Journal of Biochemistry and Molecular Biology, Vol. 38, No. 2, March 2005, pp. 206-210

JOURNAL OF Biochemistry and Molecular Biology

Cloning and Expression Analysis of Gonadogenesis-associated Gene *SPATA*4 from Rainbow Trout (*Oncorhynchus mykiss*)

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Received 22 October 2004, Accepted 3 December 2004

Gonadogenesis is a complicated process which involves multi-gene interactions. A rainbow trout (*Oncorhynchus mykiss*) gene spermatogenesis associated 4 (*SPATA*4) was cloned and characterized from adult rainbow trout testis. The cDNA sequence of rainbow trout *SPATA*4 contains an open reading frame of 1, 081 nucleatides encoding a putative protein of 259 amino acids. The putative protein from rainbow trout shares a 76.8% homology with zebrafish SPATA4. No trans-membrane regions or signal peptide were detected using bioinformatics methods. Subcellular localization analysis revealed that rainbow trout SPATA4 was a nuclear protein with highest possibility (39.1%). Multi-tissue reverse transcriptase PCR (RT-PCR) was performed to examine the distribution of rainbow trout *SPATA*4 in eleven organs of adult rainbow trout. The result demonstrated that this gene express specifically in testis and slight amount of expression was detected in ovary. Further analysis of *SPATA*4 characterization and function in rainbow trout may provide insight into the understanding of gonadogenesis process.

Keywords: Germ cell apoptosis, Gonadogenesis, Rainbow trout, *SPATA*4, Testis/ovary

Introduction

The gonadogenesis and apoptosis of germ cells is a complicated process regulated by multi-gene interactions (Eddy, 2002), which research on this process may provide insight into further understanding of gonad development, sex differentiation and physiological and pathological process of fertility (Nakanishi, 1995). Several genes have been reported to be involved in the gonadogenesis process (Knower *et al.*, 2003). It was reported that 24 expressed sequence tags (ESTs) of *SPATA*4 gene (previously named *TSARG*2) were first cloned and characterized from mouse testis spermatogenic cells in mouse cryptorchidism model and using suppression subtractive hybridization (Jiang *et al.*, 2001). Subsequently human *SPATA*4 gene, which was deduced as a candidate oncogene (Liu *et al*., 2004), was cloned using nested PCR and draft human genome searching (Liu *et al.*, 2002). The primary functional study indicated that the *SPATA*4 gene is highly conserved in various vertebrates including human, chimpanzee, mouse, rat and zebrafish (unpublished).

As one of the most widely studied model fish species, rainbow trout was utilized in researches concerning carcinogenesis, toxicology, immunology, disease ecology, physiology, nutrition and reproduction (Thorgaard *et al.*, 2002). Rainbow trout bears the advantage of relatively large size and easiness to culture, which is particularly appropriate for the extraction of large quantity of tissue samples and genetic manipulation.

Only a few gonad specific genes in rainbow trout have been identified and studied, which were demonstrated to play important roles in gonadogenesis and sex differentiation. The SRY-related cDNA sequence encoding a protein designated SOX-LZ was cloned and detected exclusively in rainbow trout testis (Takamatsu *et al*., 1995). Another gene, cytochrome P450 (11beta), was revealed to express specifically in testis and slightly in anterior kidney (Liu *et al.*, 2000). And a DMRT1 homologue was cloned in rainbow trout which expressed during testicular differentiation (Marchand *et al.*, 2000). The more in-depth study of rainbow trout gonad specific genes would be highly important and revelatory to further understanding of gonadogenesis and sex differentiation process in fishes.

In this study, *SPATA*4 gene was cloned and characterized

The cDNA sequence reported in this paper has been submitted to GenBank and has accession number AY651919.

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from rainbow trout using EST assembling methods, and the open reading frame (ORF) was validated. The sequence of rainbow trout *SPATA*4 gene and the putative protein were analyzed using bioinformatics methods. To primarily study the distribution of this gene, multi-tissue RT-PCR was applied to determine the expression pattern of *SPATA*4 in eleven organ tissues extracted from adult rainbow trout. The results indicated that the rainbow trout *SPATA*4 gene is highly conserved in vertebrates and specifically expressed in adult rainbow trout testis and slightly in ovary.

