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Author: Maruyama, Yoshihiko K.

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A Sea Cucumber Homolog of the Mouse *T-Brain-1* is Expressed in the Invaginated Cells of the Early Gastrula in *Holothuria leucospilota*

Yoshihiko K. Maruyama*

Section of Marine Biological Science, Education & Research Center for Biological Resources, Faculty of Life & Environmental Science, Shimane University, 194 Kamo, Saigo, Oki, Shimane 685-0024, Japan

ABSTRACT—A cDNA clone for a sea cucumber T-box gene was isolated and characterized. Based on molecular phylogenetic analysis it is concluded that the putative gene *Hl-Tbr*, encoded by the cDNA clone, is a T-box gene of the T-brain subfamily and hence a homolog of the mouse *T-brain-1* (*Tbr-1*) as well as of the *Xenopus Eomesodermin*. *In situ* hybridization analysis of whole mount specimens showed that *Hl-Tbr* was expressed in the invaginated cells at the early gastrula stage and the expression of the gene was scarcely detectable by the end of the late gastrula stage.

INTRODUCTION

T-box genes encode a family of transcription factors that are characterized by a putative DNA-binding domain (Herrmann and Kispert, 1994; Bollag *et al.*, 1994; Papaioannou and Silver, 1998), known as the T-domain. Studies have shown that they are conserved in a wide variety of animals and appear to play a crucial role in development (Herrmann and Kispert, 1994; Bollag *et al.*, 1994; Papaioannou and Silver, 1998; Smith, 1999). The present study first reports the isolation and characterization of a sea cucumber T-box gene, and its expression during gastrulation.

MATERIALS AND METHODS

The sea cucumber *Holothuria leucospilota* was used. Fertilization was done as noted previously (Maruyama, 1980), and embryos were reared at 27–28°C. Primers for PCR amplification were 5'-TA(C/T)AT(C/T)CA(C/T)CC(C/T)CC(A/C/G/T)GA(C/T)TC(A/C/G/T)CC-3' as the sense-strand oligonucleotide and 5'-(A/G)AA(A/C/G/T)GC(C/T)TT(A/C/G/T)GC(A/G)AA(A/C/G/T)GG(A/G)TT-3' as the antisense oligonucleotide (Tagawa *et al.*, 1998). Target fragments were amplified from a *Holothuria leucospilota* 25-hr gastrula cDNA library constructed in λ ZAPII (Stratagene). PCR products were sequenced with an ABI PRISM dye primer cycle sequencing kit (Perkin Elmer). Using PCR-derived clones, the cDNA library was screened under high stringency conditions. A cDNA clone was obtained and used for sequencing. Both strands for the cDNA clone were sequenced. *In situ* hybridization of whole-mount specimens proceeded with DIG-labeled sense (control) and antisense RNA probes, synthesized *in vitro* from a full-length cDNA clone.

Molecular phylogenetic relationships among T-box gene products were inferred by the neighbor-joining method (Saitou and Nei,

1987), analyzed using the software package PHYLIP (Felsenstein, 1985). Alignments of multiple protein sequences were made using CLUSTAL W (Thompson *et al.*, 1994). Regions of gaps and questionable homology were excluded from the analyses. Accession numbers in the DDBJ, EMBL or GenBank nucleotide sequence databases for the sequences were: ascidian As-T2, D83265; mouse m-Tbx6, U57331; *Xenopus* X-vegT, U59483; mouse m-Tbx5, U57330; mouse m-Tbx4, U57329; *Drosophila* Dm-omb, M81796; mouse m-Tbx2, U15566; Eomesodermin (*Xenopus* Eomesodermin), U75996; human hu-Eomesodermin, AJ010280; mouse m-Eomesodermin, AF013281; human hu-Tbr-1, U49250; mouse m-Tbr-1, U49251; *Drosophila* Dm-Trg, S74163; ascidian As-T, D16441; starfish ApBra, AB018527; sea urchin HpTa, D50332; *Xenopus* Xbra, M77243; amphioxus AmBra-1, X91903; acorn worm PfBra, AB004912. Accession numbers in SWISS-PROT database were: mouse m-Tbx1, P70323; mouse m-Tbx3, P70324; zebrafish Zf-T, Q07998; amphioxus AmBra-2, P80492; mouse m-T, P20293.

