Brachyury (T) Expression in Embryos of a Larvacean Urochordate, Oikopleura dioica, and the Ancestral Role of T

Susan Bassham and John Postlethwait
Institute of Neuroscience, 1254 University of Oregon, Eugene, Oregon 97403-1254

The Brachyury, or T, gene is required for notochord development in animals occupying all three chordate subphyla and probably also had this role in the last common ancestor of the chordate lineages. In two chordate subphyla (vertebrates and cephalochordates), T is also expressed during gastrulation in involuting endodermal and mesodermal cells, and in vertebrates at least, this expression domain is required for proper development. In the basally diverging chordate subphylum Urochordata, animals in the class Ascidiacea do not employ T during gastrulation in endodermal or nonaxial mesodermal cells, and it has been suggested that nonnotochordal roles for T were acquired in the cephalochordate-vertebrate lineage after it split with Urochordata. To test this hypothesis, we cloned T from Oikopleura dioica, a member of the urochordate class Appendicularia (or Larvacea), which diverged basally in the subphylum. Investigation of the expression pattern in developing Oikopleura embryos showed early expression in presumptive notochord precursor cells, in the notochord, and in parts of the developing gut and cells of the endodermal strand. We conclude that the ancestral role of T likely included expression in the developing gut and became necessary in chordates for construction of the notochord. © 2000 Academic Press

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INTRODUCTION

The phylum Chordata gets its name from the notochord, but the evolutionary origin of this mesodermal rod is as yet unclear (Peterson et al., 1999; Harada et al., 1995). The development of the notochord in vertebrate embryos requires the action of the Brachyury gene (or T), as mutations in this gene in mouse (Herrmann et al., 1990) and in zebrafish (Schulte-Merker et al., 1994) block or reduce the development of the notochord. Besides its role in notochord development, in vertebrate embryos, T is also required for correct internalization and migration of posterior mesoderm during gastrulation (Wilson et al., 1995). Vertebrate embryos express T transiently around the blastopore in presumptive endoderm and mesoderm cells as they involute and migrate, and more persistently in the notochord (e.g., Wilkinson et al., 1995; Schulte-Merker et al., 1994). Similarly, in cephalochordates, expression of at least one of two genes homologous to T (Ambra-1 or -2) occurs in a ring around the blastopore during gastrulation, in the presomatic mesoderm, and in the notochord (Holland et al., 1995). In ascidian urochordates, the role of T in notochord development is conserved (Yasuo and Satoh, 1998). In contrast, though, expression of the ascidian T ortholog was found only in cells of notochord lineage and not broadly in involuting endoderm and mesoderm or in mesoderm lateral to the presumptive notochord (Yasuo and Satoh, 1993, 1994; Corbo et al., 1997). What was the original role of T in chordate development, and how was it recruited to its current roles in vertebrate embryos?

At least two hypotheses have been suggested to explain the difference in T expression between representatives of the cephalochordate-vertebrate clade and ascidians in its urochordate sister group. In one model, the primary ancestral function of the T gene in the chordates was in notochord specification (Yasuo and Satoh, 1993; Holland et al., 1995). This hypothesis implies that a function for T in gastrulation and paraxial mesoderm formation was acquired, presumably by the evolution of positive regulatory elements driving T expression, in the cephalochordate-vertebrate clade after its divergence from the urochordates. Alternatively, both functions of T (mesendoderm formation and notochord specification) may have been present in the ancestral chordate, and ascidians retained only the notochord function. Consistent with this proposal is the identification of cis-acting regulatory regions that suppress the
larvae. Early authors in the field of chordate evolution (e.g., Christen and Braconnot, 1998; Wada, 1998; Wada and Satoh, 1994). To test whether the notochord-restricted expression of T is a pattern that is generalized to the urochordate subphylum, and therefore to address the hypothesis that notochord specification is the ancestral function for T in the chordates, we cloned a T homolog from the larvacean Oikopleura dioica and analyzed its expression during embryogenesis.

