

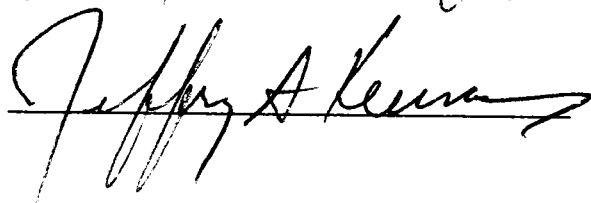
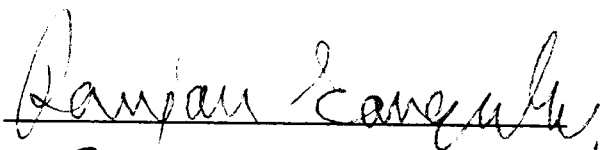
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REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION
SCREENING FOR
FSH RECEPTOR mRNA IN RAT UTERINE TISSUE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

David Andrew Wollert

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ABSTRACT

Luteinizing hormone/chorionic gonadotropin hormone receptors have been localized to extraovarian sites in the mammalian female, including uterus. In the ovary, LH receptor expression is regulated in part by the binding of FSH to its own receptor. The objective of this study was to investigate the possible uterine expression of FSH receptor mRNA in uterine tissue.

Rat ovary, uterus, liver, kidney, and hypothalamus were screened for the expression of FSH receptor mRNA by means of a reverse transcription - polymerase chain reaction (RT-PCR) amplification assay. Immature rats were primed with PMSG to stimulate follicular growth and development, followed 52 hours later by an injection of hCG to induce ovulation. Rats were then sacrificed at selected times (i.e. 2, 3, 6, 10, 13 days) following PMSG treatment.

Total cellular RNA was isolated from tissues and used as template for reverse transcription using avian myeloblastosis virus (AMV) reverse transcriptase and an oligonucleotide primer complementary to the rat FSH receptor cDNA sequence. The cDNA product of the RT reaction was then used as template for PCR.

The primers used in the PCR were designed to specifically amplify a 761 base pair region of the FSH receptor cDNA sequence. Upon electrophoretic separation

of the RT-PCR products, the 761 bp product, and thus expression of FSH receptor mRNA, was evident in ovary from each of the time points tested. The 761 bp product was not present, however, in any of the other tissue types, including human endometrium. These results suggest that FSH binding to receptor in the uterus is not involved in regulating the uterine expression of LH/CG receptors. As such, further studies are needed to determine the factors and mechanisms regulating ectopic LH/CG receptor expression.

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Chapter I

Introduction

The female reproductive system is a complex collection of organs and tissues which develop and function coordinatively and individually in response to hormones. The pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) are heterodimeric glycoprotein hormones known to regulate follicular development and luteinization in the ovary (1). Specific bioactivity of the gonadotropins is dependent upon the cell surface expression of appropriate receptor proteins by target cells. Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced by human placenta that is structurally and functionally similar, but not identical, to human LH (2). Consequently, hCG and LH bind to identical membrane receptors to regulate gonadal function.

Many of the functions and regulatory mechanisms related to gonadotropin binding have been known for some time. Still, novel sites of hormone receptor expression are being found (3-6), suggesting that unknown activities of the gonadotropins remain to be discovered.

Recently, several groups have reported the existence of LH/hCG receptors in extraovarian sites of mammalian females, including uterus, placenta, and fetal membranes (3-6). In the ovary, LH receptor expression is influenced

by gonadotropins, particularly FSH (7,8). The presence of LH receptors in the uterus suggests that uterine function may also be directly regulated by gonadotropins via endocrine, autocrine, paracrine, or juxtacrine mechanisms. Thus, it seems necessary to investigate the possible uterine expression of other gonadotropin receptors.

Classical studies of hormone receptor function and localization have relied on receptor/ligand binding assays which utilize specific radiolabeled receptor ligands (7,9,10). Receptor/ligand binding assays allow for quantitation of unoccupied receptor by measuring the amount of radioactivity present in membrane fractions of cells incubated with the labeled ligand. Such assays are effective but are limited to protein expression analysis and provide only indirect insight into the regulation of specific mRNA expression. The recent elucidation of gonadotropin receptor cDNA sequences (11,12) allows for new methods of studying receptor expression and regulation.

The polymerase chain reaction (PCR) is a recently developed technique for amplifying specific DNA sequences that has found many applications in clinical and molecular cell biology (for review see 13). Exponential amplification of a specific DNA sequence is accomplished by a cycling series of denaturation, annealing, and extension reactions involving DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), and specific sense and antisense

primers.

PCR has become a valuable technique for the generation of DNA probes, DNA sequencing, mutation analysis, and genetic screening. In addition, combining PCR with a reverse transcriptase reaction allows for the generation of cDNA libraries and mRNA analysis (14,15). This is achieved by incubating mRNA with complementary primer, dNTPs, and reverse transcriptase to generate cDNA which is then used as template for the PCR. Thus, reverse transcription-PCR (RT-PCR) can be used to identify the expression of specific mRNA using specific oligonucleotide primers in the RT and PCR reactions.

The objective of this study was to investigate the possible expression of FSH receptor mRNA in uterine tissue using RT-PCR. Evidence of FSH receptor mRNA in uterine tissue would suggest that previously unknown mechanisms of FSH regulation of uterine function may exist.

Chapter II

Review of Literature

A. The Distribution of FSH

Follicle-stimulating hormone is a dimeric glycoprotein gonadotropin essential for the development of ovarian follicles in females and testicular spermatogenesis in males. Implants of fresh anterior pituitary into hypophysectomized animals have long been shown to induce growth and maturation of the ovaries suggesting the pituitary as the site of FSH biosynthesis and secretion (16). Though there was controversy concerning the exact distribution of FSH gonadotrophs within the pituitary (17-20), it was long believed that FSH synthesis was confined to pituitary cells alone. Recently, however, novel sites of FSH biosynthesis and/or localization have been reported in such diverse tissues as prostate (21), placenta (22), testis (23), and stomach (24) suggesting that previously unknown functions of FSH remain to be discovered.

FSH In Pituitary

The pituitary gland, or hypophysis, is a small appendage at the base of the hypothalamus in the brain

stem. This organ secretes several hormones, many of which, including FSH, regulate the functions of other endocrine tissues. The pituitary tissues themselves are under the control of groups of neurosecretory cells in the hypothalamus. These neurosecretory cells respond to sensory input by secreting hormones which in turn participate in the regulation of neuroendocrine functions, including the sexual cycles. One type of neurosecretory cell secretes gonadotropin releasing hormone (GnRH) into the portal system which delivers the hormone to the anterior pituitary, whereupon GnRH acts to regulate the secretion of FSH and LH from gonadotrophs. The anterior lobe of the pituitary, or adenohypophysis, consists of the pars distalis, pars tuberalis, and pars intermedia. Gonadotrophs have been identified by immunohistochemistry in each of these regions (15,16,25,26).

Barnes (27) proposed two types of gonadotrophs in the mouse pituitary gland on the basis of electron microscopy and assumed that one type produces FSH and the other LH. Using this same classification of cell type, Kurosumi and Oota (28) reported that, in rat pituitary, FSH cells were characterized by two kinds of secretory granules, large and small, while LH cells contained only one type of small granules. Later investigations have suggested that most gonadotrophs contain both FSH and LH within the same cell, with a few containing LH alone (25,29,30).

Immunocytochemical studies have revealed the coexistence of FSH and LH in the same pituitary gonadotroph of several mammalian species (25,29,31-33). Inoue and Kurosumi (32) reported that using light- and electron-microscopic immunocytochemistry both FSH and LH are clearly present in the same gonadotrophic cells, but that the subcellular localization of these gonadotropins differed. They further reported that all pituitary gonadotrophs are characterized by both small and large secretory granules and that FSH is mainly present in large granules, whereas LH is mainly located in small granules.

Using both alpha- and beta-subunit specific antibodies for FSH and LH, Tanaka et al. (34) have shown that in bullfrog pituitary there are a number of gonadotrophs containing only the alpha subunit. Similar results have been obtained in porcine (35) and human (36) fetal pituitaries. These studies suggest that the beta- and alpha-subunits of the glycoprotein hormones may be differentially synthesized.

FSH in Prostate

With the localization of several regulatory peptides, the prostate has become a recognized gland of endocrine function (37,38). Recently, Hurkadli et al. (21) reported localization and de novo biosynthesis of FSH in human

prostatic tissue in vitro. Immunocytochemical localization revealed immunoreactive FSH in the cytoplasm of prostatic epithelial cells. De novo synthesis of FSH was demonstrated by specific immunoprecipitation of FSH detected by incorporation of [H^3] leucine.

Hurkadli and co-workers speculate that FSH, either directly or by modulating inhibin levels, may play some role in the etiologic physiology of prostate. Based on increased levels of circulating FSH in aging men and the known effects of FSH on cellular growth and differentiation in other tissues (i.e. ovary and testis), the possibility exists that FSH is involved in abnormal growth of the prostate.

FSH in Testis

Pituitary FSH specifically regulates spermatogenesis through its interaction with Sertoli cell receptors (39). Recently, Garde et al. (40) demonstrated that FSH is present in the cytoplasm of both Sertoli and Leydig cells of marmosets. This finding was of interest in view of the fact that, although FSH receptors have been demonstrated on Sertoli cells (23), their expression on Leydig cells has not been established. Though localization of FSH to Sertoli and Leydig cells could be due to receptor binding or endogenous synthesis, Garde and co-workers reported de

novo biosynthesis of FSH as demonstrated by immunoprecipitation of [H^3] labelled FSH in cultured Sertoli cell extracts.

Concentrations of FSH were found to differ in Sertoli and Leydig cells at different stages of development. From this Garde and co-workers speculate on the question of FSH modulating Leydig cell function and suggest that FSH may influence LH stimulated aromatase activity or that Leydig cell FSH may have paracrine action on Sertoli cells.

FSH in Stomach

Several active peptides originally found in the brain, including a number of hypothalamic hormones, have been demonstrated in the gastrointestinal tract (40-43). Recently, Mandrekar et al. (24) reported the immunocytochemical localization of FSH in human gastric mucosa. The histological pattern of FSH localization within the stomach suggested that FSH was expressed in specific cell types. The cytoplasmic distribution of FSH suggested that the hormone's presence was not concentrated by membrane bound receptors. The fact that thyrotropin releasing hormone (TRH) and other peptide hormones may act by endocrine, autocrine, or paracrine mechanisms differently in the gut than in the brain, suggests that an as yet undiscovered paracrine function for FSH may exist.

B. The Structure of FSH

Follicle stimulating hormone belongs to the glycoprotein family of hormones which includes FSH, the other pituitary gonadotropin LH, thyrotropin (TSH), and the placenta-derived chorionic gonadotropin (CG). This family is characterized by a heterodimeric structure, each hormone being composed of two subunits denoted alpha and beta. The interaction between the subunits is solely via noncovalent forces; No inter-chain disulfides are present. Within a species the alpha-subunits are essentially common to all four hormones while the beta-subunits are hormone specific (44). The primary structure of the alpha-subunit is encoded by a single gene and is highly conserved between different species (45). The beta-subunits are each encoded by a separate gene (the human FSH B gene is located on chromosome 11), and it is through the beta-subunits that receptor binding specificity is achieved (46). The primary structures of the individual beta-subunits, unlike those of the common alpha-subunit, have diverged significantly.

The glycoprotein hormones exhibit a complex chemical structure and possess a high degree of disulfide bridging within each subunit (46,47). Both the alpha- and beta-subunits of the four hormones consist of a single polypeptide chain to which is attached one or more oligosaccharide moieties. The structures of the

carbohydrate chains differ between the glycoprotein hormones and are believed to confer hormone specific functions. Interaction of the carbohydrate chains with putative cell membrane lectins may allow coupling of the hormone-receptor complex to adenylate cyclase (47).

Beginning in the early 1970's, the primary amino acid sequences of the gonadotropins were determined (48-51). Sairam (50) published the primary structure of the ovine pituitary FSH alpha-subunit and found it to be identical with that of ovine lutropin alpha-subunit. This laboratory also reported the primary structure of the ovine pituitary FSH beta-subunit (51). The genes for the glycoprotein subunits were isolated and sequenced throughout the 1980's and confirmed many of the primary structures previously obtained by digest methods of protein sequencing (45,52-59).

FSH-alpha

Figure 1 shows the amino acid sequence of the majority of human FSH alpha-chain molecules (53). There is some heterogeneity within the N-terminal section of the protein chain, but not in the overall sequence. Approximately 30% lack the terminal Ala-Pro dipeptide and 10% lack the Asp at position 7 (60). Thus, the minimum structure begins with Val at position 8. Variations at the N-terminus, which are

[illegible]

Figure 1 - FSH-alpha Subunit

Listed above is the amino acid sequence of the alpha-subunit of human FSH, LH and CG. Taken from Fiddes and Goodman (53).

due to post-translational changes, are of no consequence with regards to binding to beta-subunit or overall biological activity (47).

The FSH alpha-subunit contains two N-linked carbohydrate moieties attached to the asparaginyl residues at positions 56 and 82. The polypeptide sequence includes 10 cysteinyl residues which form 5 intra-chain disulfide bonds, most likely between residues 11-35, 14-36, 32-64, 63-91, and 86-88, respectively (60). The assignment of disulfide bonds is a difficult task due to the clustering of cysteinyl residues, the lack of enzymatic cleavage points within the clusters, and the problems of disulfide interchange. The disulfide bridges may be fully reduced and reoxidized to give a restored alpha-subunit with full hormonal activity. Thus, folding of the alpha-subunit to give correct disulfide bridges and a correct overall three-dimensional structure is a function of the primary sequence alone.

FSH-beta

Figure 2 shows the amino acid sequence of the human FSH beta-subunit (53). The polypeptide chain is 118 residues long and contains 12 cysteinyl residues which form 6 disulfide bridges. The FSH beta-subunit also contains two N-linked carbohydrate moieties at positions 13 and 30.

		5	10	15
CG	SER LYS GLU PRO LEU ARG PRO ARG CYS ARG PRO ILE ASN ALA THR			
LH	SER ARG GLU PRO LEU ARG PRO TRP CYS HIS PRO ILE ASN ALA ILE			
FSH	ASN SER CYS GLU LEU THR ASN ILE THR			
		20	25	30
CG	LEU ALA VAL GLU LYS GLU GLY CYS PRO VAL CYS ILE THR VAL ASN			
LH	LEU ALA VAL GLU LYS GLU GLY CYS PRO VAL CYS ILE THR VAL ASN			
FSH	ILE ALA ILE GLU LYS GLU GLU CYS ARG PHE CYS LEU THR ILE ASN			
		35	40	45
CG	THR THR ILE CYS ALA GLY TYR CYS PRO THR MET THR ARG VAL LEU			
LH	THR THR ILE CYS ALA GLY TYR CYS PRO THR MET MET ARG VAL LEU			
FSH	THR THR TRP CYS ALA GLY TYR CYS TYR THR ARG ASP LEU VAL TYR			
		50	55	60
CG	GLN GLY VAL LEU PRO ALA LEU PRO GLN VAL VAL CYS ASN TYR ARG			
LH	GLN ALA VAL LEU PRO PRO LEU PRO GLN VAL VAL CYS THR TYR ARG			
FSH	LYS ASN PRO ALA ARG PRO LYS ILE GIN LYS THR CYS THR PHE LYS			
		65	70	75
CG	ASP VAL ARG PHE GLU SER ILE ARG LEU PRO GLY CYS PRO ARG GLY			
LH	ASP VAL ARG PHE GLU SER ILE ARG LEU PRO GLY CYS PRO ARG GLY			
FSH	GLU LEU VAL TYR GLU THR VAL ARG VAL PRO GLY CYS ALA HIS HIS			
		80	85	90
CG	VAL ASN PRO VAL VAL SER TYR ALA VAL ALA LEU SER CYS GLN CYS			
LH	VAL ASP PRO VAL VAL SER PRE PRO VAL ALA LEU SER CYS ARG CYS			
FSH	ALA ASP SER LEU TYR THR TYR PRO VAL ALA THR GLN CYS HIS CYS			
		95	100	105
CG	ALA LEU CYS ARG ARG SER THR THR ASP CYS GLY GLY PRO LYS ASP			
LH	GLY PRO CYS ARG ARG SER THR SER ASP CYS GLY GLY PRO LYS ASP			
FSH	GLY LYS CYS ASP SER ASP SER THR ASP CYS THR VAL ARG GLY LEU			
		110	115	120
CG	HIS PRO LEU THR CYS ASP ASP PRO ARG PHE GLN ASP SER SER SER			
LH	HIS PRO LEU THR CYS ASP HIS PRO GLN LEU SER GLY LEU LEU PHE			
FSH	GLY PRO SER TYR CYS SER PHE GLY GLU MET LYS GLN TYR PRO THR			
		125	130	135
CG	SER LYS ALA PRO PRO PRO SER LEU PRO SER PRO SER ARG LEJ PRO			
LH	SEU			
FSH	ALA LEU SER TYR			
		140	145	
CG	GLY PRO SER ASP THR PRO ILE LEU PRO GLN			

Figure 2 - FSH-beta Subunit

Listed above are the amino acid sequences of the beta-subunits of human FSH, LH, and CG. Taken from Fiddes and Goodman (53).

Unlike the beta-subunit of LH, FSH-beta contains no O-glycosyl oligosaccharide structures. Comparison of the human FSH beta-subunit with the beta-subunits of human CG, LH, and TSH reveals two constant regions (61). One of these regions, the GAGY sequence, has been found in all beta-subunit proteins studied except for rat. Given that the beta-subunits have the common property of binding to the alpha-subunit, it has been suggested that regions of conserved sequence and structure are involved in alpha/beta interactions (62). Consequently, variable regions in the beta-subunits, being hormone specific, are most likely involved in the binding of the complete hormone to its specific receptor (47).

The cDNA and gene for FSH-beta has been isolated from human (63,64), bovine (57,58,65), porcine (66), and rat (62) species. Evidence from several studies has indicated that the gene structure for the glycoprotein hormone beta-subunits is well conserved, consisting of three exons separated by two introns. With regards to FSH, exon I codes for the 5' untranslated region, exon II for the signal peptide and 34-41 amino acids of the mature peptide, and exon III for the remainder of the mature protein and the 3' untranslated (UT) region (65). The FSH beta-subunit gene is unique among the other beta-subunit genes in that it possesses a disproportionately lengthy 3' UT region (approximately 1 kb) (65). The significance of this

feature is currently unknown, although sequences within the UT region of several genes have been shown to be important in determining RNA stability (67)

Several regions of the 5' flanking sequence of FSH-beta genes possess a high degree of similarity across species. These regions, located upstream from the promoter and transcription start sites, may represent cis-elements important in the regulation of transcription (62). Several recent studies have examined the cAMP response elements involved in expression of the glycoprotein alpha-subunit (68-71). Bokar et al. (69) found that different cis-acting elements are required for tissue specific expression of alpha-subunit in placenta and pituitary. Similar regions may be involved in tissue specific expression of beta-subunit genes.

