CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT FELINE

THYROTROPIN (fTSH): EFFECT OF GLYCOSYLATION ON IMMUNOREACTIVITY

AND BIOACTIVITY

by

SRUJANA RAYALAM

(Under the Direction of Duncan C. Ferguson)

ABSTRACT

The genes encoding the feline common glycoprotein α (CGA) and feline thyrotropin (fTSH) β

subunits were cloned and sequenced. The feline CGA gene encodes a 96 amino acid peptide and

fTSHβ sequence encodes a 138 amino acid peptide. A FLAG tag was added to the 3' end of the

CGA gene to facilitate detection and purification. A single chain analogue of fTSH termed yoked

fTSH (yfTSH) was developed by fusing C-terminus of the β subunit using a yoking peptide, CTP

to the N-terminus of the α-subunit. Expression levels of 1 µg/ml were achieved for both

heterodimeric and yoked fTSH forms in modified HEK293 (PEAK^{TM}) cells. The glycoproteins

were purified in one step using anti-FLAG immunoaffinity column chromatography to high

purity. Both heterodimeric and yoked glycoproteins were recognized with 40% detection by both

commercial canine TSH immunoassay and an in-house canine TSH ELISA. The heterodimeric

and yoked fTSH behaved immunologically parallel with the pituitary - source canine TSH in the

in-house ELISA. The heterodimeric and yoked forms of fTSH were 12.5 and 3.4 % as potent as

pituitary source bovine TSH at displacing ^{125}I-bTSH and 45 and 24 % as potent in stimulating adenylate cyclase activity in human TSH receptor-expressing JP09 cells. Also, a reduced maximal effect at maximal concentration (Emax) suggests the possibility of the recombinant peptides acting as partial agonists of the human TSH receptor. For expression of fTSH in baculovirus expression system, the signal peptide in both the subunits was replaced with the honey bee mellitin signal sequence and the intron in the fTSH beta "mini gene" construct was removed with an over-lap PCR. The expression levels as determined by immunoreactivity were 35 ± 15 ng/ml. Since purification of large quantities of recombinant fTSH for standardization by protein assay was not successful, as an alternative, enzymatically desialylated fTSH expressed in PEAK^{TM} cells was prepared and used to characterize the bioactivity. Both the insect cell-expressed and desialylated fTSH behaved immunologically parallel to pituitary-source canine TSH. No significant change in the cAMP production and binding affinity were observed with desialylation. However, the biological to immunological ratio for cAMP production increased significantly with desialylation.

INDEX WORDS: thyrotropin; TSH; feline; pituitary; sequence; expression; glycosylation; bioactivity

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DEDICATION

This thesis does not exist without the love and support of
my beloved husband Dhanunjay.

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INTRODUCTION

Thyrotropin or thyroid stimulating hormone (TSH), a 28 kilodaltons (kD) glycoprotein synthesized and secreted from anterior pituitary gland, is the primary regulator of the function and growth of thyroid gland. It is chemically and structurally similar to the gonadotropic hormones, luteinizing hormone (LH), follicle stimulating hormone (FSH), and chorionic gonadotropin (CG). Each of these hormones contain two noncovalently linked dissimilar polypeptide chains, α and β. Within the same species, the α-subunit is identical and commonly shared among all the four glycoprotein hormones, and it is the β-subunit that confers the immunological and biological specificity (Pierce and Parsons, 1981).

A number of studies have been reported on the molecular biology, biosynthesis, structure-function relationships, mechanism of action and bioactivity of thyrotropin that have helped to understand regulation of the hypothalamic-pituitary-thyroid axis. During the past-decade, molecular biology has been a powerful tool for the study of various aspects of the pathogenesis of thyroid disease. The genes encoding the α and β subunits of thyrotropin from various species as well as thyrotropin receptor from several species have been cloned and expressed (Erwin et al, 1983; Godine et al., 1982; Chin et al., 1981; O'Brien and Headon, 1995; Yang et al., 2000a; Hayashizaki et al., 1985; Maurer et al., 1984; Wolf et al., 1987; Croyle et al., 1986; Yang et al., 2000b; Parmentier et al., 1987; Nguyen et al., 2002).

Hyperthyroidism is one of the most commonly diagnosed thyroid disorders in middle to old aged cats. Over the past 20 years the prevalence of feline hyperthyroidism per veterinary clinic visit according to a recent study was 2.1 % (Edinboro et al., 2004). Furthermore,

epizootiological studies have suggested links to dietary and/or environmental associations with this disease not recognized prior to 1979. Canned cat food, cat litter, and pesticides have been identified as possible risk factors for the disease (Martin, 2000). A diagnostic tool that can sensitively detect changes in thyroid status is essential to understand the underlying thyroid pathology and also to diagnose hyperthyroidism at an early stage. Currently available ultra-sensitive human TSH assays eliminate the expensive and time consuming thyrotropin releasing hormone (TRH) stimulation tests by distinguishing euthyroid patients from hyperthyroid patients (Klee, 1987; Ehrmann, 1989). There is no commercially available source for fTSH and biochemical purification of pituitary-source TSH is complicated by the presence of much higher concentrations of LH and FSH, and only about 100 µg of TSH even in a human pituitary. Therefore, we reasoned that recombinant fTSH engineered with an immunoaffinity tag would advance the development of a recombinant peptide-based immunoassay by providing a pure standard for the glycohormones.

A commercially available canine TSH immunoassay (Williams et al., 1996)) has been evaluated for detection of feline TSH (fTSH). Although 68% of hyperthyroid cats had TSH concentrations below the detection limit, the assay was not sensitive enough to distinguish normal from low values (Graham et al., 2000). Therefore, feline-specific peptide reagents and antibodies are necessary for a clinically useful immunoassay. Measurement of endogenous fTSH would allow diagnosis of early hyperthyroidism where TSH levels are suppressed by a hyperfunctioning thyroid gland. Also, a valid feline TSH assay would help characterize chemicals which might directly or indirectly influence feline thyroid physiology, potentially leading to hyperthyroidism. Since a commercially available pituitary source of fTSH does not

exist, the approach was taken to clone sequence and express recombinant fTSH which would then allow development of a feline specific TSH immunoassay.

Ultrasensitive immunoassays, which generally utilize monoclonal antibodies to capture the glycopeptide, reveal that glycosylation of plasma hormones is immunologically distinct from pituitary stock because the ratio of circulating glycoforms appears to vary according to the pathophysiology of the pituitary axis (Zerfaoui et al., 1996). Posttranslational glycosylation produces extensive microheterogeneity of the glycopeptide with each glycovariant having a different circulatory halflife and therefore bioactivity. The oligosaccharides have also been shown to play a role in the proper folding, assembly, secretion, metabolic clearance, and the bioactivity of TSH (Magner, 1990). The degree of sialylation varies between pituitary and circulating isoforms and has been shown to influence the recognition by anti-human TSHβ antibodies (Schaaf 1997). Therefore, the nature of glycosylation patterns of TSH plays an important role in developing ultra sensitive immunoassays. Recombinant hTSH has been approved by the FDA for diagnostic use in patients to increase thyroidal isotope uptake in patients with thyroid cancer. Likewise, recombinant fTSH either in heterodimeric form or in yoked form, may have potential as a pharmaceutical designed to increase the efficiency of uptake of radioiodide by thyroid tissue in cats suffering from multinodular goiter or thyroid carcinoma.

HYPOTHESIS:

The glycosylation pattern influences not only immunological detection but also biological binding and signal transduction of recombinant feline thyrotropin.

REVIEW OF LITERATURE

1. Structure and biochemistry of TSH:

Thyrotropin (thyroid-stimulating hormone; TSH), a glycoprotein hormone is produced in the anterior lobe of the pituitary upon stimulation by hypothalamic peptide, thyrotropin releasing hormone (TRH) and is inhibited by thyroid hormones, somatostatin and dopamine (Chin et al., 1993). TSH directs the production of thyroid hormones (T3 and T4) in the thyroid gland and in turn is negatively regulated by T3 at the transcriptional level (Chin et al., 1993). Thyroid hormones are necessary for maintaining body metabolism.

TSH belongs to glycoprotein hormone family along with luteinizing hormone (LH), follicle stimulating hormone (FSH) and placental chorionic gonadotropin (CG). These glycoproteins are heterodimers with noncovalently linked α and β subunits. The α-subunit is commonly shared among all the pituitary glycoprotein hormones; however the carbohydrate structure may vary (Nilson et al., 1986). The α-subunit is produced in excess of the β-subunit in the anterior pituitary thyrotrophs (Magner, 1990). The β-subunit is different and confers the immunological and biological specificity to each hormone. The synthesis of β-subunit appears to be the rate-limiting step for TSH assembly (Taylor et al., 1995) and the alpha and beta subunit assembly is necessary for full biological activity of the hormone (Taylor et al., 1995). Regions of β-subunits of glycoprotein hormones which interact with the common α-subunit are homologous. The alpha and beta subunits are synthesized from separate mRNAs coded by DNA from genes on separate chromosomes that may differ among species (Pierce and Parsons, 1981). Thyrotropin is highly glycosylated with N-linked complex carbohydrates, which account for approximately

21% and 12% of the total weight in the α and β chains, respectively, and are very important in maintaining proper folding and bioactivity (Magner, 1990; Gesundheit et al., 1986).

The molecular weight of mammalian TSH can range from 28 to 30 kD, with the variation being associated with heterogeneity of the oligosaccharide chains. The α subunit has a molecular weight ranging from 20-22 kD, again due to differences in glycosylation, and consists of a polypeptide chain of 92 amino acids in human and 96 residues in other mammalian species (Gharib, 1990). The beta subunit has a molecular weight ranging from 15-18 kD and has one asparagine-linked oligosaccharide unit.

Crystal structure: The crystal structure of TSH has not yet been elucidated but the crystal structure of human CG (hCG) and other members of glycoprotein hormone family have been reported (Lapthorn et al, 1994; Wu et al., 1994). The crystal structure of hCG has revealed that each subunit contains a central cysteine knot and three loops, two β-hairpin loops (L1 and L3) on one side of cysteine knot, and a long loop (L2) on the other side (Fig. 1). The long loop in the α-subunit (L2) has a two turn α-helix. Because of the presence of the cysteine knot, TSH is also classified as a member of cysteine knot growth factor (CKGF) superfamily. The similarity of the folds of the α and β subunits is best visualized by superpositioning the subunits. The α and β subunits are aligned reciprocally at the interface and are associated with each other with a minimal hydrophobic core. Unlike other CKGFs that exist as homo- or heterodimers with interchain disulfide bridges, the glycoprotein hormones have noncovalently linked α and β subunits stabilized by a segment of the β-subunit termed "seat-belt," as it wraps around both the subunits maintaining the tertiary structure. This seat belt arrangement has implications for folding pathways during heterodimer formation as this process is governed by the precise order of disulfide formation (Lapthorn et al., 1994).

The cysteine residues that are involved in disulfide bond formation are critical in stabilizing the tertiary structure. The α-subunit has five disulfide bonds, while the β-subunit has six. All of the disulfide bonds are highly conserved in all the species and there is no free sulfhydryl group in either subunit (Ryan et al., 1988). The β93-100 loop is of primary importance in the expression of hormone specificity (Word and Moore, 1979; Puett et al., 1994). Site-directed mutagenesis of either Cys93 or Cys100 residues in hCGβ yields mutant forms incapable of associating with the α-subunit (Suganuma et al., 1989). Similarly residues β34-38 are important for dimer formation and mutation in this region results in a β-subunit that cannot form a dimer with the α-subunit. Likewise, the inter-cysteine loop β38-57 sequence is also important for dimerization and receptor binding (Weiss et al., 1992).

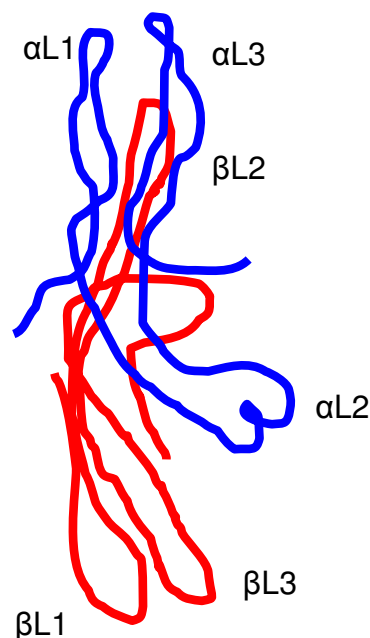Fig 1. Schematic drawing of hTSH showing α-subunit back bone in black and β-subunit chain is shown in grey. The peripheral β-hairpin loops are marked as follows: α L1 and α L3 in α-subunit; β L1 and β L3 in β-subunit. Two long loops are marked as α L2 with α-helical structure and β L2, long loop of β-subunit that is analogous to the "Keutmann loop" in the hCG β-subunit (Adapted from Szkudlinski et al., 2002).

Several regions in both subunits have been recognized to be involved in the modulation of TSH and gonadotropin functions. In the α-subunit, the α11-20 region with a cluster of basic amino acids present in all vertebrates except apes and humans (Fig. 2), has been recognized as an important motif in the evolution of TSH and gonadotropin bioactivity in primates (Szkudlinski et al., 1996). These authors also provided the first evidence that selective alterations of residues in loop domains to charged residues may permit design of analogs with increased bioactivity. The elimination of basic residues in the α L1 loop resulted in a decrease of intrinsic activity of TSH and coincided with the divergence of apes from Old World monkeys (Fig. 2). Furthermore, the presence of basic amino acids in the β-subunit sequence of L3 loop modulated the intrinsic bioactivity of TSH and gonadotropins (Grossmann et al., 1998).
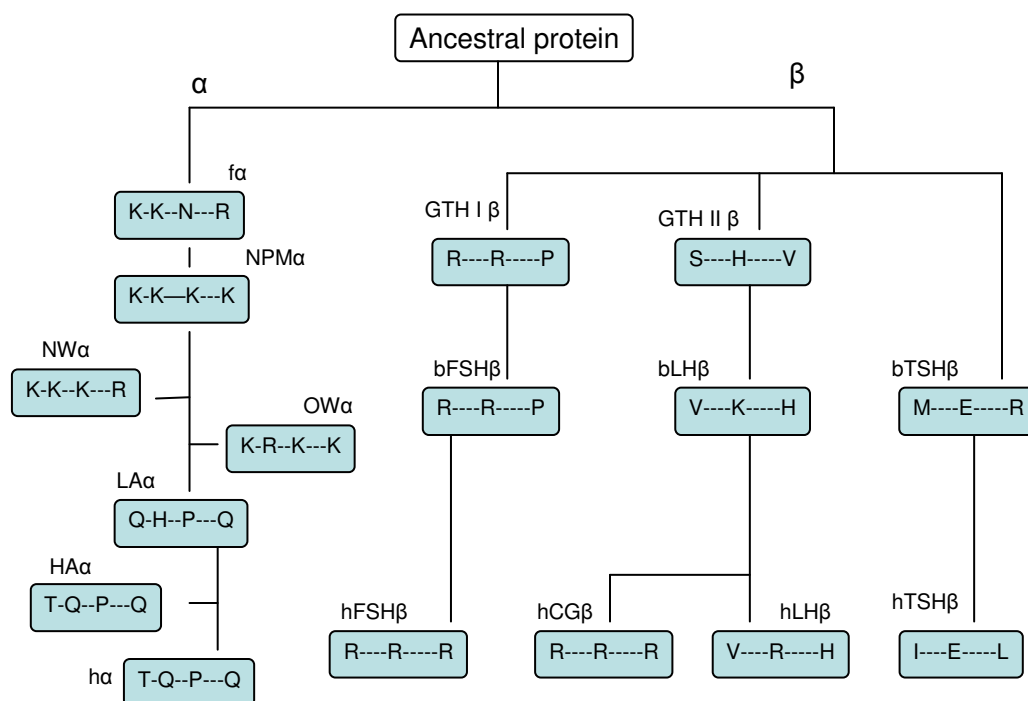

Fig.2. Sequence evolution of glycoprotein hormones. Two basic motifs in the loop 1 of α-subunit and loop 3 of β-subunit are shown. The TSHβ L3 motif resulted from a progressive substitution of basic amino acid residues (R----R-----R) with acidic or hydrophobic residues. In the loop 1 of α-subunit the substitution of basic amino acids (K-K--K---K) is seen late in the evolution. GTH,

gonadotropin; NPM, non primate mammals; NW, New World monkeys; OW, Old World monkeys; LA, Lower Apes; HA, Higher Apes; h, human; f, fish (Adapted from Grossmann et al., 1998).

Modeling of hTSH: Computer-aided modeling was attempted initially to predict a model for three-dimensional structure for hTSH and, subsequently, secondary structure prediction was made by computing the primary sequence in terms of flexibility, hydrophobicity and solvent exposure (Ronin et al., 1990; Delege et al., 1988). According to this model, the surface of interaction is made of two anti-parallel strands of β - pleated sheet running parallel to the α-helix in the β-subunit and the N- and C- termini of the α-subunit (Ronin et al., 1990). Recently, comparative modeling based on the crystal structures of hCG and hFSH has allowed construction of more complete and accurate models for human, porcine and bovine TSH (Miguel et al., 2004). These authors also reported that hTSHα chains interact with many amino acids on the Leucine Rich Domains (LRD) surface and Cleavage Domain (CD) surface of human thyrotropin receptor and the β chains do not interact with CD. Moreover, the observed higher affinity of bovine thyrotropin and porcine thyrotropin relative to hTSH for the hTSH receptor has been explained in terms of charge-charge interactions between the α chains and the receptor.

Common α subunit gene: The cDNA of the human α subunit was first cloned and reported in 1979 (Fiddes and Goodman., 1979). The common α subunit gene has subsequently been cloned from a variety of species including cow (Erwin et al, 1983), rat (Godine et al., 1982), mouse (Chin et al., 1981), horse (O'Brien M and Headon, 1995), sheep (Bello et al., 1989), pig (Rettenberger et al., 1995) and dog (Yang et al., 2000b). The gene for the α subunit has been localized to the long arm of chromosome 6 in man and chromosome 4 in the mouse and these genes are 13.7 kb (cow), 9.4 kb (man) and 7.7 kb (rat) in length. Comparison of the cDNA sequences of various species shows significant homology varying form 76 % between man and

rat to 96 % between rat and mouse (Burnside et al., 1988). The common α subunit gene in all species contains four exons and three introns, and the coding sequences span from the second exon to part of exon four. Gene mapping studies of pituitary RNA revealed a single transcription start site downstream of the TATA boxes (Wondisford et al., 1991). However, multiple regulatory elements for various hormonal and physiologic effectors can be anticipated as the α subunit is expressed in both gonadotrophs and thyrotrophs.

A cyclic AMP-response element, containing the core 8-base motif T(G or T)ACGTCA which is required for the induction of cAMP gene transcription, is present between residues 146 and 128 in the α subunit gene. The mRNAs for the common α subunits are about 800 bp in size and encode a translational product of 14 kD in molecular weight containing 116-120 residues. A 24 amino acid signal peptide precedes a 92-96 amino acid apoprotein (Fiddes and Goodman, 1979; 1981).

TSHβ subunit gene: The gene encoding TSHβ subunit has been cloned and sequenced in man (Hayashizaki et al., 1985), cattle (Maurer et al., 1984), mouse (Wolf et al., 1987), rat (Croyle et al., 1986), chicken (Gregory and Porter, 1997), sheep (Bockmann et al, 1997), rabbit (Mathee et al., 2004) and dog (Yang et al., 2000a). There are three exons and two introns in the beta subunit gene. The first is only 37 bp and untranslated followed by a 3.9 kilo base intron. The function of the first exon is unclear; however it has been speculated that exon I may interact directly with thyroid hormone and its receptor and down regulate the TSHβ gene (Wondisford et al., 1988). The second exon encodes the signal peptide and first 34 amino acids of the mature TSHβ peptide; the third exon contains the remaining coding region and 3' untranslated sequences. Unlike rat and mouse β genes that contain two transcription sites due to alteration in of the distal 5' TATA box, human TSHβ contains only one (Samuels et al., 1989).

The cyclic AMP response elements are present at the 5'- flanking region of human, rat and mouse genes and contain more than 30 nucleotides with a highly conserved core sequence, ATGACGTCAG. These regions are likely to be used for cAMP regulation (Tatsumi et al., 1988). The mRNA for TSHβ is approximately 700 bases in size and encodes a protein of 138 amino acids with 20 residues constituting signal peptide and 118 residues forming the β-subunit apoprotein (Hayashizaki et al., 1985; Whitfield et al., 1986; Wondisford et al., 1988).

Single chain or yoked analogs of glycoprotein hormones: The functional activity of TSH depends on the correct assembly of the subunits into heterodimers. The noncovalent association of the subunits is an obligatory step for the formation of biologically active hormone (3). A novel approach, namely, the construction of single chain analogs has been proven to be a promising strategy for structure-function studies and in generation of hormones with increased stability and activity (Narayan et al., 1995; Sugahara et al., 1996; Grossmann et al., 1997). Grossmann, et al, showed that the genetic fusion of hTSH α- and β-subunits using the carboxy-terminal peptide of the hCG β-subunit as a linker created a yoked form of hTSH whose receptor binding and bioactivity were comparable to native hTSH, but had higher thermostability and a longer plasma half-life (Grossmann et al., 1997).

Several features of CTP make it an ideal linker. It is rich in serine/proline content and thus lacks any secondary structure (Puett et al., 1998). At the same time CTP also provides sufficient distance and flexibility to facilitate the proper folding and interaction between the α- and β-subunits. The crystal structure of hCG suggested the flexible nature of CTP wherein the aminoacid residues 112-145, comprising entire length of CTP could not be traced (Lapthorn et al., 1994). Further, site directed mutagenesis studies have shown that the CTP is not required for subunit association or receptor binding, whereas the N-terminus of the β-subunit and C-terminus

of α-subunit appear to be important in receptor binding (Keutmann, 1992; Chen et al., 1992). The CTP contains four O-linked glycosylation sites which play and important in increasing the circulatory half life of the hCG (Matzuk et al., 1990).

Functional yoked hCG has been successfully expressed in both insect cells and Chinese Hamster Ovary (CHO) cells (Narayan et al., 1995; Sugahara et al., 1995). It was reported that yoked hormones are as effective as the heterodimer in receptor binding and activation (Narayan et al., 1995). Fundamentally for the purpose of the present study, this approach also ensures equimolar expression of the subunits and simultaneous affinity labeling of a single recombinant peptide rather than a heterodimer.

2. TSH Receptor (TSHR):

The function of TSH is mediated by binding of TSH to its specific membrane receptors and activation of signal transduction pathways. Activation of second messenger systems results in a large variety of metabolic consequences like iodide uptake and release, thyroid peroxidase generation, thyroid cell growth, thyroid hormone synthesis and release (Dumont et al., 1992). TSH receptors are located on the basal membrane of thyroid follicle cells. Due to the clinical significance of TSHR autoantibodies involved in the etiology of Graves' disease and its associated hyperthyroidism, the TSHR has been the focus of interest for past 20 years.

The human TSHR was first cloned by Parmentier et al. (1987). The feline TSHR (fTSHR) was cloned in 2002 (Nguyen et al., 2002). Species comparison revealed that the feline TSHR is closely related to the canine TSHR with 96% identity and 97% similarity in amino acid sequence. The TSHR is a single chain glycoprotein containing 744 amino acids with a molecular weight of about 100 kD. It consists of a 398-residue NH_2-terminal large putative extracellular domain, which contains six glycosylation sites and three disulfide binds followed by 346 amino

acids, comprising seven putative transmembrane segments (Libert et al., 1989; Misrahi et al., 1990). The TSHR has been studied in a variety of *in vitro* and *in vivo* systems and has been partially purified and characterized biochemically (Rees Smith et al., 1987).

The TSHR belongs to the G- protein coupled receptor (GPCR) super family having seven transmembrane domains, with similarities to receptors for LH/CG and FSH. The extracellular domain of the TSHR and the LH/CG receptors are much larger and more complex than the short extracellular domains recognized by the smaller ligands such as adrenergic and cholinergic agents. The TSHR has an additional 64 residues which are involved in autoantibody generation, in the N- and C- terminus of extracellular domain that are homologous with LH/CG receptors (Hidaka et al., 1993). However, the TSHR and gonadotropin receptor are both examples of single receptors which can couple to both the cAMP and phosphoinositide signaling cascades (Vassart and Dumont, 1992).

Together with newly discovered receptors (Nishi et al., 2000), TSHR, LHR and FSHR are now classified as leucine-rich repeats (LRR)-containing G-protein coupled receptors. Different modeling studies suggested 6-9 LRRs, each repeat containing a β - pleated sheet oriented towards the interior circumference of the horseshoe-like tertiary structure, which binds to the hormone molecule (Szkudlinski et al., 2002). TSHR is unique among glycoprotein hormone receptors in that some mature receptors on the cell surface are cleaved into two subunits (Rapoport et al., 1998; Rees Smith et at., 1988). Posttranslational modifications of TSHR include glycosylation of the six asparagine residues.

The extracellular domain of the TSHR is involved in TSH binding and signal transduction (Nagayama et al., 1992), and has been the target of a variety of autoantibodies. The binding of autoantibodies to the receptor either stimulates or inhibits thyroid cell function and/or

growth, and may have serious physiological consequences. In Graves' disease, the autoantibody is stimulatory, and in Hashimoto's disease, the autoantibody inhibits the binding of TSH to its receptor thus resulting in idiopathic atrophy (Zakarija and McKenzie, 1991).

