Gene 550 (2014) 193–199

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Identification and characterization of yak (*Bos grunniens*) *b-Boule* gene and its alternative splice variants

Bojiang Li [a], Sherry Ngo [b], Wangjun Wu [a], Hongtao Xu [a], Zhuang Xie [a], Qifa Li [a,*], Zengxiang Pan [a,*]

[a] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
[b] Liggins Institute, University of Auckland, Auckland 1142, New Zealand

ARTICLE INFO

Article history:
Received 17 May 2014
Received in revised form 11 August 2014
Accepted 15 August 2014
Available online 18 August 2014

Keywords:
Yak
b-Boule
Splice variants
Expression

ABSTRACT

Boule is responsible for meiotic arrest of sperms and male sterility during mammalian spermatogenesis. In the present study, we first identified yak *b-Boule* gene and its two alternative splice variants. The full length coding region of yak *b-Boule* is 888 bp and encodes a 295-amino acid protein with a typical RNA-recognition motif (RRM) and a Deleted in Azoospermia (DAZ) repetitive sequence motif. Two alternative splice variants of yak *b-Boule* were generated following the consensus "GT-AG" rule and named *b-Boule1* (36 bp deletion in exon 3) and *b-Boule2* (deletion of integral exon 7), respectively. In male yak, *b-Boule*, *b-Boule1* and *b-Boule2* were found to be exclusively expressed in the testes at a ratio of 81:0.1:1. Intriguingly, the mRNA expression levels of *b-Boule* and *b-Boule1* in yak testis were significantly higher than those in cattle–yak, although no significant difference was observed for *b-Boule2* expression between the yak and cattle–yak. These results suggest that *b-Boule* gene, which is partially regulated by alternative splicing, may be involved in the process of yak spermatogenesis.

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1. Introduction

Boule, a member of the *DAZ* family encoding RNA-binding protein and having a highly conserved RNA-Recognition Motif (RRM) and a DAZ repeat (Eirin-Lopez and Ausio, 2011; Lee et al., 2006; VanGompel and Xu, 2011), was first identified in *Drosophila* (Eberhart et al., 1996). Since then, its orthologues were identified in *Caenorhabditis elegans*, mouse, humans and fish (Cooke et al., 1996; Karashima et al., 2000; Xu et al., 2001, 2009). In humans, previous studies indicated that lack of *Boule* could cause meiotic arrest and spermatogenesis failure (Lin et al., 2005). Furthermore, *Boule*-null knockout mice are male azoospermic (VanGompel and Xu, 2010) whereas *Boule* transgene rescues meiotic defects in infertile *Boule* mutant flies (Xu et al., 2003). In addition, *Boule* is required for meiosis and spermatogenesis in dairy goat testicles, and its overexpression is shown to be positively correlated with meiosis-related genes in dairy goat male germline stem cells (mGSCs) (Li et al., 2013). These studies suggest that the *Boule* gene plays an important role in the process of meiosis and spermatogenesis.

Alternative splicing (AS) is a widespread mechanism for generating multiple transcripts which contributes to the increase of proteomic diversity (Brown et al., 2014; Pimentel et al., 2014; Wang et al., 2008; Yamamoto et al., 2009). It reported that differential AS have been detected in about 40% of *Drosophila* genes (Stolc et al., 2004). Moreover, AS is demonstrated to regulate many physiological processes (Pal et al., 2012; Tang et al., 2013), e.g. the differentiation of mouse embryonic stem cells (Salomonis et al., 2010) and cell apoptosis (Wu et al., 2003) and the age process (Meshorer and Soreq, 2002), while recent studies indicated that the pre-mRNA splicing defects can cause the diseases (Biamonti et al., 2014; Fan and Tang, 2013).

To date, *Boule* alternative splice variant has been found in humans and bats (Kostova et al., 2007; Yuan et al., 2010), but has not been reported in other species. In humans, three *Boule* alternative splice variants (B1, B2, B3) expressed exclusively in the testis were found in the ratio of 80:220:1 (B1:B2:B3), and B2 was thought to be strongly associated with male infertility (Kostova et al., 2007). While, two alternative splice variants (a and b) of the *Boule* gene were identified in four bat species (Yuan et al., 2010).

