エルクTSH β-鎖のcDNAクローニングと下垂体糖タンパク質ホルモン遺伝子発現の季節変動

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Cloning of the Elk TSH β-Subunit cDNA and Seasonal Expression of the Pituitary Glycoprotein Hormone Genes

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Abstract. We report the elk (*Cervus elaphus*) thyroid stimulating hormone (TSH) β-subunit cDNA cloning, nucleotide and deduced amino acid sequences. The TSH β-subunit cDNA was obtained by RT-PCR of polyadenylated pituitary RNA. The deduced elk TSH β-subunit peptide chain shares between 93 to 99% sequence similarities with the reported TSH β-subunit of a sub-set of related species. The TSH β-subunit gene is expressed in the elk pituitary gland as a mature transcript of approximately 600 bases, which corresponds to the size of the mRNA expressed in the sheep pituitary gland. Seasonal expression of the pituitary gonadotropin genes was investigated by Northern blot analyses. Samples of elk pituitary glands collected during the breeding season showed elevated steady state levels of common α-subunit and FSH and LH β-subunit gene expression, consistent with the seasonal reproductive cycling of this species. Samples collected before the breeding season demonstrated decreased expression of the gonadotropin genes. TSH, which is not directly tied to reproduction, had similar levels of expression, regardless of the animal's reproductive status.
Key words: Elk, Cervids, FSH, LH, TSH, Gene cloning, Seasonal, Gene expression

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Deer (*Cervus elaphus*) belong to an extraordinarily diverse group of ungulates, with over 25 recognized subspecies distributed across Europe, Asia and North America. They range from the smaller Scottish red deer (*C. e. scoticus*) to the much larger North American elk, or wapiti, that can be further divided into three subspecies; *C. e. nelsoni*, *C. e. manitobensis* and *C. e. roosvelti* [1]. Although sometimes considered as separate species, red deer and elk share the same karyotype (2n=68) and are capable of producing fertile offspring [2].

North American elk are temperate cervids and strict seasonal breeders, which start the mating season in autumn, in response to decreasing photoperiod [3]. Hence, most of their life-sustaining activities, such as feeding, locomotion, sleep and reproduction are photoperiod dependent. Activity of their reproductive organs, including testis, ovaries and accessory sex glands, exhibit substantial annual changes along with the circulatory levels of sex and pituitary hormones [4, 5]. In the absence of pregnancy, deer hinds are polyestrous and are capable of exhibiting between four to nine continuous 17–19 day oestrous cycles over a 3–6 month period between autumn and spring [6–8]. Thus, the pituitary gland responding to photoperiod stimulus would change expression of the pituitary gonadotropin genes according to the reproductive season. We are interested in studying expression of the pituitary glycoprotein

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genes, including the gonadotropins FSH and LH, and the structurally related TSH, as their regulation in the cervid have not been extensively studied at the molecular level. This information is fundamental for understanding the basis of seasonality in cervid species. The pituitary glycoprotein hormones are heterodimeric proteins consisting of a α-subunit, which is common to all, that is non-covalently bound to a hormone specific β-subunit [9]. In the female, FSH and LH regulate ovarian development and steroidogenesis, and in the male, regulate spermatogenesis and steroidogenesis [10]. Regulation of FSH and LH is tightly controlled by the hypothalamic gonadotropin-releasing hormone (GnRH) and by the gonadal production of inhibin, activin and steroids. TSH, although not directly related to reproduction, controls thyroid gland function and regulation of metabolism and is autoregulated by a negative feedback mechanism [11].

It is plausible to hypothesize that cervids would exhibit seasonal expression of the genes encoding the reproductive hormones FSH and LH. In contrast, TSH, a hormone that is produced regardless of reproductive status, could be expressed independently of the breeding season and/or photoperiod. We presently report the cloning of the elk pituitary TSH β-subunit cDNA and its deduced amino acid sequence. We also investigated seasonal expression of the pituitary glycoprotein common α-subunit and the FSH, LH and TSH β-subunit genes, validating the use of recently cloned probes.

Materials and Methods

Reagents

TRI®Reagent, general chemicals, antibiotics and reagents for RNA electrophoresis, were purchased from Sigma (St. Louis, MO). Oligotex® and QiaexII® kits were purchased from Qiagen (Mississauga, ON). The FirstChoice® RLM-RACE kit was purchased from Ambion (Austin, TX). Hybond-H® nylon membranes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [α-^{32}P]-dCTP was purchased from New England Nuclear (Boston, MA). Agarose, X-gal (5-bromo-4-choro-3-indolyl-B-D-galactoside), custom primers, the Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and restriction enzymes were purchased from GibcoBRL (Burlington, ON). *Taq* DNA polymerase, 10X PCR buffer, dNTPs and TOPO TA® kit were purchased from Invitrogen (Carlsbad, CA).

