Gene 544 (2014) 177–183

Contents lists available at ScienceDirect

Gene

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

The characteristics of *sox* gene in *Dugesia japonica*

Zimei Dong, Changying Shi, Haixia Zhang, He Dou, Fangfang Cheng, Guangwen Chen *, Dezeng Liu

College of Life Science, Henan Normal University, Xinxiang, 453007 Henan, China

ARTICLE INFO

Article history:
Received 22 February 2014
Received in revised form 29 March 2014
Accepted 23 April 2014
Available online 24 April 2014

Keywords:
SoxB
Planarian
Expression pattern
Regeneration

ABSTRACT

Sox genes play important roles in animal developmental processes, including embryogenesis, neural cell stemness, neurogenesis, sex determination, among others. Here, the full length *sox* gene in planarian *Dugesia japonica*, named *DjsoxB*, was cloned using rapid amplification of cDNA ends (RACE). Phylogenetic analysis demonstrates that *DjsoxB* is highly conserved evolutionarily in metazoans. Whole-mount in situ hybridization found *DjsoxB* mRNA to be mainly expressed in the head, intestine and mouth in both sexually mature and immature planarians. Moreover, *DjsoxB* transcripts were detected in the blastema after amputation and throughout the head regeneration processes. The data from real-time PCR showed that the mRNA expression levels of *DjsoxB* were distinctly up-regulated from 3 to 7 days after amputation. These results suggest that *DjsoxB* gene might be active in CNS formation and functional recovery during head regeneration, maintenance of adult CNS function and the development of other tissues (e.g. intestine) in *D. japonica*.

1. Introduction

The SOX family is one of the many multigenic families involved in regulating the development of bilateria. SOX proteins are transcription factors that contain a conserved DNA-binding domain known as the HMG (high-mobility group) domain comprised of 79 amino acids (Gubbay et al., 1990). The *sox* sequences are widely divergent outside the HMG-box, and specificity of their activity is thought to result from their temporal and spatial expression, as well as tissue-specific combinatorial interactions with other transcription factors and cofactors (Wilson and Koopman, 2002). Studies on *sox* gene expression and function in vertebrates and invertebrates have highlighted their crucial involvement in a great diversity of developmental processes such as neurogenesis, cardiogenesis, angiogenesis, chondrogenesis and endoderm development (Archer et al., 2011; Chew and Gallo, 2009; Lapan and Reddien, 2012; Lefebvre et al., 2007; Wagner et al., 2012; Wegner, 2010; Yamaguchi et al., 2011). *Sox* genes are also involved in tissue homeostasis and have been implicated in disease, notably cancer in adult animals (Ferri et al., 2004). Based on sequence analysis, the SOX family is subdivided into at least 10 subgroups: Group A contains SRY and is specific to eutherian mammals, Groups B–F are found in all higher metazoans and Groups G–J are restricted to particular lineages (Bowles et al., 2000). Among these groups, Group B *sox* genes receive the most attention due to their critical roles in proliferating neural progenitors in the central nervous system (CNS) of both embryos and adult animals. The expression patterns and functions of these genes are conserved among different species. Thus far, there have been no reports of *soxB* homologues in the planarian *Dugesia japonica*.

Planarian flatworms are free-living members of Platyhelminthes and are considered similar to the ancestral types of animals that first evolved a CNS (Agata et al., 1998). The CNS of planarians consists of an inverted U-shaped brain in the anterior region and two longitudinal ventral nerve cords (VNC) along the body (Cebria et al., 2002). Despite its apparent simplicity, the CNS of planarians displays a high degree of molecular complexity and shares evolutionarily conserved features with that of vertebrates (Pineda et al., 2002; Umesono et al., 1999). Another striking feature of planarians is their powerful regenerative ability that depends on the pluripotency of their stem cells (neoblasts). Within a week of transverse amputation at the post-auricle level, a trunk fragment will completely regenerate a head including the CNS (Dong et al., 2011; Umesono and Agata, 2009; Zhu and Pearson, 2013). In recent years, hundreds of CNS specific genes have been identified in the two most commonly studied species of freshwater planarians, *D. japonica* and *Schmidtea mediterranea* (Nakazawa et al., 2003; Nishimura et al., 2010; Umesono and Agata, 2009). However, the mechanisms behind CNS regeneration in planarians are not fully understood.