In this first investigation of the molecular embryology of any larvacean, we found that during gastrulation of O. dioica embryos T is expressed in a pattern similar to that in ascidians: in cells of the notochord lineage but not in mesoderm with lateral fates. When formation and differentiation of the larvacean tail is nearly complete, however, T is employed in nonnotochordal expression domains, notably in regions of the forming gut and cells of the endodermal strand. This may reflect a deeper metazoan ancestry of this gene's function in the patterning of posterior endoderm and suggests that in the earliest chordate T was employed in both notochord and hindgut development.

**MATERIALS AND METHODS**

**Animal culture and embryo collection.** Adult and juvenile O. dioica were collected from the plankton in coastal waters near Coos Bay, Oregon, sorted into 12–14°C seawater in glass jars (2-liter), and fed until mature on a diet of natural phytoplankton and cultured algal strains (Dunaliella tertiolecta, Isochrysis galbana, Rhodomonas lens, Nanochloropsis sp., and Micromonas sp. (strain Dw-8)). Sperm was used immediately upon spawning or was collected by rupturing ripe males by pipet and stored undiluted at 12°C for up to 24 h. Females were transferred into small fingerbowls to spawn and eggs were collected for synchronous fertilization in 0.45-μm-filtered seawater. Embryos were kept at 12°C.

**Cloning and phylogenetic analysis.** mRNA extracted from pooled embryos ranging from two-cell to late tailbud stages was converted by Superscript reverse transcriptase (GIBCO/BRL) to cDNA. A larvacean T fragment from this cDNA was amplified with nested degenerate PCR primers corresponding to the following amino acid sequences: NEMIVTK and WYVEYNWE (sense) and VTAYQNEE and NPFAKAF (antisense), corresponding to nucleotide sequences 5'AA(T/C)GGA(A/G)ATGAT(T/C/A)GT(I/C)AC(I/C)AA-3', 5'TTGAAT(A/G)TGAT(T/C/A)GT(I/C)AC(I/C)AA-3', and 5'TGAC(A/G)TGGA-3'; 5'TTCTC(I/C)ATTG(T/C/A)GT(I/C)AC(I/C)AA-3', and 5'TTCTC(I/C)ATTG(T/C/A)GT(I/C)AC(I/C)AA-3'. Respective 3' RACE products of a full-length transcript were cloned into TA-Cloning vector pcRII (Invitrogen). The open-reading frame was identified by the criteria of Kozak (1996). The 3' fragment, from corresponding amino acid
position SVYMHP through the end of the 3’ UTR, was used to generate DIG-labeled riboprobes (Boehringer Mannheim DIG/ Genius RNA Labeling Kit).

Nucleotide sequences were aligned using MegAlign software (Version 3.08, DNASTAR) and trimmed to only unambiguously alignable regions. Gaps and any ambiguous adjacent regions were excluded. Bootstrapped phylogenetic trees of trimmed sequences were calculated by the neighbor-joining method (Saitou and Nei, 1987) using Clustal X software (http://www-igbmc.u-strasbg.fr/Bioinfo/ClustalX/Top.html) and NJPLOT (http://pbil.univ-lyon1.fr/software/njpplot.html) (Perrière and Gouy, 1996).

In situ hybridization. Embryos were fixed in 1 mM EGTA, 2 mM MgSO4, 0.1 M MOPS, 0.5 M NaCl, and 4% paraformaldehyde for 1 h at room temperature before being transferred to 70% EtOH for 1 to 3 h. Embryos were transferred to fresh 70% EtOH for storage at −20°C. Prior to hybridization, prehatch embryos were rehydrated in PBT and manually dechorionated. A whole-mount in situ hybridization protocol was adapted from Thisse et al. (1993) with the following modifications: embryos were hybridized in a mixture of 50% formamide, 5× SSC, 0.1 mg/ml heparin, 0.1% Tween 20, 5 mM EDTA, 1 mg/ml tRNA, and 1× Denhardt’s reagent at 65°C with probe. Hybridized embryos were washed for 10 min in 50% hybridization buffer/50% (2× SSC, 0.1% Tween 20) at 65°C; 10 min in 2× SSC/0.1% Tween 20 at 65°C; 30 min in 0.2× SSC/0.1% Tween 20 at 65°C; 30 min in 0.1× SSC/0.1% Tween 20 at 65°C; 5 min in 50% (0.1× SSC/0.1% Tween 20/50% PBT) at room temperature. Embryos were incubated for at least 60 min at room temperature in a mixture of PBT, 2.5 mg/ml BSA, and 2% goat serum. Then embryos were incubated for 2 h at room temperature with presorbed alkaline-phosphatase-coupled antidigoxigenin antibody (Boehringer Mannheim) and treated to the standard detection described by Boehringer Mannheim. Embryos were mounted in 40% glycerol with 0.6 μg/ml DAPI. Embryos and hatchlings hybridized with a labeled sense riboprobe showed no staining at any stage (not shown).