In this study, the full length coding region of yak *b-Boule* was cloned and sequenced, and two alternative splice variants, namely *b-Boule1* and *b-Boule2* were identified. We further analyzed the structure and functional features of the DNA and protein sequences of both variants. In addition, RT-PCR was used to detect expression abundance of both splice variants in different tissues, while qRT-PCR was performed to

Abbreviations: cDNA, complementary deoxyribonucleic acid; CDS, coding sequence; ORF, open reading frame; UTR, untranslated region; AS, alternative splicing; RBD, RNA-binding domain; RRM, RNA recognition motif; DAZ, Deleted in Azoospermia.

* Corresponding authors.
E-mail addresses: liqifa@njau.edu.cn, owwa@njau.edu.cn (Q. Li).

http://dx.doi.org/10.1016/j.gene.2014.08.028
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assay their expression differences in testis tissues of yak and cattle–yak hybrid. Taken together, our results provide preliminary evidence for the role of *b-Boule* in yak spermatogenesis and male sterility.

2. Materials and methods

2.1. Tissue collection

The healthy adult yaks (male = 8, female = 2) and cattle–yaks (male = 8) were provided by the Songpan Bovine Breeding Farm, Sichuan Province. Tissue biopsies of the testis, ovary, kidney, spleen, hypothalamus, pituitary gland, heart, liver, lung, muscle and uterus were collected from the yaks and cattle–yaks after slaughter and frozen in liquid nitrogen immediately, then stored at −80 °C until total RNA extraction. The study was approved by the Animal Use Committee at Nanjing Agricultural University of China (Nanjing, China).

2.2. RNA isolation and first strand cDNA synthesis

Total RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, California, USA) following the manufacturer's instruction, and random primer was used to synthesize cDNA. The reverse transcription system in a total volume of 25 μL, contained 1 μg of random primer, 2 μg of total RNA from each sample, 20 U of RNasin inhibitor, 0.4 μL of dNTP, 200 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA), and 5 μL of 5× M-MLV Buffer (including 250 mM of Tris–HCl pH 8.3, 50 mM of $MgCl_2$, 250 mM of KCl, 50 mM of DTT, 2.5 mM of Spermidine). First, the total RNA was mixed with dNTP and the random primer, then incubated at 70 °C for 5 min followed by incubation on ice for 5 min. This was followed by the addition of RNasin inhibitor, M-MLV Buffer and M-MLV reverse transcriptase, and incubation at 37 °C for 1 h followed by 95 °C for 5 min for enzyme inactivation. The cDNAs were stored at −20 °C for later PCR.

2.3. PCR amplification and clone sequencing

Two pairs of primers (P1 and P2, in Table 1), designed based on the known cattle *Boule* gene sequence (GenBank accession no. NM_001102115.1), were synthesized to obtain the complete CDS of the yak *Boule* gene. The 10 μL PCR reactions contained the following: 0.5 μL of RT-products, 1 U of *Taq* DNA polymerase (TaKaRa, Dalian, China), 1 μL of 10× PCR Buffer, 0.25 mM of dNTP, 1.25 mM of $MgCl_2$, and 0.25 μL of 10 nM of each primer. The PCR cycling parameters were 94 °C for 5 min for the initial denaturation, 35 cycles of 94 °C for 30 s, 63.5 °C for 30 s, 72 °C for 40 s, and a final extension for 10 min at 72 °C. Reaction products were checked on 1.2% agarose gels and the target bands (PCR products) were purified with AxyPrep DNA Gel Extraction Kit (Axygen, USA) according to the manufacturer's protocol. The purified products were cloned into the pMD18-T vector (TaKaRa, Dalian, China), then transformed into the DH5α competence cells (TaKaRa, Dalian, China) and sequenced (Invitrogen, Shanghai, China). The identity and orientation of each clone were verified by sequencing with the universal primer M13 of pMD18-T vector.

Table 1
The primers used in this study.