Materials and Methods

Animals Three-year old wild-type rainbow trout were provided by Beijing Fishery Institute, and eleven organs were obtained as follows: brain (whole), eyes (whole), gill, skin (without scales), heart (whole), spleen (whole), liver, ovary (whole), intestine (total), skeletal muscle, and testis (total).

Silicon cloning and analysis of rainbow trout *SPATA*4 The full length cDNA sequence of rainbow trout *SPATA*4 was cloned and analyzed using bioinformatics methods. Full length cDNA sequence of human *SPATA*4 gene (GenBank accession no. AY040204) was obtained directly from GenBank database at the National Center for Biotechnology Information (NCBI), and was used to blast the AGENAE rainbow trout normalized testis library (tcbi). The blast results of EST CR369348, EST CR373957, EST CR368848, EST CR375384, and BX311705 partial cDNA were assembled using EST machine (http://bio.ifom-firc.it/EST_MACHINE/index.html) to obtain a 1, 081 bp nucleotide sequence. RT-PCR was then performed to clone and validate the ORF of this gene. Total RNA was extracted from adult rainbow trout testis using RNA isolation Kit (Gentra, USA) and the standard protocols recommended by the supplier. One microgram of total RNA was used as the template for the synthesis of first strand cDNA by reverse transcriptase using MMLV First Strand cDNA Synthesis Kit (Sangon, Shanghai). For PCR amplification, primers were designed and synthesized based on the cDNA sequence of rainbow trout *SPATA*4 as follows: 5'-CAA TGG CTT ACG CAC AGC CTC C-3' and 5'-CCT GGC CTT TTA AAT TGC AAT CAG C-3'. The positive control primers were designed according to rainbow trout *β-actin* (GenBank accession no. AF157514) as 5'-GGA GAT GAC GCG CCT CGG GCT G-3' and 5'-CGT AGT CCT CGT AGA TGG GTA C-3' (Sangon, Shanghai). PCR was performed using 1 U of *Taq* DNA polymerase (Sangon, Shanghai) in 10 μL reactions containing 2 μM $MgCl_2$, 5.6 μl sterile H_2O, 1 μl 10×*Taq* buffer, 250 μM of each dNTP, 0.4 μM of each primer, and 1 μL cDNA template from the reverse transcription reaction, according to the standard protocol of PCR kit (Sangon, Shanghai). PCR amplification was conducted under the following conditions: an initial cycle of denaturation step at 95°C for 1 min 30 s, 35 cycles of amplification at 94°C for 40 s, 58°C for 30 s, 72°C for 40 s, and a final extension step at 72°C for 5 min. 2 ml product of each reaction was size-fractioned by 2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The agarose gel was photographed under UV light using Imagemaster VDS (Amersham pharmacia biotech, USA). This PCR fragment was cloned into pUCm-T vectors and sequenced.

Prediction and analysis of putative protein The molecular mass and isoelectric point of the putative rainbow trout SPATA4 were predicted using Compute pI/Mw tool at the ExPASy molecular biology WWW server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/) and the deduced signal peptide was predicted using SignalP (Nielsen *et al*., 1997). The trans-membrane regions of rainbow trout SPATA4 was detected using Tmpred program (http://www.ch.embnet.org/software/TMPRED_form.html/). Possible subcellular localization was indicated by PSORT (Nakai & Horton, 1999; Emanuelsson *et al*., 2000). Motif searches were performed with PROSITE programs at the ExPASy server http://cn.expasy.org/prosite (Sigrist *et al*., 2002) to reveal potential motifs in rainbow trout SPATA4. The homology between amino acid sequence of SPATA4 from zebrafish (GenBank accession no. AY651920) and rainbow trout was analyzed using ClustalW (http://www.ebi.ac.uk/clustalw/).

RNA extraction and multi-tissue RT-PCR Total RNA was extracted from eleven organs of adult rainbow trout and the first strand cDNA was synthesized from each sample by reverse transcriptase as described previously. The PCR amplification was performed according to the standard condition and protocols provided by the PCR kit (Sangon, Shanghai) to reveal the expression pattern of *SPATA*4 gene in adult rainbow trout.

