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A comparative molecular approach to mesodermal patterning in basal deuterostomes: the expression pattern of *Brachyury* in the enteropneust hemichordate *Ptychodera flava*

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SUMMARY

This work concerns the formation of mesoderm in the development of an enteropneust hemichordate, *Ptychodera flava,* and the expression of the *Brachyury* gene during this process. *Brachyury* expression occurs in two distinct phases. In the embryo, *Brachyury* is transcribed during gastrulation in the future oral and anal regions of the gut, but transcripts are no longer detected by 2 weeks of development. *Brachyury* expression is not detected during the 5 months of larval planktonic existence. During this time, the adult coeloms begin to develop, originating as coalescences of cells that appear to delaminate from the wall of the gut. *Brachyury* expression cannot be detected again until metamorphosis, when transcripts appear in the mesoderm of the adult proboscis, collar and the very posterior region of the trunk. It is also expressed in the posterior end of the gut. At no time is *Brachyury* expressed in the stomochord, the putative homologue of the chordate notochord. These observations illuminate the process of maximal indirect development in *Ptychodera* and, by comparison with patterns of *Brachyury* expression in the indirect development of echinoderms, their sister group, they reveal the evolutionary history of *Brachyury* utilization in deuterostomes.

Key words: *Brachyury*, Hemichordate, Evolution, Mesoderm, Notochord, *Ptychodera flava*

INTRODUCTION

Most animals are classified as bilaterians and, in addition to the obvious change in symmetry properties, a hallmark of the evolutionary process that led to the bilaterians was the appearance of mesodermal structures. Mesoderm is integral to the construction of all modern bilaterian body plans. In the process of development, mesoderm is a frequent source of inductive signals; for example, in vertebrates the notochord is involved in patterning all three body axes (Danos and Yost, 1996) and also induces the development of other organs (e.g., pancreas; Kim et al., 1997). Mesodermal derivatives also constitute major components of the bilaterian body plan (Ruppert and Barnes, 1994). They define the grade of body plan organization, i.e., whether the animal is coelomate, pseudocoelomate or acoelomate. Furthermore, cell types, such as blood cells and muscle cells, and organs, such as nephridia, which are not found in sponges and cnidarians, arise from mesoderm. Finally, mesoderm conveys a third dimension to the animal's body plan with the attendant requirements for internal organization; animals that lack mesoderm are effectively 'two-dimensional' such that almost every cell is in direct contact with the ambient sea water.

We have begun a series of investigations on the origin and specification of mesoderm in basal deuterostomes. The deuterostomes comprise three monophyletic phyla: the echinoderms, the chordates and a third, lesser-known group, the hemichordates. Hemichordates consist of two subgroups, pterobranchs and acorn worms or enteropneusts. Recent phylogenetic investigations using 18S rDNA (e.g., Turbeville et al., 1994; Wada and Satoh, 1994; Eernisse, 1997) and mitochondrial codon usage (Castresana et al., 1998) suggest that, contrary to previous morphological analyses (e.g. Peterson, 1995), echinoderms and hemichordates are each other's closest relative (i.e., they are sister taxa).

Both enteropneusts and echinoderms undergo what we have termed 'maximal indirect development' (Davidson et al., 1995; Peterson et al., 1997; Cameron et al., 1998) whereby an elegant but simple larval stage precedes the development of the adult body plan. This is in contrast to the developmental mode found in chordates. Chordates are all direct developers and thus establish the adult body plan immediately without any intervening primary larval stage (see also Nielsen, 1998). The adult body plan of maximal indirect developers arises from groups of cells that are 'set-aside' from participation in the

construction of the embryo/larva. In enteropneusts and echinoderms, endomesodermal set-aside cells produce the tripartite coeloms from which the mesodermal components of the adult body plan arise. These are the anterior coelom or protocoel, paired middle coeloms or mesocoels and paired posterior coeloms or metacoels; these coeloms are called the axocoels (paired in echinoderms), hydrocoels and somatocoels, respectively, in echinoderms. The remarkable similarity in structure of hemichordate and echinoderm larval coeloms (Peterson et al., 1997) is an obvious and significant synapomorphy of these groups.

Brachyury, the canonical member of the 'T-box' family of transcription factors, is intimately tied to mesoderm specification in vertebrates (recent reviews include Herrmann and Kispert, 1994; Herrmann, 1995; Kavka and Green, 1997; Papaioannou, 1997; Smith, 1997; Papaioannou and Silver, 1998). The pattern of *Brachyury* gene expression is highly conserved amongst vertebrates. *Brachyury* is initially expressed pan-mesodermally, but is downregulated in paraxial mesoderm cells as they move through the primitive streak or equivalent. As development continues, *Brachyury* becomes restricted to the notochord, and the posterior mesoderm and gut. Several lines of evidence suggest that *Brachyury* is necessary for the proper specification of the notochord and posterior mesoderm. (1) The *Brachyury* mutation in mouse (*T*; Herrmann et al., 1990) and in zebrafish (*ntl*; Schulte-Merker et al., 1994) results in the absence or reduction of posterior mesoderm and notochord, the same tissues that express the *Brachyury* message and protein (Wilkinson et al., 1990; Schulte-Merker et al., 1992; Kispert and Herrmann, 1994). Correspondingly, the phenotype of *Brachyury* mutations in mouse can be rescued by a transgene of the wild-type *T* allele (Stott et al., 1993). (2) Studies by Kispert et al. (1995) and Conlon et al. (1996) have shown that *Brachyury* functions as a transcriptional activator. Not only do several *ntl* mutations lack this activation domain, but replacing the activation domain of *Xbra* (the *Xenopus Brachyury* orthologue) with the *engrailed* repressor domain results in phenotypes similar to the naturally occurring mutants. A normal function of *Brachyury* during vertebrate gastrulation is apparently to activate transcription of mesoderm-specific genes (Conlon et al., 1996). (3) Expression of *Brachyury* occurs as a result of mesoderm induction (Smith et al., 1991; Ding et al., 1998), possibly as an immediate-early response (Smith et al., 1991; Tadano et al., 1993; Knezevic et al., 1997). (4) Injecting *Brachyury* protein into animal caps induces mesodermal cell types (Cunliffe and Smith, 1992) in a concentration-dependent manner (O'Reilly et al., 1995). (5) *Cis*-regulatory elements in the promoter region of the *Brachyury* gene have been identified that drive reporter expression in the posterior mesoderm of mouse (Clements et al., 1996) and frog (Latinkić et al., 1997) and in the notochord of ascidians (Corbo et al., 1997).

Brachyury orthologues were earlier cloned from both sea urchins (Harada et al., 1995) and hemichordates (see Tagawa et al., 1998a for sequence and phylogenetic analysis). However, these studies were focused solely on *Brachyury* expression during embryogenesis, and nothing whatsoever was known about expression of this gene in the postembryonic processes by which the mesoderm of the respective adult body plans arises from the mesodermal set-aside cells of the embryo. We have now determined the pattern of *Brachyury* expression in the adult rudiment of a sea urchin larva (K. J. P., unpublished data) and in the larval and metamorphic stages of *Ptychodera flav*a, as described below. Furthermore, very little is known about the formation of the adult mesoderm in indirectly developing enteropneusts; prior observations are limited primarily to Morgan (1891, 1894), and no modern paper has dealt with the ontogeny of the adult enteropneust coeloms (Hadfield, 1975; see Fig. 1 for the description of these structures). Thus our goals are twofold: First, to ascertain the developmental origin of the adult coeloms of *P. flava* and, second, to examine the expression pattern of *Brachyury* throughout its process of maximal indirect development, i.e., from the early tornaria larva through metamorphosis and establishment of the adult body plan.

