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Cloning and Sequencing of the Equine Testicular Follitropin Receptor

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Summary: To investigate the possibility that specific structural determinants within the equine follitropin receptor (eFSHR) are critical to the enhanced specificity of this receptor compared to other FSHRs, we used the RACE-PCR technique to clone the eFSHR from equine testis. Sequence analysis revealed that the eFSHR is highly homologous to other mammal FSHRs, but it presents 10 unique amino acid residue replacements in the extracellular domain. Furthermore, a potential N-glycosylation site was detected at a position not encountered in other receptors. Northern blot analysis identified three transcripts of 4.2 kb, 2.3 kb and 1.0 kb in horse testis.

Follitropin (FSH) and lutropin (LH) are pituitary glycoprotein hormones that regulate functions essential for ovulation and spermatogenesis (1,2). Furthermore, primates and equidae possess a placental chorionic gonadotropin (CG) which acts as an LH. These hormones bind specifically to distinct high affinity receptors, present on gonads, which belong to the subfamily of the GTP-binding protein (G-protein) coupled receptors, characterized by a transmembrane (TM) domain and a cytoplasmic carboxyl-terminal tail (3). Glycoprotein hormone receptors are characterized by the presence of a large, glycosylated, extracellular (EC) amino-terminal domain, displaying the structural features of a leucine-rich proteoglycan and corresponding to the high affinity binding site for the hormone.

The molecular basis of the specificity of binding of glycoprotein hormones to their receptors remains challenging (4). Even though each hormone presents exquisite specificity for its corresponding receptor, the dual LH/FSH nature of both the equine CG (eCG) and the equine LH (eLH) has been recognized for a long time (5-8). Indeed, these two hormones display high FSH receptor-binding activity in various mammal species, whereas their FSH binding activity is much lower in horses. Structural properties unique

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Abbreviations used: LH, lutropin; CG, choriogonadotropin; FSH, follitropin; e, equine.

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to eCG and eLH might be in part responsible for their dual activity (9,10). However, the eFSH receptor (eFSHR) is more specific than the receptors of other species whose LH lack the unique features of the equine hormones (11). Thus, in order to investigate whether the eFSHR presents structural characteristics which would explain part of the dual activity of eCG/eLH, we designed experiments for cloning and sequencing the eFSHR.

MATERIALS AND METHODS

Isolation of equine testicular mRNA - Polyadenylated RNA was isolated from equine testes collected in a breeding stud (Lisieux, France) and purified on oligo-dT cellulose for further analysis.

Preparation of an equine FSHR specific probe - A 0.45 kb eFSHR probe was generated by the polymerase chain reaction (PCR) using degenerated primers corresponding to a human/rat consensus sequence. Two successive rounds of amplification with nested primers were performed. The FSHR specificity of the PCR fragment was confirmed after Southern blotting analysis with a human FSHR probe. This PCR fragment was purified by electrophoresis on a low melting agarose gel.

Construction of an equine testis cDNA library - Five µg equine testis polyA+RNA were reverse transcribed in the presence of linker primer. The ends of the cDNA were blunted with T4 DNA polymerase and EcoRI adaptors were added. The cDNA was ligated to the Unizap XR vector and packaged with Stratagene Gigapack Gold (Stratagene, La Jolla, CA). The library contains $1.5x10^6$ independent recombinant clones. This library was screened with the equine specific random priming [$\alpha^{32}P$] labeled probe. Duplicate nitrocellulose filters (Schleicher & Schull, Dassel, Germany) were hybridized overnight at 42° C and washed twice in 2XSSC-0.1%SDS for 15 min at room temperature and 0.1XSSC-0.1%SDS at 50°C twice for 15 min. Several clones were identified and transferred to a pBluescript phagemid for sequence analysis.

RNA blot analysis - Five µg equine testis polyA+RNA were subjected to electrophoresis in 1% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N-Amersham, Les Ulis, France) by capillarity in 10X SSC. The membrane was hybridized with the specific probe mentioned above and the extracellular domain probe.