Results

Cloning and analysis of the rainbow trout SPATA4 gene EST assembling and analysis methods were used to silicon clone the cDNA sequence of rainbow trout *SPATA*4 (Fig. 1), which has been submitted to GenBank (GenBank accession no. AY651919). The PCR fragment was cloned into pUCm-T vectors and sequenced. The complete cDNA sequence of rainbow trout *SPATA*4 is 1, 081 bp in length (Fig. 2). A

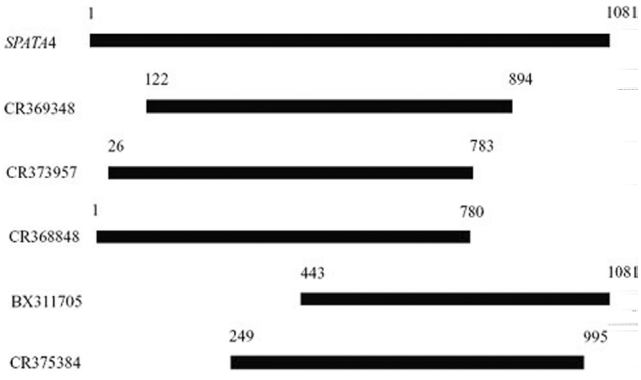

Fig. 1. Cloning of rainbow trout *SPATA*4 gene using ESTs assembling and analysis. The 1, 084 bp rainbow trout *SPATA*4 was determined by assembling EST CR369348, EST CR373957, EST CR368848, EST CR375384, and BX311705 partial cDNA using EST machine (http://bio.ifom-firc.it/EST_MACHINE/index.html).