Carbohydrates

With the amino acid and gene sequences of the glycoprotein subunits elucidated, current research has focussed on determining the structures of the carbohydrate moieties. It is believed that the protein and carbohydrate components of FSH are responsible for transmitting different physiological signals to FSH target cells. The protein domain of the FSH molecule binds to specific FSH receptors in the plasma membrane, while the carbohydrate

portion is believed to interact with putative cell membrane lectins to allow coupling of the hormone-receptor complex to adenylate cyclase (72). Binding of both components of FSH to receptor and associated structures is required for activation of adenylate cyclase and subsequent accumulation of cAMP (72-74).

FSH contains four carbohydrate chains linked through asparagine residues at positions 52 and 78 of the alpha-subunit and positions 13 and 30 of the beta-subunit. These oligosaccharides terminate with the sequence sialic acid- $\rightarrow$ Gal-beta1- $\rightarrow$ 4GlcNac-beta1- $\rightarrow$ 2Man-alpha- $\rightarrow$ (47,75). Studies with deglycosylated FSH, LH, hCG, and TSH indicate that removal of N-linked carbohydrates does not interfere with receptor binding but markedly reduces biological responses, such as steroidogenesis and stimulation of cAMP production (47).

Recombinant FSH

Exact structure determination of FSH carbohydrate moieties is necessary for the production of recombinant hormones for research and clinical use. In order to be clinically suitable, the carbohydrate structures of a recombinant product must be compatible with the immune system of the recipient. Biologically active human FSH has recently been expressed in Chinese hamster ovary cells (CHO

cells) (76). This cell line was chosen because proteins synthesized by CHO cells can have oligosaccharide chains which are closely related to those found in humans. Typical examples of human glycoproteins expressed in CHO cells are interferon-gamma (77), interferon-beta (78), erythropoietin (79), and tissue plasminogen activator (80). Hard et al. used recombinant FSH produced by CHO cells to examine more closely the structure of FSH carbohydrate chains (81). Their studies indicate that recombinant hFSH contains fewer partially sialylated oligosaccharides, which contributes to a shorter half-life in circulation relative to pituitary hFSH.

C. The Regulation of FSH Synthesis and Secretion

Follicle stimulating hormone synthesis and secretion are regulated by a complex interplay between hypothalamic gonadotropin-releasing hormone (GnRH), gonadal steroids, and the gonadal peptide hormones, inhibin, activin, and follistatin. The predominant means of regulation appears to be manifested by factors acting directly at the level of the gonadotroph in the pituitary. Only minimal amounts of GnRH are necessary to stimulate and maintain the secretion of FSH and, in contrast to LH, the release of FSH is closely linked to the rate of FSH synthesis (82).

The pituitary gonadotropins, FSH and LH, have been shown to exist within the same pituitary gonadotrophs (33).

Physiologically, however, each hormone has its own regulatory stimuli which allow for the coordinate production of the alpha- and respective beta-subunit proteins. During the rat estrous cycle there exists a simultaneous FSH and LH surge at the time of ovulation. At other times during the cycle changes in serum FSH and subunit mRNA concentrations are divergent from changes in serum LH concentrations, suggesting different mechanisms of control for the two hormones (83,84). Luteinizing hormone secretion is controlled primarily by GnRH from the hypothalamus and is modulated by gonadal steroids (85-89). The regulation of FSH secretion is less well understood.

Effect of GnRH on FSH secretion

Although GnRH is capable of stimulating the synthesis and release of both pituitary gonadotropins, the amount of GnRH required to induce and maintain FSH secretion appears to be less than for LH (90-92). This condition is most likely due to the longer half life of FSH in circulation as compared to LH (130-160 min vs 20-30 min). Similar to LH, however, the secretion of FSH can be inhibited by overstimulation with GnRH or highly potent GnRH agonists (93-97).

Rodin et al. (82) demonstrated the requirement of GnRH for expression of the FSH-beta gene by abolishing pituitary

stimulation by GnRH using passive immunoneutralization and by desensitizing gonadotropes to the actions of GnRH using receptor antagonists. Pituitary desensitization to GnRH prevented the postcastration rise in FSH-beta mRNA in castrated rats and reduced the level of FSH-beta mRNA in intact rats. Similar results have been obtained for LH-beta mRNA (98). Gonadotropin-releasing hormone has little effect on common alpha-subunit mRNA levels following castration (82,98). Together, these results suggest that FSH-beta and LH-beta gene expression are closely linked to a GnRH or GnRH receptor mediated mechanism, whereas common alpha-subunit gene expression is controlled by non-GnRH mediated mechanisms (82).

Inhibin Regulation of FSH

The existence of the protein inhibin, which acts to inhibit FSH release by the pituitary, was speculated for many years. Inhibin was finally purified from follicular fluid in the mid 1980's (99-102). Inhibin is a 32-kilodalton glycosylated protein composed of two dissimilar subunits, alpha and beta, linked by disulfide bonds. There is one form of the alpha-subunit and two forms of the beta-subunit (BA and BB), each representing a separate gene product (44).

Inhibin is synthesized by granulosa cells of the

developing follicle and by corpus luteum of the luteinized follicle and accumulates in the follicular fluid of all mammalian species so far studied (90). Following ovulation inhibin levels are high, while FSH levels are suppressed (103,104). The corpus luteum, acting under the control of LH, secretes inhibin (105) which partially accounts for the suppression of FSH during the luteal phase. Interestingly, subunit mRNAs encoding the three different inhibin subunits have been detected in ovary, testis, placenta, decidua tissue, brain, pituitary, bone marrow, adrenal, spinal cord, and kidney (106,107).

The primary effect of inhibin appears to be on FSH biosynthesis in the pituitary (108) and is mediated by a rapid and profound inhibition of steady state levels of mRNA encoding the FSH beta-subunit, and a more gradual and less extensive inhibition of alpha-subunit mRNA (109-112). It remains to be established whether inhibin decreases FSH-beta gene transcription or enhances the instability of FSH-beta mRNA. Thus, it is possible that other factors involved in the transcription, processing, and/or stability of FSH-beta mRNA may mediate the actions of inhibin (113).

Inhibin appears to inhibit the secretion of FSH with minimal effects on LH (114-116). Effects on LH secretion appear to occur only when supra-physiological doses of inhibin are administered (117,118).

Both in vivo and in vitro evidence indicate that FSH

is the major controller of inhibin secretion by the follicle (119,104). In women the concentration of inhibin rises during the early follicular phase (120). Inhibin levels also increase at the time of the pre-ovulatory FSH/LH surge. Inhibin levels decrease, however, as estradiol levels increase (121). These data suggest that during the follicular phase of women, inhibin secretion is dependent on continued FSH stimulation and that as FSH levels decline, presumably under the inhibitory effects of estradiol, inhibin secretion declines as well (90). As mentioned previously, however, during the luteal phase the corpus luteum secretes inhibin under the control of LH which, together with estradiol, suppress FSH levels. In males recent studies have shown that the effect of inhibin on FSH secretion may normally be masked by the presence of suppressive factors in Leydig cells, such as testosterone (122).

Estradiol Regulation of FSH

Estradiol, known to be responsible for the preovulatory gonadotropin surge (123), also inhibits FSH secretion at the pretranslational level. In vivo, estradiol negatively regulates the levels of all three pituitary gonadotropin subunit mRNAs (alpha, FSH-beta, and LH-beta) in gonadectomized male and female rats to levels

typical of intact rats (124-127). Though pretranslational suppression of subunit mRNAs may result from changes in mRNA synthesis or stability, Shupnik et al. (89) have demonstrated that estradiol regulation of gonadotropin levels in vivo is due in part to suppressed rates of gene transcription. Their studies also indicated that estradiol is a more potent inhibitor of LH-beta and FSH-beta mRNAs than of the common alpha-subunit mRNA, suggesting that alpha-subunit expression is differentially regulated within gonadotrophs.

Effect of Androgens on FSH Secretion

In contrast to estradiol, in vivo studies have shown that androgens, while negatively regulating LH-beta and alpha mRNA levels, have little or no effect on FSH-beta mRNA levels (124-126). These results may be explained by the absence of an androgen response element within the FSH-beta gene (128). Androgens do, however, stimulate FSH secretion by the pituitary (127,129). Consequently, Gharib et al. (128) found that androgens positively regulate FSH-beta mRNA, but not LH-beta or alpha mRNA, at level of the pituitary cell in vitro. Gharib and co-workers speculate that the absence of a stimulatory effect of androgens in vivo may be accounted for by opposing effects of androgens at the hypothalamic and pituitary levels (i.e.

negative regulation at the hypothalamus). This notion is supported by the fact that androgens decrease GnRH pulse frequency in humans and rats (130,131).

Modulatory Roles of Follistatin and Activin

Modulatory factors of FSH synthesis and secretion also exist. Follistatin is a single chain, glycosylated gonadal peptide also known to inhibit FSH secretion (132). Carroll et al. (112) have found that follistatin produces a dose dependent inhibition of FSH-beta mRNA levels with a corresponding decrease in FSH secretion.

Activin is a 24-kilodalton glycosylated protein composed of the beta-subunits of inhibin which stimulates FSH secretion and FSH-beta mRNA levels in pituitary cells (133-136). Two forms of activin have been isolated, activin A being a homodimer of the inhibin BA subunits, and activin AB a heterodimer of the inhibin A and B subunits.

Interestingly, Carroll et al. (137) found a sexually dimorphic response to recombinant human activin A in vivo. Little or no response to activin was observed in male rats. This is in contrast to a significant increase in FSH synthesis and secretion found in response to activin in female rats. Having been found that activin stimulates male rat pituitary cells in vitro (68), Carroll and co-workers suggest that in males there exists a putative

activin binding agent able to block the stimulatory effect of activin.

D. FSH Regulatory Mechanisms of Steroidogenesis

Follicle stimulating hormone has several actions in the ovary including the regulation of steroidogenesis by granulosa cells (44). Although levels of secreted steroid hormones change during follicular maturation (138,139), granulosa and luteal cells remain the principal sources of estradiol during the female reproductive cycle.

FSH Regulation of Cholesterol Availability

Cholesterol is an obligatory precursor for steroid biosynthesis. As such, processes controlling its synthesis, uptake, and utilization play important roles in granulosa cell function. Lino et al. (140) have demonstrated that in granulosa cells cultured in a serum free medium, FSH treatment enhances de novo cholesterol synthesis and increases intracellular pools of progesterone precursors. This represents an important regulatory role of FSH in granulosa cell steroidogenesis in light of the fact that progesterone production by granulosa cells in vitro is dependent on the presence of low density lipoprotein (LDL) in the culture medium (141,142). Before ovulation, the Graafian follicle is an avascular

compartment. Thus, granulosa cells in vivo do not have access to a blood supply and, consequently, LDL content within the follicle is low (143). Following ovulation the granulosa cell layer becomes vascularized and luteinized cells obtain access to circulating LDL.

FSH Regulation of Cytochrome P450_{scc}

FSH has been shown to regulate the mitochondrial cytochrome P450 cholesterol side chain cleavage enzyme (P450_{scc}) in granulosa cells (144). This enzyme converts cholesterol to pregnenolone and is the first and rate limiting enzyme of the steroidogenic pathway. Deficiency of the enzyme prevents the synthesis of sex steroids by the gonads (145).

P450_{c17} is the single enzyme mediating both 17 α -hydroxylase and 17,20-lyase activities (146). Granulosa cells have been shown to possess very little 17 α -hydroxylase activity (147,148), and it was this observation that led Short (147) to suggest the two-cell theory of steroidogenesis.

Several investigators have shown that treatment of granulosa cells with FSH increases both cholesterol side-chain cleavage activity and the total content of mitochondrial P450 protein (144,149-151). Funkenstein et al. (149) found that in cultured granulosa cells FSH

induced the synthesis of cytochrome P450scc in a concentration dependent manner with a half maximal stimulatory effect occurring at 2.5×10^{-9} M. They also found that prolonged exposure of the cells to high concentrations of FSH resulted in a decrease in cytochrome P450scc, suggesting a refractory effect. By contrast, no refractoriness of the cells to high concentrations of 8-Br-cAMP was observed in regards to cytochrome P450scc synthesis. Thus, refractoriness to FSH appears to occur at a level prior to cAMP formation.

Recently, using P450 cDNA probes and immature granulosa cells cultured in a serum free system, Urban et al. (152) demonstrated that FSH induces P450scc at the level of P450scc mRNA accumulation. This effect occurred in a time and dose dependent manner as a 100 ng/mL dose of FSH was found to significantly increase P450scc mRNA accumulation after 4 hours. Their studies, however, did not allow for distinction between an increase in the genomic transcription rate of the P450scc gene and a decrease in specific mRNA degradation. The P450scc gene does contain a cAMP responsive element within the 5'-untranslated upstream enhancer region (153,154) suggesting that FSH may act to increase P450scc transcription via induction of the adenylate cyclase system. This hypothesis is further supported by studies indicating that P450scc mRNA concentrations are also

increased following treatment with cAMP analogs (155,152).

Mechanism of P450scc Regulation

The molecular mechanisms mediating the activation of steroidogenesis by cAMP remain to be defined. In a number of cAMP regulated genes, a conserved 8-base pair cAMP responsive element (CRE) is located within the enhancer region (156). A CRE binding protein has been found which upon binding to the CRE activates transcription in vitro (157). Binding of activator protein 2 (AP-2), an enhancer binding transcription factor, has also been implicated with cAMP-dependent transcription (158). Interestingly, Ahlgren et al. (153), upon sequencing the bovine P450scc promoter/regulatory region between base pairs -183 and -83, did not find the consensus CRE sequence or the consensus AP-2 binding sequence. They did, however, identify at least one non-consensus cAMP responsive element within the region. The significance of the P450scc gene's distinct cAMP responsive element is not yet evident.

FSH Regulation of Microfilaments

The production of steroids requires the intracellular translocation of substrate and intermediate molecules. Within the granulosa cell, cholesterol is normally stored

as esters in lipid droplets and must be transported to and taken up by the mitochondria for the synthesis of pregnenolone. Pregnenolone must then be translocated to the smooth endoplasmic reticulum where specific enzymes further metabolize the substrate to progesterone.

The cytoskeleton is important in controlling the intracellular distribution of organelles (159). Microfilaments have been shown to be closely associated with mitochondria distribution in granulosa cells (160). Carnegie and Tsang (161) and Tsang et al. (162) have suggested that FSH may act on granulosa cells to regulate microfilament organization resulting in a closer association of mitochondria and cholesterol containing lipid droplets.

It has been shown that transient rounding of granulosa cell shape is influenced by hormone treatment and that this effect is mediated by a decrease in microfilament content (163,160). Tsang et al. (162) found that concentrations of FSH yielding dose dependent increases in granulosa cell progesterone synthesis also appear to decrease the synthesis and bundling of microfilaments. They also found that by inhibiting microfilament polymerization with cytochalasin B, the steroidogenic response of granulosa cells was enhanced, suggesting that steroidogenic activity is positively affected by rounding of the granulosa cell. Their data also suggest that the role played by

microfilaments is regulated in part by FSH.

FSH Regulation of Luteal Phase Steroidogenesis

While follicular maturation is significantly regulated by FSH, progesterone production in luteal phase cells is stimulated primarily by LH-dependent mechanisms (164). Devoto et al. (165) have shown that FSH does not sustain corpus luteum function in vivo and that cultured luteal cell steroidogenesis, including progesterone, estradiol, and testosterone synthesis, is LH/CG dependent.

Modulatory Roles of Androgens and Growth Factors

Follicular maturation is also characterized by an increase in granulosa cell responsiveness to FSH (166). This effect appears to be mediated by an increase in the number of granulosa cell FSH receptors (167). Androgens, however, have been shown to enhance FSH induced steroidogenesis in granulosa cells without affecting FSH receptor content (168-170). The mechanism behind androgen modulation of FSH-dependent steroidogenesis is unclear. In rats, androgens may stimulate steroidogenesis by suppressing catabolism of FSH-induced cAMP (171).

Several peptides and growth factors have been found to modulate FSH-stimulated steroidogenic activity as well (for

reviews see 172,1). Adashi et al. (173) have shown that insulin-like growth factor I (IGF-I) significantly enhances FSH-induced progesterone and estrogen biosynthesis. The activity of IGF-I appears to be at the level of cytochrome P450_{scc} induction. Transforming growth factor-beta (TGF-beta) has also been found to augment FSH-stimulated aromatase activity (174). Most recently, cytokines have been found to play important roles in suppressing FSH-stimulated steroidogenesis (175-177).

E. FSH Regulation of LH Receptor

The physiological responsiveness of both granulosa and thecal cells to the gonadotropins is dependent upon the cell surface expression of appropriate hormone binding receptors (9). LH receptors are initially expressed on thecal cells of small follicles but subsequently appear on both thecal and granulosa cells of developing follicles (10). LH receptors are present in corpora lutea (178) and in situ hybridization studies have shown that LH receptor mRNA is present in interstitial tissues as well (179).

LH Receptor Levels During the Reproductive Cycle

Induction of granulosa cell LH receptor expression is required to increase the sensitivity of granulosa cells to circulating LH during LH-induced ovulation and initiation

of luteinization. In rat, the number of ovarian LH receptors has been found to be lowest at metestrus, increasing significantly at diestrus, and maximal just prior to the LH surge. LH receptor levels decrease significantly following the preovulatory LH peak and remain low throughout estrus (178).

The induction of LH receptors that occurs during the growth of small antral follicles to preovulatory follicles has been found to be estradiol- and FSH-dependent (7-10). The ability of cycloheximide to inhibit FSH-dependent LH receptor appearance and the lack of preformed intracellular LH receptors prior to FSH exposure in cultured granulosa cells (180) suggest that FSH induction of LH receptor appearance is caused by de novo receptor synthesis. In support of this hypothesis is the recent finding that LH receptor appearance is regulated at the level of mRNA (8,179,181-184). Using Northern blots and solution hybridization, Segaloff et al. (8) demonstrated that changes in LH/CG receptor content in ovarian cells are paralleled by hormonally regulated changes in LH/CG receptor mRNA levels.

LH Receptor mRNA

Segaloff and coworkers (8) identified several LH/CG receptor specific mRNA transcripts within the rat ovary,

the most abundant being 6.7, 4.3, 2.6, and 1.2 kb in size. The nucleotide sequences of these transcripts have not been determined and, consequently, it is not known which transcript(s) encode the functional 93-KDa LH/CG receptor. The mRNAs, however, appear to be coordinatively regulated throughout all stages of granulosa/luteal cell differentiation. Interestingly, La Polt et al. (185) have recently reported that a 1.8 kb LH receptor mRNA is not coordinatively down-regulated with other LH receptor transcripts in the rat testis.

The results of Segaloff et al. (8) also confirm the synergistic effect of FSH and estradiol on LH receptor induction. Neither estradiol alone nor FSH alone was able to induce LH/CG receptor mRNAs in granulosa cells isolated from hypophysectomized rats. In contrast, when the same dose of FSH was administered to hypophysectomized rats primed with estradiol, LH/CG receptor mRNA was induced to levels comparable to that observed in cells isolated from intact rats.

