Cloning and Characterization of the SSB-1 and SSB-4 Genes Expressed in Zebrafish Gonads

Jian-Zhen Li · Ya-Ping Zhou · Yan Zhen · Yan Xu · Peng-Xiang Cheng · Huan-Nan Wang · Feng-Jiao Deng

Received: 22 April 2008 / Accepted: 8 September 2008 / Published online: 30 January 2009
© Springer Science+Business Media, LLC 2009

Abstract The protein of the *gustavus* (*gus*) gene has a typical SOCS box domain and repeats in the splA and RyR (SPRY) domains. GUS can interact with Vasa and is necessary for the specification of germ cells. We cloned two zebrafish genes, SSB-1 and SSB-4 (SPRY domain SOCS box proteins). Phylogenetic analysis shows that zebrafish SSB-1 and SSB-4 are clustered into clades of SSB-1-like and SSB-4-like genes from other species. RT-PCR analysis of tissues revealed that zebrafish SSB-1 and SSB-4 are expressed in the ovary and testis. We investigated the spatial expression patterns of zebrafish SSB-1 and SSB-4 in embryos from the two-cell stage to 72 h post-fertilization (hpf) using whole-mount in situ hybridization. SSB-1 and SSB-4 transcripts were present in all blastomeres during the early embryonic stages, but the genes differ in their expression pattern. SSB-4 mRNA was located in the region of the primordial germ cells in 24 and 72 hpf embryos, but SSB-1 mRNA was not detected at these stages. We hypothesize that SSB-4 plays a role in the early development of germ cells.

Keywords Zebrafish · SSB-1 · SSB-4 · *Gus* · Primordial germ cells

Introduction

In sexually reproducing organisms, primordial germ cells (PGCs) give rise to gametes that are responsible for the development of a new organism in the next generation. In *Drosophila* and several other species, the formation of germ cell precursors depends on a specialized cytoplasm called the germplasm, which...
contains RNAs and proteins that are required for embryonic patterning and germ cell formation (Eddy 1975; Saffman and Lasko 1999; Schisa et al. 2001; Seydoux and Strome 1999). Many studies have demonstrated that the specification, differentiation, and migration of PGCs are governed by a tightly controlled series of gene expression events. The *vasa* gene was originally identified in *Drosophila* and encodes a DEAD (Asp-Glu-Ala-Asp) box family protein, which is a putative RNA helicase and is present in both the polar granules at the posterior end of the oocyte and in the nuage structure of the germ cells (Mahowald 1992; Strome 1992).

Recently, the *gustavus* (*gus*) gene, whose protein product can interact with Vasa, has been identified in *Drosophila*. The *gus* gene is required for the localization of Vasa at the pole plasm and the specification of germ cells (Styhler et al. 2002). Two highly conserved homologs of *gus* named *SSB-1* and *SSB-4* have been identified in mice. Surprisingly, although the expression of *gus* is restricted to the germ cells in *Drosophila*, immunoblotting and in situ hybridization analysis revealed that both genes are expressed in granulosa cells at all stages of follicular development in mice, suggesting that *SSB-1* and *SSB-4* might play roles in regulating granulosa cell physiology in mice (Xing et al. 2006). Four SSB homologs have been identified in mice (Hilton et al. 1998). Also, SSB-1, -2, and -3 have been shown to be expressed in the mouse testis, and SSB-1 and -2 are expressed in the mouse ovary (Masters et al. 2005).

GUS and its homologs contain two well-conserved protein domains: a SPRY domain, which was first identified in ryanodine receptors and is thought to mediate protein–protein interactions (Wang et al. 2005), and a suppressor of cytokine signaling (SOCS) box domain, which can target proteins for ubiquitylation and/or proteasomal degradation (Kamura et al. 1998; Zhang et al. 1999).

Zebrafish is a popular model organism for studying embryonic development. Phylogenetically, zebrafish lies between invertebrates and mammals. In zebrafish, several genes related to the specification and migration of PGCs have been identified, including *vasa* (Olsen et al. 1997; Yoon et al. 1997), *nanos* (Koprunner et al. 2001), *dead end* (Weidinger et al. 2003), and *staufen* (Ramasamy et al. 2006). These genes have related expression patterns and functions among different species. Thus far, there have been no reports of *gus* homologs in zebrafish.