Ligand-receptor cross-reactivity has been investigated for a long time and it was reported that TSH binds to LH/CG receptor and increases cAMP and Inositol phosphate levels (Hidaka et al., 1993). Similarly, hCG binds to thyroid plasma membrane and displaces ^{125}I-TSH from its receptor (Azukizawa et al., 1979). Furthermore hCG can activate adenylate cyclase in Chinese Hamster Ovary (CHO) cells stably transfected with the human TSH receptor (Tomer et al., 1992) and increases iodide uptake and 3H-thymidine incorporation in FRTL-5 cells (Hershman et al., 1988).

Second messenger systems:

TSH activates both the adenylate cyclase and the phospholipase C signaling pathways (Field, 1975). Activation of phospholipase C is slower and requires larger quantities of TSH than the activation of adenylate cyclase. Adenylate cyclase mediates most of the TSH's cellular effects. Studies are necessary to evaluate the physiological significance of dual signaling system for the thyrotropin (Cho, 2002).

Adenylate Cyclase-cAMP System: Activation of adenylate cyclase leads to the production of cAMP and cAMP in turn stimulates growth as well as function and differentiation of cultured thyrocytes. It has been shown that constitutive activation of adenylate cyclase is sufficient to promote hyperplasia of thyroid gland and hyperthyroidism (Ledent et al., 1992). Accumulation of cAMP in the cytoplasm stimulates dissociation of regulatory and catalytic subunits of cAMP-dependent protein kinase and subsequent phosphorylation of various cellular proteins. Growth factors like IGF-I increase the sensitivity and maximum response of cyclic AMP to TSH

(Brenner et al., 1989). Catecholamines, which increase cAMP phosphodiesterase activity, are other factors which can modulate the cAMP response (Berman et al., 1987).

The TSHR is associated with an inhibitory guanine nucleotide (G_i) binding protein that exerts inhibitory control over adenylate cyclase activation. Adenosine diphosphate (ADP) ribosylation of G_i binding protein by pertussis toxin abolishes the inhibitory effect of Gi and may be involved in TSH stimulation of adenylate cyclase activity (Corda et al., 1987). Recently, it was reported that TSH receptor signaling via cyclic AMP stabilizes the assembly and retention of E-cadherin at the cell surface suggesting a new mechanism by which TSH supports maintenance of thyroid follicular integrity (Larsson et al., 2004).

Phospholipase C system: Bone et al., (1986) reported that TSH also activates the inositol-lipid signaling pathway. TSH stimulates hydrolysis of phosphotidylinositol-4, 5-biphosphate (PIP_2) by phospholipase C and results in the formation of diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP_3). IP_3 mobilizes intracellular Ca^{2+}, which activates Ca^{2+}- and calmodulin -dependent protein kinase (Ca-CaM). DAG activates protein kinase C (PKC) and both Ca-CaM kinase and PKC phosphorylate numerous proteins in nucleus, plasma membranes and cytosol resulting in cell responses including hormone secretion and gene expression. Interestingly, TSH-induced production of hydrogen peroxide, which is an essential process for iodide organification, a highly differentiated function of thyroid cells, is mediated not by cAMP, but by Ca2+ signaling and phospholipase-A2 activation in FRTL-5 cells (Kimura et al., 1995).

3. Regulation of TSH

TSH subunits are synthesized separately as pre-α and pre-β-subunit precursors. These precursor subunits contain signal peptide needed for intracellular passage. These precursors then undergo post-translational modifications like glycosylation, truncation, sulfation, folding and

then the two subunits associate to form a dimer. The secretion of TSH is controlled by two major factors: feedback effects of the unbound form of thyroid hormones and stimuli emanating from the central nervous system like, thyrotropin releasing hormone (TRH), somatostatin, and dopamine.

Thyroid hormones: An inverse relationship between the circulating thyroid hormone concentrations and secretion of TSH has been well established (Tong, 1974). Thyroid hormones T3 and T4 were recognized as the most important inhibitory factors in the control of TSH secretion. In the serum of most domestic species, all but about 0.1 % of T4 is bound to plasma proteins. Although it is T3 binding to nuclear receptors in the pituitary that governs the response, the circulating concentrations of both T3 and T4 play an important role in the feedback process (Larsen et al., 1981). The main action of thyroid hormones in the control of TSH secretion is to regulate its gene expression. The nuclear receptors are transcriptional regulatory proteins and binding of thyroid hormones activates the receptors. The hormone-receptor complex recognizes and binds to specific sequences of DNA. The DNA-bound receptors influence gene transcription by excluding polymerase and other DNA-binding regulatory proteins by competition. The activated T3 receptor inhibits transcription of both β and α subunit gene expression even in the absence of new protein synthesis (Shupnik et al., 1986). The nuclear receptor-T3 complex binds to the cis-acting negative thyroid hormone responsive elements in the 5'-flank region of the TSHβ gene and to a lesser extent the common α gene (Gurr and Kourides, 1985). This higher affinity binding of T3 to TSHβ gene than TSHα gene may account for the observation that thyroid hormones at different doses decreases TSH β mRNA levels more rapidly than TSH α mRNA levels in the pituitaries of hypothyroid mice (Gurr et al., 1986).

Thyroid hormones are also able to modulate the TRH effects on TSH release by decreasing the number of TRH receptors on the thyrotrophs and stimulating TRH degradation by pituitary enzymes (De Lean et al., 1977; Bauer, 1987). In addition, thyroid hormones also reduce TRH transcription in the paraventricular nuclei also contributing to the regulation of TSH secretion (Koller et al., 1987).

Thyrotropin releasing hormone (TRH): TRH is synthesized in the hypothalamic paraventricular nucleus. It is a tripeptide (pyroglutamyl-histidyl-proline-amide) and is transported to median eminence and released into hypophysial portal blood (Jackson et al., 1985). TRH is a major positive regulator of TSH secretion acting over a range of 10^{-11} to 10^{-7} M (Vale, et al., 1972). In man, the serum TSH levels in response to intravenous administration of TRH are observed within 2 to 5 minutes and peaks at 20 to 30 minutes and returns to basal levels by 2 to 3 hrs (Jackson, 1982).

TRH significantly stimulated TSH glycosylation and subunit assembly (Taylor and Weintraub, 1985). The effects of TRH are mostly post - translational, including alteration of TSH bioactivity and metabolic clearance by influencing the carbohydrate components of TSH. TSH secreted in response to TRH stimulation has a lower sulfate to mannose ratio, and a lower sialic acid to mannose ratio, than does spontaneously secreted TSH (Gesundheit et al., 1986). Presumably, TRH affects the final carbohydrate structure of secreted TSH by activating or inhibiting glycosyltransferases or by altering the intracellular secretory pathways. TRH also enhances the intrinsic bioactivity of TSH (Magner, 1990). In addition, TRH also stimulates the transcriptional activity of the TSH subunit gene three-fold to five-fold in animal models (Shupnik et al., 1990).

TRH binds to specific receptors on the plasma membrane of the pituitary cells (Hinkle and Goh, 1982) which belong to large family of G-protein coupled receptors. TRH action is mediated mainly through activation of phospholipase C (Drummond, 1986) rather than activation of adenylate cyclase (Iriuchijima and Mor, 1986). TRH also induces an immediate and rapid increase in intracellular free calcium which decays rapidly followed by an extended plateau of elevated calcium. This biphasic action correlates with induction of calcium fluxes and secretary activity in pituitary cells (Geras and Gershengorn, 1981).

Somatostatin: Somatostatin, a cyclic neuropeptide with two naturally occurring forms of 14 and 28 amino acids, is secreted from hypothalamic neurons located in the preoptic and suprachiasmatic regions and released in the median eminence. Anti-somatostatin serum administered to rats enhances the release of TSH due to cold or exogenous TRH (Vitger, 1987) supporting the inhibitory role of somatostatin in TSH regulation. Similarly in human, somatostatin reduces the elevated serum TSH levels in primary hypothyroidism, suppresses the serum TSH response to TRH and prevents TSH release after administration of dopamine antagonist (Scanlon, 1991). Somatostatin also decreases intracellular cAMP levels through binding to its specific receptors coupled to an inhibitory guanyl nucleotide-binding protein (Gi), thus reducing TSH gene expression (Ahlquist et al., 1987). Berelowitz et al (1980) have shown that T3, in addition to its direct pituitary effect, also exerts a negative feedback effect on pituitary TSH release through stimulation of somatostatin release from hypothalamus.

Dopamine: Dopamine inhibits the secretion of TSH by acting on its receptors on the thyrotrophs. Shupnik et al., (1986b) showed that dopamine treatment of cultured pituitary cells from hypothyroid rats results in decreased TSH mRNAs and suppressed transcription of both TSH subunit genes and addition of cAMP partially overcomes dopamine suppression of

transcription suggesting that dopamine may decrease the circulating TSH level by decreasing intracellular cAMP levels and thus interfering with cAMP-mediated stimulation of TSH-subunit gene expression.

4. Immunogenicity of TSH:

Within a species, the α-subunit carries similar antigenic determinants which are species specific for all the four glycoprotein hormones, TSH, LH, FSH and CG (Vaitukaitis et al., 1972). The β - subunit possess hormone specific epitopes and gives functional specificity to these hormones (Reichert et al., 1970).

Development of monoclonal antibodies (mAbs) against TSH dimer: A number of studies have been reported on production and characterization of monoclonal antibodies against TSH (Soos and Siddle 182; Soos et al., 1984; Benkirane et al., 1987a; Endo et al., 1989). The number and spatial distribution of antigenic determinants on the surface of TSH molecule was studied in detail using the monoclonal antibodies generated against intact human TSH. Using a similar approach, two- or three- dimensional mappings of epitopes were proposed for human LH (Soos and Siddle, 1983) and human CG (Schwarz et al., 1986). Also, a study of the functional role of epitope site may give important information to elucidate the mechanism of hormone action on the receptor (Hill et al., 1987).

Soos et al., (1987) characterized 23 mAbs against human TSH and postulated four antigenic determinants on TSH. Only one of these epitopes is expressed in the free TSHβ-subunit. Benkirane et al., (1987b) characterized 28 mAbs and postulated that human TSH has at least 12 different antigenic regions, 2 of them on the α -subunit, 6 on the β-subunit either in free form or in heterodimeric conformation and 4 antigenic regions expressed only on holo-hormone.

Endo et al., (1989) studied 18 mAbs generated against hTSH and proposed 11 antigenic determinants on hTSH, 5 each on α and β-subunits and one on the holo-hormone.

The cross-inhibition experiments (Benkirane et al., 1987b) revealed the relationship among the epitopes recognized by different mAbs. Each mAb was tested for its ability to inhibit the binding of radiolabeled TSH to the other mAbs. Epitope identity and proximity was interpreted as complete reciprocal inhibition and absence of inhibition as interaction of pair of mAbs with remote epitopes. Binding of the mAb to epitopes sufficiently close to each other to induce steric hindrance resulted in partial inhibition (Benkirane et al., 1987b).

Development of monoclonal antibodies (mAbs) against β-subunit of TSH: The antibodies generated against the β-subunit of TSH recognized the heterodimer as well as the isolated subunit (Endo et al., 1989). Iijima et al. (1988) raised mAbs against the isolated β-subunit of TSH and found that out of eight clones generated, only one clone was specific for free β-subunit whereas the other clones cross-reacted with the intact hormones. The mAbs generated against recombinant cTSHβ expressed in E. coli had lower affinities as assessed by their half-maximal binding. The affinities were in the range of 130-207 ng/ml for recombinant cTSHβ and 300-850 ng/ml for pituitary source bovine TSH (Yang et al., 2000a). The lower affinity was likely due to the fact that the cTSHβ produced from E.coli is not glycosylated as bacteria lack glycosylation machinery possibly resulting in different antigenic structure from the native TSH (Katakam, 1995). Even though glycosylation has no effect on binding of TSH to its receptor, sugars have been shown to be very important in the antigenic structure and immunogenicity (Thotakura et al., 1992; Sairam et al., 1990).

Antipeptide antibodies: Antipeptide antibodies were generated against various regions of hCGβ subunit (Bidart et al., 1985; Bidart et al., 1990), human LHβ subunit (Troalen et al., 1990) and

the human common α -subunit (Troalen et al., 1988). These antibodies helped in understanding the three-dimensional structure of these hormones. A similar approach was used by Birdart et al. (1991) to obtain the structural information on hTSH. Antibodies generated against the region (1-18) in the hTSHβ subunit bound weakly to native hTSH or hTSHβ indicating that only some residues in this region are accessible to the surface of the hormone. Antibodies generated against the residues hTSHβ (44-59) did not bind to hTSH or hTSHβ subunit indicating that these residues are not accessible to the surface of the hormone. However, the antibodies raised against the COOH- terminal peptide, hTSHβ (85-112), showed a higher binding to the free beta subunit than to the heterodimeric hormone. These antipeptide antibodies, on the other hand, showed higher binding to the denatured β-subunit than to its native form in western blotting (Bidart et al., 1991).

Antipeptide polyclonal antibodies were generated against peptides covering the entire primary sequence of the α and β subunit of hTSH (Julie et al., 1992) and were studies by an ELISA to compare the binding to TSH, free subunits and various glycoprotein hormones. The anti peptide antibodies raised against β(31-51) and β(92-112) showed marked recognition of TSH and anti-β(53-76) antiserum showed equal cross-reactivity with other glycoprotein hormones.

5. Glycosylation:

The oligosaccharides on the glycoprotein hormones have been implicated in several actions including the maintenance of intracellular stability, secretion, assembly, receptor binding, steroidogenesis, and modulation of plasma half-life (Ridgway et al., 1974; Parsons et al., 1983). Sugar residues in carbohydrate chains are commonly linked either to the hydroxyl oxygen of serine, threonine, or hydroxylysine (O-linked) or the amide nitrogen of asparagine (N-linked).

There are two Asn-linked sugar chains in the α-subunit, and the β-subunit has one or two chains depending on the hormone. In human TSH, the two N-linked glycosylation sites are present at asparagine residues 56 and 82 in the α-subunit, while only one glycosylation site is present at asparagine residue 23 in the β-subunit (Pierce and Parsons, 1981). There is an additional O-linked glycosylation site at threonine 43 in the free α-subunits secreted by bovine pituitaries (Parsons, et al., 1983). This additional glycosylation may prevent dimerization with the β-subunit (Pierce, 1986); however, the physiological role of the third oligosaccharide chain is not fully understood.

Glycosylation of TSH: The glycosylation of TSH subunits starts with transfer of precursor oligosaccharides rich in mannose to the nascent peptide. The high mannose precursor oligosaccharides protect nascent TSH subunits from intracellular proteolysis, aggregation and also allow proper chain folding to occur so that the correct subunit tertiary confirmation is attained (Weintraub et al., 1983). Tunicamycin, an antibiotic that blocks the reaction of UDP-GlcNAc and dolichol phosphate in the first step of glycoprotein synthesis, inhibits the formation of all dolichol carrier precursors, resulting in synthesis of nonglycosylated TSH subunits which fail to fold and assemble properly, resulting in intracellular aggregation and proteolytic degradation (Stricklant and Pierce, 1983). These high mannose precursors are later trimmed and other sugar residues were added to make complex-type N-linked oligosaccharides. The transfer of other sugar residues like fucose, β-acetylglucosamine, galactose, N-acetylgalactosamine, sulfate and sialic acid residues is believed to occur principally in the Golgi apparatus (Kornfeld and Kornfeld, 1985) and, subsequently, mature TSH is stored with in the secretory granules (Fig.3).
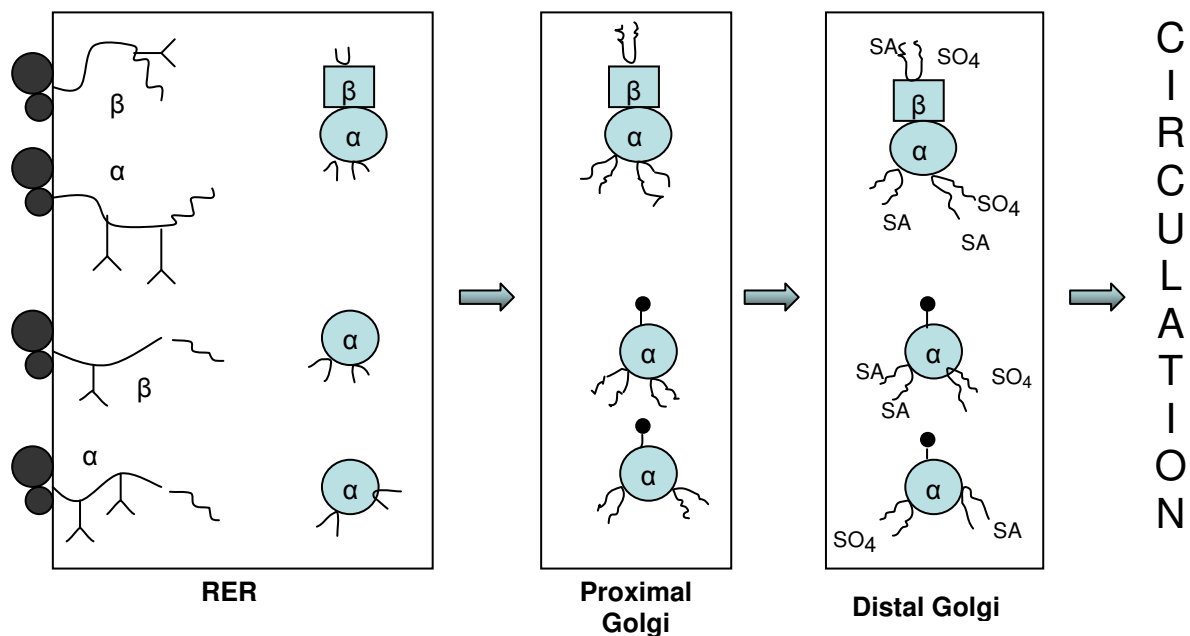
Fig.3. Biosynthesis of TSH: Cotranslational cleavage of signal peptides and glycosylation of asparagine residues present in α and β-subunits take place in Rough Endoplasmic Reticulum (RER). The combination of α and β-subunits begins in RER while subunits still contain high mannose oligosaccharides. In proximal Golgi sugars like galactose and N-acetylgalactosamine are added and free α-subunits are O-glycosylated (solid circles). Sulfate and sialic acid residues are added in distal Golgi and finally fully processed TSH and α-subunits containing heterogeneous oligosaccharides are secreted into circulation.

Glycosylation patterns in recombinant glycoproteins: Recombinant TSH is now successfully being used in clinical studies of thyroid cancer. According to Galway et al., (1990), recombinant human FSH produced by cells defective in sialic acid transport retains normal receptor binding and *in vitro* bioactivity but exhibits minimal *in vivo* activity when compared to wild type FSH, indicating the role of terminal sugars for FSH action *in vivo*. Another study demonstrated that recombinant hFSH produced in human embryonal kidney cells has biological potency 3- to 6-fold higher than pituitary FSH standards (Weintraub, 1990). Joshi et al., (1995) have constructed a longer acting analog of TSH by fusing the carboxy-terminal peptide (CTP) of hCG beta onto TSH beta called chimeric TSH which can be used as a tool to delineate the roles of sulfate and

sialic acid in the in vivo clearance and, thereby, the in vivo bioactivity of TSH. In recombination studies performed by Sairam et al., (1990), LH formed by glycosylated native alpha- and beta-subunits of the hormone was fully active but when one of the subunits was in the deglycosylated form, the receptor binding activity was greatly reduced. These results demonstrate that LH hormone glycosylation is essential for optimum receptor recognition in the sheep testis, further emphasizing the importance of correct glycosylation for ovine LH alpha subunit function. Schaaf et al (1997) showed for the first time for TSH that the two dominant intracellular signal transduction systems (cAMP formation and IP release) are activated to different degrees by hTSH glycosylation variants. A common polymorphism was recently discovered in the gene of the LH beta-subunit containing two point mutations, which introduced to LH two amino acid changes and an extra glycosylation site. The overall bioactivity of this variant is LH variant is lower than that of the wild-type LH. The pathophysiological significance of this LH variant remains open however, it is clear that clinicians monitoring LH levels in their patients have to be aware of this common polymorphic form, which behaves aberrantly in several widely applied immunoassay systems and may give rise to misleading LH levels (Huhtaniemi, 2000).

Glycosylation of glycoproteins in altered health conditions: A TSH bioassay system has been developed using the CHO cell line transfected with the recombinant human TSH receptor. Using this bioassay system, it has been shown that the secretion of TSH molecules with reduced bioactivity is a common alteration in healthy patients. In a study comparing 25 patients with central hypothyroidism, the ratio between biological(B) and immunological(I) activities of circulating TSH was shown to be only 25 % that of normal subjects (Persani et al., 2000). Cyclic AMP generation measured by radioimmunoassay assay is used to quantify the biological response to the glycoproteins in this assay. Perez and Apfelbaum (1997) suggested that

glycosylation is not an essential step in the LH secretory process since the hormone, which is normally secreted in a glycosylated form, was synthesized, transported, and released without the carbohydrate side chains. Hashim et al (1990) reported that, under basal conditions, the majority of prolactin secreted from the pituitary is glycosylated, but with hyperprolactinemia the capacity for glycosylation is exceeded.

Terminal sulfation and sialylation of complex oligosaccharides: The complex oligosaccharides of TSH and LH may terminate in either sulfate or sialic acid residues. Variations in sialic acid content as well as differences in the complexity of the glycans determine the full biological activity of FSH (Creus et al., 2001). Altered physiologic states alter the oligosaccharide structure, which influences the clearance rate and therefore the serum concentrations of TSH. For example, reduced intrinsic TSH bioactivity in pituitary hypothyroidism results from increased sialylation of TSH (Oliveira et al., 2001). Thyroid hormone and developmental factors also regulate the branching pattern and relative sialylation of TSH carbohydrate chains, which may affect TSH action *in vivo*, by modifying hormonal secretion, bioactivity and metabolic clearance (Weintraub et al., 1989).

The structure and distribution of sulfated and sialylated oligosaccharides on human TSH have indicated that 25% of the oligosaccharides contain one sulfate, 21% have one sufate and one sialic acid, 18% are neutral, 12% have two sialic acid residues, and 5 % have one sialic acid residue (Baenziger and Green, 1988). Terminal sialic acid and sulfates are of special importance because both the residues provide a net negative charge to the molecule (Drickamer, 1991). Under *in vitro* conditions, sialic acid appears to the major factor affecting the charge heterogeneity, metabolic clearance rate, and bioactivity of recombinant TSH (Szkundlinski et al., 1993). Removal of the terminal sialic acid residues markedly reduces the half-life of TSH in the

circulation by enhancing its binding to asialohepatic receptors and thus being cleared from the bloodstream (Sairam, 1983).

Extending N-glycosylation pathway: In the process of TSH biosynthesis, after the common intermediate, $Man_8GlcNAc_2$-*N*-Asn is produced, exoglycosidases and a glycosyltransferase catalyze trimming and elongation reactions, which yield, $GlcNAcMan_3GlcNAc_2$-*N*-Asn. In mammalian cells, terminal glycosyltransferases can elongate this intermediate to produce hybrid and complex *N*-glycans with terminal sialic acids. In contrast, insect cells have only extremely low levels of these terminal glycosyltransferase activities, and in some cases, have a competing exoglycosidase that can remove the terminal *N*-acetylglucosamine residue from $GlcNAcMan_3GlcNAc_2$-*N*-Asn. Hence, the major processed *N*-glycan produced by insect cells is usually the paucimannosidic structure, $Man_3GlcNAc_2$-*N*-Asn. Recently, genetically transformed insect cells with mammalian β1,4-galactosylatransferase and α2,6-sialyltransferase genes have been described (Hollister et al., 2001). Stably transformed insect cells with β1,4-galactosyltransferase can be used as modified hosts for conventional baculovirus expression vectors to produce mammalianized glycoprotein glycans which more closely resemble those produced by higher eukaryotes (Hollister et al., 1998). The commercial Mimic™ Sf9 Insect Cell line (Invitrogen, Carlsbad, CA), a derivative of the Sf9 insect cell line, was modified to stably express a variety of mammalian glycosyltransferases. Typically, insect cells are unable to process N-glycans to the extent that mammalian cells do. The addition of mammalian glycosyltransferases like α 2,6-sialyltransferase, β4-galactosyltransferase, N-acetylglucosaminyltransferase I and N-acetylglucosaminyltransferase II to the Mimic™ Sf9 Insect cells allows for production of biantennary, terminally sialyated N-glycans from insect cells (Hollister et al., 2003).

Lectins: Lectins are carbohydrate-binding proteins. Although they were first discovered more than 100 years ago in plants, they are now known to be present throughout nature (including the microbial world, wherein they tend to be called by other names, such as hemagglutinins, adhesins, and toxins). The interaction of lectin with glycans is as specific as enzyme-substrate or antigen-antibody interactions. Lectins may bind with free sugar or with sugar residues of polysaccharides, glycoproteins, or glycolipids which can be free or bound (as in cell membranes). Lectin affinity chromatography with lectins, ricin and Concovanalin A (Con A) has been used to analyze glycoprotein hormones in patients suffering with congenital disorders of glycosylation (Ferrari et al., 2001). Con A permits the separation of molecules differing in the extent of their carbohydrate branching, whereas ricin gives an estimation of the degree of their sialylation. Pituitary TSH was more retained on Con A and less sialylated than circulating hormone, suggesting that the carbohydrate chains of the pituitary form of the hormone are less mature than those present in the circulating TSH form (Papandreou et al., 1993). Glycoproteins applied to the lectin Con A are eluted in three general classes: 1) unbound glycopeptides that have bisecting, triantennary and multiantennary complex structures, with low mannose content, 2) weakly bound glycoproteins that elute with 10 mmol/L α-methylglucopyranoside and have biantennary complex or truncated hybrid oligosaccharides, and 3) firmly bound glycopeptides that elute with 300 mmol/L α-methylmannopyranoside and have high mannose or hybrid oligosaccharides, corresponding to less mature TSH molecules ((Papandreou et al 1993; Baenziger et al., 1979). A Lectin ELISA that uses biotinylated AAL (*Aleuria aurantia* lectin) and a capture antibody specific for AGP was used to study the fucosylation on AGP (α$_1$ Acid Glycoprotein) (Ryden et al., 1999).Thus, lectin ELISA and lectin blotting will provide a qualitative estimate of the sugars present in the glycoproteins.

