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Divergent functions of two ancient *Hydra Brachyury* paralogues suggest specific roles for their C-terminal domains in tissue fate induction

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Homologues of the T-box gene *Brachyury* play important roles in mesoderm differentiation and other aspects of early development in all bilaterians. In the diploblast *Hydra*, the *Brachyury* homologue *HyBra1* acts early in the formation of the hypostome, the location of the organiser in adult *Hydra*. We now report the isolation and characterisation of a second *Brachyury* gene, *HyBra2*. Sequence analysis suggests that *HyBra1* and *HyBra2* are paralogues, resulting from an ancient lineage-specific gene duplication. We show that both paralogues acquired novel functions, both at the level of their cis-regulation as well as through significant divergence of the coding sequence. Both genes are expressed in the hypostome, but *HyBra1* is predominantly endodermal, whereas *HyBra2* transcripts are found primarily in the ectoderm. During bud formation, both genes are activated before any sign of evagination, suggesting an early role in head formation. During regeneration, *HyBra1* is an immediate-early response gene and is insensitive to protein synthesis inhibition, whereas the onset of expression of *HyBra2* is delayed and requires protein synthesis. The functional consequence of HyBra1/2 protein divergence on cell fate decisions was tested in *Xenopus*. *HyBra1* induces mesoderm, like vertebrate Brachyury proteins. By contrast, *HyBra2* shows a strong cement-gland and neural-inducing activity. Domain-swapping experiments show that the C-terminal domain of HyBra2 is responsible for this specific phenotype. Our data support the concept of sub- and neofunctionalisation upon gene duplication and show that divergence of cis-regulation and coding sequence in paralogues can lead to dramatic changes in structure and function.

KEY WORDS: *Brachyury*, *Hydra*, Gene duplication, Head formation, Regeneration, Mesoderm, Evolution, Structure function analysis, *Xenopus*, Animal cap

INTRODUCTION

The question of how morphological complexity increased during animal evolution has been revitalised with the recent sequencing of genomes and transcriptomes of simple metazoans, such as cnidarians. These studies have revealed that the vast majority of important developmental genes known in vertebrate systems appeared very early during evolution in the common ancestor of cnidarians and bilaterians 600-700 million years ago (Kusserow et al., 2001; Kortschak et al., 2003; Technau et al., 2005; Miller et al., 2005; Putnam et al., 2007). In contrast to the apparent morphological simplicity of the Cnidarians it appears that there is no significant change in the gene set between basal cnidarians and vertebrates (Technau et al., 2005; Putnam et al., 2007). One factor that adds to the complexity of cnidarian genomes is the relatively high number of independent gene duplications (Technau et al., 2005; Chourrout et al., 2006). Gene duplications are thought to be one of the major driving forces of evolution. Although many of the duplicated genes are predicted to end up as pseudogenes, some contribute significantly to the evolution of developmental processes by establishing novel interactions and functions. In principle, a fully functional copy of a gene reduces the selection pressure significantly, allowing for rapid divergence of one or both copies of the gene (Ohno, 1970; Cooke et al., 1997; Krakauer and Nowak, 1999). Comparisons of paralogous genes and dominance of mutations in yeast and humans suggest that haploinsufficient genes, whose loss-of-function alleles are dominant over wild-type alleles, are more likely to be fixed after gene duplication than haplosufficient (i.e. dominant wild-type) genes (Kondrashov and Koonin, 2004). Interestingly, haploinsufficient genes include predominantly developmental regulators, structural and regulatory proteins and transcription regulators.

Predictions surrounding the fate of duplicate genes over long evolutionary time scales have been made in theoretical studies (Cooke et al., 1997; Force et al., 1999). The Duplication-Degeneration-Complementation model of Force and colleagues focuses on the evolution of cis-regulatory regions after the duplication event, although it does not explicitly exclude a divergence of the coding sequence (Lynch and Force, 2000). The detailed experimental analysis of paralogous genes and the degree of conservation and diversification of functions might therefore provide insight into metazoan evolution. Furthermore, in the case of important developmental pathways, paralogous genes might contribute to the evolution of animal body plans.

One of these important developmental genes encodes the T-box transcription factor *Brachyury* (Herrmann et al., 1990). *Brachyury* has a conserved role in mesoderm differentiation, and elongation of the posterior body axis in all vertebrates (for reviews, see Smith, 1997; Smith, 1999; Naiche et al., 2005). Comparison of basal deuterostome and protostome larvae, as well as diploblastic cnidarians, indicates that *Brachyury* has an ancestral function in defining the blastopore (Arendt

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et al., 2001; Scholz and Technau, 2003) (for a review, see Technau, 2001), although the precise cellular function can vary in the different animal lineages (e.g. Wilkinson et al., 1990; Gross and McClay, 2001; Lartillot et al., 2002). Interference with *Brachyury* function in vertebrates inhibits convergent extension, causes apoptosis, and reveals separate requirements in the Fibroblast growth factor (FGF)- and Activin-signalling pathways (Conlon and Smith, 1999).

Although *Brachyury* is considered an early panmesodermal marker in vertebrates, this 'mesodermal' gene also occurs in diploblasts, as first reported in the freshwater cnidarian *Hydra* (Technau and Bode, 1999). The *Hydra Brachyury* homologue *HyBra1* is expressed in the hypostome, the most apical part of the polyp, between the tentacle ring and the mouth opening (Technau and Bode, 1999). The hypostome is the location of the head organiser of the animal (Browne, 1909; Broun and Bode, 2002), which is considered equivalent to the Spemann organiser in amphibians (Spemann und Mangold, 1924). Comparative evolutionary studies in basal cnidarians indicate that the oral end of the *Hydra* polyp derives from the blastopore (Scholz and Technau, 2003). Under all developmental circumstances, i.e. bud formation, head regeneration, reaggregation and embryogenesis, *HyBra1* is expressed very early at the site of future hypostome formation, suggesting an early role in head organiser/head formation in *Hydra* (Technau and Bode, 1999; Technau et al., 2000; Broun and Bode, 2002).

T-Box genes have never been reported from unicellular organisms. It is therefore likely that this class of developmental control genes evolved with multicellularity. Two T-box genes, *tbx2* and *Brachyury*, have been isolated from sponges (Adell et al., 2003; Manuel et al., 2004). T-box genes fall into a number of subfamilies with Brachyury as the most distinct subfamily (Papaioannou and Silver, 1998; Papaioannou, 2001; Wilson and Conlon, 2002). Most animals appear to have only one *Brachyury* gene; however, a few organisms, including vertebrates, have two or more paralogous *Brachyury* genes. Where studied, these paralogues are highly similar in sequence and have mostly overlapping expression domains (Strong et al., 2000; Knezevic et al., 1997; Hayata et al., 1999), suggesting rather recent gene duplication events.

In this paper, we report the isolation of a second *Hydra Brachyury* gene, which appears to be the result of an ancient gene duplication event. Our phylogenetic analysis suggests that the duplication event most likely occurred after the divergence of cnidarians. We show that *HyBra1* and *HyBra2* occupy distinct expression domains in the hypostome of the polyp, and show very different dynamics of expression during head regeneration. This indicates an evolution of cis-regulatory elements. To investigate the impact of coding-sequence evolution between the two paralogues, we tested the function of both *Hydra* genes in a heterologous assay system: the *Xenopus* animal caps. This assay revealed strikingly different inductive capacities for *HyBra1* and *HyBra2*. Domain-swapping experiments indicate that their different activities are defined largely by their divergent C-terminal domains. Our data show that the *Hydra Brachyury* paralogues have undergone a mixture of subfunctionalisation and neofunctionalisation and the evolution of *HyBra* gene function occurred both at the cis-regulatory and the protein levels.