In this study, we identified a full-length *soxB* gene from *D. japonica* and investigated its temporal and spatial expression pattern in intact and regenerating planarians. Based on whole-mount in situ hybridization and real-time PCR, the results suggest that *DjsoxB* is involved in neural development. Phylogenetic analysis demonstrates that the DjSOXB coding sequence is highly conserved among SOXB proteins from other species. Our work provides basic data to aid in the elucidation of *soxB* gene functions and the molecular mechanism of planarians CNS regeneration.

Abbreviations: CNS, central nervous system; HMG, high mobility group; RACE, rapid amplification of cDNA ends; VNC, ventral nerve cords.

* Corresponding author.
E-mail address: chengw0183@sina.com (G. Chen).

http://dx.doi.org/10.1016/j.gene.2014.04.053
0378-1119/

2. Materials and methods

2.1. Animals and culture conditions

Planarians used in this work belong to the species *D. japonica*, and were collected from Yuquan spring, Henan Province, China. Both the sexually mature and immature intact worms were cultured in autoclaved tap water in the dark at 20 °C and fed once a week with fresh fish spleen. The worms were starved for at least 1 week prior to use in experiments. Regenerating fragments were obtained by transverse amputation at the post-auricle level (Fig. 4). In these experimental conditions, the fragments regenerated a complete head within about a week.

2.2. Isolation of the planarian DjsoxB gene

Total RNA was extracted using Trizol reagent (TaKaRa, China) and the first strand cDNA was synthesized from 1 μg total RNA using SuperScriptIII RNaseH-reverse transcriptase (Invitrogen, USA) according to the manufacturer's protocol. The cDNA was used as the template for PCR using a set of degenerate oligonucleotide primers (forward: 5′-ATGCYTTCATGGTDTGGTC-3′ and reverse: 5′-GGHCGATATTTGTAATC YGG-3′); these were designed to amplify the HMG box of *sox* genes on the basis of an amino-acid alignment of the SOX HMG domain sequences from various bilateria. This primer set amplified a 200 bp *DjsoxB* cDNA fragment. Based on the EST of *DjsoxB* cDNA, the 5′-gene specific primer (Inner primer: 5′-GTTCACTCAACGATTTCCACAT-3′ and Outer primer: 5′-GTTCTTTTTGATGGTTCGCTC-3′) and 3′-gene specific primer (Inner primer: 5′-TTGGGTTCAATGTGGAAATCGT-3′ and Outer primer: 5′-TGCATAATTCAGAAATTAGTAAACG-3′) were designed. The corresponding full-length transcripts were amplified by rapid amplification of complementary DNA (cDNA) ends (RACE) using both the 5′ and 3′-Full RACE kits (TaKaRa, China) according to the manufacturer's instructions. The PCR products were gel-purified and then ligated into the pUCm-T vector, which was then sequenced. The sequence reported here has been deposited into the GenBank database (accession no. KF233573).

2.3. Amplification of DjsoxB ORF sequences from genomic DNA

Genomic DNA was extracted using the genomic DNA extraction kit (TaKaRa, china) according to the manufacturer's instructions. Based on *DjsoxB* cDNA sequences, a pair of specific primers (forward: 5′-TGGT CGAGAGGACAACGTAG-3′ and reverse: 5′-CGATTCGGTTTCCAAGGTG-3′) were designed to amplify the *DjsoxB* ORF sequence from genomic DNA.

2.4. Homology analysis and phylogenetic tree reconstruction

Amino acid sequence of the HMG domains of DjSOXB was deduced from its cDNA nucleotide sequence. To align DjSOXB with other SOXB subfamily members, the GenBank protein database was searched using BLASTn and BLASTp at web servers of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Multiple SOXB protein sequences were aligned using Clustal 1.83 (http://www.clustal.org/). Phylogenetic tree analysis was carried out from the amino-acid sequence alignments by the neighbor-joining (NJ) method using Mega 4.0 program (http://www.megasoftware.net/); statistical support was provided by 1.000 bootstrap replications.