6. Glycosylation and antigenicity:

The effect of glycosylation on the antigenic structure and biological activity of hTSH was studied by Papandreou et al., in 1990. In a different study it was reported that equine pituitary glycoprotein hormones and bovine TSH are resistant to endoglycosidase action in their native forms (Swedlow et al., 1986; Lee et al., 1987). However, the oligosaccharides on β-subunit in both heterodimeric conformation and free form are easily accessible whereas, the sugars on α-subunit are hidden by subunit interaction as evident from the partial deglycosylation of native hTSH on β-subunit and complete deglycosylation of free α and β-subunits (Ronin et al., 1990).

The capacity of mAbs to bind intact and deglycosylated forms of hTSH was tested by using a panel of 14 mAbs generated against eight out of twelve known epitopes present in human TSH. All the mAbs tested recognized both the native and deglycosylated hTSH identically. In contrast, binding of fully deglycosylated hTSH to anti-β mAbs was lost while the binding to anti-α mAbs was retained. This observation suggests that the epitopes specific for subunit association as well as those present on the β-subunit are glycosylation - dependent. Deglycosylation of free subunits did not alter the recognition indicating that the effect of glycosylation is seen only in dimeric conformation. Also, mAbs raised against recombinant canine TSHβ expressed in E.coli showed lower binding affinity towards pituitary - source bovine TSH likely due to the fact that nonglycosylated recombinant canine TSHβ subunit has a different antigenic structure than the native glycosylated peptide and the antibodies raised against it might be weakly reactive towards the native heterodimeric hormone (Katakam, 1995).

To identify different epitopes contributing to the recognition of deglycosylated hTSH, competitive binding assays with radiolabeled hTSH and free subunits either in their native form or deglycosylated form were done. These experiments revealed that deglycosylated hTSH is five

times less immunoreactive towards anti-β as compared to anti- α antiserum. This finding demonstrates that all the glycosylation-independent epitopes are localized in the α-subunit of hTSH and glycosylation - dependent domains in the hTSHβ subunit (Papandreou et al., 1990).

7. Developments in TSH immunoassays:

TSH synthesis in the anterior pituitary and its secretion into the peripheral circulation are under the positive control by TRH and negative feedback by thyroid hormones. Therefore, when the hypothalamic-pituitary axis is intact, serum TSH levels reflects a direct thyroid hormone action on the pituitary thyrotrophs. On the other hand, the negative feedback of thyroid hormones on the pituitary, designed to counteract thyroid hormone fluctuations and to maintain euthyroidism, amplifies the TSH response. A minor rise or fall of thyroid hormones, particularly of free thyroxine (FT4), elicits about 10 times larger inverse change in pituitary TSH release in man. This makes TSH an extremely sensitive indicator of thyroid status (Bayer, 1991). Measurement of TSH not only replaces time consuming TSH stimulation tests, but a sensitive TSH assay also makes it possible to distinguish euthyroid from hyperthyroid patients eliminating the need for the TRH stimulation test. High sensitivity TSH tests have become the primary test for thyroid function diagnosis (Klee and Hay, 1987).

Strategies to develop TSH immunoassay: The early TSH immunoassay was too insensitive for clinical purposes (Werner et al., 1961) as it was based on cross-reaction of human and bovine TSH on a hemagglutination inhibition technique. Utiger, (1965) reported the first practical radioimmunoassay (RIA) sensitive enough for determining not only the raised but also the normal concentrations of TSH. Even though the TSH RIA was continuously improved by using more delicate methods for purification and iodination of the antigen and various solid phase separation techniques, measurement of subnormal TSH concentrations was still not achieved.

The breakthrough in the measurement in low TSH concentrations came in 1980's with the development of labeled antibody techniques. Helenium and Tikanoja (1986) used monoclonal antibodies to facilitate the development of immunoradiometric assay for TSH.

The specificity of mAbs against hTSH was determined in a liquid phase assay by comparative precipitation of radioiodinated TSH dimer, TSHα and TSHβ - subunits with each mAb (Benkirane et al., 1987a). Some of the mAbs recognized only holo-hormone and none of the subunits, showing that the conformational changes induced by the association of the two subunits modify the expression of the antigenic determinants (Benkrane et al., 1987b).

Non-isotopic TSH immunoassays based on the measurement of enzymatic activities, such as enzyme-linked immunosorbent assay (ELISA), time resolved fluorescence, enhanced luminescence and chemiluminescence have been developed and replaced a large part of the isotopic assays in the 90's (Liewendahl, 1990). However most of the above efforts have been focused on human TSH assays. A 14% difference in amino acid structure of TSH results in lack of cross-reactivity to most human assays (Yang et al., 1996; 2000a; 2000b). There is one canine TSH assay used in veterinary diagnostic laboratories (Williams et al., 1996), but there are no currently available TSH immunoassays specific for cats.

8. Feline hyperthyroidism:

Feline hyperthyroidism was first described in 1979, and since then the occurrence of this disease has reached epidemic proportions. The etiology is yet not clear, but is likely multifactorial (Ferguson and Peterson, 1990). Feline hyperthyroidism clinically resembles toxic nodular goiter in humans (Gerber et al., 1994) and is caused by excessive concentrations of circulating thyroid hormones produced by hyperplastic, benign adenomatous thyroid glands. The number of cats diagnosed for hyperthyroidism has increased markedly over the past 20 years.

Feline hyperthyroidism occurs in middle to old aged cats, with a mean age of approximately 12-13 years (Ferguson and Hoenig, 1991). The common historical and clinical signs recorded in cats with hyperthyroidism include weight loss, polyuria, polyphagia and hyperactivity approaching a prevalence of 75-100% (Hoenig et al., 1982; Peterson et al., 1983). In about 70% of the cases, bilateral thyroid lobe involvement is seen and researchers have postulated that any goitrogenic factor resulting in uncontrolled bilateral thyroid lobe growth is acting in a manner similar to TSH (Studer et al., 1989; 1991). Furthermore, epizootiological studies in cats have suggested that dietary and/or environmental factors like canned cat food, cat litter, and pesticides act as possible risk factors for the disease (Kass et al., 1999; Martin et al., 2000; Williams et al., 1996). However, it has been difficult to establish links between these factors and thyroid pathology as there are not enough diagnostic tools capable of sensitively detecting changes in thyroid status.

A commercially available canine TSH immunoassay (Williams et al., 1996) has been evaluated for detection of feline TSH (fTSH), and shown to be not sensitive enough to distinguish normal from low values (Graham et al., 2000). Therefore, we reasoned that feline -specific peptide reagents and antibodies are likely necessary for a clinically useful immunoassay. Lacking a commercially available pituitary source of fTSH, the approach was taken to clone sequence and express recombinant fTSH which would then allow development of a feline specific TSH immunoassay. Glycosylation of plasma hormones is immunologically distinct from pituitary stock because the ratio of circulating glycoforms appears to vary according to the pathophysiology of the pituitary axis (Zerfaoui et al., 1996), thus playing an important role in developing immunoassays. In the present study, we hypothesized that glycosylation pattern of recombinant feline TSH affects not only its immunoreactivity but also biological binding and signal transduction.

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CLONING AND SEQUENCING OF FELINE THYROTROPIN (fTSH): HETERODIMERIC AND YOKED CONSTRUCTS[1]

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Abstract

The genes encoding the mature common glycoprotein α (CGA) and hormone-specific β subunits of feline thyroid stimulating hormone (fTSH) were cloned and sequenced. The feline CGA gene was cloned from RNA extracted from the feline pituitary gland by the reverse transcription polymerase chain reaction (RT-PCR).The gene fragment that encodes mature TSHβ was cloned from feline genomic DNA after direct polymerase chain reaction (PCR). In both cases, primers were based on the consensus sequences from TSH in other species. The resulting 510 bp PCR product for the CGA subunit included the full coding sequence for the 96 amino acid mature subunit preceded by a 24 amino acid signal peptide. The 850 bp sequence of fTSHβ genomic DNA consisted of two coding exons, an intron of 418 bp, and a 60 bp signal sequence. The octapeptide immunoaffinity tag FLAG was added to 3' end of the α gene to facilitate detection and purification. Both genes were cloned independently downstream from the EF1α promoter of the PEAK™ transfer vector to facilitate co-expression studies in PEAK™ cells (modified human embryonic kidney (HEK) cells). A single chain analogue of fTSH termed yoked fTSH (yfTSH) was developed by fusing the nucleotides encoding the C-terminus of the β-subunit fused to the N-terminus of the α-subunit with DNA encoding the C-terminal peptide (CTP) of human chorionic gonadotropin beta subunit as a linker peptide. The resulting single chain analogue encoded from N-terminus to C-terminus: β-CTP-α-FLAG. The resulting DNA sequence was cloned, sequenced, ligated and recloned into expression vector PEAK™. This report constitutes the first cloning and sequencing of the genes encoding the subunits of feline thyrotropin.

Running Title: Cloning and Sequencing of Feline TSH

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1. Introduction

Thyrotropin (thyroid stimulating hormone, TSH), chorionic gonadotropin (CG), lutropin (luteinizing hormone, LH) and follitropin (follicle stimulating hormone, FSH) are the members of a glycoprotein hormone family. These hormones are structurally related heterodimers with a common glycoprotein α (CGA) subunit noncovalently linked to a distinct β subunit which confers immunological and biological specificity of each hormone (1). TSH is produced by the anterior pituitary gland and, through its action on the thyroid gland, plays a major role in thyroidal secretion and growth (2). The α and β subunits are each encoded by a single gene (3).

Hyperthyroidism is one of the most common endocrine disorders of cats, affecting mainly middle to old aged cats and the lack of a feline-specific TSH assay has hindered early diagnosis. A commercially available canine TSH immunoassay (4) has been evaluated for detection of fTSH and a preliminary report showed that 68% of hyperthyroid cats had serum immunoreactive TSH concentrations below the assay detection limit (5). However, regardless of the species, the assay is not sensitive enough to distinguish normal from low values. As no standard for pituitary feline TSH exists, feline-specific peptide reagents and antibodies are necessary for development of a clinically useful immunoassay. Measurement of endogenous fTSH would allow diagnosis of early hyperthyroidism where TSH levels are suppressed by a hyperfunctioning thyroid gland. Also, a valid feline TSH assay would help characterize the pathophysiological factors leading to hyperthyroidism. In 97 - 99% of the cases of feline hyperthyroidism, thyroid adenomatous hyperplasia, involving one or both thyroid lobes is noticed (6). As there is no physical connection between feline thyroid lobes, it was postulated that either circulating factors, nutritional factors, or environmental factors such as goitrogens, may interact to cause thyroid pathology in cats. A feline-specific TSH assay may also be helpful

in case of non-thyroidal illness or fluctuations of the circulating thyroid hormone levels towards the values within the reference range.

The functional activity of TSH depends on the correct assembly of the subunits into heterodimers. The noncovalent association of the subunits is an obligatory step for the formation of biologically active hormone (1). Recently, single chain or yoked (also called tethered) analogs of hTSH and hCG were constructed with the C-terminus of the β subunit fused using a yoking peptide, CTP (carboxy terminal peptide) to the N-terminus of the α-subunit. The approach has allowed more extensive structure-function studies and also has resulted in the generation of hormones with increased stability and activity (7, 8). The tandem order of subunits, β-CTP-α, was chosen based on studies suggesting the importance of the N-terminal region of hCGβ and C-terminal region of the α-subunit in receptor binding and activation (9). From the standpoint of a strategy for recombinant protein expression and purification, this approach also ensures equimolar expression, detection and purification of the single chain glycoprotein.

The CGA gene of the glycoprotein hormones has been cloned from numerous species including man (10), cattle (11), rat (12), mouse (13), horse (14), and dog (15). There are two N-linked oligosaccharide chains attached to Asn56 and Asn82 and five intramolecular disulphide bonds in the α subunit. A 24 amino acid leader sequence, which is cleaved prior to secretion, is followed by a 96 amino acid mature protein for all species except man where TSHα is a 92 amino acid mature protein (16). The gene encoding the TSHβ subunit has been cloned and sequenced in man (17), cattle (18), mouse (19), rat (20) dog (21) and equine (Genbank Accession # U51789).

There are three exons and two introns in the beta subunit gene of most species. The first exon is only 37 bp and is untranslated followed by a 3.9 kilobase intron. The function of the

first exon is unclear; however it has been speculated that exon 1 may interact directly with thyroid hormone and its receptor and down regulate the TSHβ gene (22). Since a commercially available pituitary source of fTSH does not exist, the approach was taken to clone, sequence and express recombinant fTSH which would then allow development of a feline specific TSH immunoassay. The fTSH α and β subunit sequences were independently cloned and have been submitted to Genbank and are available with accession numbers AY972823 and AY972824 respectively.

2. Materials and methods

2.1. Materials

The cloning vectors TOPO TA™ and TOPO Blunt™ were from Invitrogen (Carlsbad, CA). All the restriction enzymes were from New England Biolabs, Inc (Beverley, MA). The RNA extraction kit, RNaqueous™ kit and Retroscript™ RT-PCR kit were from Ambion Inc (Austin, TX). The DNA extraction kit, QIA Amp® DNA Blood Mini kit was acquired from QIAGEN® (Valencia, CA). High Pure Plasmid Isolation® kit was from Roche Diagnostics (Indianapolis, IN). The mammalian expression vector PEAK™ was obtained from Edge Biosystems (Gaithersburg, MD). One Shot® cells were from Invitrogen (Carlsbad, CA). The Rapid DNA ligation® kit was purchased from Roche Diagnostics Corporation (Indianapolis, IN). The ABI PRISM® 3100 automated sequencer from Applied Biosystems (Foster City, CA) was used by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. The programmable gradient thermal cycler (Model PTC-200) was the product of MJ Research, Inc (Watertown, MA).

2.2. cDNA cloning and sequencing of feline CGA gene

The RNaqueous™ kit was used for RNA extraction according to the protocol and buffers provided by supplier. Pituitary glands from two cats were obtained and processed as explained below. In brief, frozen cat pituitary gland was ground under liquid nitrogen with 100 µl of lysis buffer. Another 100 µl of lysis buffer was added to the tissue and an equal volume of 64% ethanol was added, mixed gently, and transferred to a filter cartridge provided by the suppler and centrifuged for 30-60 sec at 14000 rpm. After washing the filter cartridge twice, RNA was eluted using 40 µl of preheated elution solution. DNase buffer and DNase were added to the eluted RNA and incubated at 37°C for 30 min followed by DNase inactivation reagent and ethanol precipitation of RNA. cDNA was generated using the Retrospect RT-PCR kit following manufacturer's instructions. In summary, 5 µl of newly prepared RNA was mixed with 4 pmol of feline α signal sequence primer added up to 10 µl of nuclease free water incubated at 72°C for 3 minutes, then 2 µl of 10x RT buffer, 4 µl (200 µM) dNTPs, 1 µl RNase inhibitor and 1 µl reverse transcriptase were added, incubated at 48°C for 1 hour followed by 92°C for 10 minutes. The two primers flanking the coding sequence of the glycoprotein hormone α gene was based on the consensus sequence from human, rat, mouse, bovine, equine and canine, were used to directly amplify the α gene from the cDNA by PCR using the forward primer 5'- C AGT TAC TGA GAA ATC ACA AGA CG - 3' (feline-α-ss) and reverse primer 5' - TTA AAT CTT GTG GTG ATA GCA AGT GCT - 3'. The octapeptide immunoaffinity tag FLAG (DYKDDDDK) along with Factor XA site (ATC GAA GGT CGT) was included subsequently using the reverse primer 5' – GC TCT AGA TTA CTT ATC GTC ATC GTC CTT GTA GTC ACG ACC TTC GAT AAT CTT GTG GTG ATA GC – 3' and later a *Not I* cut site was incorporated to facilitate cloning into the expression vector using another reverse primer 5' – CAT AGC GGC CGC TTA

CTT ATC GTC ATC – 3', and in both cases feline-α-ss was used as forward primer. The *Eco RI* cut site in the vector was used for restriction digestion for later cloning into cloning and sequencing vectors. The resulting PCR band on a 1.5% agarose gel was excised and then purified. The purified PCR product was cloned into the TOPO TA™ cloning vector. The clonal DNA was checked for insert by digesting the DNA with restriction enzymes *Eco RI* and *Not I*. The positive clones were then sequenced. The CGA gene was then cloned into expression vector PEAK™ digested with restriction enzymes *Eco RI* and *Not I*, using a Rapid DNA ligation kit following the manufacturer's instructions.

2.3. Cloning and sequencing of fTSHβ gene:

The QIA Amp® DNA Blood Mini kit was used for isolating feline genomic DNA from white blood cells of two different cats separately, as per the manufacturer's instructions. In brief, the white blood cells were lysed with the lysis buffer at 56°C for 10 minutes and DNA from lysed cells was purified using spin columns provided with the kit and purified DNA was eluted with distilled water. Following the experience with the human (23) and equine TSH beta sequences (unpublished work in our laboratory), the second intron was included and the untranslated first exon and first intron were eliminated from the recombinant DNA construct. This "mini-gene" construct gave higher expression levels of recombinant human and equine TSH than constructs leaving out the second intron. Primers designed from consensus sequences of human, bovine, equine and rat TSHβ were used to amplify fTSHβ gene along with the signal peptide directly from genomic DNA as template. The forward degenerate primer 5'- GAA TTC ATG ACT GCT A(CT)C T(AT)C CTG ATG TCC - 3' was designed to recognize the DNA sequence encoding for the signal peptide. The reverse primer 5' - A TGC GGC CGC TTA GAT AGA AAC TCC TAC CAC ATC GGA CTT CT - 3' was designed to begin amplifying the

sequence upstream of TSHβ coding frame. Restriction sites for the construct were the same as for the TSHα construct with *Eco RI* on the 5' end and *Not I* on the 3' end for subsequent cloning into the PEAK™ expression vector. The subunit gene was amplified in a programmable thermal cycler with a program of 4 min at 94°C for initial denaturation followed by 1 min cycling at 94°C, 1 min at an annealing temperature of 55°C and 1 minute of elongation at 72°C for 25 cycles. The PCR products were cloned into the TOPO TA™ cloning vector after extraction from 1.5% agarose gel. The positive clones were confirmed by sequencing. The expression vector PEAK™ was linearized with restriction enzymes *Eco RI* and *Not I* and the β subunit gene was cloned into it.

2.4. Amplification of fTSH α and β constructs for developing yoked fTSH:

The nucleotide sequence encoding the full length alpha subunit excluding the signal sequence was fused in frame with the CTP to the 3' terminus of the beta subunit containing the coding sequence of the signal sequence and the secreted subunit. The second half of the CTP with an *Afl III* cut site at the 5' end was added on to the 5' end of the α gene already cloned as mentioned in 2.2. The forward primer 5' - GAG ACG CGT CTC CCG GGG CCC TCG GAC ACC CCG ATC CTC CCA CAA TTT CCT GAT GGA GAG - 3' was used along with the reverse primer 5' – CAT AGC GGC CGC TTA CTT ATC GTC ATC – 3' used in 2.2 to amplify α needed for generating yoked construct. An *Afl III* restriction site was added to the first half of the CTP sequence at the 3' end and the resulting reverse primer 5' - G ACG CGT TGG GCT TGG AAG GCT GGC GGA AGG GGC CTT TGA GGA AGA GGA GTC CTG GAT AGA AAC TCC TAC CAC ATC GGA C - 3' was added on to the 3' end of β gene and amplified in PCR using the forward primer 5'- GAA TTC ATG ACT GCT ATC TAC CTG ATG TCC - 3'.

The two gene constructs, were cloned individually into the TOPO TA™ cloning vector and the sequence was confirmed.

2.5. Construction of yoked fTSH:

The yfTSH gene was formed by three-way ligations between (i) feline CGA (isolated as about 380 bp *Afl III - Not I* fragment from a 1.5% agarose gel) (ii) fTSHβ subunit (isolated as about 880 bp Eco RI – Afl III fragment from a 1.5% agarose gel) and (iii) PEAK™ expression vector linearized by double digestion with Eco RI and Not I and isolated from a 1.5% agarose gel. The ligation reactions were carried out with T4 DNA ligase using a Rapid DNA ligation kit following the manufacturer's instructions. The ligation reaction was transformed into One Shot™ cells and the plasmids with *Eco RI - Not I* inserts of the size wanted (1260 bp) were identified by restriction enzyme digestion. The structure of the yfTSH gene was confirmed by DNA sequencing as described in 2.2 and 2.3.

3. Results:

3.1. Nucleotide sequence encoding feline pituitary α gene

PCR amplification from the first strand cDNA with a 5' primer located at the 5' end of signal peptide and a 3' primer that adds Factor Xa site, FLAG tag and a Not I site yielded a product about 0.5 kb when examined on a 1.5% agarose gel (Fig.1). The nucleotide sequence of the 510 bp DNA fragment along with the deduced amino acid sequence is shown in Fig. 2. The feline α gene encodes a 96 amino acid mature α subunit preceded by a 24 amino acid signal peptide according to the consensus sequence from other species. There are two N-linked glycosylation sites located at Asn56 and Asn82 (24), and ten cysteine residues that are involved in forming five intramolecular disulphide bonds. The alignment of feline CGA gene with its counterparts from dog, cattle, man and tiger shows that the sequence is highly conserved, but

with differences which are likely significant with regards to immunoassay detection. The projected amino acid homology of the feline alpha subunit for the secreted 96 amino acid glycoprotein was (amino acid identity, % homology): tiger (93, 96.8%), dog (92, 95.8%), cattle (88, 91.6%), horse (77, 80%) and human (63, 68%) (Fig. 3).

3.2. Nucleotide sequence encoding feline TSHβ gene

PCR amplification of feline genomic DNA yielded a product of about 850 bp when examined on a 1.5% agarose gel (Fig.1). The DNA fragment amplified represented the DNA sequences of two exons and an intron of the fTSHβ gene (Fig.4). The nucleotide sequence of fTSHβ is shown in Fig.5 along with deduced amino acid sequence. The amplified fTSHβ sequence encodes for a 138 amino acid peptide with deduced 20 amino acid signal sequence. The projected amino acid homology of the secreted feline beta subunit was (amino acids, %): dog (111, 94%), cattle (107, 90.5%), horse (110, 93.2%) and human (104, 88%) (Fig 6). The 12 cysteine residues are important in maintaining the tertiary structure of TSH molecule by forming six disulphide bridges (25, 26) and are conserved both in numbers and positions in feline, canine, human, bovine and equine TSHβ. The unique N-glycosylation site at Asn23 of the mature peptide is also conserved in all of the above species (1, 24).

3.3. Nucleotide sequence encoding yoked feline TSH construct

The PCR-amplified fTSHα and fTSHβ constructs with CTP were cloned into TOPO TA™ separately and the sequence confirmed. The ligation of fTSHα and fTSHβ constructs with CTP using T4 DNA ligase generated a 1260 bp single chain fTSH nucleotide when examined on a 1.5% agarose gel (Fig 7). The DNA fragment after ligation represented the DNA sequences encoding two exons and an intron of the mature fTSHβ, CTP and fTSHα, in the order (Fig.1). The ligated DNA fragment was cloned into the expression vector PEAK™ and the sequence

confirmed. The nucleotide sequence of yfTSH is shown in Fig.8 along with its deduced amino acid sequence.

4. Discussion:

This is the first report of the sequence of the genes encoding the α and β glycoprotein subunits of TSH in the cat and the first time a single chain construct for a pituitary hormone of a domestic animal has been prepared. It is interesting to note that sequence of the CGA gene is highly conserved between cat and dog with each gene encoding for 96 amino acid residues and only 4 residues being different in the secreted protein. Very high homology of 96.8% was observed between the feline and tiger CGA gene and 91.6% homology between feline and bovine CGA subunits. The human α subunit contains only 92 amino acids due to a four codon deletion at or near the second intron in the human sequence relative to the others and may be involved in the evolution of the α subunit gene in human (27). The sequence homology between feline and human α subunits is less than 70%. However, the ten cysteine residues which form five intramolecular disulphide bonds and play an important role in folding and stabilizing the tertiary structure of the TSH, are completely conserved in both number and position among all five species (26, 28, 29).

The fTSHβ sequence, when aligned with the TSHβ sequence of other species shows high homology with homology of the human sequence being the lowest at 88%. Intron 2, which interrupts the coding sequences of TSHβ peptide between amino acids 34 and 35, is not a highly conserved sequence containing 412 bp in feline, 448 in human and 450 bp in canine. The peptide sequence CAGYC (cysteine-alanine-glycine-tyrosine-cysteine) is highly conserved in TSHβ, LHβ, hCGβ and FSHβ and is thought to be important in subunit combination (30). The 12 cysteine residues that are important for maintaining the tertiary structure of TSH molecule are

conserved in all five species compared. The presence of a tandem dinucleotide $(GT)_n$ repeat sequence with 'n' equaling 10 was shown to be present in the canine TSHβ intron (21) whereas for the repeat sequence in feline TSHβ, 'n' equals only 5. As for canine TSH, we propose that this intron sequence may serve as an important genetic marker for mapping the canine TSH genome.