MATERIALS AND METHODS

Animals and embryological experiments

All *Hydra* experiments were carried out with polyps of the Basel and AEP strains of *Hydra vulgaris*. One-day-starved animals were used for all experiments. *Hydra* polyps were cultured as previously described (Technau and Holstein, 1996). *Xenopus* embryos were in vitro fertilised, de-jellied and cultured by standard methods (Sive et al., 1989). At the four-cell stage, each cell was microinjected with 2.5 nl volume into the animal hemisphere. RNA doses ranged from 0.1 to 1.0 ng/embryo, and were adjusted to result in roughly comparable protein amounts, as judged by western blot analysis of embryos injected with Myc-tagged protein variants. Animal caps were dissected using Dumont N°5 forceps between stages 8.5 and 9 (Nieuwkoop and Faber, 1967) and cultivated at 23°C in 0.5× MBS containing 10 μg/ml Gentamycin until stage 36 (hatching).

Cloning of *HyBra1* and *HyBra2*

Fragments of the T-box were cloned by PCR using degenerate primers (forward 5′-TTYGGNGMNCAYTGGATG-3′ and reverse 5′-RAANS-CYTTNGCRAANGG-3′) at low annealing temperatures. Random-primed probes of these *HyBra1* and *HyBra2* T-box fragments were used to screen a Lambda-Zap cDNA library (Stratagene) using standard protocols (Sambrook et al., 1989). A full-length clone of *HyBra2* was obtained from the screen of the cDNA library. To obtain the 3′ end of *HyBra1* 3′ RACE experiments were performed as described (Frohman et al., 1988). GenBank accession numbers of *HyBra1* and *HyBra2* full-length clones are (AY366371, AY366372), respectively.

DNA constructs and in vitro transcription

For RNA microinjection experiments, the *Xbra* cDNA was subcloned into the pRNA3 vector; *Hybra1* and *Hybra2* coding regions were cloned by PCR into *Bam*HI/*Xba*I sites of pCS2$^+$ and *Stu*I/*Xba*I sites of pCS2$^+$MT6 respectively. The chimeric constructs *XTH2A* and *H2TXA* were generated by subsequent subcloning of the respective PCR products. For both chimerae, the conserved amino acid motif AKAFL-DAKER was used as a junction. Capped in vitro transcripts were generated from *Sfi*I-linearised plasmids and T3 RNA polymerase (*HyBra* chimerae and *Xbra*), or by *Not*I linearisation and Sp6 RNA polymerase (wt *HyBra* paralogues).

Phylogenetic analysis

ClustalW was used to align amino acid sequences of the T-box proteins. Maximum likelihood program PUZZLE (Schmidt et al., 2002) and MrBayes (Huelsenbeck and Ronquist, 2001) were used to perform a phylogenetic tree analysis. In the Puzzle analysis 1000 replica were computed using JTT as a substitution model, assuming a strong rate heterogeneity in the gamma distribution. In MrBayes 3.1, 1,7 Mio generations were run using the WAG model of substitution and eight gamma rate categories.

RNA in situ hybridisation

Hydra in situ hybridisation was carried out as previously described (Grens et al., 1995) with minor modifications. We omitted the elevated temperature step [80°C, and for detection we used NBT/BCIP instead of BMpurple (Roche)]. *Xenopus* double in situ hybridisations were performed as described (Wittenberg et al., 1999) with a primary Fast Red stain for the fluorescein-labelled probe terminated by heat inactivation for 30 minutes at 65°C in 0.1 M EDTA-TBS buffer, followed by a BM-Purple (Roche) stain for the digoxygenin-labelled probe.

Protein synthesis assay

To assay for protein synthesis, 50 μCi [^{35}S]methionine was injected into the gastric cavity of adult polyps, and incubated for 1 hour. Thereafter, five polyps were dissolved in 100 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 2% SDS, 100 mM β-mercaptoethanol). After lysis, an equal volume of 1 mg/ml BSA was added and 100 μl lysate was spotted and air-dried on a glass fibre filter (Whatman GF/A 2.4 cm; #1820024). Then the filter was rinsed twice with 3 ml ice-cold 20% trichloroacetic acid (TCA) for 5 minutes, followed by two washes in 12.5% TCA to remove unincorporated labeled amino acids. Filters were then washed twice with 95% ethanol followed by one wash with acetone, air dried, placed in scintillation vials with 1 ml scintillation fluid and the total number of counts determined. As a negative control, five unlabeled animals were lysed and then 50 μCi [^{35}S]methionine was added to the lysate.

Inhibition of translation by cycloheximide

Polyps were incubated in 5 or 50 μg/ml cycloheximide (Sigma) in *Hydra* medium (HM) for 15 minutes, 1 hour or 2 hours. After treatment, polyps were washed five times in HM to remove traces of cycloheximide. To inhibit

translation during regeneration, animals were pretreated with cycloheximide for 15 minutes and subsequently decapitated at t_0 beneath the tentacles. Regenerates were further incubated in cycloheximide for 2 hours, washed five times and allowed to regenerate for the times indicated.

Alsterpaullone treatment

Alsterpaullone treatment was performed as described (Broun et al., 2005) with some minor modifications. Polyps were treated for 24 hours in 5 μM alsterpaullone (Sigma), then washed extensively and further incubated in HM for 15 hours and 40 hours, respectively. Specimens were then fixed and subjected to in situ hybridisation. DMSO at the corresponding concentration was used as a control with no effect.

RESULTS

Isolation and characterisation of two complete *Brachyury* orthologues from *Hydra*

A partial *HyBra1* clone has already been isolated (Technau and Bode, 1999). Using 3′ RACE we have now obtained the 1845-bp-long full-length clone with an open reading frame of 364 amino acids. By degenerate PCR and cDNA library screening, we isolated an additional full-length T-box gene. The clone consists of 1423 bp, encoding a protein of 388 amino acids (Fig. 1C). Phylogenetic analysis revealed that this gene also clearly belongs to the *Brachyury* subfamily of T-box genes and we therefore named it *HyBra2* (Fig. 2). The T-domains of the two *HyBra* genes show about 70% identity (Fig. 1A). When compared with the T-domain of vertebrate Brachyury proteins, HyBra1 and HyBra2 show about 80% and 70% identities, respectively (Fig. 1A,C). By contrast, the remaining domains are far less conserved, with an overall amino acid identity of <20% (data not shown). Interestingly, two Brachyury protein domains, one positioned N-terminally of the T-box and the other one named R1, which is embedded within the activation domain, have been gained and lost during evolution (Marcellini et al., 2003; Kispert et al., 1995). Whereas the N-terminal domain is absent in non-bilaterian Brachyury proteins, and was therefore probably acquired at the base of the bilaterians (Marcellini, 2006), the ancient R1 fragment in the C-terminal domain is already present in cnidarians, where it is found in HyBra1 and *Nematostella* Brachyury (Scholz and Technau, 2003), but not in HyBra2. Strikingly, both domains have been lost in Tunicates and arthropods but are conserved in all other bilaterian paralogues (Marcellini, 2006).