In this work, we cloned and characterized two *gus* homologs named *SSB-1* and *SSB-4* in zebrafish. Sequence analysis showed that zebrafish *SSB-1* and -4 share high sequence identity with other known and predicted *gus* sequences. We examined the expression patterns of zebrafish *SSB-1* and -4 mRNA by RT-PCR and whole-mount in situ hybridization; these findings are discussed from the viewpoint of functional and developmental integration.

**Materials and Methods**

**Experimental Animals**

Zebrafish (*Danio rerio*) were obtained from the Institute of Hydrobiology of the Chinese Academy of Science and maintained at 28.5°C on a 14 h:10 h light:dark cycle. Embryos were collected from natural spawning and cultured in egg water...
Identification, Sequencing, and Analysis of the Zebrafish SSB-1 and SSB-4 Genes

Zebrafish SSB-1 and -4 sequences were cloned by RT-PCR from zebrafish ovary-derived cDNA. Primers were designed based on predicted gene sequences (NM_001025460, XM_681061) in the zebrafish UniGene database. PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced. SSB-1 and -4 predicted protein sequences from several species were aligned using ClustalW. Phylogenetic trees were constructed using neighbor-joining (100 runs) and maximum-likelihood (100 runs) methods (Phylip). A consensus tree based on information from those two trees was also generated (Phylip).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Nonquantitative RT-PCR was used to amplify the SSB-1 and -4 genes from different tissues of adult zebrafish and embryos at various stages. PCRs were performed in a 20 μl reaction mix containing 10 mM Tris–HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 150 mM dNTP, 0.2 mM each primer, and 1 U Taq DNA polymerase. Amplification conditions were: 95°C, 30 s; 64°C, 30 s; 72°C, 1 min for 25 cycles SSB-1, SSB-4, and β-actin. Primers for SSB-1 (780 nt product) were forward 5’ TTCTGGAAGCTGCTTCTTCCATC 3’ and reverse 5’ GTATTGTGAGCCAG CACTTTC 3’. Primers for SSB-4 (822 nt product) were forward 5’ GATGGGCCAG AAAATCTCAGG 3’ and reverse 5’ CTCACCTGGTACTGGAGGTAATT 3’.

Quantitative RT-PCR

Real-time RT-PCR was used to quantify SSB-1 and SSB-4 expression using the multichannel RotorGene 3000 (Corbett Research, Australia), according to the manufacturer’s protocol. PCR cycling conditions were: 5 min at 95°C; 40 cycles of 25 s at 95°C, 20 s at 64°C, and 40 s at 72°C in a 25 μl reaction mix containing 1× Sybr Green I. Simultaneous detection of the β-actin gene was used to normalize SSB-1 and -4 expression. For robustness, each sample was analyzed at least in triplicate. Data were analyzed by RotorGene software version 4.6 and plotted in Microsoft Excel. Primers used for SSB-1 (276 nt product) were forward 5’ CCAGCTTTTCTTGAGCCCGATG 3’ and reverse 5’ TCGTCTCCTTCTCCAAAT GCCACC 3’. Primers for SSB-4 (230 nt product) were forward 5’ CAGAGGA GGCTTTTGTGCCACC 3’ and reverse 5’ CTCTGGTACTGGAGGTAATT 3’.

In situ Hybridization and Whole-Mount in situ Hybridization

SSB-1 riboprobes were prepared from a 780 bp region (nucleotides 1215–1987, GenBank accession no. NM_001025460), and SSB-4 riboprobes were prepared from a 582 bp region (nucleotides 344–926, GenBank acc. no. XM_681061).
were labeled with digoxigenin-UTP using SP6 or T7 RNA polymerase (SP6 for production of the antisense probe, T7 for the sense probe). In situ hybridization was performed at 65°C, and hybridization signals were detected using the NBT/BCIP system according to the manufacturer’s instructions (Roche).