Mechanism of Induction

The mechanisms by which FSH and estradiol act are still being resolved. The effects of FSH are due to its ability to increase cAMP levels (186-189). This hypothesis is supported by studies demonstrating that cholera toxin or

the direct addition of cAMP or its analogues induce the appearance of LH receptors on cultured granulosa cells (187-190). LH-induced down regulation of LH receptor mRNA, which occurs following the LH surge, also appears to be mediated by cAMP (8). Thus, it seems that low doses of cAMP stimulate LH receptor expression, while high doses are inhibitory.

The effects of estradiol appear to be mediated by estradiol receptors present in granulosa cells (191,192). Segaloff et al. (8) speculate that FSH, acting through protein kinase A, may phosphorylate the estradiol receptor allowing trans activation of the LH receptor gene or that estradiol may induce a trans-acting factor that is phosphorylated by protein kinase A.

Cell Specific Regulation of LH Receptor mRNA

Regulation of LH receptor cell surface expression by FSH and estradiol appears to be at the level of the LH receptor gene. Using a cloned LH receptor cDNA, Camp et al. (179) examined the cellular localization of LH receptor mRNA in rat granulosa cells at several times during the reproductive cycle. They found that LH receptor mRNA is present in relatively unchanging amounts in thecal cells and is present in granulosa cells of large preovulatory follicles, but not small antral follicles. These findings

suggest that the LH receptor gene is regulated predominantly in granulosa cells and is somewhat more constitutively expressed in thecal cells. Characterization of regulatory elements and domains within the LH receptor gene is required to further elucidate the cell specific mechanisms by which the gene is regulated.

F. FSH Action on Sertoli Cells

The Sertoli cell, the only somatic cell within the seminiferous epithelium, is of major importance in controlling several aspects of testicular function. The Sertoli cell itself is regulated by a number of factors, including FSH. Some of the events and specific processes of Sertoli cells regulated by FSH are listed in Table 1. Throughout the growth and maturation of Sertoli cells, the physiological response to FSH is continually changing. In the rat, FSH acts as an important Sertoli cell mitogen from just before birth to about 5 days of age (193). At 15-20 days of age the mitotic rate of Sertoli cells is greatly reduced and FSH serves to regulate Sertoli cell maturation (194). In the adult rat, Sertoli cells appear refractory to FSH (195,196).

Table 1 - Sertoli Cell Processes Regulated by FSH

Process	FSH and Sertoli Cells
Ion flux	Ca ²⁺ redistribution
Enzyme activity	Adenylate cyclase Phosphodiesterase Protein kinase
Protein Synthesis	General cell proteins Protein kinase inhibitors Plasminogen Activator Inhibin
Steroid synthesis	Estrogen
Cell Division	DNA synthesis and mitosis
Cell Motility	Microtubule polymerization Microfilament organization Cell shape
Cell-cell communication	Junctional complexes

Mechanism of Action

The initial event in FSH action is the specific binding of the hormone to receptors located on the Sertoli cell membrane (197). This binding is of high affinity ($K_d \sim 10^{-10}$ M) and of limited capacity ($\sim 10^4$ sites per cell) (198). The binding of FSH to its receptor is followed by the activation of adenylate cyclase and inhibition of phosphodiesterase activity (197,199,200).

FSH stimulation of adenylate cyclase and inhibition of phosphodiesterase result in a dose-dependent linear increase in intracellular cAMP levels, with subsequent activation of cAMP-dependent protein kinase (196,197,200). In addition, FSH appears to alter intracellular calcium levels of cultured Sertoli cells by a mechanism independent of the adenylate cyclase pathway or protein kinase C (201).

Sertoli Cells in Culture

FSH serves to regulate the synthesis and/or secretion of several Sertoli cell proteins, many of which have been studied in Sertoli cell cultures (202). While the measurement of biochemical functions in a culture system allows for changes to be attributed directly to the administration of hormone, a disadvantage of this approach is that the cells have been removed from their natural

environment. Given the complex intercellular mechanisms involved in testicular development and the polarized nature of Sertoli cells in vivo, cultured Sertoli cells may exhibit altered activity in an isolated environment (203).

Byers et al. (204) have combatted this problem with the development of a dual compartment (bicameral) culture chamber. The device allows Sertoli cells in vitro to establish different environments at their basal and apical surfaces. As a result, Sertoli cells cultured in this way become polarized and more closely resemble Sertoli cells in vivo.

FSH Regulation of Transferrin and Transferrin Receptor

Several studies have revealed the fundamental importance of iron delivery via transferrin to metabolically active cells and to differentiating cells in particular (for review see 205). Developing germinal cells undergo meiotic division in the adluminal compartment of the seminiferous tubule (206) and, being isolated from the interstitial lymph by Sertoli cell tight junction complexes, any iron required for meiosis or maturation of the germinal cells must arrive via the Sertoli cell.

Transferrin was first shown to be made by Sertoli cells by Skinner and Griswold (207) and has been found to comprise approximately 15% of the protein secreted by rat

Sertoli cells in culture (207,208). Skinner and Griswold (209) found that Sertoli cell secretion of transferrin was increased by FSH. Follicle stimulating hormone appears to regulate transferrin expression at the level of transferrin mRNA. Roberts and Griswold (210) found FSH to elevate transferrin receptor mRNA 2.7 fold in testes of hypophysectomized rats. Since Sertoli cells are the only cell type in the testes confirmed to possess FSH receptors, FSH induction of transferrin receptor mRNA in testes is probably mediated by the Sertoli cell.

FSH Regulation of Androgen Binding Protein

Another important role of FSH is the regulation of androgen binding protein (ABP) synthesis and secretion. Androgen binding protein was the first secreted protein product of Sertoli cells to be isolated and characterized biochemically (211). Indeed, the measurement of ABP production has been utilized as an indicator of Sertoli cell response in both whole testis and in cultured Sertoli cells. Androgen binding protein binds testosterone or dihydrotestosterone with high affinity and serves to transport the androgen to the epididymis (212).

The amount of ABP secreted by cultured Sertoli cells is quite low relative to other secreted proteins (Sertoli cells in culture secrete from 100 to several hundred times

as much transferrin as ABP) (213). In addition to FSH, ABP secretion appears to be regulated by testosterone, insulin, and vitamin A as well (214-216).

FSH Regulation of Testicular Steroidogenesis

Steroids are important regulators of spermatogenesis and testicular development. Sertoli cells have been found to synthesize estradiol in response to FSH in a dose-dependent manner (217,218). Sertoli cells do not synthesize significant amounts of estradiol, however, if incubated in the absence of exogenous steroid substrate (217), suggesting that the androgen precursor is produced elsewhere in the testes in vivo.

The amount of estradiol produced in response to FSH is also dependent upon the maturational state of the Sertoli cell, peaking at approximately 5 days of age, while decreasing significantly by 30 days (219). Rat Sertoli cell conversion of androstenedione to testosterone in response to FSH is also age dependent, peaking between 10 and 17 days of age (220).

FSH Regulation of Sertoli Cell Microfilaments

During the development and differentiation of the Sertoli cell, the response to FSH is dramatically reduced

at the time when tight junctions are formed between the Sertoli cells. These tight junctions, which constitute the blood-testis barrier, are formed in the rat by 16-19 days of age (221). Prior to junction formation, numerous microfilament bundles appear at the Sertoli cell membrane (222).

Changes in microfilament distribution can be visualized by changes in cell shape and have been implicated in the control of protein secretion and polarization of Sertoli cells (223). Consequently, morphological changes in Sertoli cells by means of microfilament redistribution appear to be influenced by FSH (224-226). This activity of FSH may be due to its ability to regulate intracellular calcium, which has been implicated in both the control of cell shape and in the regulation of protein secretion (195,201,225).

FSH Regulation of Calcium Uptake

Hormone stimulated steroid biosynthesis is critically dependent upon calcium ions. Studies on the ability of FSH to induce changes in Sertoli cell intracellular calcium levels have demonstrated that this effect is more directly related to calcium uptake than to mobilization of internal calcium (201,227,228). It is not known whether the FSH receptor acts as an ion channel or whether FSH binding to

receptor serves to modulate calcium channels by generation of a second messenger.

Grasso and Reichert (228) have shown that FSH induced uptake of Ca^{+2} can be inhibited by voltage activated and voltage-independent calcium channel blocking agents. More recently, Grasso and Reichert (201) have shown that FSH-induced uptake of Ca^{+2} is not dependent upon toxin-sensitive G-proteins or accumulation of cAMP. These findings have led them to suggest that the FSH receptor itself may function as a calcium channel in Sertoli cells.

Sertoli Cell Refractoriness to FSH

It appears that hormonal responsiveness of the Sertoli cell is dependent upon the cell's maturational status. In the rat, there is a decrease in responsiveness to FSH at 19 days of age and refractoriness by 36 days of age, by which time tight junctions have formed between Sertoli cells (195). Refractoriness can be prevented by hypophysectomy (229). Interestingly, refractoriness is only observed with respect to cAMP induced events. Effects of FSH involving Ca^{+2} distribution and cell shape are independent of age (230).

Refractoriness in regards to cAMP elevation has been attributed to changes in receptor-cyclase coupling, phosphodiesterase activity, and protein kinase inhibitor

concentration (195,231).

G. Other Physiological Roles of FSH

Follicle stimulating hormone is an essential regulator of follicular development and testicular spermatogenesis. The primary action of FSH in females is the development of ovarian follicles, while luteinizing hormone (LH) is responsible for ovulation and transformation of follicles into corpus luteum (1).

Importance of cAMP

FSH acts on specific target cells by interacting with specific receptors on the target cell surface. It is generally accepted that cAMP is an intracellular second messenger of FSH action (170). Accumulation of cAMP results in activation of the protein kinase A causing the subsequent phosphorylation of key proteins involved in gonadal function. The proposed involvement of cAMP in mediating FSH activity is owed to studies demonstrating the ability of FSH to stimulate adenylate cyclase activity (232) and intracellular cAMP accumulation (233). Other studies have shown that FSH action may be mimicked by cAMP inducers other than FSH (234,235), lipophilic cAMP analogs (234), and forskolin (236).

Recently, Adashi et al. (237) further characterized

the role of cAMP in mediating FSH activity using R_p -cAMPS, a non-degradable antagonistic analog of cAMP. In their studies R_p -cAMPS produced potent, yet incomplete attenuation of progesterone accumulation in response to treatment with FSH, thus providing additional support to the notion that cAMP acts as a mediator of FSH activity. However, to the extent that R_p -cAMPS result in incomplete neutralization of FSH activity, Adashi and co-workers suggested that the role of cAMP may be central, but not exclusive, with regards to mediating FSH activity. Indeed, FSH has also been shown to increase granulosa cell cGMP levels by 45-80% (238). Further, analogs of cGMP have been shown to stimulate FSH action with regards to LH receptor induction and stimulation of prostaglandin E biosynthesis (235).

FSH Regulation of Insulin-like Growth Factors

As mentioned previously, steroidogenesis in the granulosa cell is controlled in part by FSH, insulin and IGF-I. Although the mechanism by which FSH and insulin/IGF-I interact to promote estradiol secretion is not completely understood, recent studies suggest that FSH may act on granulosa cells so as to make them more responsive to IGF-I or insulin. FSH increases IGF-I synthesis (239), induces IGF-I receptors (240), and

decreases concentrations of IGF-I binding proteins (241,242), thereby making more IGF-I available for receptor binding.

In male Sertoli cells, the idea of IGFs acting as autocrine/paracrine amplifiers of gonadotropin action has been suggested (243). FSH stimulates the local production of testicular IGF-I at the level of mRNA and protein (244). It has also been shown that IGF-I promotes the proliferation of FSH stimulated immature rat Sertoli cells (245). Consistent with a regulatory role of FSH, Smith et al. (246) have shown that IGF binding proteins are dually regulated by FSH and IGF-I.

Experiments by Peluso et al. (247) have examined further the roles of FSH and IGF-I in controlling granulosa cell steroidogenesis. Their studies indicate that FSH is capable of both inducing and inhibiting [3 H]-thymidine incorporation, and as such, produces a biphasic response in granulosa cells. The biphasic response is characterized by an initial stimulation of [3 H]-thymidine incorporation followed by estradiol secretion. This observation is consistent with the observed stimulation of follicular growth and estradiol secretion by granulosa cells in response to FSH in vivo (1). Their observations raise the question, however, of how FSH loses its mitogenic action while retaining its steroidogenic capacity.

Peluso et al. speculate that FSH becomes uncoupled to the pathway leading to mitosis due to its ability to influence both cAMP dependent protein kinase A and, to a lesser extent, protein kinase C pathways (248). Interestingly, IGF-I may indirectly activate protein kinase C (247). Activators of protein kinase C inhibit granulosa cell differentiation and promote [³H]-thymidine incorporation (249). Thus, FSH induced activation of protein kinase C, mediated perhaps by increasing granulosa cell responsiveness to IGF-I, could account for the inhibition of mitosis observed along with FSH activation of estradiol secretion via the more defined protein kinase A pathway.

FSH Regulation of Inhibin Expression

Inhibin expression in granulosa cells appears to be predominantly controlled by FSH. Studies have shown that inhibin alpha-subunit expression is under direct control by FSH both in vitro (250) and in vivo (251). Turner et al. (252) have demonstrated the coordinate regulation of all three inhibin subunit mRNAs (alpha, beta-A, and beta-B) by FSH in primary rat granulosa cell cultures. Their observations reveal an intersexual difference in the hormonal control of gonadal inhibin production. Though Sertoli cells have also been shown to express the three

inhibin subunit mRNAs (251,253-255), only alpha-subunit expression is under FSH control. FSH does not affect Sertoli cell expression of inhibin BA or BB subunit mRNAs in vivo or in vitro (251,253,254,256). Forskolin mimics FSH induction of inhibin subunit mRNAs in granulosa cells (257) suggesting that inhibin gene expression is stimulated by the cAMP-dependent protein kinase A pathway.

Effect of FSH on Plasminogen Activator Expression

Follicle stimulating hormone has also been shown to play a role in the transcriptional regulation of plasminogen activators (PAs). Plasminogen activators are serine proteases required for the conversion of plasminogen to plasmin. As such, PAs are involved in processes requiring extracellular proteolysis (258). In the reproductive system PAs appear to play important roles in such events as ovulation, spermatogenesis, and formation of the blood testis barrier (258-265).

In rat ovary, granulosa cells synthesize both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), but only tPA is under FSH control (259-261). This same situation exists in rat Sertoli cells, the main site of PA production in the seminiferous epithelium (262-264). Surprisingly, in mouse granulosa cells, uPA, rather than tPA, is under FSH control (265).

In humans the uPA gene can be regulated by hormonal stimuli and cAMP analogs during both oogenesis and spermatogenesis (264,265). Rossi et al. (258) have shown that the uPA promoter is activated by FSH and cAMP analogs in mouse Sertoli cells. This response, however, becomes refractory with increasing age. Their data further suggest that the refractory condition results from an inability of the Sertoli cells to accumulate cAMP in response to FSH, rather than to a change in the transcriptional response of the uPA gene to cAMP.

FSH Regulation of Mullerian Inhibiting Substance

Follicle stimulating hormone has recently been shown to regulate Mullerian inhibiting substance (MIS) production in testis (266). Mullerian inhibiting substance, first recognized as a testicular protein that causes regression of the Mullerian ducts, is a member of a family of proteins which includes transforming growth factor B (TGF-B) and inhibin/activin (267,268). Mullerian inhibiting substance has been shown to affect such processes as oocyte meiosis inhibition (269,270), testicular descent (271), and inhibition of fetal ovarian differentiation and aromatase activity (272).

H. Metabolic Clearance of FSH

Follicle stimulating hormone and the other gonadotropins are complex, highly glycosylated glycoprotein hormones that remain in circulation longer than most simple peptide and glycoprotein hormones (273). Serum FSH concentrations are determined by both the rate of FSH release from gonadotrophs and the rate at which the hormone is removed from the circulation (274).

Half-lives of Isoforms

Studies of the in vivo secretion and metabolic clearance of FSH have been limited by its putatively prolonged half-life (275-278). In addition, FSH exists in multiple chromatographic isoforms making its detection by different assay systems difficult (278-280). Using agarose gel electrophoretic separation at pH 8.6 at least 20 different forms of FSH have been detected in a single human pituitary (281). In addition to this primary polymorphism, several studies have demonstrated a secondary polymorphism in FSH structure which is sex and age related (281-283). Together, this microheterogeneity of FSH contributes to observed differences in the metabolic clearance rate (MCR) of the different isoforms. Indeed, Urban et al. (284) recently reported substantial intersubject differences in

the observed ranges of endogenous FSH half-lives of normal men. Kjeld et al. (285) reported a mean single exponential FSH half-life in normal men of 414 minutes.

Significance of Sialic Residues

A correlation has been found between the MCR of a particular FSH isoform and the isoform's median charge, which is in turn influenced by the sialic acid content (281). More basic FSH isoforms are cleared more rapidly in vivo, but have greater biological activity in vitro (286,287). This is in contrast to acidic FSH isoforms which exhibit the opposite effect (288). Interestingly, Blum and Gupta (279) have speculated that FSH regulating factors may influence FSH serum concentrations by regulating the quality, rather than the amount, of FSH secreted by pituitary gonadotrophs. Increasing the relative rate of acidic FSH isoform secretion could elevate serum FSH concentrations (due to the isoform's longer half-life), while allowing FSH production rates to remain unchanged. Conversely, increasing the rate of basic isoform secretion would lower serum FSH concentrations.

Involvement of Liver

The sites and mechanisms of FSH clearance have been studied, yet many aspects of the hormone's removal from circulation remain unclear. It is widely accepted that the liver plays a role in eliminating asialo human gonadotropins from circulation (289,290). Morell et al. (291) first demonstrated that enzymatic removal of sialic acid from the glycoprotein ceruloplasmin resulted in rapid disappearance of the protein from the circulation and simultaneous accumulation within hepatocytes of the liver. These observations have been extended to asialoderivatives of FSH (292).

Glycoproteins are desialylated in vivo by the enzymes sialidase and neuraminidase. Desialylation of FSH has been shown to significantly decrease the hormone's half-life and bioactivity (279,293,294). The liver contains asialoglycoprotein receptors which are responsible for initiating the endocytotic uptake of asialoproteins (295). As such, it appears that one role of sialic acid is to mask adjacent galactosyl residues, thus preventing recognition by the liver.

Role of Kidney

Some studies have questioned the significance of hepatic asialoglycoprotein receptors in the metabolic clearance of gonadotropins (296,297). Other studies provide evidence for involvement of the kidney in FSH clearance (274,278). Gay et al. (274) have speculated that the kidney, which contains relatively high concentrations of neuraminidase, may function to remove sialic acid from gonadotropins prior to their metabolism by the liver. In support of this hypothesis, Emmanouel et al. (298) found that although renal mechanisms are responsible for 94% of the metabolic clearance of LH, only 3.5% is accounted for by urinary excretion. The remaining 90% appears to be due to degradation of the hormone within the renal parenchyma, a process that may involve removal of sialic acid residues.