Sequence comparison further revealed that *HyBra2* carries a spliced leader (SL-B) at the 5′ end (Stover and Steele, 2001), whereas no spliced leader is present in the isolated *HyBra1* cDNA (data not shown). Genome walking analysis of the publicly available shotgun sequences of the *Hydra* genome project (Craig J. Venter Institute, MD, USA) showed that the two *HyBra* paralogues are not

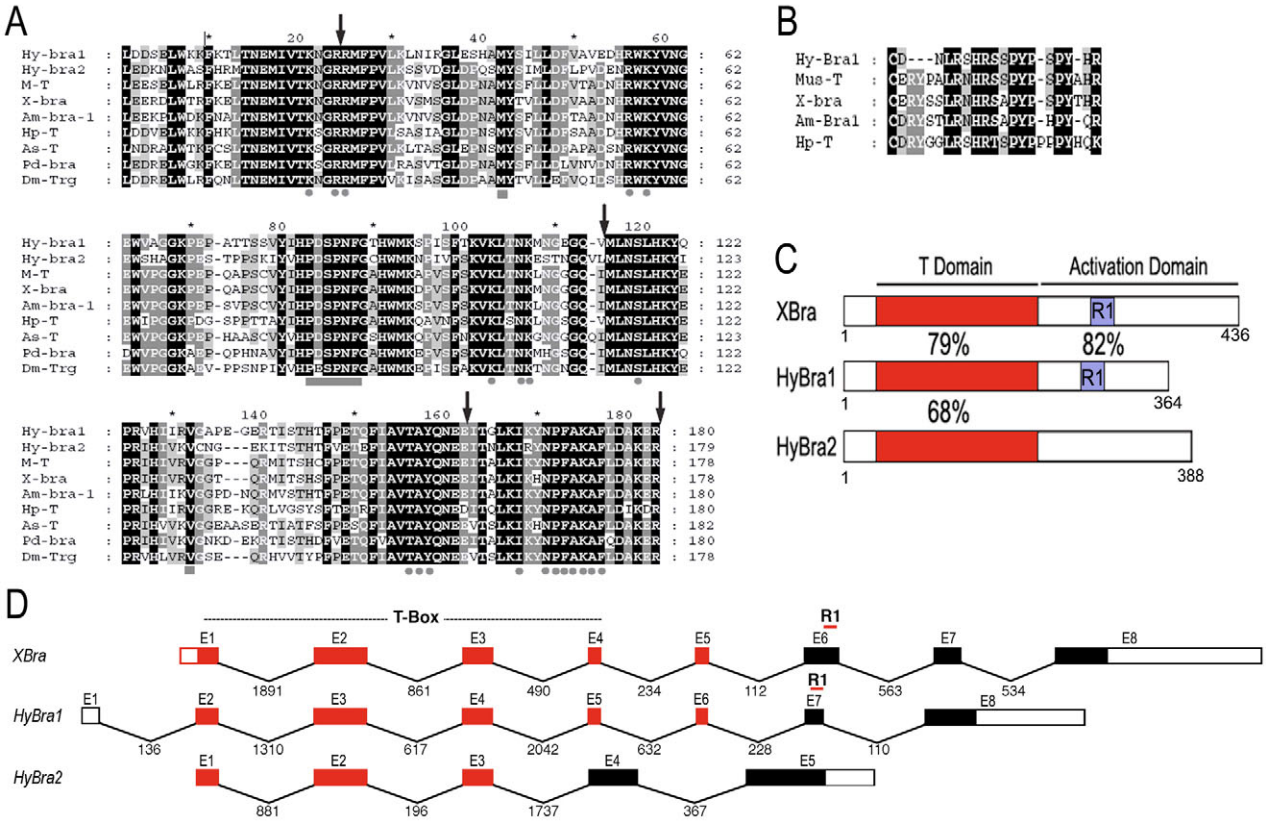

Fig. 1. Comparison of protein and DNA sequence conservation of HyBra1 and HyBra2. (**A**) Alignment of T-domains of HyBra1 and HyBra2 with Brachyury proteins from mouse (M-T), *Xenopus* (X-bra), Amphioxus (Am-bra-1), the sea urchin *Hemicentrotus pulcherrimus* (Hp-T), the ascidian *Halocynthia roretzi* (As-T), *Platynereis dumerilii* (Pd-bra) and *Drosophila melanogaster* (Dm-Trg). Amino acid identities are black, partial identities (>60%) in grey. Amino acids marked by grey dots under the sequence are involved in DNA-binding, grey bars indicate amino acids involved in dimerisation (Müller and Herrmann, 1997). Arrows indicate conserved intron sites in *HyBra1* and *mouse-T*. (**B**) Alignment of the core repression module R1 (Kispert et al., 1994) within the activation domain. *HyBra2* and *Drosophila* Trg do not have this motif. (**C**) Schematic structures of Xbra, HyBra1 and HyBra2 protein illustrate the degree of amino acid identity in the T-box and the R1 domain (blue box) in *HyBra1* and *HyBra2* compared with XBra. (**D**) Comparison of exon-intron structure *in Xbra*, *HyBra1* and *HyBra2*. Filled boxes mark the ORF, white boxes the UTR. Red boxes indicate conserved exon-intron sites, whereas black boxes indicate non-conserved regions. Black numbers represent the length of introns.

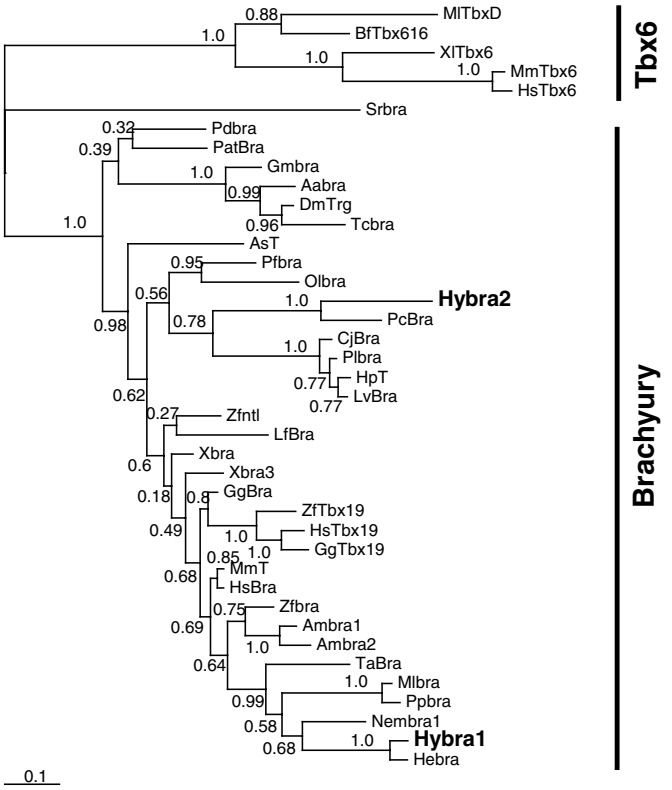

Fig. 2. Phylogenetic analysis of Brachyury proteins on the basis of their conserved T-box domain. A group of Tbx6 genes was used as an outgroup for the Brachyury family. Analysis was performed with MrBayes 3.1.2 using the WAG substitution model. Sequences were obtained from GenBank. Aa, *Aedes aegypti*; Am, Amphioxus (*Branchiostoma floridae*); As, *Halocynthia roretzi*; Bf, *Branchiostoma floridae*; Cj, *Clypeaster japonicus*; Gg, *Gallus gallus*; Gm, *Grillus maculatus*; He, *Hydractinia echinata*; Hp, *Hemicentrotus pulcherrimus*; Hs, *Homo Sapiens*; Lf, *Lampetra fluviatilis*; Lv, *Lytechinus variegatus*; Ml, *Mnemiopsis leidyi*; Mm, *Mus musculus*; Nem, *Nematostella vectensis*; Ol, *Oikopleura longicauda*; Pat, *Patella vulgata*; Pc, *Podocoryne carnea*; Pd, *Platynereis dumerilii*; Pf, *Ptychodera flava*; Pl, *Paracentrotus lividus*; Pp, *Pleurobrachia pileus*; Sr, *Scypha raphanus*; Ta, *Trichoplax adhaerens*; Tc, *Tribolium castaneum*; X, *Xenopus laevis*; Zf, *Zebrafish*.

closely linked in the genome. Interestingly, when comparing *HyBra1* and *Xbra*, five out of six intron positions in the coding sequence are conserved, four of which reside in the T-box and are conserved throughout the animal kingdom. These intron sites are also conserved in *HyBra2* except the last one linking the T-box to the C-terminal domain (Fig. 1D). In summary, the structural similarities in the T-box, the conserved R1 motif and the arrangement of introns indicate that the ancestral *Brachyury* gene structure of *HyBra1* has been conserved throughout evolution and the *HyBra2* paralogue is derivative in various aspects.

