and simplified version of the Okayama and Berg (1982) cDNA synthesis protocol. The major advantage of the improved method is the use of RNAse H nicking of hybrid template RNA for the priming of *E. coli* DNA polymerase mediated second strand synthesis. This eliminates the need for DNA hairpin formation for priming of second strand synthesis, and the associated loss of 5' cDNA sequence through subsequent S1 nuclease digestion of the hairpin structure.

First strand synthesis is mediated by oligo (dT) priming in the presence of avian myeloblastosis virus (AMV) reverse transcriptase. The second cDNA strand is primed and synthesized as above and blunt ended with T4 DNA polymerase for linker ligation and cloning.
The substitution of $^{32}$P-dATP for $^{32}$P-dCTP can result in inexplicably high levels of apparent second strand synthesis (unpublished). This is probably due to the formation of an oligo (dT) or RNA poly(A)$^+$ primed co-polymer which non-covalently associates with the enzyme DNA polymerase I (C. Wolin, personal communication). The formation of the nucleic acid/DNA polymerase complex was apparent in some cDNA synthesis reactions while being inexplicably absent in others. Results for the construction of the enriched cDNA library, in which these difficulties were not encountered, are presented in Chapter 3.

2.5.2 cDNA library enrichment by size selection chromatography of cDNA

Double stranded cDNA in TAE buffer was size selected by exclusion chromatography on a prepacked, TAE equilibrated CL-B4 Sepharose column (Pharmacia) following EcoR1 linker digestion. Fractions were collected and analysed by 1% agarose gel electrophoresis and autoradiography. Those fractions containing the majority of cDNA transcripts over 500 base pairs (bp) in length were pooled, phenol/chloroform extracted and sodium acetate/ethanol precipitated for $\lambda gt10$ ligation. Results are presented in Chapter 3.

2.5.3 Oligonucleotide probe hybridisation screening of $\lambda gt10$ cDNA library bacteriophage plaques

Recombinant bacteriophage plaques were plated with E.coli MN514 (Hfi+) host cells at a density of 30,000 plaque forming units (pfu)/150 mm LB agar (Appendix 1) petri dish and screened as duplicate nylon filter (Hybond-N, Amersham) lifts according to the manufacturers instructions. Prehybridisation, hybridisation and washing procedures were performed in an orbital shaking water bath set at 30 rpm (Paton Scientific, South Australia) and 37°C (N-
terminal degenerate oligonucleotide probe), or at 30 rpm and 50°C (C-terminal degenerate and non-degenerate oligonucleotide probes).

Prehybridisation solution containing 6x SSPE, 0.2% sodium dodecyl sulphate, 2 x Denhardt's solution and 100 µg/ml salmon sperm DNA (Appendix 1) was prepared according to Maniatis and co-workers (1982) and frozen at -20°C until required. The same solution was also used for hybridisation procedures. Filters were prehybridised for a minimum of six hours prior to overnight incubation in hybridisation solution containing the labelled oligonucleotide probe at 1 x 10⁶ cpm/ml.

Following hybridisation, filters were washed five times (10 minutes each wash) in a preheated wash solution containing 6x SSC, 0.2% SDS. Filters were blot dried prior to overnight X-ray film (XAR-5, Kodak) exposure at -70°C with intensifying screens. Positive plaques were isolated by repetitive dilution cloning. Bacteriophage DNA was prepared in liquid culture and isolated by polyethylene glycol (PEG) precipitation (Sambrook et al., 1989). Size estimates for cloned cDNA were established by EcoR1 restriction enzyme digestion of recombinant bacteriophage DNA and subsequent agarose gel electrophoresis with HindIII/EcoR1 digested bacteriophage λ DNA markers.

Hybridisation solutions were routinely re-used for up to 2 weeks for screening of secondary and tertiary plaques. Between uses, hybridisation solutions were stored frozen at -20°C.

2.6 Nucleotide sequence analysis of cloned cDNA

cDNA sequences were isolated from recombinant λgt10 bacteriophage by EcoR1 restriction enzyme digestion and LMT agarose gel electrophoresis. Inserts were then ligated into the unique EcoR1 cloning site of the plasmid
vector pUC18 (Messing, 1983). A series of overlapping truncated cDNA clones were prepared by Exonuclease III digestion (Henikoff et al., 1984) and analysed by dideoxy sequence analysis (Sanger et al., 1977). Sequencing reagents and methods were supplied in the Sequenase Kit (code 0700, United States Biochemical Corporation). Briefly, the oligonucleotide primer is annealed to the denatured template DNA to enable a short primer extension reaction incorporating a $^{32}$P-labelled nucleotide. The labelling reaction is then split into four separate reactions which further extend the labelled strand. These reactions incorporate a mixture of dNTPs and a reaction specific ddNTP which randomly terminates the extension of the labelled strand, and provides one of the four ddNTP specific DNA ladders for sequence analysis.

2.7 The preparation of competent *E.coli* cells and transformation procedures

2.7.1 Preparation of competent cells

Frozen aliquots of competent cells were prepared from 500 ml cultures as previously described (Hanahan, 1983; reviewed 1985) with the following modifications. The *E.coli* host strains JM101 (Messing et al., 1979) and XL1-Blue (Bullock et al., 1982) were prepared for M13 bacteriophage DNA transformations, while DH1 and XL1-Blue host strains were prepared for pUC18, pKC3 and pSP72 plasmid transformations. XL1-Blue and DH1 *E.coli* were grown to an OD$_{590}$ of 0.48 in LB medium (Appendix 1), while JM101 *E.coli* were grown to OD$_{590}$ of 0.225 in 2TY medium (Appendix 1). Cells were collected by centrifugation at 5,000 g for 5 minutes at 4°C, then resuspended in 200 ml Tfb I (Hanahan, 1983). The suspension was kept on ice for 15 minutes, centrifuged as above and the cells gently resuspended in 20 ml Tfb II (Hanahan, 1983) for aliquoting. Samples of competent cells were snap frozen in liquid nitrogen and stored at -70°C until required.
2.7.2 Transformation of competent cells

Frozen competent cells were incubated at room temperature until thawed. DNA (10 - 50 ng) in a volume less than or equal to 0.4 volumes of competent cells was then added. Cell suspensions were kept on ice for 45 minutes and then subjected to 90 seconds of heat shock at 42°C. Suspensions were returned to an ice bath until plating. M13 transformations were plated on 2TY agar in 0.6% 2TY top agarose (Appendix 1), containing 0.025% 5-bromo-4-chloro-3-indolyl-βD galactoside (X-Gal, BRL) and 1.2 mM isopropyl-βD thiogalactoside (IPTG, Pharmacia) to enable recombinant plaque selection on the basis of colour. Polylinker cloning sites in the M13 mp18 bacteriophage DNA are located between the lacI promoter and the lacZ gene. DNA sequences inserted into these sites disrupt expression of β-galactosidase and the subsequent cleavage of X-Gal which generates the blue colour. Plasmid based vector transformations were diluted with 2 volumes of LB growth medium, incubated for 30 minutes at 37°C and plated on LB agar containing 100 µg/ml ampicillin (Sigma).

2.8 Isolation of plasmid and bacteriophage DNA

Plasmid and RF (replicative form) bacteriophage DNA were extracted from liquid bacterial cultures according to the rapid alkaline lysis method described for mini-prep and large scale DNA extractions (Maniatis et al., 1982). Plasmid preparations for nucleotide sequence analysis and mammalian cell transfection were further purified by equilibrium centrifugation in CsCl/ethidium bromide gradients. This technique was based on the method described by Sambrook and co-workers (1989). Briefly, DNA pellets were prepared from 200 ml liquid cultures, washed with 70% ethanol, air dried and resuspended in 5.1 ml TE buffer (Appendix 1). CsCl (5.6 g) was added and dissolved in each sample prior to the addition of 510 µl 10 mg/ml ethidium bromide. This solution was loaded into 2 ultra centrifugation tubes (Quick
Seal 349621, Beckman) and centrifuged at 500,000 g for 6 hours at 20°C with the brake off. The ethidium bromide band containing the circular non-nicked plasmid DNA was removed using a 22 gauge needle attached to a syringe and repeatedly extracted with isoamyl alcohol until no ethidium bromide was visible. DNA was then precipitated by sodium acetate/ethanol precipitation.

2.9 Construction of plasmid eukaryotic expression vectors

2.9.1 Construction of pKC3 based expression constructs

The SV40-based COS cell expression vector pKC3 (Figure 2.1), was constructed by Dr. D. Hanahan (Harvard University, USA) and supplied to Dr. T. Adams (The University of Melbourne). The full length oFSH α-subunit cDNA, clone 3.2 (Bello et al., 1989), which includes an additional unpublished 5' poly T sequence (see Appendix 2), was subcloned into the EcoR1 polylinker site of the pKC3 vector. The correct orientation of the cloned cDNA fragment was confirmed by restriction enzyme digest, and the resultant vector designated pKC3-αFL (full length). An oFSH β-subunit EcoR1/Sac1 coding region fragment of 573 bp (see Figure 3.6), was cloned into the EcoR1/Sac1 polylinker sites of the pKC3 vector. The correct orientation of the cDNA fragment was confirmed by restriction enzyme digests and the construct designated pKC3-βSL (short length).

2.9.2 Construction of the FIII eukaryotic expression vector

The cloning vector pSP72 (Promega) was selected for the assembly and propagation of the FIII vector and FIII-derived eukaryotic expression vector constructs in E.coli. The hMTIIA promoter sequence was supplied in the pMTSV40-1 vector (Friedman et al., 1989) by Dr. P. Kushner (University of California). The promoter sequence contained in this vector includes the 839 bp HindIII/BamH1 3'-deletion mutant (pHS1) described by Haslinger and Karin (1985) and an additional 3' BamH1 linker. The 3' deletion mutant,
Figure 2.1
Restriction map of the pKC3 mammalian cell expression vector. Constructed by Dr. D. Hanahan (Cold Spring Harbor Laboratories, USA)
pHS1, lacks the native hMTII\textsubscript{A} ATG translation initiator codon previously described by Karin and Richards (1982). The pMTSV40-1 hMTII\textsubscript{A} fragment was directionally ligated into the Hind\textit{III}/Bam\textit{H}1 polylinker sites of the pSP72 vector (Promega) to produce the F1 construct (Figure 2.2). An 840 bp SV40 enhancer fragment, also contained in the pMTSV40-1 vector, was isolated by Eco\textit{RV}/Hind\textit{III} digestion and ligated into the unique P\textit{vul}l/Hind\textit{III} cloning sites of the F1 construct. The resultant construct was designated FII. The SV40 enhancer and hMTII\textsubscript{A} promoter sequences were separately cloned into the pSP72 vector in order to reverse the pMTSV40-1-derived orientation of the 2 fragments. The FII enhancer/promoter orientation reflects the more efficient pMTSV40-2 orientation previously described by Friedman and co-workers (1989).

Finally, the 480 bp Bam\textit{H}1 fragment of the pKC3 vector, incorporating the SV40 small t intron and polyadenylation signals, and some polylinker sequence including the Xba\textit{l}, Sal\textit{I} Pst\textit{I} and Hind\textit{III} restriction sites, was ligated into the Bgl\textit{II} site of the FII vector to produce the eukaryotic expression vector designated FIII (see Appendix 3).

2.9.3 Construction of the FIII-oFSH subunit expression vectors: FIII-\alpha FL (full length), FIII-\alpha SL (short length), FIII-\beta FL and FIII-\beta SL

The FIII-\alpha FL and FIII-\beta FL constructs were prepared by ligating the full length oFSH \alpha-subunit (clone 3.2, Appendix 2) and full length oFSH \beta-subunit (clone 6.14, see Figure 3.6) cDNA sequences, isolated as Eco\textit{R}1 inserts, into the unique Eco\textit{R}1 cloning site of the FIII vector respectively.

A truncated 607 bp Sau\textit{3}A\textit{I} \alpha-subunit fragment, encompassing the entire pre-hormone coding region sequence, was ligated into the unique Bam\textit{H}1 cloning site to produce the expression construct designated FIII-\alpha SL. The FIII-\beta SL
Figure 2.2
Diagrammatic presentation of the construction of the FIII mammalian cell expression vector.
expression construct was prepared by ligating the 600 bp EcoR1/SalI fragment of pKC3-βSL vector into the unique EcoR1/SalI sites of the FIII vector.

2.10 Construction of FIII-βSL initiation codon flanking sequence mutants FIII-βSL (M2) and FIII-βSL (M12)

2.10.1 Site directed mutagenesis of the oFSH β-subunit cDNA ATG initiation codon flanking sequences

The 573 bp EcoR1/SacI coding region fragment of the oFSH β-subunit cDNA clone 6.14 was directionally ligated into the bacteriophage vector M13 mp18 (Messing, 1983; Yannisch-Perron et al., 1985) and transformed into the E.coli host strain JM101. Single stranded template DNA for site directed mutagenesis was prepared from recombinant bacteriophage as described by Sambrook and co-workers (1989). Mutant oligonucleotide sequences were synthesized using a Gene Assembler Plus Oligonucleotide Synthesizer (Pharmacia). The 2 mutant oligonucleotides synthesized had the following sequences:

\[
3'\text{GTTCACGGGT}^\text{T}\text{GGTACCTCAAGGCCAGGT} 5' C
\]

Nucleotides differing from the native oFSH β-subunit cDNA sequence are in bold type. The nucleotides complementary to the ATG initiation codon are underlined.

All reagents and methods were supplied in the Oligonucleotide-directed \textit{in vitro} Mutagenesis System (Version-2, Amersham). Briefly, the mutant oligonucleotide is annealed to the single stranded bacteriophage template DNA. The oligonucleotide then primes DNA polymerase (Klenow fragment) mediated complementary strand synthesis. The thionucleotide dCTPαS,
which is incorporated into the complementary strand in place of dCTP, selectively protects the mutant DNA from cleavage by particular restriction enzymes including NciI. Digestion with NciI nicks the non-mutant strand and presents sites for exonuclease III digestion of this strand. The single stranded mutant DNA then provides template DNA for synthesis of the double stranded DNA and \textit{E.coli} transformation.

Mutant bacteriophage were primarily selected on the basis of \textit{NcoI} restriction enzyme digests which identified a mutant specific \textit{NcoI} restriction site. Clones identified by \textit{NcoI} restriction enzyme digestion were further analysed by 5' terminal dideoxy sequence analysis as previously described (see section 2.6).

\textbf{2.10.2 Construction of the \textit{FIII-\betaSL (M2) and FIII-\betaSL (M12) expression constructs}}

The ACCATGG (M2) and GCCATGG (M12) \betaSL mutant cDNA were isolated by \textit{EcoR1/Xba1} restriction enzyme digestion and ligated into the unique \textit{EcoR1/Xba1} cloning sites of the \textit{FIII} expression vector.

\textbf{2.11 Construction of the ovine growth hormone/oFSH \beta-subunit cDNA hybrid expression vector \textit{FIII-\betaSL (HYB)}}

Details describing the construction of the \textit{FIII-\betaSL (HYB)} construct are shown in Figure 2.3. Briefly, a 120 bp \textit{EcoR1/Ahali} (blunt ended) 5' cDNA fragment, incorporating the entire 5' non-coding and signal peptide coding region sequences of the full length ovine growth hormone (oGH) cDNA clone oGH-1 (Adams, 1989), was ligated to a 347 bp \textit{PstI} (blunt ended)/Pvull oFSH \beta-subunit mature peptide coding region cDNA fragment. The resultant hybrid cDNA encodes the entire oGH signal peptide, an intervening glycine residue, and the entire oFSH \beta-subunit mature polypeptide amino acid sequence. The
Figure 2.3
Diagrammatic presentation of the construction of the oGH/oFSH β-subunit hybrid cDNA expression construct [FIII-βSL (HYB)].
terminal oGH signal peptide amino acid residue, which flanks the intervening glycine residue, is also a glycine residue.

2.12 Mammalian cell tissue culture and DNA transfection techniques

2.12.1 COS cell culture

The SV40 transformed African green monkey kidney cell line COS (Gluzman, 1981) was supplied by Dr. Y. Gluzman (Cold Spring Harbor, USA) and maintained in Dulbecco's modified Eagle's medium (DME, Gibco) supplemented with 10% fetal calf serum (FCS, Flow) and 2 mM L-glutamine (Flow) at 37°C in a humidified atmosphere of 5% CO₂. Stocks were prepared, and stored in liquid nitrogen as 1 ml aliquots of 1-5 x 10⁶ cells in growth medium supplemented with 10% dimethyl sulfoxide (DMSO, BDH). Cells were passaged by washing cell monolayers twice with serum free growth medium and elution with trypsin-EDTA solution [0.1% trypsin (1:250, Gibco), 0.27 mM EDTA].

2.12.2 DEAE-dextran mediated COS cell transfection

On the day prior to transfection, COS cell cultures were harvested by trypsinisation and replated (40% confluent) to ensure a cell monolayer of 60-80% confluence on the day of transfection.

Routinely, 60mm culture dishes (Nunc) were transfected with 0.5-2.0 µg non-linearised vector DNA via DEAE-dextran mediated transfection (Sompayrac and Danna, 1981). A 2.5 ml transfection cocktail was prepared by first diluting approximately 10 µl of DNA containing TE solution with 500 µl Dulbecco's A PBS (Oxoid) containing 2 mg/ml DEAE-dextran (M₀ = 500,000, Pharmacia). This solution was then further diluted with 2 ml of L-glutamine supplemented (2 mM) DME.
Following two DME washes, COS cell monolayers were overlayed with the 2.5 ml of transfection cocktail and incubated at 37°C for 4-5 hours. The transfection cocktail was then aspirated and replaced with 2 ml DMSO shock solution (DME supplemented with 2 mM L-glutamine and 10% DMSO). After 2 minutes incubation at room temperature the shock solution was aspirated and carefully replaced with fully supplemented growth medium containing 100 units/ml penicillin and 100 µg/ml streptomycin (Flow). Transfected COS cell cultures were incubated for 48 hours prior to Northern blot analysis or media harvesting for recombinant oFSH estimation using the in vitro porcine testis radioreceptor assay (RRA, Maghuin-Rogister et al., 1978, see section 2.15).

2.12.3 CHO cell culture

CHO cells (CHO-K1, American Type Culture Collection) were supplied by Dr. P. Schofield (Pacific Biotechnology Ltd., Australia) and maintained as described for COS cell cultures (Section 2.12.1) in Alpha minimal essential medium (α-MEM, Gibco) supplemented with 10% FCS. Frozen cell stocks were prepared in the same medium supplemented with 10% DMSO and stored as previously described for COS cells.