The amino acid sequence comparisons of the β subunit genes from different species indicate the presence of one potential N- glycosylation site. The oligosaccharide chains attached to the protein moiety play an important role in TSH subunit folding and biologic activities (22). The N-glycosylation site in fTSHβ located at Asn23 on the N-terminus of the mature peptide is followed by two threonine residues and is well conserved (1, 24, 31). This glycosylation site is also seen in TSHβ subunits from dog, man, cow, mouse, rat and horse (17, 18, 19, 20, 21 Genbank Accession # U51789).

Human FSH, hCG and hTSH were synthesized as biologically active single chains (32, 33, 34). Following this strategy the yoked fTSH construct developed in the present study will be used for the expression of single chain fTSH where the linked α and β sequences fold properly, form correct disulphide bonds and adopt a stable native conformation (expression, purification and bioactivity reported in companion publication). The conversion of the fTSH heterodimer to a yoked form represents an important model to further investigate structure-function relationships of fTSH. This approach could also lead to the development of long acting fTSH agonists or antagonists useful as therapeutic agents.

Thyrotropin is a heterodimer where subunits assemble early in the secretory phase and only dimers are biologically active. Therefore, the subunit assembly is the rate limiting step in production of functional heterodimers. Expression of heterodimer in single chain form might

avoid the problem of dominance of α subunit usually observed in the expression system. The ability to overcome the limitations of subunit assembly and dissociation could lead to the development of analogues that have therapeutic and diagnostic applications. Thus, the β and α subunits have been genetically linked to form single chain hormones of the each member of glycoprotein hormone family. The carboxy terminal peptide (CTP) of hCGβ which is used as a linker contains several serine and proline residues. This region thus lacks significant secondary structure, is flexible, and apparently permits the α subunit to assume proper orientation with respect to the β subunit (35). Moreover, CTP has been shown to enhance the secretion rate of the hTSH single chain form (31).

In summary, the successful cloning and sequencing of feline CGA and fTSHβ genes will facilitate generation of recombinant TSH both in heterodimeric and yoked forms. Recombinant feline TSH may provide a reproducible common standard for future feline-specific immunoassays for TSH and its purity should allow development of anti-feline TSH antibodies for sensitive fTSH immunoassays. Furthermore, recombinant feline TSH in heterodimeric and yoked forms may be useful for the enhancement of radioiodide uptake during thyroid ablative therapy, an approach shown particularly useful in imaging nonfunctional adenocarcinomas in man (36), or in patients undergoing T4-suppressive therapy for thyroid tumors.
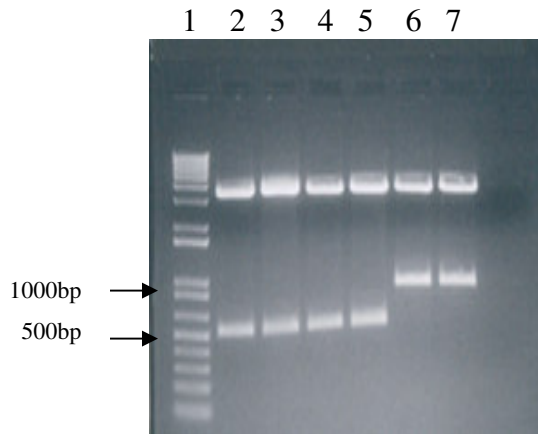
Figures:

Fig. 1. PCR amplified DNA fragments of fTSHα and fTSHβ are cloned into cloning vector TOPO TA™ separately and clonal DNA is digested with restriction enzymes Eco RI and Not I. Lane one is 1Kb plus DNA ladder, lanes 2, 3, 4 and 5 are fTSHα and lanes 6 and 7 are fTSHβ on a 1.8% agarose gel.

(GAATTC)(1) (GCCCTT)(2)

```
1                                                               45
AGT TAC TGA GAA ATC ACA AGA CGA AGC CAA AAT CCC TCT TCA GAT
46                                                              90
CCA CGG TCA ACT GCC CTG ATC ACA TCC TGC AAA AAG TCC GGA GGA
91                                                             135
AGG AGA GCC ATG GAT TAC TAC AGA AAA TAT GCA GCT GTC ATT CTG
            met asp tyr tyr arg lys tyr ala ala val ile leu
136                                                            180
GCC ATA CTC TCT GTG TTT CTG CAT ATT CTC CAT TCT TTT CCT GAT
ala ile leu ser val phe leu his ile leu his ser phe pro asp
181                                                            225
GGA GAG TTT ACA ATG CAG GGG TGC CCA GAA TGC AAG CTA AAG GAA
gly glu phe thr met gln gly cys pro glu cys lys leu lys glu
226                                                            270
AAC AAA TAC TTC TCC AAG TTG GGT GCC CCA ATT TAT CAA TGC ATG
Asn lys tyr phe ser lys leu gly ala pro ile tyr gln cys met
271                                                            315
GGC TGC TGC TTC TCC AGA GCA TAC CCC ACT CCA GCA AGG TCC AAG
gly cys cys phe ser arg ala tyr pro thr pro ala arg ser lys
316                                                            360
AAG ACA ATG TTG GTC CCA AAG AAC ATC ACC TCA GAA GCC ACA TGC
lys thr met leu val pro lys asn*ile thr ser glu ala thr cys
361                                                            405
TGT GTG GCC AAA GCC TTT ACC AAG GCC ACG GTA ATG GGA AAT GCC
cys val ala lys ala phe thr lys ala thr val met gly asn ala
406                                                            450
AAA GTG GAG AAT CAC ACA GAG TGC CAC TGC AGC ACT TGC TAT CAC
Lys val glu asn*his thr glu cys his cys ser thr cys tyr his
451                                                            492
```

```
CAC AAG ATT (ATC GAA GGT CGT)(3)(GAC TAC AAG GAC GAT GAC GAT
his lys ile  ile glu gly arg    asp tyr lys asp asp asp asp
493                             510
AAG)(4) (TAA)(5) (GCGGCCGC)(6)(TATG)(7) – 3'
Lys
```

Fig.2. Nucleotide sequence of the 510 bp cDNA fragment coding for the α subunit of the feline pituitary glycoprotein hormones. The bold letters indicate the 24 amino acid expressed signal sequence. Sequence upstream from expressed but not secreted signal sequence is italicized. The underlined sequences at 5' and 3' end of the sequence denote two primers used to clone the α gene from the first strand cDNA.   * indicates potential N-glycosylation sites. 1. *Eco R1* restriction site from TOPO Blunt vector 2. Additional sequence from TOPO Blunt vector 3. Factor XA site 4. Flag tag 5. Stop Codon 6. *Not 1* restriction enzyme site 7. Extra bases needed for restriction enzyme to work.

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        -24                          +1                         +26
Cat     MDYYRKYAAV   ILAILSVFLH   ILHSFPDGEF   TMQGCPECKL   KENKYFSKLG
Dog     MDCYRKYAAV   ILAIASVFLH   ILHSFPDGEF   TMQGCPECKL   KENKYFSKLG
Cattle  MDYYRKYAAV   ILAILSLFLQ   ILHSFPDGEF   TMQGCPECKL   KENKYFSKPD
Man     MDYYRKYAAI   FLVTLSVFLH   VLHSAPD---   -VQDCPECTL   QENPFFSQPG
Tiger   MDYYRKYAAV   ILAILSVFLH   ILHSFPDGEF   TMQGCPECKL   KENKYFSKLG
Horse   MDYYRKHAAV   ILATLSVFLD   NLHSFPDGEF   TTQDCPECKL   RENKYFFKLG
        +27                                                  +76
Cat     APIYQCMGCC   FSRAYPTPAR   SKKTMLVPKN*  ITSEATCCVA   KAFTKATVMG
Dog     APIYQCMGCC   FSRAYPTPAR   SKKTMLVPKN*  ITSEATCCVA   KAFTKATVMG
Cattle  APIYQCMGCC   FSRAYPTPAR   SKKTMLVPKN*  ITSEATCCVA   KAFTKATVMG
Man     APLYQCMGCC   FSRAYPTPLR   SKKTMLVQKN*  VTSESTCCVA   KSYNRVTVMG
Tiger   APVYQCMGCC   FSRAYPTPAR   SKKTMLVPKN*  ITSEATCCVA   KAFTKATVMG
Horse   VPIYQCKGCC   FSRAYPTPAR   SRKTMLVPKN*  ITSESTCCVA   KAFIRVTVMG
        +77                +96
Cat     NAKVEN*HTEC   HCSTCYHHKI
Dog     NAKVEN*HTEC   HCSTCYYHKS
Cattle  NVRVEN*HTEC   HCSTCYYHKS
Man     GFKVEN*HTAC   HCSTCYYHKS
Tiger   NAKVEN*HTEC   HCSTCYYHKS
Horse   NIKLEN*HTQC   YCSTCYHHKI
```

Fig.3. Sequence alignment of the CGA gene of the glycoprotein hormones from cat, dog, cattle, man, tiger and horse. The signal peptide sequences are in negative numbers. The bold/italics indicates the DNA sequences different from feline *α* gene and dashes indicate positions where the amino acid residues are missing in human *α* gene. The asterisks indicate the two potential glycosylation sites (Asn56 and Ans82).

a. fTSHα | SS | fTSH alpha | FLAG

b. fTSHβ | SS | Exon 2 | Intron 2 | Exon 3

c. yfTSH | SS | fTSH beta | CTP | fTSH alpha | FLAG

Fig.4. Constructs of feline TSH subunits and yoked fTSH 5' to 3'. a. fTSHα mature protein is preceeded by a 24 amino acid signal sequence and has a FLAG tag at the 3' end. b. fTSHβ gene includes a 20 amino acid signal sequence at the 5' end and an intron between the exons 2 and 3. c. The orientation of the feline alpha and beta subunits and the linking CTP is shown in the yfTSH construct.

```
                        1                                      33
5' (GAATTC)(1) ATG ACT GCT ATC TAC CTG ATG TCC GTG CTT TTT
                met thr ala ile tyr leu met ser val leu phe
34                                                           75
GGC CTG GCA TGT GGA CAA GCG ATG TCT TTT TGT TTT CCA ACT
gly leu ala cys gly gln ala met ser phe cys phe pro thr
76                                                          117
GAG TAT ATG ATG CAT GTC GAA AGG AAA GAG TGT GCT TAT TGC
glu tyr met met his val glu arg lys glu cys ala tyr cys
118                                                        162
CTA ACC ATC AAC ACC ACC ATC TGT GCT GGA TAT TGT ATG ACA CGG
leu thr ile asn thr thr ile cys ala gly tyr cys met thr arg
Intron 2
163      GTATGTAGTTCATCTCACTTCTTTTAGCTGAAAATTAGATAAACCTAGACT
CAGTCCATTTCTATCCAGAAAGGAAATGAGATAAATCACAACCTCATTTCACAGACCTAACGGT
CATTGGCTCCTTAGAGGTAGAGTCCCTAGGTTATAATATACGGACCTACTCCATACAGTTGGTA
CAGATAATTTTTACAATAGTTTTACTCCCAAAGTTTATTTAAACCTTATCTTGTTCCCACGATC
AAGGATAAAAGAGAGGTGTGTGTGTATGTCATTTTTTTTTGTCTCTATAGGATTCAGTGTGGAT
ATGCTGAATTGGTATTGGGGAATGGGACTAAGGAATCCTCCCCCAGTCCTATTTGTATCTATGG
GATGTAAGCGAATTAACATTTTGCTTCCTCTTCTGTGCTTCCCTCAG      580
581                                                        625
GAT ATC AAT GGC AAA CTG TTT CTT CCC AAA TAT GCT CTG TCC CAA
asp ile asn gly lys leu phe leu pro lys tyr ala leu ser gln
626                                                        670
GAT GTT TGC ACC TAC AGA GAC TTC CTG TAC AAG ACT GTA GAA ATA
asp val cys thr tyr arg asp phe leu tyr lys thr val glu ile
671                                                        715
CCA GGA TGC CCA CAC CAT GTT ACT CCC TAT TTC TCC TAC CCG GTA
pro gly cys pro his his val thr pro tyr phe ser tyr pro val
716                                                        760
GCT GTA AGC TGT AAA TGT GGC AAG TGT AAT ACT GAC TAT AGC GAC
```

```
ala val ser cys lys cys gly lys cys asn thr asp tyr ser asp
761                                                         805
TGC ATA CAT GAG GCC ATC AAG ACA AAT GAT TGT ACC AAA CCC CAG
cys ile his glu ala ile lys thr asn asp cys thr lys pro gln
806                                             835
AAG TCC GAT GTG GTA GGA GTT TCT ATC TAA(GCGGCCGC)(2)(AT)3 -3'
lys ser asp val val gly val ser ile stop
```

Fig.5. Nucleotide sequence of the 850 bp DNA fragment encoding the feline TSHβ subunit and the deduced amino acid sequence. The boundaries of exon were determined by comparisons with the consensus sequence from other species. The bold letters indicate the 20 amino acid signal sequence. Bold/italic letters denote the intron 2 sequence.

1. *Eco R1* restriction enzyme site 2. *Not 1* restriction enzyme site 3. Extra bases needed for restriction enzyme to work.

```
        +1                                                        +50
Cat     FCFPTEYMMH   VERKECAYCL   TIN*TTICAGY   CMTRDINGKL   FLPKYALSQD
Dog     FCFPTEYTMH   VERKECAYCL   TIN*TTICAGY   CMTRDINGKL   FLPKYALSQD
Human   FCIPTEYTMH   IERRECAYCL   TIN*TTICAGY   CMTRDINGKL   FLPKYALSQD
Bovine  FCIPTEYMMH   VERKECAYCL   TIN*TTVCAGY   CMTRDVNGKL   FLPKYALSQD
Equine  FCIPTEYMMH   VERKECAYCL   TIN*TTICAGY   CMTRDINGKL   FLPKYALSQD
        +51                                                       +100
Cat     VCTYRDFLYK   TVEIPGCPHH   VTPYFSYPVA   VSCKCGKCNT   DYSDCIHEAI
Dog     VCTYRDFMYK   TVEIPGCPRH   VTPYFSYPVA   ASCKCGKCNT   DYSDCIHEAI
Human   VCTYRDFIYR   TVEIPGCPLH   VAPYFSYPVA   LSCKCGKCNT   DYSDCIHEAI
Bovine  VCTYRDFMYK   TAEIPGCPRH   VTPYFSYPVA   ISCKCGKCNT   DYSDCIHEAI
Equine  VCTYRDFMYK   TVEIPGCPDH   VTPYFSYPVA   VSCKCGKCNT   DYSDCIHEAI
        +101            +118
Cat     KTNDCTKPQK   SDVVGVSI
Dog     KTNYCTKPQK   SYVVGFSI
Human   KTNYCTKPQK   SYLVGFSV
Bovine  KTNYCTKPQK   SYMVGFSI
Equine  KANYCTKPQK   SYVVEFSI
```

Fig.6. Sequence alignment of the TSHβ genes from cat, dog, man, cattle and horse. The signal peptide sequences are not shown. The bold italicized letters indicate amino acids different from cat. The asterisk indicates the putative N glycosylation site. Cysteine residues important in maintaining the hormone secondary structure by forming intramolecular disulphide bonds are underlined.
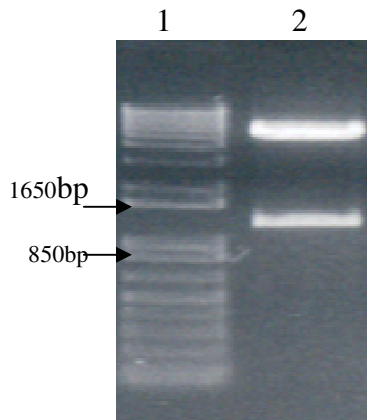
Fig.7. The DNA fragments encoding fTSHα and fTSHβ are amplified separately and the CTP encoding sequence is added as explained as explained in 2.4. The PCR amplified DNA fragments were ligated with T4 DNA ligase and cloned into PEAK™ expression vector and clonal DNA is restriction digested with enzymes EcoRI and Not I. Lane 1 is a 1Kb plus DNA ladder and lane 2 shows yfTSH.

```
                                 1                                    30
5' - (GAATTC)(1)        ATG ACT GCT ATC TAC CTG ATG TCC GTG CTT
                        met thr ala ile tyr leu met ser val leu
31                                                               75
TTT GGC CTG GCA TGT GGA CAA GCG ATG TCT TTT TGT TTT CCA ACT
phe gly leu ala cys gly gln ala met ser phe cys phe pro thr
76                                                              120
GAG TAT ATG ATG CAT GTC GAA AGG AAA GAG TGT GCT TAT TGC CTA
glu cys met met his val glu arg lys glu cys ala tyr cys leu
121                                                    162
ACC ATC AAC ACC ACC ATC TGT GCT GGA TAT TGT ATG ACA CGG
thr ile asn thr thr ile cys ala gly tyr cys met thr arg
Intron 1
163      GTATGTAGTTCATCTCACTTCTTTTAGCTGAAAATTAGATAAACCTAGACT
CAGTCCATTTCTATCCAGAAAGGAAATGAGATAAATCACAACCTCATTTCACAGACCTAACGGT
CATTGGCTCCTTAGAGGTAGAGTCCCTAGGTTATAATATACGGACCTACTCCATACAGTTGGTA
CAGATAATTTTTACAATAGTTTTACTCCCAAAGTTTATTTAAACCTTATCTTGTTCCCACGATC
AAGGATAAAAGAGAGGTGTGTGTGTATGTCATTTTTTTTTGTCTCTATAGGATTCAGTGTGGAT
ATGCTGAATTGGTATTGGGGAATGGGACTAAGGAATCCTCCCCCAGTCCTATTTGTATCTATGG
GATGTAAGCGAATTAACATTTTGCTTCCTCTTCTGTGCTTCCCTCAG      580
581                                                    625
GAT ATC AAT GGC AAA CTG TTT CTT CCC AAA TAT GCT CTG TCC CAA
asp ile asn gly lys leu phe leu pro lys tyr ala leu ser gln
626                                                    670
GAT GTT TGC ACC TAC AGA GAC TTC CTG TAC AAG ACT GTA GAA ATA
asp val cys thr tyr arg asp phe leu tyr lys thr val glu ile
671                                                    715
CCA GGA TGC CCA CAC CAT GTT ACT CCC TAT TTC TCC TAC CCG GTA
pro gly cys pro his his val thr pro tyr phe ser tyr pro val
716                                                    760
GCT GTA AGC TGT AAA TGT GGC AAG TGT AAT ACT GAC TAT AGC GAC
ala val ser cys lys cys gly lys cys asn thr asp tyr ser asp
761                                                    805
TGC ATA CAT GAG GCC ATC AAG ACA AAT GAT TGT ACC AAA CCC CAG
cys ile his glu ala ile lys thr asn asp cys thr lys pro gln
```

63