RESULTS

Phylogenetic Relationships of OdiT

We cloned a fragment of T (Brachyury) from O. dioica using degenerate primers designed from aligned protein sequences of chordate homologs. Sequencing this fragment provided information to design primers for the amplification of overlapping 3’ and 5’ fragments of a single gene by RACE PCR. The sequencing of this gene (Fig. 2) showed that it constitutes the 3’ UTR and the entire coding region of a T homolog we named O. dioica T (OdiT). In a neighbor-joining (N-J) phylogeny of alignable T-box family protein sequences (173 amino acids), OdiT falls within a strongly supported clade (bootstrap = 999) including T proteins from vertebrates, cephalochordates, urochordates, a hemichordate, echinoderms, and a cnidian (Fig. 3A). Excluded from this clade were genes representing other T-box subfamilies, as well as a second ascidian T-box gene called As-T2 which is thought to be orthologous to vertebrate tbx6 (Mitani et al., 1999). Because the T-domain itself represents nearly all of the alignable sequence and provides a limited number of informative sites, the topology of the gene phylogeny does not conform well to taxonomic relationships. In a N-J phylogeny based on 519 unambiguously alignable nucleotide positions and rooted with sea urchin T, the tree topology better reflects expected taxonomic groupings. OdiT groups strongly with its homologs in two ascidians (Fig. 3B) (for sequence alignments, see http://www.neuro.uoregon.edu/postle/mydoc.html).

Expression of OdiT from Cleaving Embryos to Hatchlings

We studied the expression of OdiT in a developmental series of O. dioica embryos. The 3’ RACE product contains 613 bp of predicted coding sequence and 374 bp of 3’ UTR; this ~1-kb fragment was used to make labeled riboprobes for whole-mount in situ hybridization (Fig. 2). Blastomeres in O. dioica embryos do not cleave synchronously after the 16-cell stage, so cell numbers were ascertained by counting DAPI-stained nuclei in early embryos. OdiT expression was first detected in a bilateral pair of blastomeres in 30-cell embryos (Figs. 4A and 4B). With the fifth cleavage (producing 30 cells), some blastomeres have already begun to overgrow their neighbors by epibolic divisions, marking the onset of gastrulation (Delsman, 1910). The two OdiT-expressing blastomeres appear at least partly submerged beneath superficial cells, and hence, gastrulation begins in Oikopleura before OdiT expression commences. These two blastomeres are the only cells expressing OdiT until after the 44-cell stage, the end of gastrulation; they can be seen in the 44-cell embryo entirely submerged beneath the closed blastopore whose perimeter consists of 6 cells arranged as petals of a flower (Figs. 4C and 4D). At the neurula stage, when the ball-shaped embryo flattens slightly (Delsman, 1910), two bilateral pairs of blastomeres are labeled by the probe (Fig. 4E). Up to this stage, expressing blastomeres were separated from one another by two intervening midline cells. Bilateral bands of expressing cells then become contiguous across the midline and form a chevron (Fig. 4F), beginning the convergence and extension of the notochord.

The tail of O. dioica has 20 muscle cells arranged in two rows of 10 cells each on either side of the 20 cells of the notochord. Just prior to extension of the notochordal rod, 19 labeled cells are lined in a staggered row at early tailbud stage (Fig. 5A). Muscle cell precursors adjacent to notochord precursors are not labeled by the OdiT probe (Figs. 5B and 5C). At hatching, 19 notochordal cells are linearly arranged (Fig. 5D) and a cell adjacent to the posteriormost cell in the row is also detected by the probe (Fig. 5E). We interpret this as notochord cell 20 which has not yet intercalated into the notochordal axis.

