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# A sea urchin homologue of the chordate *Brachyury* (*T*) gene is expressed in the secondary mesenchyme founder cells

**Yoshito Harada*, Hitoyoshi Yasuo and Noriyuki Satoh**

Department of Zoology, Kyoto University, Graduate School of Science, Sakyo-ku, Kyoto 606-01, Japan

*Author for correspondence

## SUMMARY

**Chordates are thought to have emerged from some common ancestor of deuterostomes by organizing shared anatomical and embryological features including a notochord, a dorsal nerve cord and pharyngeal gill slits. Because the notochord is the most prominent feature of chordates and because the *Brachyury* (*T*) gene is essential for notochord formation, the *T* gene is a key molecular probe with which to explore the origin and evolution of chordates. We investigated whether the sea urchin (echinoderm) conserves the *T* gene and, if so, where the sea urchin *T* gene is expressed.**

**A cDNA clone for the sea urchin *T* (*HpTa*) gene contained a long open reading frame that encodes a polypeptide of 434 amino acids. Although the overall degree of amino acid identity was not very high (52%, sea urchin/mouse), in the T domain of the N terminus the amino acid identity was 73% (sea urchin/mouse). The *HpTa* gene is present as a single copy per haploid genome. As with the chordate *T* gene, the expression of *HpTa* is transient, being first detected in the swimming blastula, maximally transcribed in the gastrula, decreasing at the prism larval stage and barely detectable at the pluteus larval stage. *HpTa* transcripts were found in the secondary mesenchyme founder cells, vegetal plate of the mesenchyme blastula, extending tip of the invaginating archenteron and, finally, the secondary mesenchyme cells at the late-gastrula stage. The results suggest that the *T* gene was organized prior to the branching of a lineage leading to chordates from that leading to echinoderms and that, during sea urchin development, the *T* gene is utilized to specify the developmental fate of embryonic cells to the secondary mesenchyme.**

Key words: sea urchin, *T* (*Brachyury*) gene, sequence conservation, transient expression, secondary mesenchyme founder cells

## INTRODUCTION

The molecular mechanisms involved not only in ontogeny but also in phylogeny need to be elucidated. The discovery of a shared function of the HOM-C/Hox cluster genes in determining the anteroposterior axis of insects and vertebrates, permits investigation of how the widely divergent animal body plans have formed during evolution (e.g. Slack et al., 1993; Akam et al., 1994; Manak and Scott, 1994). Here, we investigate the molecular developmental mechanisms involved in the origin and evolution of chordates.

The evolutionary pathway from advanced invertebrates through primitive chordates to vertebrates has been a subject of extensive investigation and vigorous discussion for more than a century (e.g. Haeckel, 1868; Garstang, 1928; Berrill, 1955; Løvtrup, 1977; Jefferies, 1986; Willmer, 1990). Chordates (urochordates, cephalochordates and vertebrates) are categorized as deuterostomes together with two invertebrate groups, echinoderms and hemichordates (e.g. Brusca and Brusca, 1990). Deuterostomes share many features, such as radial cleavage, the fate of the blastopore that does not form a mouth and an enterocoelic coelom; in addition, chordates show several common embryological and anatomical features, including a notochord, a dorsal hollow nerve cord and pharyngeal gill slits (e.g. Brusca and Brusca, 1990; Willmer, 1990). Of these features, the notochord may be the most prominent characteristic, as the name 'Chordata' is derived from this organ. Molecular phylogenetic studies (Wada and Satoh, 1994; Turbeville et al., 1994) as well as a cladistic analysis (Schaeffer, 1987) suggest that echinoderms, hemichordates and chordates form a monophyletic group of deuterostomes. Therefore, the organization of the notochord during invertebrate evolution is of salient importance in attempts to understand chordate ontogeny and phylogeny (Satoh and Jeffery, 1995; Yasuo et al., 1995).

Recent cloning and characterization of the mouse *Brachyury* (*T*) gene by Herrmann et al. (1990) have advanced the understanding of molecular mechanisms involved in the notochord formation in vertebrates (see the review by Herrmann and Kispert, 1994). The *T* gene is expressed both in the notochord and in the area from which the mesoderm is generated (Wilkinson et al., 1990). Loss of function of the *T* gene leads to a disturbance in the development of the primitive streak with the most affected region being the notochord (Chesley, 1935; Grüneberg, 1958; Yanagisawa et al., 1981). In addition, a single-copy transgene representing the wild-type *T* allele can rescue *T*-associated phenotypes expressed as a defective tail (Stott et al., 1993). Homologues of the mouse *T* gene have been

isolated from *Xenopus* (*Xbra*; Smith et al., 1991) and zebrafish (*Zf-T*; Schulte-Merker et al., 1992). Zebrafish *no tail* is a mutation of this gene (Halpern et al., 1993; Schulte-Merker et al., 1994). In addition, an ascidian *T* gene (*As-T*) is expressed exclusively in blastomeres of notochord lineage and the timing of initiation of *As-T* transcription coincides with that of a restriction of developmental fate (Yasuo and Satoh, 1993, 1994). All of these studies demonstrated that the *T* gene is conserved in primitive chordates (ascidian) and vertebrates and that it is closely associated with notochord organization. Therefore, *T* may be a key gene with which to probe the origin and evolution of chordates from a molecular-embryological perspective.

We examined whether or not the *T* gene is expressed in echinoderms, a group of deuterostomes that do not have a notochord. A *T-related gene* (*Trg*) has been identified in *Drosophila* and two other insects; it is expressed in the hindgut primordium at the blastoderm stage, then in the differentiating hindgut (Kispert et al., 1994). This finding, together with the conservation of the *T* gene in chordates, however, does not prove the presence of the *T* gene in echinoderms and hemichordates. This study seeks to show that a *T* gene homologue is present and expressed in sea urchin embryos. Both cell lineage studies and cell-type-specific gene expression analyses have demonstrated that, in the sea urchin embryo, five territories for founder cells can be distinguished (e.g. Davidson, 1989; Cameron and Davidson, 1991). Therefore, the territory expressing the *T* gene can be determined. A comparison of the embryonic regions that express the *T* gene among an echinoderm, an ascidian and vertebrates may indicate how the *T* gene changed its function during the emergence of chordates.