For in situ hybridization, gonad samples were embedded in tissue freezing medium (Leica) at -25°C, after which they were cut on an 8 µm Leica CM 1850 microtome. Testis samples were cut into 6 µm sections. For whole-mount in situ hybridization, embryonic stages were assessed according to hours postfertilization and morphological criteria (Kane and Kimmel 1993). Whole-mount in situ hybridization for embryos was performed as described (Westerfield 1994). Pictures of embryos at different stages and of sections were taken on an Olympus BH-2 microscope. Primers used for SSB-1 (780 nt product) were forward 5' TTTCTGGCAAGCTGTTTCATC 3' and reverse 5' GTATTTTGAGCCAGGC ACTTTTC 3'. Primers for SSB-4 (582 nt product) were forward 5' CAGATTTCT TCAAGCCAGCCC 3' and reverse 5' ATCTCACAGTGCCC 3'.

Cell Preparation and Transfection

pEGFP-SSB-1 and pEGFP-SSB-4 recombinant plasmids were constructed by cloning full-length cDNAs encoding SSB-1 or SSB-4 into the EcoRI and XhoI sites of pEGFP-C3 (Clontech). HeLa cells were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen) containing 10% fetal bovine serum (HyClone) in 5% CO2 at 37°C. Cells grown on coverslips were transfected with plasmids using Lipofectamine 2000 reagent following the manufacturer’s protocol (Invitrogen). Twenty-four hours after transfection, living cells were observed using a DMLA fluorescence microscope (Leica). DAPI (Sigma) was used to visualize nuclei. Primers used for SSB-1 (841 nt product) were forward 5' GCGAATTTCAG CATGGGGCAGAAGGTT 3' and reverse 5' CGCTCGAGTCATTGGTAGAGTA GATAGTTC 3'. Primers for SSB-4 (853 nt product) were forward 5' GCGAATT CCATGGGCCAGAAAAT 3' and reverse 5' CGCTCGAGTCACTGGTACT GGAGGTAATT 3'.

Results and Discussion

Cloning and Sequence Analysis of Zebrafish SSB-1 and SSB-4

Based on homology to Drosophila, we identified several gus-related zebrafish genes in the UniGene sequence database. Two of these predicted genes (NM_001025460 and XM_681061) have sequences typical of the SOCS box and SPRY domains, and we hereafter refer to these as zebrafish SSB-1 and SSB-4. They were cloned from ovary-derived cDNAs, and their sequences were identical to the predicted sequences in the UniGene database. SSB-1 contains an open reading frame of 819 bp encoding 273 amino acids, and SSB-4 contains an open reading frame of 831 bp encoding 277 amino acids. The predicted SSB-1 and -4 proteins are 76% identical to one another at the amino acid level. Multiple alignments showed that zebrafish SSB-1 and -4
have high identity to known and predicted GUS homologs in other animals; therefore, SSB-1 and -4 are evolutionarily conserved (Fig. 1). SSB-1 and -4 are located on separate linkage groups: SSB-1 on LG23 and SSB-4 on LG2.

Phylogenetic analysis using neighbor-joining and maximum-likelihood methods revealed that the zebrafish SSB-1 and -4 protein sequences lie within the SSB-1 and -4 groups of mice, Drosophila, humans, mosquitoes, cattle, dogs, chickens, and Xenopus (Fig. 1).
Tissue Distribution of Zebrafish SSB-1 and SSB-4 mRNA in Zebrafish

RT-PCR analysis of total RNAs from different tissues showed that zebrafish SSB-1 and SSB-4 transcripts are expressed in the ovary, testis, and brain, but not in the liver or kidney (Fig. 2). SSB-1 is expressed ubiquitously in the mouse, but SSB-4 is not expressed in the adult mouse (Masters et al. 2005). In Drosophila, gus mRNA is mainly expressed in the ovary (Styhler et al. 2002). Since zebrafish is a useful model animal, studies of SSB-1 and SSB-4 function in zebrafish might be relevant to understanding the function of gus homologs in mammals.

