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Molecular cloning and sequence analysis of follicle-stimulating hormone beta polypeptide precursor cDNA from the bovine pituitary gland

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ABSTRACT. Follicle-stimulating hormone (FSH) plays an essential role in mammalian spermatogenesis and follicular development. In a previous study, we demonstrated that some bulls carry numerous linked mutations in the FSH beta-subunit (*FSHB*) gene, and that these bulls have poor-quality semen, low fertility, and slightly lower serum FSH concentration compared to those without such mutations. Here, we identified the different *FSHB* mRNA transcripts in such individuals and analyzed the evolutionary pattern of the *FSHB* open reading frame (ORF) in different species. Two different lengths of *FSHB* mRNA transcripts corresponding to two different polyadenylation sites in the 3'-UTR were detected in wild-type bull pituitary glands, and four different mRNA transcripts resulting from the different polyadenylation sites and linked mutations were identified in mutation-bearing bull pituitaries. All transcripts had almost the same putative *FSHB* precursor molecule. When the ORF

sequences of wild-type and mutation-bearing genes were compared with those of other tetrapod species, the leopard frog had the lowest level of homology (57.8 and 58.1%) and the buffalo had the highest level (95.9 and 96.7%), respectively. These results indicated that the bovine *FSHB* gene transcribes at least two classes of mRNA in the wild-type and four classes of mRNA in the mutation-bearing individuals, which provides a new insight into the bovine *FSHB* evolutionary pattern. In addition, these findings lay a foundation for further study of gene expression regulation and the effects of mutations on male fertility traits in cattle.

Key words: Bovine; FSHB; RACE; mRNA transcripts

INTRODUCTION

Follicle-stimulating hormone (FSH), glycoprotein hormone derived from adenohypophyseal parenchymal cells, is a key regulator of the reproductive process in mammals. It is involved in the initiation and early stages of spermatogenesis through binding to FSH receptors in males, and follicular development and maturation in females (Dias et al., 2002; de Kretser et al., 2004; Kumar, 2005). Consistent with other members of the glycoprotein hormone family (i.e., luteinizing hormone, thyroid-stimulating hormone, and chorionic gonadotropin), functional FSH is a heterodimer formed by an α-subunit shared with other glycoprotein hormones and a specific β-subunit encoded by the *FSHB* gene (Pierce and Parsons, 1981; Gharib et al., 1990).

The cDNA sequences encoding *FSHB* polypeptide precursors have been cloned and characterized in most vertebrates, revealing that *FSHB* mRNA has a longer 3' untranslated region (UTR), which is absent in other members of the glycoprotein family. Four distinct *FSHB* mRNA transcripts were found in humans, resulting from one alternate splicing donor site in exon 1 and two different polyadenylation sites in exon 3 (Jameson et al., 1988). Three different lengths of *FSHB* mRNA transcript were found in rabbits as a result of the polyadenylation variants of one large transcript (Noguchi et al., 2006). However, it is still not clear whether there are alternative splicing and polyadenylation sites in the bovine *FSHB* mRNA transcript.

So far, some QTL associated with sperm quality were detected in bulls (Druet et al., 2009) and boars (Ren et al., 2009; Xing et al., 2009), and also some candidate genes are considered to be associated with male animal fertility traits, such as swine actin (Wimmers et al., 2005; Lin et al., 2006) and murine *Capza3* (Geyer et al., 2009). Our previous findings showed that some bulls with numerous linked mutations in the *FSHB* gene, including 13 substitutions and 1 insertion in the upstream regulation region (5'-URR) and the coding region of exon 3, exhibited slightly lower serum FSH concentration, poor-quality semen, and low fertility (Dai et al., 2009). Therefore, it is very probable that different *FSHB* transcripts exist in such particular individuals so that different expression levels of the *FSHB* polypeptide precursor exist, which further affects the FSH concentration in the serum.