RESULTS AND DISCUSSION

Using a PCR-based method (see Materials and Methods), I isolated a cDNA clone for a sea cucumber T-box gene (*Hl-Tbr*) from the *Holothuria leucospilota* gastrula cDNA library. The nucleotide and deduced amino acid sequences of the cDNA clone for *Hl-Tbr* are shown in Fig. 1. The cDNA was 3363 base pairs long and had an open reading frame that predicted a polypeptide of 679 amino acids, in which nucleotide A is present at position –3 as in most translation initiation sites in echinoderms (Mankad *et al.*, 1998). The T-domain was evident in the middle portion of the predicted protein (Fig. 1), and was highly conserved when compared with T-domains of other T-box gene products (data not shown).

On the basis of the molecular phylogenetic analysis, the T-box gene family is grouped into at least five subfamilies, Brachyury (T), Tbx1, Tbx6, Tbx2 and Tbr1 (Papaioannou and Silver, 1998): the Tbr1 subfamily will be called the T-brain

* Corresponding author: Tel. +81-8512-2-1814;
FAX. +81-8512-2-4357.
E-mail: maruyama@life.shimane-u.ac.jp

TTGAACAGCATTGATTTTGTGTTGGACTATTTAGCACGCAATTAGCGGAGCTGTTATAGCCAGCAAAATCTAAAAATTTTACAGACTGCT 90
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 M S D A Y C T Q P R S D
 ACTCTTTTCGTTGAATTATCTGAGAAATGATGGTACGACATGTCCAAGTACTGAGCCCATCGATACATTAAAAATTTGTGAGAGTGAGCATT 270
 S F V E L S E N D G T T C P S T E P I D T L K H C E S E H S
 CCATGGATCTTTCAAGTATGCGCCTGGACATATCGTATGCAAGGCTTTCACCAGAACAAATGGTACCGTCAATGCAACCAGTCTTTCCCTC 360
 M D L S S M R L D I S Y A G S S P E Q M V P S M Q P V F P P
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 S Y F N S P L A Q Q Q D V W Y N G W P R D H T L I A N E Y A
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 R S V P K S G G Q D A G V N D N A R H S Y F N S T K P V L P
 CTTTCTACAGGCGACACCTTACATGACAAATGGACTACCAATATGCGAGATTCCGGGTATCCGCTATCATATAATGCCATGCCGTTT 630
 F Y Q A T P Y M T N G L P N M Q I P G Y P L S Y N A M P F L
 TACCATATTCAGCTACAGCATGCCCTTGTGAAGTACAAGAACCCGCTTTCCTTCCAGGAGGACCATGCTCAGTTTCAAAGA 720
 P Y S A Y S M P H F E S Q E P V I S T S Q E A P C S V S K K
 AAAGAAGCAACAGCAAAATTCGGTAGCTGCCGACGACGGGGAAGACAGACGAGAGGTTGATGTACAACGCAGAGAGGAGTGTAGAGGTT 810
 R S N S K L S V A A D D G E D R R E V D V Q R R E E C R G S
 CAGCAGAAGTAAGTGCCAGAGGAGATGACGACGAACCTACAACGTCGGGTTCAGGAGGAGACACTGGCCAACAAGAGTCATCACCAGTAA 900
 A E V S A R G D D D E P T T S G Q E G D T G Q Q E S S P V K
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 L P N L N E M T I L P T K A C V F L C N Q D L W M E F D Q C
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G T E M I V T K Q G R R M F P Q L S F K F T G L D P D T K Y
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I D Y N P F A K G F R D G Y S S I S G R N K Q T V R Q Q Q G
 GTCTTGTAGGCGGATTTCCACTTAGTGTTCAGCAACGCCAACAGCATGTCTACATCCTAGCGATAAACAGCTCTTCAACTTTAATCACC 1620
 L V G G F P L S V P A N A N S M S H P S D K Q L F N F N H L
 TGATCACTGACCGTTTCACATGTTCCCATGACGACCATTACCAACCATCAAAACAGTTTCCCGCCAACCTTGGATCACAACACAATAACC 1710
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 T H D F S R Q K A V I D N F K T A F P L T S A D P P Q E I P
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 G A C S A S K S Q V P E G I Q V L N L S Q S T A P N V T I E
 AACAGAAGTACTGTACAAATGAATGGGGATCAACATGATGAGAAGACAAAGATGACGAAGTGTTCGAGCAACGACTAAAAGACAGA 1980
 Q E V L S Q M N G D Q H D E E D K D D E V F E Q T T K R Q K
 AACTAGAAGCAACAACGGAAGCGCTTCCCGTTCCTTTTACAGAAATTTTCATCAATTCAGATGCTGCTAAGAACAATGGATTTCCCTC 2070
 L E A T T E S A F P V P F Y R N F I N S D A A K N N G F P Q
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 GAATCAGAAAAAGAAAAAAAAAAAAAAAAAAAAA 3363

