Gene 572 (2015) 17–26

Contents lists available at ScienceDirect

Gene

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

Research paper

Molecular characterization and expression of buffalo (*Bubalus bubalis*) DEAD-box family VASA gene and mRNA transcript variants isolated from testis tissue

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ARTICLE INFO

Article history:
Received 24 October 2014
Received in revised form 1 June 2015
Accepted 26 June 2015
Available online 27 June 2015

Keywords:
VASA
Isoform
Spermatogenesis
RACE

ABSTRACT

VASA is a member of the DEAD-box protein family that plays an indispensable role in mammalian spermatogenesis, particularly during meiosis. In the present study, we isolated, sequenced, and characterized VASA gene in buffalo testis. Here, we demonstrated that VASA mRNA is expressed as multiple isoforms and uses four alternative transcriptional start sites (TSSs) and four different polyadenylation sites. The TSSs identified by 5′-RNA ligase-mediated rapid amplification of cDNA ends (RLM-5′-RACE) were positioned at 48, 53, 85, and 88 nucleotides upstream relative to the translation initiation codon. 3′-RACE experiment revealed the presence of tandem polyadenylation signals, which lead to the expression of at least four different 3′-untranslated regions (209, 233, 239 and 605 nucleotides). The full-length coding region of VASA was 2190 bp, which encodes a 729 amino acid (aa) protein containing nine consensus regions of the DEAD box protein family. VASA variants are highly expressed in testis of adult buffalo. We found five variants, one full length VASA (729 aa) and four splice variants VASA 2, 4, 5, 6 (683, 685, 679, 703 aa). The expression level of VASA 1 was significantly higher than rest of all ($P < 0.05$) except VASA 6. The relative ratio for VASA 1:2:4:5:6 was 100:1.0:1.6:0.9:48.

1. Introduction

The water buffalo (*Bubalus bubalis*) is the cornerstone of the livestock production-based agro-economy in many developing countries (Singh et al., 2000). Water buffalo is an inhabitant of the Asian continent and about half of its total world population exists in India. Buffaloes in Indian subcontinent play an important role as producer of milk, meat, draught power, dung and other value added products. Indian dairy industry is mainly dependent on buffalo, where nearly 95% of total milk consumed by the dairy plants comes from buffalo.

The present diversity of livestock species is the result of a combination of various processes, including domestication, migration, genetic isolation, environmental adaptation, selective breeding, introgression and admixture of subpopulations. Molecular characterization can help unravel the genetic history of a species, which is most relevant for managing the present and future genetic diversity. Different categories of genetic markers are informative for different aspects of a species' genetic history (Groeneveld et al., 2010).

VASA gene was first identified in *Drosophila* sp. as one of the components of germ plasm essential for primordial germ cell (PGC) commitment (Schüpbach and Wieschaus, 1986; Hay et al., 1988a). VASA gene encodes DEAD box family protein of ATP-dependent RNA helicase (Hay et al., 1988b; Liang et al., 1994). DEAD box proteins are characterized by nine conserved sequence motifs situated within two functional domains. The domain I (DEADc) contains six of these motifs, including the Q motif and the Walker A motif (both of which are required for ATP and RNA binding), motifs Ia and Ib (which together with motifs IV and V are responsible for RNA binding), the Walker B motif (important for ATP binding) and motif III, which may act to link ATPase and helicase activities of the protein. Domain II (HELICc) contains motifs IV, V and VI, which co-ordinate ATPase and unwinding activities as well as ATPase and RNA binding (Cordin et al., 2006; Linder and Jankowsky, 2011).

In *Drosophila*, VASA gene is responsible for mutations that cause a deficiency in the formation of germline precursor cells (Lasko and Ashburner, 1988; Styhler et al., 1998; Tomancak et al., 1998) during early stages of oogenesis and spermatogenesis, the VASA gene is required for the development of oocytes and spermatocytes (Styhler et al., 1998; Tomancak et al., 1998). In mammals, VASA is only expressed in the germline, and is widely used as a molecular marker for the study

Abbreviations: TSSs, transcriptional start sites; RLM-5′-RACE, 5′-RNA ligase-mediated rapid amplification of cDNA ends; Aa, amino acid; PGCs, primordial germ cells; ORF, open reading frame; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; piRNA, Piwi-interacting RNA.