5' RACE analysis - The different clones were isolated from the equine testicular library lacking the 5' end of the cDNA sequence. The 5' RACE (Rapid Amplification of cDNA Ends) method (5' RACE System GIBCO BRL, Gaithersburg, MA) was used for cloning full length eFSHR cDNA (12). First strand cDNA synthesis was primed using a sequence-specific (5' AGGCTGTGATGCTGCTGGCT 3') anti-sense primer. Following purification of the cDNA, a synthetic homopolymer anchor region was added to the cDNA using Terminal transferase. PCR amplification of tailed cDNA was then performed using a second nested gene-specific primer (5' AATCTACCTGTTGCTCATAGC 3'), which annealed upstream of the reverse transcription primer and an anchor primer complementary to the homopolymer tail. A second round of amplification was necessary using nested primers (5' GGAACATCATAGTGCTGGTGA 3' /Universal Amplification Primer). Following PCR, 10 µl of each reaction were electrophoresed on a 1% agarose gel in 1XTBE stained in ethidium bromide. Southern blotting analysis was used to confirm amplification of specific sequences. PCR products were cloned directly in pT7blue vector without enzymatic modifications (Novagen, Madison, WI). Different clones were isolated for sequence analysis.

DNA sequence analysis - Sequencing was performed on double-stranded DNA by the dideoxy chain termination method using a T7 sequencing kit (Pharmacia, Guyancourt, France). T3 and T7 primers were used on pBSK phagemid, U19 and T7 on pT7 blue plasmid to sequence the 5' and the 3' ends of the clones. Internal sequences of clones were obtained using oligonucleotides designed from preceding sequences. Computer analyses were carried out using Geneworks software (IntelliGenetics Inc, Mountain View, CA).

RESULTS AND DISCUSSION

Major advances in the knowledge of the structure (3), gene organization (13) and characterization (14,15) of the recombinant FSH receptor have recently been made. Identification of binding site(s) on the receptor is critical to our understanding of how glycoprotein hormones interact specifically with their corresponding receptor. In this paper, we report on the cloning and sequencing of the eFSHR, which presents a high specifity for FSH compared to other mammal FSH receptors. In addition, we describe the expression of eFSHR mRNA in the equine testis.

An equine testicular cDNA library constructed in a pBluescript SK vector was screened with a 0.45kb (nucleotide 1011 to 1460) eFSHR probe. This probe was generated by a PCR using degenerated primers corresponding to a human/rat consensus sequence. Several clones hybridizing at high stringency with this specific probe were isolated, but all of these were partial in size because they lacked varying lengths of the 5' end. Among these partial clones, the longest was sequenced to obtain about 60 % of the putative full-length eFSHR cDNA corresponding to the 3' end (nucleotide 1094 to 2319). This 3' clone includes 237 bp of 3' untranslated sequence. For cloning the full-length cDNA eFSHR, the missing 5' region was obtained by using the 5' rapid amplification of cDNA ends (5' RACE) method. After two successive rounds of amplification with nested primers and Southern blotting analysis, two different clones were obtained and cloned directly in the pT7 blue vector. One of these clones contained the extreme 5' end of the eFSHR. Reassembly of the overlapping equine testicular library and 5' RACE clone sequences revealed a 2,082 nucleotide open reading frame (Figure 1A and 1B). Computer translation of this sequence suggested that the complete receptor is composed of 694 amino acid residues, the first 17 of which correspond to a signal peptide.