For the reasons above, we report here the cloning and sequence analysis of the bovine *FSHB* mRNA transcripts from the bulls carrying *FSHB* mutations or not, which provides not only the necessary information for understanding the *FSHB* evolutionary pattern but also a useful tool for further study on gene expression regulation and elucidation of molecular mechanisms of *FSHB* mutation effects on male reproduction traits.

MATERIAL AND METHODS

Sampling

The five bulls with the linked mutations (four Simmental and one Charolais) have all been eliminated due to lower semen quality and fertility in our previous study population. Two pituitary glands belonging to these particular mutation-bearing individuals (one Simmental and one Charolais) were obtained from the local slaughter house, and another pituitary gland was from a wild-type individual (Simmental breed) to be used as a control. The tissues were frozen in liquid nitrogen immediately after dissection, and then stored at -80°C until analysis.

Preparation of RNA

Total RNA was isolated using TRIzol Reagent (Invitrogen, CA, USA) according to the standard protocol, and dissolved in 0.1% DEPC-treated water. RNA quality was checked by electrophoresis using an ethidium bromide-stained agarose gel, and RNA concentrations were determined with a UV-2802H spectrophotometer (Unico, Shanghai Optical Company, China) at 260 nm. RNA samples with $A_{260\ nm}/A_{280\ nm}$ ratios of 1.8 to 2.0 were used for further analysis.

Amplification of full-length cDNA

Full-length sequences of *FSHB* were obtained using a combination of 5'- and 3'-rapid amplification of cDNA ends (RACE). For 5'-RACE, we used a 5'-RACE cDNA amplification kit (Invitrogen) and primers GSP1 (5'-GCTGCATTTAGGACTTTC-3'), GSP2 (5'-AGCACCC TCGTGTCTGTAAGTTAAG-3'), and GSP3 (5'-TCAGGAGAGCAGAGATCAGTAGGGT-3'), based on the published mRNA sequences (GenBank No. M14853; Maurer and Beck, 1986). The locations of primers are shown in Figure 2. The first-strand cDNA was synthesized at 42°C for 50 min with 3 μg total RNA using the GSP1 primer and SuperScript™ II reverse transcriptase (Invitrogen), and tailed with dCTP using terminal deoxynucleotidyl transferase. Two consecutive polymerase chain reactions (PCR) using this cDNA as a template and Ex *Taq* polymerase (Takara, Dalian, China) were carried out as follows: the first PCRs were performed for 32 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min in a typical PCR mixture (50 μL) with GSP2 primer and 5'-RACE abridged anchor primer (AAP) (5'-GGC CACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'). The initial PCR product was diluted 1:20 with ddH_2O and used for nested PCR using the GSP3 primer and the abridged universal amplification primer (AUAP) (5'-GGCCACGCGTCGACTAGTAC-3'). The PCR conditions for nested PCR were the same as the first PCR, except for an annealing temperature of 64°C.

For 3'-RACE, we used a 3'-Full RACE Core Set kit (Takara) and primers 3F1 (5'-ACCCAGTAGCCACTGAATGTCAC-3') and 3F2 (5'-TGAGCTGCCTACCCTTATCCT AAAG-3'). The locations of the primers are shown in Figure 2. The first-strand cDNA was synthesized at 42°C for 60 min using the oligodT-3 adaptor primer and M-MLV reverse transcriptase, and two consecutive PCRs using this cDNA as a template and Ex *Taq* polymerase (Takara) were carried out as follows: the first PCR was performed under 30 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 2 min in a typical PCR mixture with 3F1 primer and 3'RACE

Outer Primer (5'-TACCGTCGTTCCACTAGTGATTT-3'). The PCR product was then diluted 1:10 with ddH_2O and used for nested PCR with the 3F2 primer and 3'-RACE Inner Primer (5'-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3'); the PCRs were the same as mentioned above.