2.12.4 Liposome mediated CHO cell transfection

On the day prior to transfection 1.2-1.8 x 10^6 cells were replated per 100 mm culture dish. Non-linearised vector DNA (5-10 µg), and 80 µl 1 mg/ml DOTMA \{N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride, BRL\} liposome reagent, were each separately diluted in 4 ml Opti-MEM medium (Gibco). The diluted DNA and DOTMA solutions were then combined. CHO cell monolayers were washed twice with Opti-MEM prior to the careful addition of the 8 ml transfection cocktail. Following 5 hours incubation at 37°C, an additional 20 ml fully supplemented growth medium containing 100 units/ml penicillin and 100 µg/ml streptomycin was added. After 24 hours the
transfected cell pools were replated on 140 mm culture dishes (Nunc) in 50 ml α-MEM medium containing 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin, and 0.8-1.0 mg/ml (active) G418 sulphate (Geneticin, Gibco). G418 resistant colonies were visible to the naked eye 6 days post transfection in all transfections incorporating the pMC1neo PolyA vector (Stratagene, catalog # 213201, U.S.A.). After 10-14 days, colonies were trypsinised and replated for expansion in fresh selection media.

Roller bottle CHO cell cultures were expanded to confluence in growth medium containing 5-10% FCS. Confluent cultures were maintained in α-MEM containing 25-75 µM ZnSO₄, 100 units/ml penicillin and 100 µg/ml streptomycin. Unless otherwise stated, serum free culture medium was replaced with fresh medium every 2 to 3 days for periods up to 2 weeks.

2.12.5 Isolation and cloning of transfected CHO cell lines expressing high levels of the recombinant oFSH heterodimer and individual oFSH α- and β-subunits
G418 resistant CHO cell transfection cell pools were diluted in G418 supplemented growth medium to enable dilution cloning distribution of approximately 1 cell/well over 2 x 96 well tissue culture plates (Nunc). After 12-14 days, individual wells were examined using an inverted microscope (Olympus) to enable the selection of single colonies. Individual colonies were scored for size and supernatants from each well were assessed for recombinant oFSH by RRA. The 12 highest producing clones were again dilution cloned and assayed. Single colonies of the 2 highest producing clones of each co-transfection were selected and expanded in non-selection media for further analysis, and the preparation of frozen stocks.

High producing cell lines expressing only α-subunit or β-subunit polypeptides, were selected from the subunit specific transfection pools (see Chapter 5).
Transfections were dilution cloned in 96 well tissue culture plates to enable the selection of twelve $\alpha$- and twelve $\beta$-subunit specific single colonies. Selected colonies were expanded and replated at 50% confluence in duplicate 24 well tissue culture plates. High producing colonies were identified by metabolic labelling, immunoprecipitation and SDS-PAGE analysis. The selected colonies were expanded from the non-labelled plate for further examination.

2.12.6 Heavy metal ion induction of transfected mammalian cell cultures

The FIII expression vector (see Section 2.9) incorporates the heavy metal ion inducible hMTII$\alpha$ promoter sequence (Karin et al., 1984a). COS and CHO cell transfection pools and cloned high producing CHO cells lines incorporating the FIII based expression constructs were induced by supplementing growth medium with 25-100 $\mu$M ZnSO$_4$.

2.13 Northern blot analysis of transfected mammalian cell cultures

2.13.1 Isolation of cellular RNA

The following method for the isolation of total RNA was adapted from Chomczynski and Sacchi (1987). Adherent COS and CHO cell monolayers were washed twice with cold Dulbecco's A PBS prior to the addition of the guanidinium thiocyanate denaturing solution (see Section 2.4). The denaturing solution was pipetted several times over the entire culture dish to ensure lysis of all cells and thorough mixing of the cell lysate. Routinely 4 ml of denaturing solution was applied per 100 mm culture dish. Culture dishes were then incubated on ice for 15 minutes prior to collecting the cell lysate.

The cell lysate solution was mixed with 400 $\mu$l 2 M sodium acetate (pH 4.0) prior to the addition of 4 ml acid (water saturated) phenol (Appendix 1). This solution was mixed prior to the addition of 800 $\mu$l of chloroform and vortexed...
for 3-5 minutes. The solution was incubated on ice for 15 minutes, vortexed for 1 minute and centrifuged at 16,000 g and 4°C for 20 minutes. The aqueous supernatant was recovered and combined with an equal volume of isopropanol. After overnight incubation at -20°C, the RNA pellet was recovered by centrifugation (as above) and rinsed with 70% ethanol. The air dried pellet was redissolved in DEPC-treated water and quantitated by spectrophotometric analysis.

2.13.2 Preparation of 32P-labelled cDNA probes

32P-labelled cDNA probes were prepared by random primer extension of denatured double stranded DNA according to the method described by Feinberg and Vogelstein (1983; 1984).

Routinely, 50 ng of cDNA was labelled overnight in the presence of 1-2 µl α32P-dATP (3000 Ci/mmol, code PB10204, Amersham) to a specific activity 3-8 x 10^8 cpm/µg. Probes were denatured prior to hybridisation by the addition of 0.1 volumes 1.0 M NaOH, or by boiling for 3 minutes.

2.13.3 Northern blotting and hybridisation procedures

RNA samples were run on 0.66 M formaldehyde/1% agarose gels according to the method previously described by Fourney and co-workers (1988). RNA was transferred by capillary blotting to Hybond-N nylon membranes (Amersham) and fixed by UV crosslinking according to the manufacturers instructions.

Prehybridisation and hybridisation solutions were prepared as described in Section 2.3. Filters were prehybridised at 65°C for a minimum of 3 hours prior to overnight incubation at the same temperature in the hybridisation solution. Filters were rinsed twice at 60-65°C in 2 x SSC, 0.2% SDS prior to high...
stringency washing at 65°C in 0.1 x SSC, 0.1% SDS. Washes (10 minutes each) were repeated a minimum of 3 times or until background radioactivity levels were no longer detectable. Filters were blot dried prior to overnight exposure to X-ray film (XAR-5, Kodak) at -70°C with intensifying screens.

2.14 SDS-PAGE analysis of transfected mammalian cell culture media and cell lysate immunoprecipitates

2.14.1 Metabolic labelling of cell cultures

In isolating and characterising the polypeptide subunits of highly purified oFSH, Grimek and McShan (1974) reported that methionine was not present in the β-subunit. Therefore, metabolic labelling experiments examining recombinant oFSH expression incorporated 35S-cysteine (>600 Ci/mmol, code SJ15232, Amersham) as the radiolabelled amino acid rather than the more routine 35S-methionine. Continuous labelling and pulse chase experiments were performed as described by Keene and co-workers (1989a) with the following modifications.

Medium free of cysteine for use in both COS cell and CHO cell labelling experiments was prepared using the RPMI Select Amine Kit (Gibco). L-serine was also excluded as a precursor substrate for cysteine. Confluent COS cell and CHO cell monolayers (35mm culture dishes) were labelled for 6-18 hours under continuous labelling conditions, or 20 minutes for time course biosynthesis analysis. Continuous cultures were labelled with 1.2 ml cysteine free RPMI containing 100 µCi/ml 35S-cysteine, 10% dialysed FCS, 100 units/ml penicillin and 100 µg/ml streptomycin. Pulse chase experiments utilised confluent cell monolayers in 24 well tissue culture plates and labelling media containing 250µCi/ml 35S-cysteine.
2.14.2 **Preparation of polyclonal mouse oFSH antisera**

oFSH for immunisation was supplied by Mr. R. Fiddes (The University of Melbourne) following purification on a monoclonal antibody [MAb, (87-70)] affinity column. This preparation is >95% pure by SDS-PAGE analysis and has a specific activity of 220-240 units/mg. Mice received primary intraperitoneal (IP) immunisations of 100 µg oFSH in Freund's complete adjuvant and subsequent secondary and tertiary IP immunisations of 100µg oFSH in Freund's incomplete adjuvant at 2 and 6 weeks post primary injection. Sera were collected 2 weeks later for Western blot analysis of oFSH heterodimer and dissociated subunit reactivity (see Appendix 4).

2.14.3 **Immunoprecipitation of metabolically labelled oFSH subunits**

Metabolically labelled cell culture media samples were centrifuged at 2,000 g and 4°C for 5 minutes in a Microfuge 11 centrifuge (Beckman) to remove contaminating cells and debris. Cell monolayers were washed twice with cold Dulbecco's A PBS, aspirated and overlayed with lysis solution containing 0.05 M Tris HCl (pH 8.0) and 1% Triton-X100 (Ajax) for 15 minutes on ice. Cell lysates were centrifuged as above to remove cell wall debris and cell nuclei. Culture media and cell lysate supernatants were precleared by overnight mixing with 4 µl normal rabbit serum and 40 µl 50% v/v protein-G Sepharose in PBS at 4°C. Precleared supernatants were collected by centrifugation prior to the addition of 5 µl oFSH antibody solution and a further 40 µl protein-G Sepharose suspension. The oFSH antibody solution contained a mixture of polyclonal and monoclonal mouse oFSH antibodies. Immunoprecipitation experiments (unpublished) had previously established the oFSH α-subunit specificity of the MAb 87-70 (supplied by R. Fiddes, The University of Melbourne), while Western blot analysis of dissociated oFSH subunits was used to establish the heterodimer and β-subunit specificity of three polyclonal mouse antisera (Appendix 4). Equal volumes of each polyclonal antiserum
and a 2.8 mg/ml purified MAb (87-70) preparation were combined to produce the oFSH antibody solution.

After overnight rotational mixing, immune complexes were isolated by centrifugation and washed 3 times in cold Dulbecco's A PBS. Labelled proteins were dissociated for SDS-PAGE analysis by elution in 0.1 M NaCl, 0.1 M glycine (pH 2.0), or by boiling in non-reducing or reducing sample buffers. Non-reducing buffer was prepared as 0.05 M Tris HCl (pH 6.8) containing 0.05% bromophenol blue, 20% glycerol and 5% SDS. Reducing buffer contained an additional 4% dithiothreitol.

2.14.4 SDS-PAGE analysis and autoradiography of immunoprecipitates

Samples were prepared as described in non-reducing buffer and analysed on 15% polyacrylamide gels according to the method described by Laemmli (1970) using the Mini-Protean II electrophoresis kit (Biorad). All gels (except those shown in Figure 4.2) were run for an additional 20 minutes after the sample dye front had run off, to enable better resolution of α- and β-subunit polypeptide bands. For autoradiography, gels were transferred from the fixing solution to a fluorographic enhancing reagent (Amplify, Amersham) and incubated for 30 minutes with gentle rocking at room temperature. Gels were dried using a slab gel drier (Biorad) and exposed at -70°C to pre-flashed Kodak XAR-5 film for periods up to 2 weeks as required.

2.15 Affinity purification of recombinant oFSH

The affinity resin utilised for the purification of recombinant oFSH was prepared by Mr. R. Fiddes (The University of Melbourne). This resin was prepared by coupling approximately 3 mg of DE52 purified MAb 87-70
Affinity extractions combined approximately 1 litre of prefiltered (0.45µm) CHO cell culture medium and 1 ml (packed gel volume) of oFSH affinity resin. The suspension was placed in a roller bottle and mixed overnight at by rolling at 60 r.p.m at 4°C. Bottles were left upright for 2 hours to permit the resin to settle and the majority of the extracted culture medium was then decanted. The remaining suspension was recovered and packed as a 1 ml column for washing and elution. Columns were washed with 15 ml Dulbecco's A PBS and eluted with 3 ml 0.1 M NaCl, 0.1 M glycine pH 2.0, or 3 ml 0.1 M NaCl, 0.1 M glycine pH 3.0 as indicated in text. The eluted samples were extensively dialysed prior to freeze drying and storage at 4°C.

2.16 Isoelectric focussing (IEF) analysis by Western transfer

Samples for IEF were prepared by affinity purification as described in Section 2.15 and focussed on 4.85% acrylamide gels over a pH gradient ranging from pH 3.0 to pH 10. Gels were prepared and run using equipment and reagents supplied in the Mini IEF Cell (model 111, catalogue # 170-2975, Biorad), according to the manufacturers instructions with the following modifications. Following polymerisation, gels were inverted and allowed to air dry in a laminar flow tissue culture hood for 15 minutes. Samples and standards were loaded in volumes of up to 2 µl and allowed to diffuse into the gel for 10 minutes prior to focussing.

At the completion of the IEF run, the gels were over-layed with dry chromatography paper (Chr 3MM, Whatman) and gently pressed to ensure adhesion of the gel to the paper. Gels were then peeled away from the IEF support film and submerged in Western transfer buffer (25 mM Tris, 0.19 M
glycine, 20% methanol). Proteins were transferred to nitrocellulose membranes (0.1 μm, reference # 402096, Schleicher and Schuell, Germany) by routine Western transfer for 1 hour at 100 volts using the Mini Protean II Trans Blot Cell (Biorad). Membranes were blocked by incubating in blotto (4 g skim milk powder per 100 ml Dulbecco's A PBS) for 1 hour at room temperature and then incubated in blotto containing a mixture of three oFSH antisera (Appendix 4) diluted 1:500 in blotto. After a further 1 hour room temperature incubation the Western blot was rinsed 3 times in blotto and placed in the second antibody solution containing horse radish peroxidase (HRP) labelled rabbit anti-mouse IgG (Silenus, Australia) diluted 1:500 in blotto. After a final 1 hour room temperature incubation blots were washed with five changes of wash buffer (0.05% Tween 20 in Dulbecco's A PBS) and developed in the presence of 0.06% diaminobenzidine.

2.17 In vitro radioreceptor assay for oFSH

The porcine testis plasma membrane RRA for oFSH was based on the method described for porcine FSH by Maghuin-Rogister and co-workers (1978) with the following modifications.

oFSH for iodination (NIDDK-oFSH/l-1, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Maryland, USA) was dissolved in 0.05 M sodium phosphate buffer (pH 7.5), at 250 μg/ml, and dispensed as 20 µl aliquots in glass capillary tubes for storage at -70°C. Routinely, 5 μg of oFSH was labelled with 0.5 mCi ¹²⁵I (100mCi/ml, code IMS 30, Amersham) using IODO-GEN iodination reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril, Pierce) coated glass tubes. Tubes for iodination reactions were prepared by evaporative drying of 5 µl IODO-GEN solution (2.4 mg IODO-GEN/ml chloroform) under dry high purity nitrogen gas (OFN Nitrogen, CIG). The ¹²⁵I solution and 20 µl 0.5 M sodium phosphate pH 7.4
were added to the reaction tube for 5 minutes equilibration at room temperature prior to the addition of the oFSH. Iodination reactions were incubated for 10 minutes at room temperature with occasional mixing. Radiolabelled oFSH was separated from free $^{125}$I by size selection chromatography on prepacked Sepharose C10 columns (Pharmacia) and stored at 4°C for up to 3 weeks.

The oFSH standard used in the assay was an in-house standard based on the NIDDK-oFSH-RP-1 standard (NIDDK, NIH, Maryland, USA) in the hCG augmentation assay described by Steelman and Pohley (1953). Porcine testis plasma membrane preparations were prepared and stored as described by Maghuin-Rogister and co-workers (1978). Briefly, fresh immature porcine testis were homogenised using a standard household blender on high speed for 2-3 minutes and filtered through cotton gauze. The filtrate is further cleared by low speed centrifugation (120 g) for 10 minutes. The plasma membrane pellet is collected by 20 minute centrifugation at 27,000 g and resuspended in 0.05 M Tris HCl pH 7.4 (10 to 20 mg wet weight / ml) by brief re-homogenisation. Membrane preparations are aliquoted and stored frozen at -20°C until required.

Duplicate standards and samples were prepared in 0.05 M Tris HCl (pH 7.4), 0.1% ovalbumin (Sigma), 3.4% sucrose (assay buffer). Where noted, standards were prepared in assay buffer containing tissue culture growth medium. Reagents were added to the assay tubes in the following order; 100 µl assay buffer, 100 µl sample or standard, 100 µl assay buffer containing 50,000-80,000 cpm $^{125}$I oFSH, and 100 µl porcine testis plasma membrane homogenate (1 volume homogenate diluted in an equal volume of assay buffer). Equilibrium binding reactions were incubated with shaking for 3 hours at room temperature. Plasma membranes were collected by centrifugation at
12,000 g and 4°C for 15 minutes, washed twice by resuspension in cold assay buffer and centrifugation. Radioactivity was measured using a Packard gamma counter (Model 500C, Packard USA). Results are presented as the mean of duplicate samples or controls.

2.1.8 In vitro Sertoli cell bioassay for oFSH

The oFSH in vitro bioassay was based on the method described by Ritzen and co-workers (1982) and was conducted by Dr. D. Robertson (Prince Henry’s Institute of Medical Research, Melbourne). Briefly, the Sertoli cells were isolated from the testis of 10 day old rats by enzymatic and mechanical digestion and incubated in DMEM medium containing 0.1% BSA in 48 well tissue culture plates at 37°C. After 24 hours the cells were washed twice with Hank’s buffered salt solution to remove non-adherent contaminating cells, and incubated for 2 hours in medium containing the oestradiol precursor 19-hydroxy-4-andosterone-3, 17-dione. Media containing a phosphodiesterase inhibitor (3-isobutyl-1-methyl-xanthine) and the diluted FSH samples were added prior to a further 24 hour incubation at 37°C. Culture supernatants were then removed and assayed for oestradiol levels by RIA.
CHAPTER THREE

ISOLATION AND NUCLEOTIDE SEQUENCE ANALYSIS OF cDNA SEQUENCES ENCODING THE OVINE FSH β-SUBUNIT
3.1 Introduction

FSH is a highly potent glycoprotein hormone of low abundance synthesized in the anterior pituitary gland. Pituitary levels of the α-subunit polypeptide are considerably higher than those of the FSH β-subunit polypeptide, reflecting the multiple role of the α-subunit which also associates with the β-subunits of the pituitary glycoprotein hormones LH and TSH (see Chapter 1). Monoclonal antibody affinity chromatography of bovine (Miller et al., 1987) and ovine (R.Fiddes, personal communication) pituitary homogenates suggest levels for the FSH heterodimer ranging from 5-22.2 mg/kg.

The low level of FSH β-subunit polypeptide suggests that β-subunit mRNA transcripts are rare, or possibly unstable, an observation supported by cDNA cloning studies. FSH β-subunit cDNA clones have been isolated and sequenced for several species including bovine (Esch et al., 1986; Maurer and Beck, 1986), porcine (Kato, 1988), rat (Maurer, 1987) and human (Bishop et al., 1990). Esch and co-workers (1986) isolated 8 positive clones from 1 x 10^6 λgt10 recombinant plaques using oligonucleotide probes and subsequent rescreening with labelled bFSH β-subunit cDNA fragments. Maurer and Beck (1986) isolated 32 hybridising clones from a λgt11 library of 600,000 plaques using a pFSH β-subunit cDNA probe, although only two clones contained confirmed FSH β-subunit sequences of sufficient size to warrant further investigation. One pFSH β-subunit clone has been isolated from 800,000 λgt11 plaques using a rabbit anti-hFSH β-subunit antiserum, and a further four pFSH β-subunit clones were isolated from 1 x 10^6 plaques using oligonucleotide probe hybridisation (Kato, 1988).