```
806                                                              850
AAG TCC GAT GTG GTA GGA GTT TCT ATC CAG GAC TCC TCA AAG GCC
lys ser asp val val gly val ser ile gln asp ser ser lys ala
851                                          892
CCT TCC GCC AGC CTT CCA AGC CCA (ACG CGT)(2) CTC CCG GGG CCC
pro ser ala ser leu pro ser pro thr  arg    leu pro gly pro
893                                                          937
TCG GAC ACC CCG ATC CTC CCA CAA TTT CCT GAT GGA GAG TTT ACA
ser asp thr pro ile ile pro gln phe pro asp gly glu phe thr
938                                                          983
ATG CAG GGG TGC CCA GAA TGC AAG CTA AAG GAA AAC AAA TAC TTC
met gln gly cys pro glu cys lys leu lys glu asn lys tyr phe
984                                                         1028
TCC AAG TTG GGT GCC CCA ATT TAT CAA TGC ATG GGC TGC TGC TTC
ser lys leu gly ala pro ile tyr gln cys met gly cys cys phe
1029                                                        1073
TCC AGA GCA TAC CCC ACT CCA GCA AGG TCC AAG AAG ACA ATG TTG
ser arg ala tyr pro thr pro ala arg ser lys lys thr met leu
1074                                                        1118
GTC CCA AAG AAC ATC ACC TCA GAA GCC ACA TGC TGT GTG GCC AAA
val pro lys asn ile thr ser glu ala thr cys cys val ala lys
1119                                                        1160
AAA GCC TTT ACC AAG GCC ACG GTA ATG GGA AAT GCC AAA GTG GAG
lys ala phe thr lys ala thr val met gly asn ala lys val glu
1161                                                        1205
AAT CAC ACA GAG TGC CAC TGC AGC ACT TGC TAT CAC CAC AAG ATT
asn his thr glu cys his cys ser thr cys tyr his his lys ile
1206                                                1241
(ATC GAA GGT GCT)(3) (GAC TAC AAG GAC GAT GAC GAT AAG)(4)
 ile glu gly ala    asp tyr lys asp asp asp asp lys
1242                     256
(TAA)(5) (GCGGCCGC)(6) (TATG)(7) – 3'
```

Fig.8. Nucleotide sequence of the DNA fragment encoding the yoked fTSH construct. The italic letters denote the intron 1 sequence of fTSHβ. The underlined sequence denotes the β specific primer sequence (reverse complement in construct) and the dotted underline denotes the α specific primer sequence (as written). Bold/Italics denote the sequence encoding the CTP linker. 1. Eco RI restriction enzyme site 2. Afl III ligation site 3. Factor XA site 4. Flag tag   5. Stop Codon 6. Not 1 restriction enzyme site 7. extra bases needed for restriction enzyme to work.

Acknowledgements

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EXPRESSION AND PURIFICATION OF FELINE THYROTROPIN (fTSH): IMMUNOLOGICAL DETECTION AND BIOACTIVITY OF HETERODIMERIC AND YOKED GLYCOPROTEINS[2]

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Abstract

The goal of this study was to express and purify recombinant feline TSH as a possible immunoassay standard or pharmaceutical agent. Previously cloned feline common glycoprotein alpha (CGA) and beta subunits were ligated into the mammalian expression vector pEAK10. The feline CGA-FLAG and beta subunits were cloned separately into the pEAK10 expression vector, and transiently co-transfected into PEAK™ cells. Similarly, previously cloned and sequenced yoked (single chain) fTSH (yfTSH) and the CGA-FLAG sequences were ligated into the same vector, and stable cell lines selected by puromycin resistance. Expression levels of at least 1 µg/ml were achieved for both heterodimeric and yoked fTSH forms. The glycoproteins were purified in one step using anti-FLAG immunoaffinity column chromatography to high purity. The molecular weights of feline CGA-FLAG subunit, beta subunit and yfTSH were 20.4, 17, and 45 kilodaltons, respectively. Both heterodimeric and yoked glycoproteins were recognized with approximately 40% detection by both a commercial canine TSH immunoassay and an in-house canine TSH ELISA. The yoked glycoprotein exhibited parallelism with the heterodimeric form in the in-house ELISA, supporting their possible use as immunoassay standards. In bioactivity assays, the heterodimeric and yoked forms of fTSH were 12.5 and 3.4 % as potent as pituitary source bovine TSH at displacing ^{125}I-bTSH and 45 and 24 % as potent in stimulating adenylate cyclase activity in human TSH receptor-expressing JP09 cells. However, in addition to reduced receptor binding affinity, the recombinant glycohormones produced a reduced maximal effect at maximal concentration (Emax) suggesting the possibility of the recombinant glycohormone constructs acting as partial agonists of the human TSH receptor.

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1. Introduction

TSH is a glycoprotein hormone with an α subunit, common to Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Chorionic gonadotropin (CG), and a hormone-specific β subunit. The α and β subunits associate noncovalently and subunit assembly is necessary for the biological activity of the hormone (1). The functional activity of these hormones is dependent on the correct assembly of the subunits into the heterodimer.

The serum concentration of TSH, which is decreased in primary hyperthyroidism, and increased in primary hypothyroidism, is a very sensitive indicator of thyroid function in man (2). Currently available ultra-sensitive human TSH assays eliminate the expensive and time consuming thyrotropin releasing hormone (TRH) stimulation tests by distinguishing euthyroid patients from hyperthyroid patients (3, 4). Therefore TSH measurement has been widely used to screen for thyroid dysfunction. Standards for human TSH (National Hormone and Pituitary Program (NHPP) and World Health Organization (WHO)) continue to be cadaver-source purified pituitary TSH. A commercial assay for canine TSH has been available since 1997 (7); however, no widely available independent standard for this or other domestic animal TSH assays have been developed.

Hyperthyroidism is the most commonly diagnosed thyroid disorders in middle to old aged cats. The hospital prevalence of feline hyperthyroidism increased from 0.06 % for the period of 1978 to 1982 to 0.3 % for the period of 1993 to 1997 and over 20 years the prevalence of feline hyperthyroidism per visit was 2.1 % (8). Furthermore, epizootiological studies have suggested links to dietary and/or environmental associations with this disease not recognized prior to 1979. Canned cat food, cat litter, and pesticides have been identified as possible risk factors for the disease (5, 6, 9). Canine TSH assays have been tried to measure TSH in feline

serum samples. In one study, 68% of hyperthyroid cats had TSH concentrations below the detection limit, as might be expected; however the commercial canine TSH assay used is generally not sensitive enough to distinguish normal from low values, particularly in the cat (10). The development of a sensitive feline TSH (fTSH) assay has been hampered by the absence of an available standard for fTSH. Biochemical purification of pituitary source TSH is complicated by the presence of much higher concentrations of LH and FSH, and only about 100 µg of TSH even in a human pituitary. Therefore, we reasoned that recombinant fTSH engineered with an immunoaffinity tag would advance the development of a recombinant peptide-based immunoassay to detect fTSH. The feline CGA subunit previously cloned and sequenced in our lab encodes for a 96 amino acid protein and contains five intramolecular disulfide bonds. The fTSHβ subunit encodes for a 138 amino acid protein and six disulfide bonds (see companion paper). The α and β subunit structure predicted two and one N-linked oligosaccharides, respectively. The oligosaccharides have been shown to play an important role in bioactivity of human TSH (11).

Recently, single chain or yoked (also called tethered) analogs of hTSH and hCG have been constructed with the C-terminus of the β subunit fused using a yoking peptide, the carboxy terminal peptide (CTP) of hCGβ to the N-terminus of the α-subunit. Expression of these recombinant single chain hormones has allowed extensive structure-function studies and also has resulted in generation of hormones with increased stability and, in some cases, activity (12, 13). Grossmann et al., showed that the genetic fusion of hTSH α- and β-subunits using the carboxy-terminal peptide of the hCGβ subunit as a linker created a yoked form of hTSH whose receptor binding and bioactivity were comparable to native hTSH, but had higher thermostability and a longer plasma half-life (12). The linker CTP is not required for the assembly of the heterodimer

nor is it required for receptor activation (14, 15). In contrast, the presence of CTP linker appears mainly important for the maximal expression of a functional single chain (17). The yfTSH-FLAG previously cloned and constructed in our lab encodes a 1260 bp glycoprotein with the sequence from N-terminus to C-terminus of β-CTP-α-FLAG.

The synthesis, purification, characterization, pharmacokinetic and toxicological properties of recombinant hTSH for human use were described for the first time in 1993 (16). In 2003, our laboratory reported the expression of recombinant canine TSH in a baculovirus expression system (18). The objective of the present study was to express higher quantities and to purify biologically and immunologically active recombinant feline thyrotropin. *In vitro* expression in mammalian cells allows the mammalian pattern of post-translational modifications of the recombinant hormone and facilitates the investigation of structure-function relationships. The ideal expression system for a mammalian glycoprotein is a mammalian cell line capable of post-translational glycosylation with a mammalian pattern of sugars. The Chinese Hamster Ovary (CHO) cells lack sulfotransferase and N-acetyl galactosamine transferase (19). Therefore the recombinant glycoproteins produced from CHO cells are not sulfated to the same degree as pituitary-derived glycoproteins. However, Human Embryonic Kidney cells (HEK 293) have been shown to sulfate pituitary glycoproteins to a greater extent (20, 21), because of the presence of higher levels of N-acetyl galactosamine transferase and sulfotransferase activities to those expressed in the pituitary gland (22). Moreover, the biological potency of recombinant FSH produced in HEK 293 cells was shown to be greater than pituitary FSH, while the biological potency of recombinant FSH produced from CHO cells was less than that of pituitary FSH (23). This work constitutes the first report of *in vitro* expression and purification of recombinant feline thyrotropin. The demonstration of immunological recognition by antibodies generated against

pituitary-source TSH, and of bioactivity confirms that recombinant glycoprotein may be used to standardize and improve clinical assays for feline TSH and has potential as a pharmaceutical agent as well.

2. Materials and methods

2.1. Materials:

All restriction enzymes necessary for cloning into pEAK10 expression vector were purchased from New England Biolabs® Inc (Beverley, MA). Expression vector pEAK10 and the PEAK™ Stable Expression Kit were obtained from Edge Biosystems (Gaithersburg, MD). The High Pure Plasmid Isolation kit™ was from Roche Diagnostics (Indianapolis, IN). One Shot™ cells, fetal bovine serum (FBS) and penicillin-streptomycin were from Invitrogen Corporation (Carlsbad, CA). The Rapid DNA™ ligation kit was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Dulbecco's Modified Eagles Medium (DMEM), Waymouth`s MB752/1 medium, Nutrient Mixture F-12 Ham, puromycin, ANTI-FLAG® M2-Agarose Affinity Gel, FLAG® Peptide, ANTI-FLAG® M2 monoclonal antibody, cAMP salt, anti-cAMP polyclonal antibody, 3-isobutyl-1-methylxanthine (IBMX), Mouse IgG, Goat anti-rabbit-Peroxidase conjugate and Tetramethyl-benzidine were all from Sigma (St. Louis, MO). Micro BCA™ Protein Assay Kit, AminoLink® kit and West Dura Super Signal™ substrate were from Pierce (Rockford, IL). Immulon™ 4HBX strips were from ThermoLabsystems (Franklin, MA), and Canine TSH was from Scripps Laboratories (San Diego, CA) and Dr. A.F. Parlow (National Hormone and Peptide Program (NHPP)). Immobilon™ transfer membrane was purchased from Millipore (Bedford, MA). Forskolin was from Fisher Scientific (Pittsburg, PA). The automated microplate reader was from Bio-Tek Instrument®, Inc (Winooski, VT). All the chemicals for making buffers and other reagents were from Sigma (St. Louis, MO). The ABI PRISM® 3100

automated sequencer from Applied Biosystems (Foster City, CA) was used by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia.

2.2. Construction of expression vectors

The expression vector pEAK10 was linearized by digesting with restriction enzymes *Eco RI* and *Not I*. Previously cloned and sequenced feline CGA was digested with the same enzymes and ligated with linearized pEAK10 vector DNA downstream of EF1α using the Rapid DNA™ ligation kit following the manufacturer's instructions. The ligated DNA was transformed into One Shot™ cells and the positive clones were checked for correct insertion by digesting with *Eco RI* and *Not I* restriction enzymes. Similarly, the previously cloned and sequenced fTSH beta subunit was cloned downstream of EF1α into the pEAK10 expression vector separately and insertion confirmed by restriction digests. The feline CGA construct for yoked fTSH previously cloned and sequenced was linearized by digesting with restriction enzymes *Afl III* and *Not I*. The fTSH beta construct for yoked fTSH was digested with *Eco RI* and *Afl III* and both the linearized subunits were double ligated with linearized pEAK10 vector as explained above. The ligated DNA was transformed into One Shot™ cells and positive clones were checked for inserts by digesting with *Eco RI* and *Not I* restriction enzymes.

2.3. Transient expression of heterodimeric fTSH

The PEAK^TM Stable Expression kit was used following the manufacturer's instructions. The PEAK^TM cells were maintained in DMEM media with 10% FBS and 1% penicillin-streptomycin. Briefly, 6 µg each of feline CGA and fTSH beta plasmid DNA were mixed with 125 µl of 1.0 M $CaCl_2$, 500 µl of 2x Phosphate buffer and the total volume was brought up to 1 ml with water. The DNA/Calcium Phosphate suspension was added dropwise to 75 cm^2 flask with 1.5 $x10^6$ PEAK^TM cells in 9 ml of media, incubated at 37^oC and 5% CO_2 for 5 hours,

washed and 10 ml of fresh media added. Five days after transfection the media was harvested, filtered and frozen at -20°C and the purification was generally performed within four weeks. A mock transfection with empty pEAK10 vector was also performed as a control.

2.4. Stable expression of feline CGA and yfTSH

The expression vector pEAK10-w/o toxic gene has a cell type independent EF-1 α promoter (24) and a puromycin resistance gene. The pEAK10 plasmid DNA checked for feline CGA gene was transfected into PEAK^{TM} cells as explained in 2.3. The transfected cells were selected by adding 0.5 µg puromycin per ml of culture medium 24-48 hrs post transfection. The cells were split at 80% confluency and 24 hrs later puromycin was added. Two weeks after transfection, puromycin was added directly while splitting and concentration gradually increased at 0.5 µg/ml weekly up to 2.0 µg/ml. Similarly a stable cell line expressing yfTSH was also developed by transfecting PEAK^{TM} cells with pEAK10 plasmid DNA containing the yfTSH gene. Mock transfections were performed in both cases as controls. The conditioned medium from stable cell lines was collected and frozen at -20°C until purified.

2.5. Purification of heterodimeric and yoked forms of fTSH and feline CGA

A column was made with 1 ml of ANTI-FLAG® M2-Agarose Affinity Gel in a pasteur pipette and equilibrated with 3 ml of 0.1 M glycine pH 3.5 and washed with 5 ml of tris buffered saline (TBS: 50mM Tris HCl, 150 mM NaCl, pH 7.4). The frozen expression media was thawed, filtered and percolated by gravity through the column 3 times. Following binding, the column was washed with 10 ml of TBS and eluted with five one-column volumes of solution containing 100 µg/ml FLAG peptide in TBS. The elution fractions were collected into glass vials and siliconized pipette tips used at every point to minimize protein losses. All the elution fractions were dialyzed against 0.03 M phosphate buffer for buffer exchange and also to remove

FLAG peptide. A small fraction was saved for a silver stain and ELISA and the rest lyophilized in aliquots and stored at -80^0C.

2.6. Purification of heterodimeric fTSH using mAb column

An affinity column was made by coupling AminoLink® coupling gel with anti-cTSH mAb (14H9.E2) following manufacturer's instructions. The development of this monoclonal antibody is described below. Recombinant heterodimeric fTSH was allowed to enter the gel bed and incubated for 60 minutes at room temperature. Bound peptide was eluted using 0.1 M glycine pH 2.5. The eluted fractions were treated in the same way as explained in 2.5.

2.7. Characterization of purified proteins

ELISA

The purified heterodimeric and yoked forms of fTSH was detected by in-house canine TSH ELISA employing a capture monoclonal antibody (14H9.E2) (mAb) previously selected for ovine/canine TSH beta and a polyclonal antibody (pAb) generated previously against pituitary-source canine TSH (25). Pituitary-source canine TSH (NHPP) was used as a standard. Immulon 4HBX strips were coated with 1.5 µg per well of 14H9.E2 monoclonal antibody in 200 µl PBS and subsequently blocked with blocking buffer (5 g/dl of sucrose and 2g/dl of bovine serum albumin (BSA) in PBS pH 7.0). A standard curve with 100 µl of solution contained cTSH ranging from 0.31 ng/ml to 20 ng/ml in an immunoassay buffer consisting of 25 mM Tris base, 10 mM EGTA, 0.15 mM KCl, 2 mg/ml BSA and 0.5% Tween pH 7.5. Anti-cTSH polyclonal antibody was diluted in the immunoassay buffer at 100 ng IgG protein per 100 µl and mouse IgG was added to the diluted polyclonal antibody at the rate of 100 µg/ml of antibody solution to block the epitopes for mouse IgG. The pAb - mouse IgG mixture was added to the wells with standards and samples. The assay was incubated at 4^oC overnight to equilibrium and washed

three times with 300 µl of immunoassay wash buffer (20 mM Tris base, 0.15 M NaCl, 5 mM EDTA, 0.5% Tween, pH 7). Goat anti rabbit- Immunoglobulin-peroxidase conjugate was then added as a tertiary antibody (1:10,000 in immunoassay buffer). The assay was incubated for 60 minutes at room temperature, washed and TMB substrate added. The reaction was allowed to proceed for 30 minutes or until a blue color develops and then stopped with 1 M HCl or 1M H_2SO_4. The absorbance was read at 450nm in an automated microplate reader.

Protein assay

Apart from measurement of immunoassayable recombinant fTSH levels with ELISA, the gravimetric measurement of total protein was performed by Bicinchoninic acid (BCA) protein assay (26). Along with bovine serum albumin standards in BCA assay, bovine TSH standards were also used for accurate measurement of recombinant TSH levels. The total protein and percentage purity of recombinant peptide as determined by silver stain were used to arrive at the gravimetric concentrations of purified recombinant fTSH.

Gel electrophoresis and immunoblotting

Purified fTSH in both heterodimeric and yoked forms (100-150 ng/lane) were heat-denatured in reducing buffer (β-mercaptoethanol) and run on a 16.5 % SDS-PAGE as previously described (27). The protein bands were subsequently transferred to an Immobilon^{TM} transfer membrane with blotting buffer containing 25 mM Tris pH 8.3, 192 mM glycine and 10% methanol. The blotted membrane was blocked with PBS pH 8.2 containing 5% nonfat dry milk and 0.05% Tween 20 for 30 minutes at room temperature. Anti-FLAG® M2 monoclonal antibody IgG protein was diluted in PBS containing 0.1% BSA and 0.05% Tween 20 (antibody incubation buffer) to 5 µg/ml and allowed to bind overnight at 4°C. The membrane was then washed three times with PBS and incubated with goat anti-mouse peroxidase conjugate in

antibody incubation buffer for 2 hours at room temperature. The protein band was visualized using West Dura Super Signal^{TM} substrate for 2 minutes and the lanes imaged and quantified with a Fluor-S Max MultiImager^{TM} (Bio-Rad Laboratories, Inc. Hercules, CA). Protein bands in the gel were also detected by silver staining and purity estimated by densitometry.

TSH Receptor Binding Assay

Competitive binding experiments were performed with a stable cell line of CHO cells expressing the recombinant human TSH receptor (rhTSHR), clone JP09 (28). Pituitary source bTSH was iodinated as described previously (36). The cells were maintained in Ham's F12 (HF-12) media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and were maintained with geneticin selection at 400 µg per ml of media. Cells were plated in 12-well plates, grown until confluency and were incubated with 1 ng (10^5 cpm) ^{125}I bTSH and various dilutions of unlabelled TSH in binding buffer (278 mM sucrose, 5 mM Hepes, 1 mM CaCl2, 5 mM KCl, 1.23 mM KH$_2$PO4, 1.2 mM MgSO$_4$, 1 mM NaHCO$_3$, 5.5 mM glucose, 0.1% BSA) and incubated at room temperature for 6 hrs. The cells were washed and subsequently solubilized with 1N NaOH and counted in an automated gamma counter (PerkinElmer, Inc., Shelton, CT).

TSH stimulation of JP09 cells

JP09 cells were grown in 12 well plates to confluence, the media removed and incubated in Waymouth`s MB752/1 medium with 0.1% BSA and 0.8 mM isobutylmethylxanthine (IBMX) for 15 min at 37$^o$C. The cells were then incubated in various concentrations of peptide for 30 min at 37$^o$C in Waymouth`s medium containing 0.1% BSA and 0.8 mM IBMX immediately following first incubation. The cells were also incubated with 0.1 mM forskolin to determine the maximal cAMP response. The incubation medium was removed, and the cells were lysed in

100% ethanol at -20°C overnight. The extract was collected, dried under vacuum, and dissolved in 1 ml of 50 mM sodium acetate pH 6.2.

Cyclic AMP Radioimmunoassay

Cyclic AMP was iodinated with ^{125}I using a standard chloramine T method (29). Briefly, 1 mCi of ^{125}I, 25 µl of deionized water, 5 µl of cAMP methyl ester (2.5 µg), 50 µl of 0.5 mM sodium phosphate pH 7.4 and 10 µl chloramine T (165 mg/10 ml of 0.5 M sodium phosphate buffer) were allowed to react for 60 seconds and the reaction terminated by adding 20 µl of sodium metabisulfite (120 mg/10 ml of 0.5 M sodium phosphate buffer). Diluted reaction mixture was added to a DEAE cellulose column. The bound labeled peptide was eluted with 5 mM, 10 mM and 50 mM concentrations of potassium phosphate pH 4.4. The first peak (in the wash) constituted free iodide and the iodinated cAMP in the second peak (usually in 10 mM potassium phosphate elution) was pooled. The radioimmunoassay standards were made using cAMP salt ranging from 50 pmol/ml to 0.1 pmol/ml in 50 mM sodium acetate buffer pH 6.2. The unknowns were diluted at 1:25 for TSH and forskolin - stimulated samples, and 1:10 for basal samples in 50 mM sodium acetate buffer pH 6.2. For the RIA, 100 µl of standard or sample was succinylated using acetylating reagent (2:1 of triethylamine and acetic anhydride) at 2.5 µl/tube and 100 µl of anti cAMP pAb was added per tube. The tubes were incubated for 4 hrs at 4°C and ~ 4-5 nCi/tube of ^{125}I – cAMP was added and again incubated 16-18 hours at 4°C. Then 100 µl of carrier 10% BSA and 2 ml of ethanol was added to tubes, spun at 2000xg for 15 min, the supernatant aspirated and the pellet counted in gamma counter.