MATERIALS AND METHODS

Collection of larvae and culture of embryos and larvae

Adult gravid worms were collected near Diamond Head, Honolulu, Hawaii and shipped overnight to Caltech's Kerckhoff Marine Laboratory (Corona del Mar, CA). Ripe animals placed at room temperature in glass dishes with sand spawned naturally within 24 hours of arrival. Eggs were fertilized, washed twice in sea water and allowed to develop for several days at 25°C. Larvae were transferred to eight-liter containers at a concentration of 1 per ml sea water with constant, slow stirring. Initially, the larvae were fed a mixture of algae consisting of *Dunaliella tertiolecta*, *Pavlova* sp. and *Phaeodactylum tricornutum* (Strathmann and Bonar, 1976) at about 3000 cells per ml sea water. However, after about 1 month of development, the animals appeared to have stopped growing. We then replaced the previous algal mixture with *Rhodomonas lens* at 3000 cells per ml sea water and renewed growth of the larvae was immediately apparent. The water was changed once a week and the animals fed once or twice a week depending on clearance rate. The larvae were cultured in this fashion for the next 6 months.

Competent larvae were obtained following the methods of Hadfield (1978). Plankton tows were conducted off Kewalo Marine Laboratory, University of Hawaii, Honolulu, at about 10 m depth using a net with a mesh size of 200 μm. Plankton was brought into the laboratory and competent *P. flava* larvae, which are easily recognized by the development of the proboscis and the large anal region posterior of the telotroch (see Fig. 5A), were isolated from other plankton under a dissecting scope. Most of the larvae collected could be confidently attributed to *P. flava* based on the developmental time table (Hadfield, 1975, 1978) and the existence of at most only two other species of enteropneust in the area (Edmondson, 1946). We did collect two specimens of another type of enteropneust which did not hybridize with the *PfBra* probe (data not shown). Competent *P. flava* larvae that were not fixed immediately (see below) were placed in a plastic Petri dish with a monolayer of freshly collected sand. By 24 hours, the animals had completed metamorphosis to the extent that a collar-trunk distinction was apparent (see Fig. 5C). The water was changed daily and the worms were fixed on 24 hour intervals for 3 days.

DNA probe isolation

A 370 base pair (bp) fragment of the *PfBra* cDNA (Tagawa et al., 1998a), which lies between a *Sac*I site (+352 from the start codon) and a *Mfe*I site (+722), was isolated and cloned into pBS II SK+ (Stratagene) that had been double digested with *Sac*I and *Eco*RI. An antisense riboprobe was made by linearizing the construct at the 5′ end with *Sac*I, removing the 3′ overhang with T4 DNA polymerase and synthesizing the riboprobe with T7 following instructions of the manufacturer (Boehringer Mannheim DIG RNA labeling kit). The sense riboprobe was made after linearization at the 3′ end of the construct with *Kpn*I.

A cytoplasmic actin coding probe previously isolated from *P. flava* (M. Kobayashi and N. S., unpublished data) was used as a positive control. Approximately 1.2 kb of the actin gene had been cloned into

pBS II SK+. The template was prepared by excision of a ~1 kb *Sma*I-*Eco*RV fragment and religation of the vector with the remaining 240 bp of the actin fragment. The antisense riboprobe was synthesized after linearization at the 5′ end of the insert with *Bam*HI.

Genomic Southern blot analysis

High molecular weight sperm DNA simultaneously collected from several individuals was digested exhaustively in separate reaction mixtures with *Eco*RI, *Eco*RV, *Hin*dIII, *Spe*I or *Xba*I. 15 µg of digested DNA were resolved by agarose gel electrophoresis, depurinated for 30 minutes in 0.2 N HCl, denatured 2× 15 minutes in 1.5 M NaCl, 0.5 M NaOH, and transferred to Hybond N+ nylon membrane (Amersham) using a TURBOBLOTTER (Schleicher & Schuell) with 0.4 M NaOH. The 370 bp *Sac*I-*Mfe*I fragment was random labeled with [^{32}P]dCTP (Pharmacia). Hybridization was done at 60°C overnight with the buffer of Church and Gilbert (1984) and washed under high stringency conditions (2× SSC, 0.2% SDS 1× 30 minutes at 60°C; 0.2× SSC, 0.2% SDS 2× 30 minutes at 60°C). The blot was exposed for about 8 hours.

To determine if the two bands in the *Eco*RI, *Eco*RV and *Hin*dIII lanes are due to allelic variation or to the presence of the respective sites in an intron (intron 3 of Wattler et al., 1998), PCR primers were designed to amplify the cDNA probe region in an isolated lambda clone containing the *PfBra* gene (K. J. P., unpublished data): forward primer: 5′-GTCTATATCCACCCAGAC-3′; reverse primer: 5′-GTACATCGTG-AGCATGGG-3′. A 2 kb fragment was amplified using standard PCR parameters and digested with *Eco*RI, *Eco*RV, or *Hin*dIII. Both the *Eco*RV and *Hin*dIII digests gave two bands; the *Eco*RI digest did not.

Fixation and in situ hybridization

Larvae and embryos were fixed in 2.5% glutaraldehyde, 0.24 M NaCl, 0.1 M PO_4 buffer, pH 7.4, for 4 hours on ice; washed in 0.4 M NaCl, 0.1 M PO_4 buffer, pH 7.4, 2× 5 minutes, and dehydrated to 70% EtOH. The field-collected competent larvae and juveniles were cleared in xylene. In situ hybridization followed the protocol of Holland et al. (1996) with the following changes: (1) embryos and larvae were digested in 7.5 µg/ml proteinase K for 5 minutes, competent larvae, 24- and 48-hour juveniles were digested for 20 minutes, 72-hour juveniles for 25 minutes; (2) anti-digoxigenin antibody was diluted to 1:5000 and preabsorbed with *Ptychodera flava* adult powder (preabsorption is necessary because the anti-digoxigenin antibody crossreacts with a muscle-specific epitope even in the absence of a riboprobe); (3) after removal of the antibody and washing in NaPBST 4× 20 minutes, the animals were washed twice in AP buffer, pH 8.0, before the AP pH 9.5 wash (see Ransick et al., 1993); (4) the staining solution consisted of 4.5 µl of 75 mg/ml NBT, 3.5 µl 50 mg/ml BCIP and 1 µl 1 M levamisole in 1 ml AP pH 9.5 buffer. Staining was apparent after 1 hour with competent larvae and juveniles, overnight with embryos and 1-week larvae. The reaction was stopped with 1 mM EDTA in NaPBST.

