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Short communication

# Transcriptional regulation of follicle-stimulating hormone β-subunit in marmoset by an alternate distal promoter

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ABSTRACT

Follicle-stimulating hormone (FSH) is essential for mammalian folliculogenesis and spermatogenesis. Common marmoset (*Callithrix jacchus*) is a New World primate which exhibits an unusual FSH profile across the ovarian cycle with a mid-follicular FSH peak that is not observed in *Catarrhini* primates like humans. Since transcription of FSH β-subunit gene (FSHβ) is a rate-limiting step in the production of mature FSH, this study aimed to investigate the regulation of marmoset FSHβ gene expression in comparison to human. In silico analysis of the FSHβ promoter sequences identified a TATA box element upstream of the conventional TATA box element in marmoset but not in human sequence. FSHβ mRNA transcript longer than the conventional transcript was detected in marmoset pituitary implying presence of a distal transcription start site. In luciferase reporter assays, the marmoset putative distal promoter had higher activity than the corresponding human region even in absence of the conventional proximal promoter. Indeed higher affinity binding of TATA box-binding protein to the putative distal TATA box element was obtained in electrophoretic mobility shift assay. This suggests existence of a differential regulation of FSHβ transcription in marmoset compared to humans.



## 1. Introduction

Follicle-stimulating hormone (FSH) secreted by the anterior pituitary is an essential regulator of mammalian reproduction which acts on the granulosa cells of the ovary to trigger folliculogenesis in the female and on the Sertoli cells of testis to regulate spermatogenesis in the male. The proper regulation of FSH synthesis, secretion and action is critical for the maintenance of normal reproduction. Like other members of the glycoprotein hormone family, FSH is produced through the heterodimeric assembly of two subunits, α-subunit and a hormone specific β-subunit. The rate-limiting step in the production of FSH is the transcription of FSH β-subunit gene (FSHβ). The FSHβ transcription by pituitary gonadotrope cells is regulated by multiple endocrine, paracrine and autocrine factors which constitute the hypothalamic pituitary gonadal (HPG) axis, such as hypothalamic GnRH, members of the transforming growth factor-β family (activin, inhibin and follistatin) and steroids (Bernard et al., 2010; Thackray et al., 2010).

The New World primates (marmosets in particular) have certain unique reproductive features compared to other primates, such as: multiple ovulation per cycle (Nubbemeyer et al., 1997), lack of lactational amenorrhea (McNeilly et al., 1981), no LHβ expression but intensive CGβ expression in pituitary (Müller et al., 2004) and primitive nature of neonatal ovary showing the presence of premeiotic germ cells (Fereydouni et al., 2014). Interestingly, the female marmoset FSH profile across the follicular phase is characterized by a mid-follicular phase (Day 6) peak (Rosenbusch et al., 1997; Gilchrist et al., 2001), which is not observed in other primates. Moreover, high dose of human FSH (hFSH) is required for efficient superovulatory response in marmoset compared to humans and Old World monkeys (Marshall et al., 2003; Grupen et al., 2007). This suggested that the FSH requirement for folliculogenesis in marmoset in terms of concentration and secretion is different than in other primates, particularly humans. The decremental FSH level during the follicular phase effectively restrict the time during which FSH remains above the threshold. This time span, called FSH window, is crucial for selection and growth of a single dominant follicle from the recruited cohort (van Santbrink et al., 1995; Schipper et al.,

*Abbreviations:* CDS, coding sequence; EMSA, electrophoretic mobility-shift assay; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; NWM, New World monkey; RT, reverse transcriptase; TBP, TATA box-binding protein; TSS, transcriptional start site; UTR, untranslated region.

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1998). Superovulation protocols applied in women during in vitro fertilization (Macklon et al., 2006) and studies in macaque monkeys (Zeleznik et al., 1985) have demonstrated that interference with the decrease in FSH levels in the mid-follicular phase overrides selection of a single dominant follicle. Although there is lack of direct evidence, the variation in FSH secretion pattern in marmoset during the follicular phase might contribute to the multiple ovulation in these species.

In order to explore the possibility of differential regulation of FSHβ gene expression in marmoset as compared to other primates, comparative analysis of marmoset and human (as a representative of *Catarrhini* primates) FSHβ gene promoter with respect to sequence and activity was carried out in this study.

