CLONING AND CHARACTERIZATION OF T-BOX AND FORKHEAD TRANSCRIPTION FACTORS FROM PORIFERA

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ABSTRACT

During evolution, the appearance of the mesoderm in Bilateria, with the exception of the Ctenophora, parallels with the appearance of the two perpendicular body axes. The effectors of bilaterian regional specification and pattern formation comprise an evolutionary conserved set of transcription factors related to the superclasses of homeoproteins, forkhead, T-box, Wnt-pathway and zinc-finger proteins. In sponges, the existence of those major regulatory genes causing the establishment of a polar patterning in bilaterians remains to be elucidated. Here we report the isolation and phylogenetic characterization of two T-box genes and four forkhead genes from the demosponge *Suberites domuncula* (Olivi, 1792).

KEY WORDS

Porifera, *Suberites domuncula*, T-box genes, forkhead genes.

INTRODUCTION

During evolution, the appearance of the mesoderm, which is involved in the differentiation and formation of tissues in adult bilaterians, parallels with the appearance of the two perpendicular body axes. The effectors of bilaterian regional specification and pattern formation comprise an evolutionary-conserved set of genes, encoding transcription factors related to the superclasses of homeoproteins (Hox and non-Hox genes), forkhead, T-box, Wnt-pathway and zinc-finger proteins (reviewed in: GALLIOT, 2000; PETERSON & DAVIDSON, 2000).

Interestingly, members of all these families have been isolated from Cnidarians (TECHNAU & BODE, 1999). In sponges the basic elements for the differentiation of pluripotent cells to distinct somatic cells through morphogenetic events as cell-cell and cell-matrix adhesion, as well as cell migration, have been identified (reviewed in: MÜLLER, 1997; WIENS *et al.,* 2001, 2003). However, the existence of those major regulatory genes causing the establishment of a polar patterning in bilaterians remains to be elucidated. Until now only a few non-Hox genes have been isolated from sponges (COUTINHO *et al.,* 1994; SEIMIYA *et al.,* 1994, 1998; HOSHIYAMA *et al.,* 1998; RICHELLE-MAURER & VAN DE VYVER, 1999; MANUEL & LE PARCO, 2000). Due to the basal position of sponges in the phylogenetic tree, the knowledge of the developmental mechanisms involved in pattern formation and morphogenesis in sponges can clarify the origin and ancestral function of these evolutionary-conserved pathways.

T-box genes conform a family of transcriptional regulators that share a highly conserved region that binds to DNA, the T-box domain. Since the first discovering of *Brachyury* or *T* gene from mouse (HERRMANN *et al.,* 1990), homologs of *Brachyury* have been isolated from all groups of metazoans, and have been grouped into different subfamilies, according to their T-box amino acid (aa) sequence. *Brachyury* is responsible for the differentiation of the notochord, the control of morphogenetic movements during gastrulation and the formation of the posterior mesoderm in vertebrates (WILKINSON *et al.,* 1990; BEDDINGTON *et al.,* 1992; O'RELLY *et al.,* 1995; CONLON *et al.,* 1996; WILSON & BEDDINGTON, 1997). However, the expression in the blastopore of *Brachyury* in Cnidaria has been recently considered (TECHNAU & BODE, 1999; TECHNAU, 2001), suggesting a more primitive function of *Brachyury* in the body axis formation. All other T-box genes are also involved in type specification and morphogenetic movements during development (HERRMANN & KISPERT, 1994; PAPAIOANNOU, 1997; PAPAIOANNOU & SILVER, 1998; SMITH, 1999; TADA & SMITH, 2001).

Forkhead proteins comprise a subfamily within the large group of helix-turn-helix proteins. They are characterized by a "winged helix domain", consisting of a 100 aa DNA binding domain that forms a modified helix-turn-helix (KAUFMANN & KNÖCHEL, 1996; GAJIWALA & BURLEY, 2000). The founding members were the mouse HNF3α, β and γ genes, activators of specific hepatic genes (CEREGHINI, 1996; COSTA, 1998), together with the *Drosophila melanogaster forkhead* gene, responsible for the formation of terminal structures that develop into the gut (WEIGEL *et al.,* 1989). Nowadays the family comprises more than 60 members, all of them related with cell differentiation and the proper formation of the embryo (KAUFMANN & KNÖCHEL, 1996).

