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Synthesis and characterization of biologically active recombinant elk and horse FSH

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ABSTRACT

The objective of this investigation was to clone and express the elk and horse common α-subunit and FSH β-subunit cDNAs, and to produce recombinant FSH from both species *in vitro*. The RNAs extracted from elk and horse pituitary glands were reverse-transcribed and amplified by polymerase chain reaction. The cDNAs corresponding to both subunits of elk and horse were cloned into the expression vector pBudCE4.1® and transfected into CRL-9096 cells. Expression of both genes was determined in the transfected cells by Northern and Western blot analysis. Recombinant elk and horse FSH secreted in culture media were characterized by an *in vitro* bioassay and RIA. When the recombinant products were assessed as activity over mass of FSH measured by RIA, the horse product was 5.6 times more potent than the elk product. The recombinant products injected to immature female Wistar rats stimulated ovarian growth. The results suggest that the products obtained correspond to recombinant versions of the native elk and horse FSH. The availability of these recombinant products may aid in the development of more predictable and efficient techniques of ovarian stimulation in cervids, equids, and other species as well.

1. Introduction

Follicle stimulating hormone is a heterodimeric glycoprotein produced by the anterior pituitary gland of all mammalian species. It consists of two different subunits, α and β. The α-subunit is common to the other glycoprotein hormones of the pituitary gland, LH and TSH, while the β-subunit is specific for each hormone. The common α-subunit and the LH β-subunit are also expressed in the placenta of primates and equids (Ward et al., 1991). Both subunits are glycosylated and achieve functional conformation by non-covalent association through a series of disulfide bonds (Ward et al., 1991).

In females, FSH binds to specific receptors located primarily in the granulosa cells and stimulates ovarian follicle growth and steroid synthesis. Follicle stimulating hormone extracted from pituitary glands has been used for ovarian stimulation in mares, cows and a variety of other species, as current gonadotropin treatments relay on the administration of an external source with FSH activity (Mapletoft et al., 2002; Squires, 2006). Other gonadotropins with predominant FSH-like activity, present in the extracts of domestic animal pituitaries, serum of pregnant mares (PMSG) and urine of postmenopausal women have also been used for ovarian stimulation. Their use has been met with a variety of successes in several species, yet heterologous exogenous sources are not highly effective in mares (McCue, 1996). Lappin and Ginther (1977) were the first to report the use of equine pituitary extracts for ovarian stimulation in mares. This was the basis for the development of a commercial pituitary derived equine FSH product (eFSH Bioniche,

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Animal Health, Belleville, ON), which was reported to induce double or triple ovulations in mares (Logan et al., 2007). However, in controlled studies, pregnancy rates postembryo transfer have been less than expected, and other problems such as failure to ovulate, and delayed return to estrus have been reported (Raz et al., 2006; Squires, 2006). There is now a great demand for products to induce multiple ovulations in mares due to the changes governing the registration of foals. Currently in most breeds, multiple foals per donor mare, derived from embryo transfer may be registered. In the North American elk (*Cervus elaphus*), or wapiti, limited research has been conducted in ovarian stimulation. The protocols and hormones designed for other species have been used in elk with limited success (Pollard and Plante, 2001). The objective of this research was to express the elk and horse FSH subunit cDNAs in CRL-9096 cells, produce both recombinant hormones, and characterize their *in vitro* and *in vivo* activity.