The low abundance of the FSH β-subunit cDNAs is made more apparent when isolation frequencies for the common gonadotrophin α-subunit cDNAs are
compared. For example, Nilson and co-workers (1983) isolated 13 bFSH α-subunit cDNA clones from 1000 plaques while Erwin and co-workers (1983) isolated 7 positive clones from an enriched library of 2500 clones. Oligonucleotide hybridisation screening of 15,000-20,000 size-selected cDNA clones identified 20 oFSH α-subunit cDNA clones (P.Bello, unpublished).

Northern blot and cDNA sequence analyses of bovine (Maurer and Beck, 1986) porcine (Kato, 1988) and rat (Maurer, 1987) FSH β-subunit transcripts indicate full length transcripts of 1.7 - 2.0 kb in length. Maurer and Beck (1986) reported the combined nucleotide sequence of 2 overlapping bovine FSH β-subunit cDNA clones as having 46 bp of 5' non-coding region sequence, 387 bp of coding region sequence encoding a 19 amino acid signal peptide and a 110 amino acid mature peptide, and 1295 bp of 3' non-coding region sequence. A discrepancy between the 1728 bp cDNA sequence and the estimated pituitary mRNA transcript of 2.0 kb established by Northern blot analysis was attributed to several hundred bp of poly (A)+ sequence associated with the pituitary mRNA but not included in the reported cDNA sequence.

This chapter reports on the oligonucleotide hybridisation screening of two ovine lamb pituitary cDNA libraries and the subsequent isolation and nucleotide sequence analysis of several oFSH β-subunit cDNA clones.
3.2 Results

3.2.1 Isolation and nucleotide sequence analysis of oFSH β-subunit cDNA sequences

The primary structures for the oFSH α- and β-subunit polypeptides have been established (Sairam, 1981; Sairam et al., 1981) and are presented for comparison with other gonadotrophin subunit amino acid sequences in Figures 1.1 and 1.2. The oligonucleotide probes described in this chapter were designed on the basis of the oFSH β-subunit amino acid sequence described by Sairam and co-workers (1981). The nucleotide sequence details and estimated melting temperatures (T_M) for the two degenerate and single non-degenerate oFSH β-subunit oligonucleotide probes are shown in Figure 3.1.

Two ovine lamb pituitary cDNA libraries were constructed in λgt10 (see Chapter 2) including a fully representative cDNA library and a size-selected cDNA library. Similar cDNA synthesis and cloning efficiencies were obtained in the construction of both libraries and as such only the results for the more technically involved size-selected library are presented. Results including efficiencies of poly (A)+ RNA extraction and purification, and cDNA synthesis, size-selection, vector ligation and packaging are presented in Figure 3.2. The optimal cDNA: λgt10 arms ligation ratio was established as 40 ng cDNA/µg λgt10 arms. At this ratio, a cloning efficiency of 1.9 x 10^6 pfu/µg size-selected cDNA resulted in a cDNA library of approximately 1.4 x 10^6 pfu. The percentage of clones incorporating cDNA inserts > 300 bp was approximately 75%. The integrity of the poly (A)+ RNA for cDNA synthesis was monitored prior to use by Northern blot analysis using an ovine growth hormone (oGH) cDNA probe (Adams, 1989) and the results are shown in Figure 3.3. A single hybridising band of approximately 1 kb is evident with
Figure 3.1

Synthetic oligonucleotide probes for the isolation of oFSH β-subunit cDNA clones from ovine lamb pituitary λgt10 cDNA libraries.

**C-terminal degenerate oligonucleotide**

Published amino acid sequence*: Glu Cys His Cys Gly Lys Cys Asp

Oligonucleotide sequence (24-mer):

\[ 3' \text{CT} \text{A} \text{AC} \text{G} \text{GTG} \text{AC} \text{G} \text{CCN} \text{TTT} \text{AC} \text{G} \text{CTG} 5' \]

Degeneracies: 512

Estimated TM: Maximum = 80°C
Minimum = 66°C

**N-terminal degenerate oligonucleotide**

Published amino acid sequence*: Asn Ile Gln Lys Thr Cys

Oligonucleotide sequence (17-mer):

\[ 3' \text{TTG} \text{TAG} \text{GTT} 5' \]

Degeneracies: 72

Estimated TM: Maximum = 50°C
Minimum = 40°C

**C-terminal non-degenerate oligonucleotide**

Amino acid sequence for the ovine* and bovine** FSH β-subunits:

\[ +47 \text{Gln Lys Thr Cys Thr Phe Lys Glu Lue Val} +56 \]

Bovine cDNA sequence**:\[ 5' \text{CAG AAA ACG TGT ACC TTC AAG GAG CTG GT-} 3' \]

Oligonucleotide sequence (29-mer):

\[ 3' \text{GTC TTT TGC ACA TGG AAG TTC CTC GAC CA} 5' \]

Non-degenerate

Estimated TM: ~ 86°C

---

* Sairam et al., (1981)
** Esch et al., (1986)
Figure 3.2
Results for the isolation of ovine lamb pituitary poly(A)+ RNA and the subsequent synthesis and size-selection of double stranded cDNA.

8.8 g ovine lamb pituitaries

\[\downarrow\]

4.5 mg total RNA

\[\downarrow\]

20 µg double poly(A)+ selected RNA

5 µg poly(A)+ RNA

\[\downarrow\]

933 ng first strand cDNA (18% incorporation)

\[\downarrow\]

1051 ng second strand cDNA (effectively 100% incorporation)

\[\Rightarrow\] 14.3% lost through extraction procedures

\[\downarrow\]

Size selection of double stranded cDNA

\[\downarrow\]

719 ng double stranded cDNA > 300 bp (45% recovered)
Figure 3.3
Autoradiograph showing Northern blot analysis of oGH* transcripts in a 2 \( \mu \)g sample of ovine lamb pituitary poly(A)+ RNA. Prehybridisation and hybridisation incubations of two and four hours respectively were performed as described in Chapter 2. Filters were exposed to X-ray film overnight with intensifying screens at -70\(^{\circ}\)C after high stringency washing.

* oGH cDNA for \( ^{32}\)P-labelling was supplied by T. Adams (The University of Melbourne) as clone oGH-1 (Adams, 1989).
no sign of mRNA degradation. First and second strand cDNA products (Figure 3.4), and fractionated cDNA samples resulting from size-selection chromatography (Figure 3.5), were analysed by agarose gel electrophoresis and autoradiography. Double stranded cDNA sequences ranging up to greater than 6.6 kb are evident in both autoradiographs.

An initial screening of 500,000 recombinant bacteriophage from the non-size-selected cDNA library with the two degenerate oligonucleotide probes identified eight hybridising clones. While the longer C-terminal oligonucleotide probe hybridised strongly to each clone, the N-terminal 17-mer was unreactive. Recombinant plaques were isolated by dilution cloning and subcloned into the bacteriophage M13 mp18 vector for nucleotide sequence analysis. Results for 5' and 3' terminal sequence analysis revealed that none of the cloned cDNAs encoded for the previously established oFSH α-subunit amino acid sequence. However, screening of 300,000 size-selected cDNA library clones with the non-degenerate oligonucleotide probe identified five hybridising clones. Each clone also hybridised with the C-terminal degenerate probe, but were again unreactive with the N-terminal probe. The three largest cDNA clones, designated 6.14, 1.1 and 1.2, were selected for further analysis.

Nucleotide sequence analysis of the oFSH β-subunit cDNA clone 6.14 (Figure 3.6) shows the clone is 1589 bp in length, and includes the entire coding region sequence for a 129 amino acid oFSH β-subunit pre-hormone. The hydrophobic signal peptide is 19 amino acids in length and includes five cysteine residues. Comparison of the predicted amino acid sequence with that previously established by amino acid sequencing (Sairam et al., 1981) shows a conservative amino acid substitution Ala for Thr at position +49, a non-conservative substitution Arg for Ser at position +88, and a single
Figure 3.4
Autoradiograph showing first (1\textsuperscript{st}) and second (2\textsuperscript{nd}) strand cDNA products run on a 1\% agarose/NaOH denaturing gel. \textit{HindIII} digested bacteriophage \(\lambda\) markers (\(\lambda\ \textit{HindIII}\)) are also indicated.
Figure 3.5
Autoradiograph showing fractionation of double stranded cDNA by size-selection chromatography on a prepacked CL-B4 Sepharose column (Pharmacia). Samples of each fraction were analysed by 1.0% agarose gel electrophoresis and overnight autoradiography. The fractions are numbered (1-8). Fractions 1-2 were pooled for cDNA library construction. EcoR1/HindIII and HindIII digested bacteriophage λ markers are also indicated.
Figure 3.6

Nucleotide sequence analysis and predicted amino acid sequence for the oFSH β-subunit cDNA clone 6.14. Amino acids with negative numbers represent amino acids of the signal peptide, while +1 represents the first amino acid of the mature oFSH β-subunit polypeptide as previously determined by amino acid sequencing (Sairam et al., 1981). The SacI restriction enzyme site used for 3’ cDNA truncation (Chapter 4) is also indicated.

<table>
<thead>
<tr>
<th>Site of N-linked oligosaccharide attachment.</th>
</tr>
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<tbody>
<tr>
<td>Amino acids differing from the published oFSH β-subunit polypeptide sequence (Sairam et al., 1981).</td>
</tr>
<tr>
<td>Site of the single amino acid exclusion.</td>
</tr>
<tr>
<td>Termination codon</td>
</tr>
<tr>
<td>ATTATA sequence motif repeats.</td>
</tr>
<tr>
<td>Potential polyadenylation signals.</td>
</tr>
</tbody>
</table>
C-terminal region amino acid exclusion at position +109. Both of the predicted amino acid substitutions and the single amino acid exclusion were confirmed by DNA sequence analysis of the coding region sequences of clones 1.1 and 1.2 (sequence data not shown). Nucleotide sequence analysis of clone 1.2 shows this clone is 180 bp shorter than clone 6.14 at the 5' end, and includes an additional 300 bp of untranslated sequence at the 3' end. The additional 3' non-coding region sequence was also present in clone 1.1. Restriction enzyme mapping and partial sequence analysis of clone 1.1 supports the conclusion that this clone is a cDNA hybrid between an oFSH β-subunit cDNA and another unidentified cDNA sequence. No internal EcoR1 sites could be identified by restriction enzyme digestion, suggesting the hybrid formed via blunt end ligation prior to linker addition. The combined nucleotide sequence of clones 6.14 and 1.2 is shown in Figure 3.7. Noteworthy features of the combined 3' non-coding region sequence include seven ATTTA sequence motifs and five possible polyadenylation signals.

In order to establish whether the combined oFSH β-subunit cDNA sequence (Figure 3.7) represents a full length mRNA transcript, the size and diversity of oFSH β-subunit mRNA transcripts in lamb pituitary poly (A)+ was established by Northern blot analysis. The probe selected for hybridisation was a 573 bp EcoR1/Sac1 coding region fragment of clone 6.14. Results show that a single hybridising band of approximately 2.0 kb was identified (Figure 3.8).

3.2.2 Comparison of the ovine and bovine FSH β-subunit cDNA sequences

Comparison of the combined oFSH β-subunit cDNA sequence with that of the bovine sequence (Figure 3.9) gives an overall homology of 85%, despite the unique 3' non-coding region sequence of 149 bp found in the ovine cDNA sequence. Comparison of ovine and bovine FSH β-subunit cDNA
**Figure 3.7**

Combined nucleotide sequence of the FSH β-subunit cDNA clones 6.14 and 1.2.

* ACAAGCGACCTGCTACGGAAAATCTCACGACTCCACAGTTACCAAGTGCCCAGGATGAAGTCCGTCCAGTTCTGCTTCCTTTTCTGTTG

*   90

* GTGGAGAGGAAYcYf;rYr( A(;AAC,rTf;çS;A(:(7ThAÇS'AACATCACCATCACGGTGGAGALAGAGGAATGTAGCTTCTGCATAAGCATUA

*   180

* CACCACGTGTTGCGACCTATTGCTACACCGCAGGCGACTTGGTGTACAAGGACCCAGCGAGGCCCAACATCCAGAAAGCATGTACCTTCPA

*   270

* GGCCAAAGTGACCGCGACACTGACTGCACCGTGCGAGGCCTGGGGCC : AAT AGAGC

*   360

* AGCAGCTGCTTTGAGCTGCCTACCCTTAAAGGACCAAAACATCCACAAGATCTCTTGCTTTCAAGGTCAAGGAAGAGTGACCTAGGCTGCAGACCAC

*   450

* GCCCGGGAGCTGGTCTACCAGACGCTrAAACTf;CSTfY;CTf;Tf;CCCACSATf; CAC;ArTCCCTf;TAf7Af7f:TACCÇAf;TACCCArTCAATCTC

*   540

* CGGCAAGTGTGACCGCGACAGCACTGACTGCACCGTGCGAGGCCTGGGGCC : AAT AGAGC

*    630

* AGCAGCTGCTTTGAGCTGCCTACCCTTAAAGGACCAAAACATCCACAAGATCTCTTGCTTTCAAGGTCAAGGAAGAGTGACCTAGGCTGCAGACCAC

*    720

* CTTCCTCTTAAAGCTAGGAAACCTGCCATGATTGTAGCCTCAATGCCTAACACTCCACATAGAATTTAGTAAGAACTTA

*   810

* ATAAAGGCTTTCTTTAAAGAAATAAGTACGAGAAGAGGATTGTTAGGCTGCAGCTGC

*    900

* GCCCGGGAGCTGGTCTACCAGACGCTrAAACTf;CSTfY;CTf;Tf;CCCACSATf; CAC;ArTCCCTf;TAf7Af7f:TACCÇAf;TACCCArTCAATCTC

*   990

* CGGCAAGTGTGACCGCGACAGCACTGACTGCACCGTGCGAGGCCTGGGGCC : AAT AGAGC

*   1080

* CTCCACGGGAGGGGGGCTACGCTCAGGTCTCGCTCGCTCCTCCTCTCTGACCAGGAGATGACAGAAGATGACGACGATGAAACATCTCTGAT

*   1170

* CAGCCCTACTGACTCTGCTCTTCCAGAAGGGGAGTCCAGGAATGAGGAGGGGTGGGCGGCGGCACACCCACCCACAGGGCTGCTTCTGAC

*   1260

* GAGAAACCAGGGGAGGCGCTCCGACCTCATGAGGTCGAGAGGCTCAAGGCGACGACGACGACCTCCAC

*   1350

* CACACTATTTAAAAAGTGGCAACAAAYTCTGTCAAGGGCTCTGGAACCCCTTCTTCCGGGAGATACATAGATCCATATCTTACCTACCCCGCTGGC

*   1440

* GTAGGCTCTACTCTTTGGAAAGAGGTTGATTGAGAGAAGAGGTAAGTTGACACTTAATGATAGAAATCTTATTATG

*   1530

* AATAACATTATGTAAGAAAGATGCCTTCTTTAAAGAAATAAGCAGCATAAACAAACCCTCCAGGTCCCCCTGC

*   1620

* CAGGCCCTACTGACTCTGCTCTTCCAGAAGGGGAGTCCAGGAATGAGGAGGGGTGGGCGGCGGCACACCCACCCACAGGGCTGCTTCTGAC

*   1710

* TGGTACCTCAGTTATGATTCTGCTCACTCCTAAACTAAATTAAATTATTATTAAAAAAAGGATCCTTACCAAGAGTCA

*   1800

* TAGGTTGGAATTTTTTTCGGCAATGAAAGGCAATCGATAAAAATGCTCTGCTTGTATACATGATTATTTTAAATAAA

*   1899

---

**Coding region sequence.**

* Translation initiation codon.

*** Termination codon.

---

**ATTTA sequence motif repeats.**

---

**Potential polyadenylation signals.**

---

**oFSH β-subunit cDNA clone 1.2 sequence.**

**Note:** The additional 3" non-coding region sequence present in FSH β-subunit cDNA clones 1.1 and 1.2, but not seen in clone 6.14, was established by unidirectional dideoxy sequence analysis only.
Figure 3.8

Autoradiograph showing Northern blot analysis of 2 µg ovine lamb pituitary poly(A)+ RNA. The blot was hybridised overnight with a $^{32}$P-labelled EcoR1/Sac1 (573 bp) oFSH β-subunit coding region fragment of clone 6.14. HindIII digested bacteriophage λ markers are also indicated (λ HindIII).
Figure 3.9
Comparison of the combined oFSH β-subunit cDNA sequence (established from cDNA clones 6.14 and 1.2) with that of the published bovine FSH β-subunit cDNA sequence (Esch et al., 1986).

Coding region sequence.

---

ATTTA sequence motif/repeats.
coding region sequences reveals 95% homology. Of the 18 nucleotide mismatches ten are silent and the remaining eight result in conservative amino acid substitutions. Amino acid sequences for the ovine and bovine FSH β-subunits, and a number of other species are presented in Figure 1.1.

3.2.3 **Isolation and nucleotide sequence analysis of oFSH α-subunit cDNA sequences**

A cDNA encoding the common α-subunit of the ovine pituitary glycoprotein hormones has also been isolated from the size-selected cDNA library and sequenced by a fellow Ph.D. student Mr. Paul A. Bello (Bello *et al.*, 1989; see Appendix 2). It should be noted however, that the clone analysed (clone 3.2) and subsequently utilised in all αFL expression vectors (see Chapter 2), has an associated 5' cloning artefact of approximately 70 dTTP residues which was not included in the published cDNA sequence (Bello *et al.*, 1989).
3.3 Discussion

Three confirmed oFSH β-subunit cDNA clones have been isolated from a size-selected ovine lamb pituitary cDNA library, cloned into the phage vector λgt10, by screening replicate filters with 32P-labelled oligonucleotide probes. Clone 6.14 has been fully sequenced and is 1589 bp in length. Partial sequence analyses of clones 1.1 and 1.2 provided additional 3' non-coding sequence not evident in clone 6.14. The combined cDNA sequence of clones 6.14 and 1.1 (Figure 3.7) suggests the short poly (A)+ tail associated with clone 6.14 is in fact an A rich region of the oFSH β-subunit mRNA which has provided sufficient poly (A)+ sequence to enable oligo (dT) priming of reverse transcriptase mediated first strand synthesis. The predicted amino acid sequence for the mature β-subunit polypeptide shows the 12 cysteine residues, maintained in both number and position in rat, human, porcine, bovine and ovine FSH β-subunits (see Figure 1.1), and necessary for the formation of six intra-chain disulphide bonds (Pierce and Parsons, 1981), have been conserved. This conservation of cysteine residues and the fact that the β-subunits of one species readily recombine with the α-subunits of other species (Pierce and Parsons, 1981), strongly indicates a conservation in tertiary structure between different β-subunits and therefore a conservation in disulphide bridge formation. In reviewing disulphide bridge assignments for cysteine residues of the gonadotrophin subunits, Ryan and co-workers (1987) established the oFSH β-subunit assignments Cys+86-Cys+93 and Cys+19-Cys+103 as correct.

