Development 129, 1411-1421 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2835

Expression pattern of *Brachyury* in the mollusc *Patella vulgata* suggests a conserved role in the establishment of the AP axis in Bilateria

Nicolas Lartillot[1], Olivier Lespinet[2], Michel Vervoort[1] and André Adoutte[1]

[1]Centre de Génétique Moléculaire, CNRS batiment 26, 1 Avenue de la Terrasse, F-91198 Gif Sur Yvette, France
[2]National Institute of Health, Building 38A, Room B2N13E, 8600 Rockville Pike, Bethesda, MD 20894, USA
*Author for correspondence (e-mail: nicolas.lartillot@cgm.cnrs-gif.fr)

Accepted 26 December 2001

SUMMARY

We report the characterisation of a *Brachyury* ortholog (*PvuBra*) in the marine gastropod *Patella vulgata*. In this mollusc, the embryo displays an equal cleavage pattern until the 32-cell stage. There, an inductive event takes place that sets up the bilateral symmetry, by specifying one of the four initially equipotent vegetal macromeres as the posterior pole of all subsequent morphogenesis. This macromere, usually designated as 3D, will subsequently act as an organiser. We show that 3D expresses *PvuBra* as soon as its fate is determined. As reported for another mollusc (J. D. Lambert and L. M. Nagy (2001) *Development* 128, 45-56), we found that 3D determination and activity also involve the activation of the MAP kinase ERK, and we further show that *PvuBra* expression in 3D requires ERK activity. *PvuBra* expression then rapidly spreads to neighbouring cells that cleave in a bilateral fashion and whose progeny will constitute the posterior edge of the blastopore during gastrulation, suggesting a role for *PvuBra* in regulating cell movements and cleavage morphology in *Patella*. Until the completion of gastrulation, *PvuBra* expression is maintained at the posterior pole, and along the developing anterior-posterior axis. Comparing this expression pattern with what is known in other Bilateria, we advocate that *Brachyury* might have a conserved role in the regulation of anterior-posterior patterning among Bilateria, through the maintenance of a posterior growth zone, suggesting that a teloblastic mode of axis formation might be ancestral to the Bilateria.

Key words: *Brachyury*, Mollusc, Gastrulation, AP axis, Teloblast, *Patella vulgata*

INTRODUCTION

Analysing the expression and function of homologous genes among species belonging to different bilaterian phyla offers the opportunity of investigating the evolution of their body plans. One of the most important results obtained in this comparative perspective is the recognition, through the conservation of the colinear expression of the genes of the *HOX* complex, that the patterning along the anterior-posterior (AP) axis might be homologous among Bilateria (Slack et al., 1993). Extending this result with the aid of other genes, a quite detailed topography of the body plan of *Urbilateria*, the last common ancestor of all bilaterians, has been proposed (DeRobertis and Sasai, 1996). Yet, the conservation, over larger evolutionary scales, of the processes of body plan formation themselves has been much less investigated, and these developmental processes are often considered to be highly divergent among distantly related Bilateria (Slack et al., 1993).

Most comparisons are based on the molecular analysis of the development of a few model systems, such as various vertebrates, the fly *Drosophila melanogaster*, and the worm *Caenorhabditis elegans*. These species belong to two of the three main groups of Bilateria that have been recognised by recent phylogenetic analyses (Aguinaldo et al., 1997; Adoutte et al., 2000), i.e. the deuterostomes and the ecdysozoans. In contrast, the third major group, the lophotrochozoans (e.g. annelids, molluscs and platyhelminths), has remained one of the weak points of the molecular survey of metazoan development.

In spite of the diversity of their body plans, many lophotrochozoans share a highly conserved and well documented early embryogenesis known as spiral cleavage (Freeman and Lundelius, 1992). Spiral cleavage is mostly characterised by an invariant cell lineage, during which stem cells for the main germ layers are rapidly segregated. In addition, it most often leads to the development of a free swimming larva, of which the trochophore, observed in many molluscs, annelids and sipunculans, is the prototype (Nielsen, 2001). Because of the conspicuous nature of the cellular aspects of spiral development, and its remarkable conservation at the interphyletic level, lophotrochozoans have been a major focus of interest in classical embryology.

One of the most important variations on the spiral theme is the difference between equal and unequal cleavage (Freeman and Lundelius, 1992). In unequal cleavers, one of the blastomeres of the 4-cell stage is larger than the others, and gives rise to most of the posterior part of the adult, so that the AP axis is specified as soon as the 4-cell stage in these species. In

contrast, equal cleavers do not depart from the four-fold symmetry until later in the cleavage period (the 32-cell stage in *Patella*). Whether this symmetry corresponds to a true developmental equivalence between the four blastomeres of the 4-cell stage has been a long standing issue (Arnolds et al., 1983; Martindale et al., 1985). A large set of experiments conducted on *Patella vulgata* have convincingly shown that the four quadrants are indeed initially truly equipotent, until the 32-cell stage (van den Biggelaar, 1977; van den Biggelaar and Guerrier, 1979; Arnolds et al., 1983; Kühtreiber et al., 1988). At this point, the four-fold symmetry is broken through a purely stochastic event, namely the competition between the four vegetalmost cells, that leads to the determination of one of them (called 3D), as the posterior pole of all subsequent morphogenesis. The bilateralisation of the embryo is soon illustrated by specific departures of the cleavage morphology of certain cells from the default, four-fold symmetrical pattern (van den Biggelaar, 1977). These developmental events following the election of 3D, in turn, most probably involve 3D as an important inductive centre, although there is little evidence to demonstrate the way in which 3D acts as an organiser in equal cleaving Spiralia (Martindale, 1986; Damen and Dictus, 1996).

The gene *Brachyury* was first identified in mouse, where it was recognised to have a fundamental role in gastrulation, axial patterning and tail formation (Beddington et al., 1992), and was then rapidly isolated in many other vertebrates (Papaioannou and Silver, 1998). In all species, *Brachyury* is expressed transiently during gastrulation around the blastopore, in the involuting mesoderm and endoderm, and its expression is subsequently restricted to notochord and tailbud (Wilkinson et al., 1990; Herrmann, 1991; Kispert and Herrmann, 1994; Smith et al., 1991; Schulte-Merker et al., 1992; Kispert et al., 1995). Genetic evidence, both in mouse (Wilson et al., 1995; Wilson and Beddington, 1997) and in zebrafish (Schulte-Merker et al., 1994; Melby et al., 1996) indicates that *Brachyury* is required for gastrulation, axial specification and caudal morphogenesis. Homologues have been identified in many other metazoan phyla. In the other diverse deuterostome lineages where it has been studied, in particular urochordates (Corbo et al., 1997; Yasuo and Satoh, 1998; Bassham and Postlethwait, 2000) echinoderms (Harada et al., 1995; Peterson et al., 1999b; Shoguchi et al., 1999) and hemichordates (Tagawa et al., 1998; Peterson et al., 1999a), *Brachyury* orthologues are always expressed from the very early stages of gastrulation, in the blastopore area, and have recurrent connections with gastrulation, morphogenetic movements, mesoderm formation and posterior patterning. Similarly, in *Drosophila*, the expression of *brachyenteron*, the *Brachyury* homologue, is restricted to the posterior part of the embryo (Kispert et al., 1994), and is involved in the morphogenesis of the caudal part of the gut (Singer et al., 1996), and of the visceral mesoderm (Kusch and Reuter, 1999). An orthologue of *Brachyury* has recently been identified in a lophotrochozoan, the unequally cleaving annelid *Platynereis dumerilii*, and its post-gastrulation expression profile is remarkably similar to that found in the larva of hemichordates (Arendt et al., 2001): in both cases, *Brachyury* is expressed in the lower part of the stomodaeum, the posterior tip of the larva and the ventral midline, highlighting a possible homology between the larval body plans between protostomes and deuterostomes.

We have isolated a *Brachyury* orthologue in *Patella vulgata*, which we named *PvuBra*. We show that *PvuBra* is expressed in the trochophore larva of *Patella* following a pattern identical to that of its orthologue in *Platynereis*. We further observed that *PvuBra* is expressed much earlier in development, first in the 3D blastomere of the 32-cell stage, and then on the posterior side of the embryo before and throughout gastrulation. We also demonstrate that the expression of *Brachyury* in 3D is controlled by the ERK MAP kinase cascade, and that ERK activation confers to 3D the ability to organise all aspects of the bilaterally symmetrical cell cleavage patterns observed in the vegetal region of the embryo, between the moment of 3D election and the start of gastrulation. Finally, a comparative analysis of *Brachyury* expression and function is given, in which we propose that *Brachyury* expression illuminates the evolution of gastrulation and AP axis specification at the scale of the Bilateria.

MATERIALS AND METHODS

Patella in vitro fertilisation and embryo rearing

Adult animals were obtained from the Station Biologique de Roscoff, France, and kept in artificial sea water. Embryos were obtained as described previously (van den Biggelaar, 1977).

Cloning and sequencing of *PvuBra*

Degenerate primers specific to *Brachyury* were designed and used in a nested PCR on *Patella vulgata* genomic DNA. A 310 pb product was amplified and cloned. 8 clones were sequenced and found to have identical sequence. Primers were forward BRA1W (NGRRMFP, nucleotide sequence 5′-AYGGNMGNMGNATGTTYCC-3′) and reverse BRA3C (PFAKAF, nucleotide sequence 5′- RAANSCYTTN-GCRAANGG-3′) for the first round of amplification; forward BRA2W (WKYVNGEW, nucleotide sequence 5′-TGGAARTAYG-TNAAYGGNGARTGG-3′) and reverse BRA4C (AYQNEE, nucleotide sequence 5′-YTCYTCRTTYTGRTANGC-3′) for the second round of amplification; forward BRA2W and reverse BRA5C (QFIAVTA, nucleotide sequence 5′-GCNGTNACNGCNATRAA-YTG-3′) for the third round.

