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HyBra1, a *Brachyury* homologue, acts during head formation in *Hydra*

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SUMMARY

A homologue of the T-box gene, *Brachyury*, has been isolated from hydra. The gene, termed *HyBra1*, is expressed in the endoderm and is associated with the formation of the hypostome, the apical part of the head in four different developmental situations. In adults, which are continuously undergoing patterning, *HyBra1* is continuously expressed in the hypostome. During budding, hydra's asexual form of reproduction, the gene is expressed in a small area that will eventually form the hypostome of the developing bud before any morphological sign of budding is apparent. The gene is also expressed very early during head regeneration and is confined to the region that will form the hypostome. During embryogenesis, *HyBra1* is expressed shortly before hatching in the region that will form the apical end of the animal, the hypostome. The absence of expression at the apical end of decapitated animals of reg-16, a head formation-deficient mutant, provides additional evidence for a role of *HyBra1* during head formation. Further, treatments that alter the head activation gradient have no effect on *HyBra1* expression indicating the role of the gene is confined to head formation. Transplantation experiments indicate that the expression occurs before head determination has occurred, but expression does not irreversibly commit tissue to forming a head. A comparison of the function of the *Brachyury* homologues suggests an evolutionary conservation of a molecular mechanism that has been co-opted for a number of developmental processes throughout evolution.

Key words: *Brachyury* homologue, *Hydra*, Head formation, Axis formation, *HyBra1*

INTRODUCTION

There is a great deal of evidence that many of the genes regulating developmental processes are common among vertebrates and arthropods. Evidence is also beginning to accumulate that these same genes arose early in metazoan evolution as they occur in the Cnidaria, the first animals with a distinct body plan. For example, members of several families of transcription factors have been characterized. These include Hox genes (Schummer et al., 1992; Shenk et al., 1993a), other homeobox genes (Schummer et al., 1992; Grens et al., 1996; Gauchat et al., 1998; Mokady et al., 1998), bHLH genes (Grens et al., 1995) and winged helix genes (Martinez et al., 1997). In addition, homologues of genes in the Wnt cascade (Hobmayer et al., 1996) as well as a number of RTK genes (Steele et al., 1996; Stover and Steele, 1998) have been isolated and characterized. Some of these genes have functions similar to their homologues in more complex animals indicating an evolutionary conservation of function throughout the metazoa. For example, the Hox genes isolated from hydra appear to have a role in anterior-posterior patterning, while the *emx* homologue in hydractinia (Mokady et al., 1998) and the *HNF-3β* homologue in hydra (Martinez et al., 1997) have roles in the patterning of the head. In addition, *Cnash*, a hydra homologue of the *achaete-scute* class of bHLH genes, which has a role in specifying the cell fate of a class of cells as do these proneural genes in vertebrates and *Drosophila* (Grens et al., 1995).

However, there are also a number of these regulatory genes that are highly conserved on the sequence level among metazoans, but in which the function has not been obviously conserved. In some cases, the gene may have been co-opted for different functions throughout evolution. In others, the complexity of the organism and the multiple roles of the gene might obscure functions that are common. Because the cnidaria have a much simpler body plan with fewer components, a given regulatory gene most likely has fewer, and plausibly only a single, function. Hence, an examination of such genes in a cnidarian could provide clues as to the common aspects of their function among the animals where they occur.

One class of genes that play important roles in a number of developmental events in several species are the T-box genes. These genes are characterized by a highly conserved DNA-binding domain, the T-box (Kispert and Herrmann, 1993; Kispert et al., 1995; for reviews see Kispert, 1995; Smith, 1997; Papaioannou and Silver, 1998). The founding member, *Brachyury*, has been shown to play a critical role in mesoderm formation in vertebrates (for review see Smith, 1997). Homologues of this gene, and other T-box genes, have been cloned in organisms ranging from *C. elegans* to humans

indicating the ancient origin of this gene family (Bollag et al., 1994; Agulnik et al., 1995). Although the T-box genes are highly conserved at the sequence level, there are clear differences in the function of these genes in terms of the tissue, the time during embryogenesis, and/or the developmental process that they are expressed in among the deuterostomes and protosotomes.

We have isolated and characterized *HyBra1*, a hydra homologue of *Brachyury*. Examination of its expression pattern in a variety of experimental circumstances indicates it plays a role in head formation in hydra, suggesting at first glance a function that is rather different from those observed in other developing embryos. However, a consideration of other genes with which *Brachyury* homologues are co-expressed suggests the evolutionary conservation of a molecular pathway that acts to invaginate or evaginate tissue during axis formation and formation of the germ layers.

MATERIALS AND METHODS

Animals and culture conditions

Unless otherwise stated, the experiments were carried out with animals of the Basel strain of *Hydra vulgaris*, strain Zürich was used for LiCl treatment. Reg-16 is a regeneration-deficient strain of *H. magnipapillata.* For DAG treatments, the 105 strain (*H. magnipapillata)* was used (Achermann and Sugiyama, 1985). Embryos were obtained by mating the female *H. vulgaris* strain AEP with the male *H. vulgaris* strain PA2. Both strains are derived from male and female strains described previously (Martin et al., 1997). Stock cultures of hydra were maintained as described previously (Martinez et al., 1997). Cultures were fed daily and starved 1 day before selection of animals for experiments.

Isolation and characterization of the *HyBra1* clone

First-strand cDNA from whole animals was used for nested PCR with three fully degenerate primers encoding conserved amino acid sequences of the T-box. Bra-1 (5′AYGGNMGNMGNATGTTYCC3′) and Bra-3 (5′RAANSCYTTNGCRAANGG3′) were used as forward and reverse outer primers, while Bra-6 (5′TTYGGNGMNCAYTGGATG3′) together with Bra-3 used for nested PCR. PCR conditions were: 3 minutes at 94°C (1 cycle); 1 minute at 94°C, 1 minute at 42°C, 1 minute at 72°C (35 cycles); 10 minutes at 72°C. Fragments of the expected size were obtained and sequenced using standard procedures (Sambrook et al., 1989). Screening of a random-primed cDNA library (as described by Sambrook et al., 1989) derived from adult hydra (Sarras et al., 1994) with the random-primed PCR fragment yielded a single 926 bp clone, which was subsequently cycle sequenced (Amersham). The sequence of the clone, termed *HyBra1 (*GenBank accession number AF105065), consists of 129 bp of 5′ UTR, a putative start codon at position 130, the full T-box as shown in Fig. 1 (bp 251-797) and 129 bp of the activation domain.

The size of the transcript of the gene was determined by northern analysis using ~2 μg of poly(A)$^+$ RNA isolated from 4000 hydra heads (Qiagen) probed with a random-primed 800 bp fragment of the *HyBra1* gene. The hybridization conditions were 7% SDS, 0.5 M sodium phosphate buffer (pH 7.2), 5% sodium dextran at 60°C for 18 hours, The blot was washed with in 2× SSC/0.2% SDS for 15 minutes at room temperature, followed by 0.2× SSC/0.2% SDS at 60°C for 30 minutes.