2.7. Statistical analysis

The statistical analysis of binding assays and cAMP assays was carried out by using GraphPad PRISM program. All graphical data were also prepared using GraphPad Prism.

Nonlinear regression was used to fit both binding and adenylate cyclase data to a sigmoidal curve when plotted against the log of the TSH concentration. The goodness of fit is reported as R and Emax and EC50 calculated. Significance of differences between Emax and EC50 for bTSH, fTSH and yfTSH in adenylate cyclase and binding assays were calculated by performing unpaired t tests. The slopes and intercepts for immunoreactivity data were also compared by performing unpaired t tests. A p value of ≤ 0.05 was considered significant. Means and standard error were reported as appropriate.

3. Results

3.1. Expression and purification of heterodimeric fTSH

The time course of expression of recombinant heterodimeric fTSH following transient transfection showed an average plateau concentration of 1500 ± 400 ng/ml (mean $\pm$ SEM, n=6) 4 days after transfection (Fig. 1). The expression media was purified using ANTI-FLAG® M2-Agarose Affinity Gel, and 47.6 ± 9.8 % (mean $\pm$ SEM, n=4 preparations) of immunoassayable TSH protein was recovered. The recovery of the protein from the column was consistent with each purification (Table 1). When the column eluate was analyzed on a 16.5 % SDS gel, protein bands with molecular weights of 21 kDa and 17 kDa for fTSHα and fTSHβ, respectively, were visualized with silver stain (Fig.2). Another band of about 23 kDa molecular weight (Fig. 2) for free alpha was also observed with fTSH purified using ANTI-FLAG® M2-Agarose affinity gel, indicating that the anti-FLAG column bound not only heterodimeric fTSH but also free alpha subunit expressed in the media. The free alpha subunit was removed by an anti-cTSHβ monoclonal antibody column as described in 2.6 (Fig. 2). This antibody has been shown in our laboratory only to bind to canine TSH in a Western blot only when non-denaturing electrophoretic conditions are used. (data not shown). A Western blot for purified protein using

anti-FLAG monoclonal antibody showed a broad band at about 21 kDa (Fig.3.) consistent with the glycosylated α – FLAG subunit in heterodimeric form.

3.2. Expression and purification of yoked fTSH and feline CGA

The final structure of the expression vector cloned with yfTSH is shown in Fig.4. The PEAK^{TM} cells were stably transfected with feline CGA and yfTSH as explained in 2.4 to develop a stable cell line expressing the recombinant protein. The peak media concentration of yfTSH averaged 800 ± 350 ng/ml (mean ± SEM, n=6) and the expression media was purified using ANTI-FLAG® M2-Agarose affinity gel with a recovery of 43 ± 3% (mean ± SEM, n=4 preparations). A western blot on the purified yfTSH using anti-FLAG monoclonal antibody showed a band at about 45 kDa (Fig.3) and that for feline CGA showed at about 21 kDa.

3.3. Immunological characterization of recombinant fTSH

The average expression concentrations in medium as determined by the in-house ELISA were 1500 ± 400 ng/ml and 800 ± 350 ng/ml (mean ± SEM, n=6 preparations) for heterodimeric and yoked forms of fTSH respectively. Both the recombinant fTSH forms were also detected by the commercially available canine TSH assay (Diagnostic Products Corp., Los Angeles, CA). However, In-house assay detected about 38.5±2.8 % and DPC assay detected 35.7±4.8 % (mean±SEM) of the recombinant fTSH in both forms as standardized by BCA protein assay.

Both the heterodimeric and yoked forms of fTSH showed immunological parallelism with pituitary source cTSH (NHPP) in our in-house ELISA (Fig.5). The IC50 (ng/ml±SE, n=3) concentrations for pituitary source cTSH (NHPP), heterodimeric and yoked forms of fTSH were 3.3±1.9, 4.3±1.2 and 3.8±1.2 and slopes were 1.36±0.2, 1.29±0.03 and 1.32±0.02 respectively. Both the IC50 values and slopes of all the three peptides were not significantly different from each other.

3.4. Biological activity of recombinant fTSH

To demonstrate the biological activity of recombinant forms of fTSH, hTSH receptor binding inhibition and adenylate cyclase activity were measured. As shown in Fig.6, the heterodimeric and yoked forms of fTSH were able to inhibit the binding of ^{125}I-bTSH to the hTSH receptor in JP09 cells but with different affinities (Table 2).

The bioactivities of recombinant fTSH constructs were also evaluated by potency to stimulate adenylate cyclase in the human TSH receptor-expressing JP09 cells. Heterodimeric fTSH was almost 2-fold more potent than yoked fTSH in stimulating cAMP production (Fig.7). However, heterodimeric fTSH was 2-fold and yfTSH was about 4-fold less potent in signal transduction than pituitary source bTSH (Table 2). The maximal cAMP concentrations upon stimulation with 100 ng/ml of TSH peptides in comparison with forskolin stimulation are significantly different from each other and also different from forskolin (Fig. 8).

The ratio between biological potency and immunoactivity has been used as an index of the overall potency of circulating TSH *in vitro* (40). The Biological to Immunological ratio (B/I) for yfTSH for binding and cAMP production was 27 % and 52 %, respectively, of that of heterodimeric fTSH (Table. 3).

4. Discussion

Previously, we cloned and sequenced feline CGA and feline thyrotropin beta genes separately (companion publication) and the present study constitutes the first report of the *in vitro* expression and purification of recombinant feline thyrotropin in heterodimeric and yoked forms. Lacking a commercially available source for pituitary fTSH, we reasoned that by demonstrating immunological parallelism to other standards, recombinant fTSH can be substituted for pituitary source fTSH as a standard. In fact, using similar purity criteria, we were

able to demonstrate that recombinant fTSH was more highly purified than available pituitary source canine TSH standards (Fig. 9).

Immunoreactive and bioactive fTSH was efficiently expressed from PEAK[TM] cells, and the expression media concentrations obtained (~1 µg/ml) were considerably higher than the concentrations obtained with recombinant hTSH expression using CHO and other eukaryotic cells (20, 30, 31). Furthermore, the synthesis of yoked fTSH indicates that the linked alpha and beta subunits presumably folded with formation of disulfide bonds and a stable conformation. The highly flexible connecting linker CTP apparently still allowed the interaction of alpha and beta subunits, with recognition by both TSH receptor and anti-TSH antibodies. Surprisingly, compared to available pituitary standards (canine for immunoassay and bovine for bioassay), the recombinant glycoproteins were detected with equal affinity by the immunoassay systems, but showed reduced receptor affinity and diminished maximal effect on the bioactivity assay, consistent with a partial agonist.

The alpha and beta subunits were previously cloned into pEAK10 expression vector separately and transiently transfected using calcium phosphate into PEAK[TM] cells. The expression vector has a single EF-1α promoter which facilitates expression of only one gene at a time. Interestingly, in a manner similar to pituitary thyrotrophs (37), equimolar expression of feline CGA and fTSH beta subunits did not occur as shown by affinity chromatography purification using anti-FLAG M2 resin (Fig.2). The biosynthesis of human recombinant glycoprotein hormones in cell cultures have shown the presence of alpha subunit immunoreactivity (32) and evidence that it is associated with 'free alpha' subunit which has a slightly higher molecular weight due to an additional O-linked oligosaccharide (32, 33). This sugar residue is located in a domain of the alpha subunit believed to normally interact with the

beta subunit during heterodimer formation (1). Therefore, the free alpha subunit expressed independently from the beta subunit is subject to glycosylation with one additional sugar moiety during post-translational processing. An affinity column made with agarose beads linked to a beta-subunit specific monoclonal antibody (14H9.E4) captured heterodimeric fTSH alone removing the free alpha subunit and increasing the purity of the recombinant fTSH preparation (Fig.2).

Having demonstrated the expression and purification of recombinant fTSH in heterodimeric and yoked forms, the next step was to assess the immunological activity of the recombinant protein. Lacking pituitary source fTSH, the immunological activity of fTSH in both forms was compared with pituitary source canine TSH (NHPP standard) and the results indicated that both forms of fTSH showed immunological parallelism to pituitary-source TSH supporting the possibility that recombinant fTSH could be substituted for pituitary source TSH as an immunoassay standard. However, the in-house ELISA detected both heterodimeric and yoked forms of the recombinant protein to an extent of about 40 % as standardized by BCA protein assay and purity determined by silver stain. The reasons for this discrepancy in both assays is not yet clear but could reflect differences in tertiary structures and/or glycosylation differences between recombinant and pituitary source-TSH.

It had previously been shown that bioactive hCG, hTSH and hFSH could be produced as single chain hormones (12, 13, 34). In this study, yoked fTSH exhibited 54% of the potency of heterodimeric fTSH and 24% of the potency of pituitary source bTSH in adenylate cyclase assays and 27% of the potency of heterodimeric fTSH and only 3.4 % of the potency of pituitary source bTSH at inhibiting ^{125}I-bTSH binding in JP09 cells. The heterodimeric fTSH showed 45 % and 12.5 % of the potency of pituitary source bTSH in adenylate cyclase and binding assays

respectively. The immunological behavior of the heterodimeric and single chain glycopeptides was parallel, supporting the possibility of using recombinant peptide as an assay standard. Yoked fTSH showed a decreased biological to immunological ratio (B/I=0.27 for binding and 0.52 for cAMP production) compared to heterodimeric fTSH (B/I defined as 1) (Table.3). However, when expressed on the basis of defined protein content, receptor binding activity and *in vitro* bioactivity of purified pituitary isoforms have been previously shown to vary over five to eight-fold (38). Because of the magnitude of this range, reported variations in isoform bioactivity (as expressed by B/I ratios) cannot be the result only of altered immunoreactivity. Moreover, it was demonstrated that sulfated/less acidic human FSH isoforms induced resumption of meiosis significantly more efficiently than sialylated/acidic isoforms regardless of the method employed to determine the concentration of the isoforms tested (radioimmunoassay, radio-receptor assay and in-vitro bioassay) (39).

Species differences might account for the reduced activity in receptor binding. Infact, others have shown that bovine and porcine TSH produced stronger interaction with hTSH receptor than hTSH (34) indicating that even homologous glycohormones do not necessarily have the highest affinity for the receptor in the same species. Glycosylation and/or subtle tertiary structure differences might also contribute to lower potency. Other studies have demonstrated that heterodimeric and yoked forms of glycoproteins expressed in the baculovirus expression system exhibited higher binding affinities than native forms presumably due to differences in glycosylation pattern which tend to favor less sialylation (13).

In summary, this is the first report of successful *in vitro* expression and purification of recombinant fTSH in heterodimeric and yoked forms. The demonstration of the immunological recognition by antibodies generated against pituitary-source TSH confirms that the recombinant

glycoprotein may be used to standardize and improve clinical assays for feline TSH, and even be utilized to prepare feline-specific immunoreagents. The diminished bioactivity relative to immunoreactivity is unusual given the generally higher structural requirements of antibodies. However, as the concentrations of the recombinant peptides never achieved the same maximal binding inhibition nor maximal effect, we postulate that these glycoproteins may act as partial agonists of human TSH receptor compared to pituitary-source bTSH.

Figures:
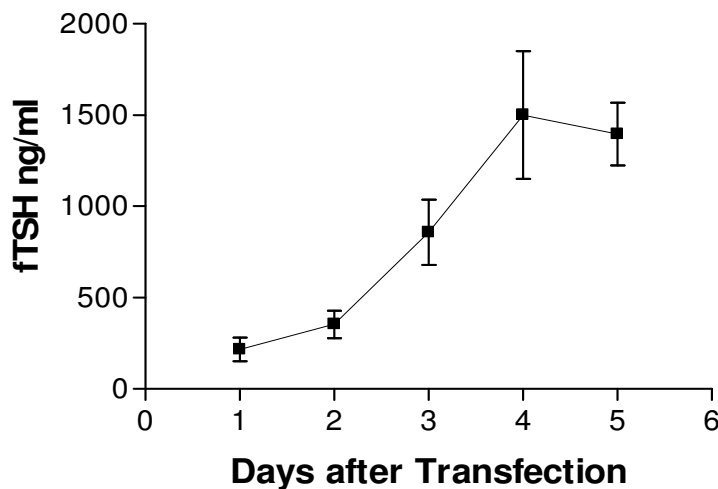
Fig.1. Expression levels of heterodimeric fTSH after transient transfection in PEAK[TM] cells. The expression levels of fTSH reached (mean ± SEM) 1500 ± 400 ng/ml 4 days after transfection. (n=4).
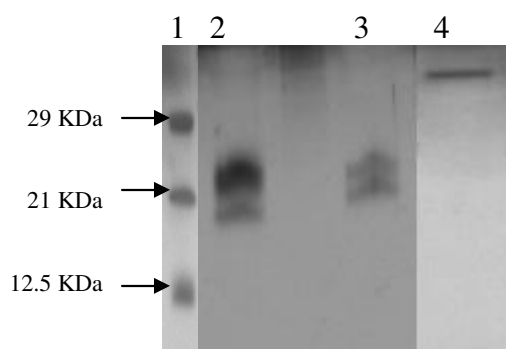
Fig.2. Silver stain of fTSH heterodimer and yfTSH. Lane 1 shows polypeptide protein standard, Lane 2 shows fTSH heterodimer with free alpha, Lane 3 shows fTSH heterodimer after free alpha subunit removal and Lane 4 shows yfTSH.
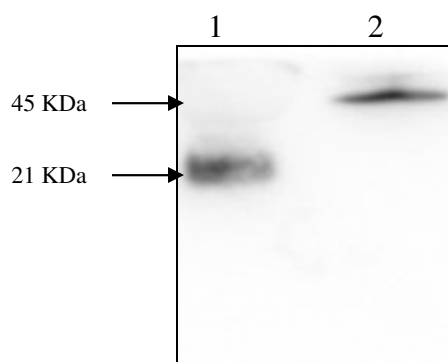
Fig.3. Western blot analysis of recombinant fTSH in heterodimeric and yoked forms using anti Flag monoclonal antibody at 5µg/ml as primary antibody and a 1:3000 of secondary goat anti-mouse HRP antibody. Lane 1 shows CGA subunit in heterodimer as the FLAG tag is present only on CGA subunit and Lane 2 shows the yoked fTSH.

PEAK Expression Vector
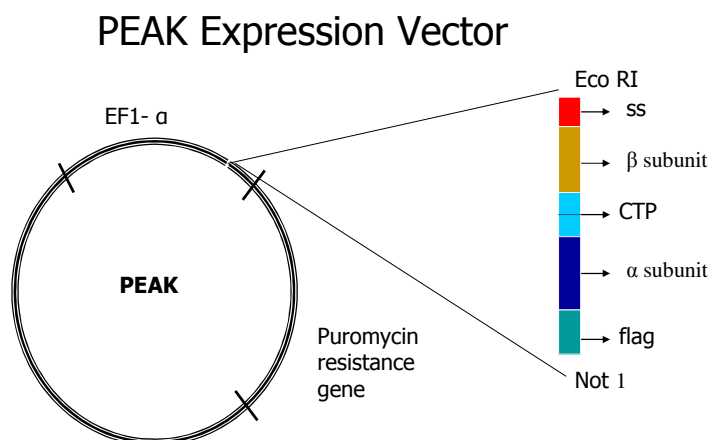
Fig.4. Schematic representation of yfTSH cloned down stream of EF1- α promoter of pEAK10 expression vector. The yfTSH construct is flanked by restriction sites Eco RI and Not I. The connecting peptide CTP serves as a linker between alpha and beta subunits.
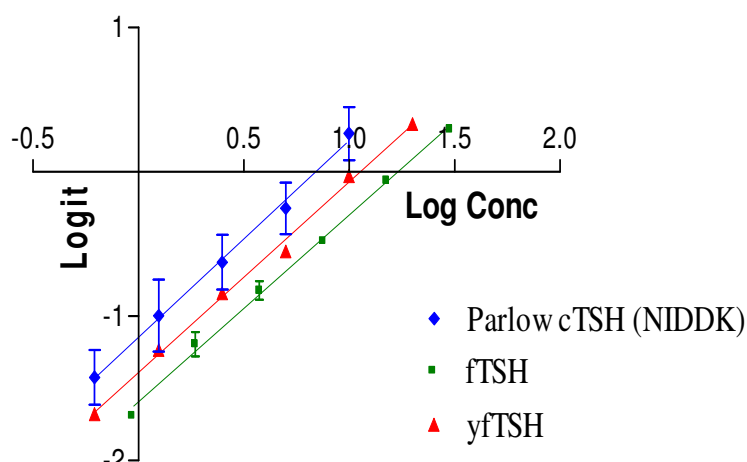
	Parlow cTSH	fTSH	yfTSH
Slope	1.36±0.2	1.29±0.03	1.32±0.02
IC50(ng/ml±SE)	3.3±1.9	4.3±1.2	3.8±1.2

Fig.5. Immunological detection using an in-house ELISA employing a polyclonal antibody and a capture monoclonal antibody developed against pituitary source cTSH detected recombinant fTSH in both heterodimeric and yoked forms. The IC50 (ng/ml±SE) values and slopes for all the three peptides were not significantly different from each other (P>0.05) indicating the immunological parallelism of recombinant fTSH with pituitary source cTSH. (n=3 for all points).
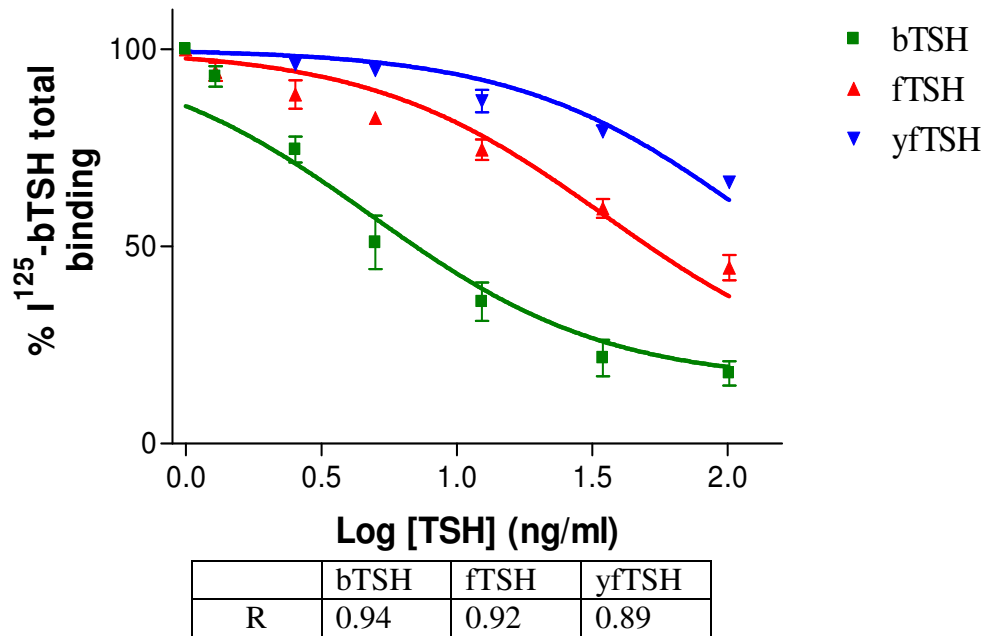
	bTSH	fTSH	yfTSH
R	0.94	0.92	0.89

Fig.6. Competitive binding assay with heterodimeric and yoked fTSH. JP09 cells were incubated with I^{125}-bTSH and increasing concentrations of unlabelled TSH to determine the binding affinity. The data was analyzed by performing a non linear regression fitting using Graphpad Prism soft ware. Each assay was performed four times, and the results are presented as the mean ± SEM. An unpaired t test was performed and analysis showed that all the groups were significantly different from each other (P<0.05). (See Table. 2)

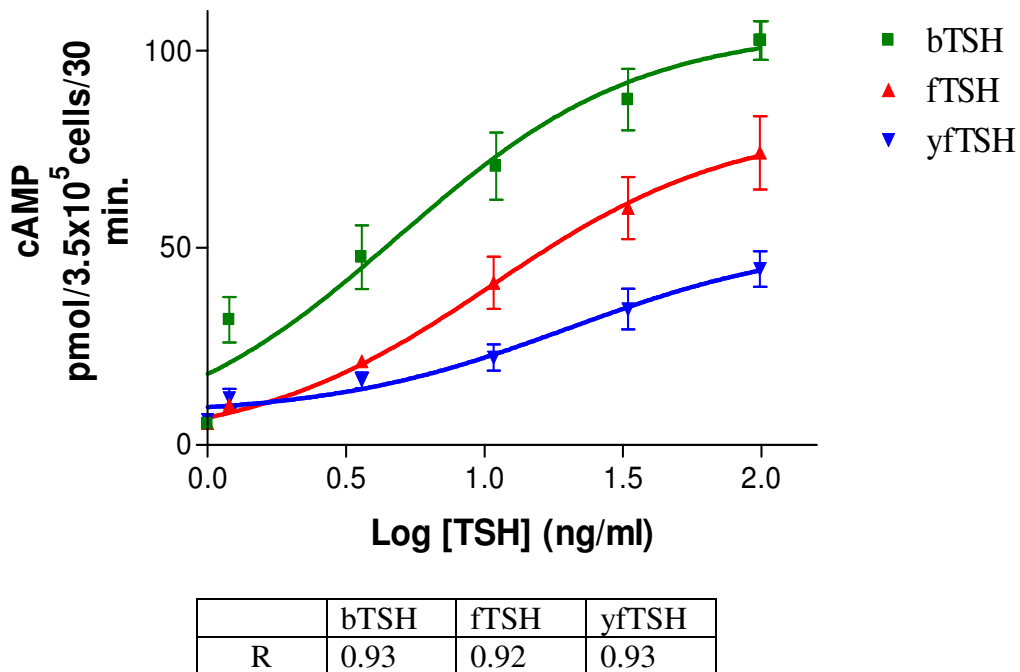|	bTSH	fTSH	yfTSH
R	0.93	0.92	0.93

91

Fig.7. Stimulation of cAMP production by heterodimeric and yoked fTSH. JP09 cells were incubated with increasing concentrations of TSH, and intracellular cAMP levels were measured by RIA. The data was analyzed by performing nonlinear regression fitting using Graphpad Prism™ software to an equation describing a sigmoidal response. Results were presented as the mean ± SEM (n=4). An unpaired t test analysis of the data showed a significant difference between bovine and feline TSH, bovine and yoked feline TSH and feline and yoked feline TSH (P<0.05). (See Table.2)
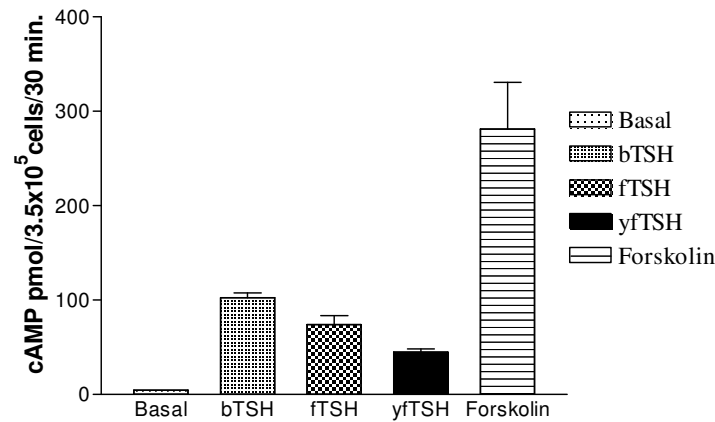

Fig.8. Basal and maximal cAMP production in JP09 cells. JP09 cells were incubated in 1 ml of Waymouth`s medium with 0.1% BSA and 0.8 mM isobutylmethylxanthine for basal cAMP concentrations, 100 ng/ml of bTSH, fTSH and yfTSH as mentioned in 2.7 for TSH stimulated cAMP levels and 0.1 mM forskolin for maximal activation of adenylate cyclase and maximal cAMP production. Results were presented as the mean ± SEM (n=4). The maximal cAMP production upon stimulation with bTSH, recombinant fTSH and yfTSH were significantly different from each other (P < 0.05).
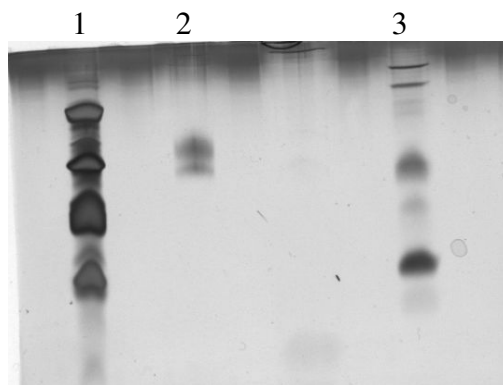

Fig.9. Silver stain of fTSH heterodimer and commercially available pituitary source cTSH standard. Lane 1 shows polypeptide protein standard, Lane 2 shows fTSH heterodimer and Lane 3 shows pituitary source cTSH.

AFFINITY CAPTURE	fTSH	yfTSH
Volume of Media (ml)	71.2 ± 2.5	82.5 ± 13.2
Conc., µg/ml	1.1 ± 0.2	0.7 ± 0.15
Initial µg in media	79.6 ± 19.1	54.3 ± 16
Flow through (µg)	19.7 ± 8.6	14.2 ± 7.5
% Captured	76.1 ± 4.6	75.1 ± 5.7
µg recovered in elutes	37.1 ± 6.9	23.0 ± 5.2
% total recovery	47.6 ± 9.8	42.9 ± 3.09
% lost in washes and dialysis	28.3 ± 6.8	31.5 ± 3.2

Table.1. Percentage recovery from immunoaffinity purification of heterodimeric and yoked fTSH. (n=4 for both constructs). The expressed media was purified by using anti-FLAG immunoaffinity chromatography. Most of the protein was obtained from elutes 1 and 2.

Binding assay	**bTSH**	**fTSH**	**yfTSH**
IC50 (ng/ml)	4.85±1.1	38.4±1.2^a	138.9±2.2^{a,b}
Adenylate cyclase assay	**bTSH**	**fTSH**	**yfTSH**
EC50 (ng/ml)	4.9±1.3	10.7±1.7^a	20.1±2.0^{a,b}
Emax (ng/ml)	105±5.4	81.2±8.1^a	51.7±6.3^{a,b}

Table.2. Summary of binding and signaling parameters of bTSH, recombinant heterodimeric and yoked fTSH. Values are the mean ± SEM of quadruplicate experiments. The EC50 values reported for each peptide correspond to their respective Emax values. 'a' represents significantly different from column one and 'b' indicates significantly different from column 2. All the three peptides behaved significantly different both in terms of binding affinity and cAMP production (P<0.05).

B/I ratio	fTSH	yfTSH
Binding assay	1	0.27
Adenylate cyclase assay	1	0.52

Table.3. Summary of Biological to Immunological ratio (B/I) of recombinant peptides. Assigning a B/I ratio of 1 to the heterodimeric form of fTSH, the B/I ratio for biological binding and cAMP production is decreased for yfTSH to 27 % of heterodimeric fTSH for binding and 52 % for adenylate cyclase activity.

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EXPRESSION OF RECOMBINANT FELINE THYROTROPIN IN BACULOVIRUS EXPRESSION SYSTEM: EFFECT OF SIALYLATION ON IMMUNOREACTIVITY AND BIOACTIVITY[3]

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Abstract

Previously cloned and sequenced feline CGA and fTSH beta subunits were expressed in insect cells using a baculovirus expression system. The signal peptide in both the constructs was replaced with honey bee mellitin signal sequence and the intron in the fTSH beta "mini gene" construct was removed with an over-lap PCR. The constructs were ligated downstream of polyhedrin promoter of baculovirus transfer vector pvl1393. The expression levels as determined by immunoreactivity (35 ± 15 ng/ml) were considerably lower than the expression levels obtained in mammalian expression system using PEAK^{TM} cells. Since the relatively low expression levels of fTSH in baculovirus expression system did not facilitate purification of large quantities of recombinant fTSH for standardization by protein assay, as an alternative, enzymatically desialylated fTSH expressed in PEAK^{TM} cells was prepared and used to characterize the bioactivity. The immunological activity of insect cell expressed fTSH and desialylated fTSH was compared with pituitary source canine TSH and non desialylated fTSH. The results from the in-house ELISA and the commercially available canine TSH assay indicated that both insect cell expressed fTSH and desialylated fTSH showed immunological parallelism to pituitary-source canine TSH and non desialylated fTSH. There was no significant change in the bioactivity of desialylated fTSH in terms of cAMP production as compared to non desialylatedfTSH. However, the biological to immunological ratio (B/I) for cAMP production increased significantly upon desialylation. Sialic acid removal did not significantly change the receptor binding affinity. Since an ideal immunoassay standard is glycosylation independent, this study supports the possibility that recombinant fTSH could be substituted for pituitary source TSH as an immunoassay standard.

1. Introduction:

TSH (Thyroid Stimulating Hormone), FSH (Follicle stimulating Hormone), CG (Chorionic Gonadotropin), and LH (Luteinizing Hormone) belong to glycoprotein hormone family. They are all structurally related heterodimers consisting of a common alpha subunit and a hormone specific beta subunit (1, 2). The molecular weight of mammalian TSH can range from 28 to 30 kilo daltons (kDa), with variation being associated with heterogeneity of the oligosaccharide chains. The alpha subunit has a molecular weight of approximately 14 kilodaltons and has two oligosaccharide units linked to asparagine residues whereas the beta subunit has a molecular weight of 15 kilodaltons and has one asparagine linked oligosaccharide unit. Like other glycoprotein hormones, TSH is highly glycosylated with N-linked complex carbohydrates, which in the human molecule, account for approximately 21% and 12% of the total weight in it's α and β chains respectively (2, 3).

Several studies have been reported on the influence of carbohydrates on the *in vivo* bioactivity of the hormone (4, 5, 6). Moreover, oligosaccharides are also required for posttranslational subunit folding and assembly, protection from intracellular degradation, and secretion of the heterodimer (4, 6, 7, 8, 9). Therefore, mammalian cells are required for recombinant glycoprotein hormone production, and attempts to achieve high level expression of glycoprotein hormones using prokaryotic systems have not been successful (7). In order to elucidate the role of carbohydrate the hormone can either be deglycosylated or can be expressed in different cell systems that have different glycosylation machinery and examine the effect of different glycosylation patterns on function. A number of studies report deglycosylation of glycoproteins chemically (10, 11), enzymatically (12), and also by using oligosaccharide processing inhibitors (13), but these modifications are not only nonspecific but may also result in

partial degradation of the protein. Therefore, for better understanding of the effect of glycosylation, recombinant feline thyrotropin (fTSH) was expressed in the baculovirus expression system. For secreted and membrane associated proteins, a signal peptide at the amino-terminus is responsible for targeting the polypeptide to the endoplasmic reticulum ER (14). The efficiency of secretion of the baculovirus system can be increased by using signal peptides of insect origin to direct the secretion of a foreign protein through the protein synthesis and secretary pathways (15, 16).