Next, PCR extensions were performed on a staged mass-zapped cDNA library (16 hours post first cleavage), kindly provided by L. Nederbragt and A. Van Loon (Van Loon et al., 1991) using non-degenerate primers corresponding to the sequence of the 310 bp PCR fragment and primers in the arm of the plasmid in which the cDNAs are cloned. In this way, we isolated a cDNA of 2019 bp, coding for a 450 amino acid protein, homologous to a T-box transcription factor. The 1.5 kb fragment corresponding to the 3′ part of the gene was used to generate a RNA probe for in situ hybridisation. The full sequence is available on the EMBL Nucleotide Sequence Database (sequence number AJ420986).

Alignment and phylogenetic analysis

Sequences of *Brachyury* homologues and other T-box genes were gathered using the NCBI PubMed database (http://www.ncbi.nlm.nih.gov). All sequences were aligned with *clustalX* (Thompson et al., 1997), and only the conserved T-domain was kept for subsequent analysis. Phylogeny reconstruction was performed by Maximum Likelihood quartet puzzling, using the software *Tree-puzzle* (Strimmer and von Haeseler, 1996). We used the JTT substitution model (Jones et al., 1992), and let rate heterogeneity across sites being modelled by two rate categories (one constant and one variable). 10,000 puzzling steps were performed, and branches supported in less than half of the quartet trees were collapsed.

A.

```
PatellaBra          LEDRSLWEKFKEFTNEMIVTKNGRRMFPVFRVSVSGVDPNAMYTLLLDFVQVDNHRWKYVNGDWVPGGKAEPAAPNCVYIHPDSPNFGAHWMKEP
PlatynereisBra      LEDRELWGKFKELTNEMIVTKSGRRMFPVLRASVTGLDPNAMYSFLLDLVNVDNHRWKYVNGDWVPGGKAEPQPHNAVYIHPDSPNFGAHWMKEP
DrosophilaByn       LDDRELWLRFQNLTNEMIVTKNGRRMFPVVKISASGLDPAAMYTVLLEFVQIDSHRWKYVNGEWVPGGKAEVPPSNPIYVHPESPNFGAHWMKEP
BranchiostomaBra2   LGEKPLWEKFKSLTNEMIVTKSGRRMFPVLKVNVSGLDPNAMYSFLLDFTAADNHRWKYVNGEWVPGGKPEPSVPSCVYIHPDSPNFGAHWMKSP
HydraBra            LDDSELWKKFKTLTNEMIVTKNGRRMFPVLKLNIRGLESHAMYSILLDFVAVEDHRWKYVNGEWVAGGKPEPATTSSVYIHPDSPNFGTHWMKSP
XenopusVEGT         LEDQDLWSQFHQEGTEMIITKSGRRMFPQCKIRLFGLHPYAKYMLLVDFVPLDNFRYKWNKNQWEAAGKAEPHPPCRTYVHPDSPAPGAHWMKDP
BrachydanioSPT      LEDPELWRSFHEIGTEMIITKPGRRMFPHCKISLSGLVPYAKYILLVDMVPEDGLRYKWNKDKWEVAGKAEPQPPYRTYLHPDSPAPGSHWMKQP
DrosophilaOMB       LEGKDLWEKFHKLGTEMVITKSGRQMFPQMKFRVSGLDAKAKYILLLDIVAADDYRYKFHNSRWMVAGKADPEMPKRMYIHPDSPTTGEQWMQKV
                                        ------>                                 ------->
                                         BRA1W                                   BRA2W
```

```
PatellaBra          LSFSKVKHTNKL-NGGGQ--IMLNSLHKYGPRIHIVRV-NSRTQ-KKSIMTFSFPETQFIAVTAYQNEEITSLKIKHNPFAKAFLDA
PlatynereisBra      VSFSKVKLTNKM-HGSGQ--IMLNSLHKYQPRIHIVKV-GNKDE-KRTISTHDFVETQFVAVTAYQNEEITALKIKYNPFAKAFQDA
DrosophilaByn       ISFAKVKLTNKT-NGNGQ--IMLNSLHKYEPRVHLVRV-GSE---QRHVVTYPFPETQFIAVTAYQNEEVTSLKIKYNPFAKAFLDA
BranchiostomaBra2   VSFSKVKLTNKL-NGGGQQ-IMLNSLHKYEPRIHIVKV-GGPDN-QRTLSTHTFAETQFIAVTAYQNEELTALKIKHNPFAKAFLDA
HydraBra            ISFTKVKLTNKM-NGEGQ--VMLNSLHKYQPRVHIIRV-GAPEG-ERTISTHTFPETQFIAVTAYQNEEITGLKIKYNPFAKAFLDA
XenopusVEGT         ICFQKLKLTNNTLDQQG---IILHSMHRYKPRFHVVQSDDMYNSPWGLVQVFSFPETEFTSVTAYQNEKITKLKINHNPFAKGFREQ
BrachydanioSPT      VSFLKLKLTNNALDQHG---IILHSMHRYHPRFHIVQADDLYSVRWSVFQTFTFPETSFTAVTAYQNTKITKLKIDHNPFAKGFRDE
DrosophilaOMB       VSFHKLKLTNNISDKHGFVSTILNSMHKYQPRFHLVRANDILKLPYSTFRTYVFKETEFIAVTAYQNEKITQLKIDNNPFAKGLRDT
                                                                                <------
                                                                                 BRA5C
                                                                                   <-----          <-----
                                                                                    BRA4C           BRA3C
```

B.

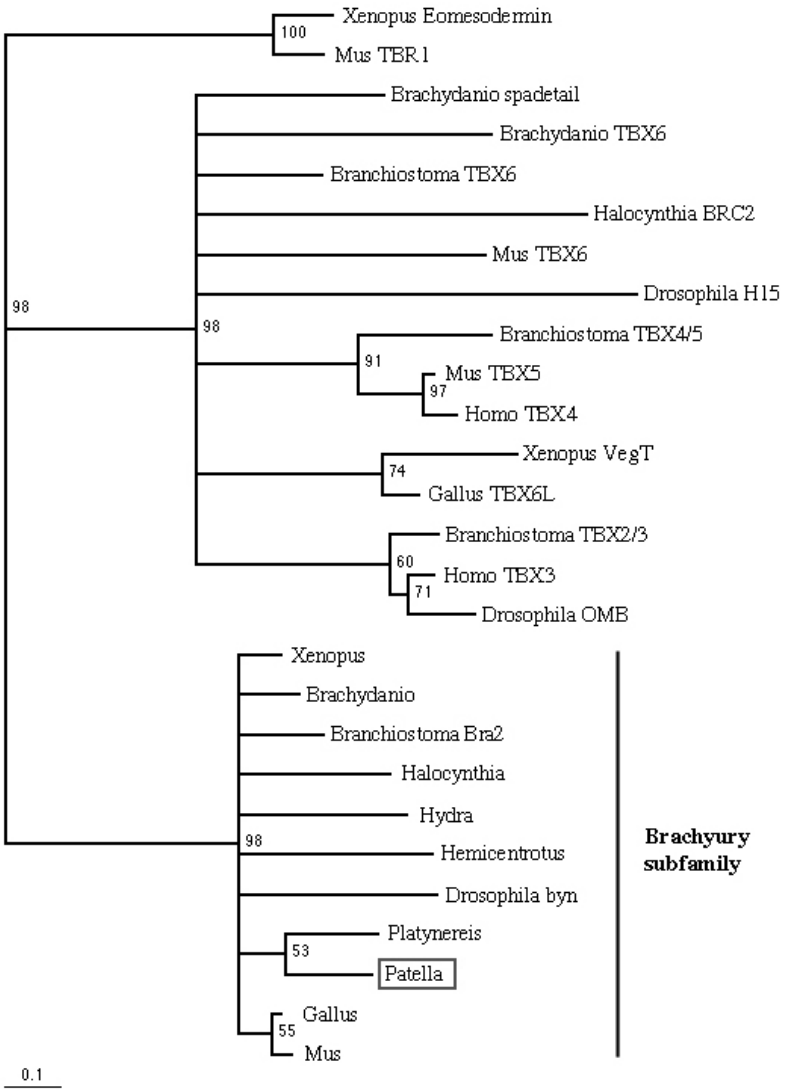

Fig. 1. Sequence alignment and phylogenetic tree. (A) The predicted amino acid sequences of the *PvuBra* fragment has been aligned with a sample of metazoan *Brachyury* and other T-box genes. Only the T domain is shown. The primers used for PCR amplification are indicated by arrows. (B) Phylogenetic tree (amino acid) of several *Brachyury* orthologues, reconstructed by quartet puzzling. A sample of other T-box genes has been taken to root the *Brachyury* subtree (see Materials and Methods).

In situ hybridisation and Hoechst staining

RNA in situ hybridisations were performed as described elsewhere (Lespinet et al., 2002), with the following modifications: probe concentration was raised to 1 μg/ml, and hybridisation was performed at 68°C. Anti-DIG antibody was used at a 1:200 dilution, and alkaline phosphatase staining was performed at 37°C. In some cases, following the in situ hybridisation, the embryos were stained with Hoechst (5 μg/ml in TBS-T; 10 minutes of incubation) to highlight the nuclei of their cells. A detailed protocol is available on request.