Mounting, sectioning and photography

Specimens were photographed using a ProgRes 3012 high-resolution digital color camera (Kontron Elektronik, GmbH) mounted on either a Zeiss Axioskop microscope equipped with DIC optics or a Zeiss dissecting microscope. To facilitate photography of live larvae, the animals were anesthetized in 10 mM sodium azide in sea water. Whole-mount in situ specimens were cleared in a graded series of glycerol solutions to 50% and then photographed. Competent larvae and juveniles were rehydrated and then dehydrated in a graded series of ethanol solutions to 100%. They were then transferred to Histosol (National Diagnostics) and embedded in paraffin wax. 15 µm sections were cut, mounted in immersion oil and photographed using a digital camera. Images were recorded using Wincam v.1.4 (Roche, Charlotte) and processed using Adobe Photoshop v.4.0.

RESULTS

PfBra is a single-copy gene

Because we are examining a discontinuous pattern of expression (see below), it was important to determine if *Brachyury* is a single-copy gene in *P. flava*. Using a genomic

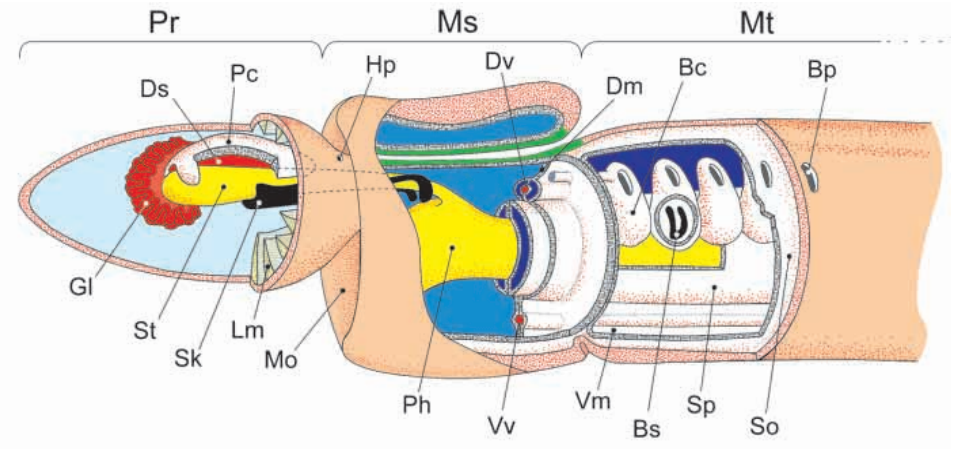

Fig. 1. Generalized anatomy of an enteropneust hemichordate. The body of an enteropneust is divided into three regions. Each body cavity or coelom, shaded in blue, is lined by mesoderm (So, somatopleura or outer lining; Sp, splanchnopleura or inner lining). The most anterior region is the proboscis or protosome (Pr) whose coelomic cavity, the protocoel, is shown in light blue. Immediately behind the proboscis is the collar or mesosome (Ms), which has a pair of cavities called the mesocoels (shown in medium blue). The rest of the body consists of the trunk or metasome (Mt) whose coelomic cavities are called the metacoels (dark blue). To see the coelomic spaces, the longitudinal musculature (Lm) of the proboscis, and the dorsal (Dm) and ventral mesenteries (Vm) of the collar are cut-away; the dorsal mesentery of the trunk is not shown. The outer ectoderm is shown in beige, the gut in yellow, the dorsal hollow nerve cord (or collar cord) in green, and elements of the circulatory and excretory systems are in red. The stomochord (St), the putative homologue of the chordate notochord, is the anterior projection of the dorsal wall of the pharynx (Ph). The stomochord is an integral component of the axial complex, the head kidney or metanephridium of the adult worm (Balser and Ruppert, 1990). It serves as a supporting element for the pericardium (Pc) whose contractions drive blood from the dorsal sinus (Ds), a continuation of the dorsal vessel (Dv). From the dorsal sinus, the blood enters the glomerulus (Gl) where it is filtered. The urine thus formed is released into the protocoel and then, after modification, it exits the body via the hydropore (Hp). Blood returns to the body through the ventral blood vessel (Vv). The stomochord and its underlying skeleton (Sk, shown in black), a thickened basement membrane, also serve to strengthen the connection between the proboscis and collar. Also of phylogenetic import are the U-shaped branchial slits (Bs) which show considerable similarity to the pharyngeal slits of chordates (Pardos and Benito, 1988; Nielsen, 1995). Water enters the gut through the mouth (Mo), and is able to pass from the gut through the U-shaped slits into the branchial sacs (Bc) and exit via the branchial pores (Bp). Note that for simplicity many structures are not indicated including most of the nervous system, circulatory system, and musculature. Redrawn from Balser and Ruppert (1990) and Benito and Pardos (1997).

Southern blot probed at high stringency with the same cDNA sequence as used for the whole-mount in situ hybridization (WMISH), we determined that, of five enzymes examined, two produced single fragments and three produced two fragments that reacted with the *Brachyury* probe (Fig. 2). Restriction digests of the probed region from a genomic lambda clone (see Materials and Methods) showed that the two bands in the *Eco*RV and *Hin*dIII lanes are due to the presence of the respective restriction sites within the probed intron (data not shown), whereas the two bands in the *Eco*RI lane are due to the heterozygous occurrence of restriction sites outside the probed region. These data indicate that the *Ptychodera flava Brachyury* gene (*PfBra*) occurs in a single copy per haploid genome. Therefore, the discontinuous expression pattern discussed below is not due to the differential regulation of two paralogues.

Early development and embryonic spatial expression pattern of *PfBra*

Both the early embryology of *P. flava* (Rao, 1954; Hadfield, 1975; Tagawa et al., 1998b) and the embryonic spatial expression pattern of *PfBra* (Tagawa et al., 1998a) have been discussed elsewhere. The salient points are as follows. By 24 hours of development, the embryo is in the process of gastrulating and an outpocketing of mesoderm from the tip of the archenteron is now readily apparent (Fig. 3A). This mesoderm with its enclosed cavity, the protocoel, will initially form part of the larval protonephridium (Fig. 3C), and later the mesoderm of the adult proboscis. WMISH of *PfBra* at these stages confirmed exactly the pattern reported by Tagawa et al. (1998a). *PfBra* expression is limited to the oral and anal regions of the embryonic gut (Fig. 3B,D). At no time is *PfBra* expressed in any component of the embryonic mesoderm. By 2 weeks of development *PfBra* can no longer be detected by WMISH (data not shown; Tagawa et al., 1998a).

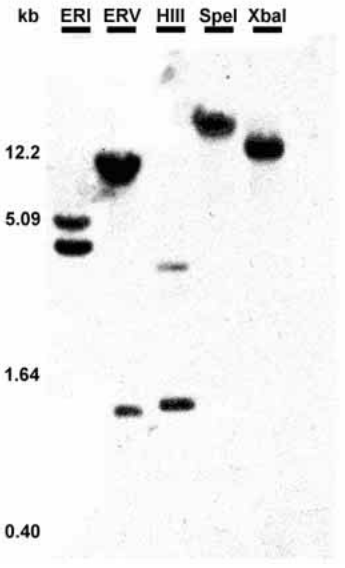

Fig. 2. Genomic Southern analysis of the *PfBra* gene. Genomic DNA prepared from sperm of several individuals was digested with either *Eco*RI (ERI), *Eco*RV (ERV), *Hin*dIII (HIII), *Spe*I or *Xba*I. 15 μg of digested DNA were resolved by agarose gel electrophoresis and transferred to an nylon membrane. The blot was hybridized with a ^{32}P-labeled cDNA probe and washed under high-stringency conditions. These data show that the *PfBra* gene is present as a single copy per haploid genome. The two bands seen in the *Eco*RV and *Hin*dIII lanes are due to the presence of the respective sites in an intron which is spanned by the cDNA probe (data not shown). The two bands in the *Eco*RI lane are due to the heterozygous occurrence of restriction sites outside the *PfBra* probed region.

