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Molecular characterization of two isoforms of ZFAND3 cDNA from the Japanese quail and the leopard gecko, and different expression patterns between testis and ovary☆

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ABSTRACT

Zing finger AN1-type domain 3 (ZFAND3), also known as testis expressed sequence 27 (Tex27), is a gene found in the mouse testis, but its physiological function is unknown. We identified the full-length sequences of two isoforms (short and long) of ZFAND3 cDNA from Japanese quail and leopard gecko. This is the first cloning of avian and reptilian ZFAND3 cDNA. The two isoforms are generated by alternative polyadenylation in the 3′UTR and have the same ORF sequences encoding identical proteins. There were highly conserved regions in the 3′UTR of the long form near the polyadenylation sites from mammals to amphibians, suggesting that the features for determining the stability of mRNA or translation efficiency differ between isoforms. The deduced amino acid sequence of ZFAND3 has two putative zinc finger domains, an A20-like zinc finger domain at the N-terminal and an AN1-like zinc finger domain at the C-terminal. Sequence analysis revealed an additional exon in the genomic structures of the avian and reptilian ZFAND3 genes which is not present in mammals, amphibians, or fish, and this exon produces additional amino acid residues in the A20-like zinc finger domain. Expression analysis in Japanese quail revealed that the expression level of ZFAND3 mRNA was high in not only the testis but also the ovary, and ZFAND3 mRNA was expressed in both spermatides of the testis and oocytes of the ovary. While the short form mRNA was mainly expressed in the testis, the expression level of the long form mRNA was high in the ovary. These results suggest that ZFAND3 has physiological functions related to germ cell maturation and regulatory mechanisms that differ between the testis and ovary.

1. Introduction

Zing finger AN1-type domain 3 (ZFAND3), also known as testis expressed sequence 27 (Tex27), is a gene originally isolated from the subtractive cDNA library of mouse testis (López-Fernández and del Mazo, 1996). ZFAND3 is known to express preferentially in post-meiotic cells in the mouse testis during spermatogenesis, and the presence of multiple transcripts has been reported (de Luis et al., 1999). These results suggest that ZFAND3 is involved in spermatogenesis, but its physiological function remains unclear because only a few studies on ZFAND3 have been conducted. Until now, research of ZFAND3 has focused on the testis, while expression has never been examined in the ovary, despite its homology to the male testis. Moreover, the existence of multiple transcripts remains to be elucidated at the molecular level.

In our research, we have been analyzing the effect of environmental factors on testicular activity of a lizard, the leopard gecko, by quantifying the mRNA of sex steroid hormone receptors (Endo and Park, 2003) and POMC (Endo and Park, 2004). During the course of these experiments, we unexpectedly isolated the partial sequences of ZFAND3 cDNA from the testis. The mRNA expressional change was observed in the testes of leopard geckos in groups reared under different environmental conditions. This result suggests that ZFAND3 has a function related to spermatogenesis and is a useful marker gene for testicular function, prompting us to identify the full-length sequences of ZFAND3 cDNA and examine its expression pattern to predict its physiological functions. The Japanese quail (*Coturnix japonica*) would provide a suitable model for exploring the physiological functions of ZFAND3, because its reproductive activity can be easily controlled by altering photoperiod conditions. Molecular cloning of ZFAND3 cDNA has been conducted in mammals, amphibians, and fish by comprehensive sequence analysis, but the cDNA sequences from birds and reptiles have not yet been identified.

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; jq, Japanese quail; lg, leopard gecko; mRNA, messenger RNA; NUP, Nested Universal Primer; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SSC, 0.15 M NaCl/0.015 M Na3·citrate pH 7.6; Tex27, testis expressed sequence 27; UTR, unstranslated region(s); ZFAND, zinc finger AN1-type domain.