HyBra1 and *HyBra2* are ancient lineage-specific paralogues

Since *HyBra1* is more similar to vertebrate *Brachyury* genes than to its cognate partner gene *HyBra2*, one possibility was that the duplication event occurred before the split of Cnidaria and Bilateria, with maintenance of one or the other (or both) among the Bilateria. So far, paralogous *Brachyury* genes have been reported for humans, *Xenopus*, chick and Amphioxus. To test the possibility that these paralogues are descendants of either *HyBra1* or *HyBra2*, respectively, we compared all paralogous Brachyury proteins in a phylogenetic analysis. We also included three other identified Brachyury proteins from different Cnidaria and one from the sponge *Suberites* (Adell et al., 2003) (Fig. 2). In this more extensive analysis, the cnidarian Brachyury proteins are divided in two separate clusters. HyBra1 clusters with Brachyury homologues from *Nematostella* (Scholz and Technau, 2003) and from a marine relative, the hydrozoan *Hydractinia echinata*, whereas HyBra2 forms a cluster with a Brachyury from the hydrozoan *Podocoryne carnea* (Spring et al., 2002). However, none of them were grouped with any of the paralogues from the chordates, nor did the bilaterian Brachyury proteins fall into two distinct (bra1 and bra2) subclasses, although lower Deuterostomes tended to cluster with HyBra2, whereas vertebrate sequences are all closer to HyBra1. However, both HyBra1 and HyBra2 are more similar to deuterostome than protostome Brachyury proteins, reflecting the divergence in the different lineages. Since the basal cnidarian *Nematostella vectensis* has only a *Bra1*-type gene (Scholz and Technau, 2003), this analysis is consistent with the view that *HyBra1* and *HyBra2* represent two ancient paralogues that evolved by a duplication event. Most likely, this event occurred early in cnidarian evolution, after the divergence of the hydrozoan lineage approximately 550 million years ago. It is also clear, that according to this scenario, *HyBra2* has undergone a more rapid rate of sequence evolution.

Expression pattern of *HyBra1* and *HyBra2* in adult polyps and during bud formation

We previously showed that *HyBra1* is expressed in the hypostome, the apical tip of *Hydra* (Technau and Bode, 1999). During various developmental conditions, such as head regeneration, bud formation (the asexual form of *Hydra* reproduction), the formation of hydra from aggregates of cells, and embryogenesis, it is expressed very early during head formation (Technau and Bode, 1999; Technau et al., 2000). In situ hybridisation showed that *HyBra2* was expressed in the hypostome of *Hydra* polyps in a very similar pattern to that of *HyBra1* (Fig. 3A,C). However, vibratome sections of adult polyps revealed that *HyBra2* was expressed primarily in the ectodermal layer whereas *HyBra1* was expressed predominantly in the endoderm (Fig. 3B,D). A comparison of various bud stages showed that *HyBra2* was expressed just as early as *HyBra1* as a spot in the ectoderm at stage 1 (Fig. 3E,H). In subsequent stages of bud formation, *HyBra2*, like *HyBra1*, remained restricted to the apical tip of the bud, which is the future hypostome (Fig. 3F,I). Early stages, as well as vibratome sections of later bud stages, confirmed that *HyBra1* was primarily expressed in the endoderm (Fig. 3G), whereas *HyBra2* was predominantly expressed in the ectoderm (Fig. 3J). This suggests that *HyBra2*, like *HyBra1*, plays an early role in head formation during bud formation, but acts mainly in the other germ layer.

HyBra1 and *HyBra2* expression is differently regulated during regeneration

We next analysed *HyBra1* and *HyBra2* expression during head regeneration in animals cut at apical and mid-gastric positions of the body column. As shown previously (Technau and Bode, 1999), *HyBra1* appears in the regenerating head of animals bisected at the apical end of the body column within 1-3 hours of head removal (Fig. 4A; Fig. 5A-C). In sharp contrast, *HyBra2* was first expressed at apical levels after roughly 8-10 hours (Fig. 4A; Fig. 5G-I). There was a substantial variability from animal to animal in the onset of *HyBra2* expression.

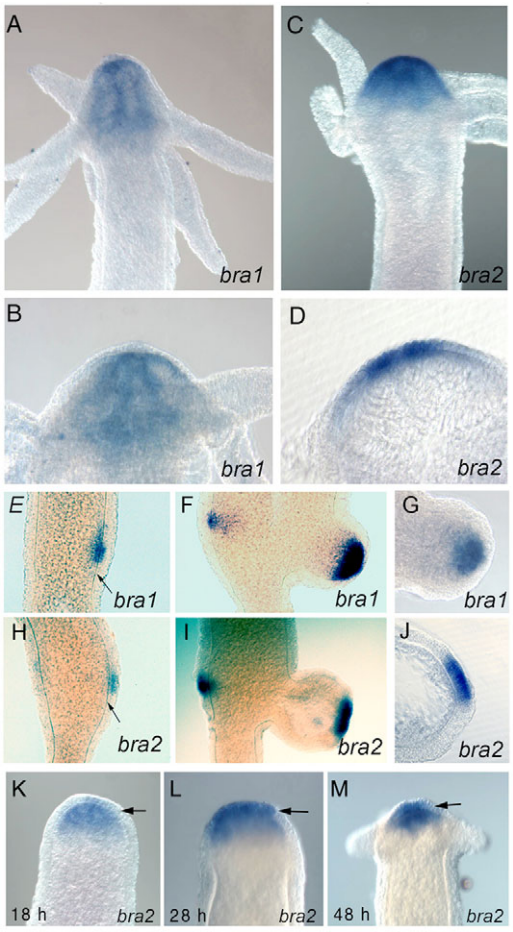

Fig. 3. Expression of *HyBra1* and *HyBra2*. *HyBra1* and *HyBra2* mRNA expression was assessed in adult polyps (**A-D**) and during budding (**E-J**). (A,B) In adult hydras *HyBra1* is predominantly expressed in the endodermal layer of the hypostome. (C,D) *HyBra2* expression was primarily detected in the ectoderm of the hypostome. (B,D) Vibratome cross-sections of the hypostome. (E-J) Both genes are expressed very early during budding, but *HyBra1* RNA is predominantly in the endoderm, whereas *HyBra2* expression is restricted to the ectoderm (arrows in E and H). (J) Vibratome section of stage 5 bud. (**K-M**) Switch of *HyBra2* expression from the endoderm to the ectoderm during head regeneration. Expression is in the endoderm at 18 hours (K), in both layers at 28 hours (L) and in the ectoderm by 48 hours (M). Arrows indicate the mesoglea, which separates ectoderm from endoderm.