In contrast to nonglycosylated peptide hormones, the urinary excretion of several glycoproteins (including FSH) contributes significantly to their overall metabolic clearance (298-301). Emmanouel et al. (298) have shown that acute exclusion of the kidney from the circulation by renal pedicle ligation reduces the MCR of exogenous FSH by 78%. Similar findings have been reported for endogenous FSH in nephrectomy experiments (274). Interestingly, upon comparison of the results obtained by acute kidney exclusion and nephrectomy it appears that urinary clearance

of FSH accounted for at most 35% of its MCR. These and other studies suggest that renal clearance of FSH is not restricted to urinary excretion, but may also involve degradation of the hormone by the renal parenchyma (274,298,302,303).

FSH Internalization by Target Cells

FSH has also been shown to be internalized and degraded by target cells (304,305). Sanford and Batten (304) found that cultured granulosa cells under receptor saturating conditions (i.e. 4630 FSH molecules bound/cell) internalized FSH at a rate of 2780 molecules of FSH/cell/hour. Granulosa cells internalized FSH by random pinocytosis at a rate of 358 molecules/cell/hour, a rate 1/8 of the receptor mediated rate. Thus, internalization of FSH by target cells, both receptor and non-receptor mediated, is insignificant with regards to the overall metabolic clearance of the receptor from circulation. However, receptor mediated internalization of FSH by target cells is very important at the cellular level in terminating the FSH binding signal, thus regulating the response of the target cell to the hormone.

1. Clinical Applications of FSH

As a form of gonadotropin therapy, FSH has been used in several clinical situations including ovulation induction and the treatment of polycystic ovarian syndrome (PCOS), cryptorchidism, and luteal phase deficiency (LPD). Human menopausal gonadotropin (hMG), due to its FSH bioactivity, has been used extensively in gonadotropin therapy (306-308). The LH content of hMG, however, often poses difficulties in the treatment of conditions such as PCOS, in which LH/FSH ratios are already abnormally high (309). With the increasing ability to produce recombinant bioactive FSH in Chinese hamster ovary (CHO) cells (76), the efficiency and economic practicality of gonadotropin therapy may be improved.

Polycystic Ovarian Syndrome

Polycystic ovarian syndrome is often used as a generic description for a broad spectrum of abnormalities in women with characteristic endocrine dysfunction. Patients with PCOS tend to display an elevation of the LH/FSH ratio, inappropriate LH pulsatility, and pathologic androgen secretion (309). Administration of an antiestrogen, most commonly clomiphene citrate (CC), is often sufficient to induce ovulation in PCOS patients (310). Clomiphene

citrate treatment, however, while generally successful at increasing ovulation rates, is often inadequate at improving pregnancy rates (311). In such CC resistant PCOS patients, hMG supplemented with hCG to induce ovulation has been the most widely used alternative (312). Still, hMG therapy can result in such complications as spontaneous abortion, multiple pregnancy, and hyperstimulation (307). Human menopausal gonadotropin contains both FSH and LH bioactivity and it is believed that the LH content of hMG is responsible for some of these problems. Due to the already elevated levels of LH in PCOS patients, administration of additional LH appears illogical.

Recent forms of PCOS therapy have utilized pure FSH and GnRH agonists (309,313). GnRH agonists, due to their longer half-life and greater pituitary GnRH receptor affinity compared to endogenous GnRH, cause down-regulation of the GnRH receptor which leads to diminished LH and FSH release (314). As such, treatment with GnRH agonists can induce a condition of 'medical hypophysectomy'. This in turn helps to normalize LH/FSH ratios, and also prevents inappropriate LH pulses that cause premature luteinization of follicles (315,316). This mode of therapy has been shown to reduce the complications often encountered with clomiphene and conventional hMG therapy (309,313). With the increasing ability to produce recombinant FSH (76) and the development of GnRH agonist implants (309), GnRH

agonist/FSH therapy appears to be a promising treatment for PCOS.

In Vitro Fertilization-Embryo Transfer

Follicle stimulating hormone is also commonly used to induce multiple follicular development for in vitro fertilization-embryo transfer (IVF-ET) (317). The success rate of IVF-ET is directly proportional to the number of oocytes transferred (318). During IVF ovulation induction, recruitment of multiple follicles is necessary to override the natural tendency in women to recruit a single dominant follicle. This can be accomplished by providing an increased level of gonadotropins, primarily FSH, early in the follicular phase (319). The timing of gonadotropin therapy is of crucial importance for successful follicular development. If the gonadotropin is given after selection of the dominant follicle, remaining follicles will have already begun the process of atresia. Though multiple follicles may still grow as a result of the gonadotropin increase, such follicles will be immature with respect to the already selected dominant follicle(s) (320).

Several IVF programs administer hMG to induce ovulation. The LH content of hMG, however, can lead to premature luteinization (320). As a result, recent IVF protocols have utilized pure FSH of pituitary or urinary

origin supplemented with LH or hMG at an appropriate time to induce ovulation (320,321). This protocol appears to be successful, though optimum doses and times of administration of the hormones remain to be determined.

Cryptorchidism

Follicle stimulating hormone has also been used in the treatment of cryptorchidism. Cryptorchidism is a condition found in 3% to 4% of full-term newborn infants characterized by delayed or improper migration of the testes into the scrotum (322). The testes need to descend into the scrotum at an early age because at two years of age degeneration of the germinal epithelium with loss of spermatogonia has been shown to have occurred in undescended testes (323). FSH appears to play a role in proper testicular descent by enhancing LH receptors in Leydig cells which leads to increased intratesticular testosterone levels (324-326).

Saggese et al. (322) have shown that combined therapy of hCG and FSH is an effective treatment for cryptorchidism with no significant side effects. The administration of exogenous gonadotropins appears to restore normal testicular descent by maintaining proper levels of testosterone.

Luteal Phase Deficiency

A recent clinical application of FSH has been in the treatment of LPD (327). Luteal phase deficiency is characterized by insufficient endometrial development due to a lack of progesterone production by the corpus luteum. As such, LPD can lead to functional infertility and first trimester miscarriage. Balasch and Vanrell (328) have reported that LPD may account for 3.5% to 20% of infertility patients and 23% to 60% of patients with repeated abortions.

Stouffer and Hodgen (329) first reported successful treatment of LPD in rhesus monkeys using FSH-rich hMG. Recently, Balasch et al. (327) have shown that some forms of LPD may be successfully treated by FSH therapy in women as well. The FSH activity of hMG serves to increase ovarian responsiveness to LH via the induction of LH receptor expression. This leads to an increase in progesterone and estradiol production. Estradiol induces the expression of progesterone receptors in the endometium. More studies are needed to explore the potential benefits of this form of treatment.

J. FSH Receptor

The ability of FSH to modulate ovarian and testicular function depends not only on the circulating level of the

gonadotropin, but also on the expression of specific cell surface receptor proteins by potential target cells (7). A great deal of information has recently been reported regarding the structure, function, and expression of this specific hormone receptor and on the gonadotropin receptor proteins in general.

Similarity of Glycoprotein Hormone Receptors

The FSH receptor is a single glycoprotein molecule present on ovarian granulosa cells and testicular Sertoli cells that mediates the folliculogenic and spermatogenic actions of FSH (330,7). Both the FSH and LH gonadotropin receptors are highly related to the TSH receptor (331,332) and all three belong to a large family of G-protein coupled receptors that includes the beta-adrenergic, muscarinic acetylcholine, dopamine, serotonin, tachykinin, angiotensin, neurotensin, and bombesin receptors (333-340). The receptors of this family are structurally characterized by the presence of seven putative membrane spanning domains.

The FSH, LH, and TSH receptors are uniquely characterized by the presence of a large, glycosylated, putatively extracellular domain at the amino terminus thought to be involved in specific hormone recognition (11,179,341). This is in contrast to other G-protein coupled receptors in which receptor activation is effected

by the binding of a small ligand to a specific site within the transmembrane domain (11).

Structure of the FSH Receptor

During the mid 1980s, several groups reported on the purification and biochemical characterization of the FSH receptor but with conflicting results (330,342-345). Using chemical cross-linking and photoaffinity approaches, one group (330) proposed that the FSH receptor contains two each of three distinct subunits with approximate molecular weights of 32, 48, and 84 kD, respectively. Another group, also using cross-linking and photoaffinity labelling techniques, suggested that the receptor consists of three distinct components with approximate molecular weights of 18, 22, and 34 kD (345). Surprisingly, recent elucidation and characterization of FSH receptor cDNA has revealed the receptor to be a single 75 kD glycoprotein (11,341). Similar to the LH/CG receptor, the FSH receptor is susceptible to proteolysis which may account for the previous confusion concerning its structure.

Isolation and characterization of a cloned cDNA encoding the FSH receptor was first reported by Sprengel et al. (11). They were able to isolate a cDNA encoding the rat Sertoli cell receptor for FSH from a cognate cDNA library using molecular probes derived from the coding

regions of the LH/CG receptor cDNA (12). Their reported nucleotide and predicted amino acid sequences of the FSH receptor are shown in Figure 3.

Starting from the translation initiation codon at position 1, the rat FSH receptor cDNA can be seen to define a 2076 nucleotide open reading frame sequentially and putatively encoding an N-terminal 17 residue signal sequence, a 348 residue and largely hydrophilic extracellular domain containing three N-linked glycosylation sites, a 264 residue domain consisting of seven transmembrane segments, and a 63 residue intracellular C-terminus with several potential phosphorylation sites. As such, Sprengel et al. predict the receptor to be comprised of 675 amino acids and to constitute a single integral membrane glycoprotein.

The work of Sprengel et al. was followed soon afterwards by the findings of Minegishi et al. (341) who reported the cloning and sequencing of human FSH receptor cDNA. Their studies indicated that the human receptor protein is 89% identical overall to the rat receptor and 95% identical within the region of the putative transmembrane segments.

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Figure 3 - FSH Receptor cDNA

Listed above is the cDNA and Predicted Amino Acid Sequence of Rat Testicular FSH Receptor as determined by Sprengel et al. (11). Amino acid numbering begins at the N-terminal sequence for the predicted mature receptor protein, with negative numbers denoting the signal sequence. The predicted signal cleavage site is indicated by an arrow. Consensus N-linked glycosylation sites are marked by triangles and the proposed seven transmembrane segments TMI-TMVII are boxed.

Similarity to LH Receptor Sequence

The FSH receptor shows strong sequence similarity to the LH receptor primary sequence which is not surprising given the similarities in structure and function of FSH and LH and the similar response mechanisms elicited by the receptors in response to hormone binding. A comparison of the amino acid sequences of rat FSH and LH receptor is shown in Figure 4.

The receptors display a 50% similarity in primary sequence within their large extracellular domains and an 80% similarity within the seven transmembrane segments. Greatest divergence is found within the N-terminus, a 40 residue segment preceeding the first transmembrane segment, and the 30 residues entailing the C-terminus (11).

Similar to the LH/CG receptor, the extracellular domain of the FSH receptor displays a composition of 14 imperfectly replicated units of approximately 20 residues each. These units contain leucine rich repeats which are similar to a leucine rich motif characteristic of other leucine rich glycoproteins (12,346). This pattern of amino acids appears to form amphipathic peptide surfaces involved in protein-protein interactions (347) and may be involved in extracellular domain-hormone interaction.

Recent studies support the proposed role of the extracellular domain in hormone recognition and binding.

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1  RELSGR PE M D APDGA R POPRAGLAA ..... SLTYLVK
1  CHNWLCMSHNVFL CQDSHTEIPTDLPANAIELAFVLTALA
41  SQA R LNEVV S S R NA D LN S LL QNTK
43  VIPAGSFAGGGLERIEISONVLEVIADVTSHLPRLKEIRERAMFL
91  E Q T R R S C RT D T S SEFNIE C L N T
91  YIMPEAFONLPRLTYLLISHTCRLPAVNRIOQLQVLLDIOQNHIN
141  TTPG A Q MHH T R YG P VOSH T IS E KE IX R
142  IVARNSFNGLSFESVILNLSKMCIEEINMCAPNGTOLDLNLSDHMLLE
191  MHSGA T S S LOA S SIOT I L S S T SR
192  LPNDVTOGASGPVILDISRTHVNSLPNGLNLRALAASTYPLRLPML
241  E TS LV S R PRKE ..... QMFSPSEFENFSKQC
242  DKFVTLNEASLTYPNCCAFANLKAQISELNFICNKSILAQDIDQNTQIG
283  EST RKA M TL SAIFEENELSGMOY GP S PK LO A E
292  DQAVSIIIDDEPSTG...RGSDMHYNEFDYDLCHNEVVVTCSPEDAFNPC
TM I
332  AF LN F L F L R S
339  EDINGYHILNIMTISILAIGNTTVLVVLTGYKLTVPRLNGLAF
TM II
382  F M L SQ Q Y N S
389  ADLCIGITLLLTASVDINTKSOYHNTAIDMOTGAGCCAGFTTVFASLS
TM IV
432  V Y V DQ LR IPI EG L STLI TH LV
439  VYTLTAITLERMHTITHAMOLECKVOLANASVNYLGNTPAFAAALPPIF
TM V
482  M VE T V ILST I V A R PA
489  GISYTHKVSICLPNDISPLSLYVHALLVNLVAFVVICGCTHITLV
TM VI
532  O ELTAPME I T AF TM
539  RNPITVSSSDTKIAKMATLIFTDFLWAPISPFATSASLKVPLITVSK
TM VII
582  S V A O LL R CKRR EL
589  ANLLVLTYPINSCAMPETATIEDNFRADFFILLSKFGCTENQAQITRT
631  ..... EF.. YTSNCKNGFPGASRP ATLRLSTVHCOOP1
639  ETSSATNMFNAKSHCSSAPRVTHSTVLVPLNHSQ.....
693  PPRALTM rat LH/CG receptor
699  ..... rat FSH receptor

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Figure 4 - Structural Comparison of Gonadotropin Receptors

Appearing above is a structural comparison of the gonadotropin receptors as depicted by Sprengel et al. (11). The FSH-R sequence is shown as the lower sequence and differences as well as substitutions in the LH/CG-R are presented above. Dots denote insertions introduced for optimal alignment. The extracellular repeats are numbered and demarked by vertical lines. Transmembrane regions TMI-TMVII are boxed.

Using full length and truncated LH/CG receptors, hormone affinity studies by Braun et al. (348) suggest that gonadotropin binding to receptor is not affected by association of the receptor with G_s protein. Braun and co-workers also constructed hybrid LH/CG-FSH receptors in which the FSH receptor transmembrane domain was combined with the LH/hCG receptor extracellular domain and vice versa. By examining the binding and activation of these chimeric receptors expressed in human embryonic kidney cells they demonstrated that hormone binding sites can be exchanged between LH/CG and FSH receptor without loss of receptor function. Specificity of the hybrid receptors was determined by the hormone binding site present. From these studies it was also shown that leucine repeats 1-11 of the FSH receptor extracellular domain confer the FSH response.

FSH Receptor Gene Structure

The rat FSH receptor gene was recently cloned and characterized by Heckert et al. (349). Their results showed the FSH receptor gene to be at least 84 kilobase pairs in length and consisting of 10 exons and nine introns (see figure 5). The first nine exons code for the receptor's putative amino-terminal extracellular domain while exon 10, the largest exon, encodes the seven transmembrane domains and the carboxy terminus.

Northern blot analysis of ovarian RNA using a cRNA probe corresponding to bases 670-1031 of the rat FSH receptor cDNA (see figure 3) revealed two predominant hybridization signals of 7.0 and 2.5 kb, as well as minor signals of 4.2 and 1.8 kb (181). The sequences and intron/exon organization of these transcripts is currently unknown.

Interestingly, the FSH receptor gene possesses two transcriptional start sites, neither of which contain a conventional upstream TATA or CAAT box. A perfect consensus sequence for an AP-1 binding protein does exist, however, at position -115. The transcription factor AP-1 has been found to interact with the promoters of phorbol diester-inducible genes (350).

Antibodies Against FSH Receptor

Specific polyclonal and monoclonal antibodies are valuable tools in studies of hormone receptor structure, function, and expression. Polyclonal antibodies have been raised against purified receptors for LH/hCG (351-353) and monoclonal antibodies against both LH and TSH receptors (354-358). Due to the difficulty experienced in purifying FSH receptor (330), preparation of specific antibodies against the receptor has been difficult as well.

Recently, Dattatreyamurty et al. (359) raised

polyclonal antibodies against FSH receptor isolated from bovine calf testis. The antibody was found to block FSH binding and to induce the conversion of androstenedione to estradiol in cultured rat Sertoli cells. Thus, their studies indicate that the antibody interacts specifically with FSH receptor and, like FSH, is capable of inducing biological effects suggesting that the hormone binding site and antibody binding site on the receptor are proximate or identical. This particular antibody was prepared prior to the cloning of FSH receptor cDNA. With the present knowledge of the FSH receptor nucleotide and amino acid sequences, receptor specific antibodies raised against synthetic or recombinant peptides should be forthcoming.

K. Localization of FSH Receptors

Several studies concerning FSH receptor expression have utilized radiolabeled FSH. In addition to quantitative studies of receptor expression, radiolabeled FSH has been used to examine the localization of FSH receptors in different tissue and cell types.

FSH Receptors in the Ovary

In the ovary, autoradiographic studies have localized FSH binding exclusively to granulosa cells (7,9,179). In contrast, LH binding has been localized to both granulosa

and thecal cells (360). The compilation of several studies has revealed the basic roles of gonadotropins, gonadotropin receptors, and granulosa and thecal cells in ovarian function. Thecal cells produce androgens under the influence of LH (361). The androgens are then converted to estradiol by the granulosa cells (362). This conversion requires induction of the granulosa aromatizing system by FSH (363,364). Consequently, estradiol acts to increase ovarian responsiveness to both FSH and LH (7). Follicular FSH receptor content remains fairly constant throughout the estrous cycle (179). This is in contrast to LH receptor content, which changes substantially (9,179).

FSH Receptors in Ovarian Tumors

There is also evidence suggesting the presence of FSH receptors in ovarian neoplasms (365,366). Nakano et al. (365) demonstrated radiolabeled FSH binding in benign tumors of both epithelial and sex cord-stromal origin. Stouffer et al. have reported radiolabeled FSH binding to a malignant granulosa cell-theca cell tumor. Although the cause of ovarian neoplasms is still unknown, some studies have suggested that gonadotropins may influence neoplastic growth and differentiation (365,367,368). Ovarian tumors have been found to develop in rodents after prolonged treatment with gonadotropin (367). Interestingly, the

incidence of human ovarian neoplasms increases around the beginning of menopause when the levels of circulating gonadotropins are high. Further studies are needed to determine the biological significance of FSH receptors in ovarian tumors.

FSH Receptors on Sertoli Cells

Whereas FSH receptors are found exclusively on granulosa cells in the ovary, localization of FSH binding in the testis is more complicated. Specific FSH binding sites have been demonstrated in the seminiferous tubules of both mature and immature rats (369-371). Although the exact role of FSH in normal seminiferous tissue is not fully understood, FSH is known to be essential for initiation of spermatogenesis at puberty and for the maintenance of spermatogenesis in adulthood (372-374). In accordance with this regulatory role of FSH, FSH receptors have been found on the plasma membrane of Sertoli cells within seminiferous tubules (23).