Fig. 1. Nucleotide and predicted amino acid sequences of the cDNA clone for *Hi-Tbr*. The T-domain is underlined.

subfamily in this study (see below). For example, the Brachyury subfamily contains two T-box genes isolated from sea urchins (*HpTa*; Harada *et al.*, 1995) and starfish (*ApBra*; Shoguchi *et al.*, 1999) of echinoderms (see Fig. 2).

To determine the subfamily to which the sea cucumber homolog (*HI-Tbr*) belongs, I aligned 117 amino acid sites of

the T-domains based upon maximum similarity, by which molecular phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987). As seen in Fig. 2, *HI-Tbr* is grouped as a T-brain subfamily gene. This subfamily is comprised of mouse and human *T-brain-1* or *Tbr-1* (Bulfone *et al.*, 1995), *Eomesodermin* (*Xenopus Eomesodermin*) (Ryan

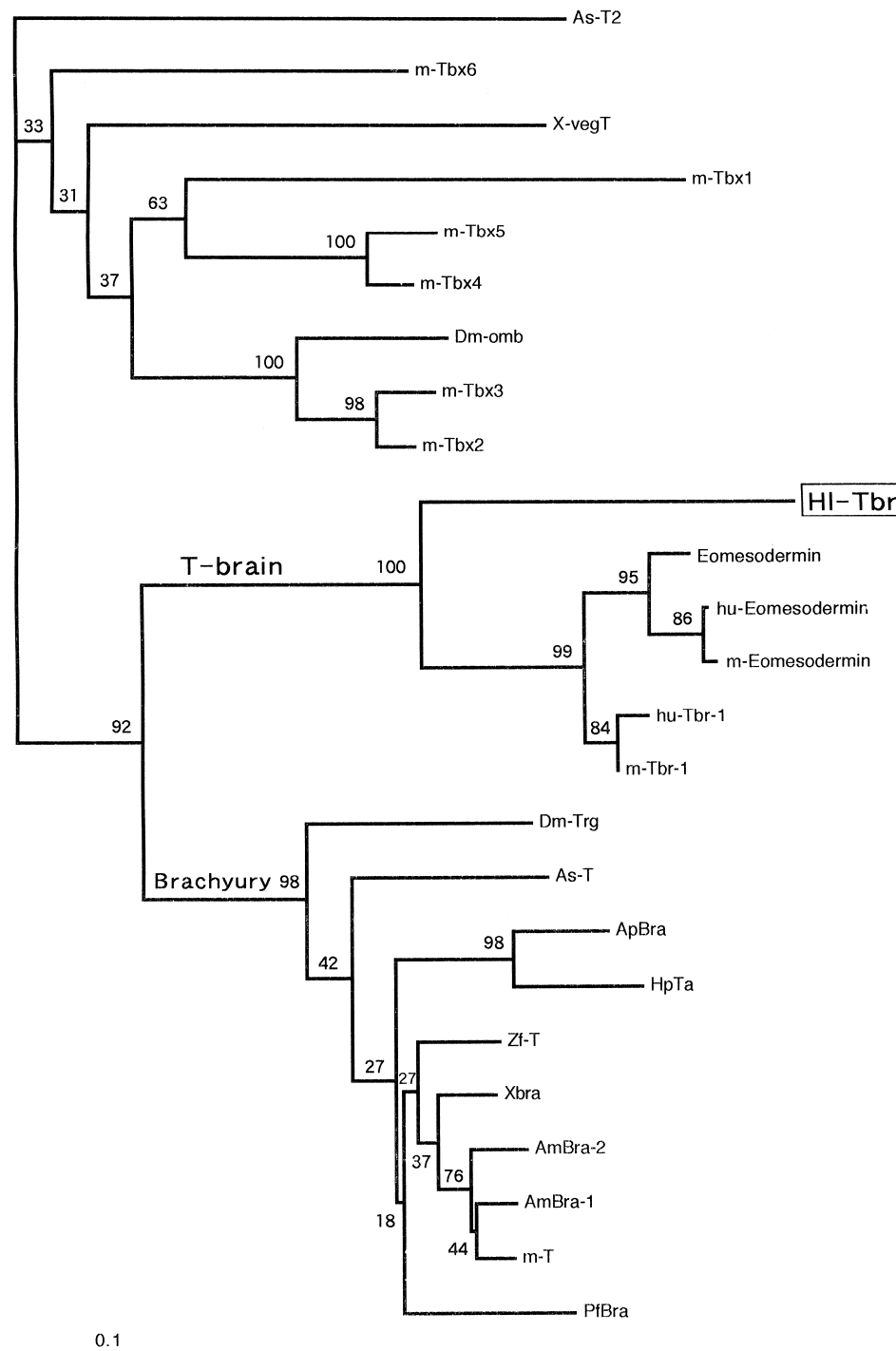


Fig. 2. Evolutionary relationship of *HI-Tbr* with other T-domain proteins. Using 117 confidentially aligned sites of T-domain amino-acid residues, a molecular phylogenetic tree was constructed by the neighbor-joining method. The branch length is proportional to the number of amino acid substitutions. The scale bar indicates 0.1 amino acid substitutions per position in the sequence. The number at each branch indicates the percentage of times that a node was supported in 100 bootstrap pseudoreplications.

et al., 1996), mouse *Eomesodermin* (Wattler *et al.*, 1998; Ciruna and Rossant, 1999; Hancock *et al.*, 1999), human *Eomes* (Yi *et al.