## MATERIALS AND METHODS

### Biological materials

*Hemicentrotus pulcherrimus* was collected during the spawning season near the Ushimado Marine Laboratory, Okayama University, Okayama, Japan. Gametes were handled and fertilized eggs were cultured according to standard procedures. Embryos were raised in filtered seawater at about 15°C. The embryos divided about every hour and became mesenchymal blastulae 24 hours after fertilization. Gastrulae, prism larvae and pluteus larvae were observed about 35, 45 and 60 hours after fertilization, respectively.

Eggs, embryos and larvae at appropriate stages were collected by low-speed centrifugation and frozen quickly in liquid nitrogen for northern blotting or they were fixed for in situ hybridization as whole-mount specimens.

### Isolation and sequencing of cDNA clones for the sea urchin homologue of the mouse *T* gene

Amino acid sequences of so-called T domain of the *T* gene products are highly conserved among mouse (Herrmann et al., 1990), *Xenopus* (Smith et al., 1991), zebrafish (Schulte-Merker et al., 1992) and ascidians (Yasuo and Satoh, 1994). The sense-strand oligonucleotide that corresponds to the amino acid sequence YIHPDSP and the antisense oligonucleotide that corresponds to the amino acid sequence NPFAKG(A)L(F) were synthesized using an automated DNA synthesizer (Applied Biosystmes Inc., Foster City, CA, USA). Using these oligonucleotides as primers, we amplified target fragments from an *H. pulcherrimus* gastrula cDNA library by means of PCR. The library was a gift from Drs H. Shimada and K. Akasaka in Hiroshima University. Probing with candidate cDNA fragments random-labeled with [$^{32}$P]-dCTP (Amersham), we screened the library at high stringency (hybridization: 6× SSPE, 0.1% SDS, 1× Denhardt's solution, 50% formamide at 42°C; washing: 2× SSC, 0.1% SDS at 65°C). Among three cDNA clones that were isolated to search for the sea urchin *T* gene, the longest was subcloned into pBluescriptII SK(+). Both strands of the clones were sequenced by dideoxy chain termination (Sanger et al., 1977) using Sequenase ver. 2.0 (USB; United States Biochemical Corp., Cleveland, OH, USA).

### RNA isolation and northern hybridization

Total RNA was extracted from specimens at various stages using acid guanidinium thiocyanate-phenol-chloroform (AGPC; Chomczynski and Sacchi, 1987). 20 μg of total RNAs were fractionated by electrophoresis on a 1% agarose gel containing 6% formaldehyde and RNA bands were transferred to a Hybond-N+ nylon membrane (Amersham). The membranes were prehybridized in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, 50% formamide, and 100 μg/ml salmon sperm DNA for 2 hours. Random-primed $^{32}$P-labeled DNA probes were then incubated with the membranes overnight at 42°C. The length of the probe for both northern and Southern hybridizations was about 2.5 kb between positions 671 and 3189 of the cDNA clone shown in Fig. 1. The membranes were washed under high-stringency conditions (twice in 2× SSC, 0.1% SDS at 65°C for 15 minutes).

### DNA isolation and genomic Southern analysis

High molecular weight *H. pulcherrimus* genomic DNA was provided from Dr H. Shimada. After exhaustive digestion with *Eco*RI, *Bam*HI and *Hin*dIII and electrophoresis, the DNA fragments were blotted onto Hybond-N+ nylon membranes (Amersham). The blots were hybridized with [$^{32}$P]dCTP-labeled DNA probes at 42°C for 18-24 hours and washed under high-stringency conditions.

### In situ hybridization

Whole-mount specimens were hybridized in situ basically as described by Ransick et al. (1993). Embryos were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS pH 7.5 on ice for overnight prior to storage in 80% ethanol at −20°C. The fixed specimens were thoroughly washed with PBT (phosphate-buffered saline containing 0.1% Tween 20), and partially digested with 2 μg/ml proteinase K (Sigma) in PBT for several minutes at 37°C. After washing with PBT, the specimens were postfixed with 4% paraformaldehyde in PBT for 1 hour at room temperature, then washed again with PBT. Specimens were then treated with PBT:hybridization buffer (1:1, v/v) for 10 minutes at room temperature and with hybridization buffer alone for 10 minutes at room temperature. After a 1 hour prehybridization at 42°C, the specimens were hybridized with digoxigenin(DIG)-labeled antisense probes for about 16 hours at 42°C. The hybridization buffer contained 50% formamide, 10% PEG#6000, 2× Denhardt's solution, 500 μg/ml yeast RNA, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.5 and 0.1% Tween 20. The probe was synthesized following the instructions supplied with the kit (Boehringer Mannheim DIG RNA Labelling kit), and the hybridized fragments were reduced to about 200-500 nucleotides by alkaline hydrolysis. The probe was used at 0.1 μg/ml in the hybridization buffer.

After hybridization, the specimens were washed in 1× SSC for 15 minutes at 65°C, three times. This solution was replaced with PBT at room temperature. The DIG label was detected as follows. The specimens were incubated with 5% goat serum in PBT for 30 minutes at room temperature, then with 1:1000 Boehringer Mannheim alkaline-phosphatase-conjugated anti-DIG in PBT. Washes proceeded in PBT (10 minutes, four times), then in alkaline phosphatase buffer (100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 100 mM Tris-HCl pH 9.5) before signal detection using NBT and BCIP following the supplier's instructions. The reactions were stopped in PBT after 30 minutes to 3 hours, then the embryos were dehydrated in a graded

series of ethanol solution and cleared by placing them in a 1:2 mixture (v/v) of benzyl alcohol:benzyl benzoate.

As a technical control, *Strongylocentrotus purpuratus* prism larvae were hybridized with *CyIIa* probe. The samples and probe are a gift from Dr Eric Davidson's laboratory in California Institute of Technology.