Larval development

The 1-week tornaria larva (Fig. 3C; Hadfield, 1975; Tagawa et al., 1998a) is equipped with both a feeding ciliated band and a locomotory ciliated band called the telotroch. The gut is clearly tripartite with an esophagus, stomach and intestine. There is an apical plate with a ciliated tuft and two eye spots (not visible in Fig. 3C but shown in the older larvae of Fig. 4). The axes of the larva are identical to that of the adult: the apical/anal axis is equivalent to the anterior/posterior axis of the adult with the eye spots visible at the tip of the proboscis during the initial stages of metamorphosis, the larval mouth, which is continuous with the adult mouth, is located ventrally, and the hydropore dorsally. The embryonic mesoderm has formed the proximal region of the protocoelomic duct, which connects to the

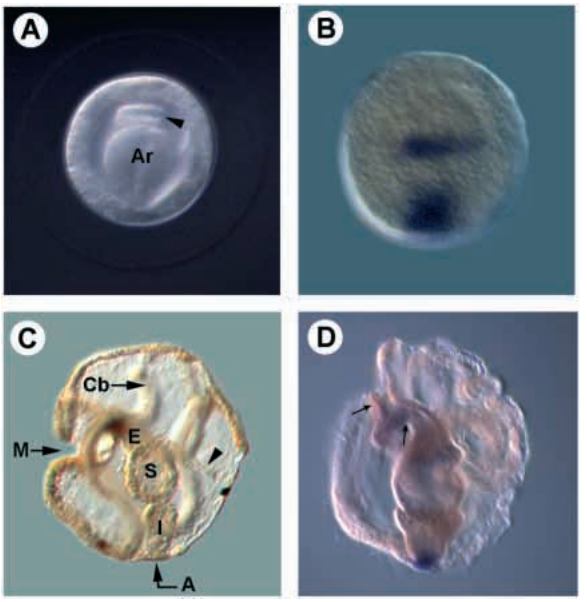

Fig. 3. Early development and spatial expression pattern of *PfBra* in embryonic- and larval-specific tissues. (A) Early gastrula of *Ptychodera flava*. Note the mesodermal sac (arrowhead) at the top of the archenteron (Ar). The enclosed coelomic cavity is the protocoel. (B) *PfBra* WMISH of an early gastrula. *PfBra* is not expressed anywhere in the embryonic mesoderm, but only in the oral and anal regions of the embryonic gut. (C) Photomicrograph of a 1-week tornaria larva. The larva is now complete with a tripartite gut (M, mouth; E, esophagus; S, stomach; I, intestine; A, anus and Cb, ciliated band). The telotroch, the locomotory ciliated band surrounding the anus, is present but not visible in the micrograph. The protocoel is continuous with the surrounding sea water via the pore canal and hydropore; this structure (coelom, canal and pore, indicated by the arrowhead) is the larval protonephridum (Ruppert and Balser, 1986). (D) *PfBra* WMISH of a 1-week larva. Note the continued expression of *PfBra* in the anal region of the intestine and in the ventral wall of the esophagus (delineated with arrows). Specimens in A and B measure about 150 μm in diameter; C and D about 250 μm in length.