Purification and Northern blot analysis of mRNA

Total RNA was extracted from female sheep and elk pituitary glands, brain and muscle. Female elk pituitary glands were collected from one representative herd animal of the anestrous to estrous transition on Sept 1st, and of the estrous breeding season on Oct 12th and Oct 28th, of 2003. A sheep pituitary gland, from a Western White Face ewe in anestrous, was collected Jan 19th, 2002. Tissue samples were collected immediately after death and stored at –80 C until processed. Tissue samples were homogenized in TRI®Reagent with a Polytron® tissue homogenizer (Brinkmann, Mississauga, ON). Total RNA was extracted following the manufacture's protocol and dissolved in a solution containing 0.5% SDS and 20 mM EDTA. Ten μg of total RNA was size-fractionated by electrophoresis in an agarose/formaldehyde gel, transferred to a nylon membrane by capillary blotting and cross-linked using a UV Stratalinker 1800® (Stratagene, La Jolla, CA). The TSH β-subunit, elk common α-subunit, FSH and LH β-subunits [12] and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; [13]) cDNA probes were labeled by primer extension with [α-32P]-dCTP (50 μCi). Membranes were hybridized and autoradiographed as previously described [14]. Arbitrary densitrometric values were assigned by measuring the mean band intensity using the Kodak Digital Science™ 1D Image Analysis Software System 120 (Eastman Kodak Company, Rochester, NY).

Synthesis, cloning and sequencing of the pituitary TSH β-subunit cDNA

Polyadenylated mRNA was obtained using the Oligotex® kit following the manufacture's protocol. First strand cDNA was synthesized from polyadenylated mRNA using a chimeric oligo (dT) adapter primer and the MMLV-RT, and amplified by PCR. The PCR mixture included 2 μl of RT-cDNA, 5 μl of 10X PCR buffer, 4 μl of 2.5 mM dNTP, 2.5 μl of 50 mM $MgCl_2$, 50 pmol of reverse and forward primers, and 1.5 units of *Taq* DNA polymerase. The cycling conditions were (1) 94 C

for 3 min, (2) 94 C for 1 min, (3) 52 C for 1 min, (4) 72 C for 1 min, (5) 25 cycles of steps 2–4, and 6) a terminal extension step at 72 C for 7 min. The forward primer 5′ AGAGCTTTT AGTCTGGGTCATTACAA 3′ and reverse primer 5′ CTATTAAAATTAGAT AGAAAATCCC 3′ were designed based on the NCBI ovine and bovine TSH β-subunit cDNA sequences, accession numbers X90775 and NM_174205, respectively. The amplified products were separated on a 1% agarose gel. The resulting band was excised from the gel and purified using the Qiaex II® kit, following the manufacturer's protocol and cloned into the TOPO TA® vector. Positive clones, carrying the insert, were sequenced with universal primers on a fluorescent automated sequencer (National Research Council, Plant Biotechnology Institute, Saskatoon, SK). Cloned cDNA were analysed and compared with sequences available in GenBank using the Clustal W alignment and Expasy translation public domain computer software (http://clustalw.genome.jp and http://ca.expasy.org).

Results

Cloning of the TSH β-subunit cDNA

A 26 oligonucleotide forward primer was designed to a site located 25 nucleotides upstream of the putative start codon of the gene, based on the reported ovine and bovine TSH β-subunit cDNA [15, 16]. A 25 oligonucleotide reverse primer was designed to the putative last 5 codons of the ovine TSH β-subunit cDNA (Table 1). By using these primers a 478 bp DNA fragment was amplified (Fig. 1), which revealed high similarity to the TSH β-subunit cDNA of sheep and other domestic animals [15–18]. This sequence included the entire coding region, with an open reading frame of 417 bp, which would encode a deduced peptide chain of 138 amino acids (Fig. 1). Comparison analysis with a subset of other species suggests that the deduced protein chain of the elk β-subunit TSH is between 93 to 99% similar to a subset of other species (Table 1). The TSH β-subunit gene is expressed in the elk pituitary gland as a mature transcript of 600 bases, which corresponds to the size of the mRNA expressed in the sheep pituitary gland (Fig. 2). Expression of the TSH β-subunit gene was not detected in samples of total RNA from elk muscle, brain, or Chinese hamster ovary cells, which were used as negative controls (Fig. 2).