Sertoli cells are the only somatic cell within the seminiferous epithelium and are thought to mediate hormonal regulation of spermatogenesis (375). Although FSH receptors are known to be located on the plasma membrane of the Sertoli cell, the distribution of receptors on the cell surface is not entirely clear. Orth and Christensen (23)

examined the localization of ^{125}I -labeled FSH in testes and found FSH receptors to be located at the basal membrane surface of the Sertoli cell.

The Sertoli cell is a morphologically complex cell that extends from the germinal epithelium to the lumen while possessing several lateral extensions enfolding adjacent germ cells. In addition, Sertoli cells possess intercellular (Sertoli-Sertoli) junctions which constitute the 'blood-testis barrier' and separate the seminiferous epithelium into a spermatogonia-rich basal compartment and an adluminal compartment in which older germ cells mature (376). Nagano and Suzuki (377) showed that in human testis there were more than 40 parallel-oriented tight junctions located around the surface of each Sertoli cell near the base of the epithelium. The tight junctions exclude the passage of many substances and thus, may prevent labelled FSH from reaching luminal receptors during receptor assays. Indeed, Setchell et al. (378) found that penetration of iodinated FSH and LH into the rat seminiferous tubules is highly restricted.

It should be noted that expression of FSH receptors at the basal membrane of Sertoli cells could have functional implications as suggested by Orth and Christensen (23). FSH stimulates Sertoli cells by means of adenylate cyclase, protein kinase, and other transcription regulating mechanisms. Localizing of FSH receptors to the basal

membrane would allow FSH to be internalized in close proximity to the Sertoli cell nucleus where transcription occurs (379).

FSH Receptors in the Lumen of Seminiferous Tubules

The physiological significance of Sertoli tight junctions is important in light of studies which have demonstrated FSH binding within the luminal area of seminiferous tubules (380,381). Using immunoperoxidase localization of FSH, Yoon et al. (380) demonstrated FSH staining in the luminal region of seminiferous tubules prepared from human testis. They speculated that free FSH was not likely to have been retained following their washing and incubation procedures and therefore suggested that the positive staining represented receptor bound hormone. Yoon and co-workers also provided two possible explanations for FSH binding in the seminiferous lumen. Loss of Sertoli tight junctions, due to the pathological conditions of sampled tissues, may have allowed hormone access to receptors in the apical portion of the Sertoli cell membrane. Alternatively, luminal staining may have represented stored or metabolized FSH internalized via receptor from the basal membrane.

FSH Receptors in Spermatogonia

Several studies have demonstrated FSH binding to interstitial cells of the testis (380,382-385). Using autoradiographic localization of labeled FSH, Orth and Christensen (381) reported the presence of FSH receptors on spermatogonia. Spermatogonia lie in the seminiferous tubules outside the Sertoli cell barrier and are thus, exposed to hormones including FSH (385). Due to the relatively narrow intercellular spaces within the rat seminiferous tubule, Orth and Christensen utilized a hyperosmotic fixation technique which, by preferentially shrinking cells of the basal compartment, enlarged the intercellular space between spermatogonia and Sertoli cells allowing distinction of positively stained cells.

FSH Receptors in the Interstitium

Yee and Hutson (382-384) demonstrated the presence of high affinity FSH receptors on testicular macrophages. Macrophages account for 20% of the cells in the interstitial region of the rat (386), yet little is known concerning their role in testicular function. Yee and Hutson have shown, however, that testicular macrophages respond physiologically to FSH in the form of increased incorporation of amino acids into secreted (but not

cellular) protein and increased uridine incorporation into cellular RNA (384). In addition, Murakoshi et al. (387) found that injections of gonadotropins into hypophysectomized rats stimulated the levels of glutathione peroxidase in the cytoplasm of testicular macrophages.

Yoon et al. (380) also demonstrated FSH binding in the interstitium of human testis, but the positively stained cells did not represent macrophages. The results of Yoon and co-workers are in agreement with the findings of Wahlstrom et al. (385) who also demonstrated specific FSH binding in the interstitium. Wahlstrom and co-workers reported that the positively stained interstitial cells were morphologically indistinguishable from Leydig cells. Although the presence of FSH receptors on Leydig cells has not been demonstrated, Leydig cell function is known to be regulated by FSH via Sertoli cells (388).

L. FSH Receptor Induction

Early studies concerning FSH receptor expression and induction relied on FSH binding analysis as there were no effective antibodies available to the receptor. Such studies revealed that FSH receptor expression is confined to granulosa cells in the female and to Sertoli cells in the male. Binding studies have also revealed that FSH responsiveness in the ovary results from an increase in the number of granulosa cells, rather than to an increase in

the amount of receptor expressed per cell (7). In contrast, FSH responsiveness in the testis appears to result from an increase in the number of receptors expressed per Sertoli cell (389).

Binding studies remain an effective and well established means of examining receptor expression and regulation. Recent elucidation of the FSH receptor cDNA sequence (11), however, has allowed receptor expression studies to be performed at the molecular level.

FSH Receptor Induction in Ovary

The reproductive cycle of the rat lasts 4 days and consists of four stages: proestrus, estrus, metestrus, and diestrus. During the ovulatory cycle, follicle maturation, ovulation, and luteinization are associated with dynamic changes in gonadotropin receptor content (181,179,7). Several studies have shown that patterns of gonadotropin receptor expression throughout the reproductive cycle are paralleled by changes in receptor mRNA levels (8,179,181,182,184).

Using a quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay, Camp et al. (179) found an increase in levels of FSH receptor mRNA during follicular development followed by a decrease in levels at the time of the LH surge. They found the pattern of LH receptor mRNA

expression to be similar but more pronounced. Results from this study are shown in figure 6 and are in agreement with results obtained by LaPolt et al. (181) using PMSG/hCG primed immature rats.

Regulation of FSH Receptor by FSH

Galway et al. (390) have shown that follicular development and ovulation can be induced in hypophysectomized estrogen-treated rats by recombinant FSH (rcFSH). Using this model, LaPolt et al. (181) have recently reported up- and down-regulation of ovarian FSH binding and FSH receptor mRNA levels by rcFSH. Results of this study are shown in figure 7.

Administration of rcFSH to induce follicular development resulted in an increase in FSH binding and FSH receptor mRNA expression. Conversely, an ovulatory injection of rcFSH significantly reduced FSH binding and receptor mRNA expression. These findings suggest that FSH regulates expression of its own receptor at the level of transcription. LaPolt and co-workers speculate that the rapid decrease in FSH receptor transcripts during homologous down-regulation may reflect decreased stability of these mRNAs and/or a decrease in the transcription rate.

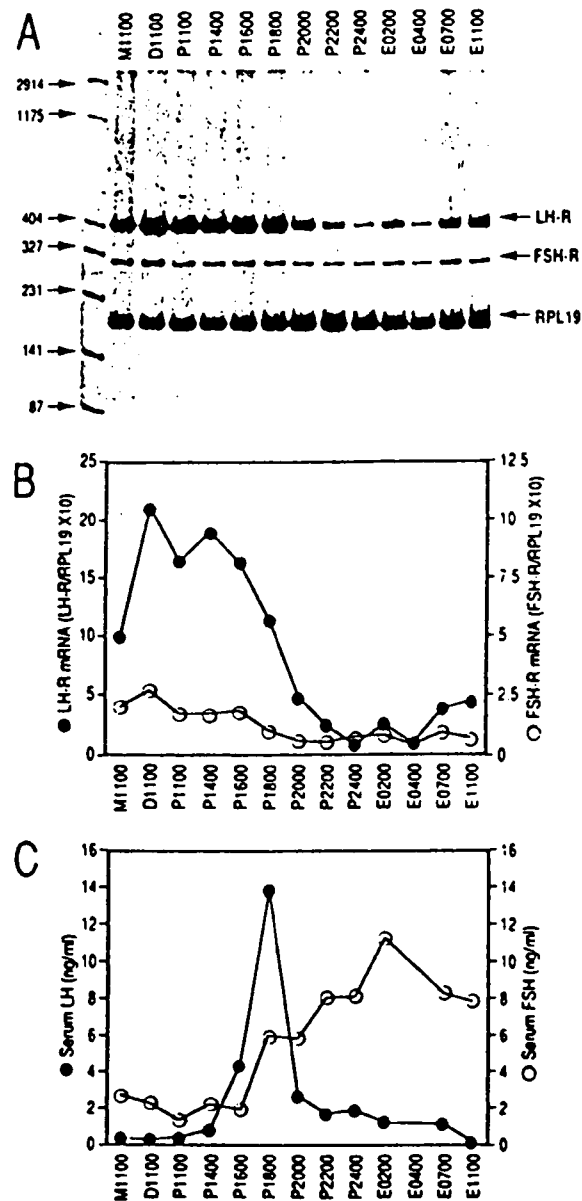


Figure 6 - RT-PCR Measurement of FSH and LH Receptor mRNAs During the Rat Estrous Cycle

Ovarian RNAs were isolated from cycling animals at the points during the estrous cycle indicated. A) an autoradiogram of a polyacrylamide gel on which the PCR products from these RNAs were separated. The corrected intensities of the LH receptor and FSH receptor bands shown in A are plotted in B. Serum LH and FSH levels in these same animals are shown in C. Taken from Camp et al. (179).

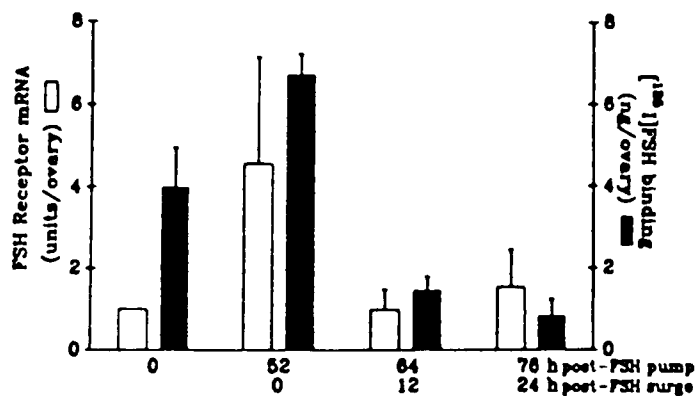


Figure 7 - FSH Binding in Rat Ovary Following rcFSH Treatment

Changes in [¹²⁵I]-FSH binding and FSH receptor mRNA levels at different times after treatment with rcFSH. Hypophysectomized rats received 4 IU/day rcFSH for 52 h, followed by an ovulatory dose (20 IU) of rcFSH. Taken from LaPolt et al. (181).

Regulation of FSH Receptor by LH

Down regulation of FSH receptors may be responsible in part for the process of atresia undergone by follicles during selection and luteinization of dominant follicles. Ovarian FSH receptors, along with receptors for LH and estradiol, appear to be down-regulated by high levels of LH at the time of luteinization (7,179,181). Using PMSG-primed immature rats, ovulatory doses of hCG have been found to decrease FSH binding and FSH receptor mRNA levels 5- and 4-fold, respectively (181). Inhibitory transcription factors (such as the recently isolated cAMP-responsive element modulator) may provide a mechanism for gonadotropin induced down-regulation of FSH receptor transcription.

Role of Estradiol in Receptor Induction

Changes in gonadotropin receptor expression and, thus, follicular responsiveness to gonadotropins appears, to be regulated at least in part by estradiol (7). It has been further shown that estradiol regulates folliculogenesis at the level of the granulosa cell (7). Estradiol acts on granulosa cells of developing follicles to increase FSH receptor content, thus increasing the cell's responsiveness to FSH (7). FSH in turn increases LH receptor content within the follicle (187,391).

It was important to determine whether the ability of estradiol to increase ovarian FSH receptor content reflected an increase in the number of FSH receptors per granulosa cell or an increase in the number of granulosa cells. Discrepancies exist between the number of FSH binding sites per granulosa cell as determined by several groups. Nimrod et al. (392) reported that granulosa cells from non-PMSG primed rats possessed 130 FSH receptors/cell. Louvet and Vaitukaitus (393) reported that granulosa cells from rats primed with or without estradiol only (no PMSG) possessed 5000 FSH receptor sites/cell. Recently, Sanford and Batten (304) found granulosa cells isolated at the preovulatory stage of folliculogenesis to possess 4630 FSH binding sites/cell. These discrepancies may be explained in part by the hormone treatment of animals prior granulosa cell isolation.

FSH binding studies by Richards et al. (7) revealed that the number of FSH binding sites per granulosa cell remains constant and thus, the estradiol induced increase in FSH binding reflected an increase in the number of granulosa cells. Their studies also found that estradiol does not act alone, but rather acts synergistically with FSH to promote follicular growth and subsequent LH receptor induction. Indeed, estradiol given alone was shown to significantly decrease LH receptor content.

Induction of FSH Receptor in Testis

In the testis, FSH is essential for the regulation of spermatogenesis. In pubertal rats FSH appears to be involved in the initiation and regulation of spermatogenesis. The role of FSH in the adult is less clear. Physiological responses to FSH are mediated by Sertoli cells which possess high affinity receptors for the hormone (23,394).

Changes in FSH receptor content during rat testicular development suggest that, while total FSH binding increases throughout the period of testicular growth (495), the density of FSH binding sites increases only during the initial phase (days 9-19) of development (496,389). In that rat Sertoli cells undergo rapid division around birth but cease to divide beyond the first 2-3 weeks of postnatal life (397), the post 20 day increase in FSH receptor content appears to reflect an increase in the number of receptors per Sertoli cell. Consequently, prepubertal increases in FSH receptor content coincide with increases in circulating levels of FSH suggesting up-regulation by FSH of its own receptor (395,396,389).

Tsutsui (395) performed hypophysectomy and hormonal replacement on male rats of various ages to identify factors involved in the developmental induction of FSH receptors. Tsutsui found FSH to be an effective inducer of

FSH receptor expression during the period of testicular growth. Testosterone was also found to induce FSH receptors but to a lesser extent. In contrast to the prepubertal rat, high doses of FSH (1.0 to 5.0 ug/mL) have been shown to inhibit expression of FSH receptors in the adult rat (389,397,398).

Tsutsui (395) found that a significant induction of both FSH receptor density and total FSH binding occurred 10 days after hypophysectomy at 9 days of age. Such an increase in FSH binding capacity following hypophysectomy suggests that factors other than pituitary hormones are involved in FSH receptor regulation.

FSH Receptor Induction in the Adult

Heckert and Griswold (399) reported a large decrease in the apparent abundance of FSH receptor mRNA in male rats 20 days of age and older. They suspect, however, that the observed decrease in mRNA was due to the dilution of Sertoli cell mRNA with germ cell mRNA which becomes more abundant at this approximate age. Thus, FSH receptor mRNA appears to be continually expressed in the adult rat allowing the testis to remain responsive to FSH.

The evolution of germ cells in the rat consists of 14 stages, each being characterized by a distinct association of cells within the seminiferous epithelium (400). The 14

stages follow a cyclic pattern with the time between two successive appearances of the same stage being approximately 12 days (401). Spermatogenesis is initiated at stage IX of the cycle when spermatogonia divide (400).

Both mRNA expression and cAMP response studies (399,402) suggest that FSH receptor protein expression is highest in stages XIII-II, which are characterized by meiotic division of spermatogonia, and lowest in stages VII-VIII of the cycle, characterized by the release of mature spermatozoa. Heckert and Griswold (399) have speculated that during the stages when FSH receptor levels are highest, the actions of FSH may be to regulate the number of germinal cells that enter meiosis and potentially differentiate into mature sperm. This hypothesis supports the proposed role of FSH in maintaining sperm output in the adult rat.

M. FSH/Receptor Interaction and Mechanisms of Signal Transduction

Follicle stimulating hormone belongs to the family of glycoprotein hormones that includes FSH, LH, and TSH. Each of these hormones is characterized by a heterodimeric structure consisting of a common alpha-subunit and a hormone specific beta-subunit (403). In that the beta-subunit confers hormone specificity in terms of bioactivity, it would seem likely that regions of FSH involved in receptor interaction would be located on the

beta-subunit.

Regions of Hormone-Receptor Interaction

Two regions of the FSH beta-subunit have been implicated in receptor binding; hFSH-B 33-53 and 81-95 (403-407). The hFSH-B 33-53 region contains the tetrapeptide sequences TRDL (positions 34-37) and KTCT (positions 49-52). A synthetic peptide corresponding to hFSH-B 33-53, encompassing both the TRDL and KTCT regions, was found to inhibit ^{125}I -FSH binding (406). Recently, synthetic tetrapeptides corresponding to the TRDL and KTCT sequences of hFSH-B were each found to inhibit ^{125}I -FSH binding to testicular FSH receptors, though to a lesser extent than the hFSH-B 33-53 peptide (405,406). Synthetic hFSH-B 33-53 has also been found to stimulate estradiol synthesis in cultured rat Sertoli cells and, thus, acts as a partial agonist of FSH (406). The hFSH-B 81-95 peptide has also been found to inhibit ^{125}I -FSH binding and is a partial agonist of hFSH (407).

Sense-Antisense Similarity Between FSH and Receptor

Interestingly, Slootstra and Roubos (408) found that the hFSH-B 33-53 and 81-95 regions exhibit sense-antisense similarity with the FSH receptor. Their findings are based

upon the molecular recognition theory (MRT) which suggests that peptides derived from complementary DNA strands interact specifically based upon hydrophathic complementarity (409). This concept has been found to apply to several peptides and their complements including ACTH (409) and insulin (410), as well as to some receptor-hormone interactions (411,412).

Hydropathy plots reveal that hFSH-B 33-53 and 81-95 represent the most hydrophilic regions of hFSH-B and show hydrophathic complementarity to four regions of the hFSH receptor, each located in the N-terminal extracellular domain (408). Within these four regions, the antisense FSH receptor and hFSH-B have six short fragments in common; AHH, CTF, CTV, RDL (twice), and TFK. All but the AHH fragment are located within the hFSH-B 33-53 and 81-95 domains.

Characteristics of the Hormone-Receptor Interaction

An understanding of FSH binding to receptor and the effects of ions and temperature on this interaction is important for the interpretation of binding data obtained from in vitro systems. To this end, the interaction of FSH with its membrane bound receptor has been studied extensively in calf-testes membrane preparations (for review see 330).

Many proteins require the presence of divalent metal ions for proper interaction with receptor. Andersen and Reichert (413) have found that FSH binding is stimulated in the presence of 5 mM Mn^{+2} , Mg^{+2} , or Ca^{+2} , while inhibited by Co^{+2} or Ni^{+2} . They also found that halide salts inhibit FSH binding in an increasing ionic radius dependent manner (i.e. $F^{-} > Cl^{-} > I^{-}$). Also, sodium salts were identified as potent inhibitors of FSH binding.

Andersen et al. (414) found FSH binding to Sertoli membrane bound receptor to be reversible at 4°C, but becoming increasingly irreversible as a function of temperature. Similar findings have been made using detergent solubilized receptor, although the interaction was more easily reversed over the same temperature range (415). This difference is most likely due to the effects of non-receptor components present in the membrane preparations used by Andersen and co-workers. FSH binding is essentially irreversible at temperatures above 30°C (414,415). The results of Andersen et al. (414) and Sanborn et al. (415) are consistent with characteristics of hydrophobic interactions, which appear to play a role in the interaction of FSH with its receptor. Still, the irreversibility of binding observed at temperatures above 30°C suggests that other mechanisms may be involved.