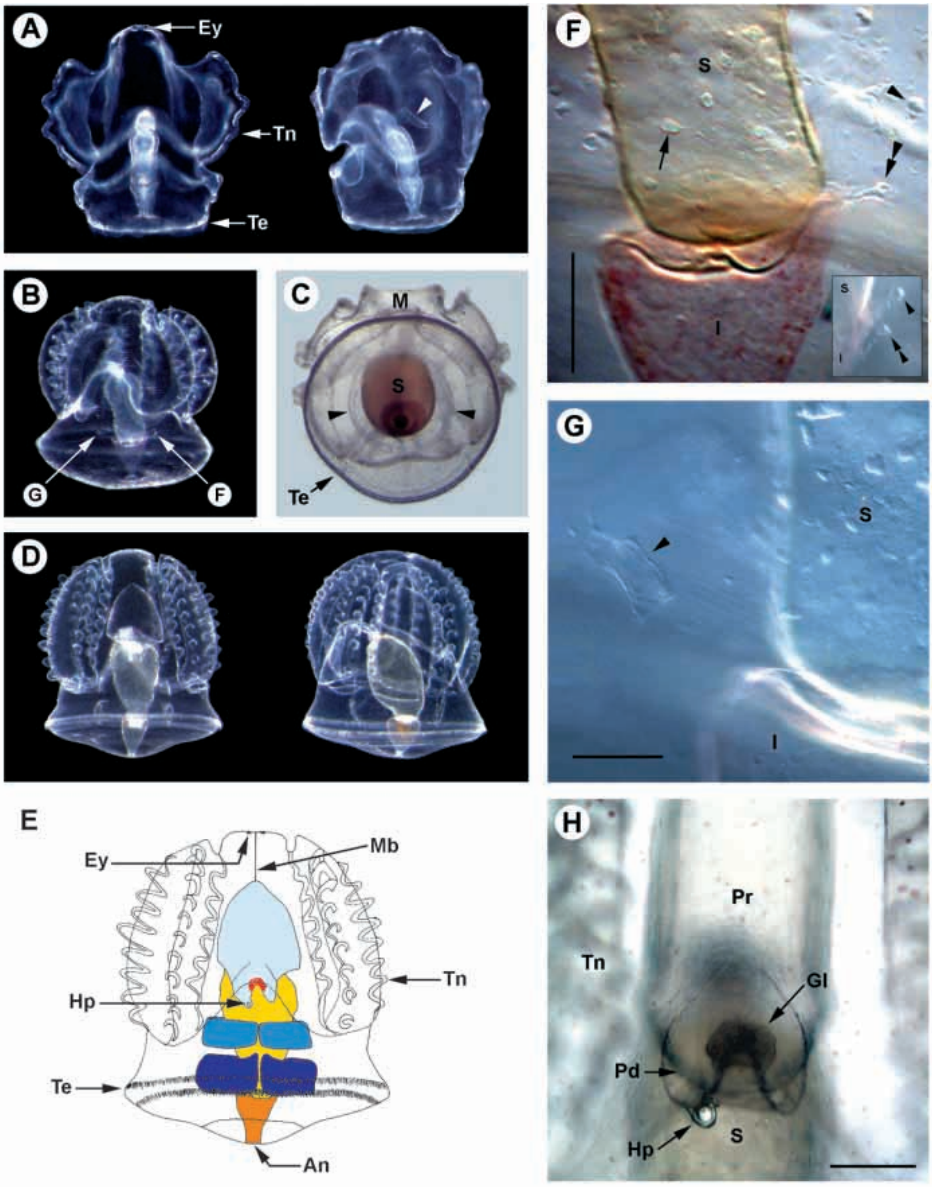

Fig. 4. Photomicrographs of anesthetized *Ptychodera flava* larvae. (A) Dark-field photomicrograph of a 2-month tornaria larva. Aside from the beginning of ciliated band specializations known as tentacles (Tn), further development of the telotroch (Te) and an increase in size to about 1 mm in length, there is little morphological difference between this stage and the one-week larva shown in Fig. 3C. The arrowhead points to the larval protonephridium. On the left is an oral view (adult ventral), on the right a side view. At the top of the animal are the two eye spots (Ey). (B) Dark-field photomicrograph of a 3-month larva shown in oral view. By this stage, the larva is about 2 mm in length and has fully developed tentacles, specializations of the ciliated band used for feeding and found exclusively in *Ptychodera* species (Hyman, 1959). It is at this stage that the mesocoels and metacoels begin to form from mesenchymal cells (arrows refer to panels F and G). (C) A 3.5-month larva shown in anal view. The coeloms are now of considerable size and are beginning to circumscribe the gut. Arrowheads point to the metacoels; S, stomach; M, mouth; Te, telotroch. (D) Dark-field photomicrograph of a 4-month tornaria larva. The larva has reached 3 mm in length, the maximum size obtained in our culture conditions. On the left is a view from the hydropore (adult dorsal aspect); on the right, side view. The coeloms are well developed with the proboscis coelom greatly expanded compared to earlier stages (cf. [A]). Also note the prominent conical bulge in the anal field and the general increase in size of the posterior region of the larva. After this stage (Hadfield, 1975), the larvae decrease in dimension, the tentacles are lost and the ectoderm around the proboscis coelom begins to take the shape of a proboscis (compare with Fig. 5A). (E) Schematic drawing of the dorsal surface of the larva pictured in D. The metacoels are shown in dark blue, the mesocoels in medium blue and the protocoel in light blue. The protocoel is connected to the apical plate, the location of the two eye spots (Ey), by a mesodermal band (Mb). Also indicated is the axial complex with the glomerulus in red and the hydropore (Hp) white (see [H] for a close-up view of this region of the larva). The stomach is in yellow, the intestine in orange, and the anus (An) indicated on the figure. Ectodermal structures include the tentacles (Tn) and the telotroch (Te). (F) Close up of the left side of the larva shown in B. The double arrowhead is pointing to cells which appear to be delaminating from the gut; S, stomach; I, intestine. Based on their relative position with respect to the stomach/intestine junction, these cells will contribute to the left metacoel. The single arrowhead is pointing to similar cells which will probably contribute to the left mesocoel. The arrow points to one of many mesenchymal cells present in the blastocoel. Scale bar is approximately 200 μm. The inset shows the same view at a slightly different angle to further illustrate the connection of the coelomic anlagen to the gut. (G) Close up of the right side of the larva shown in B. The arrowhead points to coalescing cells forming the right metacoel. Scale bar is approximately 50 μm. (H) High magnification photograph of the hydropore and axial complex (see Fig. 1) of a larva at the stage figured in D. The protocoel (Pr) communicates to the exterior via the protocoelomic duct (Pd) and hydropore (Hp). Visible in the midline is the glandular glomerulus (Gl). Tn, tentacle; S, stomach. Scale bar is approximately 250 μm.

exterior via the ectodermally derived distal duct and hydropore. The coelomic cavity, duct and hydropore form a protonephridium or larval 'kidney' (Ruppert and Balser, 1986).

By 2 months of development, the larvae have increased in size from 250 μm to about 1 mm in length (Fig. 4A). Larval growth consists of an expansion of the blastocoelar space, probably due to a net increase in ectodermal cells as the ciliated band becomes more specialized. Importantly, the epithelia of these larvae are never more than a single cell layer thick, nor is there any apparent addition of new cell types or contribution of mesoderm to the overall size of the larva. This is consistent with what is seen in other primary larva including the pluteus larva of sea urchins (Cameron et al., 1989). The telotroch has also increased in girth; it no longer extends only around the