Immunolabelling

Embryos were fixed for 1 hour in 4% paraformaldehyde in Mops, at the appropriate stage, and then stored in methanol. Next, they were transferred progressively to MNT buffer (150 mM NaCl, 100 mM maleic acid, pH 7.5, 0.1% Tween 20), blocked for 1 hour in MNT + 3% BSA, incubated in anti-dpERK (Sigma), at a dilution of 1:1000, for 1 hour, rinsed four times 10 minutes in MNT + 3% BSA, incubated in biotin-conjugated anti-mouse antibody (Jackson), rinsed four times 10 minutes in MNT. The VectaStain Biotin-Avidin amplification system was used, and the embryos were then labelled with DAB (Sigma). Washings and fixation in 4% paraformaldehyde were used to stop the staining reaction.

ERK inhibition

Erk inhibition was performed as described by Lambert and Nagy (Lambert and Nagy, 2001).

RESULTS

A *Brachyury* homologue in *Patella vulgata*

Using degenerate primers, we amplified and cloned a 310 bp fragment of a T-box gene in *Patella*. Vector anchored race PCR allowed the isolation, from a staged cDNA library (16 hours post first cleavage, h.p.f.c.), of a cDNA of 2019 bp. On this cDNA, one can predict an open reading frame of 450 amino acids encoding a putative T-box protein, which we called *PvuBra*.

Fig. 1A shows the predicted amino acid sequence of the

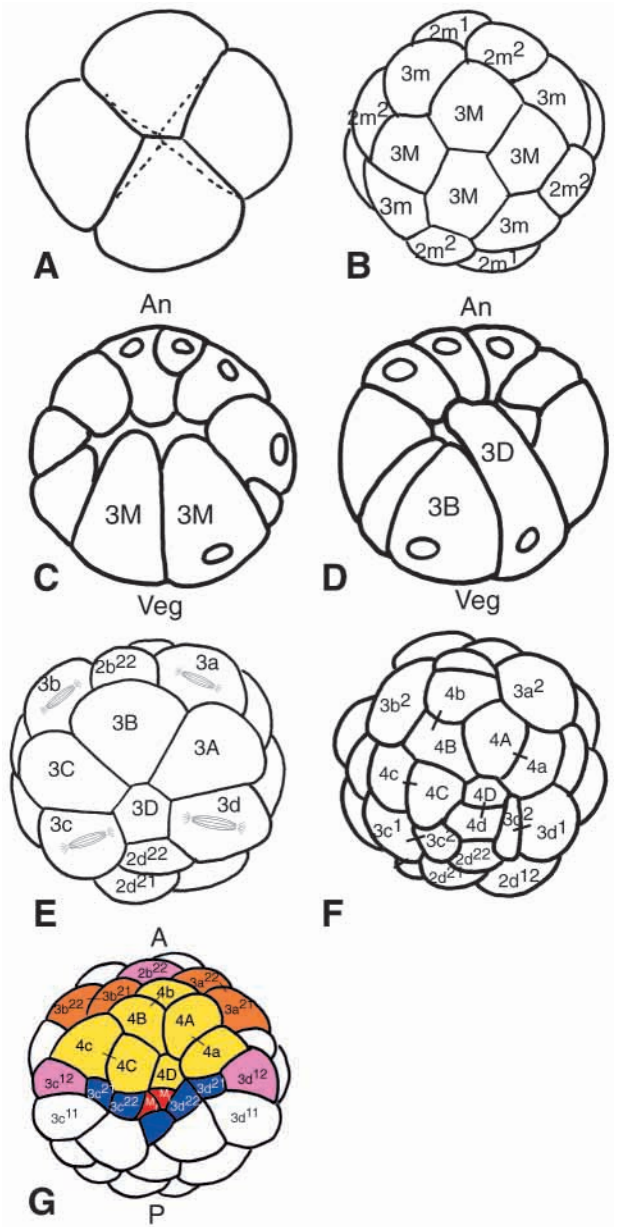

Fig. 2. Overview of *Patella* early development: 3D induction and AP axis specification. Unless mentioned explicitly, all views are taken from the vegetal pole. D quadrant (posterior) is to the bottom, so the left side of the embryo is to the right. Drawings are freely adapted from van den Biggelaar (van den Biggelaar, 1977), except A, which is adapted from Wilson (Wilson, 1904), and G. (A) 4-cell stage (30 minutes after first cleavage). The four cells are of equal size, and the progeny of each of them is called a quadrant. (B) 32-cell stage (2 hours after first cleavage (h.p.f.c.)), displaying a four-fold symmetry. (C) Early 32-cell stage (2.5 h.p.f.c.), meridional cross section, before 3D determination. Animal is to the top. Two of the four vegetal cells (macromeres) can be seen (3M). During this period, the four macromeres invade the blastocoel, and compete for contacting the animal micromeres that constitute the blastocoel roof. (D) Mid 32-cell stage (3 h.p.f.c.), same section as in C: one of the four macromeres contacts the blastocoel roof, and as a consequence, takes a 3D fate. (E) 60-cell stage (3.5 to 4 h.p.f.c.). 3D looks smaller, since most of its mass is internalised. 3a, 3b, 3c and 3d are in metaphase. Together with 3D, these latter cells' cleavage adumbrates bilateral symmetry. (F) 64-cell stage (4 h.p.f.c.): 3c and 3d have yielded smaller vegetal cells ($3c^2$ and $3d^2$) and larger animal cells ($3c^1$ and $3d^1$), whereas 3a and 3b have divided in an opposite fashion, budding off a larger cell towards the vegetal pole ($3a^2$ and $3b^2$. Note that $3a^1$ and $3b^1$ are not visible on this drawing). 3D is the last cell to divide before completion of the 6th cleavage, and yields 4d, or M, the mesentoblast, that will contribute to most of the adult mesoderm. (G) 2 hours after the 64-cell stage (6 h.p.f.c.). The segregation of the prospective germ layers is now achieved. The endodermal vegetal plate (yellow) is made from all descendants of 3A, 3B, 3C and 4D. 4d, the mesentoblast divides in a bilateral fashion, yielding M_l and M_r, the paired stem cells giving rise to the mesodermal germ bands (red). 3a and 3b derivatives also contribute to mesoderm (light orange) (Dictus and Damen, 1997). Cells coloured in dark blue are of ectodermal fate, and delineate the posterior edge of the blastopore. The pink blastomeres are the three stomodaeum founder cells, and are thus called stomatoblasts, as in *Nereis* (Wilson, 1892). During gastrulation, the vegetal plate is internalised by epiboly: the rim of ectoderm migrating over and enclosing the vegetal plate is equivalent to a blastopore edge which, in the case of gastropods, gives birth to the mouth. The dark blue cells constitute a useful landmark: they are at the edge of the vegetal plate, and will contribute to the ectoderm at the base of the larval mouth, and thus define the posterior edge of the blastopore.

PvuBra fragment that we have isolated. This has been aligned with a sample of *Brachyury* orthologues, as well as a few other T-box genes. Only the part of the alignment corresponding to the conserved T-domain (Fig. 1A) has been used to perform a Maximum Likelihood phylogenetic analysis (quartet puzzling) (Strimmer and von Haeseler, 1996). The tree that has been obtained is shown on Fig. 1B. *PvuBra* clusters reliably with other metazoan *Brachyury* homologues, showing that we have isolated a *Brachyury* orthologue in *Patella vulgata*.

PvuBra is expressed throughout gastrulation and in the young trochophore

We studied *PvuBra* spatiotemporal expression during early development, up to larval stages, by whole-mount in situ hybridisation using digoxigenin-labelled RNA probes. An overview of *Patella* early development is depicted in Fig. 2.

PvuBra is first detected at the mid 32-cell stage (3 h.p.f.c.): strong transcript accumulation can be seen in one of the four macromeres (Fig. 3A,E). In optical sections (not shown) this macromere can be seen to have a central position and to contact the blastocoel roof, and is therefore blastomere 3D.

At the end of the 6th cleavage (4 h.p.f.c.), substantial staining is still observed in 3D, and then in its daughter cells 4D and 4d (or M). In addition, *PvuBra* expression propagates to the derivatives of 3c and 3d, and $2d^2$ (Fig. 3B,F,I,J). Note that all these cells are neighbours of 3D.