Involvement of Disulfide Interchange in Hormone Binding

The irreversible binding of FSH to its receptor at physiological temperatures (i.e. above 30°C) could be the result of hormone-receptor disulfide interchange. The beta-subunit of FSH shares a region of sequence homology (CGKC) with the active site of thioredoxin (416).

Thioredoxin is an ubiquitous protein that serves as an electron donor for ribonucleotide reductase. Thioredoxin also exhibits disulfide isomerase activity as evidenced by its ability to catalyze the reactivation of reduced and denatured ribonuclease (417,418). Consequently, FSH has also been found to catalyze the reactivation of ribonuclease and with a surprisingly greater efficiency (~60 times greater) than thioredoxin (416). These findings have led some researchers to suspect that a redox process may be involved in hormone action (416,419).

Follicle stimulating hormone lacks free sulfhydryl groups so it is suspected that any redox activity of FSH would involve sulfhydryl groups available on the receptor. To investigate this possibility, Santa-Coloma et al. (419) examined the effects of N-ethylmaleimide (NEM) and glutathione on the binding of FSH to its receptor. The alkylating activity of NEM was found to reduce the affinity of the receptor for FSH without affecting binding capacity. This result suggests that sulfhydryl groups are indeed

required for a high affinity hormone-receptor complex. The presence of oxidized glutathione was found to inhibit FSH binding, most likely by cysteinylolation of receptor sulfhydryls. By contrast, the presence of reduced glutathione, a condition that favors disulfide interchange, was found to increase FSH binding up to 60%.

It appears that disulfide interchange is involved in the interaction of FSH with its receptor. Santa-Coloma and co-workers propose a model in which, following an initial reversible hormone-receptor interaction, disulfide bridge(s) form between the hormone and receptor, thus stabilizing the complex and reflecting an increased affinity.

Importance of Carbohydrates in Hormone Binding

Several studies have shown that chemically deglycosylated gonadotropins have a reduced ability to stimulate cAMP accumulation, while retaining the ability to bind to receptor (420,74,421,72). Recently, Padmanabhan et al. (422) have reported that deglycosylated FSH, while unable to increase cAMP levels in cultured rat Sertoli cells, was able to stimulate estradiol production. This result is in contrast to results obtained for deglycosylated LH and hCG which do not stimulate cAMP levels or estradiol production (420,421,423). The results

obtained by Padmanabhan and co-workers are also interesting in light of the widely accepted role of cAMP as a second messenger of FSH action, including estradiol production. The possibility remains, however, that alternate mechanisms of FSH signal transduction exist.

The Role of Calcium in FSH Signal Transduction

Evidence for the involvement of calcium in the mechanism of FSH signal transduction has recently emerged (424,425). Gorczynska and Handelsman (424) found that physiological doses (i.e. 5-10 ng/mL) of FSH stimulate a 100 nM increase in Sertoli cell cytosolic calcium concentrations. Similar results have been obtained using granulosa cells. Studies using radioactive calcium have demonstrated that the FSH-induced rise in Sertoli cell cytosolic calcium results from the transmembrane flux of extracellular calcium into the cells (228,426).

The FSH receptor is a G protein-coupled receptor and, as such, could induce calcium channel activation by phosphorylation of calcium channel proteins. The voltage-gated calcium channel blockers nicardipine and verapamil, as well as the voltage-independent calcium channel blocker ruthenium red, each prevented the FSH-induced increase in cytosolic calcium and inhibited FSH-stimulated conversion of androstenedione to estradiol

(228). These findings suggest that more than one class of calcium channel is coupled to the FSH signal transduction mechanism.

Interestingly, Grasso and Reichert (426) found that the FSH induced increase in cytosolic calcium levels does not require activation of toxin-sensitive G proteins or accumulation of cAMP. These findings have led Grasso and Reichert to speculate that the FSH receptor itself may function as a calcium channel.

Calcium fluxes have been implicated with the LH receptor signal transduction mechanism (427-430). Luteinizing hormone and FSH and their respective receptors are structurally very similar and, therefore, may share common features of signal transduction. It remains to be determined whether FSH-induced calcium fluxes represent a modulatory mechanism or an additional primary mechanism of signal transduction.

Similarity of FSH Receptor to G Protein-Coupled Receptors

Sequencing of FSH receptor cDNA has revealed the receptor to be a member of the family of G protein-coupled cell surface receptors (11). Characteristic of G protein-coupled receptors are seven membrane spanning domains and the ability to stimulate adenylate cyclase activity following ligand-dependent activation (431). This

characteristic is consistent with the widely accepted role of cAMP as a second messenger of FSH activity in both Sertoli and granulosa cells.

The FSH receptor also possesses a large N-terminal extracellular domain, a characteristic common to the gonadotropin and TSH receptors, but unique to the G protein-coupled receptor family (11,431). This domain displays leucine rich sequence repeats similar to regions of other leucine rich glycoproteins and appears to mediate hormone binding (348). The leucine rich repeats allow for the formation of amphipathic peptide surfaces suitable for protein-protein interactions.

Role of G Proteins in FSH Signal Transduction

Fletcher and Reichert (432) have shown that specific high affinity binding of GTP is required for activation of FSH-dependent adenylate cyclase. Consequently, Zhang et al. (433) have demonstrated that FSH receptors are physically and functionally associated with a guanine nucleotide regulatory protein which they suspect to be the stimulatory guanine nucleotide-binding protein of adenylate cyclase (G_s). Fletcher and Reichert (432) also found the affinity of GDP binding to be about 5 fold less than that of GTP binding. Follicle-stimulating hormone appears to enhance the high affinity binding of GTP, thus facilitating

the release of GDP. This would be consistent with the 'nucleotide exchange' mechanism of signal transduction common to other G protein-coupled receptors (431).

Interestingly, Fletcher and Reichert (432) found the number of GTP binding sites in Sertoli cell membranes to be in 50- to 100-fold excess relative to the number of FSH binding sites. This could represent the first step in amplification of the hormone signal resulting in the activation of several molecules of adenylate cyclase and increased cAMP levels.

Zhang et al. (433) found that the G_s protein coupled to FSH receptor possesses both high and low affinity GTP binding sites. More recent studies by Zhang et al. (434), using NEM and cholera toxin to uncouple receptors from GTP binding sites, have shown that only the high affinity sites are essential for FSH and guanine nucleotide activation of adenylate cyclase. The low affinity sites, although binding GTP, are not directly involved in adenylate cyclase activation.

It appears then that FSH, upon binding to its receptor, enhances the dissociation of GDP from the physically associated G_s protein thereby making GTP binding sites available. With the binding of GTP to the G_s protein, the FSH receptor appears to be uncoupled from the GTP binding sites allowing for adenylate cyclase activation and cAMP formation. Subsequent GTPase hydrolysis of GTP to

GDP terminates the signal.

Potential Models of Signal Transduction

Generally, signal transduction in G protein-coupled receptors is effected by a conformational shift within the membrane spanning segments induced by ligand binding (431). In regards to FSH-receptor interaction, Braun et al. (348) have described two possible models of signal transduction. Their hypothetical models are depicted in figure 8. In one model, a conformational change in the FSH receptor, possibly mediated by the thioredoxin-like activity of FSH, allows for interaction of the receptor with the G_s protein. In the other model, FSH binding is followed by positioning of the hormone into a receptor activation site defined by the membrane spanning domains. Further studies are necessary to elucidate the exact mechanisms by which FSH and its receptor interact to elicit a response.

N. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a simple, but extremely powerful, procedure for the in vitro amplification of specific nucleic acid sequences. Since its introduction in 1985 (435), PCR has revolutionized the field of molecular biology (for reviews see 436,13,437, 438). Its overwhelming impact on biology is due to its

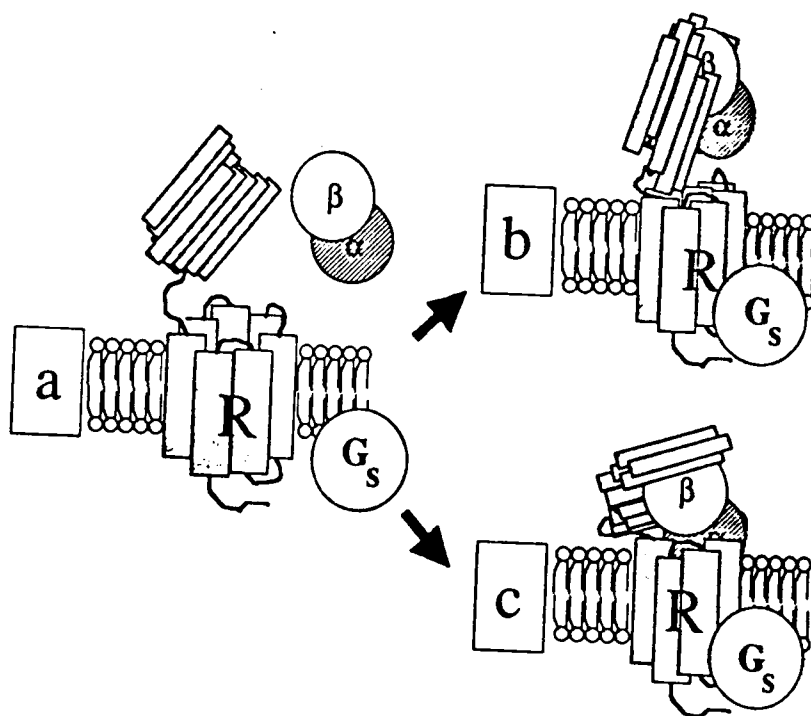


Figure 8 - Models for Gonadotropin Receptor Activation

(a) Hormone binds and (b) binding leads to a conformational change of the entire receptor molecule, or (c) bound hormone is exposed and interacts with a receptor activation site defined by the arrangement of the membrane spanning segments. The activated receptor stimulates G_s protein. R = gonadotropin receptor; G_s = G protein; alpha/beta = glycoprotein hormone. Taken from Braun et al. (348).

simplicity and broad range of applications. PCR allows for minute amounts of virtually any nucleic acid sequence to be readily generated in vitro in great abundance, so that subsequent analyses are not hindered by the presence of other nucleic acid fragments or by the lack of material to work with. PCR has allowed scientists to study DNA from embryos only a few hours old (439) to ancient DNA obtained millions of years after death (440).

Principles of the Reaction

The basic principle of PCR was conceived by Dr. Kary Mullis in 1983 while he was working at the Cetus Corporation in Emeryville, CA. The brainstorming that led Mullis to the idea of PCR is described in an interesting article authored by Mullis himself (441).

The process of DNA amplification by PCR consists of repetitive cycles of DNA denaturation, primer annealing, and extension by DNA polymerase (442). The oligonucleotide primers are designed to be complementary to a short stretch of sequence located near the 3'ends of the two strands of double stranded template. As such, when each is extended by the action of DNA polymerase, the newly synthesized strands overlap the binding site of the opposite oligonucleotide. This scheme is depicted in figure 9. As the process of denaturing, primer annealing, and polymerase

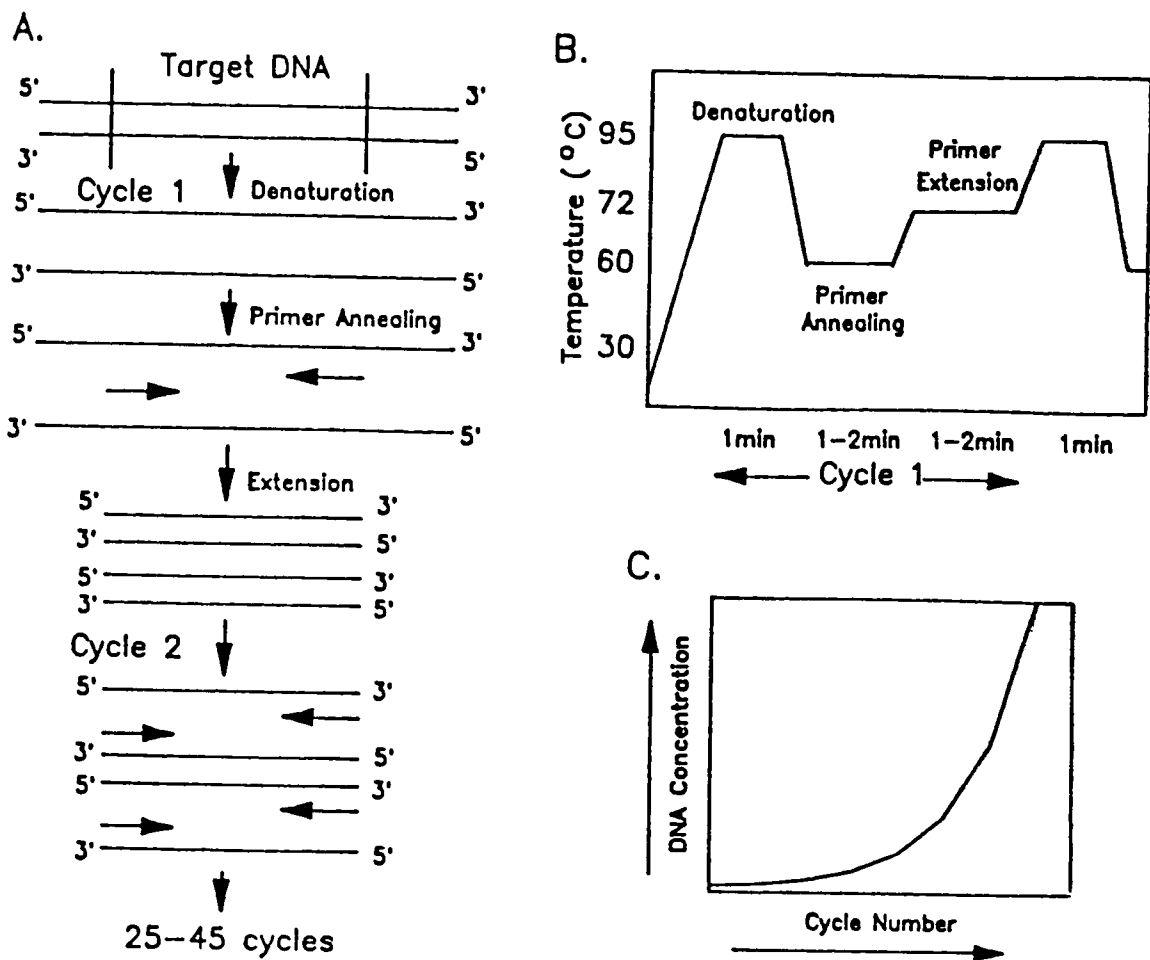


Figure 9 - The Polymerase Chain Reaction

(A) Schematic diagram of PCR amplification. Each cycle consists of denaturation of target DNA, primer annealing, and primer extension. (B) A graphic representation of the changes in temperature during a typical PCR cycle, which consists of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 to 2 min, and primer extension at 72°C for 1 to 2 min. (C) Quantitation of amplified DNA product. The amount of DNA increases exponentially as the cycle number increases. Taken from Bej et al. (13).

extension continues, the product of each PCR cycle is complementary to and capable of binding the oligonucleotide primers and, as such, serves as template for the next cycle. This leads to an exponential increase in the total number of DNA fragments that include the sequence flanked by the 5' ends of the primers (see figure 9). Thus, the theoretical abundance of amplified DNA is 2^n , with n being the number of PCR cycles performed. Ideal target sequences for PCR amplification can be up to 2 kb in length. The template DNA can be used in a pure form or as a very small fraction of a complex biological sample (i.e. cells, blood, hair roots, cytology smears, etc.). A cDNA copy of reverse transcribed RNA can also serve as template.

In addition to template nucleic acid and oligonucleotide primers, DNA polymerase, deoxyribonucleotide triphosphate monomers (dNTPs) and a magnesium source in an appropriate buffer are required to amplify DNA by PCR. The original PCR protocols of Saiki and Mullis (435,442) utilized the klenow fragment of *E. coli* DNA polymerase I to catalyze the extension of annealed oligonucleotide primers. This enzyme, however, was heat inactivated by the high temperature required for DNA denaturation and, as such, had to be replaced with fresh enzyme at each round of amplification. This problem was solved in 1988 by Saiki et al. (443) with the substitution of a thermostable DNA polymerase obtained from the thermophilic bacterium *Thermus*

aquaticus (Taq) in place of the heat sensitive klenow fragment.

In a typical PCR cycle, DNA is denatured by heating to approximately 94°C for 1 min, followed by primer annealing at 40-60°C for 1 min, and primer extension at approximately 72°C for 1 min (see figure 9). The sensitivity of PCR varies according to these parameters and with the amount and purity of template that can be reliably identified above background signal. The lower limit of detection, however, has been established. Saiki et al. (443) have amplified 2 kb DNA molecules from dilutions of cells containing a single copy of human beta-globin sequence.

Applications in Basic Research

Although a relatively new technique, PCR has already found applications in several fields of biological research including molecular biology, biotechnology, and medicine. Some of its applications in basic research are described below.

RNA Amplification

Methods for RNA analysis include Northern blotting, in situ hybridization, S-1 nuclease assays, and RNase A protection studies. Although in situ hybridization can be

used to detect as few as 10 to 100 molecules of RNA in a single cell, the other methods are much less sensitive (10^5 to 10^6 mRNA molecules) (444). By producing a cDNA copy of an RNA molecule and amplifying the cDNA by PCR, a single specific RNA molecule can be detected in a heterogenous RNA sample. This technique was first described by Veres et al. (445) and has been subsequently referred to as reverse transcription-PCR (RT-PCR). Reverse transcription PCR is accomplished by incubating RNA with complementary primer, dNTPs, and reverse transcriptase to generate cDNA, which then serves as template for the PCR.

Quantitation of mRNA

Quantitation of RNA is generally accomplished by Northern hybridization and RNase protection assays. These methods are limited, however, by the quantity of RNA required and the efficiency of RNA transfer to hybridization membranes. Thus, low abundance mRNA is often difficult to detect and quantitate by such conventional methods.

Quantitation of even low abundance mRNAs can be accomplished by 'competitive' PCR (446-448). Because PCR is an exponential process, small differences in any of the variables that control the reaction can have a significant effect on the yield of amplified product. Accurate

quantitation of a specific target sequence is accomplished by co-amplification of a competitive template that uses the same primers as those of the target cDNA but can be distinguished from the target cDNA after amplification. A mutated cDNA is often used as competitive template so as to allow for distinction based on restriction digest analysis or product size.

The target cDNA is co-amplified with a dilution series of competitor DNA of known concentration. Because the two templates are co-amplified in the same tube using the same primers, variables influencing the reaction will affect the yield of competitive template and target cDNA equally. The relative amounts of amplified DNA are measured by direct scanning of ethidium bromide-stained gels or by radio-nucleotide incorporation. Because the starting concentration of the competitive template is known, the initial concentration of the target cDNA can be determined.

Generation of cDNA Libraries

Reverse transcription-PCR can also be used to generate cDNA libraries (14). Most eukaryotic mRNAs possess a poly-A tail at their 3' end. Thus, mRNA templates can be reverse transcribed using an oligo(dT) primer. A homopolymeric tract of guanine residues can then be added to the cDNA at the 3' terminus so that subsequent PCR

amplification can be primed with oligo(dT) and oligo(dC) primers (14).