The two Asn residues necessary for the attachment of two N-linked oligosaccharide side chains have been maintained at positions +6 and +23 of the β-subunit polypeptide within the appropriate glycosylation site consensus sequences Asn-Ile-Thr and Asn-Thr-Thr respectively (for reviews see Snider, 1984; Kornfeld and Kornfeld, 1985). One other Asn residue is encoded for although this residue is not presented in the context of the described consensus
sequences. The "CAGY" sequence Cys-Ala-Gly-Tyr, which is believed to have a role in receptor recognition, or more probably subunit association (Mullin et al., 1976; Hayashizaki et al., 1989), has also been maintained at positions +27 - +30.

Previous reports describing the bovine (Esch et al., 1986; Maurer and Beck, 1986) and rat (Maurer, 1987) FSH β-subunit cDNA sequences have made reference to the long 3' untranslated sequences associated with these sequences. Northern blot analyses of bovine (Maurer and Beck, 1986), porcine (Kato, 1988) and rat (Maurer, 1987) pituitary RNAs have in each case identified a single FSH β-subunit mRNA band ranging in size from 1.7 to 2.0 kb. Similarly, Northern blot analysis of ovine lamb pituitary RNA (see Figure 3.8) has identified a single mRNA transcript of approximately 2.0 kb. These transcripts are considerably larger than mRNA transcripts encoding the LH and TSH β-subunit polypeptides which average approximately 650-700 bp (Chin et al., 1983; Gurr et al., 1983). The functional significance, if any, for the long 3' untranslated FSH β-subunit sequence has not been established.

The 3' non-coding region sequence established for the oFSH β-subunit (Figure 3.7) includes five potential polyadenylation signals. While the 2.0 kb oFSH β-subunit transcript evident in the Northern blot analysis of ovine lamb pituitary poly(A)+ RNA (Figure 3.8) would indicate only one, probably the most distal of these signals is utilised, a more recent Northern blot analysis of ovine pituitary poly(A)+ RNA has demonstrated two oFSH β-subunit mRNA transcripts of approximately 1.3 kb and 2.3 kb (Montgomery et al., 1990). Sequence analyses of several bFSH β-subunit cDNA clones (Esch et al., 1986) has also indicated multiple polyadenylation site usage. The nucleotide sequence for one bovine cDNA clone (Esch et al., 1986) has shown that, as in the human example (Jameson et al., 1988), the polyadenylation signal overlapping the translation termination codon is also active.
Seven AUUUA sequence motif repeats have been identified in the oFSH β-subunit 3' untranslated region (Figure 3.7). The sequence motif AUUUA, has also been identified in the 3' non-coding region of many cytokine genes and proto-oncogenes and may constitute a potential mRNA destabilising sequence with a functional role in post-transcriptional control of mRNA stability (Cosman 1987). An mRNA destabilising effect was first demonstrated in association with 3' non-coding region sequences of granulocyte-macrophage colony-stimulating factor (GM-CSF), by inserting a 51 bp AT-rich 3' sequence, containing six copies of the AUUUA encoding motif, into the 3' non-coding region of the rabbit β-globin gene (Shaw and Kamen, 1986). Steady state mRNA levels in transfected NIH3T3 cells show that the rabbit β-globin-3' AU-rich mRNA levels were only 3% as high as those of the GC-rich control and native β-globin mRNA levels. Furthermore, deletion or replacement of c-fos mRNA 3' AU-rich sequences has resulted in more stable mRNA transcripts (Wilson and Treisman, 1988). More recently a cystolic protein which binds specifically to RNA transcripts containing four repeats of the AUUUA mRNA sequence motif has been identified (Malter, 1989). The protein, adenosine-uridine binding factor (AUBF), demonstrates very stable rapid RNA binding and may target the protein-RNA complex for rapid cytoplasmic degradation. It is interesting to note that the bFSH β-subunit cDNA (Maurer and Beck, 1986) encodes six 3' non-coding region AUUUA sequence motif repeats. The rat genomic DNA sequence (Gharib et al., 1989) codes for an mRNA transcript containing two AUUUA sequence motifs. Published porcine (Kato, 1988) and rat (Maurer, 1987) cDNA sequences contain insufficient 3' non-coding region sequence to fully establish the presence and number of AUUUA sequence motifs.

The human FSH β-subunit gene described by Jameson and co-workers (1988) transcribes four distinct mRNA transcripts by utilising a combination of alternate
splicing and polyadenylation. The longer 1.8 kb transcripts contain three AUUUA sequence motifs and represents 80-90% of the pituitary FSH β-subunit mRNA, while the two less abundant 0.7 kb transcripts contain no AUUUA sequence motifs. If the assumption is made that the AUUUA sequence motifs have a destabilising effect, and that the short length transcripts are therefore more stable, then an enrichment for short length transcripts might be expected. As this has not been evident by Northern blot analyses of bovine, human or ovine RNA (Maurer and Beck, 1986; Jameson et al., 1988; Montgomery et al., 1990), the predominant usage of the more distal 3’ signal is further emphasised.

The eight hybridising non-FSH β-subunit clones selected from the non-enriched cDNA library were not sequenced in sufficient detail to determine whether degeneracies within the C-terminal oligonucleotide probe were responsible for their selection. The high stringency wash conditions and the lack of similar clones selected with the non-degenerate oligonucleotide probe support this possibility. The failure to isolate true oFSH β-subunit clones from the first library can most probably be explained by the low abundance of the transcript in the non-enriched library. The C-terminal degenerate oligonucleotide probe hybridised strongly with the DNA of the selected positive clones, while the N-terminal probe was unreactive under all conditions. A single amino acid residue difference between the published amino acid sequence, on which the N-terminal probe was designed, and the predicted amino acid sequence determined by cDNA sequence analysis, may have contributed to this result.

The construction and transfection of mammalian cell expression constructs incorporating the confirmed oFSH subunit cDNA sequences will enable the expression of recombinant oFSH in mammalian cell cultures.
CHAPTER FOUR

EXPRESSION OF RECOMBINANT oFSH IN
MAMMALIAN CELL SYSTEMS
4.1 Introduction

Biologically active recombinant glycoprotein hormones including hFSH (Keene et al., 1989a; Bishop et al., 1990), hLH (Corless et al., 1987), hCG (Reddy et al., 1985; Chakrabarti et al., 1989), bFSH (Chappel, 1988) and bLH (Kaetzel et al., 1985), and a variety of glycosylation defective mutant glycoprotein hormones (Matzuk et al., 1987; Keene et al., 1989b; Kaetzel et al., 1989) have been expressed in heterologous mammalian cell expression systems. These studies have enabled experimental investigation of the structure, function and biosynthesis of glycoprotein hormones, and in some instances the production of a clinically valuable product for use in reproductive management. Established veterinary applications for recombinant bFSH include the stimulation of follicular development for increased fecundity and for superovulation in embryo transfer programs (Looney et al., 1988; Wilson et al., 1989). While recombinant oFSH has not been previously described, oFSH pituitary extracts, including the commercial preparations Embryo-S (Embryo Plus, Melbourne) and Ovagen (ICP, Auckland), are available and have specific veterinary application in inducing superovulation in sheep, goats and cattle (Bindon et al., 1987; Donaldson, 1989). Glycoprotein hormones including the recombinant hCG β-subunit (Chakrabarti et al., 1989) and highly purified native oFSH (Srinath et al., 1983) may also have further applications as contraceptive immunogens in a variety of mammalian species including man.

The experiments described in this chapter include a comparison of FIII- and pKC3-based oFSH subunit expression vector constructs by Northern blot analysis and immunoprecipitation of metabolically labelled COS cell transfections. The effect of oFSH subunit cDNA truncation on recombinant oFSH expression levels in both transient COS and constitutive CHO cell transfection cultures is also examined.
4.2 Results

The vector constructs utilised in this chapter are fully described in Chapter 2. Briefly these include two pKC3 constructs, one incorporating the full length (FL) α-subunit cDNA clone 3.2 (Appendix 2) designated pKC3-αFL, and one incorporating a truncated or short length (SL) 573 bp EcoR1/Sac1 coding region fragment of the β-subunit clone 6.14 (Figure 3.6) designated pKC3-βSL. Four FIII-based constructs were also utilised including FIII-αFL and FIII-βSL which were similarly constructed, and, FIII-αSL which incorporated a 607 bp Sau3A1 coding region fragment of clone 3.2, and FIII-βFL which incorporated the entire β-subunit clone 6.14.

4.2.1 Comparison of pKC3- and FIII-based oFSH subunit expression constructs in transient COS cell transfections

Northern blot analysis: COS cell cultures were transfected with one of five combinations of pKC3- and FIII-based oFSH subunit expression constructs to establish and compare subunit transcript levels by Northern blot analysis. Two control transfections incorporating the pKC3 and FIII expression vectors without oFSH encoding cDNA sequences were also undertaken. Total cellular RNA for Northern blot analysis was recovered from transfected COS cell cultures 48 hours post transfection. Random primed 32P-labelled α- and β-subunit cDNA probes were generated from full length α-subunit and truncated EcoR1/Sac1 β-subunit cDNA fragments respectively. The vector combinations transfected and resultant autoradiographs for both α-subunit and β-subunit Northern blot analyses are shown in Figure 4.1.

Strong hybridising α- and β-subunit bands of approximately 1.4 kb are apparent for all pKC3- and FIII-based oFSH subunit transfections shown in Figure 4.1. The individual α- and β-subunit specific transfections show only α-
Figure 4.1
Autoradiograph showing Northern blot analysis of total RNA from transfected COS cell cultures (60mm culture dishes) isolated 48 hours post transfection. Cell cultures were transfected with either; 1.5 µg pKC3 vector alone, 1.5 µg FIII vector alone, 1.5 µg pKC3-αFL, 1.5 µg FIII-βSL, 1.5 µg pKC3-αFL + 1.5 µg pKC3-βSL, 1.5 µg pKC3-αFL + 1.5 µg FIII-βSL, or 1.5 µg FIII-αSL + 1.5 µg FIII-βSL. Duplicate 0.66 M formaldehyde / 1% agarose gels were loaded with 25 µg RNA/well. Blots were hybridised overnight with either α-FL (α) or the β-SL (β) 32P-labelled cDNA probes.
or β-subunit specific bands as anticipated. A slightly smaller α-subunit transcript is evident for the FIII-αSL co-transfection compared with the the pKC3-αFL co-transfections [Figure 4.1, (α)]. The α-subunit cDNA fragment incorporated in the FIII-αSL construct is approximately 180 bp shorter than the the full length cDNA incorporated in the pKC3-αFL construct. Some variation in mRNA transcript levels is apparent between transfections in both Northern blot analyses. No hybridising bands were apparent for transfections incorporating either the pKC3 or the Fill expression vectors which do not contain oFSH encoding cDNA sequences.

**SDS-PAGE analysis of metabolically labelled transfected COS cell lysate and culture medium immunoprecipitates:** One COS cell α-subunit specific transfection and two α/β-subunit co-transfections were undertaken. Transfected cell cultures were incubated for 48 hours prior to overnight metabolic labelling with 35S-cysteine. Media and cell lysate immunoprecipitate samples were prepared. Briefly, samples were precleared using normal rabbit sera as a source of non-specific Immunoglobulin and protein-G Sepharose as a carrier resin. An oFSH antibody cocktail, comprised of both monoclonal and polyclonal oFSH antibody preparations (Chapter 2), was used in conjunction with protein-G Sepharose to isolate recombinant oFSH heterodimers and individual subunits. Samples for SDS-PAGE were prepared by boiling in non-reducing buffer. Autoradiograph results for the SDS-PAGE analysis are presented in Figure 4.2.

Immunoprecipitation results for pKC3-αFL transfected COS cell lysate and media samples (Figure 4.2) revealed a single 22 kD band in the media sample only. A similar band was also evident in both pKC3- and Fill- α/β co-transfection media samples (Figure 4.2). Immunoprecipitation of lysates from the α/β co-transfections isolated two bands of approximately 21-22 kD neither
Figure 4.2

Autoradiograph showing SDS-PAGE analysis of $^{35}$S-cysteine labelled recombinant oFSH subunits from COS cell transfections incorporating 1.5 µg pKC3-αFL, 1.5 µg pKC3-αFL + 1.5 µg pKC3-βSL or 1.5 µg FIII-αSL + 1.5 µg FIII-βSL. $^{35}$S-cysteine labelled cell lysate (Lys) and culture media (Med) immunoprecipitation samples are indicated, as well as the Coomassie blue stained molecular weight markers (Mr) and native oFSH subunit polypeptides (α, β).

Note: Immune complexes isolated during the preclearing of media and cell lysate samples were also similarly analysed and no similar bands were apparent (results not shown).
of which was evident in the pKC3-αFL transfection lysate sample. No clear delineation can be seen for the recombinant oFSH subunits of α/β co-transfection media samples. Separate α- and β-subunit bands are clearly evident for the Coomassie blue stained, affinity-purified native oFSH sample (Figure 4.2).

4.2.2 The effect of oFSH subunit cDNA truncation in transient COS cell transfections

Eight COS cell transfections were performed in duplicate (Series 1 and 2 respectively) to compare recombinant oFSH expression levels and β-subunit transcript levels for full length and truncated cDNA expression constructs by RRA and Northern blot analysis respectively. The various transfections and co-transfections undertaken and the results of the β-subunit Northern blot analysis, are presented in Figure 4.3. RRA results are presented in Table 4.1.

Northern blot analysis: Total RNA was recovered from the eight COS cell transfections of Series 1 and Series 2 respectively and analysed by Northern blot analysis using the previously described 573 bp β-subunit coding region probe. Results shown in Figure 4.3 clearly indicate that COS cell transfections incorporating the FIII-BSL construct contain considerably higher levels of the FSH β-subunit mRNA than transfections incorporating the FIII-βFL construct. The FIII-BSL transcript of approximately 1.4 kb is readily apparent while the larger 2.7 kb transcript of the FIII-βFL expression construct is barely if at all detectable.

RRA of transfected COS cell culture media: Media samples for RRA were collected from transfected COS cell cultures 48 hours post transfection. The porcine testis RRA described in Chapter 2 relies on the dose dependent binding inhibition of an 125I-labelled oFSH trace by the unlabelled oFSH contained in the sample or standard. Undiluted samples were assayed in
Figure 4.3
Autoradiograph showing Northern blot analysis of total RNA from transfected COS cell cultures (60mm culture dishes) isolated 48 hours post transfection. Gels were loaded with 25 µg RNA/well. Blots were hybridised overnight with the $^{32}$P-labelled B-SL cDNA probe. Cell cultures were transfected with either; 1.5 µg FILI vector, 1.5 µg FILI-αFL, 1.5 µg FILI-αSL, 1.5 µg FILI-βFL, 1.5 µg FILI-βSL, 1.5 µg FILI-αFL + 1.5 µg FILI-βFL, 1.5 µg FILI-αFL + 1.5 µg FILI-βSL, 1.5 µg FILI-αSL + 1.5 µg FILI-βFL or 1.5 µg FILI-αSL + 1.5 µg FILI-βSL.
Table 4.1

Inhibition of $^{125}$I oFSH binding in the porcine testis oFSH RRA by recombinant oFSH expressed in transient COS cell transfection cultures. COS cell cultures [2 x 60 mm culture dishes (Series 1 and 2)] were transfected with either; 1.5 µg FIII-αSL, 1.5 µg FIII-βSL, 1.5 µg FIII-αFL + 1.5 µg FIII-βFL, 1.5 µg FIII-αFL + 1.5 µg FIII-βSL, 1.5 µg FIII-αSL + 1.5 µg FIII-βFL or 1.5 µg FIII-αSL + 1.5 µg FIII-βSL. Media samples were collected 48 hours post transfection and assayed undiluted.

<table>
<thead>
<tr>
<th>Control / COS cell culture medium</th>
<th>Series 1 (CPM bound)</th>
<th>Series 2 (CPM bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (negative control)</td>
<td>1340</td>
<td></td>
</tr>
<tr>
<td>Saline + excess oFSH (positive control)</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td><strong>Media samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIII-αSL</td>
<td>1160</td>
<td>–</td>
</tr>
<tr>
<td>FIII-βSL</td>
<td>1070</td>
<td>–</td>
</tr>
<tr>
<td>FIII-αFL + FIII-βFL</td>
<td>1030</td>
<td>907</td>
</tr>
<tr>
<td>FIII-αFL + FIII-βSL</td>
<td>930</td>
<td>807</td>
</tr>
<tr>
<td>FIII-αSL + FIII-βFL</td>
<td>913</td>
<td>893</td>
</tr>
<tr>
<td>FIII-αSL + FIII-βSL</td>
<td>793</td>
<td>790</td>
</tr>
</tbody>
</table>
duplicate, as were the saline negative and the saline plus excess native oFSH positive controls. While the limited response range displayed by the controls was not indicative of a good assay, two general trends in the α/β co-transfection results were noted. Firstly, FIII-αSL co-transfections resulted in higher levels of recombinant oFSH expression than FIII-αFL co-transfections, and secondly co-transfections incorporating the FIII-βSL construct also resulted in higher recombinant oFSH levels than those incorporating the FIII-βFL construct.

Considerable inhibition of \(^{125}\text{I} \) oFSH binding was also associated with the single α- and single β-subunit transfections although this inhibition was less than that seen for the α/β co-transfections. Possible explanations for this result are presented in the Discussion.

4.2.3 RRA of recombinant oFSH expression levels for G418 resistant CHO cell transfection pools

Six CHO cell transfections were undertaken using liposome mediated DNA transfer to establish permanent cell lines for the expression of recombinant oFSH and individual oFSH subunits. The vector combinations transfected and RRA results for the G418 resistant CHO cell transfection pools are presented in Table 4.2. The G418 resistance marker was supplied for co-transfection as the pMC1neo PolyA vector (Stratagene, catalog # 213201, U.S.A.).

As expected, only transfections incorporating the pMC1neo PolyA vector resulted in G418 resistant colonies. CHO cell transfection pools, representing the 200-300 resistant colonies of each pMC1neo PolyA co-transfection, were replated at 25% confluence and cultured in selection medium for 96 hours prior to collecting samples for RRA. Duplicate samples were assayed undiluted and at a dilution of 1:4 in assay buffer. oFSH standards for the RRA
Table 4.2

Inhibition of $^{125}$I oFSH binding in the porcine testis RRA by media of transfected CHO cell culture pools. CHO cell cultures (100 mm culture dishes) were transfected with 2.5 µg FIII-α + 5.0 µg FIII-β + 0.5 µg pMC1 neo PolyA, while individual subunit transfections received 5.0 µg of FIII-oFSH subunit + 0.5 µg pMC1 neo PolyA. Subconfluent (25%) G418 resistant CHO cell culture pools cultures were replated in fully supplemented selection media and cultured for 96 hours prior to harvesting samples for RRA.