Here we report the isolation and phylogenetic characterization of two members of the T-box family in the sponge *Suberites domuncula* (*Sd-Bra* and *Sd-Tbx*), as well as four genes belonging to the forkhead family (*Sd-Fox1-4).*

MATERIAL AND METHODS

Sponge material

Live specimens of *S. domuncula* (Porifera, Demospongiae, Hadromerida) were collected by SCUBA diving near Rovinj (Croatia) from depths between 15 and 35 m. The sponges were brought to Mainz (Germany) and there kept in 10^3 l tanks at 17° C before use in the experiments.

Full-length cDNAs cloning and sequencing

A sample of one sponge living in the aquarium was shock-frozen, pulverized in liquid-nitrogen and RNA extracted using the TRIzol Reagent (GibcoBRL, Grand Island, N.Y.). Total RNA was reverse transcribed to synthesize pooled cDNA for RACE PCR using the "Invitrogen GeneRacer Kit" (Invitrogen, Groningen, The Netherlands). In order to amplify T-box related genes, nested PCR with fully degenerate primers encoding the conserved amino acid sequences of the T-box domain was performed with this cDNA; NEMIVTK (5'-AAYGARATGATHGTN ACNAA-3') and NPFAKAF (5'-AANGCYTTNGCRAANGGR TT-3') were used as a forward and reverse outer primers, while WKYVNGE (5'-TGGAARTAYGTNAAYGGNGA-3') and TAYQNEE (5'-TCYTCRTTYTGRTANGCN GT-3') were used for nested PCR reaction. PCR conditions were: 4 min at 94° C (1 cycle) and 30 s at 94° C, 30 s at 42° C, 20 s at 72° C (35 cycles). In order to amplify forkhead related

genes, nested PCR with fully degenerate primers encoding the conserved amino acid sequences of the forkhead domain was performed; KPPYSY (5'-AARCCNCCNTAYTCNTA -3') and MFENGS (5'-ATGTTYGANAAYGGNW-3') were used as a forward and reverse outer primers, while KPPYSY and WQNSIR (5'-TGGGARAAYTCNATHMG-3') were used for nested PCR reaction. PCR conditions were: 4 min at 94° C (1 cycle) and 30 s at 94° C and 30 s at 40° C (35 cycles). Fragments of the expected size were obtained and sequenced using standard procedures. The corresponding full-length transcripts were amplified by RACE from the same cDNA used above, according to the Producer's Manual.

Phylogenetic analysis

The deduced aa sequences corresponding to the T-box and forkhead domains of *S. domuncula* proteins reported here were compared with those from other organisms using the neighbor-joining method (SAITOU & NEI, 1987). Accurate multiple protein sequence alignments were made using the software CLUSTAL W (THOMPSON *et al.,* 1994).

RESULTS AND DISCUSSION

Full-length cloning of *S. domuncula* T-box genes: *Sd-Bra* and *Sd-Tbx*

A PCR product of the expected size was amplified from cDNA of the sponge *S. domuncula* using degenerate oligonucleotide primers corresponding to shared sequences of T-box genes as described under "Materials and Methods". After sequencing and sending several independent clones to BLAST (BLAST, 1997), two different cDNAs could be identified as T-box containing genes. One of them showed the highest similarity with the T-box domain of members of the Brachyury subfamily, and was named *Sd-Bra*; the other showed the highest similarity with vertebrates *Tbx4* and *Tbx5* T-box domains and was termed *Sd-Tbx*.

Applying the RACE technique with the same cDNA used above, we obtained the corresponding full-length *Sd-Bra* and *Sd-Tbx* cDNAs. *Sd-Bra* cDNA was 1315 base pairs long, with an open reading frame that predicted a protein of 318 aa. *Sd-Tbx* full length cDNA was 2373 base pairs long, with a single open reading frame that predicted a polypeptide of 501 aa, containing the T-box domain in the 3' part of the protein.