☆ The nucleotide sequences reported in this paper have been deposited in the DDBJ/EMBL/GenBank with accession numbers JF740026 (jqZFAND3-S), JF740027 (jqZFAND3-L), JF740028 (lgZFAND3-S), JF740029 (lgZFAND3-L), BK008021 (green anole ZFAND3), and BK008022 (zebra finch ZFAND3).

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In this study, and for the first time, we cloned avian and reptilian ZFAND3 from the Japanese quail and the leopard gecko, and characterized the unique molecular features of the two mRNA isoforms. Avian and reptilian ZFAND3 genes had unique genomic structures and amino acid residues compared with those of other vertebrate classes. Also, a phylogenetic tree of ZFAND3 and other members of the zinc finger AN1 super family was constructed. Further, expression analysis of ZFAND3 mRNA in the Japanese quail was conducted using RT-PCR and *in situ* hybridization.

2. Materials and methods

2.1. Animals

Mature male and female Japanese quail, *C. japonica*, were used in this study. They were treated according to the guideline of the Bioscience Committee at the University of Tokyo. Fertilized Japanese quail eggs were obtained from a local supplier (Motoki Corporation, Saitama, Japan). Newly hatched Japanese quail were reared in mixed-sex groups under a long-day condition of 16-h light and 8-h dark (lights on at 6:00, lights off at 22:00) for 4 weeks. After 4 weeks of age, male birds were isolated in individual cages, and female birds were kept in groups, and both sexes were reared under the same long-day condition for an additional 4 weeks until sexually mature. Birds were provided feed and water *ad libitum*. Animals were killed by rapid decapitation, followed by complete bleeding. Tissues and organs were immediately dissected, frozen in liquid nitrogen, and stored at −80 °C until use. Testes and ovaries used for *in situ* hybridization were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline overnight.

The fertile male leopard geckos (*Eublepharis macularius*) of 15 months of age used in this study were treated according to our previous report (Endo and Park, 2003).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). The cDNA used for rapid amplification of cDNA ends (RACE) was synthesized from 3 μg of total RNA using a SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Palo Alto, CA) according to the manufacturer's instructions. The cDNAs used as templates for RT-PCR were synthesized from 3 μg of denatured total RNA using 5 μM oligo(dT) primer and 100 U of M-MLV reverse transcriptase (Promega, Madison, WI) in a 20-μl reaction volume with incubation at 42 °C for 1.5 h.

2.3. Molecular cloning of Japanese quail and leopard gecko ZFAND3 cDNA by RACE and RT-PCR

Sense and antisense gene-specific primers were designed based on the partial sequence of leopard gecko ZFAND3 cDNA. RACE was carried out to obtain the complete sequence. 3′ and 5′ RACE was performed from testis cDNA using SE02 and Nested Universal Primer (NUP), and NUP and lg-AS01 (Table 1), respectively. All of the following PCR amplifications were performed in a 20-μl reaction mixture containing each primer at 1 μM, 0.25 U of TaKaRa Ex Taq (TaKaRa, Shiga, Japan), each dNTP at 250 μM, and Ex Taq Buffer (TaKaRa). PCR condition was as follows: 94 °C for 5 min, 30 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min. The amplified products were separated by electrophoresis on 1.5% agarose gel and visualized using ethidium bromide staining. The DNA fragments were extracted using phenol and chloroform, cloned into a pGEM-T vector (Promega) and sequenced using a dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Tokyo, Japan).

Sense and antisense gene-specific primers for confirming the ORF were designed based on the results of 3′ and 5′ RACE. RT-PCR was carried out to confirm the entire ORF of leopard gecko ZFAND3 cDNA from testis cDNA using SE01 and AS01 (Table 1) as described above.