About 1 hour after the 64-cell stage (5 h.p.f.c., Fig. 3C,G), transcripts are weak in 4D and in M (Fig. 3C,G). $3c^1$ and $3d^1$

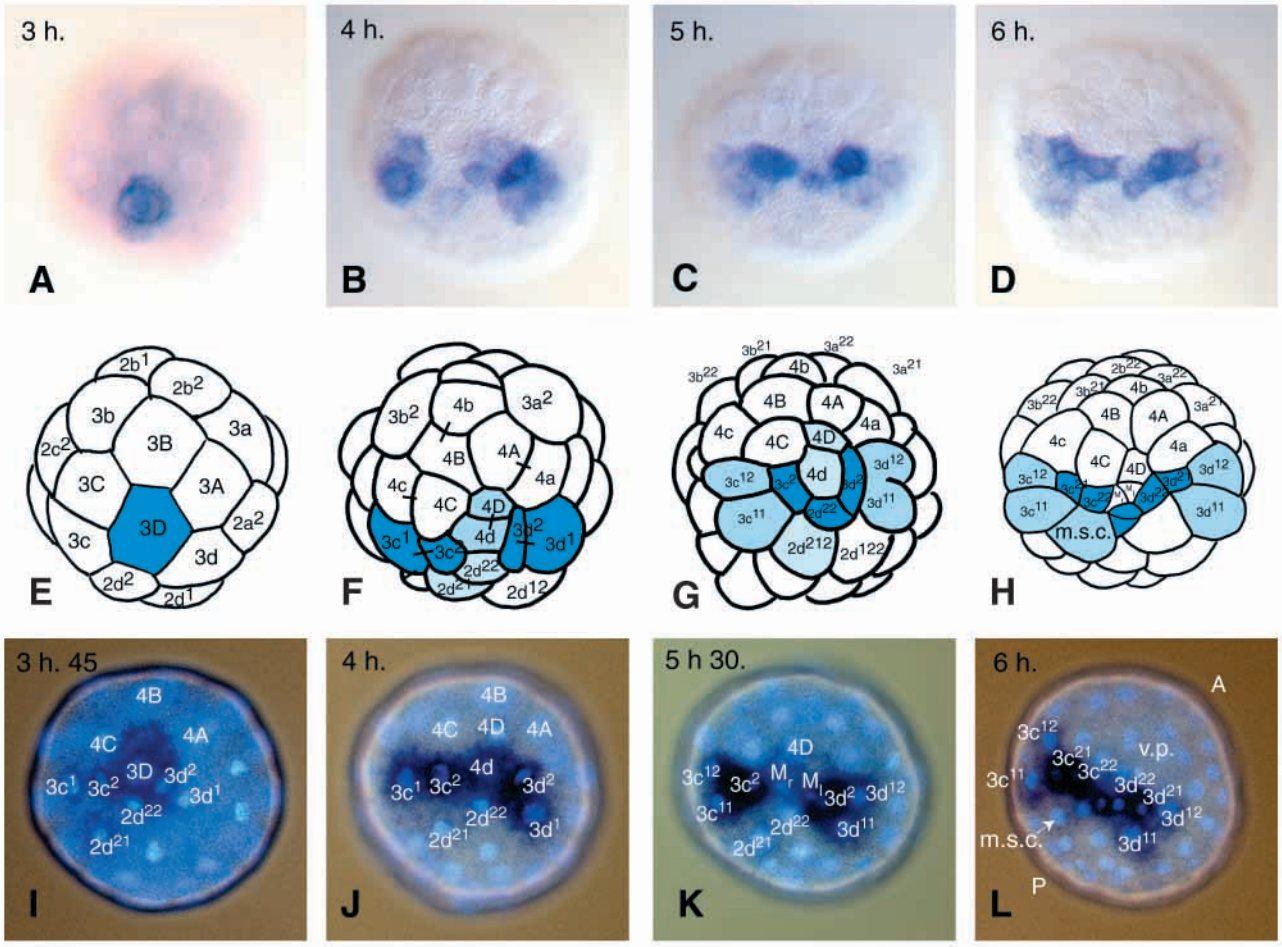

Fig. 3. *PvuBra* expression during early development. (A-D) In situ hybridisation using *PvuBra* antisense probe. (E-H) Schematic pictures indicating lineage of expression. (I-L) In situ hybridisation combined with Hoechst staining of the nuclei. (A,E) Between late 32- and 40-cell stage (3 h.p.f.c.). *PvuBra* is strongly expressed in 3D. (B,F) 64-cell stage (4 h.p.f.c.), showing expression in 3D daughter cells (4D and 4d), as well as a spread of expression in 3c, 3d and $2d^2$ daughter cells. Sister cells are linked by a short straight line. (C,G) Beginning of the 88-cell stage (5 h.p.f.c.). $3c^2$ and $3d^2$ have not divided yet, and show strong *PvuBra* expression. $3c^1$ and $3d^1$ have divided, with their spindles approximately parallel to the plane of bilateral symmetry. Their daughters express weaker levels of *PvuBra* transcripts, compared to $3c^2$ and $3d^2$. The resulting pattern takes on the shape of two bilateral wings. Expression is also strong in $2d^{22}$, fainter in $2d^{212}$, and has nearly vanished in 4D and 4d. (D,H) 6 h.p.f.c. The two wings ($3c^1$ and $3d^1$ derivatives) still express *PvuBra* at a medium level. $3c^2$ and $3d^2$ have divided and, in contrast to their sister cells, they have their spindles parallel to the posterior edge of the vegetal plate, so that, together with $2d^{22}$, they are the five darkly stained cells at the posterior edge of the blastopore. Behind, $2d^{212}$, the midline stem cell (arrowhead, m.s.c.; see text for details), has budded off a new small cell situated just next to $2d^{22}$, and which also strongly expresses *PvuBra*. The midline stem cell itself shows much weaker expression. Notice its typical asymmetrical position, markedly to the right (for us, to the left). Transcripts are no longer detected in 4D, or in M_l and M_r. Gastrulation is about to start. (I) 63-cell stage. (J) 64-cell stage. (K) 30 minutes after the 88-cell stage, the paired mesoteloblasts can be seen below the focal plane, with their spindles in metaphase. (L) 6 h.p.f.c.. The two wings are apparent.

have divided, and their daughters weakly express *PvuBra*. Their sister cells, $3c^2$ and $3d^2$, closest to the vegetal pole, show a notably stronger expression, and their cleavage is delayed, compared to $3c^1$ and $3d^1$. $2d^{22}$ shows a strong expression. $2d^{21}$ has divided, and a weak signal can be detected in $2d^{212}$. 15 to 30 minutes later; M has divided, and the paired mesentoblasts (M_l and M_r) have sunk into the blastocoel (Fig. 3K).

Later on (6 h.p.f.c.), transcripts have completely disappeared from 4D and M_l and M_r. From now on, expression of *PvuBra* is restricted to 3c, 3d and $2d^2$ derivatives (Fig. 3D-H). $3c^2$ and $3d^2$ have eventually divided, with their spindles aligned with the presumptive left-right axis. Their daughter cells display a high level of transcripts. Between $3c^2$ and $3d^2$ daughter cells, two cells can be seen that express *PvuBra* and, more posteriorly and markedly displaced to the right of the embryo (to the left on the picture), a cell derived from $2d^{212}$ shows a very weak, but consistently detectable expression (Fig. 3D-H). These three cells derive from $2d^2$, although it is very difficult to determine their exact lineage and cleavage schedule. Our interpretation is that $2d^2$ behaves like a stem cell, that will bud off the cells of the ventral midline. Subsequently, we will call it the 'midline stem cell' (m.s.c.; see Discussion).

Thus, right at the start of gastrulation, the pattern becomes stabilised as follows (Fig. 3D,H,L). Six cells, descended from 3c, 2d and 3d, show strong *PvuBra* expression, and constitute the posterior edge of the blastopore. In addition, two pairs of bilateral cells, posterior to the vegetal plate, and originating from 3c1 and 3d1, express PvuBra at lower levels. The $3d^1$ and $3c^1$ daughter cells, respectively on the left and right posterior side of the vegetal plate, express *PvuBra* at lower levels. On each side, the anteriormost cells ($3c^{12}$ and $3d^{12}$) are stomodaeum founder cells, or 'stomatoblasts', as in *Nereis* (Wilson, 1892), whereas the posteriormost cells ($3c^{11}$ and $3d^{11}$) will end up in the terminal posterior region, and will contribute to the anus. $3c^{11}$ and $3d^{11}$ will thus be called the 'terminal cells'. More posteriorly, on the left, is a larger cell, displaying still lower levels of *PvuBra* transcripts, the 'midline stem cell'.

During the first phase of gastrulation (Fig. 4A), *PvuBra* expression is nearly lost by the midline stem cell and the two lateral wings. In contrast, the cells of the posterior edge of the blastopore remain positive for *PvuBra* during the whole period. Often, a faint expression is seen in the nuclei of endodermal macromeres migrating to the apical pole (not shown).

During the second phase of gastrulation (blastopore closure), the cells of the posterior edge migrate anteriorly over the vegetal plate, and take on a V-shape (Fig. 4B). Meanwhile, expression appears on the midline posterior to the migrating edge, as well as in a spot more posterior, to the right side of the embryo. This latter spot of expression likely corresponds to the midline stem cell, that starts re-expressing *PvuBra*, and from which the stained cells on the midline might be budding off.

In the young free swimming trochophore (13 h.p.f.c., Fig. 4C), *PvuBra* is expressed in a strip of ectoderm below the stomodaeum, continuous with a strand of stained cells along the ventral midline and, at the posterior tip of the embryo, in two bilateral cells (terminal cells). This pattern is reminiscent of that seen for the *Brachyury* homologue in the trochophore larva of *Platynereis* (Arendt et al., 2001). In 24 hour larvae, the expression on the midline and in the terminal region has faded away (Fig. 4D). The ectoderm below the stomodaeum still expresses *PvuBra*. Four cells in the posteriormost part of the dorsal mantle edge have started to transcribe *PvuBra* RNA.