*, 1999), and *Hl-Tbr*. This clade was supported by the highest bootstrap value (100%; Felsenstein, 1985). In addition, it appears that the Tbr1 group or the T-brain subfamily except for the sea cucumber gene is comprised of two subgroups, Tbr-1 and Eomesodermin (see Fig. 2). From these results, it is concluded that the sea cucumber gene, *Hl-Tbr*, is a homolog of Tbr-1 genes (mouse *Tbr-1* and human *Tbr-1*) as well as of Eomesodermin genes (*Xenopus Eomesodermin*, mouse *Eomesodermin* and human *Eomes*).

It is well-known that these vertebrate T-brain genes are expressed in a region of the developing forebrain; in mouse *Tbr-1* (Bulfone *et al.*, 1995), in *Xenopus Eomesodermin* (Ryan *et al.*, 1998) and in mouse *Eomesodermin* (Ciruna and Rossant, 1999; Hancock *et al.*, 1999). In addition, a transient expression mainly during gastrulation is also reported in the Eomesodermin subgroup (Ryan *et al.*, 1996; Ryan *et al.*, 1998;

Ciruna and Rossant, 1999; Hancock *et al.*, 1999), while no such expression is reported in the Tbr-1 subgroup (mouse *Tbr-1*).

Expression of the sea cucumber T-brain gene, *Hl-Tbr*, in embryos under gastrulation was examined by whole-mount *in situ* hybridization (Fig. 3). No signals above background levels were detectable when control (sense) probes were used. Transcripts of *Hl-Tbr* were detected in the invaginated cells of early gastrulae (Fig. 3A, B). It appears that the territory of the gene expression may demarcate that of the initial invagination (*cf.* Fig. 3A, B). In advanced gastrulae (Fig. 3C), the signal is seen only around the middle-to-upper portion of the archenteron. I note that some archenteric cells persisted in exhibiting more or less intense signals (see Fig. 3C). Then, by the end of the late gastrula, signals were barely detectable in the embryos (Fig. 3D). In addition to the unambiguous expression in the initially invaginated cells of the archenteron, a very weak staining, sometimes, that of background level,