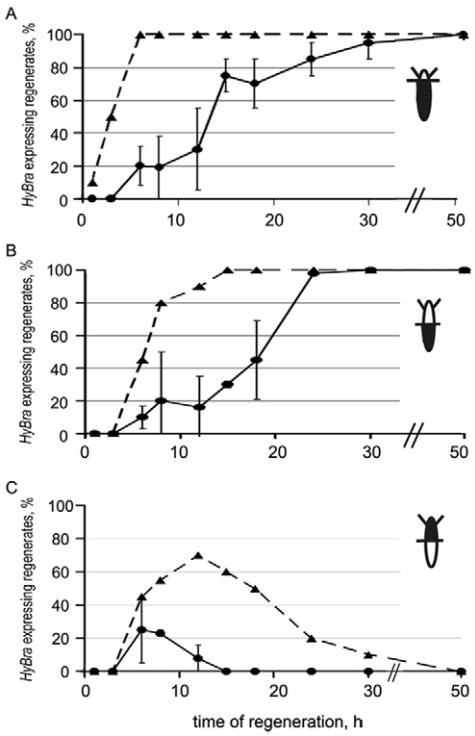

Fig. 4. Kinetics of *HyBra1* and *HyBra2* expression during regeneration. Expression of *HyBra1* (dashed line) and *HyBra2* (solid line) during head regeneration following bisection at (**A**) the upper end of the body column, and (**B**) in the middle of the body column. (**C**) Expression of the two genes during foot regeneration following bisection in the mid-body column. Data points are means (± s.d.) of 40-80 animals resulting from 2-5 independent experiments. *HyBra1* data modified after Technau and Bode (Technau and Bode, 1999).

Interestingly, *HyBra1* initially appears several hours later in animals bisected in the middle of the body column (Fig. 4B) compared with animals bisected at the apical end (Fig. 4A) (Technau and Bode, 1999). This delay is consistent with the delay in head formation at this level and a lower level of the head activation gradient (Bode and Bode, 1984). By contrast, there was only a slight delay in the onset of *HyBra2* expression in regenerating heads following mid-column bisection compared with those bisected near the head (Fig. 4B). Furthermore, the apical half of these bisected animals transiently, but strongly expressed *HyBra1* at the site of foot regeneration, whereas *HyBra2* is expressed very weakly, and only in a few individuals (Fig. 4C; H.B. and U.T., unpublished). Hence, *HyBra1* is among the earliest genes expressed during head regeneration, and is transiently upregulated during foot regeneration, whereas *HyBra2* appears somewhat later and is expressed only where a head is being formed.

Another striking difference between the two genes occurs in the tissue layer in which they are expressed during head regeneration. At about 10 hours after bisection, the initial expression of *HyBra2* occurred in the endodermal layer (Fig. 3K). Ectodermal expression first became detectable at the most apical tip after about 24 hours, and then slowly spread over the hypostome (Fig. 3L,M). Endodermal expression persisted for 2-3 days of regeneration and eventually vanished. This shows that *HyBra2* expression is differently regulated during head regeneration compared with expression in budding and adult *HyBra2* expression. It indicates that the two processes, although sharing many common features, differ at the molecular level.

HyBra1 is an immediate-early response gene during head regeneration

HyBra1 is one of the earliest genes expressed during head regeneration and bud formation (Technau and Bode, 1999). This raised the question whether initiation of *HyBra1* expression depends on the synthesis of another factor to activate its expression. To address this question we analysed the expression of *HyBra1* and *HyBra2* during regeneration in the presence of the translational inhibitor cycloheximide (CHX) (Cascio and Gurdon, 1987). We first established that a treatment with 5-50 μg/ml CHX for up to 2 hours leads to slight but reversible phenotypic effects. Under these conditions, the reappearance of tentacles after head removal is delayed by 12-18 hours (data not shown). Treatment for up to 6 hours was also tolerated by the animals, but had more severe effects.

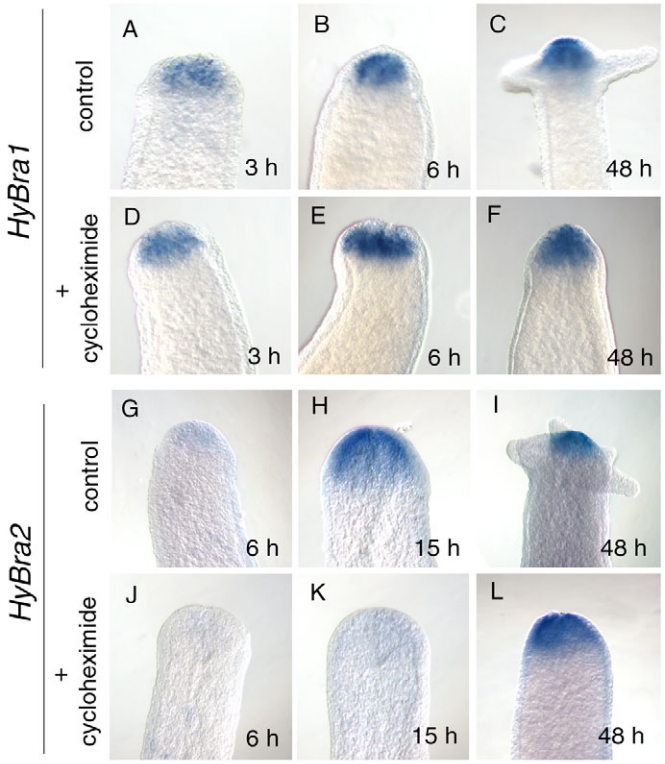

Fig. 5. Expression of *HyBra1* and *HyBra2* during regeneration in the presence or absence of cycloheximide. Animals were pretreated for 15 minutes in 5 μg/ml cycloheximide, decapitated and allowed to regenerate for the times indicated. The first two hours of regeneration were carried out in the presence of the cycloheximide. (**A-C**) *HyBra1* expression under control conditions. (**D-F**) The onset of *HyBra1* is not affected by cycloheximide. *HyBra2* expression in cycloheximide-treated animals (**J-L**) starts later than in control animals (**G-I**).

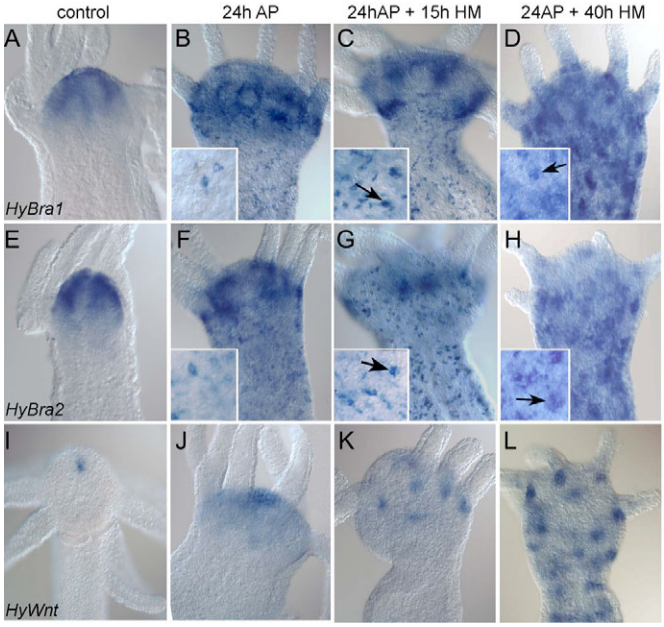

Fig. 6. Ectopic expression of *HyBra1* and *HyBra2* in response to alsterpaullone treatment. Animals were exposed to 5 μM alsterpaullone (AP) for 24 hours and subsequently transferred to Hydra media (HM) for 15 hours and 40 hours respectively. *HyBra1* (**A-D**), *HyBra2* (**E-H**) and *Wnt* (**I-L**) are affected by the GSK-3 inhibitor alsterpaullone. Ectopic *HyBra1* and *HyBra2* expression starts 24 hours after AP treatment in single cells (B,F, high-magnification inset) whereas normal hypostome expression declines rapidly and is shifted to the tentacle bases (compare A,B with E,F); elevated levels of expression are seen over the uniform endodermal expression (compare A,E with B,F). In later stages, the single cell expression in the body column begins to cluster (arrows in C and G) and subsequently peaks in a stochastic pattern of spots of 10-15 epithelial cells (arrows in G and H). *HyWnt* hypostome expression is lost 15 hours after completion of AP treatment and clusters along the body column in ectopic expression domains are narrower than those of *HyBra1* and *HyBra2*.