Seasonal expression of the elk pituitary glycoprotein genes

The relative seasonal expression of the elk pituitary glycoprotein genes is shown in the Northern blot autoradiograms of Fig. 2. The steady state levels of FSH β-subunit mRNA were 12- and 2-fold higher in the Oct 12th and Oct 28th samples, respectively, as compared to the Sept 1st sample. Steady state level of LH β-subunit mRNA was 2-fold higher in the Oct 12th sample as compared to the Sept 1st sample. In contrast, on Oct 28th the LH β-subunit mRNA was reduced to 1/3 the levels exhibited by the Sept 1st sample. Steady state levels of gonadotropin α-subunit mRNA were 3-

Table 1. TSH β-subunit deduced amino acid sequence similarities between elk and a sub-set of related species

	Number of aa (% of identity)	TSH β-subunit aa position										
		5	15	33	43	69	76	88	109	120	121	122
Elk	138	A	**T**	I	V	A	R	I	T	V	V	G
Ovine [15]	137 (99%)	A	M	I	V	A	R	I	T	V	V	G
Bovine [16]	134 (97%)	A	M	**V**	V	A	R	I	T	**M**	V	G
Porcine [17]	131 (94%)	A	M	I	**F**	V	**H**	I	T	V	**L**	E
Equine [18]	129 (93%)	**T**	M	I	**I**	V	**D**	**V**	**A**	V	V	E

Similarities, shown in brackets, are expressed as percent of the identical amino acids over the total for the species. Amino acid positions where the elk sequence differs is shown. Non-identical amino acids are bolded.

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-52  AGAGCTTTTAGTCTGGGTCATTACAACATCAGCTCACCAATGCAAAGTAAGCATG ACT GCT ATC  12
-13                                                        M   T   A   I  -10

 13  TTC CTG ATG TCC ATG ATT TTT GGC CTT GCA TGT GGA CAA GCA ATG TCT TTT  63
 -9   F   L   M   S   M   I   F   G   L   A   C   G   Q   A   M   S   F    8
                                    -1 / +1
 64  TGT ATT CCA ACT GAG TAT ACT ATG CAT GTC GAA AGG AAA GAA TGT GCT TAC 114
  9   C   I   P   T   E   Y   T   M   H   V   E   R   K   E   C   A   Y   25

115  TGC CTA ACC ATC AAC ACC ACC ATC TGT GCT GGA TAT TGT ATG ACA CGG GAT 165
 26   C   L   T   I   N   T   T   I   C   A   G   Y   C   M   T   R   D   42

166  GTC AAC GGC AAG CTG TTT CTT CCC AAA TAT GCC CTG TCT CAG GAT GTC TGT 216
 43   V   N   G   K   L   F   L   P   K   Y   A   L   S   Q   D   V   C   59

217  ACA TAC AGA GAC TTC ATG TAC AAG ACT GCA GAA ATA CCA GGA TGC CCA CGC 267
 60   T   Y   R   D   F   M   Y   K   T   A   E   I   P   G   C   P   R   76

268  CAT GTT ACT CCT TAT TTC TCC TAC CCT GTA GCT ATA AGC TGT AAG TGT GGC 318
 77   H   V   T   P   Y   F   S   Y   P   V   A   I   S   C   K   C   G   93

319  AAG TGT AAT ACT GAC TAT AGT GAT TGT ATA CAT GAG GCC ATC AAA ACA AAC 369
 94   K   C   N   T   D   Y   S   D   C   I   H   E   A   I   K   T   N  110

370  TAC TGT ACC AAA CCT CAG AAG TCC TAT GTG GTG GGA TTT TCT ATC TAA TTT 420
111   Y   C   T   K   P   Q   K   S   Y   V   V   G   F   S   I Stop     125

421  TAATAG                                                             426
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Fig. 1. cDNA nucleotide and deduced amino acid sequences of elk TSH β-subunit. The nucleotides of the 5′ UTR are designated with negative numbers and the first nucleotide of the ATG translational start codon as +1. The forward custom primer was designed from nucleotide positions –52 to –27. The reverse custom primer was designed from nucleotide positions 401 to 426. The end of the signal peptide and the beginning of the mature protein amino acid sequence is designated as the –1/+1 boundary. The proposed leader sequence is 13 amino acids in length. The TSH β-subunit is N-glycosylated at Asn30.

fold higher in the Oct 12th sample as compared to the Sept 1st sample. In contrast, on Oct 28th the gonadotropin α-subunit mRNA was reduced to 1/2 the levels exhibited by the Sept 1st sample. The mRNA levels of pituitary glycoprotein hormones was similar between the anoestrous sheep pituitary gland collected Jan 19th and the elk pituitary gland collected Sept 1st. Irrespective of the date of sample collection, all animals had equal expression of TSH β-subunit mRNA. No change in the expression of the GAPDH gene, used to correct for RNA loading was observed between samples.