The carbohydrates of the common alpha subunit are most important for biological activity (17). As shown by deglycosylation studies of hTSH , the sialic acid residues at Asn 52 attenuate *in vitro* hormonal activity whereas they play a stimulatory role in hCG and FSH (18,5,4). Interestingly, removal of terminal sialic acid residues of recombinant hTSH increased *in vitro* bioactivy of hormone (5). The biological activity of resulting peptide showed a reduced ability to stimulate adenylate cyclase, despite still binding to its receptor with high affinity (4). There is an unusual sulfate group that terminates certain chains in TSH and LH which is not seen in CG and found only to some extent in FSH. The sulfate moiety is covalently linked to N-acetylgalactosamine in contrast to the usual terminal structure in which sialic acid is bound to galactose. Pituitary TSH and LH contain predominantly sulfated oligosaccharides, due to the presence of GalNAc-transferase and GalNAc-4-sulfotransferase in the pituitary thyrotrophs and lutrophs, and terminate mostly in SO_4-4GalNAcß1–4GlcNAcß1–2Manα (19). On the other hand hTSH expressed in CHO cells terminates with complex sialylated oligosaccharides (20, 21). However, Human Embryonic Kidney cells (HEK 293) sulfate pituitary glycoproteins to a greater extent (18, 22), because of the presence of higher levels of N-acetyl galactosamine transferase and sulfotransferase activities similar to those expressed in the pituitary gland (23).

In this study, we have tested the feasibility of using a baculovirus system to express recombinant fTSH in quantities suitable for immunological studies. Enzymatic desialylation with neuraminidase and lectin binding studies were also designed to investigate the role of sialylation in the immunoreactivity and bioactivity of recombinant fTSH. The present study describes the first attempt to express recombinant fTSH in insect cells using a baculovirus vector. Further, this is the first report investigating the carbohydrate compositional analysis of recombinant fTSH and elucidating the role of sialic acid on immunoreactivity and bioactivity. The demonstration of immunological recognition by antibodies generated against pituitary-source TSH confirmed that sialic acid does not influence immunoreactivity of recombinant fTSH. As previously reported for hTSH (24), desialylation on the other hand, increased the biological to immunological (B/I) ratio for cAMP production where as the B/I ratio for binding was unaltered. Recombinant fTSH derived from insect cells will be useful in defining structure-function relations of hormone analogs.

2. Materials and methods:

2.1. Materials:

All the restriction enzymes were from New England Biolabs, Inc (Beverley, MA). High Pure Plasmid Isolation® kit and Rapid DNA ligation® kit were purchased from Roche Diagnostics Corporation (Indianapolis, IN). One Shot[TM] cells, Sf 9 cells, Hi 5 cells, penicillin-streptomycin, Express Five[TM] media and Sf 900 media were from Invitrogen Corporation (Carlsbad, CA). Dulbecco's Modified Eagles Medium (DMEM), Waymouth`s MB752/1 medium, Nutrient Mixture F-12 Ham, puromycin, ANTI-FLAG® M2-Agarose Affinity Gel, FLAG® Peptide, ANTI-FLAG® M2 monoclonal antibody, cAMP salt, anti-cAMP polyclonal antibody, 3-isobutyl-1-methylxanthine (IBMX), Mouse IgG, Goat anti-rabbit-Peroxidase

conjugate, Tetramethyl-benzidine, neuraminidase, β-galactosidase and N-acetylhexosaminidase were all from Sigma (St. Louis, MO). The BaculoGold Transfection Kit was purchased from PharMingen (San Diego, CA). All the biotinylated lectins were obtained from Vector Laboratories (Burlingame, CA). ConA Sepharose™ was obtained from Amersham Biosciences (Piscataway, NJ). Micro BCA™ Protein Assay Kit, AminoLink® kit and West Dura Super Signal™ substrate were from Pierce (Rockford, IL). Immulon™ 4HBX strips were from ThermoLabsystems (Franklin, MA), and Canine TSH was from Scripps Laboratories (San Diego, CA) and the National Hormone and Pituitary Program (NHPP, Torrance, CA). Immobilon™ transfer membrane was purchased from Millipore (Bedford, MA). Forskolin was from Fisher Scientific (Pittsburg, PA). The automated microplate reader was from Bio-Tek Instrument®, Inc (Winooski, VT). All the chemicals for making buffers and other reagents were from Sigma (St. Louis, MO).

2.2. PCR to add Honey Bee Mellitin (HBM) signal sequence:

The signal peptide in the previously cloned and sequenced feline CGA and fTSHβ DNA constructs (25) were replaced with the honey bee mellitin (HBM) signal sequence (atgaaatt cttagtcaac gttgcccttg tttttatggt cgtgtacatt tcttacatct atgcg, Genbank ACCESSION # NP 001011607) in two PCR reactions using two forward primers one at a time. The second part of the HBM sequence was first added on to 5' end of fTSHα with forward primer 5' TTT ATG GTC GTA TAC ATT TCT TAC ATC TAT GCG TTT TGT TTT CCA 3' and subsequently the first part of HBM signal sequence was added with forward primer 5'- GAA TTC ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTA - 3'. In both cases, the reverse primer 5' – CAT AGC GGC CGC TTA CTT ATC GTC ATC – 3' was used to amplify fTSHα-FLAG. The same forward primers with the reverse primer 5' - A TGC GGC CGC TTA GAT

AGA AAC TCC TAC CAC ATC GGA CTT CT - 3' were used to replace the signal sequence in the fTSHβ DNA construct. Appropriate restriction sites, Eco RI at the 5' and Not I at the 3' end were included for the subsequent cloning into baculovirus transfer vector pvl1393.

2.3 Construction of transfer vector for fTSHα and fTSHβ:

Previously cloned and sequenced fTSHα was ligated into baculovirus transfer vector pvl1393. The baculovirus transfer vector pvl1393 was linearized by digesting with restriction enzymes *Eco RI* and *Not I*. Previously cloned and sequenced feline common alpha was digested with the same enzymes and ligated with linearized pvl1393 vector DNA downstream of the polyhedrin promoter using the Rapid DNA™ ligation kit following the manufacturer's instructions. The ligated DNA was transformed into One Shot™ cells and the positive clones were checked for correct insertion by digesting with *Eco RI* and *Not I* restriction enzymes. For development of the fTSH beta subunit construct, the intron was removed of the "mini-gene" construct with an overlap PCR before ligating into the pvl1393 transfer vector. Internal primers were designed on either side of the intron and two pieces of fTSHβ were amplified separately. To amplify the fTSHβ gene towards the 5' end of intron, the primers used were:

1. Forward primer 5'- GAA TTC ATG ACT GCT ATC TAC CTG ATG TCC - 3'

2. Reverse primer 5'- GCC ATT GAT ATC CCG TGT CAT ACA ATA - 3'

To amplify fTSHβ gene towards the 3' end of intron the primers used were:

3. Forward primer 5'- A TGC GGC CGC TTA GAT AGA AAC TCC TAC CAC ATC GGA CTT CT - 3'

4. Reverse primer 5'- TGT ATG ACA CGG GAT ATC AAT GGC AAA - 3'

The two pieces were then overlapped by using both the amplified pieces as templates and primers 1 and 2 shown above (Fig.1). The fTSH beta subunit was then cloned downstream of the

polyhedrin promoter into the pvl1393 transfer vector separately and insertion confirmed by restriction digests.

2.4. Generation of recombinant baculoviruses:

The recombinant baculoviruses were generated by using BaculoGold[TM] Transfection Kit following the manufacturer's instructions. In brief, 2 µg of recombinant pvl1393 plasmid DNA containing fTSHα and fTSHβ genes were mixed separately with 0.5 µg of linearized viral DNA and incubated for 5 minutes. One milliliter of transfection buffer was then added and the mixture overlaid on Sf9 cells cultured in a 60 mm plate in serum-free medium, and incubated at 27^0C for 4 hr. After incubation, the cotransfection plate was rinsed and 3 ml of fresh medium was replaced. The cells were allowed to grow for 4-5 days at 27^oC, and the medium was harvested. The recombinant viruses in the culture medium were amplified 2 to 3 rounds and the viral titer was determined by plaque assay. Single plaques were isolated from the plaque assay and amplified four rounds before being used for expression.

2.5. Expression of recombinant heterodimeric fTSH:

For expression, Hi 5[TM] cells were used instead of Sf9 cells. The Hi 5 cells were seeded at 2×10^7 cells/ 75 cm^2 flask and the recombinant viruses with a titer of 10^7 plaque forming units/ml were used for infection at a virus to cell ratio of 10. The recombinant viruses for fTSHα and fTSHβ were added at 1:1 ratio for generation of recombinant heterodimeric fTSH. The cells were allowed to grow at 27^0C for 4 days and the medium was harvested for testing the levels of expression. The expression levels were determined in in-house canine TSH ELISA employing a capture monoclonal antibody previously selected for ovine/canine TSH beta and a polyclonal antibody generated previously against pituitary-source canine TSH as explained previously (26).

2.6. Purification of fTSH expressed in baculovirus expression system:

The expressed media was concentrated down 40 fold using a Vivacell 250 (Vivascience AG, Hannover, Germany) gas pressurised ultrafiltration system. Anti-FLAG^{TM} column chromatography was not successful in purifying the insect cell expression medium. The FLAG^{TM} immunoaffinity tag cleavage was suspected and hence as affinity column was made by coupling AminoLink$^®$ coupling gel with anti-cTSH mAb (14H9.E2) (26) following manufacturer's instructions. The concentrated media was allowed to enter the gel bed and incubated for 60 minutes at room temperature. Bound peptide was eluted using 0.1 M glycine pH 2.5. The eluted fractions were dialyzed against 0.03 M phosphate buffer for buffer exchange.

2.7. Enzymatic deglycosylation of fTSH expressed in PEAK^{TM} cells:

Heterodimeric fTSH previously expressed in PEAK^{TM} cells was enzymatically deglycosylated by using exoglycosidase like neuraminidase. One µl of *A. ureafaciens* sialidase (10 U/ml) was added to 20 µg of recombinant fTSH in 100 µl of 50 mM sodium phosphate (pH 5.0) and incubated overnight at 37^0C. The deglycosylated fTSH is then dialyzed against 0.03M phosphate buffer (pH 7.2) for buffer exchange and ran over Con A column as described in 2.8.

2.8. Lectin binding studies:

One milliliter of Con A Sepharose was loaded into Pasteur pipettes and the column was equilibrated with TBS (20 mM Tris HCL and 0.5 M NaCl, pH 7.4) and deglycosylated fraction described in 2.7 was loaded onto the column. Con A - bound fTSH was eluted with 0.2 M methyl- α-D mannopyranoside, pH 7.4 for first four fractions and pH 4.5 for last two fractions. The elution fractions were collected into glass vials and siliconized pipette tips used at every point to minimize protein losses. All the elution fractions were dialyzed against 0.03M phosphate buffer for buffer exchange. The fractions were checked for the presence of sugars N-

acetylglucosamine, galactose and sialic acid by performing dot blots with biotinylated lectins WGA (Wheat Germ agglutinin), RCA (Ricinus Communis agglutinin) and SNA (Sambucus Nigra agglutinin) respectively. In brief, 2 µl of the elutes and non desialylated fTSH, a positive control were blotted on a nitrocellulose membrane. and incubated with biotinylated lectins (10 µg/ml) for 2 hrs and after washing with TSS-T (20 mM Tris HCL, 0.5 M NaCl and Tween20 0.05%, pH 7.4) membrane was subsequently incubated with peroxidase conjugated Streptavidin (1:1000) for one hour and the dot blot was visualized using West Dura Super Signal^{TM} substrate for 2 minutes and the dots imaged and quantified with a Fluor-S Max MultiImager^{TM} (Bio-Rad Laboratories, Inc. Hercules, CA).

2.9. Immunological detection:

The enzymatically desialylated fTSH and fTSH expressed in the baculovirus expression system were detected by in-house canine TSH ELISA employing a capture monoclonal antibody (14H9.E2) (mAb) previously selected for ovine/canine TSH beta and a polyclonal antibody (pAb) generated previously against pituitary-source canine TSH (30). Pituitary-source canine TSH (NHPP) was used as the immunoassay standard. Immunological parallelism of desialylated fTSH and fTSH expressed in the baculovirus expression system with pituitary-source canine TSH and recombinant fTSH expressed in mammalian cells (PEAK^{TM} cells) was evaluated as explained previously (26).

2.10. Bioactivity studies:

TSH Receptor Binding Assay

Competitive binding experiments were performed with a stable cell line of CHO cells expressing the recombinant human TSH receptor (rhTSHR), clone JP09 (28). Pituitary source bTSH was iodinated as described previously (29). The cells were maintained in Ham's F12 (HF-

12) media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and were maintained with geneticin selection at 400 μg per ml of media. The radioreceptor assay was performed as described previously (26). The receptor-binding activity of the deglycosylated fTSH in comparison to recombinant fTSH expressed in mammalian cells and pituitary source bTSH was determined by their ability to displace ^{125}I-bTSH from binding to hTSH receptors expressed on the JP09 cells.

TSH stimulation of JP09 cells

JP09 cells were grown in 12 well plates to confluence, the media removed and incubated in Waymouth`s MB752/1 medium with 0.1% BSA and 0.8 mM isobutylmethylxanthine (IBMX) for 15 min at 37$^{o}$C. The cells were then incubated in various concentrations of peptide for 30 min at 37$^{o}$C in Waymouth`s medium containing 0.1% BSA and 0.8 mM IBMX immediately following first incubation. The cells were also incubated with 0.1 mM forskolin to determine the TSH-independent maximal cAMP response. The incubation medium was removed, and the cells were lysed in 100% ethanol at -20°C overnight. The extract was collected, dried under vacuum, and dissolved in 1 ml of 50 mM sodium acetate pH 6.2. cAMP radioimmunoassay was performed as described previously (26).

2.11. Statistical analysis

The statistical analysis of binding assays and cAMP assays was carried out by using GraphPad PRISM program**.** All graphical data were also prepared using GraphPad Prism. Non linear regression was used to fit both binding and adenylate cyclase data to a sigmoidal curve when plotted against the log of the TSH concentration. The goodness of fit is reported as R and Emax and EC50 calculated. Significance of differences between Emax and EC50 for bTSH, fTSH and desialylated fTSH in adenylate cyclase and binding assays was calculated by

performing unpaired t tests. The slopes and intercepts for immunoreactivity data were also compared by performing unpaired t tests. *P* value of ≤ 0.05 was considered significant. Means and standard errors were reported as appropriate.

3. Results

3.1. Replacing signal sequence with HBM in both α and β-subunits and removal of intron in fTSHβ:

PCR addition of HBM to previously cloned and sequenced (25) feline CGA DNA constructs yielded a product of 420 bp when examined on a 1.5% agarose gel (Fig.1). The Intron in fTSHβ was removed by an overlap PCR as explained in 2.3 (Fig.2) and the final PCR product with HBM signal sequence of 432 bp was obtained as shown in Fig 3.

3.2. Expression of fTSH α and β-subunits and purification of heterodimeric fTSH:

Hi 5^{TM} cells were infected, separately and concurrently, with fTSHα and fTSHβ producing recombinant virus at an MOI of 10.0. Figure 4 shows a dot blot with anti-FLAG^{TM} monoclonal antibody on fTSHα expressed in Hi 5^{TM} cells. The medium from mock-infected Hi 5^{TM} cells was used as a negative control and recombinant fTSH expressed in PEAK^{TM} cells (26) was used as positive control. The expression levels of fTSHβ glycopeptide, as evaluated by the in-house ELISA reached 35±10 ng/ml (n=4) four days after infection. Infection with fTSHα and fTSHβ producing recombinant baculoviruses at a ratio of 1:1 resulted in expression of fTSH (Fig.6) as evaluated by anti-FLAG western and ELISA (maximal expression: 35±15 ng/ml, n=4). The expressed media was concentrated approximately 40 fold and purified as outlined in 2.6. A silver stain showing purified heterodimeric fTSH expressed in Hi 5 cells along with fTSH expressed in PEAK^{TM} cells is shown in Figure 5. It is clear from the silver stain that the alpha subunit is not present in equimolar amounts to the beta subunit in the purified sample. In fact, in

some preparations no alpha band was seen on silver stain and no-anti-FLAG immunoreactivity seen on western blot.

3.3. Deglycosylation and lectin binding studies:

The desialylated fTSH was checked for the absence of sialic acid by doing dot blots using different biotinylated lectins (Fig.7). The dot blots with lectins WGA, RCA and SNA showed the presence of N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and absence of sialic acid in neuraminidase treated recombinant fTSH expressed in PEAK^{TM} cells.

3.4. Immunological parallelism of fTSH expressed in insect cells and desialylated fTSH:

The average expression concentrations of fTSHβ in medium as determined by the in-house ELISA was 35 ± 10 ng/ml and that of heterodimeric fTSH was 35 ± 15 ng/ml (mean ± SEM, n=4). Both the recombinant fTSH expressed in insect cells and desialylated fTSH were also detected by the commercially available canine TSH assay (Diagnostic Products Corp., Los Angeles, CA). However, In-house assay detected about 33±1.8 % (n=3) and DPC assay detected 32.7±2.8 % (mean±SEM) (n=3) of the desialylated fTSH in both forms as standardized by BCA protein assay.

Both fTSH expressed in insect cells and desialylated fTSH showed immunological parallelism with pituitary source cTSH (NHPP) and non desialylated fTSH in our in-house ELISA (Fig.8). The IC50 (ng/ml±SE, n=3) concentrations for pituitary source cTSH (NHPP), heterodimeric fTSH expressed in PEAK^{TM} cells, fTSH expressed in insect cells and desialylated fTSH were 5.3±1.9, 7.6±1.0, 6.96±1.5 and 8.8±2.2 and slopes were 1.40±0.07, 1.47±0.08, 1.39±0.09 and 1.42±0.05 respectively. Both the IC50 values and slopes of all three peptides were not significantly different from each other.

3.5. Biological activity of recombinant fTSH:

The bioactivity of desialylated fTSH was compared with that of recombinant fTSH expressed in PEAK^{TM} cells and bovine TSH by comparison of hTSH receptor binding inhibition and stimulation of adenylate cyclase activity. As shown in Fig. 9, desialylated fTSH was also able to inhibit the binding of ^{125}I-bTSH to the hTSH receptor in JP09 cells with an affinity not significantly different from non desialylated fTSH (P>0.05) (Table 1).

The bioactivity of desialylated fTSH was evaluated by potency to stimulate adenylate cyclase in the human TSH receptor-expressing JP09 cells. Desialylated fTSH was 2.3 -fold less potent than bovine TSH in stimulating cAMP production (Fig.10). However, desialylation did not alter the potency for stimulation of adenylate cyclase (Table 1). The EC50 (ng/ml) values for desialylated and non - desialylated fTSH are not significantly different from each other (Fig. 8). The Biological to Immunological ratio (B/I) for desialylated fTSH for binding was 96 % of that of fTSH and is not significantly different from fTSH whereas, for cAMP production, the B/I ratio was 16 % more for desialylated fTSH than non desialylated fTSH and is significantly different (P<0.05) (Table. 2).

4. Discussion

The present study describes the first attempt to express previously cloned and sequenced fTSHα and fTSHβ genes (25) in insect cells using a baculovirus expression vector. Furthermore, this study reports on the effect of glycosylation on the immunoreactivity and also bioactivity of the recombinant fTSH.

In the baculovirus system, fTSH was secreted but the expression levels determined by immunoreactivity (35 ± 15 ng/ml) were considerably lower than the expression levels obtained in mammalian expression system (~ 1 µg/ml) using PEAK^{TM} cells (26). In contrast, others had

shown that the expression levels of recombinant human TSH were higher in the insect cells than the levels obtained in eukaryotic cells (30). A study reported the substitution of natural signal sequences with the signal sequences from honeybee mellitin promotes a high level of expression of a glycosylated form of gp120 and efficient secretion (16). However, use of honeybee mellitin signal peptide does not ensure proper processing of recombinant proteins (31).

Insect cells lack the capacity to process carbohydrate residues to complex-type, terminally sialylated oligosaccharides, especially when a vector like pvl1393, containing the very late viral polyhedrin promoter, was used for expression. As a result, glycoproteins produced under such conditions usually contain high mannose-type precursor oligosaccharides (32, 33). In contrast, hTSH expressed in CHO cells terminates with complex, sialylated oligosaccharides (20, 21). Also, other studies that analyzed insect cell-expressed glycoprotein carbohydrates by compositional analysis suggested that the glycosylation machinery of insect cells does not possess the same capacity to process N-linked oligosaccharides to complex-type carbohydrates as mammalian cells (34).

The relatively low expression levels of fTSH in baculovirus expression system together with the possible cleavage of the immunoaffinity tag did not facilitate purification of large quantities of recombinant fTSH for standardization by protein assay. The observation that the FLAG[TM] immunoaffinity tag was being cleaved also suggests problems with intracellular processing or suggests the presence of protease cleaving the FLAG peptide. Parenthetically, even with different baculovirus vectors and or yoking of the subunits, expression levels were no higher for equine or canine TSH (unpublished data). Insect cells lack the capacity to process sugar moieties to complex sialic acid terminated oligosaccharides especially when a very late promoter like polyhedrin is used to express genes of interest, resulting in production of

glycoproteins with high mannose-type precursor oligosaccharides (34, 34, 38). Therefore, as an alternative to insect cell expressed fTSH, we prepared enzymatically desialylated fTSH expressed in mammalian expression system. Complete deglycosylation without denaturing the protein was not possible because the oligosaccharides on the β-subunit in both heterodimeric conformation and free form have been reported to be easily accessible whereas, the sugars on α-subunit are hidden by subunit interaction (36). Moreover it is well-known that under *in vitro* conditions, sialic acid appears to be the major factor affecting the bioactivity of recombinant TSH (21). *In vivo* sialylation influences metabolic clearance rate as removal of the terminal sialic acid residues markedly reduces the half-life of TSH in the circulation by enhancing its binding to hepatocyte lectins responsible for clearance from the bloodstream (37).

Lacking pituitary source fTSH, the immunological activity of insect cells expressed fTSH and desialylated fTSH was compared with pituitary source canine TSH (NHPP standard) and non desialylated recombinant fTSH. The results from the in-house ELISA and the commercially available canine TSH assay indicated that desialylated fTSH showed immunological parallelism to pituitary-source canine TSH and recombinant fTSH supporting the conclusion that the immunological reagents in both of these assays are not sialylation dependent. These results do not rule out the immunological and biological impact of other sugars. Glycosylation of plasma hormones has been shown to be immunologically distinct from pituitary stock because the ratio of circulating glycoforms appears to vary according to the pathophysiology of the pituitary axis (38), thus playing an important role in immunoreactivity with many antibodies. For our recombinant fTSH expressed in PEAK[TM] cells to be used as a standard in feline specific TSH assay, the immunoreactivity would ideally be independent of glycosylation. Since recombinant fTSH, even after desialylation, showed immunological