Cloning and DNA sequencing

The final PCR products were cloned into pMD-18T vector (Takara), and recombinant plasmids were transformed into *Escherichia coli* competent cells (strain DH5α). About 30 positive clones of 5'-RACE and 50 positive clones of 3'-RACE, from the three independent amplifications, respectively, were sequenced. Sequencing was conducted on both strands using the pMD-18T vector primers at the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China. Nucleotide sequence alignments, translations, and comparisons were carried out using DNAMAN (version 5.2.10)

Multiple sequence alignment and phylogenetic analyses

Multiple sequence alignments of tetrapodian *FSHB* open reading frame (ORF) were performed with the Clustal X program, version 2.0 (Larkin et al., 2007). Values used for pairwise alignments were gap opening penalty 30 and gap extension penalty 0.8. Values for multiple alignments were gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences 30%. The phylogenetic and molecular evolutionary analysis based on the aligned ORF sequences were constructed by the Molecular Evolution Genetic Analysis software (MEGA, version 4.0) (Kumar et al., 2008). Maximum parsimony, minimum evolution, and neighbor-joining methods with p-distance model for multiple nucleotide substitutions produced very similar topologies, and the tree inferred from the neighbor-joining method is shown. For deriving confidence values of this analysis, bootstrap trials were replicated 1000 times. The GenBank accession numbers and references of selected species used for ORF sequences alignment and phylogenetic analysis in this study are indicated in Table 1.

RESULTS AND DISCUSSION

Two PCR products, approximately 650 and 1300 bp, were obtained from the bovine *FSHB* cDNA using 5'- and 3'-RACE (Figure 1). The results were repeated twice and confirmed for the three different individuals. The cloned nucleotide sequences were validated to encode the bovine *FSHB* subunit precursor molecule by comparing with the reported sequences (GenBank Nos. M14853 and NM_174060). Two different full-length cDNAs, 1679 and 1763 bp, respectively, resulting from different polyadenylation sites were identified in the wild-type bull pituitary. One canonical polyadenylation signal (AATAAA) was present at 17 bp upstream of the poly (A) tail in the short transcript, and another AATAAA was present at 12 bp upstream of the poly (A) tail in the long transcript (Figure 2). However, we did not find a different alternate splicing site in exon 1 from 29 positive clones of 5'-RACE, which indicated that it may be different from the human *FSHB* precursor molecules.

Table 1. Species and references of *FSHB*s used for ORF sequence alignment and phylogenetic analysis in this study.

Animal class/species	Scientific name	Order	Accession No.	Reference
Mammals				
Cattle	*Bos taurus*	Cetartiodactyla	GQ163481	Present study
Cattle	*Bos taurus*	Cetartiodactyla	M14853	Maurer and Beck, 1986
Buffalo	*Bubalus bubalis*	Cetartiodactyla	EF710660	-
Deer	*Cervus nippon*	Cetartiodactyla	AY156688	-
Sheep	*Ovis aries*	Cetartiodactyla	X15493	Mountford et al., 1989
Swine	*Sus scrofa*	Cetartiodactyla	AF134151	Li et al., 2000
Horse	*Equus caballus*	Perissodactyla	AB029157	Saneyoshi et al., 2001
Tiger	*Panthera tigris altaica*	Carnivora	AF540937	-
Panda	*Ailuropoda melanoleuca*	Carnivora	AF448454	Liao et al., 2003
Human	*Homo sapiens*	Primates	NM_000510	Jameson et al., 1988
Squirrel monkey	*Saimiri boliviensis boliviensis*	Primates	DQ143873	Scammell et al., 2008
Owl monkey	*Aotus nancymaae*	Primates	DQ200807	Scammell et al., 2008
Macaque	*Macaca fascicularis*	Primates	AJ781395	Schmidt et al., 1999
Possum	*Trichosurus vulpecula*	Diprotodontia	AF008550	Lawrence et al., 1997
Rabbit	*Oryctolagus cuniculus*	Glires	AY614704	Noguchi et al., 2006
Rat	*Rattus norvegicus*	Rodentia	NM_001007597	Maurer, 1987
Armenian hamster	*Cricetulus migratorius*	Rodentia	AB235911	-
Chinese hamster	*Cricetulus griseus*	Rodentia	AB248599	-
Djungarian hamster	*Phodopus sungorus*	Rodentia	AB252645	-
Syrian hamster	*Mesocricetus auratus*	Rodentia	AB241062	-
Mastomys	*Mastomys coucha*	Rodentia	AY458603	Takano et al., 2004
Mongolian gerbil	*Meriones unguiculatus*	Rodentia	AY376457	Koura et al., 2004
Mouse	*Mus musculus*	Rodentia	BC061159	Strausberg et al., 2002
Birds				
Chicken	*Gallus gallus*	Galliformes	AY029204	Shen and Yu, 2002
Quail	*Coturnix japonica*	Galliformes	AB086952	Kikuchi et al., 1998
Duck	*Anas platyrhynchos*	Anseriformes	DQ232890	Shen et al., 2006
Goose	*Anser cygnoides*	Anseriformes	EU563910	-
Reptiles				
Reeves' turtle	*Mauremys reevesii*	Testudinoidea	AB085201	Aizawa and Ishii, 2003
Softshell turtle	*Pelodiscus sinensis*	Testudinoidea	DQ234263	Chien et al., 2005
Amphibians				
Toad	*Bufo japonicus*	Anura	AB085668	Komoike and Ishii, 2003
Leopard frog	*Rana pipiens*	Anura	DQ054790	-