## RESULTS

### The sea urchin conserves a homologue of the mouse *T* gene

The chordate *T* gene products consist of two distinguishable domains (Herrmann et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992; Yasuo and Satoh, 1994). The N-terminal half is highly conserved among the T proteins, while the degree of conservation is not high in the C-terminal region. In particular, a stretch of about 200 amino acids, known as the T domain, in the N terminus show extensive similarity. This T domain confers specific DNA binding (Kispert and Herrmann, 1994). Using oligonucleotide primers corresponding to the shared sequences, we amplified target fragments from *H. pulcherrimus* gastrula cDNA by the PCR reaction. Sequencing the amplified 300 bp-long fragments after subcloning them into pBluescriptII SK(+), revealed that the sea urchin library contains at least two independent cDNA clones, with sequences similar to that of the chordate *T* gene (data not shown). Genomic Southern analysis suggested that these sequences corresponded to two independent genes in the sea urchin genome (data not shown; cf. Fig. 3). We therefore designated the genes for the clones, *HpTa* and *HpTb*, respectively. Northern analysis indicated that *HpTa* and *HpTb* transcripts were about 3.7 kb (cf. Fig. 4) and 5 kb (data not shown) in length respectively. In this study, we further analysed *HpTa*, because the nucleotide sequence of the *HpTa* gene resembled that of the mouse *T* gene more than *HpTb*.

Screening of $2.7\times10^5$ pfu of *H. pulcherrimus* gastrula cDNA library with the random-labeled *HpTa* fragment, yielded three positive cDNA clones. Sequencing the longest showed that it consisted of about 3,700 nucleotides, suggesting that the clone was nearly the entire length of the transcript. As shown in Fig. 1, the cloned fragment contained a single long, open reading frame of 1,302 nucleotides that encode a polypeptide of 434 amino acids. The open reading frame extended from nucleotides 349 to 1,651 of the fragment. The calculated relative molecular mass ($M_r$) of the protein was $46\times10^3$. A putative polyadenylation signal was found at position 3,659-3,664.

Fig. 2 shows a comparison of the amino acid sequences of the T domain of *HpTa* with those of the putative proteins encoded by the mouse *T* (Herrmann et al., 1990), *Xenopus Xbra* (Smith et al., 1991), zebrafish *Zf-T* (*no tail*; Schulte-Merker et al., 1992), ascidian *As-T* (Yasuo and Satoh, 1994) and *Drosophila Trg* (Kispert et al., 1994) genes. Although the overall degree of amino acid identity was not very high (52%, sea urchin/mouse; 41%, sea urchin/ascidian), in the T domain shown in this figure, the full extent of amino acid identity was 73% (sea urchin/mouse), 73% (sea urchin/frog), 75% (sea urchin/zebrafish) and 68% (sea urchin/ascidian). The high degree of identity in the T domain between the sea urchin protein and the mouse T, Xbra, Zf-T and As-T proteins demonstrated that this cDNA clone corresponds to the sea urchin homologue of the chordate *T* gene.

### *HpTa* presents as a single copy gene

We determined the number of different sequences that corresponded to *HpTa* in the sea urchin genome by means of genomic Southern hybridization. As shown in Fig. 3, one major band and a few minor bands were detected in each lane of DNAs digested with *Eco*RI, *Bam*HI and *Hin*dIII, suggesting that *HpTa* is present as a single copy gene and that the sea urchin genome contains T domain genes other than *HpTa*. In addition, *HpTa* and *HpTb* had completely different hybridization patterns (data not shown).

### Expression of *HpTa* during sea urchin embryogenesis is transient

Northern blotting of the *HpTa* gene during embryogenesis of *H. pulcherrimus* revealed that the *HpTa* transcripts are transient.

Hybridization signals were undetectable in unfertilized eggs and early embryos at the cleavage and early blastula stages (Fig. 4). This result was confirmed by longer exposure. A weak but distinct hybridization signal was first detected in hatching blastulae. A distinct band was found at the mesenchymal blastula and gastrula stages, at which time the intensity of the band was maximal. The intensity of the band decreased after the gastrula stage. Although a band was detected in prism larvae, it was barely detectable at the pluteus-larva stage (Fig. 4). This pattern of transient *HpTa* expression coincides with that of the chordate *T* genes.

### *HpTa* is expressed in secondary-mesenchyme founder cells

Cell lineage analyses of sea urchin embryos have demonstrated that unique sets of founder cells generate five territories of gene expression by means of an invarient pattern of complete cleavage (e.g. Davidson, 1989; Cameron and Davidson, 1991). The five territories and cell types are aboral ectoderm, oral ectoderm, ectoderm-derived neurons, primary skeletogenic mesenchyme cells and the definitive vegetal plate. The vegetal plate cells after gastrulation, contribute to the endoderm and four types of secondary mesenchyme, namely basal cells, coelomic pouches, pigment cells and circumesophageal muscle. We studied which territories or cell types express *HpTa* by means of in situ hybridization of whole-mount specimens.

Control embryos hybridized with a sense probe did not show signals above the level of background (Fig. 5B,D,G,I). Sea urchin embryos processed for in situ hybridization were fragile, particularly those up to the swimming blastula stage. Most embryos broke down during the later half of hybridization process. The first stage at which we obtained a clear result was that of the mesenchyme blastula, when the distribution of hybridization signals was not even but restricted to cells of the vegetal plate (Fig. 5A). At the early gastrula stage, the signals were found in cells of the invaginating tip of the vegetal plate or newly formed archenteron (Fig. 5C). Neither aboral and oral ectoderm nor primary mesenchyme cells that have delaminated from the vegetal wall into the blastocoel showed signals above the background (Fig. 5A,C).