<table>
<thead>
<tr>
<th>Standard/Sample</th>
<th>CPM bound</th>
<th>Recombinant oFSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standards</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0 µg/ml native oFSH</td>
<td>701</td>
<td></td>
</tr>
<tr>
<td>5.0 µg/ml native oFSH</td>
<td>813</td>
<td></td>
</tr>
<tr>
<td>1.0 µg/ml native oFSH</td>
<td>958</td>
<td></td>
</tr>
<tr>
<td>200 ng/ml native oFSH</td>
<td>1204</td>
<td></td>
</tr>
<tr>
<td>40.0 ng/ml native oFSH</td>
<td>2061</td>
<td></td>
</tr>
<tr>
<td>8.0 ng/ml native oFSH</td>
<td>3937</td>
<td></td>
</tr>
<tr>
<td>1.25 ng/ml native oFSH</td>
<td>5953</td>
<td></td>
</tr>
<tr>
<td>Buffer only (negative control)</td>
<td>6465</td>
<td></td>
</tr>
<tr>
<td><strong>Media Sample</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) FIII-αFL (undiluted)</td>
<td>5494</td>
<td></td>
</tr>
<tr>
<td>FIII-αFL (1:4)</td>
<td>6358</td>
<td></td>
</tr>
<tr>
<td>(2) FIII-βSL (undiluted)</td>
<td>5637</td>
<td></td>
</tr>
<tr>
<td>FIII-βSL (1:4)</td>
<td>6442</td>
<td></td>
</tr>
<tr>
<td>(3) FIII-αFL + FIII-βFL (undiluted)</td>
<td>4446</td>
<td></td>
</tr>
<tr>
<td>FIII-αFL + FIII-βFL (1:4)</td>
<td>5669</td>
<td>8.0</td>
</tr>
<tr>
<td>(4) FIII-αFL + FIII-βSL (undiluted)</td>
<td>4500</td>
<td></td>
</tr>
<tr>
<td>FIII-αFL + FIII-βSL (1:4)</td>
<td>5800</td>
<td>7.5</td>
</tr>
<tr>
<td>(5) FIII-αSL + FIII-βSL (undiluted)</td>
<td>3290</td>
<td></td>
</tr>
<tr>
<td>FIII-αSL + FIII-βSL (1:4)</td>
<td>5000</td>
<td>16.0</td>
</tr>
</tbody>
</table>
were prepared in assay buffer containing an equal proportion of non-
transfected CHO cell culture media as the diluted samples. Results for the
RRA are presented in Table 4.2.

RRA results for the three FIII-α/β CHO cell co-transfections (Table 4.2) show
the highest levels of recombinant oFSH expression are found in association
with the FIII-αSL + FIII-βSL co-transfection. By extrapolation from the standard
curve (not shown), this transfection pool produces 5-10 ng/10^6 cells/day.
Little or no difference in expression levels is apparent between the FIII-αFL +
FIII-βFL and FIII-αFL + FIII-βSL co-transfections which produce approximately
half this amount. The media from control and individual subunit transfections
were negative.

The inhibition of ¹²⁵I oFSH binding, seen in association with the single
subunit transfections of the COS cell transfections (Table 4.1) and not found in
the CHO cell series of transfections, can be attributed to non-specific inhibition
of ¹²⁵I oFSH receptor binding by the high media content of the undiluted COS
cell culture samples assayed. Results shown for diluted media samples show
no non-specific inhibition when compared to the negative control. Controls
and standards for this experiment were prepared in sample buffer containing
an equivalent proportion (1:4) of negative control transfection media. RRA
results for undiluted culture media samples of the CHO cell transfection (Table
4.2) cannot be quantitated for the same reason.
4.3 Discussion

Northern blot analysis and immunoprecipitation results for the COS cell transfection experiments comparing the pKC3- and FIII- expression constructs, confirmed that both expression vectors are effective for transient COS cell expression of individual oFSH-subunit cDNA.

Northern blot analysis results for the α- and β-subunit transcripts [Figure 4.1, (α) and (β)] show slight differences in mRNA transcript levels for the two vectors. This may reflect differences in the strengths of the pKC3 and FIII promoters, however variations in transcript levels between FIII-βSL transfections would suggest that efficiency of transfection and variations in sample processing are equally responsible. While transcript levels often reflect promoter strength, slight differences in promoter strength in the transient COS cell expression system may be hidden by the extremely high vector copy number. Expression vectors incorporating the SV40 origin of replication can achieve copy numbers in COS cells in excess of $1 \times 10^5$ copies per cell (Mellon et al., 1981).

A further explanation for the slightly lower transcript levels associated with the FIII-αSL co-transfection, compared with pKC3-αFL transfections (Figure 4.1), may lie in the α-subunit 5′ poly(T) sequence artefact present in the cDNA of the pKC3-αFL construct but not in the truncated α-subunit FIII-αSL construct (see Appendix 2). This poly(T) sequence, also present in the $^{32}$P-dATP-labelled cDNA probe used for Northern blot hybridisation, would hybridise exclusively with the pKC3-αFL transcript and thereby generate an apparent discrepancy in the α-subunit transcript levels. The discrepancy would be further highlighted by the use of $^{32}$P-dATP in the generation of labelled cDNA probes.

The α-subunit 5′ poly(T) sequence may also result in the formation of RNA secondary structures, including circularised RNA molecules or RNA concatamers,
through 5' poly(T) - 3' poly(A) hybridisation. As circular mRNA transcripts do not bind eukaryotic ribosomes (Konarska et al., 1981), and excess mRNA secondary structure has been associated with decreased translational efficiency (Kozak, 1980), the formation of these structures may account for the lower expression levels associated with the pKC3- and FIII-αFL construct co-transfections (Tables 4.1 and 4.2). Both immunoprecipitation (Figure 4.2) and RRA (Tables 4.1 and 4.2) results show that translation of full length α-subunit mRNA transcript has not been completely inhibited.

Immunoprecipitation experiments examining the secretion of recombinant hFSH and individual hFSH-subunits in CHO cells (Keene et al., 1989a) have shown that while the hFSH heterodimer and the free α-subunit are efficiently secreted, the free β-subunit is not. Similarly, immunoprecipitation results for the pKC3-αFL transfection (Figure 4.2) show that a strong oFSH α-subunit band of approximately 22 kD is present in the COS cell media sample, but absent from the cell lysate sample. This finding is in keeping with the short intracellular half-life of the recombinant hFSH α-subunit (Keene et al., 1989a) and depletion of 35S-cysteine during the overnight metabolic labelling period used in this transfection experiment. Conclusive evidence for oFSH β-subunit translation has not been demonstrated in these experiments. However, the two 21-22 kD bands present in both α/β co-transfection lysate samples which are not evident in the lysate sample of the α-subunit specific transfection (Figure 4.2) probably represents non-secreted forms of the β-subunit and/or stabilisation of some α-subunit polypeptide. A β-subunit specific transfection was not undertaken on the basis of earlier native oFSH SDS-PAGE analyses which indicated that individual oFSH subunit polypeptides, expressed in the α/β co-transfections, would be easily identified. However the results presented in this and subsequent chapters clearly indicate that the identification of recombinant oFSH α- and β-subunit polypeptides is difficult by SDS-PAGE analysis.
RRA results examining expression of the recombinant oFSH heterodimer (Tables 4.1 and 4.2), show that both COS and CHO cells co-transfected with oFSH α- and β-subunit cDNA expression constructs, assemble and secrete an oFSH heterodimer displaying bioactivity in the porcine testis RRA. While productivity estimates have been established using a native oFSH standard in the in vitro RRA, it is possible that each preparation tested may have a different activity in the assay, and that true productivity estimates (ng/ml) may better be represented in units/ml. For the purposes of the discussion it is assumed that the recombinant oFSH products have identical specific activities to the affinity purified native oFSH standard used in the RRA.

The higher oFSH expression levels of α/β co-transfections incorporating the FIII-βSL construct, can be partially explained by the higher mRNA transcript levels associated with this construct (Figure 4.3). The suggestion raised in Chapter 3, that the multiple AUUUA sequence motif repeats of the 3' untranslated region may cause destabilisation of the full length β-subunit mRNA transcript, is supported by this finding. The removal of the FSH β-subunit cDNA 3' non-coding region which encodes several AUUUA mRNA sequence motifs, most probably results in the transcription of a more stable mRNA transcript, higher steady state mRNA levels and higher FSH β-subunit polypeptide expression. In vitro mutagenesis of the specific ATTTA cDNA sequences would establish the role of these sequence motif repeats in oFSH β-subunit mRNA stability more fully. A number of other 3' and 5' untranslated and coding region sequences have been implicated in regulating mRNA stability. These include unidentified sequences within the c-fos, c-myc, histone and β-tubulin mRNA transcripts (for review see Cleveland and Yen, 1989).

While RRA results for the CHO cell transfection experiments failed to demonstrate a difference in expression levels between FIII-αFL + FIII-βFL and FIII-αFL + FIII-βSL
co-transfections, results for the FIII-αSL + FIII-βSL co-transfection did reveal higher associated αFSH expression levels (Table 4.2). The higher levels of recombinant αFSH expression evident in association with the FIII-βSL COS cell co-transfections, and anticipated but not seen in the subsequent CHO cell transfection experiments, may be attributed to the different vector ratios utilised in the two experiments. Early experiments examining de novo TSH synthesis in mouse pituitary tumour cells (Weintraub et al., 1980) and in vitro βLH synthesis (Fetherston and Boime, 1982) indicated that the level of β-subunit expression controlled heterodimer assembly. More recently conflicting evidence has been provided in experiments showing that methotrexate induced α-subunit gene amplification in αβ LH co-transfected CHO cells can increase expression of the heterodimer eleven-fold (Kaetzel and Nilson, 1988). COS cell co-transfections incorporated equal proportions of each subunit vector while the CHO cell co-transfections incorporated twice as much β-subunit vector as α-subunit vector. If the FIII-αFL expression levels in the CHO cell transfection series were rate limiting for heterodimer formation, no difference in FIII-βFL and FIII-βSL co-transfections would be anticipated.

In summary, the results described in this Chapter show that the FIII expression vector is capable of recombinant αFSH-subunit expression from cloned αFSH subunit cDNAs in both transient COS cell and G418 resistant CHO cell transfections. RRA results comparing FIII vectors which incorporate either full length or truncated αFSH α- and β-subunit cDNAs, have shown that higher expression levels are found in association with constructs utilising the truncated cDNA sequences. Northern blot analysis comparing FIII-βFL and FIII-βSL transcript levels has shown higher β-subunit transcript levels are associated with the FIII-βSL construct and provided supportive evidence for the likely destabilising effect of the 3' non-coding region AUUUA sequence motifs repeats encoded for by the full length β-subunit cDNA sequence (Chapter 3).
CHAPTER FIVE

ENHANCED oFSH EXPRESSION BY
β-SUBUNIT MUTAGENESIS
5.1 Introduction

Comparison of over 200 cellular (Kozak, 1984a) and 50 viral mRNA 5' non-coding region sequences (Kozak, 1981), has identified tentative mRNA initiation codon flanking region consensus sequences. To establish whether AUG initiation codon flanking sequences influence ribosomal recognition of the translational start site, the translational efficiencies of wild-type and single base pair mutant rat preproinsulin mRNA transcripts in transient COS cell transfections were compared (Kozak 1984b; 1986). Expression levels for various constructs were found to vary 20-fold despite comparable levels of the transcripts. The optimal sequence identified for the initiation of translation by eukaryotic ribosomes was -3ACCAUGG+4, while the previously described consensus sequence -3GCCAUGG+4 (Kozak, 1984a) was found to be only 33% as active (Kozak, 1986). The importance of an A residue at position -3 has been further emphasised in clinical studies which have associated a deficiency in globin synthesis and resulting α-thalassaemia with a two nucleotide deletion in the AUG flanking sequence of the α-globulin gene (Morle et al., 1985). This deletion results in a conversion of the α-globin gene AUG flanking sequence from CACCAUG to CCCCAUG. Results of previous experiments (Kozak, 1984b) have shown that an A residue in position -3 is at least 15-fold more active in initiating translation than a C residue in the same position. The importance of a G residue in position +4 was initially investigated by measuring synthetic oligonucleotide binding to wheat germ ribosomes in vitro (Kozak, 1981) and later confirmed using site directed mutagenesis in COS cell transfection experiments (Kozak, 1986).

This chapter describes strategies adopted to increase the translatability of the oFSH β-subunit mRNA transcript, including the synthesis of two AUG flanking sequence mutants, and the construction of a single hybrid oGH/oFSH β-subunit cDNA construct. Flanking sequence mutants, incorporating the optimised
ACCAUGG (Kozak, 1986) and the less efficient GCCAUGG (Kozak, 1984a) sequences were generated using site directed mutagenesis. The hybrid cDNA was constructed by ligating the oGH cDNA 5' non-coding and signal peptide coding sequence, to the oFSH β-subunit mature peptide coding sequence. This particular hybrid cDNA construct was considered worthy of investigation on the basis of high levels of expression of >200 µg/ml, previously described for recombinant hGH expression in an equivalent CHO cell expression system (Friedman et al., 1989).
5.2 Results

5.2.1 Confirmation of FIII-BSL (M2) and FIII-BSL (HYB) β-subunit transcription and translation in transient COS cell transfections.

Six COS cell transfections were undertaken in triplicate to confirm and compare oFSH β-subunit transcript levels for heavy metal ion induced and non-induced transient transfection cultures, and to examine recombinant oFSH subunit polypeptides by SDS-PAGE analysis of 35S-labelled immunoprecipitates. Details of the COS cell transfections undertaken are presented in Figure 5.1.

Northern Blot Analysis: Autoradiograph results of the β-subunit Northern blot analysis (Figure 5.1) confirm that the previously untested FIII-BSL (M2) and FIII-BSL (HYB) vector constructs actively transcribe oFSH β-subunit mRNAs and that the rate of transcription mediated by these constructs, and the original FIII-BSL construct, can be increased by heavy metal ion induction. Photodensometric analysis of the induced and non-induced COS cell mRNA indicates a 3-4 fold increase in transcript levels following the addition of zinc to the culture media. The 1.2-1.4 kb transcripts detected are consistent with those previously identified in Chapter 4. The weaker hybridisation signal associated with the induced FIII-BSL (HYB) co-transfection, is attributable to the comparatively low sample content of this lane as evident by ethidium bromide staining of the gel (Figure 5.1).

SDS-PAGE analysis of 35S-cysteine labelled COS cell lysate and culture media immunoprecipitate samples: COS cell cultures were incubated for 48 hours post transfection prior to overnight labelling with 35S-cysteine. Immunoprecipitates were isolated and dissociated for SDS-PAGE analysis by resuspending the carrier protein-G Sepharose pellet in 0.1 M NaCl, 0.1 M glycine pH 2.0. The Sepharose free eluate was then
**Figure 5.1**

Autoradiograph showing Northern blot analysis of oFSH β-subunit mRNA in transfected COS cell cultures. Cultures (35 mm culture dish) were transfected with 1.6 µg FIII vector alone, 1.6 µg FIII-αSL, 1.6 µg FIII-βSL, 1.6 µg FIII-αSL + 1.6 µg FIII-βSL, 1.6 µg FIII-αSL + 1.6 µg FIII-βSL (M2), or 1.6 µg FIII-αSL + 1.6 µg FIII-βSL (HYB). Cells were cultured in routine growth media (Medium) or growth media containing 100 µM ZnSO₄ (Medium + Zn) for 48 hours post transfection. Gels were loaded with 30 µg cellular RNA/well. Ethidium bromide staining of the 28S ribosomal RNA band (28S) is shown beneath the Northern blot autoradiograph. The β-subunit probe for hybridisation was the 32P-labelled EcoR1/Sac1 βSL cDNA fragment as described in Chapter 3.
diluted with an equal volume of 2x non-reducing sample buffer and analysed on a 15% polyacrylamide gel. To enable better separation of the dissociated oFSH subunits, electrophoresis was continued for an additional 20 minutes after elution of the loading buffer dye front. Samples were not boiled prior to loading in an attempt to visualise non-dissociated or re-associated α/β heterodimers. Results of the SDS-PAGE analysis are presented in Figure 5.2.

Free oFSH α-subunit polypeptide bands of 20 kD and 22 kD are evident in the FIII-αSL transfected COS cell lysate and media samples respectively (Figure 5.2). Similar bands are also evident in the α/β co-transfection lysate and media samples (Figure 5.2) although these bands are not easily distinguished from associated β-subunit bands. Free β-subunit bands, of approximately 22 kD and 24-26 k, while not clearly apparent in FIII-βSL transfection samples, are evident in the α/β co-transfection cell lysate and media samples respectively (Figure 5.2). The free α- and β-subunit bands shown in the samples of the co-transfection incorporating the non-mutated β-subunit expression construct are also visible in the samples of co-transfections incorporating the mutant and hybrid β-subunit cDNAs (Figure 5.2). Only the FIII-αSL + FIII-βSL (HYB) co-transfection lysate and media samples show the higher molecular weight bands of the non-dissociated oFSH heterodimer being approximately 35 kD and 40 kD respectively.

5.2.2 Recombinant oFSH expression in transfected CHO cell culture pools and cloned high producing CHO cell lines

Six CHO cell α/β co-transfections were undertaken to compare recombinant oFSH expression levels for wild-type and mutated β-subunit cDNA sequences. Two individual subunit transfections and an additional control α/β co-transfection, incorporating human FSH α- and β-subunit cDNA
Figure 5.2

Autoradiograph showing SDS-PAGE analysis of $^{35}$S-cysteine labelled recombinant oFSH subunit polypeptides transiently expressed in transfected COS cell cultures (35 mm culture dish). Cell lysate (Lys) and media (Med) immunoprecipitate samples for cell cultures transfected with 1.6 µg FIII vector alone, 1.6 µg FIII-αSL, 1.6 µg FIII-βSL, 1.6 µg FIII-αSL + 1.6 µg FIII-βSL, 1.6 µg FIII-αSL + 1.6 µg FIII-βSL (M2), or 1.6 µg FIII-αSL + 1.6 µg FIII-βSL (HYB) are shown. Molecular weight markers (M_r) are also indicated.
expression constructs, were also undertaken. The human α- and β-subunit constructs were supplied by Dr. P. Schofield (Pacific Biotechnology, Sydney), and were designed like FIII, on the pMTSV40-2 eukaryotic expression vector (Friedman et al., 1989).