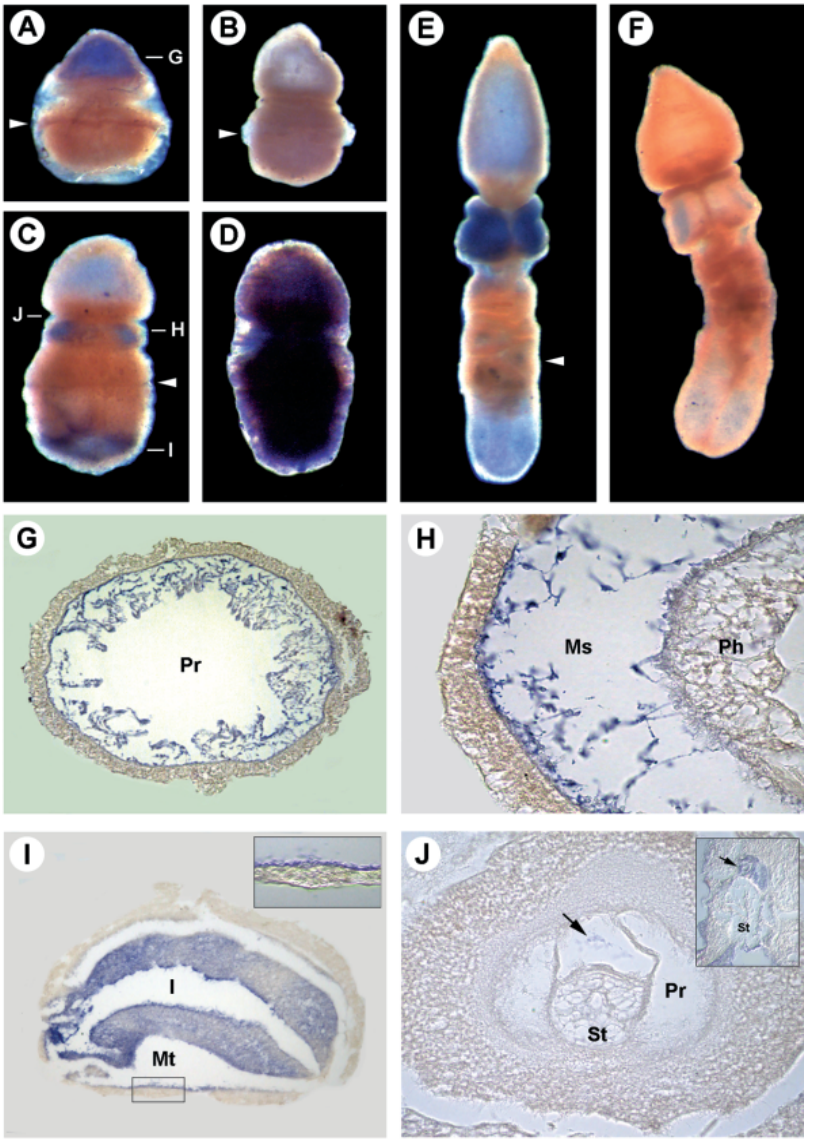

Fig. 5. Metamorphosis and adult spatial expression pattern of *PfBra*. Competent larvae collected out of the plankton will complete metamorphosis when placed on sand. Competent larvae and juveniles were fixed at 24 hour intervals for WMISH. (A-F) Dorsal view with anterior to the top; (G-J) cross sections in which dorsal is to the top. (A) *PfBra* WMISH of a competent larva from the plankton. Note the formation of the proboscis, which is expressing *PfBra*. The horizontal dash indicates the plane of section shown in G. The arrowhead indicates the position of the telotroch, which is apparent as a line across the body. (B) Negative (sense) control of the same stage as A. Again the telotroch is indicated with the arrowhead. (C) WMISH of a competent larva 24 hours after contact with sand. The mesosome and metasome are now clearly delineated and both express *PfBra*. The telotroch (arrowhead) has lost most of its cilia but can still be identified. Horizontal dashes indicate the planes of section shown in H-J. (D) Positive 24-hour control showing the ubiquitous expression of a cytoplasmic actin gene. (E) WMISH of a 3-day juvenile. All three body regions are still expressing *PfBra*. The dark spots in the trunk region are ingested sand grains. The arrowhead indicates the relative position of the telotroch. (F) Negative (sense) 72-hour control. (G) Transverse section of the proboscis of the larva shown in A. *PfBra* is expressed in the mesoderm, specifically the lining of the protocoel (Pr) and the cells delaminating from this lining which will eventually fill most of the coelomic space; these cells are, or are in the process of becoming, the longitudinal musculature of the proboscis (see Fig. 1). (H) Transverse section through the collar region of the larva shown in C. *PfBra* is strongly expressed in the somatopleura of the mesocoel (Ms) and the cells delaminating from this layer which will form the collar musculature. There may also be expression in the splanchnopleura. Note the histology of the pharynx (Ph); it consists of large vacuolated cells and is histologically very similar to the stomochord (Fig. 5J) (see also Ruppert, 1997). (I) Transverse section of the larva shown in C through the intestine region. *PfBra* is expressed in the posterior region of the intestine (I) and the coelomic lining of the metacoel (Mt) (boxed region of the metacoel is shown in the inset). (J) Transverse section of the larva shown in C through the base of the proboscis. The stomochord (St) does not express *PfBra*. However, a group of cells located dorsal to the stomochord are positive for *PfBra* (arrow); inset shows the same area from the 72-hour animal shown in E. The exact nature of these cells is unclear at this time and was not studied further. The animals figured in A and B are about 1 mm in length; in C and D about 1.5 mm in length; in E is about 2.5 mm; and in F about 2 mm in length.

anus, but now surrounds the whole posterior edge of the larva. The ciliated band has begun to generate specializations called tentacles, structures which appear to be limited to the genus *Ptychodera* within the enteropneusts (Hyman, 1959; Hadfield, 1975). These are first seen as small ridges along the edge of the ciliated band in Fig. 4A. They seem to serve to enhance feeding and may be necessitated by the large size and extended planktonic existence of *Ptychodera* larvae (Strathmann and Bonar, 1976), though their exact function has not been studied in detail (Gilmour, 1982). Aside from the protonephridium and wandering mesenchyme cells in the blastocoel (see Fig. 4F, and Tagawa et al, 1998b), no other mesoderm is apparent in these larvae.

At 3 months of development tentacles are now evident and there is a further widening of the plane of the telotroch (Fig. 4B). The most important change relevant to this study is the appearance of the mesocoels or collar coeloms and the metacoels or trunk coeloms. These arise from cells which appear to delaminate from the gut (Fig. 4F) and coalesce to form the bilateral mesodermal sacs (Fig. 4G). In contrast to other indirectly developing hemichordates (summarized in Hadfield, 1975), both pairs of coeloms form at the same time, following the development of tentacles. Cells that form the metacoels appear to arise from the stomach/intestine junction; those that form the mesocoels arise from the wall of the stomach (Fig. 4F).

After about two more weeks of development the larvae are still expanding in size; a representative animal measures about 2.5 mm in length (Fig. 4C). The coeloms are conspicuous in the blastocoel and are now beginning to circumscribe the gut (Fig. 4C). Their position contrasts sharply with that described by Morgan (1894) for *Ptychodera bahamensis* (c.f. Hyman,

1959), in which the wandering mesenchymal cells aggregate against the epidermis in the position of the presumptive coeloms. Also making their appearance at this stage are the pericardium and glomerulus (Fig. 4H). These structures are components of the axial complex, the metanephridium or adult 'kidney' (see Fig. 1, and Ruppert and Balser, 1986).

At 4 months of development the larvae have reached the maximum size obtained in our culture conditions, about 3 mm in length (Fig. 4D,E). The tentacles are well developed and the coeloms have further increased in size. The protocoel is now very conspicuous, having taken the shape that will be assumed by the adult proboscis. After this stage, the larvae decrease in size and become more elongate, the tentacles are gradually lost, the gut is shifted posteriorly, and the whole preoral part of the larva is reshaped into a proboscis (Hadfield, 1975). The larvae are competent to undergo metamorphosis when they have completed these morphological changes. We observed the whole process of larval development from the beginning, with the exception of the last stages of proboscis and gut morphogenesis antecedent to acquisition of competence.

We performed WMISH for *PfBra* on all larval stages figured in Fig. 4. At no time could any signal above background be detected that is attributable to *PfBra* (data not shown). Expression of this gene is renewed only when the larvae attain metamorphic competence.

Metamorphosis and expression pattern of *PfBra* in formation of adult body plans

Competent *P. flava* larvae were collected from plankton tows off Waikiki Beach, Honolulu, Hawaii. Hybridization of *PfBra* with competent larvae showed expression in the mesoderm of the protosome (Fig. 5A,G), including cells delaminating from the lining of the coelomic cavity and the lining itself. This lining will form the circular musculature of the proboscis and the delaminating cells form the longitudinal musculature (Morgan, 1894; Hyman, 1959). Expression was not seen in the region where the stomochord will form or anywhere else at this stage. The negative (sense) control showed no expression (Fig. 5B).