During the course of the experiments, we found that the ZFAND3 mRNA isoform of other species (human, mouse, and African clawed frog) had a longer 3′UTR (long form) than the sequence we identified (short form) in the nucleotide database (NCBI). An antisense probe, specific to the 3′UTR of the long form, was designed based on the nucleotide homology of other species. RT-PCR was carried out to obtain the entire ORF of leopard gecko ZFAND3 cDNA long form from testis cDNA using SE01 and AS02 (Table 1). The reaction condition for RT-PCR was as follows: 94 °C for 5 min, 30 cycles of incubation at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 3 min, and finally 72 °C for 10 min. 3′RACE was carried out to confirm the polyadenylation sites of the long form with SE03 and NUP (Table 1) as described above. The sequence of the entire ORF was conducted independently three times to avoid potential PCR amplification errors.

Molecular cloning of the two isoforms of Japanese quail ZFAND3 cDNA was performed based on the results of cloning of the leopard gecko and was performed in the same manner.

2.4. Comparison of the 3′UTR sequences of ZFAND3 long form cDNA from various vertebrates

The CLUSTAL X program (version 2.0.12) (Larkin et al., 2007) was used with default settings to align the 3′UTR sequences of the ZFAND3 long form cDNA from various vertebrates. The GenBank accession numbers of ZFAND3 cDNAs used in the comparison are as follows: Japanese quail, JF740027; Leopard gecko, JF740029; Green anole, BK008021; Zebra finch, BK008022; Human, NM_021943; Mouse, BC083124; African clawed frog, BC081022; and Chicken, XM_419483.

2.5. Prediction of the microRNA target sites in the 3′UTR

The putative microRNA target sites in the 3′UTRs of mouse and human ZFAND3 mRNA were predicted using the microRNA.org website (http://www.microrna.org/; Betel et al., 2008). Target predictions were based on a development of the miRanda algorithm. The target sites predicted by miRanda are scored according to the likelihood of mRNA downregulation using mirSVR, a regression model that focuses on the sequence and contextual features of the predicted miRNA:: mRNA duplex (Betel et al., 2010). Target sites with good (<= −0.1) mirSVR scores found in mouse and human ZFAND3 were compared with the 3′UTR sequences of ZFAND3 long form cDNA from various vertebrates to examine whether such sites are conserved.

Table 1
Oligonucleotide primers used for RT-PCR, RACE, and sequencing.

Name	Nucleotide sequence	Usage for
SE01 (common)	5′-GACTCCAGCTGACCGGCCTGGAAT-3′	RT-PCR
AS01 (common)	5′-GGCTGACTTTGTCCTAAATAAGGCT-3′	RT-PCR
AS02 (common)	5′-AACAAACTTCACAGAACAGATACTG-3′	RT-PCR
SE02 (common)	5′-CACTAATGTTAGCCTTATTTAGGAC-3′	3′RACE
SE03 (common)	5′-AGTTCGTGTGAATTATGGACAATGTTCACT-3′	3′RACE
NUP (adaptor primer)	5′-AAGCAGTGGTATCAACGCAGAGT-3′	5′ RACE, 3′RACE
jq-SE01 (jq specific)	5′-AGCAGCAACTGCAGCTGCTTAAG-3′	Sequencing
jq-AS01 (jq specific)	5′-GCTGCTGGCTTCGTATTCCTGAATA-3′	Sequencing
jq-SE02 (jq specific)	5′-GTACAGCAGGAACTGGGATCATGTC-3′	Sequencing and RT-PCR
jq-AS02 (jq specific)	5′-GTGGCTAGCAGGCACCTTAGTAAC-3′	RT-PCR
jq-AS03 (jq specific)	5′-AGTTCCCAGCGTAAGGCTTTTAGAGTG-3′	RT-PCR
lg-SE01 (lg specific)	5′-GAGGTTAGGTAGCATCACCAGCA-3′	Sequencing
lg-AS01 (lg specific)	5′-TTTGCTGCGCTCGCTGCCGGCGTCT-3′	5′RACE
lg-AS02 (lg specific)	5′-AGTGACAGGAGGGTAATCCACTCT-3′	Sequencing
GAPDH SE	5′-TGTGACTTCAATGGTGACAG-3′	RT-PCR
GAPDH AS	5′-CAGATCAGTTTCTATCAGCC-3′	RT-PCR