The presence of *HyBra1* in embryos was determined using RT-PCR. Total RNA was isolated from 30-40 embryos at several embryonic stages (unfertilized egg, blastula, gastrula/postgastrula, cuticle formation stage, cuticle stage 2-10 days after fertilization), reverse transcribed with AMV or M-MLV as described by the

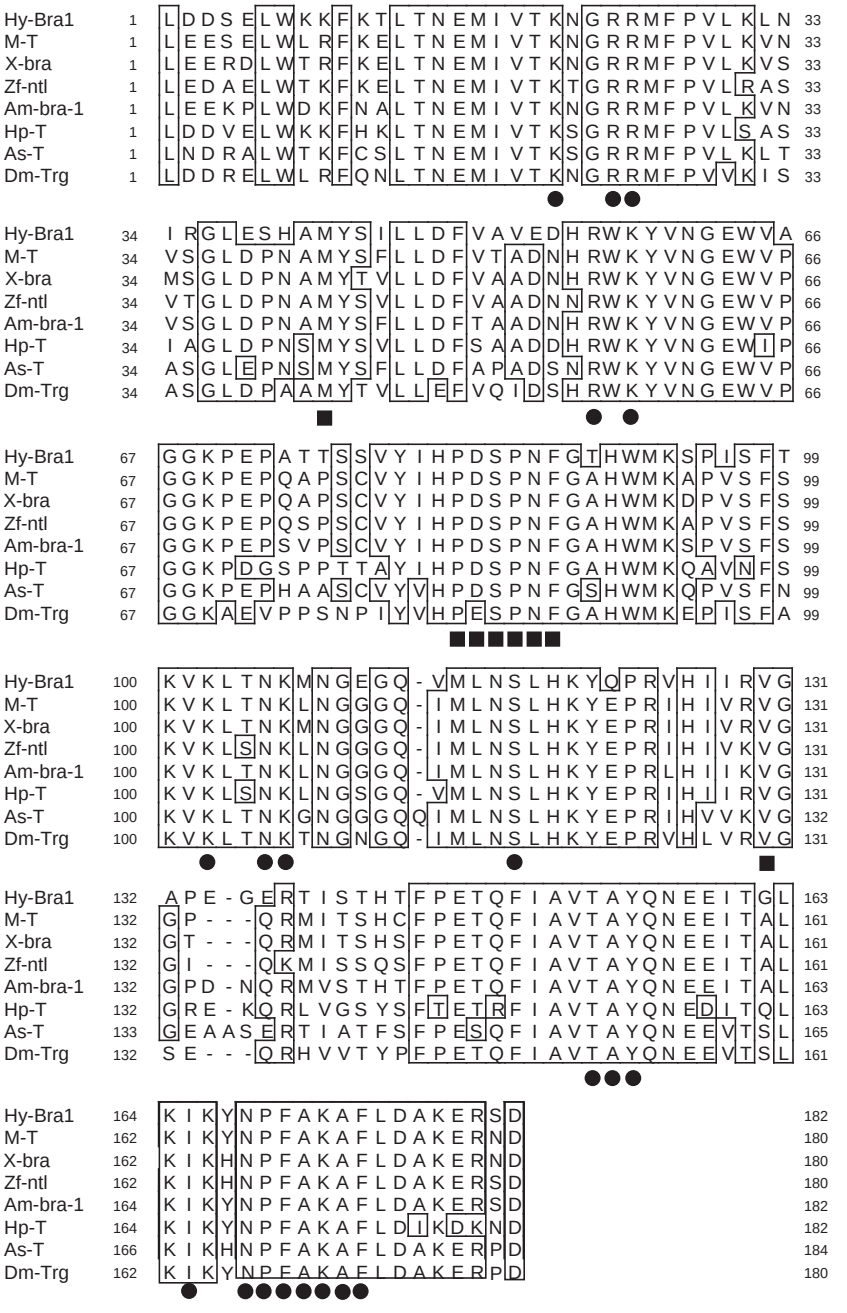

Fig. 1. Comparison of the *HyBra1* T-box with other members of the T-Subfamily of T-box genes. Boxed sequences are domains of amino acid identicalness. Squares indicate residues involved in dimerization, circles indicate those involved in DNA binding (Müller and Herrmann, 1997). Abbreviations: As, Ascidia; Am, *Amphioxus*; C, Chick; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; M, *Mus musculus*; Hu, human; Hp, *Hemicentrotus pulcherrimus*; Hy, *Hydra vulgaris*; X, *Xenopus laevis*; Zf, *Danio rerio.*

manufacturer (Gibco), and 1 μl of the first-strand cDNA (corresponding to half an embryo) used as a template in 20 μl PCR. *HyBra1*-specific primers were used alone, or together with primers for the hydra EF-1α gene (GenBank accession number Z68181) as an internal control (forward primer: 5′GTTTGAAGCTGGTATTTC3′; reverse primer: 5′TGTTTACCAGTATCTTTG3′). PCR conditions were: 3 minutes 94°C (1 cycle), 1 minute 94°C, 1 minute 46°C, 1 minute 72°C (35 cycles), followed by 10 minutes 72°C.

Phylogenetic analysis of the T-box family

T-boxes from GenBank database were aligned using *ClustalW* and a phylogenetic analysis was performed using the program *Puzzle* (Strimmer and von Haeseler, 1996), a maximum likelihood-based method applying quartet puzzling. Blosum 62 was used as a substitution model and, because of blocks of high conservation within the sequence, we assumed a rate heterogeneity along the sequence. The required parameter alpha of the gamma distribution was estimated by the program from the data set and found to be 0.6, justifying our initial assumption. 1000 replicate analyses were performed. Branch lengths correspond to the number of changes. Numbers at each node correspond to the percentage of replicates in which the node was supported, a measure of statistical support (Strimmer and von Haeseler, 1996). An analysis using the maximum parsimony program *PAUP* (Swofford, 1996) yielded basically the same tree topology (analysis not shown).

In situ hybridization

In situ hybridization analysis on whole mounts of animals or late-stage embryos was carried out as described previously (Grens et al., 1995, 1996) with some minor modifications. Inserts of *HyBra1* plasmids were amplified by PCR using T3 and T7 primers and 500 ng of the PCR product was directly labeled with digoxygenin (DIG; Tautz and Pfeifle, 1989). Hybridization was carried out over 36 hours with approximately 0.025 ng/μl DIG-labeled RNA probe. Samples were incubated in a 1:2000 preabsorbed dilution of anti-DIG-antibody (Boehringer) in sheep serum overnight, washed in MAB (100 mM maleic acid, pH 7.5) and visualized with the alkaline phosphatase substrate BM Purple (Boehringer). The color reaction was stopped with 100% ethanol and animals were mounted in Euparal (Asco Laboratories).

Treatment with diacylglycerol (DAG)

Treatment of hydra (105 strain, *H. magnipappillata*) with DAG (0.1 mM dioctanoyl-sn-glycerol) was carried out as described by Müller (1989). The working solution was prepared in 3 ml hydra medium, sonicated for 30 seconds and cooled on ice for 5 minutes. On the first day, animals were treated for 30 minutes in a 6 cm Petri dish and, on subsequent days, they were exposed to DAG for 2 hours. After each treatment, they were washed 5 times in hydra medium. Animals were fed 3× per week during the treatment.

Regeneration and transplantation experiments

Animals were bisected either directly beneath the tentacle ring or in the middle of the body column, and allowed to regenerate. Periodically thereafter, samples were fixed in fresh 4% paraformaldehyde in hydra medium and subsequently analyzed for expression of *HyBra1* with in situ hybridization. In parallel samples, the regenerating tip was isolated and grafted laterally into the 2-region (as defined by Bode and Bode, 1984) of a host animal. Host and donor tissue were strung onto a 0.25 mm diameter fish line and held in contact with one another with flanking pieces of parafilm for about 2 hours in order to heal. Secondary head formation was analysed 2 days after grafting. A secondary head was defined by the presence of a hypostomal dome surrounded by at least two tentacles, while partial heads were defined by a single distal tentacle.