In larvaceans, the tail is twisted 90° relative to the trunk, dorsal to the left, ventral to the right. In hatchlings whose tails have just straightened, two T-expressing cells lie adjacent and to the right of the notochord (Fig. 5F). Their position and shape suggest these are the subchordal cells, products of the caudal endoderm, which migrate back into the tail after retracting into the trunk with rest of the endodermal strand (Delsman, 1912).
In hatchlings 6 h postfertilization, as organogenesis is being completed in the trunk, OdiT signal rapidly decreases in the notochordal cells and new expression domains appear in dorsal and ventral cells of the posterior trunk. Initially, a flattened cell immediately rostral and ventral to the notochord and underlying the ectoderm is labeled by the probe (Fig. 6A), as are a series of cells at a posterio-lateral position in the trunk and lying just left of the midline under the epidermis (Figs. 5F and 6A); based on their positions, these cells may be of the developing hindgut and esophagus, respectively. In the latest stage hatchlings we examined ("Stage II"; Galt and Feniaux, 1990) the ventral expression domain was a smooth-bordered oblong lying against the epidermis and surrounded by the expanding body lumen (Fig. 6B). When fully developed, a sausage-shaped rectum lies in this position.

**DISCUSSION**

In order to explore the evolutionary origins of developmental innovations in the phylum Chordata, we have begun to investigate the molecular embryology of O. dioica. This organism occupies an important position in chordate phylogeny, and larvacean anatomy suggests that it may facilitate detection of homologies between urochordates and vertebrates on the basis of both morphological and molecular criteria. To investigate the ancestral function of a gene important in the development of a novel chordate character, the notochord, we have cloned the larvacean ortholog (OdiT) of the vertebrate T gene and analyzed its expression during embryogenesis. We isolated the complete coding region of the OdiT message as well as the entire 3' UTR. Gene phylogenies established the orthol-
ogy of OdiT to the T (Brachyury) subfamily of T-box genes by analysis of both predicted amino acid sequence and nucleotide sequence (Fig. 3). In a phylogeny of T-domain sequences from a broad range of T-box proteins, OdiT groups unambiguously within the T clade. And in a phylogeny of alignable DNA sequences using a sea urchin T as the outgroup, OdiT falls within the chordate sequences and groups strongly with the ascidian Ts. Orthologs are genes in two species that can trace their ancestry back to a single gene in the last common ancestor of those species (Fitch, 1983).

FIG. 3. Gene phylogenies indicate OdiT is a Brachyury ortholog. (A) Neighbor-joining (N-J) tree of alignable tbx family protein sequence. Included in the tree are T homologs from a broad phylogenetic range as well as representative members of the other tbx subfamilies (Wattler et al., 1998). (B) N-J tree of alignable nucleotide sequence from chordate T genes, with two deuterostome outgroups, a hemichordate and an echinoderm. Bootstrap values greater than 50% are shown. GenBank accession numbers are X91903 (Ambra-1), P80492 (Ambra-2), AB018527 (Ap-Bra), D16441 (As-T), D83265 (As-T2), AF123247 (Ciona Bra), AB001939 (Cynops brachyury), U67086 (Ch-T), U67087 (Ch-TbxT), D50332 (HptA), AF105065 (HyBra), AB001871 (Me-Tam), Q07998 (ntl), AF204208 (OdiT), AB004912 (PfBra), NM_003181 (Homo T), XM_36831 (Mus T), U57331 (tbx6), NM_005149 (TBX19), P55965, S74163 (byn), and M 77243 (Xbra). For alignments, see http://www.neuro.uoregon.edu/postle/mydoc.html.
We conclude that OdiT is a true T ortholog and not a different member of the T-box gene family.