CHO cell cultures (100 mm culture dish) were transfected with the following vector combinations:

1. FIII vector alone (10µg)
2. FIII-αFL (5µg) + FIII-BSL (5µg) + pMC1neo PolyA (0.05µg)
3. FIII-αSL (5µg) + FIII-βFL (5µg) + pMC1neo PolyA (0.05µg)
4. FIII-αSL (5µg) + FIII-BSL (5µg) + pMC1neo PolyA (0.05µg)
5. FIII-αSL (5µg) + FIII-βSL (M2) (5µg) + pMC1neo PolyA (0.05µg)
6. FIII-αSL (5µg) + FIII-βSL (M12) (5µg) + pMC1neo PolyA (0.05µg)
7. FIII-αSL (5µg) + FIII-βSL (HYB) (5µg) + pMC1neo PolyA (0.05µg)
8. Human FSH α (5µg) + hFSH β (5µg) + pMC1neo PolyA (0.05µg)
9. FIII-αSL (10µg) + pMC1neo PolyA (0.05µg)
10. FIII-βSL (10µg) + pMC1neo PolyA (0.05µg)

RRA results for G418 resistant CHO cell culture pools:
Approximately 150-200 G418 resistant CHO cell colonies were generated per 2 x 10⁶ cells transfected. A negative control transfection, which did not incorporate the pMC1neo PolyA vector generated no G418 resistant colonies. G418 resistant CHO cell pools were re-plated at equal cell densities to enable comparative evaluation of recombinant oFSH expression levels. Confluent CHO cell pools were incubated for 48 hours in selection media supplemented with 75mM ZnSO₄ and assessed for recombinant oFSH expression levels by RRA. Standards were prepared in media free assay buffer, and samples were diluted (1:3 and 1:6) in assay buffer for RRA. Media samples assayed at the 1:6 dilution do not display
non-specific inhibition in the RRA (results not shown). The results of the RRA are presented Table 5.1.

While low recombinant oFSH expression levels, ranging up to approximately 2 ng/ml, do not permit accurate quantitation from the standard curve, a clear trend in expression levels is apparent (Table 5.1). The poor expression levels previously associated with the FIII-αFL construct in COS cell co-transfections (Chapter 4), are again evident when the FIII-αFL + FIII-βSL and FIII-αSL + FIII-βSL co-transfections are compared (Table 5.1). Similarly, higher oFSH expression levels are again evident in association with the FIII-βSL co-transfection when compared with the FIII-βFL co-transfection (Table 5.1).

A further improvement in recombinant oFSH expression levels has been achieved through the utilisation of the FIII-βSL (HYB) hybrid construct. To examine whether this improvement is attributable to higher mRNA transcript levels, RNA was isolated from each of the transfection pools and compared by Northern blot analysis. Results for both α- and β-subunit Northern blot analyses are presented in Figure 5.3.

The β-subunit Northern blot analysis [Figure 5.3, (β)] shows similar mRNA transcript levels for the three highest producing α/β co-transfection pools. These co-transfections incorporated the FIII-βSL, FIII-βSL (M2) and FIII-βSL (HYB) expression constructs respectively. The FIII-βSL (M12) co-transfection shows a weaker β-subunit mRNA band. The larger FIII-βFL transcript, which includes the long 3' untranslated region and five associated AUUUA sequence motifs is not apparent. While high background levels are shown in the autoradiograph of the α-subunit Northern blot analysis [Figure 5.3, (α)], α-subunit bands of similar intensity are evident for three of the four α/β
Table 5.1

Inhibition of $^{125}$I oFSH binding in the porcine testis RRA by recombinant oFSH expressed by G418 resistant CHO cell transfection pools.

<table>
<thead>
<tr>
<th>Standard / Transfection sample</th>
<th>CPM Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000ng/ml Standard</td>
<td>1411</td>
</tr>
<tr>
<td>200 &quot; &quot;</td>
<td>1590</td>
</tr>
<tr>
<td>40.0 &quot; &quot;</td>
<td>1989</td>
</tr>
<tr>
<td>8.0 &quot; &quot;</td>
<td>3219</td>
</tr>
<tr>
<td>1.6 &quot; &quot;</td>
<td>5860</td>
</tr>
<tr>
<td>0.3 &quot; &quot;</td>
<td>7959</td>
</tr>
<tr>
<td>'0' Assay buffer only (negative control)</td>
<td>9107</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Dilution (1:3)</th>
<th>Dilution (1:6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2) FIII-αFL + FIII-βSL</td>
<td>8619</td>
<td>9144</td>
</tr>
<tr>
<td>(3) FIII-αSL + FIII-βFL</td>
<td>7700</td>
<td>8282</td>
</tr>
<tr>
<td>(4) FIII-αSL + FIII-βSL</td>
<td>7300</td>
<td>8213</td>
</tr>
<tr>
<td>(5) FIII-αSL + FIII-βSL (M2)</td>
<td>7265</td>
<td>8175</td>
</tr>
<tr>
<td>(6) FIII-αSL + FIII-βSL (M12)</td>
<td>7855</td>
<td>8511</td>
</tr>
<tr>
<td>(7) FIII-αSL + FIII-βSL (HYB)</td>
<td>7124</td>
<td>7858</td>
</tr>
<tr>
<td>(8) h-αFSH + h-βFSH constructs</td>
<td>8037</td>
<td>8281</td>
</tr>
</tbody>
</table>
Figure 5.3

Autoradiograph showing α- (α) and β-subunit (β) Northern blot analyses of 25 µg/well cellular RNA isolated from G418 resistant CHO cell culture pools. Hybridising 32P-labelled cDNA probes were generated from Sau3A1 α-subunit and EcoR1/Sac1 β-subunit fragments as described in Chapter 2.
co-transfections. The hybridising band for FIII-αSL subunit specific transfection, which incorporated twice as much FIII-αSL vector DNA as the α/β co-transfections cultures, is approximately twice as intense [Figure 5.3, (α)].

Isolation and RRA analysis of cloned CHO cell lines expressing high levels of recombinant oFSH: The three highest producing α/β co-transfection pools, incorporating the FIII-αSL and FIII-βSL, FIII-αSL and FIII-βSL (M2) or FIII-αSL and FIII-βSL (HYB) constructs (see Table 5.1), were dilution cloned in 96 well plates to enable selection of high producing single colonies by RRA. RRA results for the six highest producing colonies from 48 randomly selected single colony wells of each transfection are presented in Table 5.2. The two highest producing colonies identified for each transfection were again dilution cloned, expanded and assessed for oFSH expression levels by RRA. RRA results for the two highest producing clones of each transfection are presented in Table 5.3. Clones isolated from the FIII-βSL (HYB) α/β co-transfection show the highest expression levels ranging from 100-155 ng/10⁶ cells/day, based on the median cell number over the 48 hour culture period and oFSH media concentrations determined by extrapolation from the RRA standard curve (not shown). Clones isolated from the FIII-βSL and FIII-βSL (M2) α/β co-transfections express between 42-70 ng/10⁶ cells/day.

Isolation and cloning of transfected CHO cell lines expressing either oFSH α-subunit or β-subunit polypeptides: Two high producing α-subunit specific and two high producing β-subunit specific cell lines were selected from 12 randomly selected single colony wells of the FIII-αSL and FIII-βSL CHO cell transfections respectively (Section 5.2.2), by SDS-PAGE analysis of metabolically labelled immunoprecipitates. Subunit
Inhibition of $^{125}$I oFSH binding in the porcine testis RRA by recombinant oFSH. The assay assessed 48 single colony containing wells for each of the three highest producing co-transfections, co-transfections FIII-αSL + FIII-βSL [αSL-βSL], FIII-αSL + FIII-βSL (M2) [αSL-βSL (M2)] and FIII-αSL + FIII-βSL (HYB) [αSL-βSL (HYB)] (see Table 5.1). Results for the six highest producing clones [1-6] identified for each transfection are presented.

<table>
<thead>
<tr>
<th>Order of productivity of isolated colonies</th>
<th>αSL-βSL (CPM bound)</th>
<th>αSL-βSL (M2) (CPM bound)</th>
<th>αSL-βSL (HYB) (CPM bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5773</td>
<td>5914</td>
<td>5088</td>
</tr>
<tr>
<td>2</td>
<td>5983</td>
<td>5916</td>
<td>5409</td>
</tr>
<tr>
<td>3</td>
<td>6286</td>
<td>5929</td>
<td>5460</td>
</tr>
<tr>
<td>4</td>
<td>6357</td>
<td>6014</td>
<td>5542</td>
</tr>
<tr>
<td>5</td>
<td>6582</td>
<td>6042</td>
<td>5645</td>
</tr>
<tr>
<td>6</td>
<td>6607</td>
<td>6247</td>
<td>5720</td>
</tr>
</tbody>
</table>

**Assay controls**

- Positive control (200 ng/ml oFSH).............1030 (CPM bound)
- Negative control (Assay buffer only).........6990 (CPM bound)
Table 5.3
Recombinant oFSH expression levels for six high-producing cloned CHO cell lines as determined by RRA. Cloned cell lines were isolated by dilution cloning of the two highest producing single colony wells identified in Table 5.2 and replated at 50% confluence. Cell cultures were grown for 48 hours in fully supplemented growth medium containing 75 µM ZnSO₄ prior to harvesting the culture media for RRA.

<table>
<thead>
<tr>
<th>Transfection/Cloned Cell Line</th>
<th>Recombinant oFSH ng/ml Media</th>
<th>Recombinant oFSH ng/10⁶ Cells/Day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIII-αSL + FIII-βSL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αSL-βSL/1.5</td>
<td>17</td>
<td>42</td>
</tr>
<tr>
<td>αSL-βSL/9.10</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td><strong>FIII-αSL + FIII-βSL (M2)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αSL-βSL (M2)/ 4.6</td>
<td>28</td>
<td>70</td>
</tr>
<tr>
<td>αSL-βSL (M2)/30.1</td>
<td>22</td>
<td>55</td>
</tr>
<tr>
<td><strong>FIII-αSL + FIII-βSL (HYB)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αSL-βSL (HYB)/14.4</td>
<td>62</td>
<td>155</td>
</tr>
<tr>
<td>αSL-βSL (HYB)/30.4</td>
<td>40</td>
<td>100</td>
</tr>
</tbody>
</table>
specific cell lines were isolated to examine the biosynthesis and secretion of individual oFSH subunits. Autoradiograph results which enabled the selection of the subunit specific clones are presented in Figure 5.4. The clones selected were designated αSL/4, αSL/8, βSL/4 and βSL/7.
Figure 5.4
Autoradiograph showing SDS-PAGE analysis of $^{35}$S-cysteine labelled recombinant oFSH subunit polypeptides expressed individually in G418 resistant CHO cell lines. Cell lysate (Lysate) and media (Media) Immunoprecipitates for each of twelve $\alpha$- [(\(\alpha\)), (1-12)] and twelve $\beta$-specific [(\(\beta\)), (1-12)] cell lines. Cell lines were isolated as single colony wells by dilution cloning of $\alpha$- and $\beta$-subunit specific CHO cell transfections. Cultures were labelled for six hours as previously described (Chapter 2). Molecular weight markers (Mr) are indicated.
5.3 Discussion

Results of the COS cell transfection experiments have confirmed the transcription and translation of both the optimised (Kozak, 1986) AUG translation initiation codon flanking sequence mutant FIII-βSL (M2), and the oGH/oFSH β-subunit hybrid cDNA construct FIII-βSL (HYB).

The 20 kD cell lysate and 22 kD media bands evident in the FIII-αSL transfection samples (Figure 5.2) are consistent with the molecular weights described for recombinant human α-subunit polypeptides expressed in transfected CHO cells (Keene et al., 1989a). SDS-PAGE analysis of the labelled FIII-βSL transfection samples (Figure 5.2) did not clearly identify β-subunit bands. This is not an unexpected result considering the limited 35S-cysteine in the media, the duration of the metabolic labelling (15 hours), and the previously described inefficient secretion (<20%/12 hours) and intracellular degradation (t1/2 ~ 5 hrs) of the hFSH β-subunit polypeptide in transfected CHO cells (Keene et al., 1989a).

The β-subunit bands present in each COS cell α/β co-transfection sample (Figure 5.2) are in keeping with the α-subunit mediated "rescue" of the β-subunit polypeptide described by Keene and co-workers (1989a). That is, while free β-subunits are very slowly secreted, β-subunits expressed in conjunction with the α-subunit polypeptide are more efficiently secreted (>90%/12 hours) in the form of the hFSH heterodimer. Shorter metabolic labelling incubations, as used in the selection of the high producing β-subunit specific CHO cell lines (Figure 5.4), enabled detection of free β-subunit polypeptides in cell lysate preparations. The α- and β-subunit polypeptide bands shown in the non-mutated β-subunit co-transfection are also evident in the FIII-βSL (M2) and FIII-βSL (HYB) co-transfections (Figure 5.2), indicating that the structural characteristics of the wild-type βSL cDNA product have been retained in the βSL (M2) and βSL (HYB) cDNA products. Less obvious differences attributable to the post-translational
processing of the oGH signal peptide, including the site of signal peptide cleavage, require further investigation.

The 35 kD and 42 kD bands of the FIII-βSL (HYB) co-transfection (Figure 5.2) may represent non-dissociated or reassociated α/β heterodimers. The lack of similar bands in the FIII-βSL and FIII-βSL (M2) co-transfections could be attributable to differences in the β-subunit polypeptides, resulting in stronger α/β subunit interactions, or more probably variation in sample preparation. Similar sized bands were not evident for the FIII-βSL (HYB) co-transfection when samples were analysed under reducing conditions (data not shown). Samples for SDS-PAGE analysis were prepared in 0.1 M NaCl, 0.1 M glycine pH 2.0 to enable dissociation of the FSH-antibody immune complexes without total dissociation of the oFSH heterodimers, as would be the case if routine sample boiling were employed. Some dissociation of the oFSH heterodimer in 0.1 M NaCl, 0.1 M glycine pH 2.0 is to be expected considering previous reports describing dissociation in 1 M propionic acid pH 2.4 (Papkoff and Ekbland, 1970) and partial dissociation in 0.1 M NaCl, 0.1 M glycine pH 3.0 (R. Fiddes personal communication). It is interesting to note that both acid and urea dissociated FSH subunits readily recombine under less stringent conditions to form biologically active heterodimers (Papkoff and Ekbland, 1970; Grimek and McShan, 1974).

The increase in molecular weight associated with the secreted forms of the α- and β-subunit polypeptides is attributable to the addition of sugar residues prior to secretion (Snider, 1984). Membrane bound glycosidases and glycosyltransferases of the endoplasmic reticulum and golgi complex, remove terminal mannose residues of the high mannose N-linked oligosaccharide precursor and replace them with galactose, N-acetylglucosamine and sialic acid to form the complex-type oligosaccharides of the mature glycoprotein (see Figure 1.3). The secretion-associated shift in molecular weight has been blocked by
culturating hFSH transfected CHO cells in the presence of tunicamycin (Matzuk and Boime, 1988a). Tunicamycin blocks N-linked glycosylation by preventing synthesis of the lipid-linked oligosaccharide carrier complex (Struck and Lennarz, 1977).

RRA results for the G418 resistant transfected CHO cell pools (Table 5.1) confirm earlier RRA results for both the COS and CHO cell transfection experiments described in Chapter 4. While overall the expression levels achieved in this series of CHO cell transfections (Table 5.1) appear lower than those previously described (see Chapter 4), the highest levels of recombinant oFSH expression are again associated with the truncated α- and β-subunit cDNA expression constructs. However α-subunit expression levels associated with the FIII-αSL construct were not sufficiently rate-limiting to prevent a comparative evaluation of the wild-type and modified FIII-βSL cDNA construct expression levels.

Site directed mutagenesis of the wild-type β-subunit AUG initiation codon flanking sequence to the optimised FIII-βSL (M2) sequence ACCAUGG, has not altered recombinant oFSH expression levels. The FIII-βSL (M12) initiation codon flanking sequence GCCAUGG, previously established as being only 33% as efficient as the optimised ACCAUGG sequence (Kozak, 1986), was predictably less efficient than the FIII-βSL (M2) sequence. The lower expression levels associated with this construct may be partially explained by the lower FIII-βSL (M12) transcript levels evident in the β-subunit Northern blot analysis (Figure 5.3), although reasons for the low FIII-βSL (M12) transcript levels are not obvious. It is unlikely that the mutations described for the FIII-βSL (M2) construct would affect the rate of promoter mediated transcription. Furthermore, differences in transfection efficiency or RNA sample loading are also unlikely considering similar α-subunit transcript levels (Figure 5.3) and similar numbers of G418 resistant colonies generated in each transfection (data not shown).
The FIII-βSL (HYB) construct co-transfection has produced the highest levels of recombinant oFSH expression in CHO cell transfection pools, and in isolated high producing colonies and cloned cell lines (Tables 5.1, 5.2 and 5.3). While the oGH translation initiation codon flanking sequence GCTATGATGG (Adams, 1989) presents two possible translational start sites, the second ATG codon utilises the optimised A(-3) and G(+4) flanking sequence and appears the more efficient of the two. In vitro transcription experiments to determine which of the two initiation codons is utilised have not been reported. Other sequence differences between the oGH and oFSH β-subunit mRNA signal peptide and 5' non-coding region sequences may also effect translatability by altering the tertiary structure of the mRNA transcript (Kozak, 1980; 1988). Experiments examining the effect of leader length and secondary structure on mRNA function have found that lengthening the leader sequence enhanced translation of mRNA sequences containing secondary structure (Kozak, 1988). The enhancement was proportional to the leader length when the leader was extended up stream of the site of secondary structure. No enhancing effects were observed if the leader length was lengthened downstream of the site of secondary structure or in the absence of hypertonic stress. Similarly, the inhibitory effect of secondary structure was only evident under conditions of stress. While the utilisation of the oGH mRNA leader sequence does not represent a large change in leader length, secondary structure including the degree of hairpin formation may have a role in regulating expression of the hybrid and wild-type mRNA transcripts.

SDS-PAGE analysis of 35S-cysteine labelled subunit-specific cell lines from the α- and β-subunit specific CHO cell transfections, has enabled the selection of high producing cell lines and demonstrated the variability in expression levels between individual cell lines (Figure 5.4). These, as well as the high producing oFSH heterodimer cell lines of α/β co-transfections incorporating the FIII-αSL+FIII-βSL, FIII-αSL+FIII-βSL (M2) and FIII-αSL+FIII-βSL (HYB) constructs, will
enable the synthesis of sufficient quantities of recombinant oFSH polypeptides for more detailed analysis.