At the mid-gastrula stage, the archenteron has extended to

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   1 GGAATTCCGGCGTAGATCGTCCATTTAAACTATTCTGGAGGCTTCATAAAACGGTGTTTAACAGTCCCTTGGGAT   75
  76 CACTTAGTAACTATTTCGTGACTTTTTTTTTTCTTTCTCAAACTGTGGTTTCGCCGTCAGTCACTTGCATTTCGG  150
 151 TTTGCAAGTTTTATTTTCCTCGTGTAACACGACATTGCCATACTTCGGAACATCCTGATTTTATGTCACTTTGAA  225
 226 TTTCCAACACGGAAGATCTTCGAGCTTTATCCAAGCGAACTTTATCCAAGCGAAAGGCGGAATCGACTGCCCTGC  300
 301 CTGCCCGAGCGCGCCCGCGGAAAACTCTCCACACCCATCACCACCACTATGCCTGCAATGAGCGCCGACGCTCTG  375
   1                                             M  P  A  M  S  A  D  A  L    9
 376 CGTGCCCCGTCCTACAACGTTTCGCATCTTCTCAACGCCGTACAGTCGGAGATGAACCGAGGGAGTGAGAAGGGA  450
  10  R  A  P  S  Y  N  V  S  H  L  L  N  A  V  Q  S  E  M  N  R  G  S  E  K  G   34
 451 GACCCCAGCGAGGAAGGACTCAAAGTTAGGCTGGACGATGTGGAATTGTGGAAAAAGTTTCACAAGTTAACCAAC  525
  35  D  P  S  E  E  G  L  K  V  R  L  D  D  V  E  L  W  K  K  F  H  K  L  T  N   59
 526 GAGATGATCGTGACCAAAAGTGGGAGGCGAATGTTCCCCGTCCTCTCCGCCAGCATCGCCGGTCTCGACCCCAAT  600
  60  E  M  I  V  T  K  S  G  R  R  M  F  P  V  L  S  A  S  I  A  G  L  D  P  N   84
 601 TCCATGTACTCCGTCCTGCTCGACTTCTCCGCCGCCGACGATCACCGCTGGAAGTACGTCAACGGCGAGTGGATC  675
  85  S  M  Y  S  V  L  L  D  F  S  A  A  D  D  H  R  W  K  Y  V  N  G  E  W  I  109
 676 CCCGGTGGCAAGCCCGACGGCTCGCCTCCGACCACCGCCTACATCCATCCCGACTCGCCCAACTTCGGGGCACAC  750
 110  P  G  G  K  P  D  G  S  P  P  T  T  A  Y  I  H  P  D  S  P  N  F  G  A  H  134
 751 TGGATGAAGCAAGCCGTCAACTTCAGCAAAGTGAAGTTGTCGAATAAACTCAACGGAAGCGGGCAGGTGATGCTA  825
 135  W  M  K  Q  A  V  N  F  S  K  V  K  L  S  N  K  L  N  G  S  G  Q  V  M  L  159
 826 AACTCCCTTCACAAGTACGAACCACGTATCCACATCATACGCGTCGGAGGCAGGGAGAAGCAGAGACTGGTTGGA  900
 160  N  S  L  H  K  Y  E  P  R  I  H  I  I  R  V  G  G  R  E  K  Q  R  L  V  G  184
 901 AGTTACTCCTTCACAGAAACCCGCTTCATTGCCGTCACAGCATACCAGAACGAAGATATAACACAGCTTAAGATC  975
 185  S  Y  S  F  T  E  T  R  F  I  A  V  T  A  Y  Q  N  E  D  I  T  Q  L  K  I  209
 976 AAGTACAACCCCTTTGCAAAAGCTTTCCTCGATATCAAAGACAAAAACGACGGACATGATCTTTTCGATGATGTG 1050
 210  K  Y  N  P  F  A  K  A  F  L  D  I  K  D  K  N  D  G  H  D  L  F  D  D  V  234
1051 CACGATTTCCAGGGTTCAAAGTATCCCCAATTTGGTGGCTGGTTTCTGCCCGGCTCTGGAGCTTTCGGCCCCACA 1125
 235  H  D  F  Q  G  S  K  Y  P  Q  F  G  G  W  F  L  P  G  S  G  A  F  G  P  T  259
1126 CCCCATCAGTTCAACCCGTCTATCGGTCTACCGTCCCATGCAGGATGCGATAGGTACGGCGGGTTAAGGAGCCAT 1200
 260  P  H  Q  F  N  P  S  I  G  L  P  S  H  A  G  C  D  R  Y  G  G  L  R  S  H  284
1201 AGGACATCCCCATACCCACCCCCGCCCTACCACCAGAAGTACTCCGCCGCAGGTGCAGGCTACGGTGCCGAAGCT 1275
 285  R  T  S  P  Y  P  P  P  P  Y  H  Q  K  Y  S  A  A  G  A  G  Y  G  A  E  A  309
1276 TCGGCTGGTCTCTCTAGCAGTATCTCGCTTCTCGCTGCCGACAGCTGGTCTTCTCTCGCTAACTCTACGTCGGCA 1350
 310  S  A  G  L  S  S  S  I  S  L  L  A  A  D  S  W  S  S  L  A  N  S  T  S  A  334
1351 GCTAGCTCCATGCCAGCCTGCAGTCAATACGGCAGCATGTGGCCCTCCACCGCCGCAACCTCCGGGTTCTCTCAC 1425
 335  A  S  S  M  P  A  C  S  Q  Y  G  S  M  W  P  S  T  A  A  T  S  G  F  S  H  359
1426 GTCAGCTCCCCGCAGTCACCGCTCCCAACCGGCTTGTTCCGAAACCCTCACCCAACCTCGTCACACCAGCACAAC 1500
 360  V  S  S  P  Q  S  P  L  P  T  G  L  F  R  N  P  H  P  T  S  S  H  Q  H  N  384