In cephalochordates and vertebrates, T is expressed in presomitic mesoderm, in the notochord, and in involuting mesodermal and endodermal cells surrounding the closing blastopore (or equivalent) (e.g., Wilkinson et al., 1990; Schulte-Merker et al., 1992). In previously investigated basal chordates (ascidians), T is expressed in cells that have become segregated during cleavage to a purely notochordal lineage, but not in mesoderm lateral to the presumptive notochord or the nearby endoderm (Yasuo and Satoh, 1993, 1994; Corbo et al., 1997). In light of this result in ascidian embryos, it has been proposed that the ancestral role for T in chordates was in notochord specification (Yasuo and Satoh, 1994). If the notochord-only hypothesis is true, then the nonnotochordal expression domains in cephalochordate and vertebrate embryos represent functions of the T gene that were acquired after the evolutionary split between the urochordates and the cephalochordate–vertebrate lineage. Alternatively, the expression pattern in the cephalochordate–vertebrate clade—in the notochord, surrounding mesoderm, and endoderm—could have been the ancestral pattern, and ascidian embryos might have lost T function in endoderm and paraxial mesoderm formation.

Peterson et al. (1999) favor the notochord-plus-surrounding-cells hypothesis and propose a parsimonious model for the evolution of T function based on gene expression in arthropods and the deuterostome phyla. According to this model, prior to the protostome/deuterostome split, T served a function in posterior gut development. Kusch and Reuter (1999) additionally propose that T had an ancestral role in mesoderm development in the last common ancestor of protostomes and deuterostomes. The chordate lineage acquired notochord expression after divergence from the echinoderm-hemichordate clade. Although vertebrates and cephalochordates express T in posterior endoderm and paraxial mesoderm early in development, neither of these proposed ancestral expression domains has been demonstrated in the normal development of ascidians. To evaluate these alternatives, we examined T expression in a basally diverging urochordate. Recent molecular phylogenetic analyses have placed the class Appendicularia at a basal node within the urochordate clade (e.g., Wada, 1998). This contradicts the prevailing hypothesis

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that larvaceans arose by paedomorphosis from the thaliaceans (Garstang, 1928) and are the most derived of the urochordates. Instead, the molecular data support the work of morphologists who argued that the motile body plan of the adult larvacean is a plesiomorphy of Chordata (e.g., Lankester, 1882; Lohmann, 1933). If the notochord-restricted expression pattern of As-T is the ancestral condition for Urochordata, the larvacean homolog is predicted to be expressed only in notochord lineage cells. If the ascidian pattern is an apomorphy of the ascidian clade and larvaceans are basal within the urochordates, the larvacean homolog might share an expression pattern with the out-group, the cephalochordate-vertebrate clade. In other words, OdiT might be expressed in endoderm and nonnotochordal mesoderm.

**Early Expression of Larvacean T Is Restricted to the Developing Notochord**

Expression of OdiT was detected neither in endodermal cells while these were internalized by epiboly during gastrulation nor in paraxial mesoderm during tail formation.
Early OdiT expression appeared to be restricted to cells of notochordal fate, as identified by comparison with Delsman’s fatemap (Delsman, 1910) and as deduced by the continuity of T expression in a developmental series that included hatchlings with differentiated notochords. We interpret expression in a single cell adjacent to the end of the 19 cells lined up in the notochord as a 20th late interdigitating notochord cell. This would agree with ascidian development where interdigitation of notochord cells is not synchronous (Cloney, 1964) and notochord elongation is completed in the rostral end earlier than in the caudal end of the extending tail (Miyamoto and Crowther, 1985; Corbo et al., 1997). This cell, however, remains distinctive; Galt (1972) noted a 20th cell in O. dioica that is histologically notochord-like but it remains physically separate from the rest of the notochord.

Our results support some of the observations made by Delsman in O. dioica embryos and his predictions of cell fate which he based on comparison with ascidian embryos (Delsman, 1910). There is no modern cell-lineage map for this or any larvacean. The first pair of cells expressing OdiT, however, are in the position of A21/A21, left and right side progeny of blastomeres A2/A2, which Delsman (1910) predicted would give rise to the notochord (Figs. 4A and 4B). At early neurula stage, 4 cells are detected by the probe (Fig. 3E). Two are likely to be the original A21 pair, while the second pair may be progeny of B21/B21. The B21 pair are proposed to correspond approximately to ascidian B6.2/B6.2 (Delsman, 1910; Galt and Fenaux, 1990). In ascidians, progeny of these B-line (posterior, vegetal) blastomeres express As-T a cleavage later than do A-line (anterior, vegetal) notochord precursors (Yasuo and Satoh, 1994) and produce the posteriormost notochord cells (Nishida, 1987; Satoh, 1994).