Interestingly, in some *Sd-Bra* independent clones we found 51 base pairs missing, which resulted in a 17 aa shorter polypeptide product, allowing us to predict two different protein products, Sd-Bra1 and Sd-Bra2, of 318 and 335 aa respectively.

Phylogenetic analysis of *S. domuncula* T-box genes

The sequence of the T-box domain have been taken as a basis for grouping the T-box genes into 5 different subfamilies: T or Brachyury, Tbrain, Tbx2, Tbx1 and Tbx6. In general, the different subfamilies are also distinguished by their pattern of expression and their function (PAPAIOANNOU & SILVER, 1998; reviewed in: SMITH, 1997; PAPAIOANNOU, 2001).

To address the question to which subfamily the sponge T-box domain proteins can be grouped, the T-box domains of Sd-Bra and of Sd-Tbx where aligned with T-box domains of members of different metazoan phyla, and molecular phylogenetic analyses were performed by the neighbor-joining method (SAITOU & NEI, 1987). As seen in Fig. 1, Sd-Bra groups with members of the Brachyury or T family and Sd-Tbx groups within Tbx2 subfamily, but more closely related to the Tbx4 and Tbx5

subgroup. According to the basal position of Porifera in the phylogenetic tree of Metazoans, the two sponge T-box genes are also in the root of each subfamily.

Alignment of the T-box domain of Sd-Bra and Sd-Tbx with other T-box domain proteins shows a high degree of conservation between the diverse T-box proteins and the ones from *S. domuncula*. Even more, residues involved directly in DNA binding (according to MÜLLER & HERRMANN, 1997) are totally conserved (Fig. 2).

Interestingly, in most taxa of metazoans a member of the Brachyury subfamily has been found. However, Tbx2 subfamily includes the chordate *Tbx2-3-4-5* genes (BOLLAG *et al.,* 1994; AGULNIK *et al.,* 1996; BASSON *et al.,* 1997), and, until today, only two members from protostomians [*C. elegans Tbx2* (AGULNIK *et al.*, 1995, 1997) and *D. melanogaster omb* (PFLUGFELDER *et al.,* 1992)]. In cnidarians as well as in calcareous sponges only T-box genes belonging to the Brachyury subfamily have been reported [*H. vulgaris Hybra1* and *H. equinata* Brachyury (TECHNAU & BODE, 1999; KROIHER, pers. comm.); *Sycon raphanus Sybra* (MANUEL, 2001)]; and in echinoderms and hemichordates only T-box genes belonging to the Brachyury (PETERSON *et al.,* 1999; SHOGUCHI *et al.,* 1999), and to the Tbrain subfamilies have been identified (TAGAWA *et al.,* 1998, 2001; SHOGUCHI *et al.,* 2000; CROCE *et al.,* 2001) .

Concerning the sequence outside the T-box region, only a few clear homologs and orthologs from very related animals have high similarities. No conserved domains or high homology was found between Sd-Bra or Sd-Tbx and other T-box genes. However, their C-terminal regions are rich in Ser, Thr and Pro residues, a common feature for transactivation domains.

Cloning and characterization of *S. domuncula* forkhead genes

A PCR product of the expected size was amplified from cDNA of the sponge *S. domuncula* using degenerate primers corresponding to conserved sequences of forkhead genes (see Materials and Methods). After sequencing and sending several clones to BLAST, four different cDNAs could be identified as winged helix containing genes (*Sd-Fox1-4*).

To elucidate to which subfamily the sponge forkhead proteins can be grouped, their forkhead domains were aligned with members of the different metazoan phyla belonging to different forkhead subfamilies (according to KAESTNER *et al.,* 2000), and molecular phylogenetic analyses were performed by the neighbor-joining method (SAITOU & NEI, 1987). Phylogenetic analysis grouped Sd-Fox1 and 4 to the I and F subfamily, respectively. Sd-Fox2 falls at the root of the I and G groups, and Sd-Fox3 in groups D and E (Fig. 3).