2. Materials and methods

2.1. Reagents

General chemicals and molecular biology reagents including TRI®Reagent, Luciferin, cholera toxin (CT), Lipofectamine 2000®, 3-isobutyl-1-methyl-xanthine (IBMX) and rabbit immunoglobulin G (IgG) from serum were purchased from the Sigma Chemical Co. (St. Louis, MO). *Thermus aquaticus* (*Taq*) polymerase, Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT), dNTPs, T4 DNA ligase, random primers, restriction endonuclease enzymes, agarose, Klenow fragment of the DNA polymerase-I, oligonucleotide custom primers and tissue culture reagents including trypsin, gentamicin reagent solution, Geneticin®, fetal bovine serum (FBS), Fungizone® and α-minimum essential medium (α-MEM) were purchased from Canadian Life Technologies (Burlington, ON). [α-^{32}P]-dCTP was purchased from New England Nuclear (Boston, MA). Plastic tissue culture plates were purchased from Falcon (Lincoln Park, NJ). The pCR-Script® Amp SK(+) cloning kit was purchased from Stratagene (La Jolla, CA). Oligo (dT) cellulose Type 7, Hybond-H® nylon membranes were purchased from Amersham Biosciences (Piscataway, NJ). Oligotex®, pDRIVE polymerase chain reaction (PCR) cloning, QiaexII, Qiagen® plasmid DNA purification columns and QIAquick nucleotide removal kits were purchased from Qiagen (Mississauga, ON). The TOPO TA® PCR cloning kit, pBudCE4.1® cloning kit and Zeocin™ were purchased from Invitrogen (Carlsbad, CA). FirstChoice® RNA Ligation Mediated-Rapid Amplification of cDNA ends (RLM-RACE) kit was purchased from Ambion (Austin, TX). The dihydrofolate reductase deficient (DHFR) Chinese hamster ovary (CHO) cells, CRL-9096, were obtained from the American Type Culture Collection (Rockville, MD). Molecular weight standard Precision Plus Protein™ standards and Immun-Blot® PVDF membrane were purchased from Bio-Rad Laboratories Canada (Mississauga, ON). Polyclonal antibodies against equine FSH (AFP 2062096), rabbit anti-ovine FSH (AFP 5288113), NIDDK-anti-human FSH-6, and purified equine FSH were provided by Dr. A. Parlow from the National Hormone and Peptide Program (Torrace, CA). ImmunoPure® goat anti-rabbit IgG (H + L) alkaline phosphatase conjugated was purchased from Pierce Biotechnology Inc. (Rockford, IL). Equine pituitary gland FSH was obtained from Bioniche Animal Health USA, Inc. (Athens, GA). Recombinant human FSH (Gonal-F™) was obtained from Serono Canada (Oakville, ON). Inmobilion™ Western chemiluminescent AP substrate and the Amicon® Ultra-15 centrifugal filter devices were purchased from Millipore Corporation (Billerica, MA) and the X-OMAT Blue XB-1 from Kodak (Rochester, NY).

Table 1
List of oligonucleotide primers used to clone the elk common glycoprotein α-subunit and FSH β-subunit.

Primer	Sequence
Forward α-subunit	5′-AGGCAGAGGACGAAGAGCCATGGA-3′
Reverse 3′-RACE inner	5′-CGCGGATCCGAATTAATACGACTCACTATAGG-3′
Forward FSH β-subunit	5′-AGCATCCACAGTTACCAAGTGC-3′
Reverse FSH β-subunit primer 1	5′-CTGAAGGAGCAGTAGCTGGGC-3′
Genomic forward FSH β-subunit	5′-AGCAGTATTCAATCCCTGTCTCA-3′
Genomic reverse FSH β-subunit	5′-CATGTACACACAGACAGCTTGGATG-3′
Forward FSH β-subunit	5′-AGCATCCACAGTTACCAAGTGC-3′
Reverse FSH β-subunit primer 2	5′-TTACTCTCTGACGTCGCTGAAGGAGC-3′
Forward FSH β-subunit	5′-AGCATCCACAGTTACCAAGTGC-3′
Reverse FSH WFSHBTh-1	5′-TCGAGGCACCAGCTCTCTGACGTCGCTGAAG-3′
Reverse Thrombin XhoI	5′-CTCGAGATGCTTCCTCGTGGCACCAG-3′

2.2. cDNA cloning

Pituitary glands from elk and horse were removed immediately after slaughter. Tissue was homogenized with a Polytron® tissue homogenizer and total RNA was extracted using TRI®Reagent. Polyadenylated RNA was obtained using the Oligotex® kit. The common elk and horse α-subunit cDNAs were amplified by polymerase chain reaction (PCR), using cDNA synthesized by RLM-RACE. The elk and horse FSH β-subunit cDNAs were amplified by PCR, using first strand cDNA synthesized with a chimeric oligo (dt) adapter primer and MMLV-RT. The custom primers were designed based on sequence homology to GenBank sequences (Tables 1 and 2). The PCR mixture included 2 μl of RT-cDNA, 5 μl of 10× PCR buffer, 4 μl of 2.5 mM dNTP, 2.5 μl of 50 mM $MgCl_2$, 50 pmol of forward and reverse primers and 1.5 units of *Taq* DNA polymerase.

Table 2
List of oligonucleotide primers used to clone the horse common glycoprotein α-subunit and FSH β-subunit.

Primer	Sequence
Forward α-subunit	5′-AGG CAG AGG ACG AAG AGC CAT GGA-3′
Equine FSH α2	5′-GAA GTA GAT GGT GGG CAG GAA GAT-3′
Equine FSH β-RT	5′-GCC AGG AGA GCA GAG GTC AG-3′
EF-1α	5′-TCAAGCCTCAGACAGTGGTTC-3′
BGH	5′-TAGAAGGCACAGTCGAGG-3′
T7	5′-TAATACGACTCACTATAGGG-3′
Equine FSH β-rev	5′-TGTCAGCGTTCTTTATTCCTTCAT-3′