While the fatemaps of larvaceans and ascidians are likely to share similarities, the two classes of urochordates differ significantly in timing and pace of gastrulation. Delsman observed that the gastrulation of O. dioica is precocious and succinct; O. dioica begins gastrulating with the 5th cleavage (during the transition from 16 to 30 cells) and completes it by the end of the 6th wave of cleavage resulting in 44 cells (Delsman, 1910; Galt and Fenaux, 1990; Figs. 4A–4D). OdiT expression persists into late organogenesis in the trunk, after expression is no longer detected in the notochord. (A) Larva, 1 h after hatching, lateral view. Black and clear arrows indicate ventral and dorsal trunk expression domains, respectively. (B) Larva 2 h after hatching. Ventral expression in developing rectum (r). L, trunk lumen. Scale bar represents 20 μm in (A) and 50 μm in (B).
expression is first detected at the 30-cell stage, the early gastrula (Figs. 4A and 4B). As-T expression, in contrast, is first detected in 64-cell ascidian embryos at least one cleavage before the onset of gastrulation which begins after 110 cells (Yasuo and Satoh, 1994). The onset of T expression in these urochordates is apparently independent of the timing of gastrulation. In ascidians, As-T is not detected in blastomeres until they become segregated to a purely notochordal fate (Yasuo and Satoh, 1994). Expression of T in larvaceans is probably also constrained by the timing of segregation of cell fates with the result that, while it is detected at an earlier cleavage stage than in ascidians, it first appears during, rather than before, gastrulation.

While these results, taken alone, do not contradict the hypothesis that T’s role in the ancestral chordate was in notochord specification, rather than in broader mesodermal domains, OdiT expression in endoderm cells (discussed below) supports the hypothesis that T also played a function in endoderm development in the chordate ancestor.

Larvacean T Is Expressed in Posterior Endoderm

Most oikopleurids have in the tail a characteristic cell type that varies in number between species, O. dioica having two such “subchordal cells” (Lohmann, 1933). The subchordal cells are named for their position ventral to the notochord; these cells appear on the animal’s right because the tail is twisted 90° relative to the trunk, and thus, caudal right corresponds to ventral. Although their function is not understood, the subchordal cells may have a secretory role (Fredriksen and Olsson, 1991), and Delsman (1912) described them as a product of the caudal endoderm, the endodermal strand. This one-cell wide strand runs ventral to the notochord in a position similar to vertebrate hypochord cells (Kimmel et al., 1995) which express many of the same genes expressed in notochord (e.g., Yan et al., 1995). As the caudal endoderm retracts into the trunk during extension of the tail, the presumptive subchordal cells detach themselves from the endodermal strand and migrate back into the tail (Delsman, 1912). Shortly after hatching, two cells to the right of the notochord are detected with the OdiT probe (Fig. 5F). Their position relative to the notochord, their elongate shape, and the fact that they are seen sometimes at the base of the tail or sometimes further caudal suggest they are the two migrating subchordal cells later found in the tailfin of adult O. dioica. The putative subchordal cells apparently express T as they migrate. What could be the role of T in these cells? In mouse, T is necessary for migration of cells through the primitive streak (Wilson et al., 1995), and the authors propose that genes downstream of T mediate cell-surface changes necessary for migration of cells away from the midline after ingestion. Similarly, in fly embryos mutant for the T homolog, byn (brachyenteron), caudal visceral mesodermal cells are retarded in their ability to migrate, a deficiency the authors attribute to a defect in the cells’ adhesive properties (Kusch and Reuter, 1999). The cells of the endodermal strand, including subchordal cells, are the only cells described as migratory in larvacean development (Delsman, 1912), and T may function to alter adhesion properties for the segregation and migration of these subchordal cells from the endodermal strand.