RESULTS

Characterization of *HyBra1*

Using nested PCR and first-strand cDNA derived from whole hydra, fragments of two genes, each with a high level of similarity to the T-box of *Brachyury* were isolated. One of them, termed *HyBra1* was used to screen a hydra cDNA library (Sarras et al., 1994) and yielded a single clone 926 bp in length that included some of the 5′UTR, the entire T-box and approximately 100 bp on the 3′ end of the T-box. Northern analysis using stringent conditions indicated the presence of a single transcript of approximately 2.3 kb (data not shown). Sequence comparison with other *Brachyury* homologues indicates that *HyBra1* shows a a very high degree of conservation in the conserved T-box (about 75-80% amino acid identity; Fig. 1). In general, most domains that are conserved among these homologues are also conserved in *HyBra1*. This includes those residues that have been shown to be implicated in dimerization and direct contact with the palindromic binding site by crystallographic studies on Mouse *Brachyury* (Müller and Herrmann, 1997).

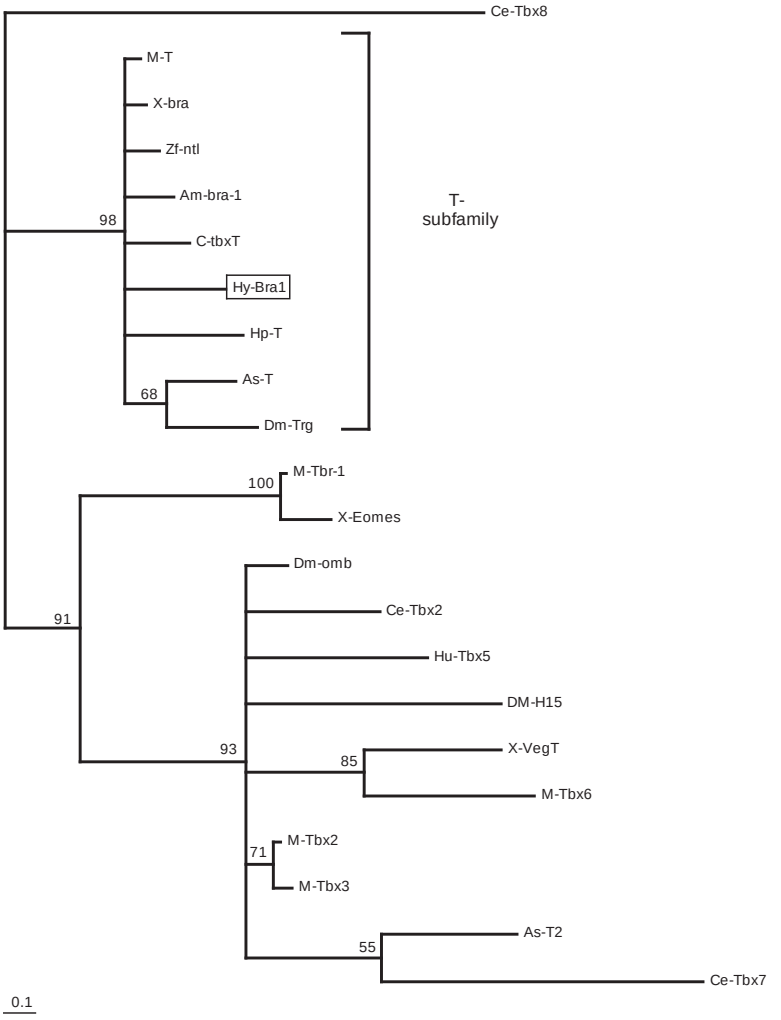

Fig. 2. Phylogenetic analysis of T-box proteins. A Maximum Likelihood Method (PUZZLE) was used to reconstruct the phylogenetic tree of CLUSTALW aligned protein sequences comprising the entire T-Box. See Materials and Methods for details.

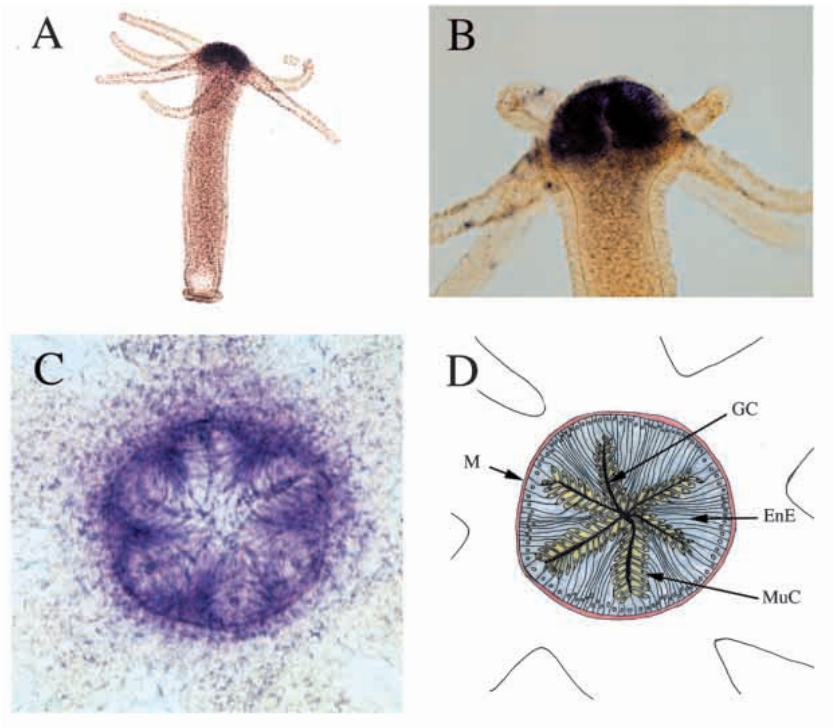

Fig. 3. Expression pattern of *HyBra1* in adult hydra as analyzed with in situ hybridization on whole mounts as observed in A, a budless adult, and at higher magnification in B, the head. (C) A transverse optical section through the hypostome. (D) A schematic of the cross-section of the hypostome. EnE, Endodermal epithelial cells; GC, gastric cavity; M, mesogleoa; MuC, mucus cells.

Phylogenetic analyses indicate that T-box genes fall into one well-defined subfamily, the *Brachyury* (T) subfamily, and 2-6 other subfamilies that are quite distinct from the *Brachyury* subfamily. A maximum likelihood analysis shows that *HyBra1* clearly clusters with the *T/Brachyury*-like genes (Fig. 2) as indicated by the level of statistical support (95%). The branching order within the T-subfamily, however, cannot be resolved with certainty. Hence, we conclude that *HyBra1* is a true orthologue of *Brachyury*.

HyBra1 is expressed in the hypostome of adult hydra

Because of the tissue dynamics of an adult hydra, the developmental processes that were active in setting up the body plan remain continuously active to maintain the form and regional distribution of cell types in the adult (for review see Bode and Bode, 1984). In situ hybridization analysis in adult hydra revealed that *HyBra1* is strongly expressed throughout the hypostome, the apical half of the head (Fig. 3A). Expression drops sharply in the tentacle zone, the lower half of the head (Fig. 3B), and can not be detected below the tentacles.

Of the two tissue layers, the ectoderm and endoderm, *HyBra1* is always strongly expressed in the endoderm of the hypostome, and occasionally some faint staining in the ectoderm of the hypostome was observed. The endoderm of the hypostome is folded into wedges that protrude into the gastric cavity. Correspondingly, the gastric cavity is reduced to a canal with branches that separate neighboring wedges or protrusions (Fig. 3D). In the hypostome, the endoderm contains two cell types, endodermal epithelial cells and mucous cells. The endodermal epithelial cells extend from the base of the tissue layer next to the basement membrane to the edge of the tissue layer facing the gastric cavity. The mucous cells are located among the epithelial cells on the outer edge of each protrusion facing the gastric cavity (see Fig. 3D). The unstained areas are the location of the mucous cells, thus, expression of *HyBra1* is confined to the epithelial cells of the endoderm (Fig. 3C).