1501 CTCGCATCAACGGCACACGGAATGGCTCCGGTAGCGAGCGGTCTCCCATCCGCCGCGGTCACAACCGCGAACTCG 1575
 385  L  A  S  T  A  H  G  M  A  P  V  A  S  G  L  P  S  A  A  V  T  T  A  N  S  409
1576 TCAGAAGCGCACGCACTGAGTCAGTCCGTCATGGCGCCAGGAGAATGCAGAGCTTCAGACAATGCTGGGTACCTT 1650
 410  S  E  A  H  A  L  S  Q  S  V  M  A  P  G  E  C  R  A  S  D  N  A  G  Y  L  434
1651 TGAGAGGTTCTTTGGATCGCTCCTGGTGGATAACCCTGATGCCTTTGACCAGACAAGAGGTGCCGTCGAACGTAT 1725
      *
1726 AGACAGCCTCAATGCCTTCCTCGGGAACAGAGCGGCAATCCTTGCAGGATTGCGACCTGACCCGCCAACCGTTCC 1800
1801 AACCAATGACACCGTTGACCAGGAAGAACTTGGTCGGGTATTGACCCAGCTGTCGTCGGTGAACAGCAACCTGGA 1875
1876 ACAGAAGGTCAGCAAGCTGGAAAGACGGGTAGACAACGGGGCGGAGGCTGCGGGAGACAGAAGCTTGACCGGAGG 1950
1951 AACCAATGACATCTTTACCCTGGATAACTGCAGAGATGGTTTAGTGACTTTAGAGGCAACTGTCAACGCGCTCAC 2025
2026 AGCCACGCAACTTGACAAGGACAACGCCGACCGCCTTGCTGGGTCACGTGATGTTATCTCAGCAGGAGACCGCGC 2100
2101 CATCCTGGAGGGGTCTGTCTACGGATCTGCCAGGGGTGTCAACAGCGTCCGCAAGTCTGTGTTCTCGGTGGCCAG 2175
2176 TACACACCCTCTGCTTGGTAGCACCGACCCCACGACCGTCCCCTTTGATTACACTTTCGTCAATAAAGGCAATCA 2250
2251 CTGGAATGGCACAAGCCAGCAGTTCGTATGCGCCATCACCGGTTACTATTGGTTCCACTTTTCACTGCGATCGTA 2325
2326 CGATGGTCATTACATGGGCGTGACGATGATGAAGAACAACGAGGTGGTCACGGCAATGTTCACGGAGAAGAGCGC 2400
2401 CCGGAACGTTATGGAGAGCCAAGGTGTGGTGCTGTCACTCGAGATCGGGGACACGGTTTACCTTCGACTGGGACC 2475
2476 GTCCACGGAGTACGCCGTCCACTCCGACGCCTACAAGTACGCGACCTTTTCCGGGTTCCTGGTCTATAAAGGCCT 2550
2551 CTGAAGAAGAAAAAGATGTGTAATAGCGGAATCGAAAATGGCCCCCAAATTGCATTATTTCAAGAAGATTTTATA 2625
2626 CAAAATGTGACATCCATTTTTGTAAAAGCAGGTACTAAGTATGCTCTGATTTTAGCCTATATGCGTCTTTTCGAG 2700
2701 TTAGCATTGAAGAGAGATACCGTACTATGCAAAAACAGTTACATGCATTCAAATAATTGCTAACATCGGGAAAAT 2775
2776 ATGACCCCATTTGGTGAAATTTCTTCGTCTTTACGCAGAAATATCTGGTACCATGAGTTCACACAGTCATACTAG 2850
2851 TTGGATGACCGTGAGACCTATTCTGTGTGATCATTTCATACATTTAACATAATTATGTTAACGATTTTCAACGAA 2925
2926 TCTATTTCATTGGTGCGATGTGGTTCTTAGTGCGATTAAAGGACAGTCACGGAGCTTTATGGAATATTAAGTACG 3000
3001 TAATTACTGAATTGCTTCAGAATTGATCTATATATTGGTCAAATCCAAGTGCTCGCAATATTGTCTGCTGAATGT 3075
3076 ATACATTTAGAATTTTGTCTTCATTACATTCCAAAGTGAGCGTATCCTTTTTTATTATTGATTTATCAAATCGAA 3150
3151 AAAGATAATAATCCAAACAATTCAGAGAATGGTGAATTCCATAACAATGATTGTTGTTGAGTTATTAAACATATT 3225
3226 TTCATAGAGCACTATGAAAGTTTAAACAAATATCCCAAAGTGTTTGTATATTTCGATTGTCATTTCATTAGATGC 3300
3301 CCTCATTTGCAAAGAAAAGTAAAGATTGACGTTTATTGTCTCCATTTGTGTCTTCATCTGATGATTGTTGTAACG 3375
3376 GTTCTTAAAGAGGCTAGATTGCAATTCCATCGTTAACATAATGGGCGACGATTTACGGAGCAATCATTGGAGGAA 3450
3451 TTTGAAAACGGCAGATATTTTTTCCCCTTTTTTAATTTAATCCATCATCATGATATGTTTTAAGTATTTTGTCAA 3525
3526 CCTTAGTCTAGATTTGTACATGGTCAAGCTTTGATTCACGTCAAACAAACAAAAATCATTTTAATGAATGTAATG 3600
3601 ACATTCATTCTGTCTCAGTTTGTAATGTCCAGATACAGGGGATTAATTTTTTTATATGAATAAAGAGTACGAAAA 3675
3676 AGATAAAAAAAAAAAAAAAA                                                        3695
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**Fig. 1.** Nucleotide and predicted amino acid sequences of a cDNA clone for the sea urchin *HpTa* gene. The sequence of the cDNA encompasses 3,695 bp including 16 adenylyl residues at the 3′ end. The ATG at the position 349-351 represents the putative start codon of the *HpTa*-encoded protein. A potential signal sequence for polyadenylation is underlined. An asterisk indicates the termination codon.