No.	Primer sequence (5′–3′)	Length of product/bp	Tm/°C
P1	F: GAAAGTCTCCGTCGGGAAGCGT R: GGCAGCTTCTAGCCGGTTCATTG	1091	63.5
P2	F: AATTGCACAAGATGGTGGATGT R: CCAAATGGCTTATGTTATAGGGATAC	907	63.5
P3	F: GTGAAGGTGCTTACGAACT R: ATAAGGCTCTGGAACTGAA	838	54.4
P4	F: CAAGTGCCATTGCTATGCCTGC R: GGTTCATTGAAGCTGGATCTCGG	157	60.0
P5	F: GACTTTAAGTATGGATCTGTA R: AGTAACCTCTGGAGTATGAA	303	47.2
P6	F: CACCACCTTGGCTGCCCCT R: ATGGCACTTGAAGCATAAACC	250	60.4
P7	F: TCCAGCCTTCCTTCCTGGGCAT R: GGGGCGCGATGATCTTGATCTTC	213	56.0

2.4. Cloning and identification of Boule gene alternative splice variants

Boule alternative splice variants were amplified using primer P3 with the testis cDNA of yak. The PCR reaction was performed and the target bands were detected, purified, cloned and sequenced as described previously in Section 2.3.

2.5. Bioinformatics analysis

The splicing of sequences and the identification of ORFs were analyzed using DNAStar 5.02 (DNASTAR Inc.). Analysis of the genetic structural domains was performed with Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan); amino acid sequences were analyzed using the Protparam program (http://www.expasy.org/tools/protparam.html); and hydrophilicity and hydrophobicity of proteins were analyzed using kyte-Doolittle Hydropathy Plots (http://fasta.bioch.virgrinia.edu/fasta_www/grease.htm). Prediction of signal peptide sequences was performed using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). Predictions of protein transbilayer helices and conserved domains were performed using the TMpred Server (http://www.ch.embnet.org/software/TMPRED_form.html) and the Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), respectively. Proteinic tertiary structures were predicted using SWISS-MODEL software (http://swissmodel.expasy.org/SWISS-MODEL.html). The modification of locus and the functional groups were analyzed using ELM-Functional Sites in Proteins (http://elm.eu.org/links.html). Transcriptional response elements were predicted with MatInspector software (http://www.genomatix.de/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) using appropriate weight/substitution matrices via the TRANSFAC 7.0 database (http://www.gene-regulation.com) of transcription factor binding sites and DNA binding profiles.

2.6. Semi-quantitative RT-PCR analysis

Primers (P4–P7; Table 1) were designed based on yak *b-Boule* cDNA and its alternative splice variants. *β-actin* housekeeping gene was used as an internal standard; the mRNA sequence (GenBank accession no. NM_173979) was used for primer design. Semi-quantitative RT-PCR was used to determine the expression of *b-Boule* in various yak tissues (testis, ovary, kidney, spleen, hypothalamus, pituitary gland, heart, liver, lung, muscle and uterus). The PCR reaction was performed and the PCR products were detected as described previously in Section 2.3.

2.7. Quantitative real-time PCR analysis

Real-time PCR was performed to determine the expression level of the *b-Boule* gene in testis tissues. The PCR reaction (20 μL) consisted of 1 μL of RT-products, 1 U of EX *Taq* HS DNA (TaKaRa, Dalian, China), 4 μL of 5× PCR Buffer, 0.3 mM of dNTP, 3.75 mM of $MgCl_2$, 0.5 μL of 10 nM of each primer, and 1 μL of 20× SYBR green I. The cycling parameter is as follows: 95 °C for 1 min followed by 45 amplification cycles each comprising of 95 °C for 10 s; 60 °C for 10 s and 72 °C for 15 s (Plate Read) and a final extension for 10 min at 72 °C. A melt curve analysis was performed from 60 °C to 95 °C by reading plate every 0.2 °C (read plate after homoiothermism 1 s), and finally at 72 °C for 5 min. Each reaction was performed three times. Plasmids containing the *b-Boule* gene or splice variants were diluted 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10, and used to generate standard curves. Statistical difference between the expression level of splice variants and *b-Boule* gene in yak testis was performed by ANOVA using SPSS 13.0 software. Statistical difference in testicular expression of *b-Boule* gene

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