Expression of *HyBra1* in the adult head suggests it would also have a role in the development of the head. Since head development occurs during budding, hydra's form of asexual reproduction, during head regeneration, as well as during embyrogenesis, this possibility can be examined in three different circumstances.

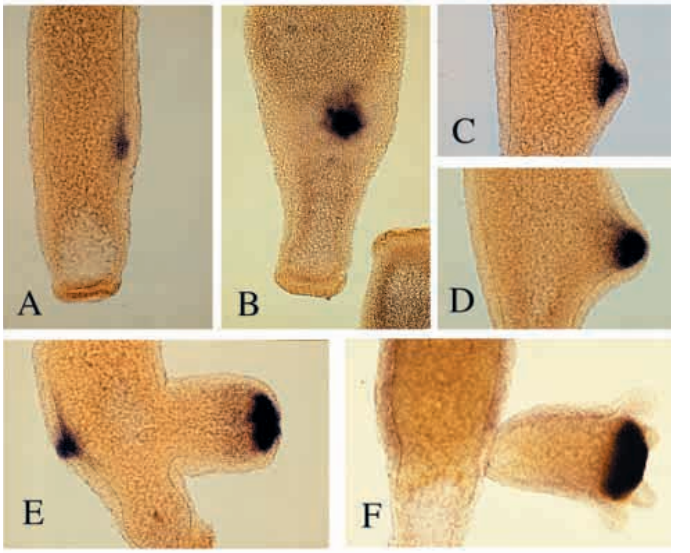

Fig. 4. Change in the expression pattern of *HyBra1* expression during budding. (A) Before stage 1; (B) stage 1 (placode stage); (C) stage 2-3; (D) stage 3-4; (E) stage 5; (F) stage 7. Bud stages are defined as described in Otto and Campbell (1977). The spot on the left side of the animal in E is a second bud at stage 2.

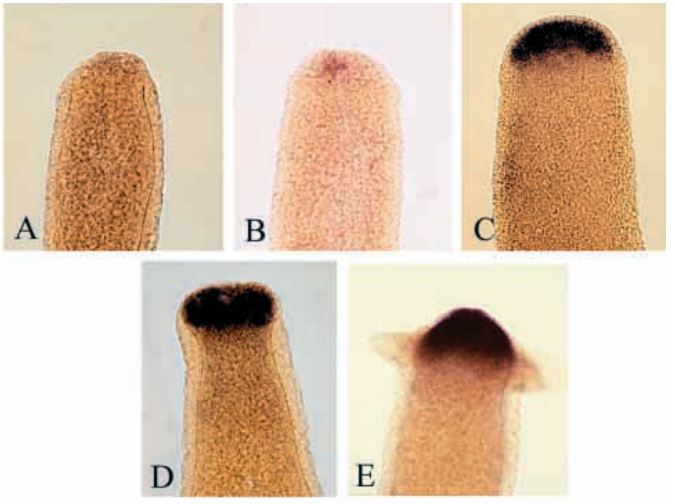

Fig. 5. *HyBra1* expression in the regenerating head at several times following bisection beneath the tentacles. Samples were examined at (A) 1 hour, (B) 3 hours, (C) 4 hours, (D) 6 hours and (E) 48 hours.

HyBra1 is expressed in a developing head during budding

During asexual reproduction, the first sign of a developing bud is the formation of a circular ectodermal placode in the lower third of the body column. The placode evaginates and then elongates into a cylindrical protrusion, which subsequently forms a head at the distal end and a foot at the proximal end. *HyBra1* expression is first observed as a small distinct spot comprising 10-15 endodermal epithelial cells in the region where a bud will form, but before any morphological sign of bud formation is visible (Fig. 4A). By bud stage 1 (placode stage; bud stages as described by Otto and Campbell (1977)), when the ectoderm thickens locally, the underlying endoderm expresses *HyBra1* in a slightly larger, perfectly round spot (Fig. 4B). As the placode protrudes and elongates into a cylinder, *HyBra1* expression of the gene expands to encompass the area of the future hypostome, but does not extend further in a proximal direction (Fig. 4C-F).

Thus, *HyBra1* expression remains strictly confined to the distal tip forming a cap of expression throughout all stages of bud evagination. As the gene is expressed very early during budding, the hypostome appears to be specified at a very early stage. We also note that expression during budding always appears to be much stronger than in the hypostome of the adult suggesting that the requirement for the gene during head formation is higher than for the maintenance of the head in the adult.

HyBra1 expression occurs during head regeneration

When hydra are bisected, the apical end of the lower half regenerates a head in 2-3 days. We monitored *HyBra1* expression during head regeneration in animals bisected at two axial locations. When bisected directly beneath the tentacles, *HyBra1* expression was first detected 3 hours later (Figs 5A,B, 6A), and rapidly increased in intensity during the next 1-2 hours (Fig. 5C) reaching a maximum by 6 hours (Figs 5D, 6A) since no appreciable increase was detected at 12 hours (data not shown). By 36-48 hours, tentacle buds emerged precisely

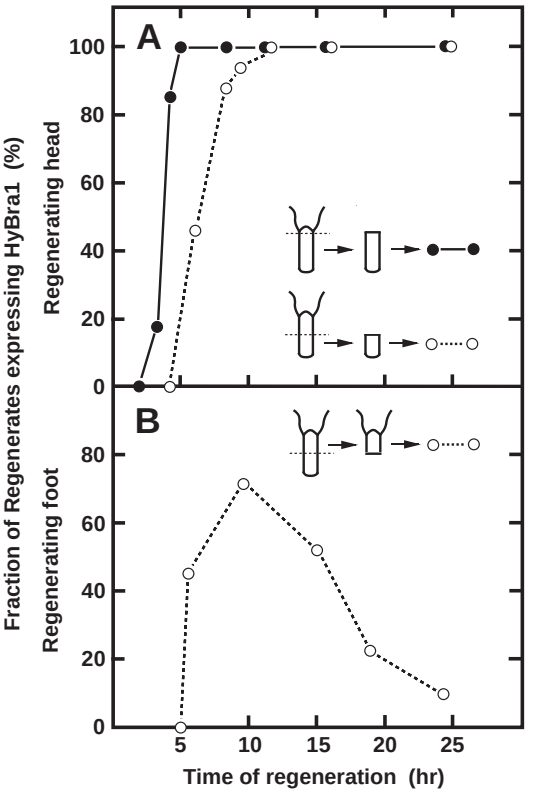

Fig. 6. Time course of *HyBra1* expression during head and foot regeneration. (A) Rate of increase in expression during head regeneration as a function of the axial level of bisection. Once initiated *HyBra1* was expressed continuously in the hypostome of the animals (B) Transient expression during foot regeneration. Axial level of bisection for each type of regenerate is indicated in figures in A and B.

at the basal margin of the expression domain (Fig. 5E) indicating that *HyBra1* expression is confined to the developing hypostome. Thereafter, expression tended to decrease reaching the somewhat lower levels typical of an intact animal by 72 hours.

When animals were bisected in the middle of the body column, *HyBra1* expression was first detectable at the apical tip by 6-9 hours, reaching a maximum expression around 12-18 hours (see Fig. 6A). Since the rate of head formation is graded down the body column, the slower appearance of *HyBra1* is consistent with the slower rate of head formation when animals are bisected at this level (Wilby and Webster, 1970; MacWilliams, 1983b; Technau and Holstein, 1995).