nearly a half of the blastocoel and two islands of skeletalgenetic sites are formed by the primary mesenchyme cells (Fig. 5E,F). Hybridization signals were found in cells of extending tip of the archenteron and secondary mesenchyme cells migrating from there (Fig. 5E,F). In contrast, signals were not so evident in cells of the middle and posterior parts of the archenteron (Fig. 5E,F).

At the late gastrula stage, the archenteron has almost reached the wall of the most anterior part of the oral ectoderm (Fig. 5H) and some secondary mesenchyme cells have separated from the archenteron wall into blastocoel. Intense hybridization signals were detected in the secondary mesenchyme cells and cells of the archenteron tip, whereas the cells of the archenteron showed barely detectable signals (Fig. 5H).

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HpTa          45-LDDVELWKKFHKLTNEMIVTKSGRRMFPVLSASIAGLDPNSM
mouseT        46-LEESELWLRFKELTNEMIVTKNGRRMFPVLKVNVSGLDPNAM
Xbra          44-LEERDLWTRFKELTNEMIVTKNGRRMFPVLKVSMSGLDPNAM
no tail       39-LEDAELWTKFKELTNEMIVTKTGRRMFPVLRASVTGLDPNAM
As-T          19-LNDRALWTKFCSLTNEMIVTKSGRRMFPVLKLTASGLEPNSM
Trg           91-LDDRELWLRFQNLTNEMIVTKNGRRMFPVVKISASGLDPAAM
                 * .  ** .*  ********* *******.    .**.* .*

HpTa      YSVLLDFSAADDHRWKYVNGEWIPGGKPDGSPPTTAYIHPDSPNFGAHWM
mouseT    YSFLLDFVTADNHRWKYVNGEWVPGGKPEPQAPSCVYIHPDSPNFGAHWM
Xbra      YTVLLDFVAADNHRWKYVNGEWVPGGKPEPQAPSCVYIHPDSPNFGAHWM
no tail   YSVLLDFVAADNNRWKYVNGEWVPGGKPEPQSPSCVYIHPDSPNFGAHWM
As-T      YSFLLDFAPADSNRWKYVNGEWVPGGKPEPHAASCVYVHPDSPNFGSHWM
Trg       YTVLLEFVQIDSHRWKYVNGEWVPGGKAEVPPSNPIYVHPESPNFGAHWM
          *. **.*   * .*********.**** .       *.**.*****.***

HpTa      KQAVNFSKVKLSNKLNGSGQ-VMLNSLHKYEPRIHIIRVGGRE-KQRLVG
mouseT    KAPVSFSKVKLTNKLNGGGQ-IMLNSLHKYEPRIHIVRVGG---PQRMIT
Xbra      KDPVSFSKVKLTNKMNGGGQ-IMLNSLHKYEPRIHIVRVGG---TQRMIT
no tail   KAPVSFSKVKLSNKLNGGGQ-IMLNSLHKYEPRIHIVKVGG---IQKMIS
As-T      KQPVSFNKVKLTNKGNGGGQQIMLNSLHKYEPRIHVVKVGGEAASERTIA
Trg       KEPISFAKVKLTNKTNGNGQ-MMLNSLHKYEPRVHLVRVGS---EQRHVV
           *  . * ****.** ** **  .***********.*...**    .. .