We then measured the inhibition of translation by incorporation of [^{35}S]methionine. We found that after treatment with either 5 or 50 μg/ml CHX for 15 minutes, translation was inhibited by 80-90%. Thereafter, the rate of protein synthesis slowly recovered over the next 2 days (data not shown). Treatment for 2 hours resulted in the 80-90% level of inhibition being maintained for 6 hours, followed by a slow recovery (data not shown).

We then performed a regeneration experiment in the presence of CHX. After a pre-treatment of 15 minutes, polyps were decapitated at apical levels and allowed to regenerate in the presence of CHX for 2 hours. Thereafter, they were allowed to regenerate in *Hydra* medium. Periodically, after end of the CHX treatment, polyps were fixed and analysed for *HyBra1* and *HyBra2* expression by in situ hybridisation. We found that *HyBra1* was expressed as early and strongly as in untreated controls (2-3 hours; Fig. 5A-F). By contrast, the onset of *HyBra2* expression was delayed compared with the controls by about 8-10 hours (Fig. 5G-L). This shows that transcriptional activation of *HyBra2*, but not of *HyBra1* requires protein synthesis during head regeneration.

One possible candidate protein to regulate *HyBra1* expression is β-catenin (Hobmayer et al., 2000). It is expressed ubiquitously, transcriptionally upregulated during regeneration and stabilised in the nucleus at the apical end of *Hydra* (Hobmayer et al., 2000; Broun et al., 2005). Indeed, we obtained support for such a role of β-catenin by treatment with the Glycogen synthase kinase 3 β (GSK3β) inhibitor alsterpaullone (Broun et al., 2005). Inhibition of GSK3β results in stabilisation and nuclear translocation of β-catenin, where it can act, in concert with T-cell factor (TCF), as a transcriptional regulator (Leost et al., 2000; Bain et al., 2003). Treatment with alsterpaullone for 24 hours followed by incubation in *Hydra* medium for 15-40 hours leads to an ectopic upregulation of both *HyBra1* and *HyWnt* genes in the body column (Fig. 6), confirming earlier results (Broun et al., 2005). Like *HyBra1*, *HyBra2* is also upregulated broadly in response to alsterpaullone treatment (Fig. 6E-H). *HyWnt* and both *HyBra* genes are downregulated at the same time in the hypostome (Fig. 6; data not shown). Interestingly, 15 hours after treatment ectopic upregulation of *HyBra1* and *HyBra2*, but not of *HyWnt*, in the body column is detectable in single cells, which appear to be interstitial cells (Fig. 6B,C,F,G). After 40 hours of alsterpaullone treatment, both *HyBra1* and *HyBra2* are strongly upregulated throughout the endoderm of the gastric cavity, with peaks of expression of regular spacing consisting of 10-15 epithelial cells also visible in the ectoderm (Fig. 6D,H). Ectopic *HyWnt* expression is found in similar spots but does not expand to ubiquitous upregulation in the endoderm (Fig. 6L) (Broun et al., 2005). This suggests that *HyBra1* and *HyBra2* are direct or indirect targets of the Wnt–β-catenin pathway.

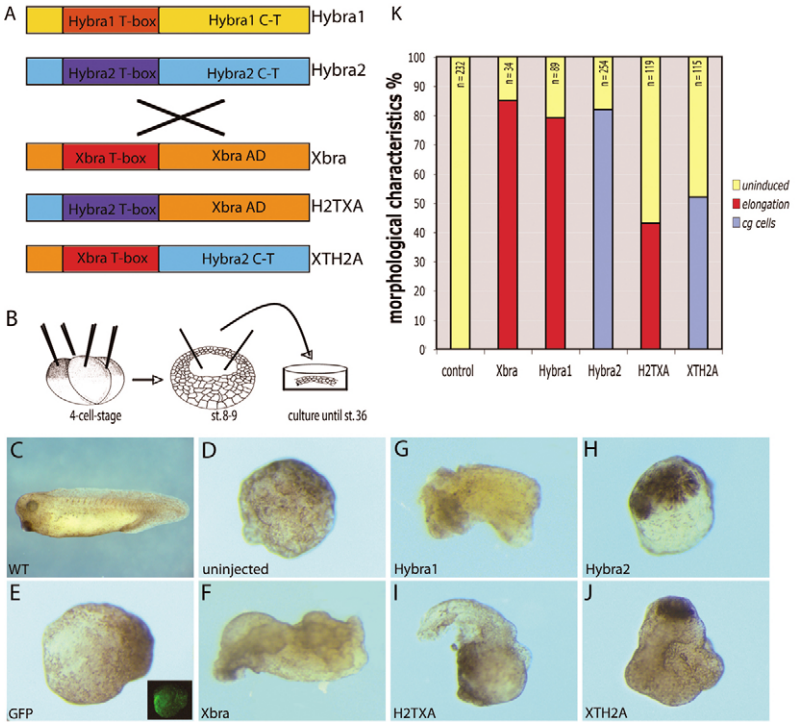

Fig. 7. Distinct morphological phenotypes induced in *Xenopus* animal caps by *Hydra Brachyury* proteins. (**A**) Schematic view of the microinjected constructs (AD, activation domain; C-T, C-terminal domain). (**B**) Sketch of the animal cap experiment (st, stage). (**C-E**) Control specimens: (C) wt sibling embryo at NF36, (D) uninjected and (E) *GFP*-injected animal caps with default epidermal morphology. Animal caps injected with *Xbra* (**F**), *HyBra1* (**G**) or *H2TXA* (**I**) with elongated morphology, indicating mesoderm formation. (**H**) *HyBra2*-injected animal cap with two darkly pigmented cement gland areas. (**J**) *XTH2A*-injected explant with single cement gland area. (**K**) Statistical overview of morphological characteristics [uninduced, elongated and cement gland celltype-specific (cg cells)] induced by different *Brachyury* paralogues (3-10 independent injections). RNA doses were titrated from 0.1 to 1.0 ng/embryo, resulting in consistent, qualitatively different phenotypes represented by the displayed specimen.

Structure-function relationship in animal cap assays for mesoderm induction

We were interested whether the high degree of sequence divergence between the two *HyBra* paralogues reflects two different functions. To test this, we used a well-defined in vivo system, the *Xenopus* animal cap. Untreated animal caps differentiate into epidermal tissue, but can be forced to form mesoderm if injected at the two- to four-cell stage with *Xenopus bra* mRNA (Smith et al., 1991). Injection of mRNAs encoding other T-box genes such as *VegT* or *eomesodermin*, induces endoderm in addition to mesoderm, indicative of the endogenous functions of these proteins (Marcellini et al., 2003; Conlon et al., 2001; Zhang and King, 1996; Zhang et al., 1998). Other factors, such as *otx2* and *ath-3* have been shown to induce differentiation of neural tissue in animal caps (Gammill and Sive, 1997; Takebayashi et al., 1997). Hence, animal caps can display a variety of very distinct and specific differentiation fates in response to an exogenous transcription factor provided by mRNA microinjection. Notably, *Brachyury* mRNAs from a wide range of bilaterian species induce mesoderm under these assay conditions (Marcellini et al., 2003). Thus, this assay can be used to rapidly identify functionally crucial domains or motifs generated by sequence divergence during animal evolution.

We first injected *Xbra*, *Hybra1* and *Hybra2* mRNA into four-cell-stage embryos, dissected animal caps and cultured them until the hatching stage, when we scored morphological (Fig. 7) and molecular phenotypes (Fig. 8). Uninjected or *GFP*-preloaded explants were round and lacked any sign of non-ectodermal differentiation (Fig. 7D,E; Fig. 8A,B). The large majority of *HyBra1*- or *Xbra*-injected caps were elongated (Fig. 7G,F) and expressed the striated muscle marker *cardiac actin* (Fig. 8C,E). Under these conditions, *Xbra* and *HyBra1* demonstrate comparable mesoderm inducing activities. By contrast, *HyBra2* never induced elongation or mesodermal marker gene expression, even when very high amounts of mRNA were injected, suggesting that *HyBra2* lacks a common mesoderm-inducing activity (Fig. 7K; Fig. 8M).