The RT-PCR allows for the generation of large cDNA libraries from as few as 1 or 2 mammalian cells. Also, the primers can be designed to contain potential restriction sites at their 5' termini, which facilitates cloning into appropriate vectors.

Generation of Nucleic Acid Probes

One of the most important applications of PCR has been the generation of nucleic acid probes for cloned and uncloned gene sequences. A cloned gene can be partially sequenced so as to allow for the synthesis of appropriate primers. A nucleic acid probe can then be generated by PCR using the cloned gene fragment as template in conjunction with the synthesized primer pair.

Mixed oligonucleotide primed amplification of cDNA (MOPAC) is used to generate nucleic acid probes for uncloned genes (449). Degenerate pools of oligonucleotides are used to amplify cDNA produced by reverse transcription of mRNA obtained from cells expressing the protein of interest. Only a limited amount of amino acid sequence is necessary to create the degenerate pools of oligonucleotide primers. One pool of primers corresponds to all possible combinations of coding sequences over a short span of amino

acids, while the other pool corresponds to all possible complementary combinations of coding sequence over another span of amino acids. Following PCR, amplified products are cloned into appropriate vectors, sequenced, and used as probes for library screening or Southern or northern hybridization.

Cloning of PCR Products

PCR-amplified DNA is readily cloned into expedient vectors (450,451). PCR amplification also allows for the introduction of promoter sequences and restriction sites into DNA by incorporating the specific sequences into the primers. PCR can also be used to identify positive clones, eliminating the need to perform mini-preps of bacterial plasmid DNA (452,453). Bacterial cells obtained by touching a toothpick to a colony can be used directly in a PCR amplification to identify a plasmid insert.

DNA Sequencing

Asymmetric PCR can be used to generate several copies of single stranded DNA template suitable for di-deoxy sequencing (454,455). By using one of the primers in excess during the reactions, double stranded products will not accumulate exponentially, but rather, multiple copies

of single stranded product (the strand homologous to the primer in excess) are produced. This product can then be sequenced by conventional di-deoxy methods, or directly by PCR using *Taq* polymerase (456). As already mentioned, PCR eliminates the need for mini-preps of plasmid DNA. Plasmids and inserts can be sequenced directly by placing intact bacterial cells into the PCR reaction (452).

Mutagenesis

PCR directed mutagenesis utilizes oligonucleotides synthesized with point mutations to introduce the mutation into the amplified product. The product can then be cloned into vectors and the mutation analyzed. This method of mutagenesis is simple and eliminates the need to grow and screen several bacterial colonies to identify mutations of choice.

Limitations

Despite the versatile nature of PCR, the process is not without limitations. Of particular importance is the relatively high rate of nucleotide misincorporation. *Taq* DNA polymerase lacks an editing function and has been found to incorporate an incorrect nucleotide at a rate of about 2×10^4 nucleotides per PCR cycle. This translates into an

overall error frequency of 0.25% over 30 cycles of amplification (443).

The frequency of error associated with PCR is generally not a problem for the generation of nucleic acid probes to be used in hybridization or the generation of DNA templates to be used in sequencing. The misincorporation rate of *Taq* DNA polymerase has even been used to generate random mutations in mutagenesis experiments (457). The error frequency, however, does imply that the sequence obtained for an individual DNA molecule cloned from an amplified pool is unreliable.

Another problem encountered with PCR is contamination and non-specific amplification which can produce false positives. The PCR is so sensitive that even a single contaminating molecule can be amplified so as to produce a visible product upon electrophoretic analysis. The same holds true for a non-specific molecule with sufficient homology to hybridize with the primers. False positives can only be avoided by careful technique and the inclusion of appropriate controls. Non-specific amplification can be reduced by increasing the stringency of the PCR (i.e. raising annealing temperatures).

False negatives are also a potential problem when using PCR. Amplification can fail for technical reasons or simply as a result of human error. Thus, both positive and negative results obtained by PCR must be interpreted

carefully.

O. Clinical Applications of PCR

The application of in vitro DNA amplification by PCR in research and diagnostics has accelerated the discoveries of the molecular basis of several infectious, malignant, and genetic diseases. Much of this is due to the versatility of the reaction. Hair roots, buccal washings and scrapes, paraffin embedded tissue, cytology smears, and body fluids can all be used directly for DNA amplification and analysis by PCR (458-462).

Analysis of Genetic Disease by PCR

The PCR has found an increasing number of applications in clinical research. One area of widespread use is in the analysis of genetic diseases. In general, a target sequence is amplified from genomic DNA or cDNA and examined for mutations or polymorphisms by sequencing, hybridization with allele-specific oligonucleotides, or restriction analysis. In addition, deletions in genomic DNA can be identified by determining whether a specific segment of DNA can be amplified (463).

Once the gene responsible for a genetic disease is identified, PCR can be used to rapidly characterize genetic defects in individual patients. For example, PCR and

automated sequencing have made it possible to define the nucleotide mutation causing Lesch-Nyhan syndrome (464). Lesch-Nyhan syndrome is an X-linked genetically lethal disease that results from severe deficiency of the purine salvage enzyme hypoxanthine-guanine-phosphoribosyl transferase (HPRT). PCR has been used to characterize many altered sequences from patients with HPRT deficiency (465).

Disease Diagnosis by PCR

The first reported use of PCR in disease diagnosis was described by Saiki et al. (435) in 1985. In vitro DNA amplification was used to discriminate between beta(A)- and beta(S)-alleles for the direct diagnosis of sickle cell anemia. The precise base-pair mutation responsible for the sickle cell disease phenotype had been known for some time. Thus, Saiki and co-workers were able to analyze amplified DNA by oligonucleotide discrimination hybridization. In this particular study, PCR was simply used to confirm diagnostic results obtained by Southern hybridization. Since this original publication, however, PCR has been applied for the direct diagnosis of several human genetic diseases (for review see 463,466).

Of particular significance is the diagnosis of cystic fibrosis (CF), the most common and severe inherited disease in caucasians. With the recent cloning of the underlying

CF gene (467), prenatal diagnosis for CF can be carried out using PCR on samples of fetal chorion obtained in the first trimester (468).

PCR carrier testing for cystic fibrosis, sickle cell anemia, and several other genetic diseases can be performed on hair roots and buccal washings (460,461) allowing for rapid and non-invasive mass screening for genetic disease carriers.

HLA Typing by PCR

In addition to genetic disease diagnostics, PCR has facilitated the analysis of disease susceptibility. PCR-based sequencing studies have led to a better understanding of the evolution of human leukocyte antigen (HLA) polymorphisms and provide a rapid and precise method of HLA typing (469,470). Human leukocyte antigen typing is valuable not only in the analysis of genetic susceptibility to autoimmune diseases but also in the tissue typing of donors and recipients for successful transplantations (471).

PCR Detection of Bacterial Pathogens

PCR has also been used for the rapid detection of pathogens including bacteria and viruses (for review see

13). The PCR-based technique is particularly beneficial for the detection of pathogens whose in vitro cultivation is difficult, lengthy, or unavailable. For example, *Treponema pallidum*, the pathogen causing syphilis, cannot be cultivated on artificial medium. A sensitive PCR-based assay for *T. pallidum* that amplifies the gene encoding a pathogen specific immunogen has been described by Burstain et al. (472).

PCR has also been used for the detection of *Mycobacterium tuberculosis*. Owing to a long generation time, conventional means of identifying the pathogen can take up to six weeks. Wit et al. (473) have developed a PCR-based assay for the detection of repetitive DNA sequences present in the *M. tuberculosis* genome sensitive to as few as 10 organisms.

PCR Detection of Viral Pathogens

PCR has been used to detect viral pathogens as well. Conventional virus diagnosis has relied on the direct demonstration of virus in clinical samples or on direct detection of a specific antibody response. The elucidation and characterization of virus-specific nucleic acid sequences has allowed for the development of PCR-based detection assays, which can be several orders of magnitude more sensitive than detection by hybridization or antibody

response (474).

Significantly, PCR has been applied to HIV diagnosis. Kwok et al. (475) demonstrated that PCR could be used to detect HIV DNA sequences in established infected cell lines, in cells cultured from infected individuals, and in semen mononuclear cells and supernatants from AIDS patients. It was later reported that PCR could be used to detect HIV DNA in peripheral blood mononuclear cells of seropositive, but not seronegative patients (476). A reverse transcription step has been incorporated into the technique to allow for the detection of HIV RNA as well as DNA, thus enabling the detection of free virus in blood (477,478).

PCR in Cancer Research

Several cancers have been associated with RNA or DNA tumor viruses. The PCR has been used to detect Epstein-Barr virus, human T-cell leukemia virus, hepatitis B, and human papilloma virus, which are implicated with Burkett's lymphoma and nasopharyngeal carcinoma, adult T-cell leukemia, hepatocellular carcinoma, and cervical carcinoma, respectively (479,471).

The PCR has also been used to amplify regions of known oncogenes or anti-oncogenes for further analysis by sequencing, restriction digest, and allele-specific

hybridization. Such analysis allows for the detection of somatic mutations in tumor tissue or cells and has been used extensively to detect known mutations in the *ras* oncogenes in a variety of cancers (for review see 471).

DNA Fingerprinting with PCR

The PCR has become a valuable tool in forensic and paternity checks. Human DNA contains hypervariable regions called minisatellites which can give an individual 'fingerprint' (480). DNA suitable for fingerprinting analysis can be amplified by PCR from traces of material, such as blood spots on clothing and individual hairs. As such, identification by PCR-based DNA fingerprinting can be used for paternity checks and has even been used as evidence in court (Pennsylvania vs Pestinikis, 1989).

Chapter III

Materials and Methods

A. Materials

Given below is a description of the preparation and storage of reagents and oligonucleotides used in this study.

Reagents

Pregnant mare serum gonadotropin (PMSG; 1780-2570 IU/mg) and human chorionic gonadotropin (hCG; 2570-3861 IU/mg) were obtained from Sigma Chemical Company (St. Louis) and diluted into 0.1% BSA/PBS to a concentration of 250 and 125 IU/mL, respectively. Avian myeloblastosis virus (AMV) reverse transcriptase (7500 U/mL), Taq DNA polymerase (10,000 U/mL), RNasin (5000 U/mL), reverse transcriptase (RT) buffer (10X), Taq polymerase amplification buffer (10X), dATP (100 mM), dCTP (100 mM), dTTP (100 mM), and dGTP (100 mM) were obtained from Promega (Madison, WI) and stored at -80°C. All other chemical reagents used were of fine or molecular grade. Buffer compositions are listed in the Appendix.

Oligonucleotides

Two 19-mer oligonucleotide primers were designed using the published rat and human FSH receptor cDNA sequences (11,341) and synthesized and purified by the University of Tennessee Molecular Biology Resource Facility. Typical primers used in PCR are 18 to 28 nucleotides in length and 50% to 60% G+C in content (481). Primer A (5' GCAAGGTGACAG AGATTCC 3') is homologous to bases 101-119 of the rat FSH receptor cDNA sequence and 100% homologous to the corresponding human cDNA sequence. Primer C (5' GAAGTTCAGA GATTGCCG 3') is complementary to bases 862-844 of the rat FSH receptor cDNA sequence and 95% complementary to the human cDNA sequence possessing a mismatch at base 15 (G/A).

The melting temperature (T_m) of each primer was calculated by the "2°C for A or T and 4°C for G or C" rule outlined by Thein and Wallace (482). Primer A is 53% GC with a T_m of 60°C. Primer C is 47% GC and has a T_m of 56°C. Each primer was diluted in TE buffer, quantitated by spectrophotometric analysis at a wavelength of 260 nm, and 20 uL aliquots diluted in double distilled H₂O to a concentration of 10 picomol/uL and stored at -20°C.

Owing to the observed similarity between the FSH receptor and LH receptor cDNA sequences (11,12), the specificities of the primers were examined relative to the LH receptor cDNA sequence using the 'compare' function of

the GCG genetic analysis software program (483). Primers A and C were each found to be less than 68% homologous to the LH receptor cDNA sequence. The primers were also analyzed against the 'GenEMBL' nucleic acid database using the 'FASTA' function of the GCG genetic analysis program (483). Due to their relatively short length, both primers exhibit a high degree of homology to several reported nucleic acid sequences (see table 2).

B. Methods

Given below is a description of experimental procedures used for the collection and preparation of tissues and for the screening of samples by RT-PCR.

Hormone Priming of Animals and Tissue Collection

Animals were housed and treated with hormones as described by Solano et al. (178). Immature female Sprague-Dawley rats (~25 days old) were bred and housed in the University of Tennessee Animal Facility. Rats were housed at room temperature in a 14 hour light, 10 hour dark cycle and fed rat chow and water ad libitum.

Rats were injected with 50 IU PMSG to stimulate follicular growth and development. This was followed 56 hours later with an injection of 25 IU hCG to supplement

Table 2 - Sequence Homologies to Primers A and C

Primer A	Primer C
Human Na/K ATPase subunit alpha	Human (CA)-n repetitive
Human alpha-1 antichymotrypsin	Drosophila Z600/rev
Mouse T200 glycoprotein	H. glutamyl T-peptidase
Human ribosomal protein S14	Rat RJG-gene for c-jun
Human T-cell receptor V-beta	Human rotavirus
Human transforming protein	D. discoideum myosin Hvy
Tomato chlorophyll a/b-protein	Mouse CF transmembrane
Rat cytochrome P450scc gene	E. coli sppA gene
H. brasiliensis HMGR2 mRNA	Rat P450(1) variant
Viral haemorrhagic septicaemia	Rat cytochrome P450 gene

the endogenous LH surge. The LH-like activity of hCG causes rupture of the mature follicle and release of the oocyte. All injections were administered subcutaneously. Rats (29 total) were then sacrificed by decapitation at various times post PMSG injection including days 1, 2, 3, 6, 10, and 13. Untreated rats were also sacrificed for use as controls.

Uterine horns were removed from each rat, fat trimmed, and stored at -80°C . Ovary, kidney, heart, spleen, pituitary, and hypothalamus were also removed from rats and stored at -80°C .

Human uterine biopsy samples were obtained from Dr. Jeffrey Keenan at the University of Tennessee Medical Center, transported to the lab on dry ice, and stored at -80°C .

RNA Extraction

All glassware used throughout the project were baked for 4 hours at 150°C and autoclaved before use. All plasticware were either autoclaved or obtained in sterilized packaging. All solutions were prepared with diethyl-pyrocabonate (DEPC) treated H_2O .

Total RNA extractions were performed as described by Chomczynski and Sacchi (484). Frozen tissue samples were homogenized on ice in 1 mL solution D using a 15 mL glass

homogenizer [Solution D is a denaturing solution containing 4 M guanidinium isothiocyanate, 25 mM Na citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol]. A stock solution without 2-mercaptoethanol was prepared and stored up to 3 months. The 2-mercaptoethanol was then added to 25 mL of stock solution as needed). The homogenate was then transferred to a 3 mL polypropylene centrifuge tube on ice. Sequentially, 0.1 mL of 2 M sodium acetate (pH 4) and 1.5 mL phenol/chloroform/isoamyl alcohol (Tris saturated) were added to the homogenate, with thorough mixing by vortexing following the addition of each reagent. The final suspension was vortexed for 10 seconds and chilled on ice for 15 minutes. Samples were then centrifuged at 10,000 rpm for 20 minutes at 4°C. The top aqueous phase contained the RNA and was transferred to a clean 3 mL polypropylene tube. The RNA was then precipitated by the addition of 1 mL isopropanol followed by incubation at -20°C for 2 hours. The suspension was centrifuged at 10,000 rpm for 20 minutes at 4°C and the resulting pellet resuspended in 0.3 mL solution D using a plastic disposable pasteur pipette. The suspension was then transferred to a 1.5 mL sterile microfuge tube and the RNA precipitated by the addition of 0.3 mL isopropanol followed by incubation at -20°C for 1.5 hours. The precipitated RNA was pelleted by centrifugation in an Eppendorf centrifuge at 12,000 rpm for 10 minutes. The RNA pellet was washed with 65 μ L 75% ethanol, vacuum

dried for 15 minutes, and dissolved in 75 μ L DEPC-treated H_2O .

Total cellular RNA was extracted from ovary, uterus, liver, kidney, hypothalamus, and heart. Initial attempts at RNA extraction were unsuccessful most likely due to RNase contamination of equipment and the inability to homogenize tissues rapidly and effectively. Precautions were taken to decrease RNase contamination (i.e. baking the homogenizer between each extraction) and tissues were homogenized while frozen. As a result, later extractions were successful. Homogenization of uterine and heart tissue remained difficult, however, most likely due to their high muscle and connective tissue content.

Assessment of RNA Quality

Extracted RNA was quantified by spectrophotometric absorbance analysis (Abs) at 260 nm and the relative purity of the RNA determined by its Abs_{260}/Abs_{280} ratio with a value of 1.7-1.8 considered ideal. Absorbance ratios between 1.5 and 1.8 were consistently obtained. Integrity of extracted RNA was determined by formaldehyde/agarose gel electrophoresis. A 1% gel was prepared by melting 1 g of agarose into 10 mL 10X MOPS buffer and 85 mL DEPC H_2O . In a fume hood, 5.4 mL of 37% formaldehyde and 6 μ L ethidium bromide (10 mg/mL) were mixed into the solution. The gel

was poured into a plastic gel former and allowed to solidify. Samples consisting of twelve micrograms extracted RNA were loaded into the gel along with 15 uL of RNA gel loading dye.

The gel was then run at 100 V in 1X MOPS buffer for approximately 3 hours. Separated RNA was observed using a UV transilluminator. Eukaryotic RNA contains two abundant rRNA species (28S ~5 kb in length and 18S ~2 kb) which serve as internal molecular weight standards.

Reverse Transcription

Both reverse transcription and PCR amplification were performed as described by Sambrook et al. (485). Reverse transcription constitutes the synthesis of a cDNA strand using an RNA template. Each reverse transcription reaction was carried out in 20 uL mixture containing 2 uL 10X RT buffer, 0.3 uL DEPC H₂O, 3.2 uL 25 mM MgCl₂, 4 uL 5 mM dNTPs, 4 uL primer C (10 pm/uL), .5 uL RNasin, 2 uL AMV RT, and 4 uL RNA (1.25 ug/uL) in a 500 uL polypropylene microfuge tube. A master mix containing all the reagents except RNA was prepared as needed and 16 uL aliquots added to 4 uL (5 ug) RNA samples. The reaction mixture was incubated at 40°C in a water bath for 45 minutes, heated at 95°C for 10 minutes, and then chilled on ice for 15 minutes. Primer C was designed to anneal specifically to

rat FSH receptor mRNA and thus served to prime the synthesis by AMV RT of the complementary cDNA strand.