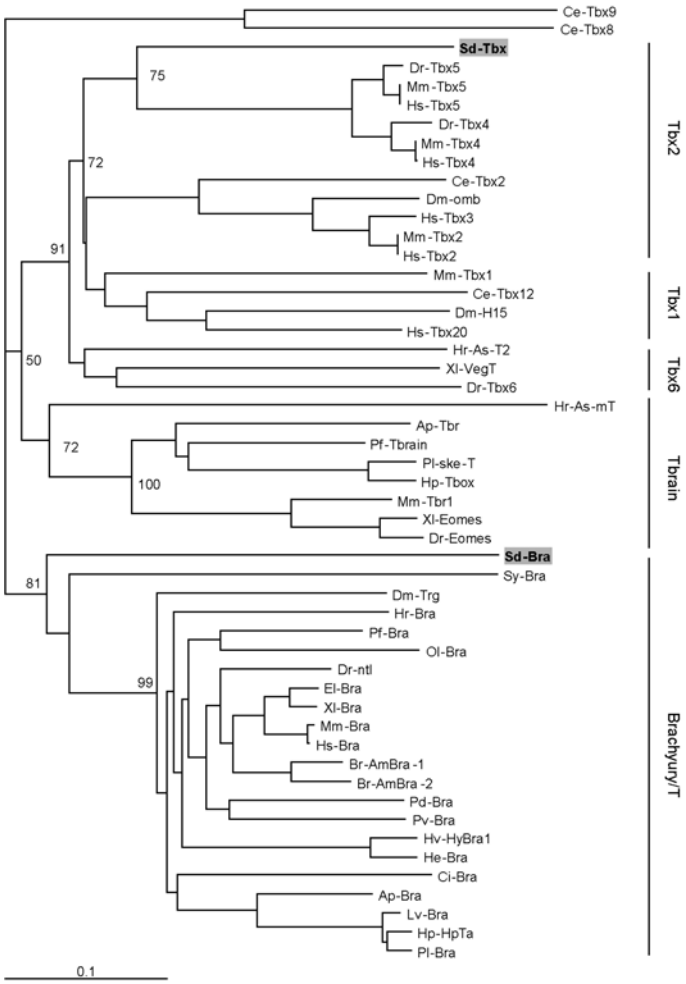

Fig. 1. Molecular phylogenetic analysis of T-box proteins. T-box domains from members of all T-box families were aligned with the Clustal W program. The different T-box subfamilies are indicated. T-box proteins from *S. domuncula* are shaded; Sd-Bra is grouped within the Brachyury / T subfamily and Sd-Tbx is grouped within the Tbx2 subfamily. The *C. elegans* sequences Ce-Tbx8 and Ce-Tbx9 were used as outgroup. The numbers at the nodes are an indication of the level of confidence, given in percentage, for the branches as determined by bootstrap analysis. Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence. (Ap, *Asterina pectinifera*; Br, *Branchiostoma floridae*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; El, *Eleutherodactylus coqui*; He, *Hydractinia echinata*; Hp, *Hemicentrotus pulcherrimus*; Hr, *Halocynthia roretzi*; Hs, *Homo sapiens*; Hv, *Hydra vulgaris*; Lv, *Lytechinus variegatus*; Mm, *Mus musculus*; Ol, *Oikopleura longicauda*; Pd, *Platynereis dumerilii*; Pf, *Ptychodera flava*; Pl, *Paracentrotus lividus*; Pv, *Patella vulgata*; Sd, *Suberites domuncula*; Sy, *Sycon raphanus*; Xl, *Xenopus laevis*). (*Sd-Bra* and *Sd-Tbx* cDNA sequences are listed in the GenBank / EMBL / DDBJ databases and in a recent original paper ADELL *et al.,* 2003).