HpTa      SYSFTETRFIAVTAYQNEDITQLKIKYNPFAKAFLDIKDKND-226
mouseT    SHCFPETQFIAVTAYQNEEITALKIKYNPFAKAFLDAKERND-225
Xbra      SHSFPETQFIAVTAYQNEEITALKIKHNPFAKAFLDAKERND-223
no tail   SQSFPETQFIAVTAYQNEEITALKIKHNPFAKAFLDAKERSD-218
As-T      TFSFPESQFIAVTAYQNEEVTSLKIKHNPFAKAFLDAKERPD-202
Trg       TYPFPETQFIAVTAYQNEEVTSLKIKYNPFAKAFLDAKERPD-270
          .  * *..**********..* **** ********* *.. *
```

**Fig. 2.** A comparison of the amino-acid sequence of the *HpTa* T domain with those of the mouse *T* (Herrmann et al., 1990), the *Xenopus Xbra* (Smith et al., 1991), the zebrafish *no tail* (Schulte-Merker et al., 1992), the ascidian *As-T* (Yasuo and Satoh, 1994) and the *Drosophila Trg* (Kispert et al., 1994). Asterisks (*) indicate that all of the six *T* gene products have an identical amino acid. Similar amino acids at a given position in all six proteins are shown by dots. For maximum similarity, gaps have been introduced.

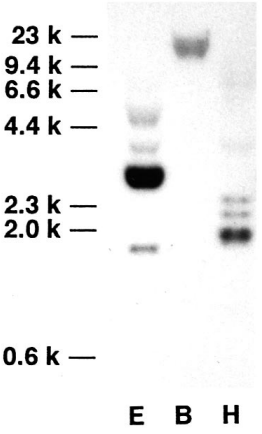


**Fig. 3.** Genomic Southern analysis of the *HpTa* gene. 10 μg of genomic DNA prepared from a single *H. pulcherrimus* was digested with *Eco*RI (E), *Bam*HI (B) and *Hin*dIII(H), resolved by electrophoresis and transferred to a nylon membrane. Blots were hybridized with a $^{32}$P-labeled DNA probe and washed under high stringency conditions.

At the prism larval stage, the secondary mesenchyme cells are scattered over the inner wall of blastocoel. At this stage, hybridization signals were not detected above the background level (data not shown). Fig. 5J is a control in which the *CyIIa* gene in *Strongylocentrotus purpuratus* embryos is expressed in the primary mesenchyme cells. As development proceeded, we could not detect the distinct signal above the background level at the late prism and the pluteus larval stages (data not shown).

Thus, we concluded that the *HpTa* gene is expressed in embryonic cells that give rise to the secondary mesenchyme

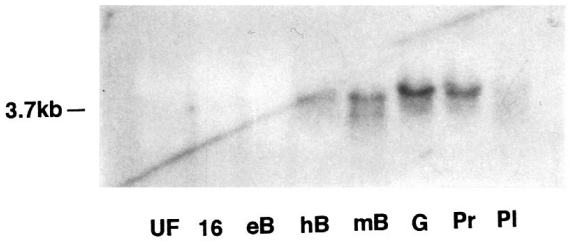


**Fig. 4.** Temporal expression pattern of *HpTa*. Northern blots of total RNA prepared from the unfertilized eggs (UF), 16-cell-stage embryos (16), early blastulae (eB), hatching blastulae (hB), mesenchymal blastulae (mB), gastrulae (G), prism larvae (Pr), and pluteus larvae (Pl) were hybridized with a $^{32}$P-labeled DNA probe and the membrane was washed under high stringency conditions. Each lane was loaded with 20 μg total RNA. The transcripts were undetectable in unfertilized eggs and in early blastulae. Transcripts of about 3.7 kb first appeared at the hatching blastula stage and the accumulation of the mRNAs was maximal at the gastrula stage. The amount of the mRNA decreased after the prism larva stage and the mRNAs were barely detectable in the pluteus larvae.

cells. As mentioned before, the vegetal plate cells give rise to the archenteron (larval gut) and the secondary mesenchyme cells. *HpTa* was expressed in the secondary mesenchyme founder cells, but not in the gut cells. In addition, the secondary mesenchyme cells give rise to four types of cells, circumesophageal muscles, pigment cells, basal cells and coelomic pouches. However, further differentiation of the secondary mesenchyme cells into these four types occurs after the prism stage. At this stage, the hybridization signals were undetectable. Therefore, we were unable to identify which components of the secondary mesenchyme are the positive founder cells of all four types in this study.

## DISCUSSION

This study revealed that the sea urchin *Hemicentrotus pulcherrimus* contains homologues (*HpTa* and *HpTb*) of the chordate *T* gene. *HpTa* is expressed transiently at the blastula and gastrula stages and the distribution of *HpTa* transcripts is restricted to the secondary mesenchyme founder cells.

### The sea urchin conserves homologues of the chordate *T* gene

Molecular phylogenetic studies based upon a comparison of 18S rDNA sequences (Wada and Satoh, 1994; Turbeville et al., 1994) and a cladistic analysis based on the comparison of embryological and morphological features (Schaeffer, 1987) suggest that echinoderms, hemichordates and chordates are monophyletic and, thus, have evolved from a common ancestor. As shown in this study, *T* gene expression is conserved in the sea urchin embryo. Recent studies in our own laboratory (Terazawa and Satoh, 1995) and from Herrmann's group (Dr B. G. Herrmann, personal communication) have identified an amphioxus homologue of the *T* gene, which is expressed in the mesoderm of the gastrula. Although a hemichordate homologue of the chordate *T* gene should be identified, it is highly likely that the *T* gene is conserved and expressed in embryos of all deuterostome groups. In other

words, the *T* gene was organized in the common ancestor of deuterostomes.

Therefore, the *T* gene is unlikely to have originated along with notochord organization during the emergence and evolution of chordates. Instead, the gene had some functions in embryogenesis, for example in the formation of mesoderm, and the gene or a duplicate was used for notochord formation during chordate evolution (Yasuo et al., 1995). In addition, the *T-related gene* (*Trg*) has been identified in *Drosophila*, *Tribolium* and *Locusta* (Kispert et al., 1994). This suggests that the root of this gene extends to more primitive animals. Because the T domain is involved in DNA binding and in the regulation of trascriptional activities of downstream genes, the function of this gene might be more fundamental than previously assumed (Kispert et al., 1994).