Discussion

The purpose of this study was to generate probes to investigate expression of the TSH β-subunit gene in comparison with the common pituitary glycoprotein α-subunit, and FSH, LH β-subunit genes in the female elk at different time points during the year. The elk TSH β-subunit cDNA reported was amplified by PCR using *Taq* polymerase. Although *Taq* polymerase does not have proofreading capability, we consider that the information generated is sufficient to speculate on the nucleotide differences in the elk cDNA and the deduced structure of the protein, in comparison with other species. The amplified TSH β-subunit cDNA is 98% similar, but 70 bases shorter than the ovine TSH β-subunit cDNA reported by Brockmann et al. (1997). This discrepancy is due to the design of the forward primer, which did not target the complete 5′ UTR sequence. Although this cDNA fragment does not have the entire 5′ UTR, it does include 6 out of the 10 nucleotides of the Kozak consensus sequence needed for translation [19]. Thus, we consider that this fragment corresponds to the elk TSH β-subunit cDNA. This cDNA would encode a peptide chain of 138 amino acids, of which 125 correspond to the mature protein. The leader sequence for the elk TSH β-subunit is proposed to be 13 amino acids in length, determined by aligning the conserved Asn^{30}

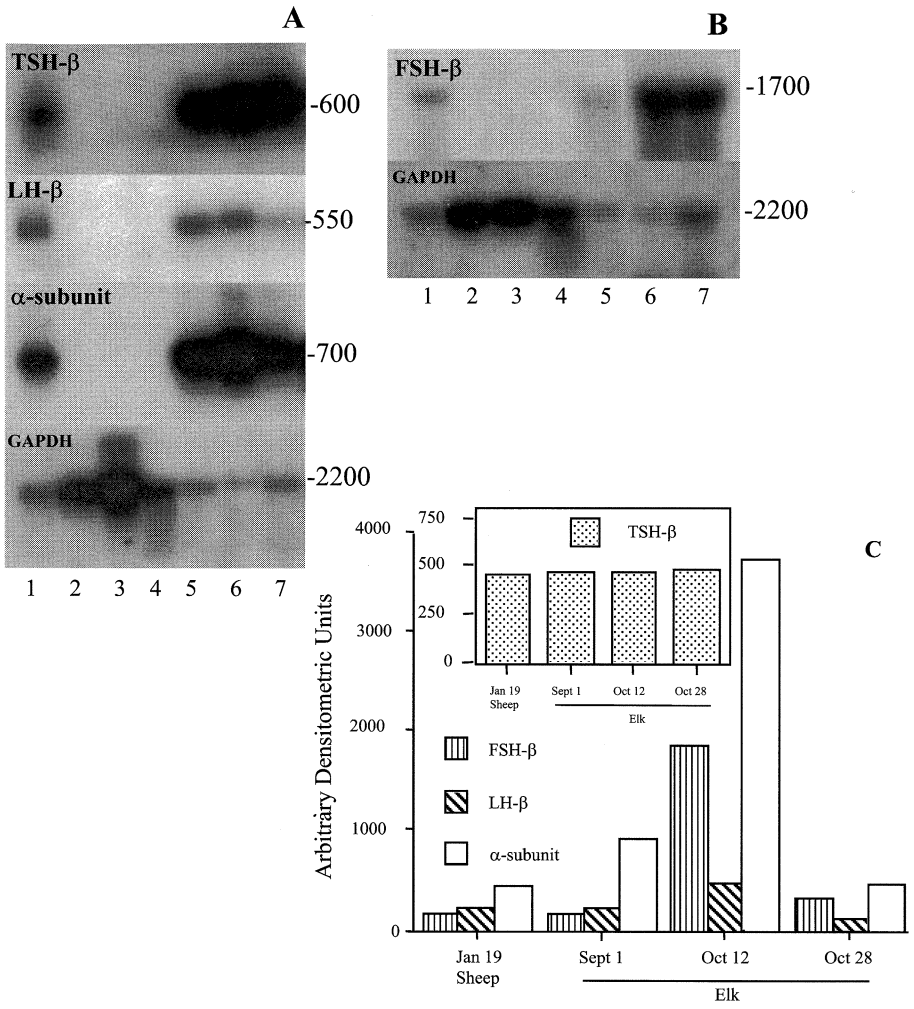

Fig. 2. Autoradiograms of Northern blot analyses of sheep pituitary gland (1), elk muscle (2), Chinese hamster ovary cells (3), elk brain (4), and female elk pituitary glands collected Sept 1st, Oct 12th and Oct 28th, (5, 6, and 7), respectively. Membranes were probed with the cDNAs of TSH and LH β-subunits, the common α-subunit (Panel A) and the FSH β-subunit (Panel B). All membranes were reprobed with GAPDH cDNA. The numbers at the right of the figure indicate the approximate sizes in bases of the mature mRNA transcripts. Data were normalized for GAPDH mRNA levels in each sample and expressed relative to the control value (sheep). Panel C is a graphic representation of the values obtained, represented as arbitrary densitometric units.