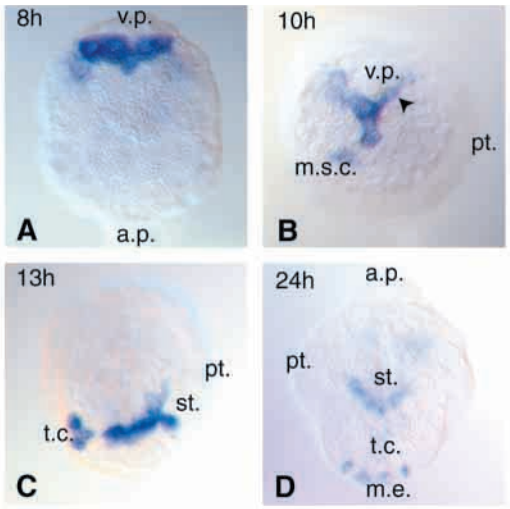

Fig. 4. *PvuBra* in situ hybridisation, later stages. (A) Posterior view of a 8 h.p.f.c. embryo. The vegetal plate (v.p.) is just visible above the five stained cells marking its posterior edge. The apical pole (a.p.) is below. Staining is now barely visible in the wings, and not apparent in the midline stem cell. (B) 10 h.p.f.c. embryo, ventral view. The posterior edge of the blastopore takes a V-shape (arrowhead). Posteriorly, the ventral midline is being formed, and also expresses *PvuBra*. A spot of expression appears terminally, consistently on the right side. It probably corresponds to the midline stem cell (m.s.c.). The prototroch (pt.) is visible in the background. (C) Young trochophore (13 h.p.f.c.), three quarter view, from the right-ventral side. Gastrulation has proceeded to its end. Stomodaeum (st.) is to the right, ventral midline to the bottom, and terminal cells (t.c.) to the left. (D) Older trochophore (24 h.p.f.c.). ventral-posterior view. The ectodermal cells underlying the stomodaeum (st.) still show transcripts. Midline and terminal expression has fainted away. More dorsally, right at the mantle edge (m.e.), four new spots of expression can now be seen. sb., stomatoblast; pt., prototroch; m.e., mantle edge.

The overall process can now be summarised as follows (Fig. 5). *PvuBra* is transiently expressed in 3D (Fig. 5A), and then rapidly propagates to neighbouring 3c, 3d and $2d^2$ derivatives (Fig. 5B). These latter cells cleave in an orderly and bilateral fashion (Fig. 5C,D), and their derivatives mark the posterior edge of the blastopore. Then, during gastrulation (Fig. 5E-G), the posterior edge, still expressing *PvuBra*, migrates anteriorly, over the vegetal plate. The anteriormost cells derived from $3c^1$ and $3d^1$, the lateral stomatoblasts, are carried along, and finally contribute to the stomodaeum. Meanwhile, the posteriormost cells derived from $3c^1$ and $3d^1$, the terminal cells, stay behind and contribute to the anus. During the whole period, the midline stem cell, derived from $2d^{21}$, lags posteriorly as well, and, following our interpretation, buds off the cells of the midline. In this way, the pattern seen in the young trochophore (Fig. 5H) is completely derived from three founder cells: 3c, 3d and $2d^2$. Cells on the ventral midline originate from $2d^2$, whereas both the anterior and the terminal domains of expression are made up of 3d and 3c derivatives, on the left and right, respectively.

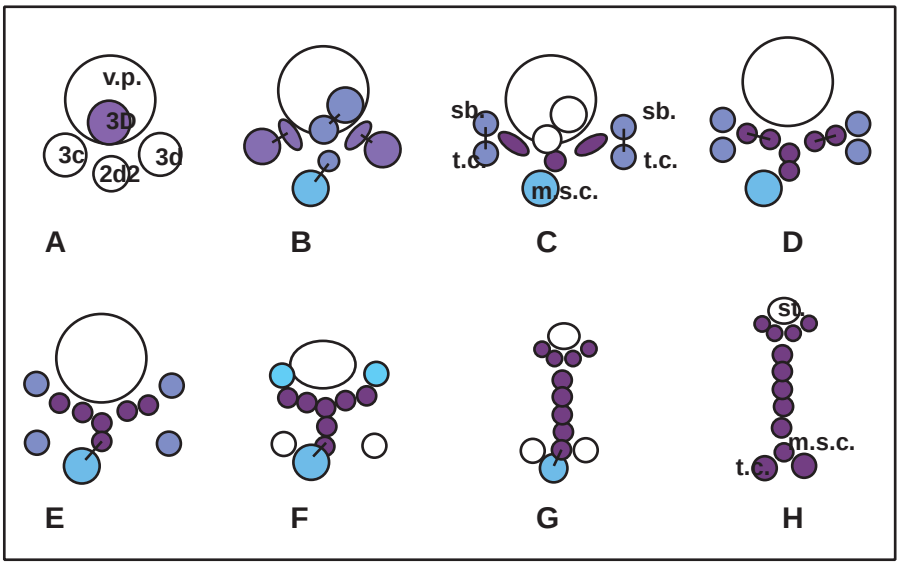

Fig. 5. Summary of *PvuBra* expression, from 3D induction to the end of gastrulation. Color shading corresponds to observed *PvuBra* RNA expression. (A) 32-cell stage (3 h.p.f.c.). (B) 64-cell stage (4 h.p.f.c.). (C) 88-cell stage (5 h.p.f.c.). Division of $3c^1$, $3d^1$ and $2d^2$ (see text for details). (D) 6 h.p.f.c.. Division of $3c^2$, $3d^2$ and the midline stem cell, so that the midline is now made of two cells. 3D derivatives are now omitted. (E) 10 h.p.f.c.. The posterior edge of the blastopore migrates anteriorly, together with the two lateral stomatoblasts. Terminal cells have lost *PvuBra* expression. A third small cell is added to the midline by the stem cell. (F,G) Late gastrulae, showing the growing midline, and the closing blastopore. (H) 13 h.p.f.c. young trochophore expression pattern. v.p., vegetal plate; t.c. teminal cell; m.s.c. midline stem cell; sb. stomatoblast; st. stomodaeum.

ERK MAP kinase is specifically expressed in 3D during DV induction

ERK MAP kinase activation is involved in early development in spiralian embryos. In *Ilyanassa*, which cleaves unequally, ERK is activated in 3D, and then propagates, in a vegetal to animal succession, in the micromeres of the D quadrant (Lambert and Nagy, 2001). In contrast, in the equal cleaver *Chaetopleura*, it is activated only in 3D (J. D. Lambert, personal communication). In order to investigate the role of ERK activation in *Patella*, and to test a possible connection between its activation and *Brachyury* expression, immunostainings were performed on *Patella* early embryos, using an antibody raised against the activated, diphosphorylated form of ERK (dpERK) (Gabay et al., 1997).

Labelling is only seen between the 32- and the 40-cell stages, in 3D (Fig. 6A), as in *Chaetopleura*. Staining is observed throughout the cytoplasm and, somewhat more intensely, in the nucleus and at the boundary that 3D shares with the roof of the blastocoel (Fig. 6B). Weak labelling is also detected in the three other macromeres (not shown). No labelling is seen in any other cell of the embryo, until after gastrulation. As ERK MAP kinase is known to be a mediator of receptor tyrosine kinase (RTK) signalling in metazoans (Bier, 1998), this strongly suggests that a RTK is involved in the process of 3D determination, and furthermore, that high level of ERK activity might be necessary to mediate a 3D fate. In embryos simultaneously labelled with anti-dpERK antibody and *PvuBra* antisense probe, *PvuBra* was always detected in 3D together with dpERK (not shown). Thus, ERK MAP kinase and *PvuBra* expression are activated concomitantly, at least within a 20-minute window. This is compatible with *PvuBra* being a target of ERK MAP kinase signalling.

MAP kinase inhibited embryos loose 3D-specific *PvuBra* expression and display a radial cleavage pattern

A way to assess the epistatic relationships between ERK and *PvuBra* and, more generally to determine the role played by 3D-specific ERK activity in subsequent development, would be to interfere with the ERK pathway. The drug U0126 (Promega) is known to specifically inhibit the MAPKK that phosphorylates ERK MAPK, and has been used several times to address the role of ERK during development (see Lambert and Nagy, 2001, and references therein) (Lambert and Nagy, 2001). We thus investigated the consequences of ERK inhibition by the drug U0126 during the early development of *Patella*.

Embryos were selected at the 2- and 4-cell stage, raised until the 32-cell stage, and kept in U0126 during the following 5 hours. Two alternative doses of U0126 were used: a medium dose as in Lambert and Nagy (10 μM) (Lambert and Nagy, 2001), and a high dose (50 μM). Next, the embryos were fixed at regular intervals, examined for their cell cleavage pattern and their *PvuBra* expression profile.

In all embryos treated with U0126, the exclusive contact of one of the macromeres with the blastocoel roof is still observed, although it is less conspicuous when a higher concentration of the drug is applied (not shown). This quantitative dose-response behaviour suggests that residual MAP kinase activity is still present: indeed, when medium doses of U0126 are used, some labelling of 3D can be detected with the anti-dp-ERK antibody (not shown). No such labelling could be observed at high doses of ERK inhibitor. Although 3D determination seems to occur, the pattern of the 6th and 7th

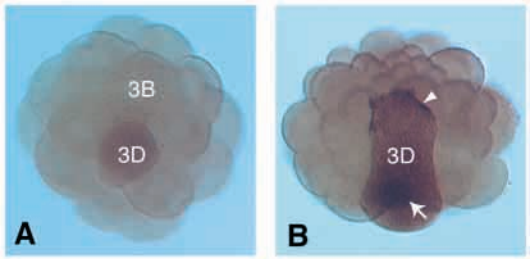

Fig. 6. Localisation of activated ERK in a 32-cell stage embryo. (A) Vegetal view. The labelled cell is one of the four macromeres. (B) Side view of the same embryo. The labelled cell is contacting the blastocoel roof, and thus can be identified as 3D. Labelling is strong in the nucleus (arrow) and along the zone of contact with the cap of micromeres (arrowhead).