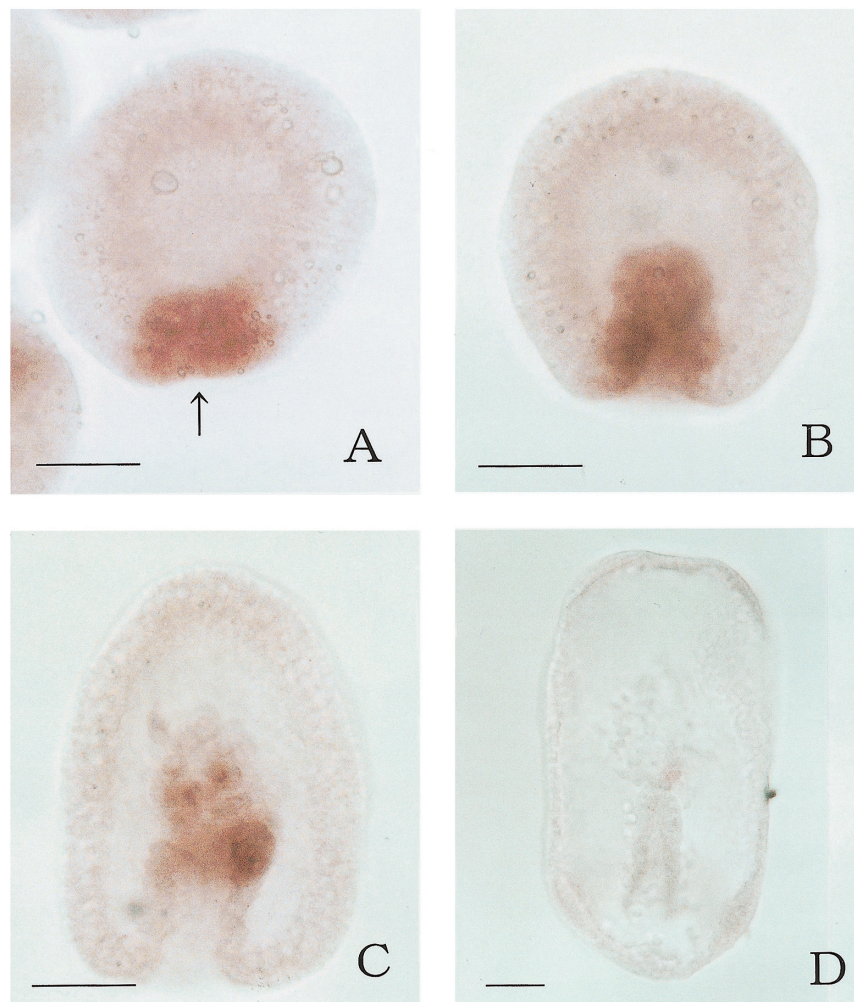


Fig. 3. Expression of *Hl-Tbr* in sea cucumber embryos. (A) Early gastrula 15 hr post-fertilization (pf), showing the signal in the cells at the vegetal/posterior end, where invagination begins. Arrow, blastopore. (B) Early gastrula 18 hr pf, showing the signal in the invaginated cells. (C) Gastrula 24 hr pf, showing the signal in the middle-to-upper portion of the archenteron. (D) Mouth-forming stage embryos 37 hr pf. Signals are scarcely detectable. Scale bar = 50 μ m.

was noticed in animal hemispheres, especially on one side of the lateral gastrula-wall (data not shown); this pattern of staining disappeared in advanced gastrulae, too. An animal polar region in the gastrulae did not exhibit such signals which may suggest a specialized region (for example, the apical plate region as is well-known in sea urchin embryos).

The expression of the sea cucumber T-brain subfamily T-box gene during gastrulation was described in this study. The predominant expression in the invaginated cells at the initial step of archenteron formation may suggest a role for the gene in this morphogenetic process as well as fate decision processes of the cells, giving rise to larval mesoderm and probably some portion of the larval endoderm. In addition, I note that the expression pattern of the starfish T-brain gene, *Ap-Tbr*, during gastrulation has been revealed (Shoguchi *et al.*, 2000), and that the predominant expression pattern of *Ap-Tbr* at the early gastrula stage quite resembles that of *Hl-Tbr*.

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