PCR Amplification of the RT Products

To each heat-treated reverse transcription reaction mixture 80 μ L of PCR mix was added. The PCR mix was prepared by combining 8 μ L 10X PCR buffer, 60.5 μ L DEPC H₂O, 5 μ L 25 mM MgCl₂, 3 μ L primer A (10 pm/ μ L), 3 μ L primer C (10 pm/ μ L), and 0.5 μ L Taq DNA polymerase. To prevent evaporation during thermal cycling, 65 μ L of sterile mineral oil was placed on top of each reaction. Samples were placed in a Perkin/Elmer thermal cycler and 25 PCR cycles performed. Each cycle consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute.

Primers A and C were designed to amplify a 761 bp fragment of the rat FSH receptor cDNA sequence. Presence and size estimation of RT/PCR products were determined by electrophoresis in a 1.5% agarose gel using Hae III cut PhiX-174 DNA as a marker (Phi-X174/HaeIII contains resolvable fragments of 1353, 1078, 872, 603, 310, and 271 bp). Marker fragments and PCR products were observed with a UV transilluminator and photographed using an f-stop of 4.5 and a shutter speed of 1/2 second.

Chapter IV

Results

A. RNA Integrity

Integrity of RNA samples was assessed by formaldehyde-agarose gel electrophoresis. Total eukaryotic RNA contains two abundant rRNA species (28S and 18S) which serve as internal molecular weight standards and signify intact RNA. Following electrophoretic separation of extracted RNA samples, bands of approximately 5 kb and 2 kb, representing the 28S and 18S rRNA species, respectively, were visible in the gels upon UV illumination. Photographing the gels, however, was difficult. The formaldehyde and ethidium bromide in the gels caused each gel to fluoresce intensely over the UV light, thus masking the nucleic acid bands in the photograph. This problem could potentially be avoided by adding ethidium bromide to the RNA samples directly, rather than to the entire gel.

B. Reverse Transcription - Polymerase Chain Reaction

Methods commonly used to detect specific RNA molecules include northern and in situ hybridization and dot or slot blot analysis. However, the difficulty in quantitating the low abundance of FSH receptor mRNA by northern analysis

encountered by Camp et al. (179) suggested that the sensitivity of RT-PCR would be more suitable for detecting FSH receptor mRNA in tissues where it is potentially less abundant.

Initial attempts at RT-PCR were unsuccessful, most likely due to the absence of magnesium ions in the RT and PCR reaction mixtures. Both AMV reverse transcriptase and Taq DNA polymerase require magnesium ions, the concentration of which affects the efficiency of their polymerizing activities (481). A $MgCl_2$ concentration of 4 mM for both the RT and PCR reactions was found to yield successful results. Agarose gel electrophoretic separation of RT-PCR products are shown in Figures 10 and 11. A total of 5 ug of RNA were used in each RT reaction followed by 25 cycles of PCR amplification (denaturation at 95°C, annealing at 55°C, and extension at 72°C, each for 1 minute).

In figure 10 the expected 761 bp product, representing the amplified target sequence of the FSH receptor cDNA, is visible only in the ovarian samples (lanes 2, 4, and 6) and not in uterus (lanes 3, 5, and 7) or liver (lane 8). A 761 bp product was expected in the ovarian samples as ovary has been shown to contain FSH receptor mRNA (7,181) and thus, serves as a positive control. The intensity of the 761 bp band is greatest in the 48 hr post PMSG ovary sample (lane 4). The 761 bp band is least visible in the day 7 post hCG ovary (lane 6). There are unexpected products of 450 and

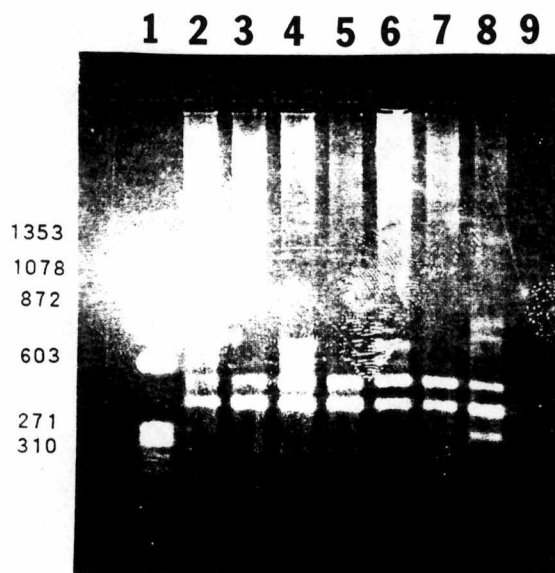


Figure 10 - Reverse Transcription-PCR of Rat Tissues

5 ug RNA of each sample were reverse transcribed and amplified by PCR as outlined in materials and methods. PCR products are shown here as separated by electrophoresis in a 1.5% agarose gel.

<u>Lane</u>	<u>Sample</u>
1	Phi-X174/Hind III DNA marker
2	Ovary 24 hr PMSG
3	Uterus 24 hr PMSG
4	Ovary 48 hr PMSG
5	Uterus 48 hr PMSG
6	Ovary day 7 hCG
7	Uterus day 7 hCG
8	Liver day 7 hCG
9	No RNA

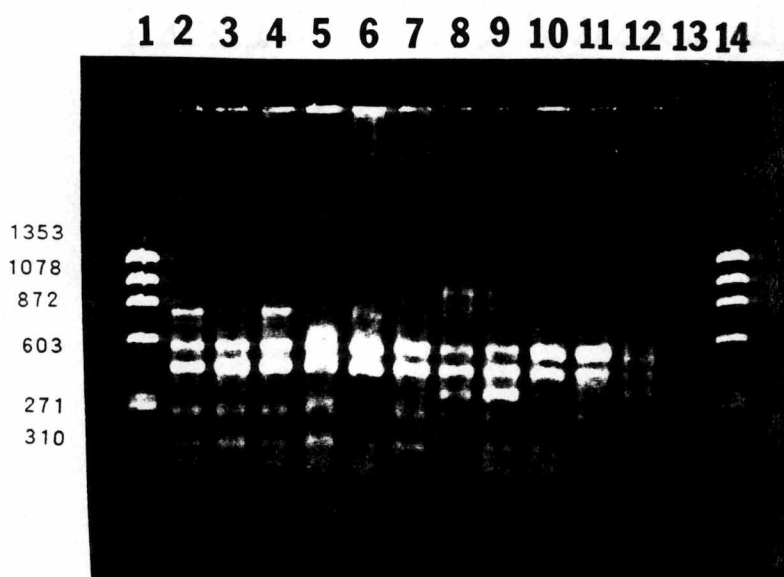


Figure 11 - Reverse Transcription-PCR

5 ug RNA of each sample were reverse transcribed and amplified by PCR as outlined in materials and methods. PCR products are shown here as separated by electrophoresis in a 1.5% agarose gel.

<u>Lane</u>	<u>Sample</u>	<u>Lane</u>	<u>Sample</u>
1	Phi-X174/Hind III	8	Liver day 7 hCG
2	Ovary 24 hr PMSG	9	Liver (ut)
3	Uterus 24 hr PMSG	10	Hypothalamus (ut)
4	Ovary 48 hr PMSG	11	Kidney (ut)
5	Uterus 48 hr PMSG	12	Human Endometrium
6	Ovary day 7 hCG	13	No RNA
7	Uterus day 7 hCG	14	Phi-X174/Hind III

550 bp visible in each tissue sample (lanes 2-8). The identity and source of these bands are unknown, but they most likely represent the RT-PCR amplification of an ubiquitous mRNA with strong sequence homology to primers A and C (see table 2). Alternatively, they could represent alternatively spliced FSH receptor or LH receptor mRNA products. These possibilities are addressed in the Discussion. The liver (lane 8) contains an additional band of approximately 950 bp. The origin of this band is also unknown.

Figure 11 shows the electrophoretic separation of additional RT-PCR products. Lanes 2-8 are essentially identical to figure 10 as they represent ovary and uterus from 24 and 48 hr post PMSG, ovary and uterus 7 days post hCG, and liver on day 7 post hCG. Once again the expected 761 bp FSH receptor cDNA product is present only in the ovary samples (lanes 2, 4, and 6) and not in uterus (lanes 3, 5, and 7), liver (lanes 8 and 9), hypothalamus (lane 10), kidney (lane 11), or human endometrium (lane 12).

Lane 9 corresponds to liver from an untreated immature control rat. Upon comparison, there appears to be no difference between results obtained from liver of treated versus untreated rats. Distinct bands of approximately 950 and 320 bp are visible in both liver samples (lanes 8 and 9). These products appear to be unique to the liver as they are not evident in the other lanes. The 450 and 550

bp bands found in each sample in figure 10 are visible in lanes 2-11 of figure 11 as well. Thus, these additional products are present in ovary, uterus, liver, hypothalamus, and kidney suggesting that they represent amplification of an ubiquitous sequence. Another band of approximately 260 bp is visible in lanes 2-11 as well.

Lane 12 represents a human uterine biopsy sample and does not contain the 761 bp FSH receptor cDNA product. However, the lane does contain three distinct bands, two of which may be equivalent to the 450 and 550 bp products found in lanes 2-11.

Chapter V

Discussion

Many of the functions and regulatory mechanisms related to gonadotropin binding have been known for some time. Still, novel sites of hormone receptor expression are being found (3-6) suggesting that unknown activities of the gonadotropins remain to be discovered.

Recently, several groups have reported the existence of LH/hCG receptors in extraovarian sites of mammalian females, including uterus, placenta, and fetal membranes (3-6). The physiological significance of these ectopic receptors is currently unknown. Changes in receptor concentration, however, appear to be associated with the hormonal status of the animal suggesting a possible involvement in reproductive processes (3).

The induction of LH receptors that occurs during the growth of small antral follicles to preovulatory follicles in the ovary has been shown to be dependent in part on FSH (7-10). The fact that LH receptor expression is induced by FSH in the ovary suggests that FSH, via FSH receptor, may influence LH receptor expression in the uterus as well. FSH itself has recently been reported in such diverse ectopic tissues as prostate (21) and stomach (24). This study was designed to investigate the possible expression of FSH receptor mRNA in uterine tissue by RT-PCR.

The reproductive cycle of the rat lasts 4 days and consists of four stages: proestrus, estrus, metestrus, and diestrus. Serum levels of FSH and LH and expression of their respective receptors during the rat estrous cycle are shown in figure 6. The concentration of uterine LH/hCG receptors in the rat was found to be low or immeasurable in the follicular phase of the estrous cycle and maximal after ovulation (see figure 12).

The PMSG/hCG primed pseudopregnant immature rat utilized in this study is an established model for the study of ovarian function regulation by gonadotropins. The intrinsic FSH and LH activities of PMSG stimulate the granulosa cells of small preantral follicles to grow and differentiate. The subsequent administration of an ovulatory dose of hCG induces the Graffian follicles to ovulate and luteinize. Approximately 8-10 days after PMSG treatment, the ovulated follicles become heavily luteinized and express maximal amounts of LH receptors. Thus, if FSH were to regulate LH receptor expression in the rat uterus, evidence of FSH receptor mRNA would likely be present in uterine samples of day 7 hCG treated rats.

The RT/PCR results in figures 10 and 11, however, indicate that FSH receptor mRNA is not expressed in uterine tissue at any of the times tested during the hormone treatment period. In both figures the 761 bp product, representing the amplified target sequence of FSH receptor

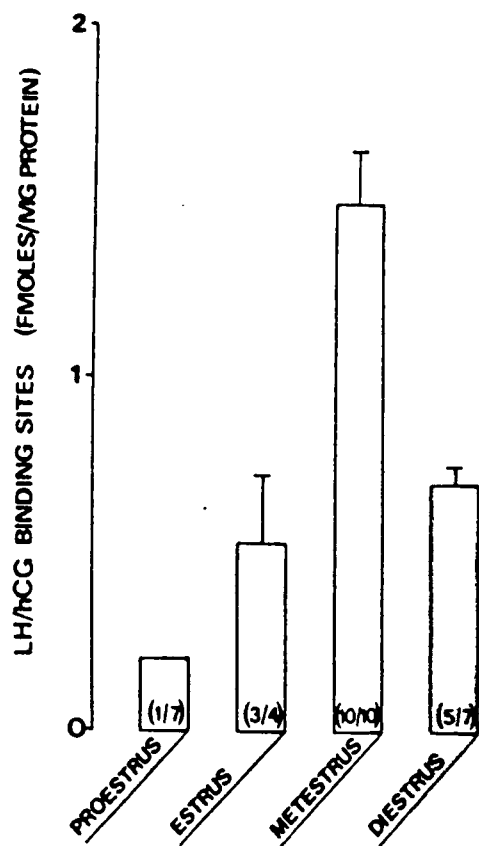


Figure 12 - LH/hCG Binding in Rat Uterus

Concentration of rat uterine LH/hCG receptors throughout the estrous cycle of the rat. Taken from Bonnamy et al. (3).

cDNA, is present only in ovarian samples.

The presence of FSH receptor and FSH receptor mRNA in the ovary is well established (7,9,179). Binding studies using radiolabeled FSH and hybridization studies using recently derived cDNA probes have revealed that FSH receptor protein and mRNA expression is confined to granulosa cells in the ovary (10,179,181) and that receptor and receptor mRNA expression change according to the reproductive cycle (see figure 7).

The absence of FSH receptor mRNA in liver, hypothalamus, and kidney, which served as negative control tissues for the study, was expected and is in agreement with results obtained by others (179,181). LaPolt et al. (181), who examined the regulation of FSH receptor gene expression in the rat ovary, also demonstrated the lack of FSH receptor transcripts in rat hypothalamus, pituitary, lung, liver, and kidney by Northern hybridization.

In figures 10 and 11, the unexpected RT/PCR products of approximately 450 and 550 bp detected in several samples most likely represent non-specific amplification of an ubiquitous sequence with strong homology to the primers used in the RT/PCR. These products were not evident, however, in the human endometrium sample suggesting that the ubiquitous sequence may be species specific. Although the origin of these products is unknown, their presence in each of the samples actually serves as an internal control,

suggesting that failure of the RT-PCR cannot account for the absence of the 761 bp product in the uterine samples.

Non-specific amplification is a potential limitation of any PCR experiment (437). The PCR is so sensitive that a single non-specific molecule with sufficient homology to the primers can be amplified in the PCR so as to yield a product visible upon electrophoretic analysis. Indeed, mutated primers can be used to incorporate mutations into specific amplified products. Only the nucleotides at the 3' end of the primers MUST be homologous to the template. This ensures a properly oriented 3'OH group for the DNA polymerase to initiate polymerization.

Alternatively, the unexpected 450 and 550 bp products may represent the amplification of alternatively spliced FSH receptor mRNAs. The FSH receptor gene was recently cloned and characterized by Heckert et al. (349). Their results showed the FSH receptor gene to be at least 84 kilobase pairs in length and consisting of 10 exons and nine introns (see figure 5). The first nine exons code for the receptor's putative amino-terminal extracellular domain while exon 10, the largest exon, encodes the seven transmembrane domains and the carboxy terminus.

Northern blot analysis of ovarian RNA using a cRNA probe corresponding to bases 670-1031 of the rat FSH receptor cDNA (see figure 3) revealed two predominant hybridization signals of 7.0 and 2.5 kb, as well as minor

signals of 4.2 and 1.8 kb (181). The sequences and intron/exon organization of these transcripts is currently unknown. Interestingly, multiple mRNA transcripts of 7.0, 2.5, 4.2, and 1.8 kb have been identified for LH receptor as well, and these transcripts appear to be coordinatively regulated (8).

Striking similarities between the sequences, intron/exon structures, and mRNA transcript sizes of the pituitary gonadotropin receptors suggest that FSH receptor mRNAs may be coordinatively regulated as well. This presumption and the established tissue specificity observed for FSH receptor expression strongly suggest that the 450 and 550 bp amplification products do not represent alternatively spliced FSH receptor mRNAs.

The results of this study indicate that FSH receptor mRNA is not expressed in uterine tissue of the rat. This finding would suggest that FSH binding to receptor in the uterus is not involved in regulating uterine expression of LH/hCG receptors. The possibility exists that LH/hCG receptor expression is regulated by different mechanisms in the uterus than in the ovary. Several genes have been found to be differentially regulated (487). In addition, the existence of multiple transcriptional start sites, a feature of both the LH and FSH receptor genes, could provide a means for differential regulation. Still, the mechanism of regulation, as well as the physiological

significance of LH/hCG binding sites in the uterus remain unknown at present. Noting the preferential localization of LH/hCG binding sites in myometrium of the pig uterus, Ziecick et al. (4) have postulated a role for LH/hCG binding in regulating myometrium relaxation.

Bonnamy et al. (3) have speculated that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) could be involved in regulating the depletion of uterine LH/hCG receptor binding sites observed at the end of the estrous cycle. They noted that the decline of uterine LH/hCG receptor concentration in the rat occurs at a stage of the estrous cycle when uterine $PGF_{2\alpha}$ content has been found to increase (3,487). In addition, in vivo injection of $PGF_{2\alpha}$ has been previously shown to decrease the concentration of luteal LH/hCG binding sites (488).

Further studies are needed to determine the physiological significance and regulatory mechanisms of uterine LH/hCG receptors. With the recent elucidation and characterization of both the FSH receptor and LH receptor genes (349,489), interesting studies concerning molecular aspects of gonadotropin binding and receptor expression should be forthcoming.

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APPENDIX

APPENDIX

1. 0.1% DEPC H₂O: .5 g di-ethyl pyrocarbonate
Bring to 500 mL with DDI H₂O
Let sit 12 hours then autoclave
2. 5 mM dNTPs: Combine 10 uL of each nucleotide (ATGC)
from 100 mM stock
Bring to 200 uL with 160 uL DDI H₂O
3. Gel Dye: 50% glycerol
.05% bromophenol blue
1X TEA buffer
4. 10X MOPS buffer: .2M MOPS (3-[N-morpholino]propane-
sulfonic acid)
.05 M sodium acetate
.01 M EDTA
5. 10X Reverse Transcriptase buffer: 100 mM Tris-HCl
(pH8.8)
500 mM KCl
1% Triton X-100
6. RNA Gel Dye: .72 mL deionized formamide
.16 mL 10X MOPS buffer
.26 mL formaldehyde (37%)
.18 mL DEPC H₂O
.10 mL 80% glycerol
.08 mL bromophenol blue (saturated)
7. Solution D: 4 M guanidinium isothiocyanate
25 mM sodium citrate (pH 7)
0.5% sarcosyl
0.1 M 2-mercaptoethanol
8. Taq Polymerase PCR buffer: 100 mM Tris-HCl (pH 9)
500 mM KCl
1% Triton X-100
9. 25X TEA buffer: 40 mM Tris (pH 7.6)
20 mM sodium acetate
2 mM EDTA
10. TE buffer: 10 mM Tris-Cl
1 mM EDTA (pH 8)

VITA

David Andrew Wollert was born on May 16, 1968 in Louisville, Kentucky. He graduated from Ballard High School in May 1986 and enrolled at the University of Louisville in the fall. During his undergraduate education David spent three semesters at Washington University in St. Louis and graduated from the University of Louisville with a B.A. in biology in May of 1990. He received a Science Alliance Fellowship from the University of Tennessee for the following summer and accepted a Teaching Assistantship from UT to support his work in graduate school. David began his graduate education at UT in the fall of 1990 and received the Master of Science degree with a major in Life Sciences in August of 1992.