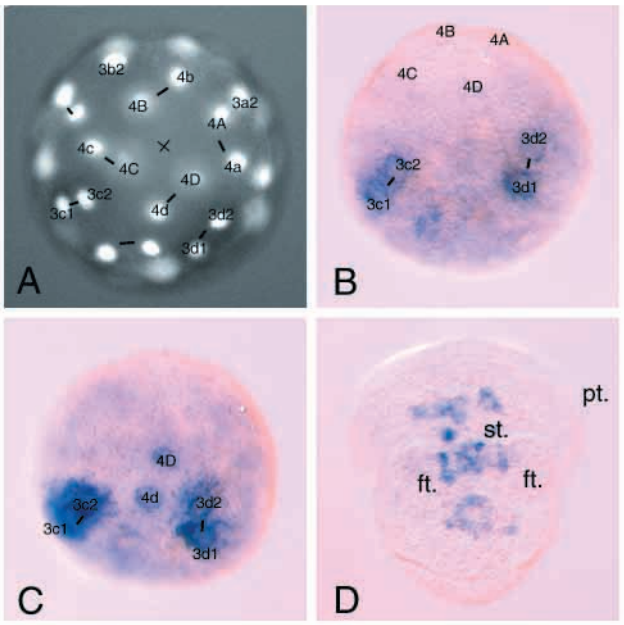

Fig. 7. Mitotic pattern and *PvuBra* expression in ERK inhibited embryos (medium dose). Unless specified otherwise, all views are from the vegetal pole. Presumed D quadrant to the bottom. (A) In situ hybridisation, combined with Hoechst staining of the nuclei, on a 64-cell stage embryo, showing an equalised cleavage pattern (compare with Fig. 3I,J). Sister cells are indicated by a line. (B) 64-cell stage, side views, showing *PvuBra* expression in $3c^1$, $3c^2$, $3d^1$, $3d^2$, and at a weaker level, in $2d^{22}$ and $2d^{21}$. No staining is seen in 4D and 4d (the cleavage is equal: compare with Fig. 3B). (C) Late 64-cell stage: some *PvuBra* expression is now seen in 4D and 4d. (D) Early trochophore: *PvuBra* expression is detected in cells scattered on the ventral side of the trochophore. pt., prototroche; ft., foot lobe; st., stomodaeum.

cleavage is clearly equalised, in the sense that, in all four quadrants, third quartet macromeres and micromeres divide synchronously, according to the A and B quadrant pattern (Fig. 7A). This was true in more than 75% of the embryos, whatever the dose of U0126 that had been applied (n=40 and n=19, for medium and high dose, respectively).

PvuBra expression is undetected in 3D by in situ hybridisation in U0126-treated embryos (100% of the analysed embryos, in both low and high dose experiments, n=12 and n=18, respectively). This shows that *PvuBra* expression in 3D is under the regulation of the MAP kinase pathway. 30 minutes after the 64-cell stage, some *PvuBra* expression is seen, following a pattern of expression reminiscent of what is usually observed in normal embryos, albeit superimposed on a fourfold symmetrical cleavage pattern: *PvuBra* RNA is first detected in $3c^1$, $3c^2$, $3d^1$ and $3d^2$, and less often in $2d^2$ derivatives as well (Fig. 7B). The overall level of transcription is lower than in control embryos, and furthermore, is delayed (approximately 30 minutes) at high doses of U0126. Then, *PvuBra* transcripts appear in 4D and 4d, slightly before the 7th cleavage (Fig. 7C). We were not able to perform a detailed investigation of the cleavage pattern nor of the expression of *PvuBra* during later stages of gastrulation.

The overall morphology of the 24-hour trochophore larvae is somewhat disturbed (Fig. 7D), but the main traits of the body plan are present: foot, prototroch, and apical organ (n=8). Furthermore, *PvuBra* is expressed on the ventral side of the larvae, although not restricted to the midline (Fig. 7D). This indicates that no gross axial and gastrulation defects have resulted from ERK inhibition.

DISCUSSION

We have isolated a *Brachyury* homologue (*PvuBra*) in the gastropod *Patella vulgata*, and analysed its expression pattern during embryogenesis. *PvuBra* is first transcribed as soon as the fourfold (spiral) symmetry is broken and an anterior-posterior (AP) axis is specified. Its expression is restricted to the posterior quadrant: it is first seen in 3D, then propagates to the first cells that switch to a bilaterally symmetrical pattern (3c and 3d), and finally marks the posterior edge of the blastopore.

Possible roles for *PvuBra* in *Patella* early development

Since *PvuBra* is expressed in 3D, and then in the mesentoblast 4d, which gives rise to most of the adult mesoderm, one may wonder about a possible role of *PvuBra* in mesoderm specification. In vertebrates, *Brachyury* is expressed in mesodermal and endodermal derivatives, and has been claimed to have a central role in mesoderm formation (Smith et al., 1991; Isaacs et al., 1994). However, genetic studies in mouse (Beddington et al., 1992) and zebrafish (Schulte-Merker et al., 1994) have not clearly established that *Brachyury* is specifically required for mesoderm specification, and have instead raised the interesting alternative that *Brachyury* might be involved in the morphogenetic movements of gastrulation (Wilson et al., 1995; Wilson and Beddington, 1997; Melby et al., 1996).

In *Patella*, *PvuBra* expression in the mesentoblast 4d is very transient. In addition, besides 4d, the 3a, 3c and 2b cells also have a mesodermal fate in *Patella* (Dictus and Damen, 1997), and they were never seen to express *PvuBra*. Thus, *PvuBra* is not panmesodermal, which suggests that it does not have a fundamental role in mesoderm formation in *Patella*. In contrast, the expression of *PvuBra* in 4d is reminiscent of what is observed in *Drosophila:* at the blastoderm stage, *brachyenteron* is expressed in a posterior ring around the embryo, only the ventral part of which is fated to mesoderm, the caudal visceral mesoderm (CVM) (Kusch and Reuter, 1999), giving rise to the longitudinal visceral muscles (LVM) (San Martin and Bate, 2001). Upon invagination, this anlage soon looses *brachyenteron* transcripts, but the protein remains stable in these cells for quite a long time thereafter, and is required for most of the aspects of LVM morphogenesis and signalling. Such a stability at the protein level cannot be excluded in *Patella*'s mesentoblast, and for that reason, *PvuBra* protein detection should be implemented.

The pattern of expression of *PvuBra* on the posterior edge of the blastopore of *Patella* gastrulating embryos is consistent with a role of *PvuBra* in morphogenetic movements. Furthermore, the behaviour of the mitotic spindles of 3c and 3d, right after 3D determination, also suggests that *PvuBra* might be involved in the regulation of unequal cell cleavages. As radialisation experiments in *Patella* have shown, all asymmetries and

asynchronies of cell cleavages in the dorsal quadrant after the 64-cell stage can be interpreted as departures from a spiral default state, which are induced, directly or indirectly, by 3D. *Brachyury* is expressed at the right place and time to be involved in such modulations of cell cleavage morphology.

MAP kinase and the induction of 3D in *Patella vulgata*

ERK MAP kinase is activated at a low level in the four macromeres of the early 32-cell stage embryo, and then at a much higher level in 3D. These observations suggest two distinct roles for ERK activation: it could be required for the process of symmetry breaking proper, and in addition, high levels of ERK activity in 3D might be necessary for determining 3D's fate, and in particular, its organising properties in the pre-gastrula.

Inhibition of ERK signalling, even partial, leads to an equalised cell division pattern. The cleavage is equalised as far as we have been able to analyse it (up to the 88-cell stage). This strongly indicates that part of 3D organising function in *Patella* early development, i.e. the regulation of cell cleavage asymmetries, requires ERK activity in 3D. Very similar cell cleavage modifications have also been observed in *Ilyanassa* as a result of ERK inhibition, including the suppression of 3c- and 3d-specific mitotic behaviour as opposed to 3a and 3b, and the effect of ERK inhibition on 4d (Lambert and Nagy, 2001). All these alterations are more pronounced in *Patella*, where for instance 4d divides synchronously with, and cannot be distinguished from the three other micromeres of the third quartet. This is probably due to the fact that, in *Patella*, the cleavage is perfectly equal until the 32-cell stage, and thus offers the ideal background to observe the lack of its 3D mediated regulation. Otherwise, the overall impression is that the role of ERK in the regulation of cell cleavage induced by 3D is well conserved in gastropods, irrespective of the differences observed between equal and unequal cleavers.

In addition, in ERK-inhibited embryos, *PvuBra* transcription is suppressed in 3D, which shows that *PvuBra* is a target of the ERK signalling cascade in 3D. This is all the more likely to be a direct epistatic relationship as no time lag could be resolved between dpERK detection and *PvuBra* transcripts localisation. More importantly, the correlation between the 3D-specific suppression of *PvuBra* activation and the equalisation of the cleavage pattern raises the interesting possibility that *PvuBra* expression in 3D might mediate at least part of ERK-dependent regulations of cell cleavage patterns.