Competent larvae placed on appropriate sand will complete metamorphosis within 24 hours such that a clear mesosome/metasome distinction is apparent (Fig. 5C). Growth is also observed: most competent larvae will increase in length between 0.5 mm and 1.0 mm during the first 24 hours, and this size increase involves the accretion of multilayered structures including those derived from mesoderm, not just a simple increase in blastocoelar space as seen in the monolayered larvae. Because the position of the telotroch is marked with a pigmented band (Hadfield, 1975, 1978; arrowheads in Fig. 5) and its position can be followed for several weeks, it is clear that growth occurs both in front and behind the telotroch, confirming Morgan's (1894) observations. *PfBra* is now expressed in the mesoderm of the collar and trunk, and continues in the proboscis musculature. A section through the mesosome (Fig. 5H) shows strong expression in the somatopleura of the mesocoel as well as in cells that appear to be delaminating from this layer and forming the collar musculature (see Hyman, 1959). Expression in the splanchnopleura is weak to absent. Expression was also seen in the very posterior region of the metacoelomic somatopleura and in the wall of the gut (Fig. 5I). Note that the anterior limit of expression in both the gut and the mesoderm is coincident. A section through the base of the proboscis is shown in Fig. 5J. It is clear that there is no *PfBra* expression in the stomochord, nor did stomochordal sections at any other level ever display *PfBra* expression, over the time period studied, i.e., the period from the onset of metamorphosis (Fig. 5A) through 72 hours postsettlement (Fig. 5E) during which the stomochord forms (Hadfield, 1975; K. J. P., unpublished observations). However, a group of cells dorsal to the stomochord appear positive for *PfBra*; although they could be cells in the dorsal sac (see Fig. 1), their exact nature is unclear and requires further study. A positive control embryo at this stage shows ubiquitous expression of a cytoplasmic actin gene as expected (Fig. 5D). By 72 hours, juveniles show continued expression of *PfBra* in the mesoderm of all three body regions and also in the posterior gut (Fig. 5E). Except for a broader

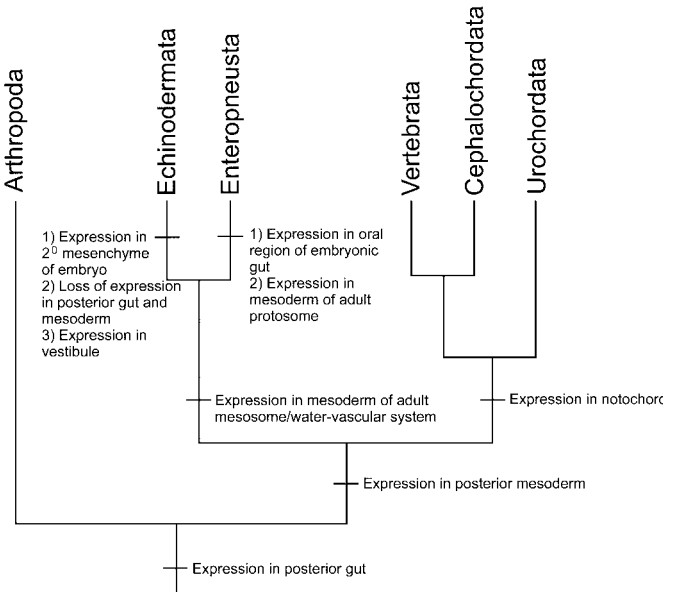

Fig. 6. A proposed evolutionary history of *Brachyury* utilization, plotted on a diagram displaying phylogenetic relationships of the taxa in which the expression pattern of *Brachyury* is known (see text for sources of phylogeny). Since *Brachyury* is expressed in the posterior gut of arthropods, chordates and enteropneusts, this is the primitive role of *Brachyury* in deuterostomes. We propose that *Brachyury* was recruited for the specification of mesoderm within the deuterostomes, initially mesoderm of the posterior trunk. In indirectly developing echinoderms and enteropneusts *Brachyury* has separate embryonic and adult roles. Both phyla share the expression pattern of *Brachyury* in the mesoderm of the middle coelom (i.e., mesocoel in hemichordates, hydrocoel in echinoderms). Each phylum also shows its own unique adult patterns: The enteropneust expresses *Brachyury* in the forming mesoderm of the protosome; the euechinoid expresses *Brachyury* in the vestibule, but has lost expression in the posterior mesoderm (somatocoel derivative) and gut. *Brachyury* plays unique roles during the embryogenesis of both phyla: it is expressed in the oral region of the hemichordate embryo and is expressed in the secondary mesenchyme of the echinoderm embryo. Within the chordates *Brachyury* was recruited for the specification of the notochord. Note that in ascidian urochordates the expression in mesoderm has been divided between two paralogues, one expressed in notochord and the other expressed in posterior mesoderm (Yasuo et al., 1996). See text for details and references.

domain of expression dorsal to the stomochord (Fig. 5J inset), there is little difference in expression between this stage and the 24 hour juvenile figured in Fig. 5C. Hybridization of 48 hour juveniles showed an identical pattern of expression as the 72 hour animals (data not shown). A negative (sense) control again displays no staining (Fig. 5F).

DISCUSSION

Biphasic expression of *PfBra* and the process of maximal indirect development

Ptychodera flava is a hemichordate that undergoes maximal indirect development. This is a phylogenetically widespread mode of development found in many lophotrochozoans (e.g., annelids, entoprocts, ectoprocts, nemerteans), and deuterostomes (echinoderms and enteropneusts), and probably primitive for both clades, but conspicuously absent in the ecdysozoans (e.g., arthropods, nematodes; Peterson et al., 1997; Nielsen, 1998; for discussion of the phylogeny of Bilateria, see Balvoine and Adoutte, 1998). Maximal indirect developers are characterized by the development of a primary larva (Jägersten, 1972), which is the immediate endproduct of embryogenesis and which spends several days to months feeding in the plankton. The adult body plan, which bears little resemblance to that of the larva, is formed from groups of cells that are 'set-aside' from the process of embryonic specification, retaining an indefinite capacity for cell division, in contrast to the embryonic cells that constitute the larva itself (Davidson et al, 1995; Peterson et al., 1997). The process by which *P. flava* generates its adult body plan, as documented in Figs 4 and 5 of this study, is a dramatic illustration of maximal indirect development. We have focused on the mesodermal set-aside cell components, here observed for the first time under laboratory conditions, from their appearance in the embryo and feeding larva to their enormous expansion and metamorphic differentiation as the coelomic mesoderm of the juvenile worm.

The significance of the pattern of *PfBra* expression must be appreciated in terms of its indirect development. That is, early and late patterns belong respectively and exclusively to the embryonic phase when the larval structures are generated, and to the postlarval metamorphic phase when the structures of the adult body are generated. These two phases of expression are separated by months in time. The early phase lasts from midgastrulation through about 10 days of development. The *PfBra* gene is then downregulated and expression is not detected again until after 5 months of planktonic existence. In these two phases, *PfBra* is expressed in different cellular components, which largely give rise to different structures. Initially the gene is expressed in the oral and anal regions of the embryonic gut. Ultimately these regions are incorporated in the oral and anal regions of the juvenile. At metamorphosis, however, *PfBra* is expressed in entirely different cell types, viz. the mesodermal derivatives of each of the three body regions. The only overlap between the embryonic and adult expression patterns is in the posterior gut. The biphasic expression pattern, separated by months in time and located in widely disparate cell types of disparate fate, implies that the *Brachyury* must have at least two separate *cis*-regulatory modules, one directing embryonic expression and another expression in the mesoderm of the metamorphic coeloms. Furthermore, it is likely also to have at least two different sets of downstream target genes. Expression of *Brachyury* is also biphasic in the sea urchin, where its expression again occurs transiently in the embryo, the transcripts disappear, and then reappear in the context of adult body plan formation (K. J. P., unpublished data). We have also studied a second transcription factor, *Not*, which is similarly utilized in a biphasic manner in the sea urchin (K. J. P., unpublished data). Many other transcription factors, which like *Brachyury* play developmental roles, are probably also utilized in a biphasic fashion in the process of maximal indirect development (see also Davidson et al., 1995).