The mature equine FSH receptor consists of a 677 amino acid protein (≈74,470 daltons) and displays 91 %, 90 % and 88 % identity to the human (16,17), ovine (18), and rat FSHR (19), respectively (Figure 2). Careful examination of the positions at which the eFSHR sequence differs from those of the other FSH receptors indicated several interesting features in the EC domain. In exon 4 (13), Pro^{113}, highly conserved in all glycoprotein hormone receptors, was substituted by a His in the eFSHR. Interestingly, all other changes were found within leucine-rich repeats 6-11 which appear to be critical in FSH binding (20). These replacements are located at positions Trp->Arg^{176}, Asp->Tyr^{202}, Arg->Gly^{227}, Ser->Asn^{268}, His-Pro-Ile->Gln^{289}-Thr-Thr^{291} and Gln^{310}->Glu^{309}. A deletion was also observed in leucine-rich repeat 12, at position Tyr/Asp^{303}. No significant changes

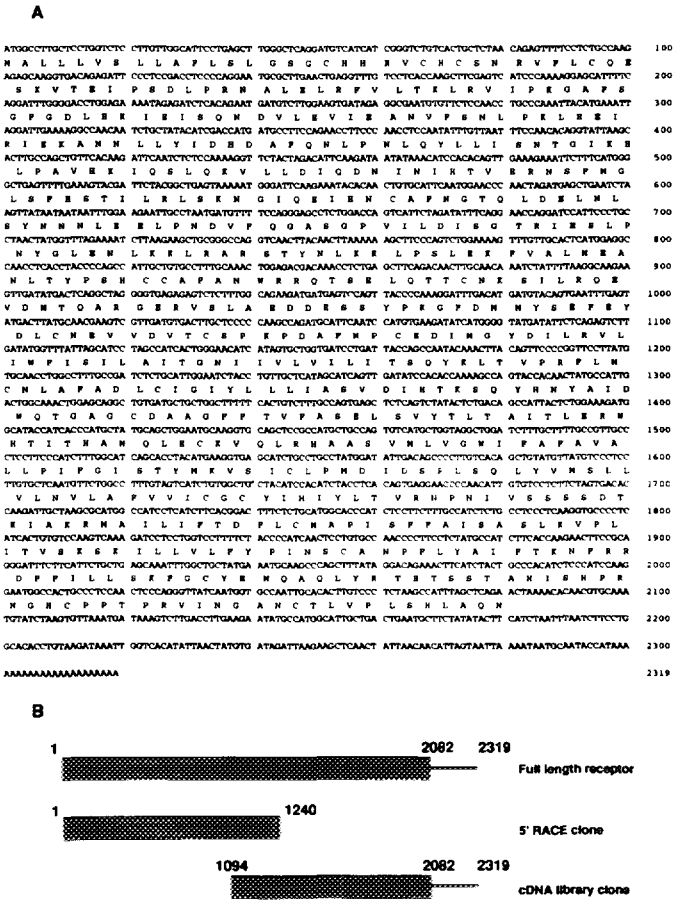

Figure 1. (A) **Nucleotide and deduced amino acid sequence of the eFSHR** : Position +1 is assigned to the first nucleotide of the putative initiator codon. The full length receptor sequence was obtained by re-assembly of the 5' RACE and cDNA library clone sequences.

(B) **Schematic representation of the full length eFSH receptor, 5' RACE and cDNA library clones** : the boxes indicate sequences of the coding region. Untranslated regions are given as lines.

were detected within the TM domain of the eFSHR, as an overall identity of about 99 % was detected. Computer analysis revealed the presence of four putative N-linked glycosylation sites in the EC domain of the eFSHR. All the FSHR presented highly conserved potential sites at positions 191, 199 and 293. In equidae, a fourth site was created by the Ser->Asn^{268} replacement, while FSHR from human and primates also possessed a fourth potential site located at position 318 (21). The presence, as well as the significance, if any, of the rearrangement of this particular N-linked glycosylation site warrant further investigation.