Intriguingly, it turned out that *HyBra2* is a highly potent cement gland inducer, even when very low RNA concentrations were injected (Fig. 7H,K; Fig. 8G,M). The *Xenopus* cement gland, which is temporarily formed during the tadpole stage, consists of palisade-like epithelial cells. These cells have a distinct morphological structure and are therefore easy to identify. Of all *Hybra2*-injected caps, 86% contained one or several cement gland-like areas characterised by their regular arrangement of strongly pigmented cells (Fig. 7H,K). Additional evidence that these structures are cement glands comes from two observations. First, like their endogenous counterparts at the anterior end of sibling embryos (Fig. 7C), their surface was highly adhesive, indicating mucus secretion (data not shown). Second, 84% of *Hybra2*-injected caps expressed *Xcg-1* (Sive et al., 1989), a cement-gland-specific marker (Fig. 8G,M). Since there is evidence that cement glands, although epithelial in nature, develop as a consequence of neural induction (Sasai and De Robertis, 1997), we analysed the animal caps for expression of the pan-neural marker *N-CAM* (see Fig. S1 in the supplementary material) and *β-tubulin*, a marker for differentiated neurons (Fig. 8). Although these neural mRNAs were hardly detectable in *Hybra1*- and *Xbra*-injected caps, both markers were strongly expressed in a significant fraction of the *Hybra2*-injected explant (Fig. 8F,H,M and see Fig. S1 in the supplementary material). The absence of muscle and endoderm suggests that this induction results from the direct conversion of ectoderm into neural tissue in response to *HyBra2*. Together, the *Xenopus* animal cap experiments reveal that the activities of *HyBra1* and vertebrate *Xbra* are very similar, but that *HyBra2* has significantly diverged and acquired cement gland and neural-inducing properties.

This unexpectedly distinct, qualitative difference in activity motivated us to look into the domains of the proteins in more detail. We therefore constructed two chimeras, swapping the T-Box and C-terminal domains of Xbra and HyBra2, as shown in Fig. 7A. Surprisingly, animal caps injected with the *H2TXA* chimera contained not a single cement gland, whereas 40% of them were

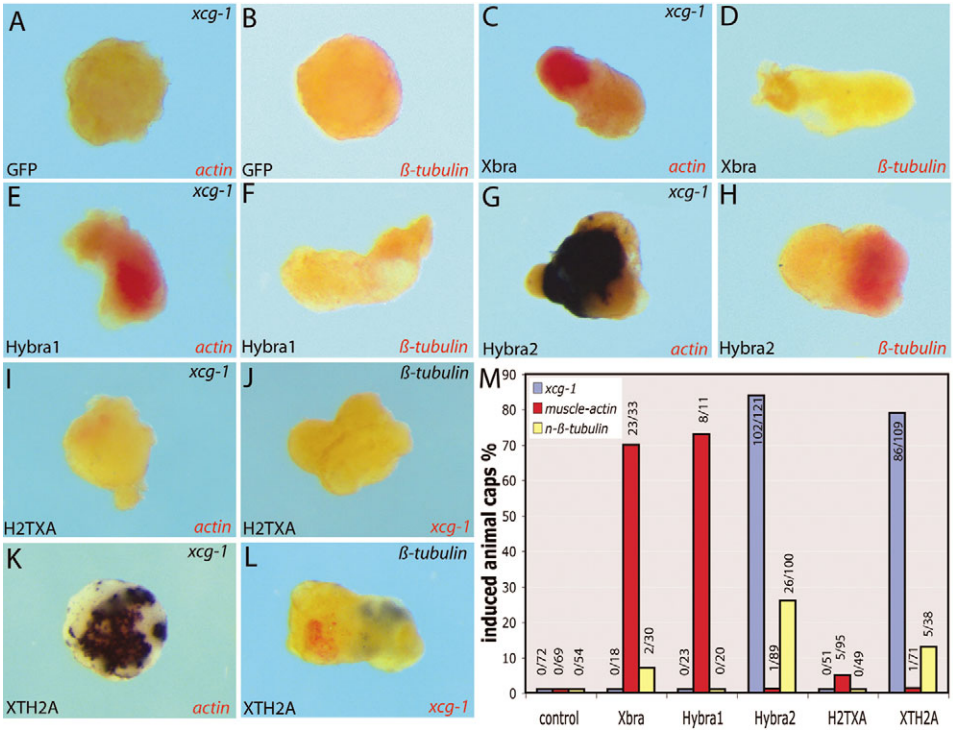

Fig. 8. Molecular analysis of the differentiated animal cap phenotypes induced by HyBra proteins. Marker analysis by double (**A**,**C**,**E**,**G**,**I-L**) or single (**B**,**D**,**F**,**H**) RNA in situ hybridisation; probes are listed within panels, black letters indicate BM-Purple stain, red letters indicate Fast-Red stain ($n \geqslant 3$ independent repeats). (A,B) *GFP*-injected control explants showed no staining of tested marker genes. Injections of *Xbra* (C) and *HyBra1* (E) induced *muscle actin*, but not *xcg-1* expression, whereas *HyBra2* (G) triggers the converse gene expression pattern. Furthermore, mRNA of the differentiated neuronal marker *β-tubulin* was often present in *HyBra2*- (H), but never in *HyBra1*- (F), and only rarely in *Xbra*-injected explants (D). (I-L) Double in situ staining of animal caps injected with the Hybra2/Xbra chimerae. (I,J) *H2TXA*-injected animal caps show no *xcg-1*, *β-tubulin* induction and very limited *muscle actin* expression. (K,L) By contrast, *XTH2A*-injected caps clearly show strong induction of *xcg-1* and *β-tubulin*, but no *muscle actin* mRNA. (**M**) Statistical overview of induced markers (*xcg-1*, *muscle actin*, *β-tubulin*) by injection of particular mRNAs (2-6 independent single and/or double in situ hybridisation experiments).

elongated (Fig. 7I,K). Although the latter suggests the induction of mesoderm, muscle actin was only rarely induced (Fig. 8I,M). Moreover, no *Xcg-1* or *β-tubulin* expression was detected (Fig. 8I,J,M). The converse fusion, *XTH2A*, induced cement glands and neural *β-tubulin* approximately to the same extent as *HyBra2*, but neither induced elongation or *muscle actin* expression (Fig. 7J,K; Fig. 8K,L,M). Thus, the *XTH2A* variant phenocopies the activity of *HyBra2* and is unable to induce mesoderm.

The results described above suggest that it is primarily the part located C-terminally of the T-box that accounts for the qualitatively distinct protein activities of HyBra1 and HyBra2, rather than the divergence of the T-Box domains. However, the minuscule mesoderm-forming activity contained within the H2TXA chimera indicates that the two domains cannot be freely exchanged, but also have to cooperate to a certain extent. From this we conclude that the specific activity, which in the *Xenopus* animal cap assay induces cement gland and neural cell fates, is confined to the C-terminal domain of HyBra2 and probably involves more than constitutive transcriptional activation or repression.