Reg-16, a mutant strain of *Hydra magnipapillata*, which is defective in head regeneration (Achermann and Sugiyama, 1985), provides another approach for examining the role of this gene in head formation. When reg-16 is bisected, most animals do not regenerate a head, while some form a partial head and a few regenerate a complete head. Expression of *HyBra1* was followed for 6 days in animals bisected beneath the tentacles. In contrast to the wild type, where strong *HyBra1* expression is observed by 6 hours, none of the reg-16 animals expressed *HyBra1* before 48 hours. By 6 days, most of the animals had not formed head structures and no *HyBra1* expression was detected (Fig. 7A). A few regenerates (about 5%) formed a

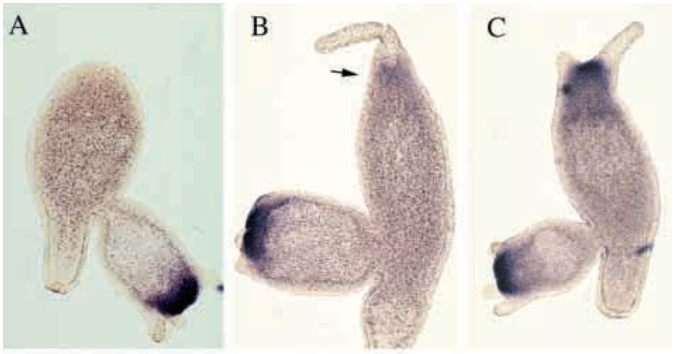

Fig. 7. *HyBra1* expression in regenerating animals of the regeneration-deficient mutant reg-16. The three types of regenerates observed 6 days after decapitation were (A) no head regeneration, (B) partial regeneration with a single medial tentacle, and (C) a head with hypostome and two or more tentacles. Arrow in B indicates low level of *HyBra1* expression.

single medial tentacle at the apical tip. In these animals, *HyBra1* expression occurred either at a very low level (Fig. 7B) or was not detected. A small fraction (10-20%) of the regenerates developed a complete head (i.e. a hypostomal dome plus at least two tentacles) and, in every one of these, *HyBra1* was expressed (Fig. 7C).

These results obtained with reg-16 coupled with those obtained for normal regenerates and budding indicate that the expression of *HyBra1* is tightly correlated with development of the head and specifically with hypostome development.

HyBra1 expression precedes but does not irreversibly commit tissue to head formation

The early expression of *HyBra1* during budding and head regeneration suggests that it might be involved in the process of head determination. One approach is to compare the time of the appearance of *HyBra1* expression in a regenerating tip with the time that tip was determined to form a head. A standard grafting procedure (e.g. MacWilliams, 1983a,b) involving the formation of a secondary head was used to measure when this determination had taken place. As shown in Fig. 8A, animals were bisected beneath the tentacles, allowed to regenerate and periodically the regenerating tip was transplanted to the upper body column of another hydra (see Materials and Methods for details). 2 days after grafting, the fraction of transplants forming a secondary head was measured. As shown in Fig. 8B, the fraction forming a secondary head rose from a value of 15% at T=0 to 75% by T=5-6 hours with a T_{50} of 4-5 hours. Since all of the regenerates expressed *HyBra1* by 4-5 hours (Fig. 6A), and only half were committed to head formation by that time, expression of the gene is consistent with a role in the head determination process.

However, though *HyBra1* is expressed before head determination, this expression does not imply the tissue is irreversibly determined to form a head. Four results support this view. (1) Only 40-50% of the 4-5 hours regenerating tips formed heads upon transplantation after 2 days even though all regenerating tips expressed the gene. (2) In a second experiment (see Fig. 9A), apical tips regenerating for 4-5 hours were transplanted to a host, and periodically samples examined for *HyBra1* expression. As shown in Fig. 9B, the fraction expressing the gene drops from 100% to 50-60% in 2 days which is similar to the fraction that will form heads. Hence, those transplants that do not form heads lose *HyBra1* expression. (3) In a variation of the same experiment, we transplanted apical tips immediately after decapitation to a host. These grafts did not express *HyBra1* at the time of transplantation (Fig. 9B). Surprisingly, 6 hours after grafting almost all (96%) of the grafts strongly expressed the gene at their distal ends in a manner characteristic of regenerating tips. During the next 2 days, however, most of the transplants (60%) ceased expressing the gene, which correlates reasonably well with the fraction (15%) of these transplants that were expected to eventually form heads (see Fig. 8B). (4) We also examined *HyBra1* expression during foot regeneration at the basal end of the upper half of animals bisected in the middle of the body column. Surprisingly, we found that *HyBra1* is transiently expressed reaching a maximum by 9 hours following bisection and then slowly disappearing thereafter (Fig. 6B). As with head regeneration, expression was confined to the regenerating tip.

Expression of *HyBra1* is not correlated with the head activation gradient

The head activation level of the tissue, i.e. its potency to form a head, is graded along the body axis of the adult animal and has a maximum in the head (MacWilliams, 1983b). The expression of *HyBra1* in the adult head as well as during head regeneration could be a response to a high level of this gradient. The overall level of the gradient along the body column can be raised by treatment with diacylglycerol (DAG) (Müller, 1989), or lowered with LiCl (Hassel and Berking, 1990). Hydra were

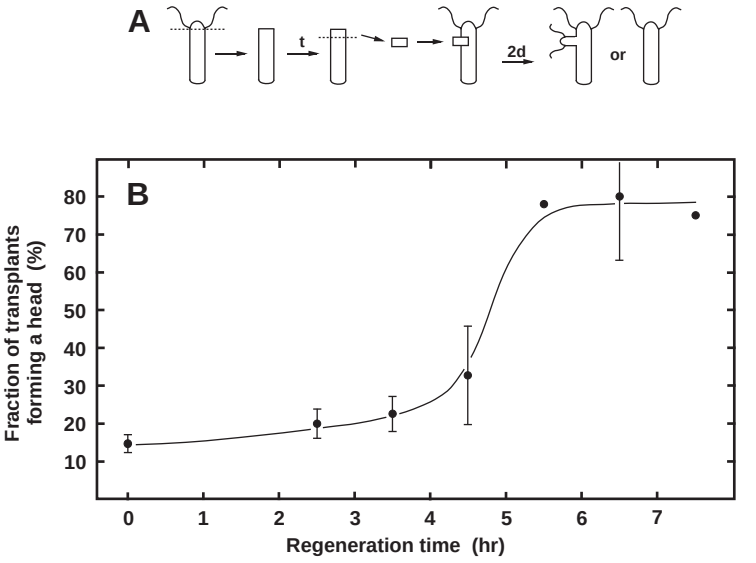

Fig. 8. Time course of head determination in the regenerating tip of animals bisected beneath the tentacles. (A) Scheme of the transplantation experiment; t, time of isolation of the regenerating tip after decapitation; 2d, 2 days. (B) Time course of head determination in terms of transplants forming a secondary head. Each time point represents 1-5 experiments each with a sample size of n=13-24.

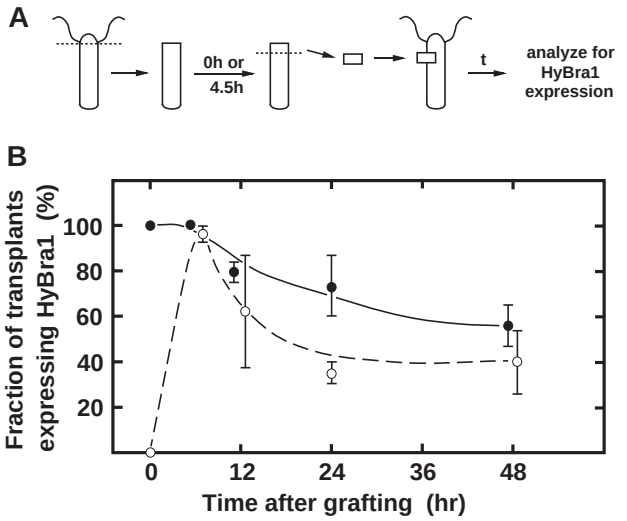

Fig. 9. Effect of transplantation into the vicinity of a head on *HyBra1* expression in a regenerating tip. (A) Scheme of the transplantation experiment. (B) Changes in the fraction of transplants expressing the gene as a function of time. Transplanted after a time of regeneration of 0 hour (open circles), or 4.5 hours (closed circles). Data points are mean values (±s.d.) of 2-5 independent experiments.