The results described in this chapter show recombinant oFSH expression levels have been increased by substituting the 5' non-translated region and polypeptide leader sequence of the oFSH β-subunit cDNA, with the equivalent sequence from the oGH cDNA. Preliminary Northern blot analyses show that differences in mRNA transcript levels are unlikely to account for the higher expression levels. Further polypeptide analyses including β-subunit N-terminal amino acid sequencing, SDS-PAGE, isoelectric focussing (IEF), and RRA and bioassay estimations of activity are required to more fully characterise and compare the recombinant and native oFSH heterodimers and individual subunits.
CHAPTER SIX

CHARACTERISATION OF WILD-TYPE AND MUTANT oFSH cDNA PRODUCTS EXPRESSED IN CHO CELL LINES
6.1 Introduction

A variety of bioactive recombinant glycoproteins including bovine LH (Kaetzel et al., 1985), human interferon-β (Kagawa et al., 1988), hFSH (Keene et al., 1989a) and human tissue plasminogen activator (Spellman et al., 1989) have been expressed in CHO cells. CHO cells are considered suitable for the expression of human glycoproteins because the oligosaccharide moieties synthesized by these cells closely resemble those found in man (Mutsaers et al., 1986; Kagawa et al., 1988). Nevertheless, oligosaccharides associated with the recombinant human glycoproteins expressed in CHO cells differ from their native counterparts to varying degrees (Mutsaers et al., 1986; Kagawa et al., 1988; Hard et al., 1990). These differences can result in the synthesis of a recombinant product having improved biological activity over its native counterpart, as in the case of hFSH (P. Schofield, personal communication), or conversely having less bioactivity or even deleterious properties in the recipient (Kagawa et al., 1988).

This chapter describes experiments undertaken to compare the recombinant oFSH glycoproteins derived from both native and mutant cDNA sequences, with pituitary-derived oFSH heterodimer and dissociated α- and β-subunits. These experiments include: (i) N-terminal amino acid sequence analysis of the recombinant oFSH α- and β-subunit polypeptides derived from the high producing cell line αSL-βSL (HYB)/14.4 (see Chapter 5), (ii) SDS-PAGE and (iii) isoelectric focussing (IEF) analyses comparing pituitary-derived oFSH with the recombinant αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 CHO cell products and (iv), time course analysis of the biosynthesis of individually expressed α- and β-subunit cDNA (non-mutated) products, and native and mutant cDNA derived heterodimers. Finally the activities of the native and recombinant oFSH heterodimers expressed by the αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 CHO cell lines are compared in the in vitro RRA (Maghuin-Rogister et al., 1978) and Sertoli cell bioassay (Ritzen et al., 1982).
6.2 Results

6.2.1 **N-terminal amino acid sequence analysis of the recombinant oFSH α- and β-subunit polypeptides derived from the high producing cell line αSL-βSL (HYB)/14.4**

Recombinant oFSH was isolated from αSL-βSL (HYB)/14.4 culture media by MAb 87-70 affinity chromatography as described in Chapter 2. Briefly, one litre of fully supplemented growth medium was collected from routine αSL-βSL (HYB) 14.4 cell culture over a period of 2-3 weeks and extracted with 1 ml (packed gel volume) MAb 87-70 affinity resin (approximately 3 mg purified antibody/ml resin). Approximately 30 μg recombinant oFSH (as determined by RRA) was recovered from the affinity column. One microgram of affinity purified recombinant oFSH was utilised for N-terminal sequence analysis. The sequence analysis was performed by Dr. B. Classon (The University of Melbourne) using the Edman degradation technique (Edman and Begg, 1967) on an automated amino acid sequencer (model 471A, Applied Biosystems) with an on-line pTH amino acid analyser (model 120A, Applied Biosystems).

The N-terminal amino acid sequence established for the recombinant oFSH α-subunit polypeptide [Figure 6.1, (α)] confirmed that the native site of prepeptide cleavage is utilised in FIII-αSL transfected CHO cells. Two β-subunit amino acid sequences, each utilising a different signal peptide cleavage site, were established for the recombinant oGH/oFSH β-subunit hybrid cDNA product [Figure 6.1, (β)]. Approximately 30% of the β-subunit polypeptides utilised what would be the equivalent native oFSH β-subunit signal peptide cleavage site (see Figure 1.1), while the remaining 70% utilised what would be the equivalent of the native oGH signal peptide cleavage site. Signal peptide cleavage at the "oGH-directed" site results in the inclusion of an extra glycine residue at the N-terminus of the mature β-subunit
Figure 6.1
Signal peptide cleavage sites established for the recombinant oFSH α- and β-subunit polypeptides expressed by the FIII-αSL and FIII-βSL (HYB) expression constructs in the high producing CHO cell line αSL-βSL (HYB)/14.4.

(α)
Recombinant oFSH α-subunit prepeptide

Signal peptide cleavage site...........↓
-25 ~ -5 -1 +1 +5 ~ +96
Met ~ Gln Ile Lue His Ser Phe Pro Asp Gly Glu ~ Ser
|------------------------ oFSH α-subunit prepeptide sequence-------------------------|

(β)
Recombinant oGH/oFSH β-subunit hybrid prepeptide

[70%] [30%]

Signal peptide cleavage sites........ ↓  ↓
-25 ~ -4 -1 +1 +5 ~ +109
Met ~ Gln Val Val Gly Gly Ser Cys Glu Leu Thr ~ Ser
|------------ oGH signal peptide----------|  |---------------- oFSH mature β-subunit----------|
|-----------------|  |-----------------|
| Intervening glycine residue |
polypeptide sequence. This residue is not derived from the oGH or oFSH cDNA sequences, but encoded as a result of the hybrid cDNA construction (see Chapter 2, section 2.11),

6.2.2 SDS-PAGE and IEF of monoclonal antibody affinity purified recombinant oFSH heterodimers and dissociated α- and β-subunits

**SDS-PAGE:** Samples containing approximately 500 ng of MAb affinity purified αSL-βSL/9.10 and αSL-βSL(HYB)/14.4 recombinant oFSH, and affinity purified pituitary oFSH, were prepared as non-boiled/non-reduced and boiled/non-reduced samples and analysed by extended SDS-PAGE electrophoresis on 15% polyacrylamide gels. A non-transfected CHO cell supernatant control was prepared by MAb affinity purification of an equal volume of culture medium to that required for the isolation of the 500 ng αSL-βSL/9.10 sample, was also prepared and included. The native oFSH preparation was isolated from pituitary extracts by MAb 87-70 affinity chromatography (Mr. R. Fiddes, The University of Melbourne) and contained BSA (Fraction V, Boehringer) as a bulking agent.

The results of both the Coomassie blue and silver staining analyses are presented in Figure 6.2. Interestingly, the recombinant heterodimers and dissociated oFSH subunits display considerably slower electrophoretic mobilities than the native glycoproteins. Both gels show recombinant oFSH heterodimers of approximately 35-42 kD and dissociated α- and β-subunit bands ranging from approximately 18-24 kD (Figure 6.2). The native heterodimers and dissociated oFSH subunits are approximately 33-34 k, and 16-20 kD respectively (Figure 6.2). Slight differences between the two recombinant products were evident using the silver staining technique although less apparent by Coomassie staining. Bands equivalent to the oFSH heterodimers and dissociated subunits are not evident in the negative
Figure 6.2

SDS-PAGE analyses comparing oFSH heterodimers and dissociated subunits of MAb affinity purified pituitary oFSH with the affinity purified recombinant oFSH products expressed by the high producing CHO cell lines αSL-βSL/9.10 and αSL-βSL(HYB)/14.4. Samples were prepared as both non-boiled/non-reduced and boiled/non-reduced samples to enable visualisation of oFSH heterodimers and dissociated oFSH subunits respectively. Staining techniques, samples and molecular weight markers are as indicated.
control sample, although a contaminating albumin band (Mr = 50 k) is apparent in each of the CHO cell derived media samples and the non-transfected CHO cell supernatant control (Figure 6.2).

**IEF/Western transfer:** Samples containing approximately 2.0 µg of each recombinant oFSH preparation, a similarly prepared non-transfected CHO cell supernatant control (see above), and a MAb affinity purified pituitary oFSH preparation (containing no additional BSA bulking protein) were analysed by IEF and subsequent Western transfer (see Chapter 2). IEF experiments using the Coomassie Blue G-250 quick stain technique (IEF cell instruction manual, model # 111, Biorad, USA) did not detect any protein other than the contaminating BSA band (results not shown). The IEF gel focussing gradient ranged from pH 3-10. The results of the IEF Western transfer are presented in Figure 6.3.

Results of the IEF Western transfer show a considerable difference in pl values between the recombinant and native oFSH preparations (Figure 6.3). Some slight differences are also evident between the two recombinant products which show a number of tight and diffuse bands with pl values ranging from 4.5-6.5. The native oFSH sample does not show any clearly defined bands but rather a single broad diffuse band ranging in pl from 6.2-8.2 with the major proportion focussing around pH 7.0. No staining is evident for the non-transfected CHO cell supernatant control (Figure 6.3).

**6.2.3 Biosynthesis of oFSH α- and β-subunit polypeptides, and wild-type and mutant cDNA derived oFSH heterodimers**

CHO cell lines expressing oFSH α- or β-subunit polypeptides, or wild-type or mutant cDNA derived oFSH heterodimers, were labelled for 20 minutes with 35S-cysteine (150 µCi/ml) and chased with unlabelled cysteine for 0.33, 1, 2,
Figure 6.3

Western transfer of an isoelectric focussing gel (pH 3-10) comparing MAb affinity purified pituitary oFSH and recombinant oFSH glycoproteins expressed by the high producing CHO cell lines αSL-βSL/9.10 and αSL-βSL(HYB)/14.4. A control MAb affinity purified non-transfected CHO cell supernatant and Coomassie blue stained IEF standards are also indicated.
4, 6 or 12 hours as indicated in Figure 6.4. Cell lysate and media samples were immunoprecipitated for SDS-PAGE analysis and analysed by extended runs on 15% acrylamide gels as boiled/non-reduced samples.

Results presented in Figure 6.4 (αSL), show that recombinant oFSH α-subunit polypeptides are secreted independently of the oFSH β-subunit in the α-subunit specific CHO cell line αSL/8. Results for the β-subunit specific CHO cell line βSL/7 [Figure 6.4, (βSL)], show that the free oFSH β-subunit is not efficiently secreted although some β-subunit bands are evident in the media samples within 2 hours of biosynthesis.

Band intensities for the α- and β-subunit specific cell lysate samples [Figure 6.4, (αSL) and (βSL)], are considerably darker than their respective α- and β-subunit specific media samples [Figure 6.4, (αSL) and (βSL)]. The difference in band intensities between the secreted and non-secreted forms of the individually expressed oFSH subunits, may be indicative of polypeptide degradation or saturation of the precipitating antibody. The smaller band sizes evident in the 2 and 4 hour cell lysate samples of both the α- and β-subunit specific cell lines [Figure 6.4 (αSL) and (βSL)] suggests intracellular degradation is at least partly responsible.

Co-expression of both wild-type and mutated β-subunit cDNA sequences with the wild-type α-subunit sequence, in each case resulted in efficient secretion of both oFSH subunits [Figure 6.4, αSL-βSL, αSL-βSL (M2) and αSL-βSL (HYB)]. While clear delineation between individual α- and β-subunit polypeptides is difficult, the multiple bands evident in all 0.33-6.0 hour cell lysate and 2-12 hour media samples are consistent with the combined α- and β-subunit bands of the subunit specific cell lines. As samples were boiled for SDS-PAGE analysis, no oFSH heterodimers are apparent. Band intensities
Figure 6.4

Time course analyses showing the biosynthesis of oFSH heterodimers and individually expressed oFSH subunits in CHO cell lines. Cell cultures were labelled with $^{35}$S-cysteine for 0.33 hours (0.33) and chased with unlabelled cysteine for the times indicated (1-12). Cell lysate (Lysates) and culture media (Media) immunoprecipitates for the previously described $\alpha$SL/8 ($\alpha$SL), $\beta$SL/7 ($\beta$SL), $\alpha$SL-$\beta$SL/9.10 [$\alpha$SL-$\beta$SL], $\alpha$SL-$\beta$SL (M2)/4.6 [$\alpha$SL-$\beta$SL (M2)] and $\alpha$SL-$\beta$SL (HYB)/14.4 [$\alpha$SL-$\beta$SL (HYB)] cell lines were prepared and analysed by SDS-PAGE extended runs on 15% polyacrylamide gels (Chapter 2).
shown for the three α/β time course analyses [Figure 6.4, αSL-βSL, αSL-βSL (M2) and αSL-βSL (HYB)] indicate that wild-type and mutant cDNA derived α- and β-subunits are extracellularly stable and that little if any saturation of the precipitating antibody has occurred.

6.2.4 RRA and in vitro bioassay of recombinant oFSH expressed by both wild-type and mutated cDNA sequences

RRA: CHO cell lines αSL-βSL/9.10, and αSL-βSL (HYB)/14.4, and a non-transfected negative control, were grown to confluence in 850 cm² roller bottles in fully supplemented growth medium. Briefly, confluent cells from a single 175 cm² flask were eluted with trypsin and added to a single 850 cm² roller bottle in 250 ml fully supplemented growth medium. Roller bottles were rotated at 0.25 rpm for 48 hours by which time cell monolayers were confluent. Cultures were then maintained in 250 ml α-MEM medium supplemented with 0.12% FCS and 50 µM ZnSO₄ for 10 days. Culture supernatants were harvested, concentrated 25-fold and dialysed against fresh α-MEM medium prior to RRA and in vitro bioassay analyses. The results of both assays are presented in Table 6.1.

The RRA incorporated an in-house oFSH standard based on the NIDDK-oFSH-RP-1 standard (NIH, NIDDK, USA) as determined using the rat ovarian weight hCG augmentation bioassay (Steelman and Pohley, 1953). Recombinant oFSH activities for the concentrated media preparations were established from the mean results for three sample dilutions assayed in duplicate (see Table 6.1). The concentrated medium from cell line αSL-βSL/9.10, which incorporates the truncated wild-type oFSH subunit cDNA sequences, contained 11.1 x 10⁻³ units (NIH)/ml (46.1 ng/ml) recombinant oFSH. The concentrated medium from cell line αSL-βSL (HYB)/14.4, which incorporated the wild-type α-subunit and hybrid oGH/oFSH β-subunit cDNA...
Table 6.1

Comparison of the recombinant oFSH activities of the concentrated αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 culture supernatants as determined in the RRA and *in vitro* Sertoli cell bioassay. RRA results were established by extrapolation from an oFSH standard curve. *In vitro* bioassay results are presented as calculated on the basis of the same oFSH standard in the RRA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RRA Activity (oFSH NIH units)</th>
<th><em>In vitro</em> Bioactivity (oFSH NIH units)</th>
<th>Ratio RRA:Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSL-βSL/9.10</td>
<td>1.11 x 10^{-3}</td>
<td>1.33 x 10^{-3}</td>
<td>1:1.20</td>
</tr>
<tr>
<td>αSL-βSL (HYB)/14.4</td>
<td>1.79 x 10^{-3}</td>
<td>2.50 x 10^{-3}</td>
<td>1:1.39</td>
</tr>
<tr>
<td>Negative control</td>
<td>ND</td>
<td>1.4 x 10^{-5}</td>
<td>1</td>
</tr>
</tbody>
</table>
constructs, contained $1.79 \times 10^{-3}$ units (NIH)/ml (78.0 ng/ml) recombinant oFSH. The non-transfected CHO cell supernatant control was included in the assay to eliminate the possibility of bovine FSH contamination from the FCS used in the medium. The control was negative in the assay. The unexpectedly low levels of recombinant oFSH expression are probably attributable to nutrient exhaustion of the media within the first few days of culture.

**In vitro Sertoli cell bioassay:** The *in vitro* Sertoli cell bioassay was based on the method described by Ritzen and co-workers (1982) and conducted by Dr. D. Robertson (Prince Henry's Institute of Medical Research, Melbourne). This assay system determines FSH bioactivity on the basis of oestrogen biosynthesis in *in vitro* Sertoli cell cultures. The amount of oestrogen secreted by the Sertoli cell cultures in response to exogenous FSH is then determined by RIA. The assay included the same oFSH standard and non-transfected CHO cell negative control as utilised in the RRA, as well as the World Health Organisation human FSH standard (hFSH 83/575, WHO, Switzerland). The results for the *in vitro* Sertoli cell bioassay are presented in Table 6.1.

The recombinant oFSH bioactivities established for the αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 supernatants on the basis of the oFSH standard in the *in vitro* Sertoli cell bioassay were $1.33 \times 10^{-3}$ and $2.50 \times 10^{-3}$ units (NIH)/ml respectively. These estimates are approximately 1.20 and 1.39 times higher than estimates established for the same concentrated αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 supernatants in the RRA.
6.3 Discussion

The results described in this chapter have shown clear physicochemical and biological differences between the recombinant and pituitary-derived oFSH heterodimers and individual subunits. Some slight differences were also demonstrated between the two recombinant oFSH products.

N-terminal amino acid sequence analysis of oFSH subunit polypeptides expressed in CHO cells by wild-type α-subunit and hybrid oGH/oFSH β-subunit cDNA expression constructs, confirmed the accurate translation and signal peptide cleavage of the recombinant α-subunit prepeptide in CHO cells, and demonstrated a probable difference between the wild-type and oGH/oFSH hybrid β-subunit cDNA products. The use of multiple signal peptide cleavage sites within the oGH/oFSH β-subunit prepeptide could be eliminated by site-directed mutagenesis of the hybrid cDNA sequence to remove the codon encoding the intervening glycine residue.

Results of the SDS-PAGE analysis confirmed that the αSL-βSL/9.10 and αSL-βSL (HYB)/14.4 CHO cell lineages secrete a multimeric form of oFSH that can be dissociated into subunits by boiling. The higher molecular weights of the recombinant heterodimers and dissociated subunits compared to the native proteins, are most probably attributable to differences in the associated oligosaccharides. IEF Western transfer results, which show that the recombinant oFSH heterodimers have considerably lower pI values than the native preparation, further support this possibility. The lower pH range established for both the recombinant products is in keeping with the high levels of sialylation described for hFSH expressed in CHO cells (Hard et al., 1990). A similar increase in molecular weight has been demonstrated for recombinant bLH expressed in CHO cells, when compared with pituitary derived bLH (Kaetzel et al., 1985). No such difference has been described for recombinant hFSH (Keene et al., 1989a) although hFSH
isoforms isolated from patients treated with GnRH antagonists display lower molecular weights and higher pl values consistent with reduced sialic acid content (Dahl et al., 1988). It is of interest to note that while native oFSH and bLH have ratios of %sialylated:%sulphated oligosaccharides of less than 1.0, native hFSH has a ratio greater than 12.6 (Green and Baenziger, 1988b). It is also interesting that while the recombinant oFSH heterodimers and dissociated oFSH subunits expressed in CHO cells showed slower electrophoretic mobilities compared with the pituitary derived subunits (Figure 6.2), recombinant oFSH subunits expressed by the same expression constructs in COS cells did not show such differences (see Figure 4.2).