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http://dx.doi.org/10.1016/j.gene.2015.06.067
0378-1119/

of gametogenesis and origin, migration and differentiation of primordial germ cells (PGCs) (Reynolds et al., 2005; Gustafson et al., 2011). VASA is an essential protein for spermatogenesis (Soultanas et al., 1999; Fujiwara et al., 1994; Anderson et al., 2007).

VASA is required for the formation of germ cells and abdominal segments in the fruit fly (Johnstone and Lasko, 2004). VASA is also required for the embryonic stem cells differentiating into primordial germ cells and spermatogonial stem cells (Geijsen et al., 2004; Hübner et al., 2003). On the basis of structural conservation, genes homologous to VASA have been cloned and characterized in many animal species: *Mus musculus* (Fujiwara et al., 1994), *Homo sapiens* (Castrillon et al., 2000), and *Bos taurus* (Pennetier et al., 2004a,b; Luo et al., 2013). Up to present, VASA expression provides the most reliable molecular marker for the germ cell lineage in most animal species. Here, we sequenced, and characterized the VASA transcript variants isolated from testis tissue, as well as, identified different transcription start sites (TSSs) in 5′UTR and polyadenylated sites in 3′UTR region.

2. Materials and methods

2.1. Materials

Buffalo adult tissues like testis, ovary, kidney, liver, spleen, heart, and muscle were dissected from the slaughtered buffalo, immediately dipped in liquid nitrogen, and stored at −80 °C until used for total RNA isolation.

2.2. RNA extraction and reverse transcription

Total RNA was isolated from testis tissue using the Tri Reagent (Sigma-Aldrich, GmbH, US) according to the manufacturer's instructions and verified the integrity of total RNA by analyzing about 1 μg RNA sample on 1% (w/v) formaldehyde denaturing agarose gel. The reverse transcription reaction mixture for first strand cDNA synthesis included 2 μg of total RNA, 1 μL of random primer, 200 U RevertAid M-MLV reverse transcriptase, 20 U RiboLock RNAse inhibitor, 4 μL of 5× RT buffer and 2 μL of dNTP (10 mM) in a final volume of 20 μL. The reverse transcription reaction was performed according to the manufacturer's instructions of RevertAid First Strand cDNA Synthesis Kit (Fermentas, Maryland, USA).

2.3. Amplification and cloning of open reading frame

At present, the ORF region of VASA gene has not been sequenced in buffalo. Considering the close phylogenetic relationship between buffalo and cattle, a pair of primers (VASA-F1 and VASA-R1) were designed according to the published cattle sequences available in GenBank (Accession numbers NM_001007819.1) to amplify the full-length ORF of buffalo VASA cDNA. Primers were designed with the primer3 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primer sequences used for the full length ORF amplification of VASA were 5′CACGTGCAGCCGTTTAAG3′ (sense primer) and 5′TGCTTTAATCTTTCTGGTTAACACC3′ (antisense primer). They were synthesized commercially by Sigma-Aldrich chemical Pvt. Ltd (Bangalore, India). PCR amplification was done with Long PCR Enzyme Mix (Fermentas, Maryland, USA) under the following conditions: initial step of 93 °C (3 min), then 93 °C (15 s), 60 °C (30 s), 68 °C (2.5 min) for 35 cycles using the full-length ORF primers. PCR products were separated by electrophoresis using 1.5% agarose gel with ethidium bromide staining, and was purified using Agarose Gel DNA Purification Kit (Fermentas, Maryland, USA).