## 2. Materials and methods

### 2.1. Experimental animals

Blood and pituitary tissues were collected for DNA and RNA isolation respectively at the Experimental Animal Facility, National Institute for Research in Reproductive Health (Mumbai, India) from captive-bred common marmosets, rats and mice that were sacrificed for other studies approved by the Institutional Animal Ethics Committee recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

### 2.2. Sequences and in silico tools

Marmoset and human DNA sequence data were obtained from the Ensembl genome browser (www.ensembl.org). For the nucleotide numbering throughout this article, the conventional TSS (transcriptional start site) of FSHβ was designated as +1. Prediction of potential transcription factor binding sites (TFBS) in the promoter was performed by MatInspector (Cartharius et al., 2005).

### 2.3. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from pituitary tissues using TRIzol (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into complementary DNA (cDNA) using Superscript III RT and Oligo$(dT)_{20}$ primers (Invitrogen). The presence of different transcripts was analyzed using the cDNA preparation as template and with specific primers in conventional PCR. The primer sequences are given in Supplementary data Table S1.

### 2.4. Generation of reporter plasmid construct

The −1042/+7, −485/+7 and −126/+7 hFSHβ promoter reporter constructs in firefly luciferase vector pGL3-Basic was a kind gift from Dr. D. Bernard (McGill University, Québec, Canada) and are denoted as HP1, HP2 and HP3 respectively. The −482/−40 segment of hFSHβ promoter was subcloned from the above mentioned HP2 construct into pGL3-Baisc vector (Promega Corp Madison, WI) via *MluI* and *XhoI* restriction sites and verified by sequencing. The resulting construct is denoted as HP2M. The −1028/+7, −482/+7, −482/−40 and −125/+7 segments of the marmoset FSHβ gene promoter were amplified from marmoset genomic DNA, similarly cloned into pGL3-Basic vector and verified by sequencing and are denoted as MP1, MP2, MP2M and MP3 respectively. The sequences of the primers are outlined in Supplementary data Table S1.

### 2.5. Transfections and luciferase reporter assays

Murine immortalized LβT2 gonadotrope cell line was provided by Dr Pamela Mellon (University of California, San Diego, CA). LβT2 cells ($4 \times 10^5$ cells) were plated in 24-well plates and 48–72 h later co-transfected with X-tremeGENE HP reagent (Roche, Mannheim, Germany) following the manufacturer's protocol using 450 ng of reporter constructs and 45 ng of renilla luciferase vector (pRL-TK) (Promega) for normalization of transfection efficiency. After overnight co-transfection, the cells were treated with 1 μM of GnRH agonist (Lupride, Sun Pharmaceutical, India) and processed for reporter assay as described elsewhere (Wang et al., 2010). The firefly luciferase and renilla luciferase activity in the cell lysate was estimated using Luciferase Assay Substrate (Promega) and Renilla Luciferase Assay Substrate (Promega) respectively as per the manufacturer's instructions.

### 2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using digoxigenin (DIG)-end-labeled double-stranded oligonucleotides and LβT2 cell nuclear extract. The nuclear protein extract was prepared as described elsewhere (Beg et al., 1993). The binding reactions and detection were performed according to the manufacturer's instructions (DIG gel shift kit, second generation; Roche). The sequences of the probes are outlined in Supplementary data Table S1.

### 2.7. Statistical analysis

All experiments were performed at least three times. The data presented are from representative experiments. Differences between means were compared employing one-way analysis of variance (ANOVA) followed by *post-hoc* comparisons (Tukey) using 'GraphPad Prism 5.0' software (GraphPad Software Inc., CA).

## 3. Results and discussion

### 3.1. Prediction of an alternate TATA box element in marmoset FSHβ promoter

Using MatInspector program differential TFBS between the −1000/+1 bp gene sequences of marmoset and human FSHβ were identified. In the marmoset promoter sequence a TBP (TATA box-binding protein) binding site (TATA box element) was predicted on the +strand at position −353/−346 (matrix similarity of 0.855), in addition to the conventional proximal TATA box element at −32/−25 (matrix similarity of 0.986). This distal TATA box element was not predicted for the corresponding human promoter (Supplementary data Fig. S1). Additionally, on sequence alignment of FSHβ promoter from different mammalian species, it was found that the in silico predicted distal TATA box sequence was present only in the marmoset sequence (Supplementary data Fig. S1).

### 3.2. Presence of longer FSHβ transcript specifically in marmoset pituitary

In order to evaluate if the distal TATA box observed in marmoset is functional, we attempted to detect presence of longer FSHβ transcript in addition to the conventional transcript, in marmoset pituitary and compared it to human, rat and mouse pituitary. RT PCR amplification of the FSHβ coding region (Fig. 1A) confirmed presence of conventional FSHβ transcript in all pituitary RNA samples (Fig. 1B, middle panel). The presence of longer FSHβ transcript was observed only in marmoset pituitary and not in human, mouse and rat pituitary when primers spanning a region

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