The *PfBra* gene apparently performs several functions during adult body plan formation. First, *PfBra* is expressed in the posterior gut, the same group of cells (or their progeny) that expressed *PfBra* in the embryo (see Hyman, 1959). Second, *PfBra* is probably intimately involved in adult muscle cell specification. *PfBra* is not detected during the initial stages of endomesodermal set-aside cell delaminations, nor is it expressed at all in the early stages of coelom development. *PfBra* is detected when cells begin to delaminate from the somatopleura, and both the lining and the delaminating cells are known to form the adult musculature (see Hyman, 1959). The expression of *PfBra* in body wall muscle cells is consistent with the mode of muscle development in enteropneusts, which are unusual in that the adult muscles are derived from cells that delaminate from the somatopleura (Hyman, 1959). Thus *PfBra* is expressed in the somatopleura and in the cells delaminating from this lining, but expression is barely observed in the inner lining or splanchnopleura. *PfBra* may be involved either in specifying the prospective muscle cells of the adult, or in controlling their epithelial-to-mesenchymal transition (Wilson et al., 1995), or both. In addition, *PfBra* could be involved in both the specification of cells in the dorsal sac (see Fig. 5J) and in morphogenesis. The coincident expression pattern in the posterior mesoderm and intestine in hemichordates is reminiscent of the expression pattern in vertebrates, where functional analysis suggests that *Brachyury* is involved in axial elongation (see Kispert and Herrmann, 1994).

Evolutionary history of *Brachyury* utilization

There now exists sufficient comparative data on *Brachyury* expression to permit a hypothetical reconstruction of the evolutionary history of *Brachyury* utilization (Fig. 6). Data from arthropods establishes the primitive role of *Brachyury* in the latest common ancestor of deuterostomes; i.e., arthropods may be considered the outgroup. The expression pattern of *Brachyury* has been examined in three insect taxa, *Drosophila*, *Tribolium* and *Locusta*, and all three taxa show expression in the posterior gut and derivatives (e.g., anal pads; Kispert et al., 1994; Murakami et al., 1995; Singer et al., 1996; reviewed in Reuter, 1995). *Brachyury* is also expressed in the posterior gut of chordates (see Introduction for references) and in enteropneusts, both embryo and adult (Tagawa et al., 1998a; and this work). Thus it is most parsimonious to postulate that the primitive role of *Brachyury* was specification of the posterior gut.

Brachyury is not expressed in the mesoderm of any arthropod so far examined (Kispert et al., 1994). However, *Brachyury* was evidently recruited for mesoderm specification at the base of the deuterostomes. We hypothesize that the expression seen in the trunk mesoderm of developing vertebrate embryos is homologous to the expression described

here in the posterior metacoel of enteropneusts. The coincident expression in gut and mesoderm suggest an inductive relationship between the two (Davidson, 1991). Hence, the link between expression in posterior mesoderm and gut may be a phylogenetically old but intimate one. Nonetheless, this hypothesis demands examination of at least one more outgroup taxon, preferably a member of the Lophotrochozoa (e.g., a polychaete worm).

Within the deuterostomes each major clade recruited *Brachyury* for the specification of novel mesodermal structures: Chordates recruited *Brachyury* for the specification of the notochord and in the process must have evolved new *cis*-regulatory apparatus distinct from that directing posterior mesoderm expression (see Clements et al., 1996; Latkinkić) et al., 1997) The latest common ancestor of echinoderms and enteropneusts recruited *Brachyury* for specification of the mesoderm of the middle coelom or mesocoel (the hydrocoel in echinoderms). This expression in the mesoderm of the mesocoel can be thought of as a shared derived character (i.e., synapomorphy) of Echinodermata + Hemichordata. Each phylum also shows its own unique expression patterns. In echinoderms *Brachyury* is expressed in secondary mesenchyme of the embryo (Harada et al., 1995; see also K. J. P., unpublished data) and, as indicated in Fig. 6, loss of expression in the posterior mesoderm and gut is inferred from the phylogenetic distribution of the character and the phylogenetic relationship among these taxa. Furthermore, euechinoids express *Brachyury* in a structure that is unique with respect to other echinoderms, viz. the larval vestibule, which provides the ectodermal component of the adult rudiment, including the anlagen for the adult body wall epithelium and central nervous system (K. J. P., unpublished data). Enteropneusts display a unique pattern of expression in the oral region of the embryonic gut (Tagawa et al., 1998a and this work), and in the mesoderm of the adult protosome, as we show here. The protosome of hemichordates is very muscular and is the primary propulsive organ of the adult. The homologue in echinoderms, on the contrary, plays no role in locomotion and functions solely as an excretory organ.

Thus the evolution of *Brachyury* utilization took place in many stages. Its evolutionary history illustrates retention of a primitive, pan-bilaterian function, several instances of recruitment in development of phylum-specific structures, and instances of loss of function, some of which are exemplified by the expression pattern of *PfBra* in *Ptychodera flava*. This species displays the primitive expression pattern in the posterior gut of both the larva and adult. Recruitment of *Brachyury* for mesoderm specification, a shared character of the deuterostomes, is seen in the metamorphosing juvenile, where *PfBra* is expressed in the posterior mesoderm of the metasome. The expression seen in the mesoderm of the mesosome is shared with echinoderms. Finally, the expression seen in the mesoderm of the protosome is unique to enteropneusts. What is compelling about these patterns is that the evolutionary history of the enteropneust body plan is illuminated by the evolutionary history of *Brachyury* utilization.

The origin of the notochord

Several authors (e.g., Peterson, 1994; Reuter, 1995; Yasuo et al., 1995; Holland, 1996) have suggested that an examination of the expression pattern of *Brachyury* might provide a simple test of the proposed homology between the stomochord and notochord (Bateson, 1884). The histological similarity between the two is striking: both are relatively stiff rods consisting of usually vacuolated cells surrounded by a sheath of extracellular material (Welsch and Storch, 1970; Balser and Ruppert, 1990). The primary dissimilarity is that the stomochord is an anterior projection off the dorsal roof of the foregut, as shown in the diagram of the enteropneust body plan provided in Fig. 1. This study shows unequivocally that the stomochord does not express *Brachyury*, at least during the stages that we examined when the stomochord is developing. The oral expression in the embryo led Tagawa et al. (1998a) to suggest a possible relationship between this pattern of expression and development of the stomochord. However, it is now evident that this embryonic expression pattern is irrelevant to stomochord specification. Expression of *PfBra* in the embryonic esophagus is ventral, not dorsal; and about 5 months separate the downregulation of embryonic expression and the appearance of the stomochord. Furthermore, *PfBra* is not expressed in the stomochord in any case. While these results do not support the hypothesis of homology between stomochord and notochord, it is important to emphasize that an absence of expression cannot disprove it. The relationship between the stomochord and notochord could exist at a different level in the specification hierarchy, e.g., downstream, at the level of cell type, or even upstream of *Brachyury*. A test of this hypothesis would be to examine in the hemichordate expression of a diagnostic downstream target of *Brachyury* in notochord specification and for stomochord expression of transcription factors such as *forkhead* that operate upstream of *Brachyury* in notochord specification (Ang and Rossant, 1994; Weinstein et al., 1994).

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