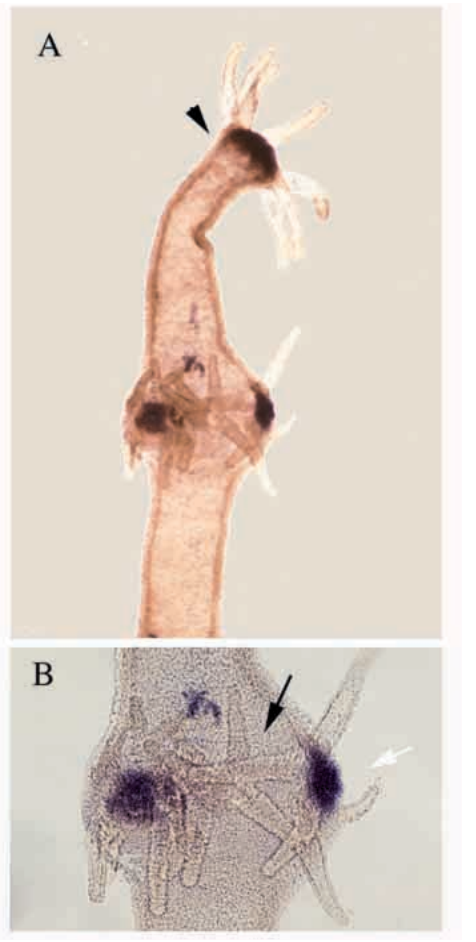

Fig. 10. *HyBra1* expression in a DAG-treated animal that developed an ectopic head and ectopic tentacles. (A) Expression in the whole DAG-treated animal. The basal border (arrowhead) of *HyBra1* expression remains unaltered and does not extend into the body column. (B) Higher magnification of the region with ectopic structures shown in (A). White arrow indicates an ectopic head with hypostome and tentacles, while the black arrow indicates an ectopic tentacle.

treated with both compounds to determine if the range of *HyBra1* expression was altered.

Treatment of animals of the 105 strain of *Hydra magnipapillata* with DAG for up to 14 days resulted in the formation of ectopic tentacles and heads, which has been shown to coincide with a rise in the head activation gradient (Müller, 1990). Fig. 10A shows a DAG-treated animal that formed an ectopic head and several ectopic tentacles. Three observations can be made. (1) Secondary heads characterized by a hypostomal dome and at least 2 tentacles surrounding it, always strongly express *HyBra1* in the developing hypostome. (Fig. 10B). (2) Formation of single tentacles was not accompanied by the expression of *HyBra1*, suggesting that its expression is tightly correlated with hypostome, but not tentacle, formation (Fig. 10B). (3) The range of expression of *HyBra1* in the adult head of a DAG-treated animal remained unaltered, that is, it did not extend basally into the body column (see Fig. 10A), as would be expected if the expression of this gene were readily influenced by an altered head activation gradient in the body column.

Treatment with 1 mM LiCl for 7 days leads to the formation of ectopic feet along the body column (Hassel and Berking, 1990) as well as a lowering of the head activation gradient (Maggiore and H. R. B., unpublished results). Animals of the Zürich strain of *Hydra vulgaris* were treated for 7 days and analyzed for *HyBra1* expression. No changes were observed (data not shown). Hence, the absence of changes in the expression of *HyBra1* along the body column of the adult with either treatment indicates that expression of this gene is not governed by the head activation gradient.

HyBra1 expression during embryogenesis

Finally, we examined the expression pattern of *HyBra1* during embryogenesis. Embryogenesis in hydra is quite simple (e.g. Martin et al., 1997). In brief, the fertilized egg undergoes 6-7 cleavage divisions resulting in a blastula consisting of a single layer of cells. Then, gastrulation occurs by ingression of single cells from all parts of the blastula into the blastocoel thereby forming a solid stereo gastrula. Subsequently, the cells of the outer layer, the presumptive ectoderm, secrete a thick cuticle and the embryo enters a relatively quiescent stage. 2-3 weeks after fertilization, the mass of epithelial cells in the center of the embryo organize into an epithelial layer, the endoderm. Within a day thereafter, the cuticle cracks at the apical end, and the hatchling emerges and elongates from a spherical form into the cylindrical shape of the adult animal forming a head and foot during and shortly after elongation.

The expression pattern of *HyBra1* during this process was examined in two ways. In one, embryos at several stages were collected, RNA isolated and RT-PCR carried out. As shown in Fig. 11, *HyBra1* was detectable in adult animals, but not during early or middle stages of embryogenesis. Other experiments indicated that it is also not expressed during the late cuticle stages (data not shown).

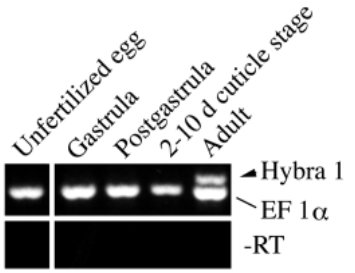

Fig. 11. Expression of *HyBra1* during several stages of embryogenesis as measured with RT-PCR. EF-1α was used as an internal loading control.

To gain more precise information on the timing and location of expression of the gene, in situ hybridization analysis was carried out on whole mounts of embryos. Consistent with the RT-PCR results *HyBra1* expression was not detected during the early stages of embryogenesis (early cleavage stages, blastula, gastrula) before cuticle formation (data not shown), whereas control in situ hybridizations with EF-1α showed ubiquitous expression in these stages (data not shown). During the final stages, the endoderm forms, the cuticle becomes porous and, subsequently, the cuticle cracks open at the anterior end of the embryo (Martin et al., 1997). Just prior to hatching, a small patch of *HyBra1* expression was found in the endoderm at the presumptive apical pole (Fig. 12A). This onset of expression occurred most likely shortly after the formation of the endodermal layer. As the embryo expanded and elongated during hatching, the expression of the gene increased in intensity as well as in area covering the apical end (Fig. 12B,C). 5-10 hours later the tentacles emerge indicating that *HyBra1* expression was confined to the developing hypostome (Fig. 12D).

Thus, the expression pattern of *HyBra1* during embryogenesis is similar to that found during budding. In both modes of reproduction, the gene is expressed shortly before the onset of both head formation, and continues to be expresssed in the formation of the hypostome.

DISCUSSION

HyBra1 is the first T-box gene isolated from a diplobast

The positional cloning of T or *Brachyury* as the gene responsible for the T mutation in mice (Herrmann et al., 1990) marked the isolation of the founding member of a novel class of transcription factors which are all implicated in important developmental processes. This class of genes is characterized by a conserved DNA-binding domain, the T-box, which spans 180-200 amino acids and is usually located at the N terminus of the gene (Kispert and Herrmann, 1993). A number of other T-box-containing genes, which fall into several subfamilies, have been isolated from metazoans ranging from *C. elegans* to humans indicating the ancient origin of the gene family (Agulnik et al., 1995; Papaioannou and Silver, 1998). However, to date no T-box genes have been identified in diploblasts. Thus, *HyBra1* is the first member of this gene family identified in a basal metazoan lacking the mesoderm.