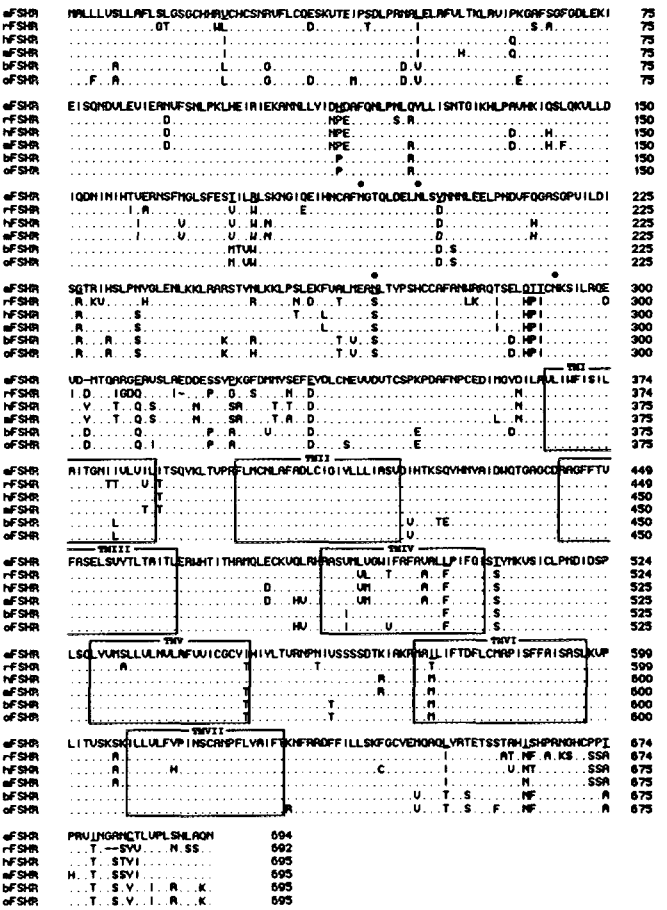

Figure 2. **Alignment of the amino acid sequences** of rat (r), human (h), *Macaca Fascicularis* (m), bovine (b), ovine (o) and equine (e) FSH receptors. The putative transmembrane domains are boxed. (•) Indicate potential N-glycosylation sites. Amino acids that are unique to the eFSHR are underlined.

Two probes were used in Northern blot analysis of RNA prepared from equine testis (Figure 3). Probe A (nucleotide 1011-1460) revealed the presence of two major transcripts of 2.3 and 4.2 kb . Three distinct transcripts sized 4.2 kb, 2.3 kb and 1.0 kb were detected with probe B (nucleotide 1 to 978), corresponding to the EC domain. Multiple transcripts of the FSHR mRNA have also been detected in rat (22), mouse (23), sheep (24), non-human primate (21) and human testis (25). Such transcripts may arise from different sites and lengths of polyadenylation and/or from alternative splicing. Although the function and the mechanisms of regulation of these different transcripts remain to be determined, the observation of multiple transcripts seems to be a general phenomenon for the glycoprotein hormone receptor.

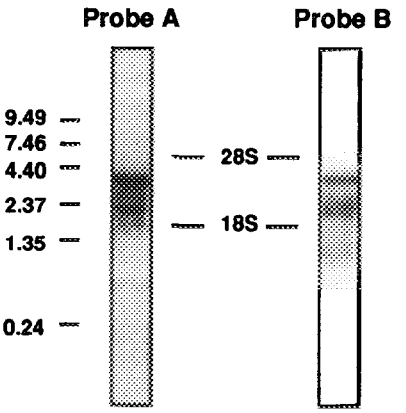

Figure 3. **RNA blot analysis of eFSHR mRNA from horse testis**. Polyadenylated RNA (5µg) extracted from 18 month-old horse testes were hybridized with two different probes, probe A corresponding to nucleotides 1011 to 1460 and probe B corresponding to nucleotides 1 to 978. RNA ladder molecular weight markers are shown on the left side of the figure.

Taken together, our results indicate that the equine FSH receptor is highly homologous to the FSHR from other species, as it presents only about 10 unique replacements in the EC domain. These features will facilitate attempts using site-directed mutagenesis, to determine whether any of these changes is important in the capacity of the eFSHR to better distinguish eLH/eCG from FSH.

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