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1    tgataaacatgtcaaatgtgccatatgagctagtaagctcgttagcttagctagttcgttt
62   accacaaatgttctataagacacagtgttatatagcaacaacttaacgaaatactgaacc
122  agaaaagttagaccaatagtaacttatcttatcattttgtaagcgtgtgtggctggttca
182  tagacgctagctgaattaacattacagctgacaatggcttacgcacagcctcccaaaaag
 1   *                              M  A  Y  A  Q  P  P  K  K
242  acaggactgccacgagaagttctgaagtggctccaaagcctggatttgtcattttctcca
 10  T  G  L  P  R  E  V  L  K  W  L  Q  S  L  D  L  S  F  S  P
302  aagaatatgcgcagggatttctccaatggctaccttgtggcagagatgttctcctggtat
 30  K  N  M  R  R  D  F  S  N  G  Y  L  V  A  E  M  F  S  W  Y
362  tatcatgaagacttccccatgcactcctacaacaatgggacatcacttccaaccaaacag
 50  Y  H  E  D  F  P  M  H  S  Y  N  N  G  T  S  L  P  T  K  Q
422  ggcaactgggcacaaatagagaggtttctagtgaaacagaacatccatctgcaaaaggaa
 70  G  N  W  A  Q  I  E  R  F  L  V  K  Q  N  I  H  L  Q  K  E
482  gttctggacgggaccatccattgcaagccaggagcagcggagcttctagtgcaagagatt
 90  V  L  D  G  T  I  H  C  K  P  G  A  A  E  L  L  V  Q  E  I
542  tacaccatcttaactaacaggaggatcaaagggatccagggcagggagattgacttcaca
110  Y  T  I  L  T  N  R  R  I  K  G  I  Q  G  R  E  I  D  F  T
602  gacagggactaccaggaccagctgcccatgctagctcgggccacagcctccaaggccatc
130  D  R  D  Y  Q  D  Q  L  P  M  L  A  R  A  T  A  S  K  A  I
662  aagaacaacctgcggctgaccgaggtcatagccgagcccaacatctccaccaatcagagg
150  K  N  N  L  R  L  T  E  V  I  A  E  P  N  I  S  T  N  Q  R
722  aaggtgcaggccatcatccacatgcacctggagcagagggcggccgagagagtccagaac
170  K  V  Q  A  I  I  H  M  H  L  E  Q  R  A  A  E  R  V  Q  N
782  cccaagcgcttcaacgtgaagcccaccttgggagaactggccgtgagactgcccccatct
190  P  K  R  F  N  V  K  P  T  L  G  E  L  A  V  R  L  P  P  S
842  tctcaccacggagatgacagctccgatagcaacgcctcagtacaaagtggaaccgcaaag
210  S  H  H  G  D  D  S  S  D  S  N  A  S  V  Q  S  G  T  A  K
902  tcatgtgaaccatccatcaggagtaaagccagtgtccatttcaaggagattgaggtgcgt
230  S  C  E  P  S  I  R  S  K  A  S  V  H  F  K  E  I  E  V  R
962  cagatggacagacgctcgctgattgcaatttaaaaggccaggagattcagccgtttggtg
250  Q  M  D  R  R  S  L  I  A  I  *
1022 tgtatttgtgagagatgcctctgatacgatacctctaataaaagtagctttatacttttt
```

Fig. 2. cDNA and predicted protein sequence of rainbow trout *SPATA*4 gene and potential motifs. Polyadenylation signal (AATAAA) is underlined; Stop codon is indicated by asterisk (*). Blue: Protein kinase C phosphorylation site; Red: N-glycosylation site; Green: N-myristoylation site; Framed: Tyrosine sulfation site; Shadowed: Casein kinase II phosphorylation site.

potential polyadenylation signal (AATAAA) was detected at nucleotide position 844.

Analysis of the putative protein The rainbow trout *SPATA*4 gene consists of an open reading frame encoding 259 amino acids with a theoretical molecular weight of 29, 578 KD and the isoelectric point of 9.47 using ORF finder at NCBI and Compute pI/Mw tool at the ExPASy server. No signal peptide or transmembrane domain could be detected in the amino acid sequence of rainbow trout SPATA4 using SignalP version 3.0 and TMpred program. The subcellular

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zebrafish      MAYSLSPKKAGLPREVLKWLQSLDLSFFPKNVRRDFSNGYLVAEIFSWYFPRDFQMHSFD 60
rainbow trout  MAYAQPPKKTGLPREVLKWLQSLDLSFSPKNMRRDFSNGYLVAEMFSWYYHEDFPMHSYN 60
               ***: .***:***************** ***:***********:****: .** ***::

zebrafish      NGTSLAAKQSNWSQIEKFFVKQNISLVKEMIDGTIHCKPGAAELLVQEIYTILTNRSIQA 120
rainbow trout  NGTSLPTKQGNWAQIERFLVKQNIHLQKEVLDGTIHCKPGAAELLVQEIYTILTNRRIKG 120
               *****.:**.**:***:*:***** * **::************************** *:.

zebrafish      IQRVEQGFTDKPYQDQLPMVARATASVSIKSNLSLTEVKAEPNIITNQRKVLAIIHRHLE 180
rainbow trout  IQGREIDFTDRDYQDQLPMLARATASKAIKNNLRLTEVIAEPNISTNQRKVQAIIHMHLE 180
               **  *.***: *******:****** :**.** **** ***** ****** **** ***

zebrafish      QRKEERVQDPKRFNVKPTLGEQAVRLLAHQHEPN--------LQMNTSQAAC-------- 224
rainbow trout  QRAAERVQNPKRFNVKPTLGELAVRLPPSSHHGDDSSDSNASVQSGTAKSCEPSIRSKAS 240
               **  ****:************ **** . .*. :        :* .*:::.

zebrafish      ------------------
rainbow        VHFKEIEVRQMDRRSLIAI 259
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Fig. 3. Alignments between the putative zebrafish and rainbow trout SPATA4 protein. " * " indicates that the residues or nucleotides in that column are identical in all sequences in the alignment. " : " indicates that conserved substitutions have been observed, and " . " indicates the semi-conserved substitutions.

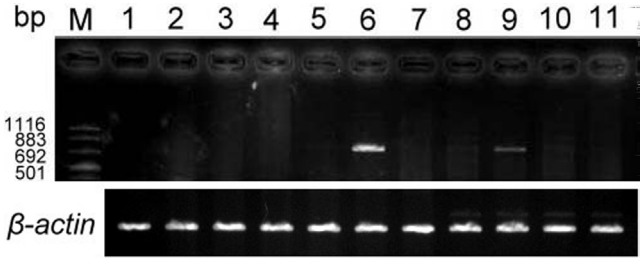