residue with related species. The length of the elk signal peptide is shorter than the porcine signal peptide, which was reported to be 20 amino acids long (Li et al., 1996). For the 15th amino acid we report a tyrosine for elk, whereas methionine was reported for sheep [15]. This is not a conserved change, as tyrosine is an aromatic amino acid while methionine is a sulfur amino acid, which occurs in the mature protein. Moreover, the reverse primer used compromises the last 5 deduced amino acids and the putative stop codon. Therefore, we cannot speculate on the nature of these last five amino acids in the elk TSH β-subunit. The differences in amino acid sequence detailed in Table 1 may result in differences in the functionality of the hormone between the species.

By using the cDNAs of the α-subunit, the FSH and LHβ-subunits reported in the preceding paper

[12], and the presently reported TSH β-subunit, as probes, we investigated the seasonal expression of the pituitary glycoproteins in the female elk. The relative changes in the β-subunit mRNA levels observed were consistent with the reproductive status of the animals investigated. The Sept 1st sample showed low levels of FSH and LH β-subunit mRNA, which were similar to the levels reported for the anoestrous sheep [20]. This observation is consistent with the information indicating that the sample corresponds to an animal that did not show evidences of estrous, and was taken before the beginning of the elk reproductive season.

The Oct 12th sample shows elevated mRNA levels of the FSH and LH β-subunits, and this sample was taken from a cycling animal during the middle of the reproductive season for the species. Elevated levels of FSH and LH β-subunits mRNAs were observed in this animal, which is coincident with the increased transcriptional activity of the pituitary gonadotropes during the reproductive season [21, 22]. These observations are consistent with the reported changes in the gonadotropic cells in the pituitary gland of the wild Sika deer and other temperate cervids. Changes in the activity of the pituitary gonadotropes were reported to be correlated with the high ovarian activity observed during the mating period of the Sika deer [23, 24].

The sample from Oct 28th was taken from an animal that was likely pregnant, as it was in the same pen with a bull. We know that with adequate nutrition and when other environmental and social factors are not limiting, elk exhibit high fertility, with >95% of mature females bearing singleton calves each year [25]. Thus, it is reasonable to speculate that the elevated levels of steroid during pregnancy resulted in a negative feedback on the hypothalamus-pituitary axis, resulting in decreased expression of the of the gonadotropin genes [22].

Expression of the FSH and LH β-subunit genes is also consistent with the observed elevated levels common glycoprotein α-subunit mRNA. The highest steady state level of glycoprotein α-subunit mRNA was observed in an animal during the breeding season, which also demonstrated the highest levels of FSH and LH β-subunits mRNA. Expression of the glycoprotein α-subunit gene is needed not only for the synthesis of FSH and LH, but also for synthesis of TSH. Therefore, it is reasonable to speculate that the elevated levels of the common α-subunit observed during the reproductive season respond to the requirements for the synthesis of all the glycoprotein hormones produced in the pituitary gland. Moreover, it is known that an excess of α-subunit mRNA is normally produced in pituitary gland [26]. Overall the results of the expression of the gonadotropin genes are consistent with cycling nature of the elk reproductive season.

TSH β-subunit steady state mRNA levels were shown to have similar expression in all the samples studied. TSH is not directly involved in regulating reproduction; therefore it was speculated that TSH β-subunit expression would not be altered by the reproductive status of the animals under study. When the expression of TSH β-subunit mRNA was investigated among ovariectomized female rats receiving estrogen treatments, little to no change was detected [27].

In summary, the cloned elk TSH β-subunit cDNA presently reported shares high similarity with the bovine and sheep published sequences. This information represents an important addition to the collection of sequences for the cervids in the gene database [28]. The transcript levels of TSH were similar regardless of the reproductive status of the animal. Overall, this descriptive study demonstrates transcriptional quiescence of the pituitary gonadotropes during anoestrous and pregnancy, while there is a dramatic increase in expression of the gonadotropic genes during the breeding season in the elk.

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