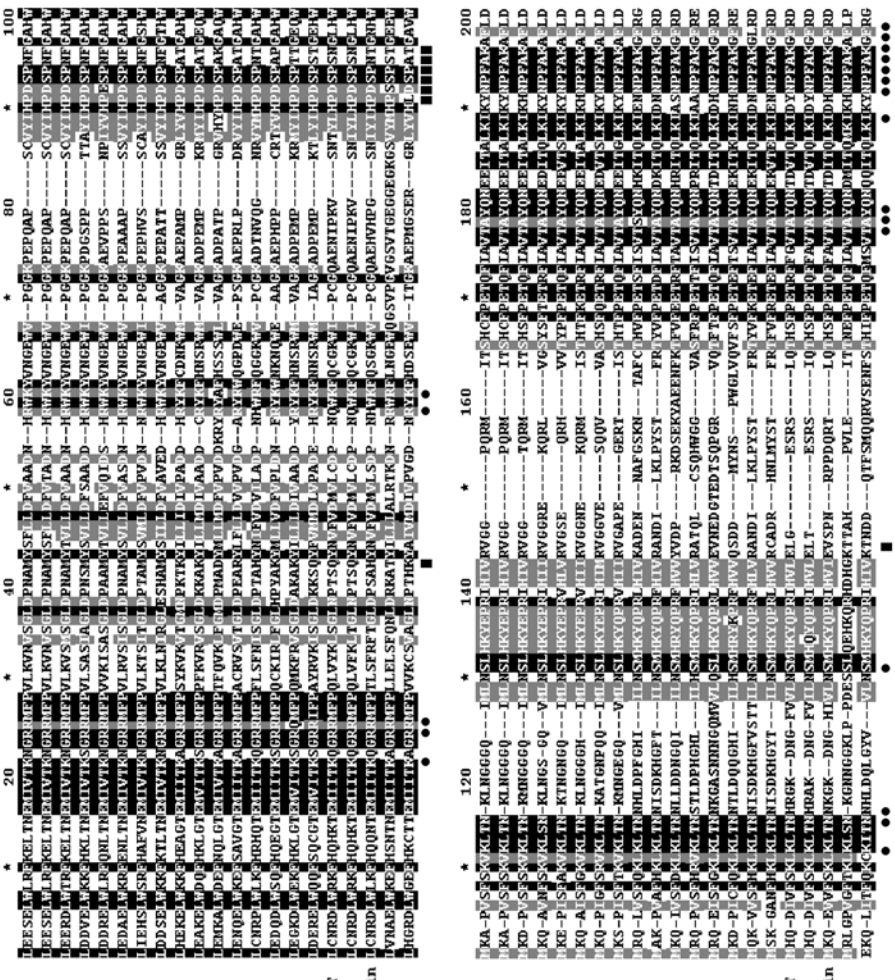

Fig. 2. Alignment of Sd-Bra and Sd-Tbx T-box domains with other representatives of T-box domain proteins. Identical and conserved amino acids are in black and gray, respectively. Amino acids directly involved in contacting DNA and in dimerization are marked with a solid circle and a rectangle, respectively (according to MÜLLER & HERRMANN, 1997); the numbers above the alignment indicate the position within this domain.