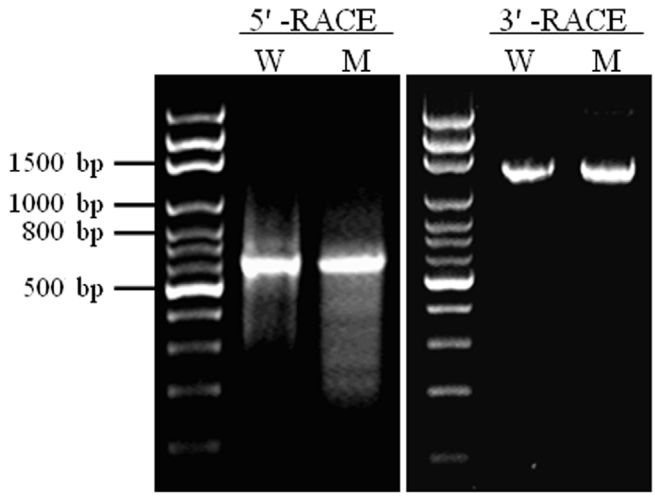

Figure 1. The products of 5'- and 3'-RACE were resolved on 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination. *Lane W* = wild-type. *Lane M* = mutation.

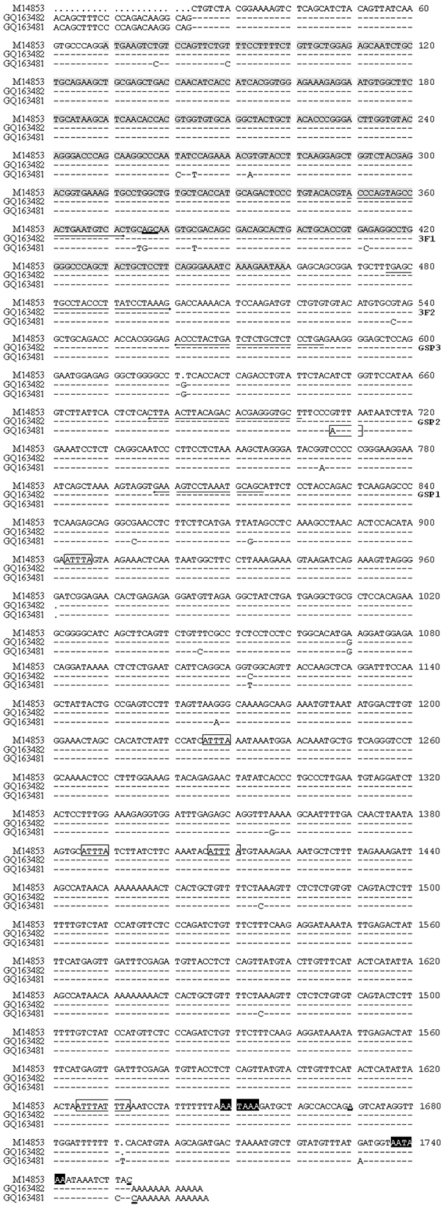

Figure 2. Sequence alignment of the bovine *FSHB* cDNA comparing that cloned in this study and that previously reported. The open reading frame is shown in a shaded box, the bases missing in M14853 are indicated by dots, and the identical nucleotides are indicated by hyphens. Arrows indicate primer positions (3F1, 3F2, GSP3, GSP2, and GSP1). The substitution 376A > G led to the amino acid replacement Ser84Gly, underlined here. Potential polyadenylational signals are indicated with white letters, and putative polyadenylation sites are underlined. Motifs of AUUUA in the 3'-UTR are boxed. The nucleotide sequences of the bovine *FSHB* cDNA obtained from this study are available in the GenBank database under accession numbers GQ163481 to GQ163484, and the sequence was the same between Simmental and Charolais breeds.

Although the two different transcripts could not be validated using Northern blot analysis due to the close length, we could verify and confirm this in the 48 positive clones of 3'-RACE, which included 31 long and 17 short mRNA transcripts, and these two different polyadenylation signal (AATAAA) locations were basically consistent with the reported species such as rabbit (Noguchi et al., 2006) and toad (Komoike and Ishii, 2003). The 3'-UTRs of many mRNAs, especially those of the early responsive genes (ERGs), are involved in posttranscriptional regulation (Pesole et al., 2001; Rabani et al., 2008). It is interesting that among the gonadotropin hormone subunits, only *FSHB* mRNA contains a unique 3'-UTR, which is highly conserved across the species, but the role of the 3'-UTR in the physiological regulation of *FSHB* gene expression is still not very clear. The discovery of different transcripts will provide very important information for further study of the role of the longer 3'-UTR in *FSHB* mRNA.