SSB-1 and SSB-4 mRNAs Detectable in Developing Germ Cells

RT-PCR analysis of total RNAs from different stages of oocyte development showed that SSB-1 and -4 expression can be detected in all stages of this process in zebrafish (Fig. 3). To investigate in detail whether SSB-1 and -4 localize to germ cells or other cell types within zebrafish gonads, we performed in situ hybridization using digoxigenin-labeled RNA probes corresponding to regions of the zebrafish SSB-1 and -4 cDNAs. In germ cells of the testis, signals were detected for both genes. SSB-4 expression was detected in all oocyte development stages. Ovaries hybridized to the SSB-1 probe showed that a large amount of SSB-1 transcript began to be detectable at stage IB (follicle phase of primary growth) and was highly abundant and diffuse in the cytoplasm between stage IB and stage II (cortical alveolus stage), then began to decrease after stage II. It is generally recognized that oocyte growth occurs in two distinct phases; the primary growth phase involves primary oocyte growth concomitant with nuclear changes, and the secondary growth phase generates many proteins (Fausto et al. 2004). For the waning expression levels of SSB-1 during oocyte development, we suggest that SSB-1 mRNAs are synthesized in large amounts in the primary growth phase, and then, part of mRNAs might be degraded or translated to proteins in the secondary growth phase.

These patterns of SSB-1 and -4 expression are distinct from those of mouse SSB-1 and -4 and Drosophila gus. In Drosophila, gus expression is restricted to the...
developing oocyte and nurse cells in the ovary, with no expression in the testis (Styhler et al. 2002). In mice, SSB-4 cannot be detected in the testis, and both SSB-1 and SSB-4 are expressed in granulosa cells but not in the oocyte (Xing et al. 2006).

Expression Patterns of Zebrafish SSB-1 and SSB-4 in Different Developmental Stages

RT-PCR was used to determine the temporal expression profile of SSB-1 and -4 in different zebrafish embryonic stages. SSB-1 and -4 transcripts appeared throughout development from the two-cell stage to 72 hpf (Fig. 4). Real-time quantitative PCR analysis further indicated that SSB-1 and -4 transcripts are present from the one-cell stage to 72 hpf, by comparing with the control, β-actin (Fig. 4). Expression of SSB-1 and -4 was high at the one-cell stage and the eight-cell stage, then rapidly decreased from the eight-cell stage to the sphere stage, and remained at stable levels from the shield stage onward.

![Fig. 3](image_url) Expression of SSB-1 and SSB-4 in developing germ cells. RT-PCR analysis of SSB-1 and SSB-4 expression in comparison to β-actin in oocytes (a, stages indicated above lanes). Expression analysis of zebrafish SSB-1 and SSB-4 by in situ hybridization to gonad sections (b-k). Antisense probes for SSB-4 (b) and SSB-1 (d) show expression in developing germ cells of the testes of adult zebrafish. Ovary of adult zebrafish hybridized with digoxigenin-labeled SSB-4 (f) and SSB-1 (h) antisense mRNA shows expression of these transcripts in developing oocytes. SSB-4 (b, f) and SSB-1 (d, h) mRNA in situ hybridization with sense and control RNA probes. HE staining in female and male gonad samples is shown below (j, k). Stages of developing oocytes are indicated by Roman numerals IB, II, and III. Scale bars, 50 μm.
Using whole-mount in situ hybridization, we further investigated the spatial expression patterns of zebrafish SSB-1 and SSB-4 genes in embryos from the two-cell stage to 72 hpf (Fig. 5). Our results showed that SSB-1 and SSB-4 transcripts were ubiquitous in early cleavage and gastrula stage embryos. Both SSB-1 and SSB-4 are expressed in the blastomere at the two-cell, 256-cell, and sphere stages and also are widely expressed at the 75% epiboly stage. At 24 hpf, there was weak SSB-4 expression in PGCs, and labeling with a zebrafish PGC marker (vasa) as a control in the same stage indicated the PGC localization of SSB-4. In 72 hpf embryos, SSB-1 RNA was restricted to the otic vesicle of 72 hpf embryos. Unlike SSB-1, however, SSB-4 expression was detected in both PGCs and heads.

In zebrafish, early embryonic development is controlled by maternally contributed factors (Pelegri et al. 1999). Data from analysis of whole-mount in situ hybridization and RT-PCR showed that SSB-1 and SSB-4 mRNAs are present from the one-cell stage to 72 hpf. The abundant expression in the one-cell stage and continuous presence of SSB-1 and SSB-4 mRNAs suggest that these transcripts might be maternally supplied. SSB-4 expression in the genital ridge also implies its potential involvement in the formation of germ cells.