Expression of OdiT after hatching further supports the hypothesis that T’s function originally included a role in patterning posterior endoderm. O. dioica hatches at 6 h after fertilization (at 12°C) while organogenesis is still in progress in the trunk and there are no open lumens in the gut or body cavity (Galt and Fenaux, 1990). Shortly after hatching, new expression of OdiT appears in the trunk while detectable transcript rapidly disappears from the notochord. The elongate cluster of expressing cells just rostral to the notochord is likely the developing hindgut. In the latest stage of the hatchlings we examined, the borders of this expression domain had smoothed to a sausage shape apposed to the epidermis where Delsman (1912) shows that the anus forms (Fig. 6B). The identity of an expressing row of cells in the posteriodorsal trunk is less certain. Given its position in the dorsal trunk slightly left of the midline, we interpret it as the developing esophagus which curves left to feed dorsally into the left half of the two-lobed stomach. Alternatively, these posteriodorsal cells may be of the developing left stomach lobe itself which later is distinguished from the right lobe by a row of gland cells extending along the left wall to the esophagus (Fenaux, 1998).

Expression of OdiT in the larvacean hindgut provides an interesting parallel to the expression of T in an enteropneust hemichordate, Ptychodera flava. PfBra (a T homolog in P. flava) is expressed in the anal region of the developing larval hindgut and in the intestine of the metamorphic juvenile (Tagawa et al., 1998; Peterson et al., 1999). The hemichordate expression as well as similar expression of T in an embryonic seastar, however, appears to be in structures derived from invaginating ectoderm, rather than in the endodermally derived portion of the gut (Peterson et al., 1999; Shoguchi et al., 1999). The expression of T in the hindgut of larvaceans and hemichordates, therefore, can not be homologized without violating the distinction between two embryonic germ layers. Peterson et al. (1999) infer a broader homology of T expression in posterior gut between the deuterostome phyla and a protostome outgroup (Arthropoda). In their model for evolution of T utilization, the ancestral role for T in bilaterians was to help form the posterior gut. In arthropods, the apparent T ortholog, byn, is expressed in the developing hindgut (Kispert et al., 1994). Kispert et al. (1994) note that it is somewhat arbitrary to define this part of the arthropod gut as ectodermal because this label is based on epithelial properties shared with epidermis rather than on the hindgut’s internalized position after gastrulation. If one regards hindgut development in these bilaterians as homologous, the larvacean pattern can be reconciled with that described in outgroups to the chordates (hemichordates, seastars, and insects). Therefore, our data support the model that T had a role in posterior gut
formation in the protostome/deuterostome ancestor and that this role has been conserved in larvacean urochordates. An apparent absence of T expression in vertebrate and cephalochordate hindgut development might represent a shared evolutionary loss of late expression in posterior endoderm in this lineage. The absence of reported T expression in the ascidian hindgut might also represent an evolutionary loss. Alternatively, there might have been a heterochronous shift in T expression to a later developmental stage than has yet been experimentally examined in ascidians. This explanation predicts that T expression will resume at ascidian metamorphosis when the endoderm is patterned to make the juvenile gut. Finally, in the urochordate lineage, absence of early expression of T during gastrulation in presumptive endoderm and mesoderm might be a loss correlated with the mosaic nature of urochordate early development or with a change in the mechanics of gastrulation due to small cell number.

This is to our knowledge the first publication on the molecular embryology of any member of the class Appendicularia. Larvaceans have been largely ignored, in part because of the difficulties in culturing them, but also because of the dominance over the last 7 decades of Garstang's hypotheses concerning the evolution of the chordates. Garstang (1928) proposed that the cephalochordate-vertebrate lineage and the larvaceans arose by separate events of paedomorphosis; both lineages independently evolved from motile larvae of different sessile chordate ancestors. Ascidians are the most primitive of the urochordates, he argued, while larvaceans are the most derived, having sprung from a larval stage in the thaliacean lineage. Because molecular data suggest that larvaceans diverged basally in the urochordate clade (e.g., Wada, 1998), and because some aspects of ascidian molecular embryology may not be generalizable to all of Urochordata, investigation of developmental mechanisms in larvacan urochordates could be vital to our understanding of the common ancestor of the chordates.

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