parallelism with pituitary-source cTSH, it supports the possibility that recombinant fTSH could be substituted for pituitary source TSH as an immunoassay standard.

Previous researchers have shown the *in vitro* activity of hTSH expressed in insect cells to be higher than the hTSH expressed in Chinese Hamster Ovary cells (CHO). Also the *in vitro* activity of CHO-hTSH increased upon enzymatic desialylation indicating that the terminal sialic acid residues are responsible for the reduced *in vitro* signal transduction (30). However, there was no significant change in the bioactivity of desialylated fTSH in terms of cAMP production as compared to non desialylated fTSH as HEK 293 cells both sulfate and sialylate glycoproteins as opposed to CHO cells which primarily sialylated. Accordingly, desialylation will not have as great an effect on glycoproteins expressed in HEK 293 cells as those expressed in CHO cells. Infact, the desialylated recombinant human TSH expressed in CHO cells was more potent than pituitary hTSH whereas, the activity of pituitary hTSH was not altered by neuraminidase digestion, since it contains a minimal amount of sialic acid (39).

Oligosaccharides modulate glycoprotein hormone signal transduction at a post-receptor binding step (8, 9). The binding affinities for desialylated fTSH and non - desialylated fTSH are not significantly different from each other whereas there is a significant difference between binding affinity for pituitary source bovine TSH and desialylated fTSH. Despite the differences in *in vitro* activity, insect cell - expressed recombinant hTSH and CHO-hTSH bound to the TSH receptor with similar affinity (30). Similarly, this observation was in good agreement with studies on hTSH expressed in glycosylation mutant cell lines, lacking N-acetyl glucosamine transferase I activity (18) and also in study using sequential enzymatic deglycosylation (39). In contrast, the biological to immunological ratio, an index of the overall potency of circulating TSH *in vitro* (40), is decreased for binding assay with desialylation, however; the decrease is not

significant. The B/I ratio of fTSH increased significantly with desialylation for cAMP production

by about 16 % and is in agreement with a previous report that indicated that desialylated hTSH

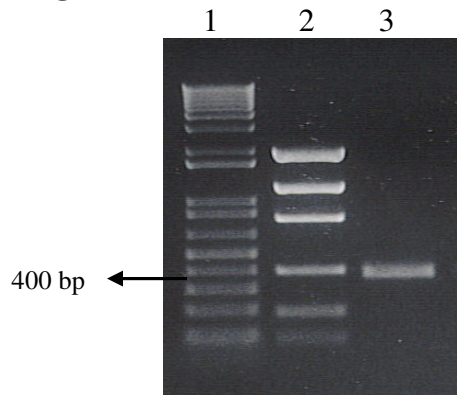showed an increased B/I ratio for cAMP production (24).

Figures:

Fig.1. The signal sequence in fTSHα was replaced with honey bee melittin signal sequence and the resulting DNA fragment was amplified in PCR yielding a product of 420 bp. Lane 1 is a 1Kb plus DNA ladder and lane 2 is low mass DNA ladder. Lane 3 shows fTSHα with HBM signal sequence.
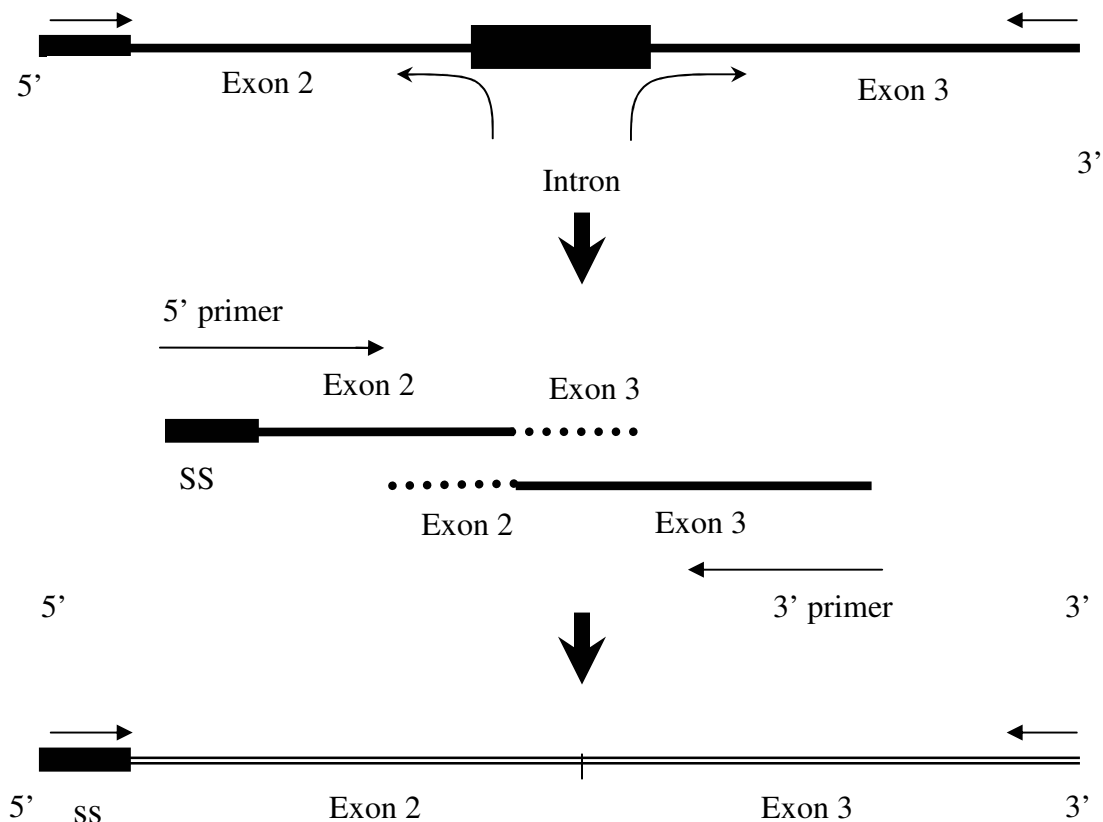
Fig.2. Overlap PCR to remove intron in fTSHβ subunit. Two internal primers were designed to amplify exons 2 and 3 separately and two exons were then joined together by dong an overlap PCR using both the pieces as template DNA and using 5' and 3' primers as explained in 2.3.
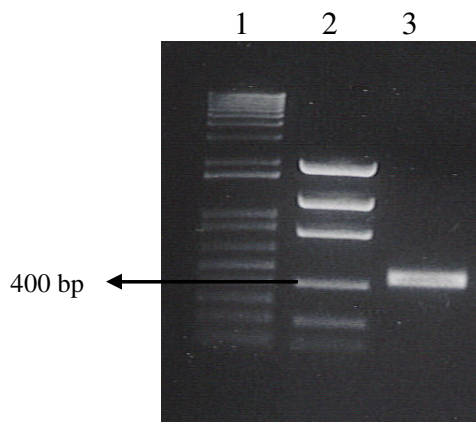

Fig.3. The Intron in fTSHβ was removed by doing an over lap PCR as described in 2.4. The signal sequence was replaced with the honey bee Melittin signal sequence and the final PCR yielded a product of 432 bp. Lane 1 is a 1Kb plus DNA ladder and lane 2 is low mass DNA ladder. Lane 3 shows fTSHβ (without intron) with HBM signal sequence.

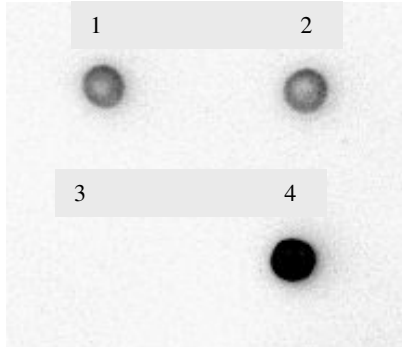Fig.4. Dot blot for fTSHα expressed in insect cells. 2µl of fTSHα expressed media is blotted onto nitrocellulose membrane and incubated with anti Flag monoclonal antibody at 5µg/ml as primary antibody and a 1:3000 of secondary goat anti-mouse HRP antibody. Dots 1 and 2 show fTSHα expressed in media, dot 3 is media from mock infection of HI 5 cells and dot 4 fTSH expressed in PEAK^TM cells.
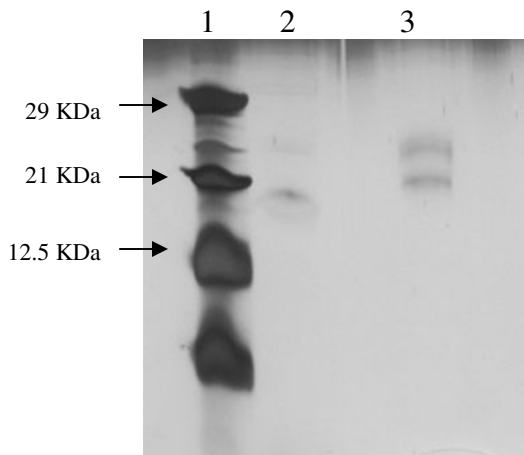

Fig.5. Silver stain of heterodimeric fTSH expressed in baculovirus expression system. Lane 1 shows polypeptide protein standard, Lane 2 shows purified fTSH expressed in High Five cells, Lane 3 shows fTSH heterodimer expressed in PEAK^TM cells.
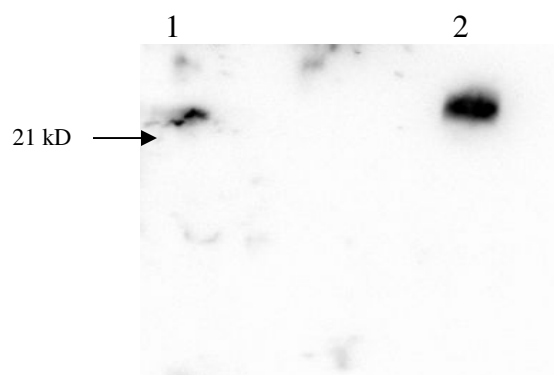
Fig.6. Western blot analysis of purified recombinant fTSH expressed in baculovirus expression system using anti Flag monoclonal antibody at 5µg/ml as primary antibody and a 1:3000 of secondary goat anti-mouse HRP antibody. Lane 1 shows CGA subunit in baculovirus expressed fTSH as the FLAG tag is present only on CGA subunit and Lane 2 shows the fTSH expressed in PEAK^{TM} cells.
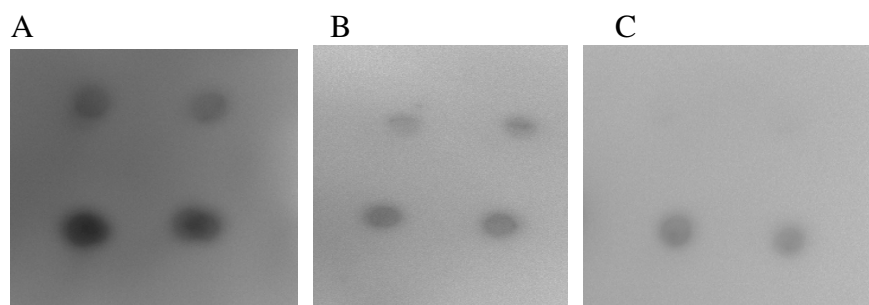

Fig.7. Dot blots with lectins A. WGA B. RCA and C. SNA. Second row in all the three blots shows recombinant heterodimeric fTSH indicating the presence of sugars corresponding to lectins like N-acetylglucosamine for WGA, N-acetylgalactosamine for RCA and sialic acid for SNA. First row in all blots shows Neuraminidase treated fTSH showing the absence of signal when blotted with SNA.
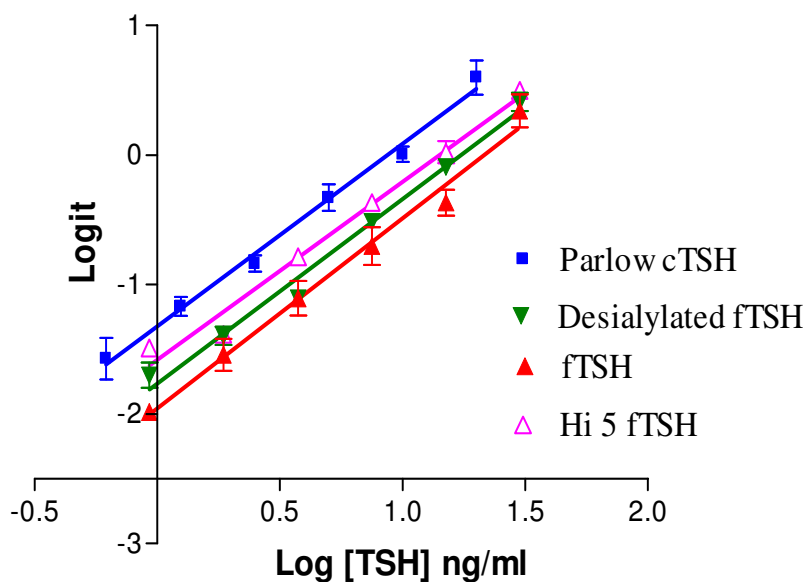
	cTSH	fTSH	Hi 5 fTSH	Desialylated fTSH
Slope	1.40±0.07	1.47±0.08	1.38±0.09	1.42±0.05
IC50 (ng/ml)	5.09±1.3	7.65±1.4	6.96±1.5	8.85±1.2

Fig.8. Immunological detection using an in-house ELISA employing a polyclonal antibody and a capture monoclonal antibody developed against pituitary source cTSH detected both Hi 5 expressed fTSH and desialylated fTSH. The IC50 (ng/ml±SE) values and slopes for all the three peptides were not significantly different from each other (P>0.05) indicating the immunological parallelism of fTSH expressed in insect cells and desialylated fTSH with pituitary source cTSH. (n=3 for all points).
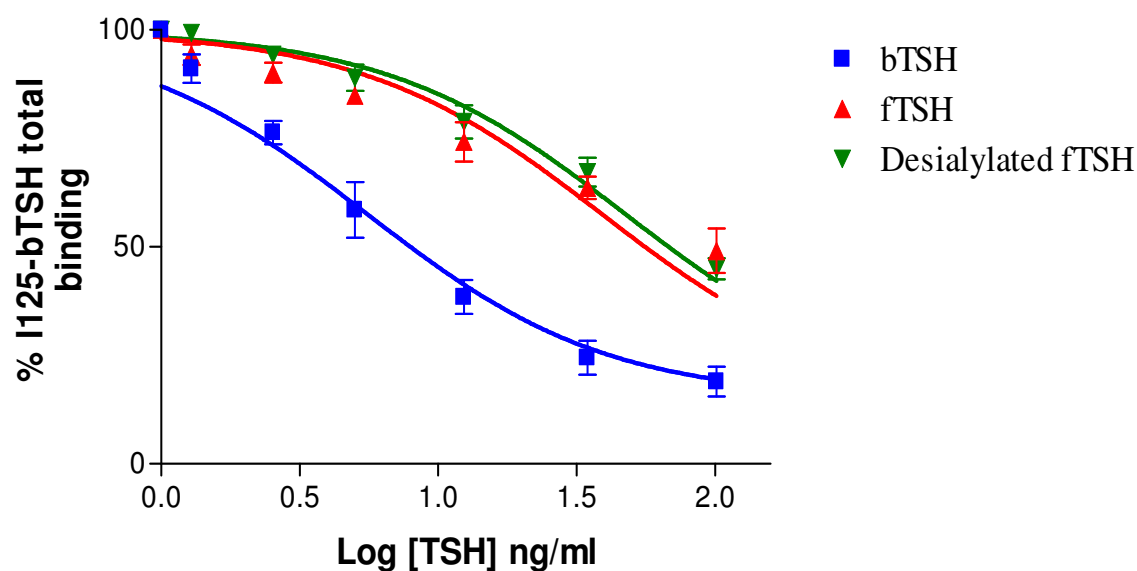
Fig.9. Competitive binding assay with desialylated fTSH. JP09 cells were incubated with I^{125}-bTSH and increasing concentrations of unlabelled TSH to determine the binding affinity. The data was analyzed by performing a non linear regression fitting using Graphpad Prism soft ware. Each assay was performed four times, and the results are presented as the mean ± SEM. An unpaired t test was performed and analysis showed that fTSH and desialylated fTSH groups were not significantly different from each other (P>0.05). (See Table. 1)
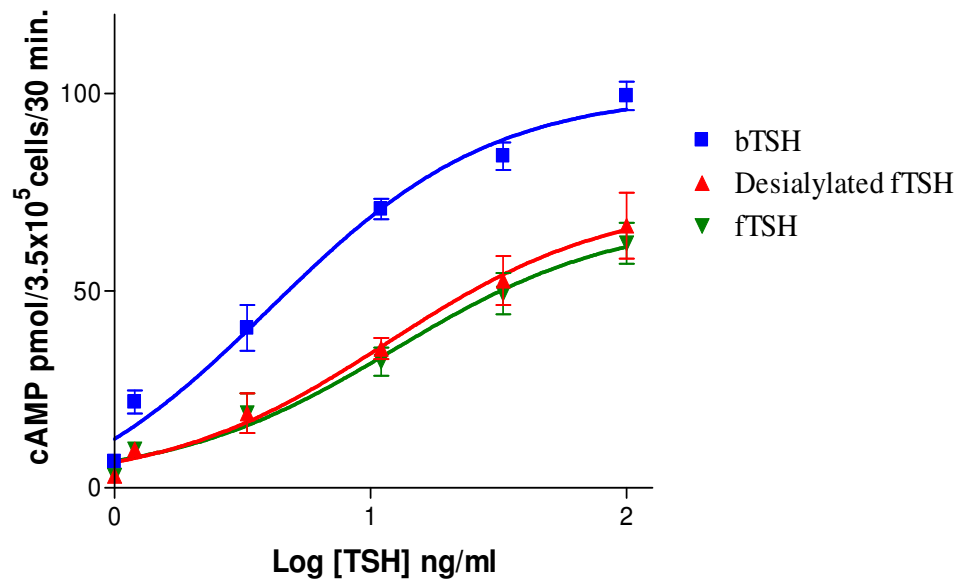

Fig.10. Stimulation of cAMP production by desialylated fTSH. JP09 cells were incubated with increasing concentrations of TSH, and intracellular cAMP levels were measured by RIA. The data was analyzed by performing nonlinear regression fitting using Graphpad Prism™ software to an equation describing a sigmoidal response. Results were presented as the mean ± SEM (n=4). An unpaired t test analysis of the data showed a significant difference between bovine and feline TSH, bovine and desialylated feline TSH (P<0.05) and no significant difference between feline and desialylated feline TSH (P>0.05) (See Table.1)

Binding assay	bTSH	fTSH	Desialylated fTSH
IC50 (ng/ml)	5.6±0.8	39.95±2.2^a	47.13±6.2^a
R	0.98	0.99	0.99
Adenylate cyclase assay	bTSH	fTSH	Desialylated fTSH
EC50 (ng/ml)	4.0±1.3	12.42±1.5^a	11.7±1.4^a
Emax (ng/ml)	99.2±5.4	68.0±8.1^a	72.8±6.3^a
R	0.97	0.95	0.94

Table.1. Summary of binding and signaling parameters of bTSH, recombinant fTSH and desialylated fTSH. Values are the mean ± SEM of quadruplicate experiments. The EC50 values reported for each peptide correspond to their respective Emax values. 'a' represents significantly different from column one and 'b' indicates significantly different from column 2. Desialylated fTSH behaved significantly different from pituitary source bTSH both in terms of cAMP production and binding affinities (P<0.05) whereas, there is no significant difference between desialylated and non desialylated fTSH (P>0.05) both in terms of binding affinities and cAMP production.

B/I ratio	fTSH	Desialylated fTSH
Binding assay	1	0.96
Adenylate cyclase assay	1	1.16^a

Table.2. Summary of Biological to Immunological ratio (B/I). Assigning a B/I ratio of 1 to the heterodimeric form of fTSH, the B/I ratio for biological binding is decreased for desialylated fTSH by 4 % than fTSH and is not significantly different. For cAMP production, the B/I ratio is increased for desialylated fTSH to more than 16 % than fTSH and is significantly different (P<0.05). 'a' represents significantly different from column one.

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SUMMARY AND DISCUSSION

Thyrotropin (TSH) along with Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Chorionic gonadotropin (CG) belong to the glycoprotein hormone family. All these glycoprotein hormones share a common α subunit, and a hormone-specific β subunit. The α and β subunits associate noncovalently and subunit assembly is necessary for the biological activity of the hormone (Pierce and Parsons, Annu Rev Biochem, 50:465-495, 1981). It has been reported that the α-subunit carries species-specific antigenic determinants, while the β-subunit possess hormone-specific evolutionarily conserved determinants (Vaitukaitis et al., Endocrinology 91:1337-1342, 1972). TSH plays an important role in maintaining the normal circulating levels of thyroid hormones through its action on the thyroid gland.

Hyperthyroidism is one of the most common endocrine disorders of cats, affecting mainly middle to old aged cats and the lack of a feline-specific TSH assay has hindered early diagnosis. Since a commercially available pituitary source of fTSH does not exist, we reasoned that cloning, sequencing and expression of recombinant fTSH would allow development of a feline specific TSH immunoassay. Feline pituitary RNA was extracted and an RT-PCR followed by a PCR was done to amplify feline CGA gene along with signal peptide. The feline CGA gene encoded a 96 amino acid protein and is highly conserved between cat and dog with only 4 residues being different in the secreted protein. Very high homology of 96.8% was observed between the feline and tiger CGA gene and 91.6% homology between feline and bovine CGA subunits. The sequence homology between feline and human α subunits is less than 70% because of the four codon deletion in human CGA gene. The ten cysteine residues which are involved in

the formation of five intramolecular disulphide bonds are completely conserved in both number and position among all species. The fTSH α subunit sequence has been submitted to Genbank and is available with accession number AY972823.

For the fTSHβ gene feline genomic DNA was isolated and the "mini-gene" construct along with signal peptide was amplified. The fTSHβ gene encoded for a protein of 118 amino acids and showed high sequence homology of 94% with dog and low sequence homology of 88% with human. The 12 cysteine residues, involved in formation of six disulphide bridges are conserved in feline, canine, human, bovine and equine TSHβ. Intron 2, is not a highly conserved sequence containing 412 bp in feline, 448 in human and 450 bp in canine. The peptide sequence CAGYC is highly conserved in TSHβ, LHβ, hCGβ and FSHβ and is thought to be important in subunit combination (Hayashizaki et al., EMBO J 8:2291, 1989). The fTSHβ subunit sequence has been submitted to Genbank and is available with accession number AY972824.

There are two N-linked glycosylation sites located at Asn56 and Asn82 in feline CGA and are conserved in all other species. The unique N-glycosylation site at Asn23, followed by two threonine residues in the mature TSH beta peptide, is also well conserved in all the species. The oligosaccharide chains attached to the protein moiety have been shown to play an important role in TSH subunit folding and biologic activities (Magner, J.A. Endocr. Rev. 11:345-385, 1990). This is the first report of the sequence of the genes encoding the α and β glycoprotein subunits of TSH in the cat. It is clinically relevant that the predicted amino acid homologies of feline CGA and fTSH beta subunits with dog TSH sequences were high 96 % and 94 % respectively. Such homology likely accounts for the recognition of TSH in cat samples by antibodies in canine TSH assays. However, it is interesting to note that even though the predicted

amino acid homology of fTSH beta subunit with human TSH beta was 88 % the human TSH assay does not detect fTSH.

The correct assembly of the alpha and beta subunits noncovalently into heterodimers is an obligatory step for the formation of biologically active hormone. Single chain or yoked analogs of hTSH and hCG were constructed with the C-terminus of the β subunit fused using a yoking peptide, CTP (carboxy terminal peptide) to the N-terminus of the α-subunit. CTP contains several serine and proline residues and thus lacks significant secondary structure, apparently, permitting the α subunit to assume proper orientation with respect to the β subunit. The approach has resulted in the generation of hormones with increased *in vivo* stability and activity (Grossmann et al.,   J Boil Chem. 272:21312-21316, 1997). The tandem order of subunits,   β-CTP-α, was chosen based on studies suggesting the importance of the N-terminal region of hCGβ and C-terminal region of the α-subunit in receptor binding and activation. From the standpoint of a strategy for recombinant protein expression and purification, this approach also ensures equimolar expression, detection and purification of the single chain glycoprotein. Therefore, the nucleotide sequence encoding the full length alpha subunit excluding the signal sequence was fused in frame with the CTP to the 3' terminus of the beta subunit containing the coding sequence of the signal sequence and the secreted subunit. We demonstrated that the yoked fTSH may act as a substitute for either pituitary source fTSH or recombinant heterodimeric fTSH as a standard in immunoassay. The development of a permanent cell line expressing yfTSH facilitates production of this recombinant peptide. Furthermore, recombinant feline TSH in heterodimeric or yoked forms may be useful for the enhancement of radioiodide uptake during thyroid ablative therapy.

The ideal expression system for a mammalian glycoprotein is a mammalian cell line capable of post-translational glycosylation with a mammalian pattern of sugars. The most commonly used Chinese Hamster Ovary (CHO) cells lack sulfotransferase and N-acetyl galactosamine transferase resulting in the expression of glycoproteins that are not sulfated to the same degree as pituitary-derived glycoproteins. However, in these studies we expressed the recombinant hormones in human Embryonic Kidney cells (HEK 293) which have been shown to sulfate pituitary glycoproteins to a greater extent (Grossmann et al., J. Biol. Chem. 270:29378–29385, 1995), because of the presence of higher levels of N-acetyl galactosamine transferase and sulfotransferase activities to those expressed in the pituitary gland. The feline CGA-FLAG, fTSH beta and yoked fTSH constructs were ligated into pEAK10 expression vector separately and expressed in PEAK^{TM} cells. The molecular weights of feline CGA subunit, beta subunit and yfTSH with glycosylation were 20.4, 17, and 45 kilodaltons, respectively. This is the first report of the *in vitro* expression and purification of recombinant feline thyrotropin in heterodimeric and yoked forms.

Expression levels of about 1 µg/ml were obtained for both heterodimeric and yoked forms of fTSH and the levels obtained were considerably higher than the concentrations obtained with recombinant hTSH expression using CHO and other eukaryotic cells (Grossmann et al., Mol Endocrinol 10:769-779, 1996). Furthermore, the synthesis of yoked fTSH indicates that the linked alpha and beta subunits presumably folded with formation of disulfide bonds and a stable conformation. However, in a manner similar to pituitary thyrotrophs, equimolar expression of feline CGA and fTSH beta subunits did not occur owing to a single EF-1α promoter which facilitates expression of only one gene at a time in the expression vector pEAK10. The 'free alpha' subunit which has a slightly higher molecular weight due to an additional O-linked

oligosaccharide is removed by percolating the media though an affinity column made with agarose beads linked to a beta-subunit specific monoclonal antibody (14H9.E4), which captured heterodimeric fTSH alone removing the free alpha subunit and increasing the purity of the recombinant fTSH preparation.

Lacking pituitary source fTSH, the immunological activity of fTSH in both forms was compared with pituitary source canine TSH (NHPP standard) and the results indicated that both forms of fTSH showed immunological parallelism to pituitary-source TSH supporting the possibility that recombinant fTSH could be substituted for pituitary source TSH as an immunoassay standard. However, the in-house ELISA detected both heterodimeric and yoked forms of the recombinant protein to an extent of about 40 % as standardized by BCA protein assay and purity determined by silver stain. Surprisingly, the recombinant glycoproteins showed reduced receptor affinity and diminished maximal effect on the bioactivity assay.

The yoked fTSH exhibited 24% of the potency of pituitary source bTSH in adenylate cyclase assays and only 3.4 % of the potency of pituitary source bTSH at inhibiting ^{125}I-bTSH binding in JP09 cells. The heterodimeric fTSH showed 45 % and 12.5 % of the potency of pituitary source bTSH in adenylate cyclase and binding assays respectively. Yoked fTSH showed a decreased biological to immunological ratio compared to heterodimeric fTSH. However, receptor binding activity and *in vitro* bioactivity of purified pituitary isoforms have been previously shown to vary over a five to eight-fold range when expressed on the basis of defined protein content. Glycosylation and/or subtle tertiary structure differences might also contribute to lower potency. Other studies have demonstrated that heterodimeric and yoked forms of glycoproteins expressed in the baculovirus expression system exhibited higher binding affinities than native forms presumably due to differences in glycosylation pattern which tend to

favor less sialylation. The demonstration of the immunological parallelism with pituitary source canine TSH confirms that the recombinant glycoprotein may be used to standardize and improve clinical assays for feline TSH, and even be utilized to prepare feline-specific immunoreagents. Since the concentrations of the recombinant peptides never achieved the same maximal binding inhibition nor maximal effect, we postulate that these glycoproteins may act as partial agonists of human TSH receptor compared to pituitary-source bTSH. The reduced activity in receptor binding might be accounted for species differences as well, since it was reported that homologous glycohormones do not necessarily have the highest affinity for the receptor in the same species.

Ultra-sensitive immunoassays will give misleading results when the glycosylation pattern of the standard used in immunoassay is different from pituitary and circulating isoforms. For recombinant fTSH expressed in PEAK^{TM} cells to be used as a standard in feline specific TSH assay, the immunoreactivity would ideally be independent of glycosylation. The glycosylation machinery of insect cells does not possess the same capacity to process N-linked oligosaccharides to complex-type carbohydrates as mammalian cells resulting in secretion of high mannose-type precursor oligosaccharides (Luckow V.A, Curr Opin Biotechnol 4:564–572, 1993).

For expression of recombinant fTSH in baculovirus expression system, the intron in fTSH beta subunit was removed with an over lap PCR and the signal peptide on both CGA and beta subunits was substituted with insect cell specific honey bee melittin signal sequence. The expression levels of only about 35 ng/ml were achieved as opposed to 1 µg/ml with PEAK^{TM} cells. This is in contrast to other studies which reported expression of recombinant hTSH at 1µg/ml in insect cells and 10-50 ng/ml of recombinant hTSH in mammalian cells. It is

interesting to note that the beta subunit expression seemed to dominate in insect cells, but it appeared that the alpha subunit may have been partially degraded to release the C-terminal FLAG tag. Since the relatively low expression levels of fTSH in the baculovirus expression system did not facilitate purification of large quantities of recombinant fTSH for standardization by protein assay, as an alternative, enzymatically desialylated fTSH expressed in PEAK^{TM} cells was prepared and used to characterize immunoreactivity and bioactivity. Complete deglycosylation without denaturing the protein was likely not possible because the oligosaccharides on β-subunit in both heterodimeric conformation and free forms have been reported to be more easily accessible whereas, the sugars on α-subunit are hidden by subunit interaction. Moreover it is well known that under *in vitro* conditions, sialic acid appears to be the major factor affecting the bioactivity of recombinant TSH (Szkudlinski et al., Endocrinology 133:1490–1503, 1993).

The immunological activity of insect cell - expressed fTSH and desialylated fTSH was compared with pituitary source canine TSH (NHPP standard) and non - desialylated fTSH and the results indicated that both insect cell expressed fTSH and desialylated fTSH showed immunological parallelism to pituitary-source TSH and non desialylated fTSH supporting the possibility that the antibodies used in our in-house ELISA and the commercially available canine TSH assay are not glycosylation dependent. There was no significant change in the bioactivity of desialylated fTSH in terms of cAMP production as compared to non - desialylated fTSH. However, the biological to immunological ratio (B/I) for cAMP production increased significantly upon desialylation. The receptor binding affinity was also not significantly changed by desialylation and in agreement with previous reports for human TSH.

The cloning of feline CGA and beta subunits enabled the use of the PEAK^{TM} expression system, which gives higher expression. The recombinant fTSH expressed in PEAK cells behaved immunologically parallel to pituitary source canine TSH and changes in glycosylation pattern did not affect the immunological recognition in both in-house ELISA and commercially available canine TSH assay. However the biological to immunological ratio for cAMP production was significantly increased upon desialylation of fTSH expressed in PEAK^{TM} cells indicating that sialic acid attenuates *in vitro* bioactivity in terms of cAMP production. The receptor binding affinity was not affected upon neuraminidase treatment. Therefore, the recombinant glycopeptide provides an ideal feline-specific TSH immunoassay standard which is not at least sialylation dependent. However, more studies like determining the carbohydrate compositional analysis, bioactivity studies using pituitary source fTSH and recombinant fTSH expressed in insect cells and the generation of higher titer antibodies specific for fTSH will further advance the goal of development of an ultrasensitive immunoassay for feline TSH.