However, interference with ERK signalling does not lead to a phenotype as extreme as that observed in embryos treated with monensin: following such a treatment, embryos are completely equalised, and in particular, no 3D blastomere is specified (Kühtreiber et al., 1988). In contrast, in the case of ERK inhibition, a 3D macromere is still determined, even when high doses of U0126 are applied, as indicated by the exclusive contacts of one of the macromeres to the blastocoel roof. In addition, *PvuBra* is still expressed asymmetrically, in 3c and 3d but not in 3a nor 3b. It is quite possible that ERK inhibition be only partial. Another interpretation would be that part of 3D organising role, that of inducing *PvuBra* expression in 3c, 3d and $2d^2$, is independent of ERK activity in 3D. A way to test this possibility is to identify and interfere with the molecular signals emitted by 3D. In *Ilyanassa*, in contrast, ERK signalling has been shown to be required in most aspects of embryonic patterning attributed to the organising role of the D quadrant (Lambert and Nagy, 2001). An important fact that might explain why the consequences of ERK inhibition in *Ilyanassa* are stronger than in *Patella* is that, in *Ilyanassa*, ERK is activated not only in 3D, but also in all blastomeres that require induction by 3D.

At any case, the experiments shown here demonstrate that different aspects of bilateral symmetry specification are differentially controlled in a spiral cleaving embryo: the regulation of cell cleavage timing and morphology requires high level of ERK signalling, whereas transcriptional regulation, except for 3D-specific *PvuBra* activation, seems to be partly independent of ERK, or to require only low levels of its activity. Surprisingly, ERK-inhibited embryos show substantial regulative abilities, and seem to accomplish a fairly complete gastrulation, as witnessed by their more or less normally patterned trochophore, in spite of the suppression of all the early bilateral cleavage patterns.

Finally, the combined involvement of the ERK MAP kinase signalling cascade and *PvuBra* in 3D induction in *Patella* raises interesting questions about a possible conservation of their interactions among *Bilateria*. Indeed, *Brachyury* is also activated by FGF in vertebrates (Smith et al., 1991), while in *Drosophila*, the *Brachyury* homologue *brachyenteron* is downstream of the Torso RTK (Singer et al., 1996; Kusch and Reuter, 1999). A crucial point here is to identify the RTK involved in the process of 3D election in *Patella*. Two likely candidates would be FGF and EGF receptors.

Brachyury as a marker of the blastopore

In the young trochophore, *PvuBra* is expressed in a domain encompassing the ectoderm right below the stomodaeum, as well as two bilateral terminal cells and, in-between, the ventral midline. This expression pattern is nearly identical to that of the orthologue of *Brachyury* in the young trochophore larvae of *Platynereis* (Arendt et al., 2001). In order to interpret this expression profile, it should be stressed that a different mode of gastrulation prevails in the two species. In *Platynereis*,

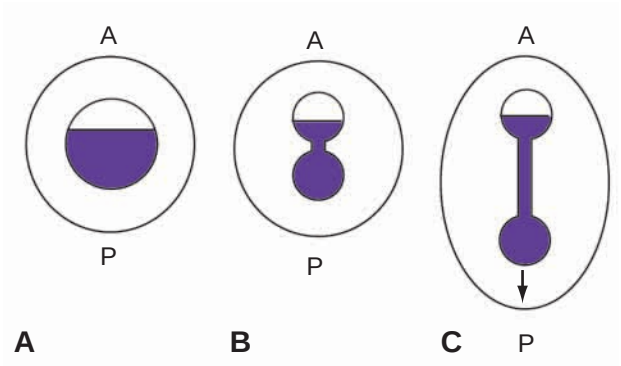

Fig. 8. Amphistomy, AP axis formation, and *Brachyury* expression (blue) in the common ancestor of the Bilateria. (A) Following AP axis specification, the vegetal region of the embryo becomes subdivided into an anterior, *Brachyury* negative, and posterior, *Brachyury* positive, domains. (B) The blastopore closes in an amphistomous fashion. (C) The posteriorly located growth zone is responsible for the AP axis elongation (arrow). As a result of the whole process, *Brachyury* is expressed posteriorly and on the ventral midline.

gastrulation is amphistomous (as in *Nereis*) (Wilson, 1892): when closing, the blastopore takes the shape of an '8', the anterior and posterior loop of which give birth to mouth and anus opening, respectively. Only in a second step is the ventral midline formed, by invasion of the space in-between the two loops by more lateral cells (Fig. 8). In *Patella*, in contrast, gastrulation is of a protostomous type, the blastopore giving rise to the mouth only, and the anus breaking through secondarily. However, results from classical comparative embryology tend to show that gastrulation was probably amphistomous in the common ancestor of the so-called 'protostomes' (Nielsen, 2001), so that *Patella*'s protostomous gastrulation can be considered a secondary modification.

The domain of expression of *Brachyury* in *Platynereis* young larva can be described very briefly: it merely delineates the closed blastopore, except for its anteriormost part (Fig. 8). This might indicate that *Brachyury* has, or has had, some functions in the very process of blastopore closure and/or ventral midline extension. Now, the fact that, in *Patella*, *PvuBra* expression profile is so reminiscent of the expression pattern of its orthologue in *Platynereis*, in spite of a secondarily modified gastrulation, could be interpreted as a 'molecular vestige' of the ancestral amphistomous blastopore: in other words, *PvuBra* marks what used to be blastopore-derived structures, before gastrulation evolved to a protostomous mode.

One can go further along this line of reasoning: indeed, as in *Patella* and *Platynereis*, *Brachyury* orthologues are expressed in the ventral part of the mouth and around the anus in the larvae of the hemichordate *Ptychodera* (Tagawa et al., 1998) and the echinoderm *Asterina* (Shoguchi et al., 1999). By extension of our argument, this would tend to indicate, not only a one to one homology between foregut and hindgut in protostome and deuterostome larvae, as proposed previously (Arendt et al., 2001), but also the important fact that amphistomy is ancestral to Bilateria (Jägersten, 1955; Arendt and Nubler-Jung, 1997; Technau, 2001). One could suppose that, in the lineage leading to the last common ancestor of all Bilateria, *Brachyury* was expressed around the posterior half of the closed blastopore. There, its primitive function could have been in the regulation of morphogenetic movements of gastrulation and convergent extension, as well as the maintenance of the posterior growth zone.

Posterior functions of *Brachyury* among Bilateria

Another striking aspect of *PvuBra* expression profile is that it illuminates the progressive growth of the AP axis during gastrulation in *Patella* (see Fig. 5). *PvuBra* is expressed in 3D, and then in $2d^2$ which, according to our interpretation, behaves like a stem cell, budding off the cells of the ventral midline. This interpretation is mostly based on the assumption of cellular continuity of *PvuBra* expression, and awaits further experimental confirmation. Another report of stem cells for the ventral midline ectoderm was made by Robert (Robert, 1902), in *Trochus*, although in this case, not only $2d^2$, but also $2d^{12}$ contributes to the midline ectoderm. More generally, posterior stem cells, or *teloblasts*, are seen in other spiralians, such as annelids (Weisblat and Shankland, 1985), where they give rise to most of the ectoderm and the mesoderm of the trunk. Significantly, these teloblasts are derived from the D quadrant exclusively. Thus, in spiralians, the D quadrant seems to play a conserved role in AP axis formation, in that it gives birth to the posterior growth zone*. The results we have obtained in *Patella* underline the teloblastic mode of AP axis formation that also prevails in molluscs and, more importantly, suggest that *Brachyury* might be involved in the developmental activity of the posterior pole.

On a broader scale, it appears that the progressive growth of the AP axis, in an anterior to posterior sequence, and through the activity of a posterior growth zone, is observed in diverse phyla across Bilateria as, for instance, in short-germ insects, polychaetes and chordates. In vertebrates, the posterior pole of the developing axis is the organiser, in the form of the superior lip of the blastopore of fish and frog, or the node of amniotes, and later on, the chordo-neural hinge of the tail bud. *Brachyury* is expressed in the vertebrate organiser during the major part of embryonic development, and genetic studies in mouse and fish show that this expression is necessary for AP axis formation (Wilson and Beddington, 1997; Melby et al., 1996). Similarly, in *Patella*, as soon as the four-fold symmetry has been broken, *PvuBra* is a expressed in the D quadrant, and remains a marker of the posterior pole of the AP axis up to the end of larval development, until the whole axis is laid down. This striking similarity is in favour of the hypothesis that the progressive growth of the AP axis represents a conserved developmental process which, already in Urbilateria, would have taken the form of an organising posterior growth zone, controlled by a genetic system involving *Brachyury*, among others. This posterior pole would have evolved into the organiser of vertebrates, and into the set of teloblasts in Spiralia.

We wish to thank David Lambert and Lisa Nagy for communicating unpublished information, as well as Lex Nederbragt, Hans Goedemans and Andre van Loon for making available the cDNA libraries, and for all the work that has been done with their help. We are grateful to Martine Le Gouard for helping to rear the embryos, Peter Damen for helping us to sort out many cell lineage issues, Bernadette Limbourg-Bouchon for her help with microscopy and imaging, Amer Ghandour for image edition, and Jo van den Biggelaar, Benjamin Prudhomme, Renaud de Rosa, Jean-François Julien and Guillaume Balavoine and three anonymous referees for their useful comments on the manuscript. Work in the Centre de Génétique Moléculaire is financially supported by the Centre National de la Recherche Scientifique and the Université Paris-Sud. This work was also supported by the Genome Project (CNRS), the Fondation de la Recherche Médicale, and the Institut Français de la Biodiversité.

REFERENCES

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B. and de Rosa, R. (2000). The new animal phylogeny: reliability and implications. *Proc. Natl. Acad. Sci. USA* **97**, 4453-4456.

Aguinaldo, A. M., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. and Lake, J. A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387**, 489-493.

Arendt, D. and Nubler-Jung, K. (1997). Dorsal or ventral: similarities in fate maps and gastrulation patterns in annelids, arthropods and chordates. *Mech. Dev.* **61**, 7-21.