Detailed molecular studies on *Brachyury* established that the T-box is a DNA-binding domain, which recognizes a palindromic 20 nucleotide sequence in vitro (Kispert and Herrmann, 1993). In a recent crystallographic study, Müller and Herrmann (1997) suggested that *Brachyury* binds as a homodimer, based on their identification of the critical residues implicated in the dimerization of the *Brachyury* protein production as well as those involved in the binding of the DNA. Interestingly, *HyBra1* shares all of these critical residues suggesting that it might have similar dimerization and DNA-binding properties. These diagnostic residues as well as our phylogenetic analysis of the gene family demonstrates that *HyBra1* is a clear orthologue of *Brachyury*.

HyBra1 has a role in hypostome formation

In adult hydra, the sequence of events in the development of a head during budding or regeneration, that is the formation of the hypostome and tentacle zone, has been followed using markers that are hypostome-specific (Technau and Holstein, 1995; Hermans-Borgmeyer et al., 1996; Martinez et al., 1997), tentacle-specific (Bode et al., 1988; Weinziger et al., 1994), or mark the entire head (Javois et al., 1986). However, none of these markers appears during the early stages of head formation. *Budhead*, a hydra homologue of the *fork head/HNF-3*β members of the winged helix family of transcription factors, is expressed in a broad area during the initial stage (stage 0/1), of bud formation, and only much later is expression reduced to the presumptive hypostome (Martinez et al., 1997). However,

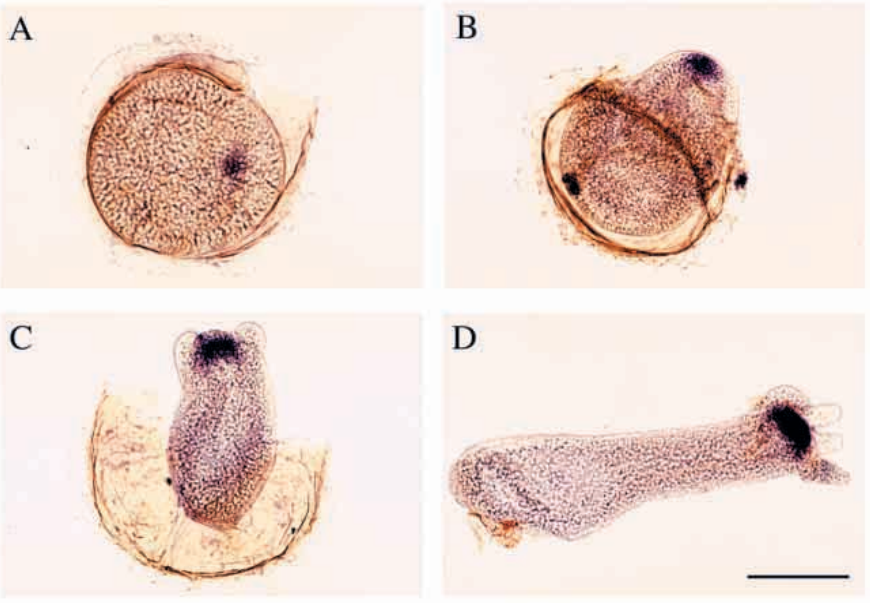

Fig. 12. *HyBra1* expression at several stages during the hatching of a hydra embryo. (A) An embryo 18 days after fertilization in which the cuticle is cracking. (B) An early hatching stage as the embryo begins to elongate. (C) A later hatching stage in which the tentacle buds begin to emerge. (D) A young animal 5 hours after hatching. Bar, 200 μm.

during head regeneration, the gene is expressed considerably later than *HyBra1*. It appears weakly and broadly in the upper half by 4-8 hours following decapitation and is not confined to the apical tip until 12 hours (Martinez et al., 1997).

In sharp contrast, *HyBra1* is strongly expressed during the initial or very early stages of head formation during both budding and regeneration. Before the first morphological sign of bud formation [placode formation], a small circular spot with a strong level of expression appears. During head regeneration, expression of the gene begins by 3 hours following bisection, or shortly after wound healing is complete, and reaches a maximal level by 4-6 hours. In both cases, expression of the gene is confined to the region that will form the head. Hence, the expression of *HyBra1* provides evidence that the gene plays a role early in head formation.

This conclusion is strengthened by two other results. (i) In the adult, the tissues are continually undergoing patterning to maintain the form of the animal (see Bode and Bode, 1984 for review). Correlated with this ongoing process, *HyBra1* expression is continuous in the head, and only in the head of the adult. (ii) Expression of the gene is directly correlated with the level of head regeneration in the head regeneration-deficient mutant, reg-16 (Achermann and Sugiyama, 1985). Should no head form, the gene is never expressed. If regeneration results in a partial head, a low level of *HyBra1* is detected while, if a complete head forms, then the normal level is found in the head.

Expression of *HyBra1* is confined to the hypostome, the apical half of the head. This is true in the fully formed heads of adults as well as in the developing heads during bud formation and regeneration. In developing heads, expression of the gene is in all the tissue apical to the level where the tentacles emerge, hence, in the developing hypostome. This was confirmed by the DAG results. In animals that formed ectopic tentacles and ectopic heads, *HyBra1* expression was always found in the developing hypostome of an ectopic head, but never observed in the vicinity of individual ectopic tentacles. Hence, the gene plays a role in the formation of a specific part of the head, the hypostome.

Since *HyBra1* is expressed very early during head formation, it may have a role in the head determination process. Consistent with this is a strong level of *HyBra1* expression in regenerating tips before these tips are committed to head formation. However, the expression of *HyBra1* is reversible. It vanishes in a regenerating tip in which the process of head formation has been initiated, but is subsequently repressed by transplanting it to a location in the body column not conducive for head formation. Hence, this reversible expression indicates that *HyBra1* expression may be part of the head determination process, but is not the critical step leading to irreversible determination.

Relationship of *HyBra1* to the head patterning processes

A large body of transplantation and regeneration experiments have defined three patterning processes governing head formation in hydra predicted earlier on theoretical grounds (Gierer and Meinhardt, 1972; MacWilliams, 1983a,b; for review see Bode and Bode, 1984). One is the source density gradient (or head activation gradient; MacWilliams, 1983b), which is maximal in the head and graded down the body column. Head activation is defined by the ability of a piece of tissue to induce the formation of a secondary head when grafted into the body column of another animal. Hence, this gradient, which is a stable tissue property (MacWilliams, 1983b), is the basis of the regeneration polarity of the body column. As part of the tissue dynamics of an adult hydra, tissue of the body column is continuously displaced apically from the body column into the head and basally into the foot (Campbell, 1967; Otto and Campbell, 1977). Hence, the head activation gradient is also dynamic, and is maintained by a signal[s] produced in the head and continuously transmitted down the body column.

Although it is plausible that *HyBra1* expression is a response to a high level of head activation in the hypostome region, this is unlikely. Raising or lowering the overall level of the head activation gradient with DAG or LiCl, respectively, had no effect on the range or location of *HyBra1* expression. Were expression of the gene responsive to the gradient level, DAG treatment should have led to an extension in a basal range of its expression into the tentacle zone and possibly into the body column.