With respect to the wide distribution of the *T* gene in various animal groups, the T domain genes may constitute a family as pointed out by Kispert et al. (1994). In *Drosophila*, the T domain was identified in the gene *optomoter blind* (*omb*) (Pflugfelder et al., 1992). Here, we isolated another T domain gene, *HpTb*, from the sea urchin gastrula cDNA library. We are now examining *HpTb* gene expression to obtain more exact understanding of the sea urchin *T* genes. In addition, an ascidian genome contains two T domain genes other than the *As-T* gene (Yasuo and Satoh, unpublished). A preliminary molecular phylogenetic tree constructed by a comparison of sequences of about 50 amino acids in the highly conserved T domain among chordates and insects, suggests that in the *T*-gene family, a group of *T* genes (chordate *T* and insect *Trg*) branched from that of *omb* (ascidian *omb-like gene* and *omb*) (Yasuo et al., 1995). The *HpTa* gene was included in the former group while the *HpTb* was diverged one. Because some *T* genes such as *HpTb* have diverged from these two groups, there should be other groups within the *T*-gene family.

## Relationship between the secondary mesenchyme of the sea urchin embryo and chordate notochord

In vertebrates, *T* gene expression begins in both prospective posterolateral mesoderm and notochord cells (Herrmann and Kispert, 1994). In contrast, the spatial expression of the ascidian *As-T* is restricted to the primordial notochord cells and *As-T* was not expressed in other mesodermal precursor cells that give rise to mesenchyme and muscle (Yasuo and Satoh, 1993, 1994). What this difference means is uncertain at present. Regardless, it is highly likely that *HpTa* is orthologus with the chordate *T* genes. Because *HpTa* is expressed in the secondary mesenchyme founder cells and the chordate *T* is expressed in the notochord, we question whether there is any phylogenetic relationship between the secondary mesenchyme and notochord.

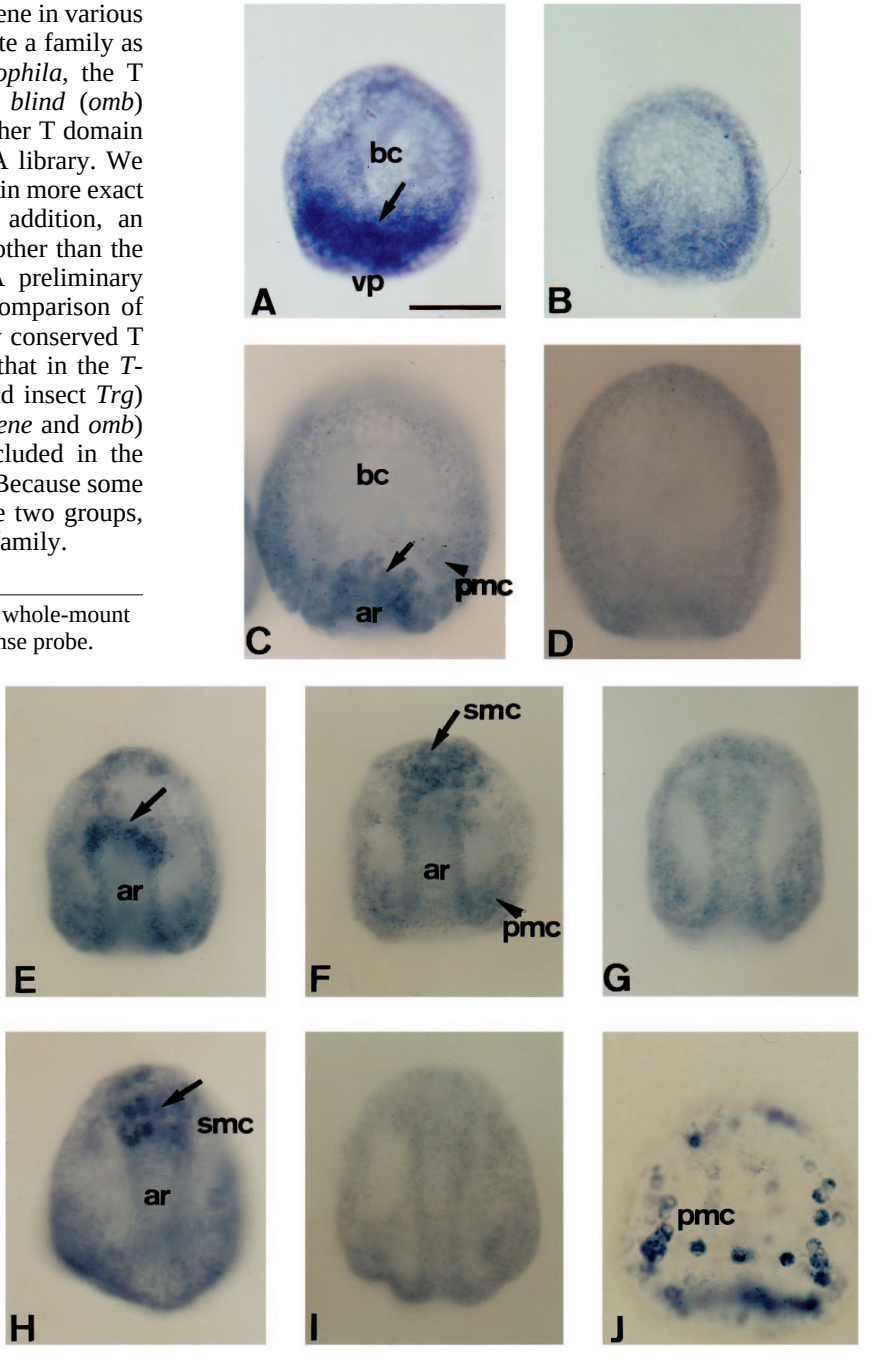


**Fig. 5.** The spatial expression of *HpTa*, as revealed by whole-mount in situ hybridization with a digoxigenin-labeled antisense probe. (A) A mesenchyme blastula hybridized with an antisense probe showed distinct hybridization signals in cells of the vegetal plate (vp; arrow). bc, blastocoel. Scale bar represents 50 μm and applies to all panels. (B) The same stage embryo hybridized with a sense probe (control) showed no detectable signals. (C) An early gastrula hybridized with the antisense probe showed intense signals in cells at the tip of the invaginating archenteron (ar; arrow). Primary mesenchyme cells (pmc; arrowhead) do not show signals above the background. (D) The same stage embryo hybridized with the sense probe. (E,F) Mid-gastrulae showing distinct hybridization signals in cells of secondary mesenchyme precursor cells (smc; arrows) around the tip of the archenteron, but not in cells of primary mesenchyme cells (pmc; arrowhead). (G) A mid-gastrula hybridized with the sense probe. (H) A late gastrula hybridized with the antisense probe showed signals in secondary mesenchyme precursor cells (smc; arrow). (I) A late gastrula hybridized with the sense probe. (J) A control *Strongylocentrotus* prism larva hybridized with a *CyIIa* antisense probe showing signals in the primary mesenchyme cells (pmc).

However, secondary mesenchyme cells have no known features of molecular differentiation in common with notochord cells, and no developmental function or fate in common with notochord. It will require analysis of genes that are upstream and downstream with respect to *T* gene in these organisms. In addition, we should analyze sea urchin homologues of genes that are expressed in the vertebrate notochord, for example, the *HNF3*-related genes (e.g. Sasaki and Hogan, 1993; Ang and Rossant, 1994), to support further argumentation.

## Developmental roles of the *HpTa* gene in sea urchin embryogenesis

*HpTa* gene expression begins around the time of hatching of the embryo. At this stage, the secondary mesenchyme founder cells are buried in the vegetal plate. The earliest feature of the differentiation of secondary mesenchyme cells reported to date is the separation of the cells from the anterior tip of the invaginating archenteron. Specific antigens are expressed in various derivatives of the vegetal plate territory. For example, an endoderm antigen is expressed prior to archenteron invagination throughout the vegetal plate and later in the cells of intestine and stomach (Wessel and McClay, 1985). *Endo16* is expressed in cells of the vegetal plate of *S. purpuratus* blastulae and in cells of the archenteron of gastrulae (Ransick et al., 1993). Therefore, *Endo16* expression is an early vegetal plate marker. However, the archenteron tip cells did not express *Endo16* (Ransick et al, 1993), where *HpTa* was expressed. Because *HpTa* expression is restricted to secondary mesenchyme founder cells and it begins early, *HpTa* transcripts might be used as an early marker of differentiation of these cells.

The mechanisms of specification of sea urchin secondary mesenchyme cells are not fully understood (Davidson, 1989), although the vegetal plate cells are specified by cell-cell interaction (e.g. Ransick and Davidson, 1993). The secondary mesenchyme cells take the place of the primary mesenchyme cells when sea urchin embryos are manipulated to deprive the primary mesenchyme cells from the blastocoel (Ettensohn, 1992). However, the detailed mechanism of secondary mesenchyme specification remains to be determined. As demonstrated with the vertebrate *T* gene (Herrmann and Kispert, 1994), the product of the *HpTa* gene may function as a gene regulatory factor involved in the specification of the secondary mesenchyme cells. The ectopic expression of the *HpTa* gene or the injection of antisense RNA into the vegetal plate cells may help answer this question.

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (D50332).

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