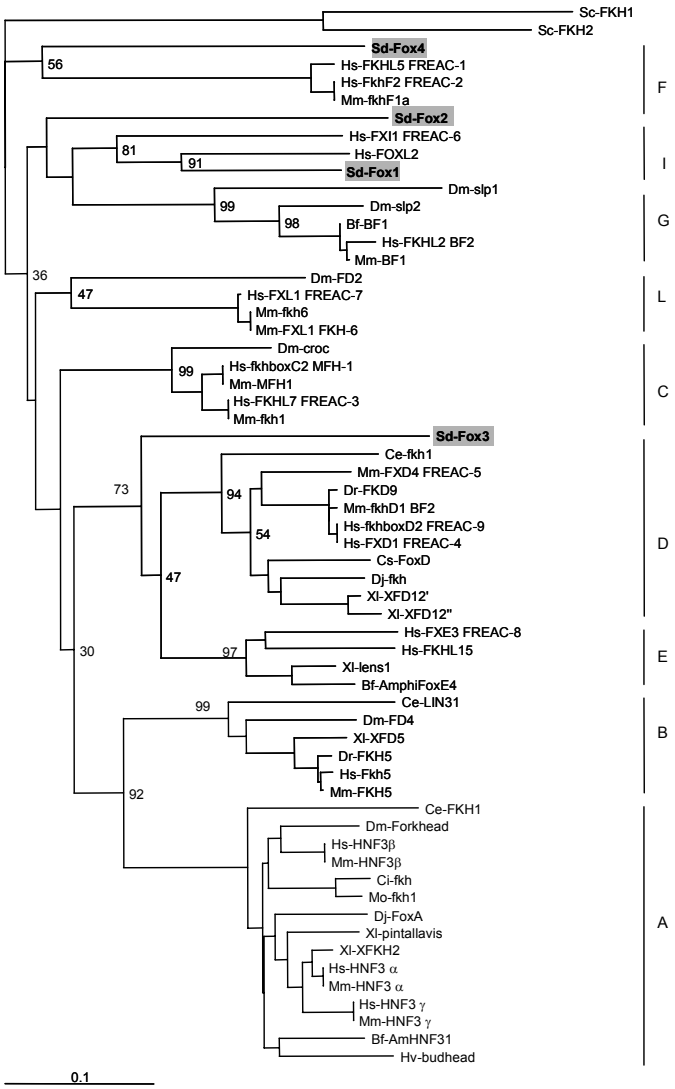

Fig. 3. Molecular phylogenetic analysis of forkhead proteins. Forkhead domains from members of all forkhead families were aligned with the Clustal W program. The different forkhead subfamilies are indicated (according to KAESTNER *et al.,* 2000). The forkhead proteins from *S. domuncula* are shaded. Sc-FKH1-2 were used as outgroup. The numbers at the nodes are an indication of the level of confidence, given in percentage, for the branches as determined by bootstrap analysis. Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence. (Bf, *Branchiostoma floridae;* Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis;* Cs*, Ciona selvatgii; Dj, Dugesia japonica; Dm, Drosophila melanogaster; Dr, Danio rerio; Hs, Homo sapiens; Hv, Hydra vulgaris*; Mm, *Mus musculus*; Mo, *Molgula oculata*; Sc, *Saccharomyces cerevisiae*; Sd, *S. domuncula*; Xl, *Xenopus laevis*).

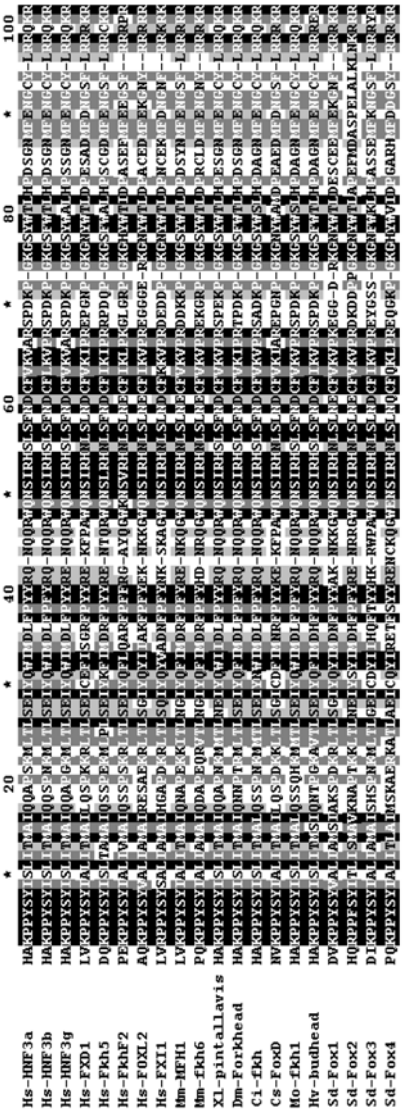

Fig. 4. Alignment of *S. domuncula* forkhead domains with other representatives of forkhead proteins. Identical and conserved amino acids are in black and gray, respectively. The numbers above the alignment indicate the position within this domain.

In Fig. 4 alignment of forkhead domains of the four sponge genes with members of different forkhead proteins demonstrates the high degree of sequence conservation between all of them.

The corresponding full-length cDNAs of the four sponge forkhead molecules were obtained by RACE technique. *Sd-Fox1* cDNA was 1260 base pairs long, with an open reading frame that predicted a protein of 275 aa; *Sd-Fox2* cDNA had 786 base pairs, and an open reading frame of a putative protein of 218 aa; the *Sd-Fox3* cDNA comprised 1850 base pairs and a predicted protein of 444 aa and the *Sd-Fox4* cDNA with 1880 base pairs and a protein of 470 aa. All of them contained the forkhead in the N-terminal part of the protein. The potential role of the sponge forkhead genes is under investigation.

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