Four different transcripts were detected combined with the linked mutations with different polyadenylation sites in the mutation-bearing pituitaries. Among them, two different cDNA sequences resulted from 23 linked mutations, including 9 substitutions in the coding region, of which 376A > G led to the amino acid replacement Ser84Gly in the mature protein (Figure 2). As the *FSHB* cDNA sequences obtained were based on clones derived from PCR amplification, we considered the possibility that some of the sequence results obtained in this study might have been due to PCR or sequencing errors. However, scores of positive clones of one or two independent PCR amplifications from the same bulls were sequenced, and all the sequences reported here were confirmed by sequence alignment for the same individual. Thus, we ascertained that these sequences represent genuine bovine *FSHB* cDNA sequences and not PCR artifacts or sequencing errors.

Sequence analyses of the rat, mouse, human, ovine, and bovine *FSHB* 3'-UTR had revealed the presence of elements implicated in mRNA instability and translational control such as AU-rich element (AUUUA, ARE) and lipoxygenase differentiation control elements (Manjithaya and Dighe, 2004). Thirteen substitutions and one-base insertion were found in the *FSHB* 3'-UTR of mutation-bearing bulls, of which 707 G > A created a new ARE. Thus, there were seven "AUUUA" motifs after six original ARE in such special *FSHB* mRNA transcript (Figure 2). It is known that ARE can be described as a signal for rapid mRNA degradation (Jarrousse et al., 1999; Zhang et al., 2002; Barreau et al., 2005), so this single nucleotide substitution may be one of the most important reasons for explaining the lower serum FSH concentration in the mutation-bearing individuals. However, further studies are still needed to elucidate the significance and mechanism of the "AUUUA" motif in bovine *FSHB* expression.