The purified PCR products were ligated to the pTZ57R/T Vector (Fermentas, Maryland, USA), and transformed into competent *Escherichia coli* XL1 blue cells. Recombinant clones were selected by colony PCR. Plasmid DNA was isolated from the positive clones and was further subjected to PCR amplification using the same set of primers used for RT-PCR reaction for confirmation of the insert. Three identified positive clones with a DNA fragment of interest were sequenced by the Xcelris Genomics (Ahmedabad, Gujrat, India).

2.4. The 5′- and 3′-rapid amplification of cDNA ends

The 5′- and 3′-RNA ligase-mediated rapid amplification of cDNA ends (5′- and 3′-RLM-RACE) was performed with GeneRacer kit (Life Technologies, NY, USA) according to the manufacturer's protocol. Briefly, 1 μg of total RNA from adult buffalo testis was treated with calf intestinal phosphatase to remove the 5′-phosphates from any degraded or non-capped mRNA, followed by treatment with tobacco acid pyrophosphatase (TAP) to remove the 5′-cap structure from full-length mRNA, leaving a 5′-phosphate. A 5′-GeneRacer RNA oligo was ligated to the TAP-treated mRNA using T4 RNA ligase. After oligo ligation, mRNA was reverse-transcribed using SuperScript III RT and the GeneRacer Oligo dT primer. The regions corresponding to the legitimate 5′-ends of the capped mRNA species were amplified by two consecutive PCR amplifications. The first round of PCR was performed using a sense GeneRacer 5′- outer primer (5′CGACTGGAGCACGAGGAC ACTGA3′) and an antisense VASA-specific primer (5′ACGGCAACCTCG GAAACTACCTCTT3′). Nested PCR amplification was carried out using 2 μL outer PCR products as a template DNA with a sense GeneRacer 5′-Nested primer (5′GGACACTGACATGGACTGAAGGAGTA3′) and a nested antisense VASA-specific primer (5′CCTCTTCCACCTCTTCTGGACGAT3′).

To obtain 3′-ends of VASA, 1 μg total RNA was reverse transcribed using GeneRacer Oligo dT primer. After cDNA synthesis, the 3′-end of RNA was obtained by two rounds of PCR amplification. The first round

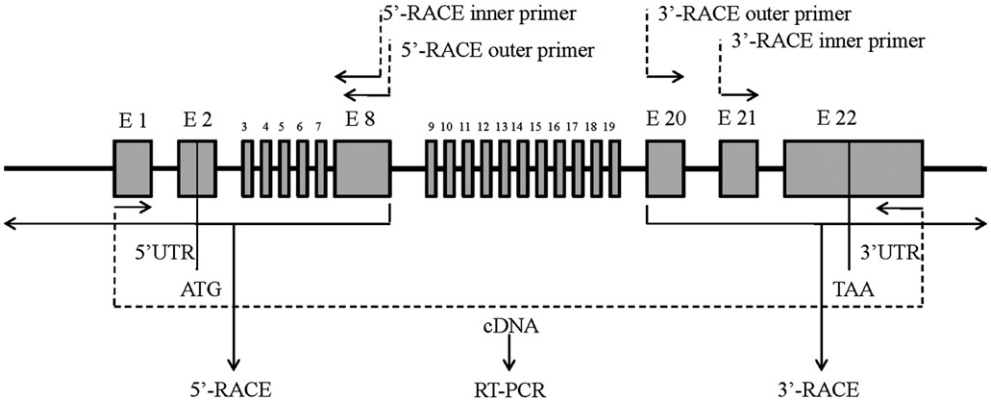

Fig. 1. Schematic representation of the strategy used to determine the structural organization of the buffalo VASA gene. The coding region was amplified using cDNA by RT-PCR. The genomic DNA is depicted with exons as rectangular boxes and the introns as a solid line; 5′- and 3′-UTRs were determined by RLM 5′-RACE and 3′-RACE; primer positions for RACE are shown by arrows. RT-PCR, reverse transcription-polymerase chain reaction; UTRs, untranslated regions; 5′- and 3′-RLM-RACE, 5′- and 3′-RNA ligase-mediated rapid amplification of cDNA ends.