Arendt, D., Technau, U. and Wittbrodt, J. (2001). Evolution of the bilaterian larval foregut. *Nature* **409**, 81-85.

*It should be mentioned that, since the bilateral symmetry appears along with the specification of the D quadrant, a dorsal-ventral axis can in principle be defined at the very same moment as the AP axis is visible. However, describing the process in terms of AP axis only, as we do here, leads to a simpler description, at least in a comparative perspective. A more complete analysis of the implications of the expression of *Brachyury* on the comparison of DV axis formation at the scale of Bilateria will be developed elsewhere.

Arnolds, J. A., van den Biggelaar, J. A. M. and Verdonk, N. H. (1983). Spatial aspects of cell interactions involved in the determination of dorsoventral polarity in equally cleaving gastropods and regulative abilities of their embryos, as studied by micromere deletions in *Lymnea* and *Patella*. *Roux's Arch. Dev. Biol*. **192**, 75-85.

Bassham, S. and Postlethwait, J. (2000). *Brachyury (T)* expression in embryos of a larvacean urochordate, *Oikopleura dioica*, and the ancestral role of T. *Dev. Biol*. **220**, 322-332.

Beddington, R. S., Rashbass, P. and Wilson, V. (1992). *Brachyury* – a gene affecting mouse gastrulation and early organogenesis. *Development* **Suppl.** 157-165.

Bier, E. (1998). Localized activation of RTK/MAPK pathways during *Drosophila* development. *BioEssays* **20**, 189-194.

Corbo, J. C., Levine, M. and Zeller, R. W. (1997). Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. *Development* **124**, 589-602.

Damen, P. and Dictus, W. J. (1996). Organiser role of the stem cell of the mesoderm in prototroch patterning in *Patella vulgata* (Mollusca, Gastropoda). *Mech. Dev*. **56**, 41-60.

DeRobertis, E. M. and Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* **380**, 37-40.

Dictus, W. J. and Damen, P. (1997). Cell-lineage and clonal-contribution map of the trochophore larva of *Patella vulgata* (mollusca). *Mech. Dev*. **62**, 213-226.

Freeman, G. and Lundelius, J. W. (1992). Evolutionary implications ofthe mode of D quadrant specification in coelomates with spiral cleavage. *J. Evol. Biol*. **5**, 205-247.

Gabay, L., Seger, R. and Shilo, B. Z. (1997). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.

Harada, Y., Yasuo, H. and Satoh, N. (1995). A sea urchin homologue of the chordate *Brachyury* (*T*) gene is expressed in the secondary mesenchyme founder cells. *Development* **121**, 2747-2754.

Herrmann, B. G. (1991). Expression pattern of the *Brachyury* gene in whole-mount *TWis/TWis* mutant embryos. *Development* **113**, 913-917.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. (1994). *eFGF* regulates *Xbra* expression during *Xenopus* gastrulation. *EMBO J*. **13**, 4469-4481.

Jägersten, G. (1955). On the early phylogney of the Metazoa. *Zool. BiDr. Uppsala* **30**, 321-354.

Jones, D. T., Taylor, W. R. and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci*. **8**, 275-282.

Kispert, A. and Herrmann, B. G. (1994). Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. *Dev. Biol*. **161**, 179-193.

Kispert, A., Herrmann, B. G., Leptin, M. and Reuter, R. (1994). Homologs of the mouse *Brachyury* gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*. *Genes Dev*. **8**, 2137-2150.

Kispert, A., Ortner, H., Cooke, J. and Herrmann, B. G. (1995). The chick *Brachyury* gene: developmental expression pattern and response to axial induction by localized activin. *Dev. Biol*. **168**, 406-415.

Kühtreiber, W. M., van Til, E. H. and van Dongen, C. A. M. (1988). Monensin interferes with the determination of the mesodermal cell line in embryos of *Patella vulgata*. *Roux's Arch. Dev. Biol*. **197**, 10-18.

Kusch, T. and Reuter, R. (1999). Functions for *Drosophila brachyenteron* and *forkhead* in mesoderm specification and cell signalling. *Development* **126**, 3991-4003.

Lambert, J. D. and Nagy, L. M. (2001). MAPK signaling by the D quadrant embryonic organizer of the mollusc *Ilyanassa obsoleta*. *Development* **128**, 45-56.

Lespinet, O., Nederbragt, A. J., Cassan, M., Dictus, W. J. A. G., van Loon, A. E. and Adoutte, A. (2002). Characterization of two *snail* genes in the gastropod mollusc *Patella vulgata*. Implications for understanding the ancestral function of the *snail*-related genes in *Bilateria*. *Dev. Genes. Evol.* (in press).

Martindale, M. Q. (1986). The 'organizing' role of the D quadrant in an equal-cleaving spiralian, *Lymnea stagnalis*, as studied by UV laser deletion of macromeres at intervals between third and fourth quartet formation. *Int. J. Inv. Reprod. Dev.* **9**, 229-242.

Martindale, M. Q., Doe, C. Q. and Morrill, J. B. (1985). The role of animal-vegetal interaction with respect to the determination of dorsoventral polarity in the equal-cleaving spiralian, *Lymnaea palustris*. *Roux's Arch. Dev. Biol.* **194**, 281-295.

Melby, A. E., Warga, R. M. and Kimmel, C. B. (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* **122**, 2225-2237.

Nielsen, C. (2001). *Animal Evolution. Interrelationships of the Living Phyla.* Second Edition. Oxford: Oxford University Press.

Papaioannou, V. E. and Silver, L. M. (1998). The T-box gene family. *BioEssays* **20**, 9-19.

Peterson, K. J., Cameron, R. A., Tagawa, K., Satoh, N. and Davidson, E. H. (1999a). A comparative molecular approach to mesodermal patterning in basal deuterostomes: the expression pattern of Brachyury in the enteropneust hemichordate *Ptychodera flava*. *Development* **126**, 85-95.

Peterson, K. J., Harada, Y., Cameron, R. A. and Davidson, E. H. (1999b). Expression pattern of *Brachyury* and *Not* in the sea urchin: comparative implications for the origins of mesoderm in the basal deuterostomes. *Dev. Biol*. **207**, 419-431.

Robert, A. (1902). Recherche sur le développement des Troques. *Arch. Zool. Exp. 3e Série*. **10**, 269-538.

San Martin, B. and Bate, M. (2001). Hindgut visceral mesoderm requires an ectodermal template for normal development in *Drosophila*. *Development* **128**, 233-242.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Schulte-Merker, S., van Eeden, F. J., Halpern, M. E., Kimmel, C. B. and Nusslein-Volhard, C. (1994). *no tail* (*ntl*) is the zebrafish homologue of the mouse *T* (*Brachyury*) gene. *Development* **120**, 1009-1015.

Shoguchi, E., Satoh, N. and Maruyama, Y. K. (1999). Pattern of *Brachyury* gene expression in starfish embryos resembles that of hemichordate embryos but not of sea urchin embryos. *Mech. Dev*. **82**, 185-189.

Singer, J. B., Harbecke, R., Kusch, T., Reuter, R. and Lengyel, J. A. (1996). *Drosophila brachyenteron* regulates gene activity and morphogenesis in the gut. *Development* **122**, 3707-3718.

Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993). The Zootype and the phylotypic stage. *Nature* **361**, 490-492.

Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Strimmer, K. and von Haeseler, A. (1996). Quartet Puzzling: A quartet maximum-likelihood method for reconstructing tree topologies. *Mol. Biol. Evol*. **13**, 964-969.

Tagawa, K., Humphreys, T. and Satoh, N. (1998). Novel pattern of *Brachyury* gene expression in hemichordate embryos. *Mech. Dev*. **75**, 139-143.

Technau, U. (2001). *Brachyury*, the blastopore and the evolution of the mesoderm. *BioEssays* **23**, 788-794.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. **25**, 4876-4882.

van den Biggelaar, J. A. (1977). Development of dorsoventral polarity and mesentoblast determination in *Patella vulgata*. *J. Morphol*. **154**, 157-186.

van den Biggelaar, J. A. and Guerrier, P. (1979). Dorsoventral polarity and mesentoblast determination as concomitant results of cellular interactions in the mollusk *Patella vulgata*. *Dev. Biol*. **68**, 462-471.

Van Loon, A. E., Colas, P., Goedemans, H. J., Neant, I., Dalbon, P. and Guenier, P. (1991). The role of cyclins in the maturation of *Patella vulgata* oocytes. *EMBO J.* **10**, 3343-3349.

Weisblat, D. A. and Shankland, M. (1985). Cell lineage and segmentation in the leech. *Philos. Trans. R. Soc. Series B* **312**, 39-56.

Wilkinson, D. G., Bhatt, S. and Herrmann, B. G. (1990). Expression pattern of the mouse *T* gene and its role in mesoderm formation. *Nature* **343**, 657-659.

Wilson, E. B. (1892). The cell-lineage of *Nereis*. A contribution to the cytogeny of the annelid body. *J. Morphol*. **6**, 361-480.

Wilson, V. and Beddington, R. (1997). Expression of T protein in the primitive streak is necessary and sufficient for posterior mesoderm movement and somite differentiation. *Dev. Biol*. **192**, 45-58.

Wilson, V., Manson, L., Skarnes, W. C. and Beddington, R. S. (1995). The *T* gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. *Development* **121**, 877-886.

Yasuo, H. and Satoh, N. (1998). Conservation of the developmental role of *Brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. *Dev. Biol*. **200**, 158-170.