All transcripts had an ORF of 309 bp, beginning with the first translation initiation codon at position 70 and ending with the stop codon at position 459, which encoded the same precursor protein. The signal peptide cleavage site was at position 126, yielding a signal peptide of 19 amino acids and a mature peptide of 110 amino acids. The phylogenetic tree of tetrapodian *FSHB* was presented based on their alignment in ORF sequences (Figure 3). In general, species for the same animal classes are clustered in groups. The homology of two different ORF sequences of the bovine *FSHB* precursor protein resulting from linked mutations was 97.7%. When the ORF sequences of wild-type and mutation-bearing genes were compared with those of other tetrapodian species, respectively, a closer phylogenetic relationship with buffalo (95.9 and 96.7%) and ovine (95.4 and 96.7%) than with deer (92.3 and 93.6%) and swine (90.8 and 91.8%) was observed in the Cetartiodactyla. Intermediate identities with the birds and reptiles were about 70%, and the lowest level of homology was found with the

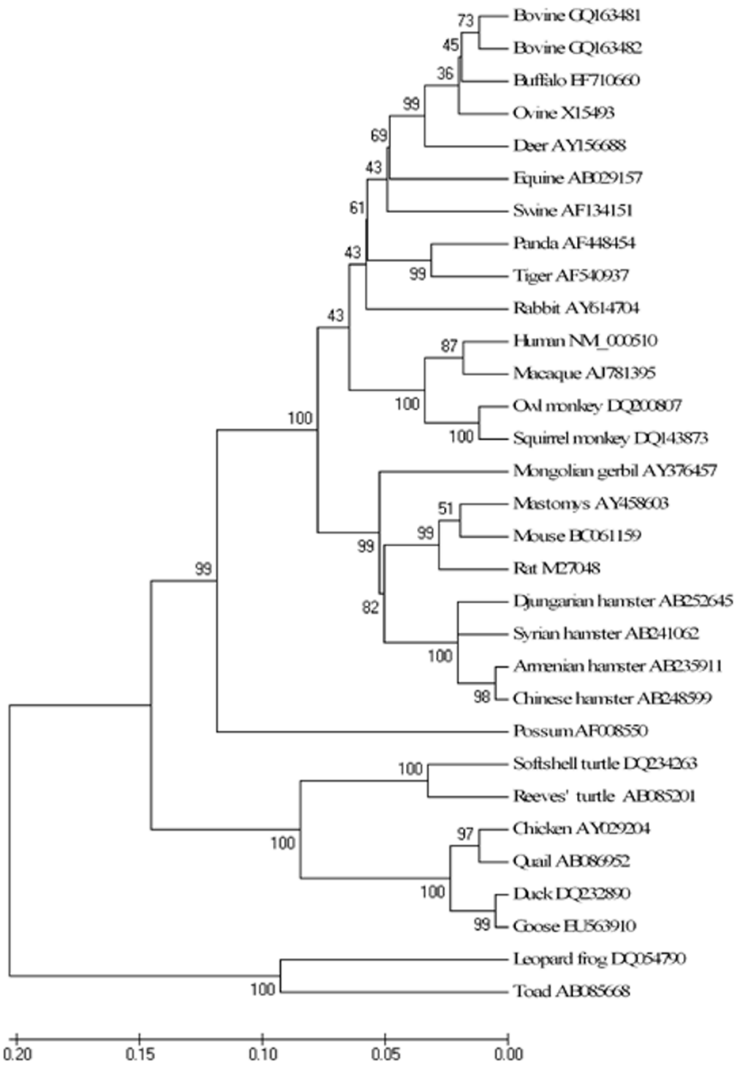

Figure 3. A phylogenetic tree based on the aligned ORF sequences of the tetrapodian *FSHB*s. GenBank accession numbers and references of *FSHB* sequences analyzed in this study are indicated in Table 1.

leopard frog (57.8 and 58.1%). The high genetic diversity of bovine *FSHB* mRNA implies that it has undergone substantial alteration of nucleotide sequence during the course of evolution, which provided the necessary information for understanding the *FSHB* evolutionary pattern even though the mutations are a negative change for frozen semen production.

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