The in vitro and in vivo bioactivities of recombinant hFSH heterodimers expressed in wild-type and glycosylation defective mutant CHO cell lines have been compared with pituitary derived hFSH (Galway et al., 1990). The mutant CHO cell lines used in this study were deficient in either the glycosylation enzyme N-acetylgalactosamine transferase-1, or sialic acid transport in the golgi complex. The resultant oligosaccharides synthesized by the mutant cell lines were therefore predicted to be simple mannose-type core structures deficient in both sialic acid and N-acetylgalactosamine, or wild type oligosaccharides deficient in sialic acid only. Results of the in vitro bioassay showed all hFSH preparations were equipotent demonstrating sialic acid and N-acetylgalactosamine moieties do not influence bioactivity in vitro. Results of the in vivo bioassay showed that both glycosylation deficient CHO cell products were essentially inactive, further demonstrating the pivotal role sialic acid moieties play in ensuring bioactivity in vivo. Somewhat conflicting results have been described in another report indicating that acidic isoforms of recombinant hFSH are less active in in vitro bioassays, and that basic isoforms have higher receptor affinities as determined by displacement of radiolabelled pituitary hFSH in a rat testis radioreceptor assay (Cerpa-Poljak et al., 1990).
While the apparently higher sialic acid contents of the two recombinant oFSH preparations are not likely to account for the higher *in vitro* Sertoli cell bioassay activities (see Table 6.1), such differences may result in significant increases in *in vivo* bioactivity by prolonging the circulatory half-life of the recombinant hormone (Hard *et al*., 1990). A more likely explanation for the apparent increase in recombinant oFSH activity in the *in vitro* bioassay may lie in a higher receptor binding affinity of the less extensively glycosylated native oFSH heterodimer, in the RRA. The higher receptor binding affinity of the native oFSH standards may result in lower recombinant oFSH estimations in the RRA, although, this effect would probably be offset by accompanying higher *in vitro* bioactivity for the oFSH standards. While low levels of associated sialic acids may improve *in vitro* bioactivity (Cerpa-Poljak *et al*., 1990), experiments described by Sairam and Bhargavi (1985) have shown that deglycosylation of the glycoprotein hormone α-subunit effectively abolishes signal transduction.

In summary, results described in this chapter have shown that the recombinant oFSH products are biologically active *in vitro*. Furthermore, IEF results showed that the recombinant oFSH heterodimers expressed by the CHO cell lines display characteristically low pl values previously associated with recombinant hFSH expressed in CHO cells (Keene *et al*., 1989a). Further experiments are required to confirm the *in vivo* bioactivity of the recombinant oFSH products.
CHAPTER SEVEN

GENERAL DISCUSSION
The experiments described in this thesis have enabled the cloning and characterisation of the full length oFSH β-subunit cDNA sequence, and derivation of the predicted amino acid sequence for the oFSH β-subunit prehormone. While some coding region amino acid sequence disparities are apparent when the predicted oFSH β-subunit sequence and the sequence established by amino acid sequence analysis (Sairam, 1981) are compared, such disparities were not unexpected considering the polymorphic nature of FSH β-subunit genes shown by Southern blot analysis (Adams and Gogolin, unpublished; Montgomery et al., 1990).

Mammalian cell transfection experiments incorporating the oFSH α- and β-subunit expression constructs have enabled the biosynthesis and secretion of a recombinant oFSH heterodimer displaying biological activity in the in vitro Sertoli cell bioassay. Transfection experiments incorporating truncated oFSH β-subunit cDNA sequences have shown that the 3' non-coding region sequences of the β-subunit mRNA transcript, in part, regulate the stability of the mRNA in transfected mammalian cells. Northern blot analysis comparing β-subunit transcript levels in cell cultures expressing either full length or 3' truncated cDNA sequences have shown that the removal of the 3' non-coding region sequence greatly increases mRNA transcript levels. The large increase in β-subunit transcript levels was, however, accompanied by only a small increase in oFSH heterodimer expression levels. Definitive experiments demonstrating that the 3' non-coding region AUUUA sequence motifs, and not other non-coding or coding region sequences (Cleveland and Yen, 1989), are responsible for destabilising the oFSH β-subunit mRNA transcript have yet to be undertaken. Site directed mutagenesis of the seven cDNA sequence motifs encoding the AUUUA sequence, and subsequent transfection of expression constructs incorporating these mutant sequences should provide further insight into their role in regulating mRNA stability.
Given that the AUUUA mRNA sequence has a functional role in regulating gene expression, it is tempting to postulate that multiple polyadenylation site usage, which results in mRNA transcripts having either multiple or no AUUUA motifs, may form a basis for endogenous FSH regulation. Both ovine and human FSH β-subunit genes utilise multiple polyadenylation sites which result in mRNA transcripts having either multiple or probably no AUUUA motifs (Esch et al., 1986; Jameson et al., 1988; Montgomery et al., 1990). While results of Northern blot analyses described in this thesis and other reports have shown that the longer 2.0 kb FSH β-subunit transcript is dominant in ovine pituitary mRNA, preferential transcription of the shorter more stable transcript could result in higher FSH expression and higher fecundity. Higher oFSH serum levels have been described in the high fecundity breed of Merino sheep known as Booroola (Bindon, 1984), although results of oFSH β-subunit gene segregation and Northern blot analysis have indicated that at least in this instance the increased fecundity cannot be attributed to differences in the β-subunit gene sequences (Montgomery et al., 1990). Reports describing two ovine FSH β-subunit genomic sequences [P. Bello, (unpublished); Guzman et al., 1990] may further complicate this finding.

Results described in Chapter 5 have shown that substitution of the 5' non-coding and signal peptide coding region sequences of the oFSH β-subunit mRNA transcript also affects the level of recombinant oFSH heterodimer expression. While mRNA secondary structure analysis has not been performed for the native or mutant oFSH β-subunit transcripts, such an analysis may provide some insight into the regulation of oFSH expression. Further improvements in recombinant oFSH expression levels could be achieved through similar modifications to the α-subunit cDNA sequence. While most efforts in achieving high level expression of oFSH have centred on improving β-subunit expression, results of at least one experiment in which an 11-fold increase in bovine LH expression was achieved following copy
number amplification of only the α-subunit expression construct (Kaetzel and Nilson, 1988) warrant further optimization of α-subunit expression levels.

Post translational modifications such as glycosylation can greatly influence the in vivo bioactivity of the glycoprotein hormone heterodimer (Sairam and Bhargavi, 1985; Galway et al., 1990). Experiments described in Chapter 6 have shown substantial differences in native and recombinant oFSH glycosylation patterns. As the recombinant product appears by far the more extensively sialylated of the two, and a high sialic acid content is believed to prolong circulatory half-life and therefore bioactivity in vivo, one would anticipate that the recombinant product would be more active. While Hard and co-workers (1990) made the same observation and prediction in examining native and recombinant hFSH, it is interesting to note that the difference in in vivo bioactivity between native and recombinant oFSH is potentially much greater than the difference between native and recombinant hFSH. Hard and co-workers (1990) have reported that native and recombinant hFSH preparations differ primarily in the extent of core mannose fucosylation, the type of sialic acid linkage, and the percentage of neutral oligosaccharides, 5%-10% (native) vs 0% (recombinant). While these differences predictably result in some charge difference between the native and recombinant glycoproteins, results of earlier chromatofocusing experiments have shown that the two forms of hFSH are "very similar if not identical" (Keene et al., 1989a). Results of the isoelectric focussing experiment comparing the charge heterogeneities of native and recombinant oFSH (Chapter 6), have shown that the two oFSH preparations are very different. These results are supported by the characteristically high levels of sialylation associated with CHO cells and the low ratio (≈0.95) of %sialylated:%sulphated oligosaccharides described for native oFSH (Green and Baenziger, 1988b). The ratio of %sialylated:%sulphated oligosaccharides described for native hFSH is 12.58 (Green and Baenziger, 1988b).
While some increase in in vivo bioactivity can be expected for recombinant oFSH, the extent of this "improvement" and the benefit to veterinary medicine is yet to be established. It is possible that the higher levels of sialylation found in association with the recombinant oFSH heterodimer may result in an excessively prolonged circulatory half-life, as is often the case when the highly sialylated gonadotrophic hormone eCG [equine chorionic gonadotrophin, or pregnant mare's serum gonadotrophin] is used for stimulating reproductive performance (Dohnt et al., 1978; Bindon and Piper, 1981). The circulatory half-lives for eCG in sheep and cattle are 21.2 hours and 50-120 hours respectively (McIntosh et al., 1975; Schams et al., 1977). As the highly sialylated carboxy-terminal amino acid extension, unique to the eCG β-subunit is probably primarily responsible for the eCG heterodimer's long half-life, it is unlikely that recombinant oFSH will have as long a half-life. Nevertheless, the higher sialic acid content of oligosaccharides associated with the recombinant oFSH heterodimer may increase the oFSH circulatory half-life and therefore reduce the dose of oFSH required for a given veterinary application. Similarly, differences in endogenous oFSH glycosylation patterns may provide an explanation for differences in fecundity such as those described for Booroola and non-Booroola Merinos, despite identical FSH α- and β-subunit genes.

Despite many reports describing the successful over-production of recombinant proteins the challenge of making such technology routinely applicable to all target molecules still exists. Why for example, have the maximum expression levels achieved for recombinant human and ovine FSH been in the order of 1 μg/10^6 cells/day (Bishop et al., 1990) while hGH expression levels achieved using an equivalent expression vector and the same host cell system have been at least 200-fold higher (Friedman et al., 1989)? Obviously there are many contributing factors affecting the level of expression, including mRNA stability and efficiency of translation, and the biological and physicochemical nature of the recombinant
protein. Certainly, FSH and the heterodimeric nature of the glycoprotein hormones provides an excellent example of the complex systems controlling both endogenous and heterologous gene expression.

Possible future efforts aimed at achieving higher recombinant oFSH expression levels, should centre on increasing the translatability of the abundant oFSH α- and β-subunit mRNA transcripts. One possible approach would be to incorporate 5' mRNA non-coding region sequences, such as the adenovirus late mRNA tripartite leader sequence. Heterologous mRNA transcripts incorporating the adenovirus tripartite leader sequence have been shown to produce 10- to 50-fold higher levels of translation products in the presence of viral-associated (VA) RNA (Kaufman, 1985). The VA RNA, which can be provided by adenovirus infection or co-transfection of plasmids containing the VA genes, is believed to bind to the leader sequence and facilitate mRNA transport, cytoplasmic localisation, or ribosome binding (Kaufman, 1985). Less significant increases in translation have also been described for tripartite leader-containing mRNA sequences independently of VA RNA (Kaufman, 1985; Dolph et al., 1988).

Assuming higher levels of recombinant oFSH expression can be achieved through the application of these and other new technologies, the scale-up of mammalian cell cultures for the over expression of recombinant oFSH may provide an alternative source of oFSH for use in veterinary medicine.
APPENDICES
Appendix 1

Reagents:

**Phenol (pH > 7.8)**

Analytical grade phenol (cat # 10188, BDH) was liquefied by heating to 65°C and dispensed into 25 ml lots for storage at -20°C until required. As required, phenol was thawed at 65°C and 8-hydroxyquinoline (Fluka) added to 0.1% (w/v). Liquefied phenol was then equilibrated to a pH > 7.8 by adding and mixing an equal volume of 1 M Tris HCl (pH 8.0) 0.2% β-mercaptoethanol. The aqueous phase was then removed and the equilibration procedure repeated twice more with 0.1 M Tris HCl (pH 8.0) 0.2% β-mercaptoethanol. Equilibrated phenol was stored at 4°C for up to one month.

**Acid phenol (water saturated)**

Frozen phenol (25 ml) was thawed at 65°C and 8-hydroxyquinoline added to 0.1% (w/v). Liquefied phenol was then mixed with an equal volume of H2O and allowed to settle. Once separated the aqueous phase was aspirated and the procedure repeated twice more. The water saturated phenol was stored at 4°C for up to one month.

**Chloroform / isoamylalcohol**

Chloroform / isoamylalcohol was prepared at a ratio of 24:1 and stored in an air-tight bottle.

**Phenol / chloroform / isoamylalcohol**

Equal parts of equilibrated phenol and chloroform / isoamylalcohol were combined and stored for up to one month at 4°C.
Denhardt’s solution (100x)

- 2% Ficoll (type 400, Pharmacia)
- 2% polyvinylpyrrolidone
- 2% BSA (Fraction V)

0.5 M Ethylenediaminetetra-acetic acid (EDTA, pH 8.0)
Dissolve EDTA in H₂O whilst constantly adjusting the pH to 8.0 with NaOH pellets. Sterilize by autoclaving.

Ethidium bromide 10 mg/ml
Dissolve ethidium bromide in H₂O and filter through a 45 μm disposable filter. Store in the dark.

LB (1 litre)

Media: 10 g bacto-tryptone
5 g yeast-extract
10 g NaCl
pH to 7.5 with NaOH
Sterilize by autoclaving

LB Agar (1 litre):
1 litre LB media +15 g bacto-agar
Sterilize by autoclaving.

Salmon sperm DNA (10 mg/ml)
Salmon sperm is dissolved by boiling for up to 30 minutes with stirring. DNA is then sheared by repeatedly passing the solution through an 18 gauge needle for up to 10 minutes. Prehybridisation and hybridisation solutions are heated to 65°C prior to the addition of the concentrated salmon sperm stock.
3 M Sodium acetate (pH 5.2)
Dissolve sodium acetate in H₂O and adjust pH to 5.2 with glacial acetic acid. Sterilize by autoclaving.

10% Sodium dodecyl sulphate (SDS)
Dissolve SDS (cat # 1028693, Boehringer) in H₂O at 65°C. Adjust pH to 7.2 with HCl.

20x SSC (standard sodium citrate)
3 M NaCl
0.3 M Tri-sodium citrate
Sterilize by autoclaving.

20x SSPE
3 M NaCl
0.2 M NaH₂PO₄
0.02 M EDTA (pH 8.0)
pH to 7.4 with NaOH
Sterilize by autoclaving.

50x TAE
2 M Tris acetate (484 g Tris base + 114.2 ml glacial acetic acid / litre)
0.05 M EDTA (pH 8.0)

TE
10 mM Tris HCl (pH 7.4)
1 mM EDTA (pH 8.0)
Sterilize by autoclaving.
0.6% Top agar (1 litre)
  10 g casamino acids
  5 g yeast extract
  3 g NaCl
  2 g KCl
  0.6 g agarose
  10 ml 1 M Tris-HCl (pH 7.5)
  10 ml 1 M MgCl

Sterilize by autoclaving.

1 M Tris-HCl (various pHs)
  Dissolve Tris base in H₂O and adjust pH with concentrated HCl accordingly. Sterilize by autoclaving.

2TY media (1 litre)
  16 g bacto-tryptone
  10 g yeast extract
  5 g NaCl

Sterilize by autoclaving.

2TY Agar (1 litre)
  1 litre 2TY media + 15 g agar

Sterilize by autoclaving.

2TY Top agarose (1 litre)
  1 litre 2TY media + 6 g agarose

Sterilize by autoclaving.
Appendix 2

Nucleotide sequence and the predicted amino acid sequence for the ovine glycoprotein hormone α-subunit cDNA clone 3.2 (Bello et al., 1989)*. Amino acid residues of the signal peptide are indicated by negative numbers while those of the mature polypeptide are indicated by positive numbers. The predicted amino acid sequence agrees entirely with the published ovine α-subunit amino acid sequence (Sairam, 1983). Restriction enzyme sites of interest are indicated.

---

**CHO**

Sites of N-linked oligosaccharide attachment.

***

Termination codon.

---

Polyadenylation signal.

---

Cloning sequence artefact.

---

The published amino acid sequence (Bello et al., 1989) does not include the 5' cloning sequence artefact of approximately 70 dTTP residues.

---

*
Appendix 3

Restriction enzyme cloning sites for the FIII eukaryotic expression vector.
Appendix 4

Western immunoblot analysis of three oFSH mouse antisera. Two 60 µg samples (boiled/non-reduced and non-boiled/non-reduced) of MAb affinity purified oFSH (supplied by R. Fiddes, The University of Melbourne) were prepared and combined as a single sample to enable SDS-PAGE separation of the intact oFSH heterodimer and individual oFSH subunits. A single gel width sample and prestained molecular weight markers (Mr) were separated by 15% SDS-PAGE and transferred to a nitro-cellulose membrane by Western transfer. The oFSH portion of the membrane was cut into strips for amino black staining and immunoblot analysis. Three oFSH antisera (1-3) and a normal mouse serum negative control were diluted to 1:500 in blotto. A HRP-labelled, rabbit anti-mouse IgG second antibody (Silenus, Australia), was diluted to 1:1000 in blotto. Immunoblots were developed in the presence of 0.06% diaminobenzidine.
ABBREVIATIONS
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>b</td>
<td>Bovine</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CG</td>
<td>Chorionic gonadotrophin</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary (cell)</td>
</tr>
<tr>
<td>COS</td>
<td>Simian virus 40 transformed African green monkey kidney cells</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>e</td>
<td>Equine</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem (cell)</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FL</td>
<td>Full length</td>
</tr>
<tr>
<td>FRP</td>
<td>Follicle stimulating hormone releasing hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin releasing hormone</td>
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<tr>
<td>gpt</td>
<td>Guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>h</td>
<td>Human</td>
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<tr>
<td>HGPRT</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>hMTIIA</td>
<td>Human metallothionein IIA</td>
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<tr>
<td>HPD</td>
<td>Hypothalamo-pituitary disconnected (HYB) Hybrid</td>
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<td>kD</td>
<td>Kilodaltons</td>
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<td>Kilobases</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
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</table>
M     Moles per litre
MAb   Monoclonal antibody
(M2)  Mutant 2
(M12) Mutant 12
Mr    Molecular weight in kilodaltons
MTX  Methotrexate
Neo'  Neomycin resistance (gene)
N-linked Nitrogen-linked
o     Ovine
O-linked Oxygen-linked
OVX   Ovariectomised
p     Porcine
P/S   Penicillin/streptomycin
RIA   Radioimmunoassay
RRA   Radioreceptor assay
RSV   Rous sarcoma virus
Ser   Serine
SL    Short length
SV40  Simian virus 40
Thr   Threonine
TK    Thymidine kinase
TM    Melting temperature
tPA   Tissue plasminogen activator
tRNA  Transfer ribonucleic acid
Tt    Testosterone
TSH   Thyroid stimulating hormone
XGPRT Xanthine-guanine phosphoribosyl transferase
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Mercer, J.E. and Clarke, I.J. (1988). Regulation of pituitary levels of messenger RNA (mRNA) for the subunits of follicle stimulating hormone (FSH) and luteinising hormone (LH) by gonadotrophin-releasing hormone in the ewe. *Proceedings of the Australian Society for Reproductive Biology, Annual Conference.* Newcastle, Australia.


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