Fig. 4. Expression of *SPATA*4 gene in eleven organ tissues from adult rainbow trout. Total RNA was extracted from eleven adult rainbow trout organs for multi-tissue RT-PCR using the *SPATA*4 specific primers. From left to right were: M. pUC 8 marker ladder; 1. brain; 2. heart; 3. liver; 4. gill; 5. skeleton muscle; 6. testis; 7. intestine; 8. eye; 9. ovary; 10. skin; 11. spleen. The expected 791 bp band was observed in rainbow trout testis and ovary (upper panel). The same samples were assayed for *â-actin* mRNA expression as a positive control (lower panel).

location prediction using PSORT indicated that rainbow trout SPATA4 is more possible to be a nuclear protein (39.1%). Four motifs were identified using PROSITE within the sequence of SPATA4 as follows: protein kinase C phosphorylation site, N-glycosylation site, N-myristoylation site, casein kinase II phosphorylation site (Fig. 2). The amino acid sequence of rainbow trout SPATA4 was aligned with zebrafish SPATA4 and a 76.8% homology was detected (Fig. 3).

Spatial expression of *SPATA*4 gene in adult rainbow trout RT-PCR was performed with total RNA extracted from eleven organs of adult rainbow trout to determine the tissue-specific expression pattern of *SPATA*4 gene, which appeared as a band of 791 bp in 2% agarose gel. Abundant amplification product of *SPATA*4 gene was detected using rainbow trout *SPATA*4

specific primers in adult rainbow trout testis and slight amount of PCR products were observed in ovary. No visible PCR product was detected with cDNA derived from the other nine organs (Fig. 4).

Discussion

*SPATA*4 gene has been cloned in various vertebrates such as human, mouse, rat and chimpanzee and demonstrates a testis specific expression pattern in human (Liu *et al.*, 2002), mouse (Liu *et al.*, 2003) and rat (Liu *et al.*, 2004). To further study the characteristic and distribution of this gene, *SPATA*4 was cloned from rainbow trout, a widely studied model fish species. ESTs analysis, an efficient and powerful approach for identifying and profiling new gene in various organisms (Douglas *et al.*, 1999), was used to silicon clone rainbow trout *SPATA*4 gene from tcbi. The amino acid sequence of putative protein encoded by rainbow trout *SPATA*4 shares a 76.8% homology with zebrafish SPATA4, and our recent study demonstrated that this gene is highly conserved in various vertebrates (unpublished). Muti-tissue RT-PCR was carried out to investigate the distribution of rainbow trout *SPATA*4 gene, and a spatial specific expression pattern was detected. The result revealed that rainbow trout *SPATA*4 gene expressed specifically in testis and slightly in ovary, with no expression observed in the other nine organ tissues. Similar expression pattern of this gene was discovered in zebrafish (unpublished), but in human and mouse, *SPATA*4 expression was exclusively detected in testis (Liu *et al.*, 2002; Liu *et al.*, 2003). The difference of the expression pattern between fish and mammals might relate to the different evolutionary ranks and the different mechanism of sex differentiation and determination. As a gonochoristic iteroparous fish model, rainbow trout is one of the most appropriate fishes to study reproductive biology (Bromage *et al.*, 1992), which was suggested to have a genetically determined X/Y sex determination system like mammals (Thorgaard, 1977; Thorgaard and Gall, 1979) but was modulated by environmental factors (Baroiller and Guiguen, 2001). It differs from the zebrafish during sex determination in the way that there is a sex reversal process of zebrafish during which both sexes pass through an ovary-like stage before differentiating into the sexual phenotypes (Bull, 1983). However, the analysis of sex chromosome reveals that the rainbow trout is in the early stages of sex chromosome differentiation (Thorgaard *et al.*, 2002). Hence, the specific evolutionary stage of sex differentiation of rainbow trout may be a possible explanation to the differences of *SPATA*4 expression pattern between fish and mammals. Further more, it has been revealed that some fish species, including zebrafish and rainbow trout, have a high incidence of duplicated genes, which may complicate the interpretation of genetic information (Allendorf and Thorgaard, 1984; Venkatesh, 2003). Therefore, it is concluded that this gene is associated with the gonadogenesis of rainbow trout. The mechanism and significance of gonad specific expression of *SPATA*4 in rainbow trout are under further research.

Acknowledgments This work was supported by research grants from the Tsinghua-Yue-Yuen Medical Sciences Fund (Grant THYY20040008), the National High Technology Research and Development Program of China (No. 2001AA621140) and the China Postdoctoral Science Foundation (No. 2004035052).

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