In Drosophila, GUS is necessary for Vasa function, and the mechanism of Vasa localization most likely involves direct interaction between Vasa and GUS (Styhler et al. 2002). SSB-1 binds to the tyrosine kinase domain of MET via its SPRY domain and enhances transcriptional activation following MET stimulation by hepatocyte growth factor (Wang et al. 2005). Moreover, MET is expressed in granulosa cells of the mouse ovary (Parrott and Skinner 1998; Yang and Park 1995). According to our analysis of SSB-1 and -4 expression patterns, it is possible that

---

Fig. 4  a Expression of zebrafish SSB-1 and SSB-4 in embryos. RT-PCR analysis of SSB-1 and SSB-4 in comparison to β-actin (top panel) in developing embryos (stages indicated above lanes). A control using no reverse transcription as a template was also included as a negative control. M: marker. b Analysis of SSB-1 and SSB-4 expression in developmental stages using real-time quantitative RT-PCR. Expression of SSB-1 and SSB-4 relative to β-actin in embryos (stages indicated below lanes). White column indicates SSB-1 and black column indicates SSB-4.
Fig. 5  Expression patterns of *SSB-1* and *SSB-4* in embryogenesis and larval development analyzed by whole-mount in situ hybridization (shown in side views of embryos). The signals of *SSB-1* transcripts are detected at the two-cell stage (a), 256-cell stage (b), sphere stage (c), 75% epiboly stage (d), and 72 hpf (e), using *SSB-1* sense probe as a control for 72 hpf (f). The signals of *SSB-4* transcripts are also detected at the two-cell stage (g), 256-cell stage (h), sphere stage (i), 75% epiboly stage (j), 24 hpf (k), and 72 hpf (n), using vasa antisense probe as a control for 24 hpf (l). Sense probe of *SSB-4* as control for 24 hpf (m) and 72 hpf (o). Regions are boxed and magnified in (k) and (l). The black arrows point to clusters of *SSB-1*- or *SSB-4*-expressing cells. *ov* otic vesicle, *ge* genital ridge, *h* head
SSB-4 function is similar to that of GUS and that this protein interacts with Vasa in zebrafish. The possibility, however, that zebrafish SSB-1 or -4 interacts with Vasa remains to be tested, and further investigation is required to understand the functions of SSB-1 and -4 in zebrafish germ cells.

SSB-1 and SSB-4 Cytoplasmic Proteins in Transfected HeLa Cells

The subcellular localization of *SSB-1* and *SSB-4* were examined by transient transfection of HeLa cells with plasmids encoding fusion proteins with full-length *SSB-1* or *SSB-4* fused to EGFP. As shown in Fig. 6, both fusion proteins were expressed in the cytoplasm but were not detectable in nuclei. The subcellular localization of SSB-1 and SSB-4 coincides with the *Drosophila* GUS protein, which localizes to the cytoplasm of nurse cells in the ovary (Styhler et al. 2002).

In summary, we cloned two zebrafish orthologs (*SSB-1* and *SSB-4*) of the *Drosophila gus* gene. Phylogenetic analysis shows that zebrafish *SSB-1* and *SSB-4* are clustered into clades of *SSB-1*-like and *SSB-4*-like genes from other species. *SSB-1* and -4 transcripts can both be detected in the ovary and testis of zebrafish. In embryos, *SSB-1* and -4 are highly expressed from the zygote stage to the blastula stage. *SSB-4* mRNA was located in the region of PGCs in 24 and 72 hpf embryos, and *SSB-1* mRNA was not detected at these stages, indicating that *SSB-1* differs from *SSB-4* in its expression pattern. These data suggest that *SSB-4* might play a role in the early development of germ cells.
Acknowledgments  We thank Wenyan Li for thoughtful reading of the manuscript. We also thank members of the Deng lab for helpful discussions and technical assistance. This work was supported by the National Natural Science Foundation of China (grant nos. 30570968 and 30871407), from the “863 Program” (grant no. 2007AA06Z407), and the National personnel training base of basic science education (grant no. J0630648).

References

Yoon C, Kawakami K, Hopkins N (1997) Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124:3157–3165