While the head activation gradient provides a specific level of a potential to form a head at a given axial level along the body column, a second patterning process termed ‘unstable head activation’ (MacWilliams, 1983b), or ‘head formation process’ (HFP) is the actual process of head formation. It has several properties that coincide with the pattern of *HyBra1* expression. (i) As is *HyBra1* expression, the HFP is confined to the apical regenerating tip of the lower half of a bisected animal that will form a head. (ii) The half-life of HFP as measured by transplantation experiments is about 12 hours (MacWilliams, 1983a,b), which is also the half-life for the loss of *HyBra1* expression in a regenerating tip that is prevented from forming a head. (iii) The variable level of *HyBra1* expression, which corresponds to the variable extent (complete, partial or none) of head regeneration in decapitated reg-16 animals, also correlates well the level of HFP in heads, partial heads and upper body columns (=no head formation) (Rubin and Bode, 1982). (iv) Finally, the time required for a regenerating tip to become determined to form a head increases with increasing distance from the head, which implies a decreasing level of head activation (MacWilliams, 1983a,b; Technau and Holstein, 1995). Similarly, the timing of the initial appearance of *HyBra1* increases with increasing distance from the head. Thus, the level of expression of the gene is intimately linked with the level of the HFP.

Head inhibition, the third process governing head formation, is continuously produced in the head and transmitted basally to prevent tissue of the body column, which is always capable of forming a head, from doing so (MacWilliams, 1983a). Three results indicate that *HyBra1* expression is inversely correlated with the level of head inhibition. (i) Removal of the head of normal animals leads to a rapid drop in the level of head inhibition and a rise in *HyBra1* expression. (ii) The head inhibition level remains high following decapitation of a reg-16 adult and, concomitantly, the gene is not expressed. (iii) When a regenerating tip is grafted into a region of high head inhibition, expression of *HyBra1* is down regulated. Hence, expression of this gene is actively repressed by head inhibition.

In sum, these considerations indicate that, of the three head patterning processes, *HyBra1* expression is intimately linked

with two of them, the HFP and head inhibition. It provides molecular evidence for the distinct effects and properties of the local, rapidly changing head activation and the slowly changing source density gradient as predicted by a reaction-diffusion theory (Gierer and Meinhardt, 1972). Finally, the transient expression of *HyBra1* during foot regeneration provides more evidence for a complication in the patterning processes. Since a head invariably regenerates at an apical end, it is commonly assumed that the HFP alone is initiated at this end resulting in the formation of a head. However, the transient expression of *HyBra1* in a regenerating foot suggests that both head and foot formation processes are initiated at the basal end. Other evidence is also consistent with this idea. *Cnox-2*, the hydra homologue of the *Deformed* gene, which is expressed in epithelial cells of both layers throughout the animal except in the head (Shenk et al., 1993b), transiently vanishes during foot regeneration. Hence, both head and foot formation appear to be initiated at the basal end, but the head formation process is eventually suppressed, plausibly by head inhibition from the existing head.

The role of *HyBra1* in head formation during embryogenesis

During embryogenesis, *HyBra1* is initially expressed very late, shortly before hatching, in a small area that will form the hypostome. To our knowledge, this is the first gene isolated from a cnidarian, for which the expression pattern during embryogenesis has been determined. The pattern provides insight into two aspects of reproduction in hydra. One has to do with the timing of the initiation of head formation in hydra. In hydra, the sperm entry point, which is established before fertilization is also the location of the anterior end of the developing embryo (Martin et al., 1997; Freeman, 1981). This implies that during oogenesis an anterior-posterior polarity is set up early, plausibly in the oocyte. However, the very late expression of *HyBra1* suggests that formation of the head and the adult body axis is only initiated in a second later step and much later than the establishment of the anterior-posterior polarity in the embryo.

Another issue that is poorly understood is the relationship between the patterning events that occur in the sexual (embryogenesis) and asexual (budding) modes of reproduction. Certainly the early stages are quite different. Embryogenesis in hydra proceeds as is common in animal embryos starting with cleavage divisions and gastrulation, eventually leading to the formation of two embryonic layers, the ectoderm and endoderm (e.g. Martin et al., 1997). In contrast, budding begins with the two layers already formed. Thereafter, the processes are similar in that elongation of the spherical shell of the embryo into a cylinderical shell is fairly similar to the evagination and subsequent elongation of the body wall into a cylindrical protrusion during budding. In both processes, *HyBra1* expression first occurs in a small spot in the part of the developing embryo or bud that will be the apical tip of the elongating cylindrical shell. Hence, this pattern provides the first information that the initial stages of elongation and head formation are similar in the two modes of reproduction.

The evolution of *Brachyury* function

The identification of a *Brachyury* orthologue in hydra shows that *Brachyury* is a member of an ancient gene family. The high degree of conservation in the T-box including the dimerization and DNA-binding domains points to a strong selection for this functional part of the gene. However, the function of the homologues in different phyla is not obviously conserved since the homologues are expressed in different tissues and locations. In vertebrates, *Brachyury* initially has a pan-mesodermal expression in the marginal zone of the blastula, but later becomes restricted to the axial mesoderm during gastrulation (Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1994). Expression is related in the protochordate ascidians, where *Brachyury* is expressed in the precursors of axial mesoderm, but not in an early pan-mesodermal fashion (Yasuo and Satoh, 1994; Corbo et al., 1997). However, in another group of deuterostomes, the sea urchins, the differences are greater. The *Brachyury* homologue is expressed in the precursors of secondary mesenchyme, which are derived from the anterior end of the invaginating endoderm during gastrulation and give rise to a variety of cell types (Harada et al., 1995). Among the arthropods, the expression patterns are different again. In *Drosophila*, the *T-related gene* (*Trg*) is expressed in the hindgut primordia during blastoderm stage and later in the differentiating hindgut (Kispert et al., 1994; Singer et al., 1996). Similarly, the *Brachyury* homologue in *Locusta*, a short-germ insect, is expressed in the posterior tip of the gastrulating embryo, a region that continues to grow and add segments at the posterior end (Kispert et al., 1994). Clearly, these patterns are quite different from the anterior expression of the hydra homologue in the developing head.

However, the gene may participate in a common molecular pathway in all these organisms. It is striking that, where examined in vertebrates, ascidians, sea urchins, insects and hydra, the expression of the *Brachyury* homologue coincides with the expression of a *fork head*/*HNF-3*β homologue (Weigel et al., 1989; Dirksen and Jamrich, 1992; Harada et al., 1996; Shimeld, 1997; Shimauchi et al., 1997; Terazawa and Satoh, 1997; Olsen and Jeffrey, 1997). In the several organisms examined, this coordinated expression occurs in a variety of developmental circumstances such as different tissues, at different times during embryogenesis, as well as in tissues composed of single cells or epithelial layers. The same pairing of expression holds true for hydra. *HyBra 1* and the hydra *fork head*/*HNF-3*β homologue *budhead* are expressed in an overlapping pattern in the endodermal epithelial cells of the hypostome. Whereas *HyBra1* is expressed throughout the hypostome, *budhead* is expressed in the lower half of this structure (Martinez et al., 1997).

Not only are these two genes expressed together in many organisms, there is some evidence that they function in the same pathway. In animal cap assays in *Xenopus*, it has been shown that *Brachyury* and *pintallavis*, the *fork head*/*HNF-3*β homologue, have a synergistic effect on the differentiation of dorsal mesodermal fates (O'Reilly et al., 1995). These considerations suggest that, early in metazoan evolution, a molecular pathway involving a *Brachyury* homologue and a *fork head*/*HNF-3*β homologue arose. Subsequently, this pathway was conserved, but co-opted for a variety of functions throughout metazoan evolution.

Finally, the expression of *HyBra1* in the endoderm provides evidence for the question of the evolutionary origin of the mesoderm. From comparative embryological studies, it has

been proposed that the mesoderm arose from the endoderm in the diploblast-to-triploblast transition (e.g. Siewing, 1969). We note that the expression of the hydra orthologue in the endoderm is consistent with this idea.

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