DISCUSSION

The diploblastic metazoan hydra has two ancient paralogues of *Brachyury*

Among non-bilaterian animals, the first T-box gene described was the *Brachyury* homologue *HyBra1* of the diplobast *Hydra* (Technau and Bode, 1999). We now report the identification of a second *Brachyury* gene from this basal metazoan, termed *HyBra2*. The phylogenetic analysis strongly suggests that the duplication event occurred at the base of the Hydrozoan lineage, at least 550 million years ago (Chen et al., 2002). Hydrozoa have retained two *Brachyury* genes, *Bra1* and *Bra2*. Whether the duplicated *Brachyury* genes in *Hydractinia* (Bra2-type) and *Podocoryne* (Bra1-type) have been preserved as in *Hydra* or have been lost is not yet clear. Our sequence analysis further suggests that bilaterian, in particular all vertebrate Brachyury proteins, share more features with HyBra1 than with HyBra2. This suggests that a HyBra1-like molecule was present in the common ancestor of cnidarians and bilaterians and that it has retained more of its ancestral structural features than HyBra2. Interestingly, *HyBra1* shares a small conserved motif in the activation domain with the vertebrate Brachyurys, corresponding to the core of the repression module 1 (R1) as described previously (Kispert et al., 1995). The conservation of this motif suggests that it might play a role as a conserved interface for protein-protein interaction.

The role of *Brachyury* during head formation in *Hydra*

HyBra1 and *HyBra2* are expressed in endoderm and ectoderm, respectively, of the hypostome, which is considered equivalent to the Spemann organiser (Browne, 1909; Broun and Bode, 2002). Interestingly, although *HyBra1* and *HyBra2* mRNA expression is activated at the same stage during budding, the kinetics of expression

of *HyBra1* and *HyBra2* differs significantly during regeneration: *HyBra1* is an immediate-early gene, in the sense that its onset of expression does not require protein synthesis, whereas *HyBra2* expression does require protein synthesis and occurs significantly later. This points to some crucial differences in the molecular regulatory network between regeneration (for reviews, see Holstein et al., 2003; Galliot et al., 2006; Bosch, 2007) and budding, despite the fact that overall the same genes are used. Head formation is thought to be suppressed in the body column of a normal polyp by an inhibitor that forms a concentration gradient from the head to the budding region (MacWilliams, 1983). While the nature of this postulated inhibitor is still unclear, we propose that decapitation rapidly removes the head inhibitor from the tissue, which leads to a local post-translational modification of a transcriptional regulator present in the tissue that activates *HyBra1* (and other immediate-early response genes). Members of the MAPK and CREB pathway are implicated in early head regeneration and possibly involved in the regulation of *HyBra1* (Cardenas et al., 2000; Cardenas and Salgado, 2003; Manuel et al., 2006; Kaloulis et al., 2004). Another candidate molecule to activate *HyBra1* expression upon post-translational modification is β-catenin. *β-catenin* is expressed throughout the animal, but upregulated at the apical tip during regeneration (Hobmayer et al., 2000) and stabilised in the apical region in nuclei (Broun et al., 2005). In accordance with a role in regulating *HyBra* expression, *HyBra1* and *HyBra2* expression is ectopically upregulated in animals where β-catenin has been ectopically stabilised by alsterpaullone treatment.

Hence, inhibition of early head-specific genes in the body column might be lifted in response to regeneration signals, preparing the tissue for rapid regeneration, irrespective of its future fate. By contrast, *HyBra2* does not belong to the immediate-early-response genes during head regeneration and is not detectable during foot regeneration. After the immediate-early response, additional factors, which stabilise the polarity of the tissue and tell the regenerating end whether to differentiate a head or a foot, must be involved.

The evolutionary divergence of the *Hydra Brachyury* paralogues occurred at diverse levels

It has been repeatedly proposed that divergence and evolution of cis-regulatory regions may drive such increasing complexity as observed in the lineage of the vertebrates (Levine and Tijan, 2003; Davidson and Erwin, 2006). The duplication-degeneration-complementation (DDC) model provides a theoretical framework for the fate of paralogues upon duplication (Force et al., 1999; Lynch and Force, 2000). Essentially, paralogues that are retained over long evolutionary time scales underwent either subfunctionalisation or neofunctionalisation. Subfunctionalisation mainly emphasises the divergence and subdivision of the cis-regulatory elements of the parental gene. Indeed, there are cases where the gene expression domains of both duplicates together make up for the expression domain of a single orthologue in a species, which has branched off before the duplication event (De Martino et al., 2000). In the case of *HyBra1* and *HyBra2* the acquired cis-regulatory elements resulted in a paralogue-specific spatial and temporal regulation of the two *Hydra Brachyury* genes. If the combined expression of both paralogues reflects the expression of the ancestral gene, this would mean that *Brachyury* is marking a domain in *Hydra*, rather than a particular germ layer.

In addition to subfunctionalisation, divergence of coding sequences in paralogues can lead to neofunctionalisation. In the case of ancient duplications, the paralogues may have diverged significantly and possibly taken up novel functions. Therefore, ancient lineage-specific paralogues in particular provide the opportunity to study the levels and target sites of the diverging mutations. They also provide us with evolutionary variants of proteins, which can then be studied for structure-function relationships and help to rapidly identify crucial motifs for specific subfunctions. In the absence of functional assays in *Hydra* with specific read-outs for distinct cellular fates, we used a heterologous system – the *Xenopus* animal caps – as an assay system to test whether both *Hydra* paralogues have functionally diverged. This assay system is ideal, because this naive tissue, which otherwise develops normally into undifferentiated epidermis, but can be pushed to differentiate endoderm, mesoderm or neural ectoderm, depending on the input. It is well established that *Brachyury* induces mesoderm, both on the molecular level and in terms of tissue properties such as convergent extension (Smith et al., 1991). Although *Hydra* does not contain mesoderm, *HyBra1* is able to induce mesoderm just like the *Xenopus Brachyury* gene (Marcellini et al., 2003) (this study). In contrast, *HyBra2* behaves differently to other bilaterian *Brachyury* genes in this assay by strongly inducing formation of cement glands and neural tissue. Domain-swapping experiments demonstrate that the HyBra2 C-terminal domain is necessary and sufficient (when fused to a Brachyury T-box) to induce neural tissue formation. Interestingly, Hybra2 is not the only Brachyury protein with neuralising activity. The *Xbra* paralogue *Xbra3* activates both mesodermal and neural differentiation (Strong et al., 2000). In animal caps, however, it induced only posterior neural tissue and cement glands were not observed (Hartmann et al., 2002). It is therefore not clear, whether this is a secondary effect of the neuralising effect of embryonic FGF (eFGF), which forms a feedback loop with Brachyury (Schulte-Merker and Smith, 1995). In addition, specific truncations of the *Xbra* activation domain can convert the mesoderm inducer into a neuralising factor (Rao, 1994). However, C-terminal truncations of HyBra2 either did not alter the neuralising phenotype or had no effect at all (data not shown). Further, the HyBra2 C-terminal domain is even longer than that of HyBra1, which is a mesoderm inducer. We conclude that the similar phenotypes obtained with the HyBra2 and with truncated version of Xbra in the study by Rao (Rao, 1994) must be of different origin, yet might reveal a common mechanism.

Brachyury has previously been shown to be a transcriptional activator, so one possibility to explain the radical difference in the inductive behaviour of HyBra2 in animal caps is that the C-terminal activation domain evolved into a repression domain. However, this does not seem to be the case, as an *H2T-EnR* variant (*HyBra2* T-box fused to the *Engrailed* repressor domain) did not mimic the neuralising activity of *HyBra2* (data not shown). Furthermore, repression of Brachyury function by injection of a comparable *XbraT-EnR* construct caused mesodermal defects in convergent extension and notochord formation and induction of anterior endoderm (Conlon et al., 1996; Conlon and Smith, 1999); however, the induction of cement glands had not been reported in these studies. Therefore, it is unlikely that Hybra2 simply represents an antimorphic variant of mesoderm-inducing Brachyury proteins. Future studies aimed to identify proteins interacting with variants of Brachyury might be key for our understanding of how transcriptional activity and specificity is modulated.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/23/4187/DC1

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