2.5. Whole-mount in situ hybridization

A fragment of *DjsoxB* (from position 336 to position 835) was used as templates for the sense and antisense digoxigenin-labeled RNA probes, which were synthesized using the RNA in vitro labeling kit (Roche). Whole-mount in situ hybridizations were performed with both sexually mature and immature worms, which were intact and at different regenerative stages as described previously (Pearson et al., 2009), with some modifications: Prior to fixation, planarians were treated with 2% HCl in 5/8 Holtfreter's solution to kill and remove mucus at 4 °C for 5 min, followed by Proteinase K treatment (20 mg/ml) for between 10 and 20 min depending on the size and stage of regeneration. Hybridization was performed at 57 °C for 28 h in a solution containing 50% formamide, 5 × SSC, 50 × Dehardent's, 1% Tween 20, 0.1 mg/ml heparan sulfate, 1 mg/ml yeast tRNA and 10% dextran sulfate with digoxigenin-labeled RNA probes. Samples were observed with a Leica DMLB stereomicroscope and images were captured with a Leica DFC300FX camera.

2.6. Quantitative real-time PCR analysis of gene expression

Quantitative real-time PCR was carried out as described previously (Dong et al., 2012). The following primers were used for real-time PCR: *DjsoxB* 170 bp (forward primer: 5′-ATAATACCACAACCCCAGTC GG-3′ and reverse primer: 5′-TGATGATGGGAAAGTGAATGCC-3′); A 121 bp *Djβ-actin* housekeeper gene was used as the qRT-PCR internal control (GenBank accession number AB292462) (Forward primer: 5′-ACACCGTACCAATCTATG-3′ and Reverse primer: 5′-GTGAAACTGTAA CCTCGT-3′). Primers were designed by Oligo 6.0 software and all primer pairs generated a single PCR band of the expected size; PCR products were verified by DNA sequencing.

3. Results

3.1. Isolation and characterization of a planarian soxB gene cDNA clone

Initial isolation and partial characterization of a planarian *soxB* homologue were achieved by PCR amplification with a set of degenerate oligonucleotide primers. A full-length cDNA of 1939 bp was identified by RACE-PCR from both ends, which included a 5′-terminal untranslated region (UTR) of 16 bp and a 3′-terminal UTR of 841 bp. The 3′ UTR was found to contain a canonical polyadenylation signal sequence AATAAA and a poly (A) tail; the ORF was 1082 bp, which encoded a polypeptide of 361 amino acids with a predicted molecular mass of 39.90 kDa and a theoretical isoelectric point of 9.46 (Fig. 1).

3.2. Homology analysis of DjsoxB

The deduced amino acid sequence contains a HMG domain and the landmark RPMNAFMVW motif, which is conserved in *sox* gene members of the larger HMG family. The HMG domain constitutes 79 amino acids at positions 6–84 in the predicted open reading frame of DjSOXB (Fig. 1), and it is highly similar to other SOXB members in invertebrates and vertebrates. Fig. 2 shows the comparison of the HMG domain amino acid residues of DjSOXB with those of *soxB* gene products from other organisms. In these sequences, 54 residues in the HMG domains of all species are identical and 12 are similar (Fig. 2). Outside of the HMG domain, the *DjsoxB* sequence shares little homology with other *soxB* genes. For example, *DjsoxB* shares only 34.90% and 19.81% identity with its same family *S. mediterranea soxB* and *soxp-3*, respectively. In addition, sequence analysis of cDNA and genomic DNA revealed a single transcription unit of 1939 nucleotides without introns (a feature of *soxB* genes).

To better understand evolutionary relationships among *sox* family genes and the grouping of *DjsoxB*, the NJ method was used to construct a phylogenetic tree using the full-length protein sequences of *DjsoxB* and *sox* genes from other organisms. As shown in Fig. 3, DjSOXB was found to cluster with Group B members. Vertebrate SOXB proteins clustered together and resided at the terminus of the phylogenetic tree, whereas invertebrate SOXB proteins used in this analysis clustered at the base of the tree in Group B. Not surprisingly, the *DjsoxB* from *D. japonica*, *soxB* from *S. mediterranea* and *sox1/2/3* from *Clonorchis simensis* belonging to phylum Platyhelminth were clustered together (Fig. 3). This pattern reflects the evolutionary history of this gene family