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ORIGINAL ARTICLE

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Novel and conserved roles for *orthodenticle/otx* and *orthopedia/otp* orthologs in the gastropod mollusc *Patella vulgata*

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Abstract The *orthodenticle*/*otx* and *orthopedia*/*otp* classes of homeobox gene families have been identified in all three major classes of bilaterians: deuterostomes, lophotrochozoans, and ecdysozoans. *Otx* genes have been studied extensively and play a role in the development of anterior neural structures. *Otp* genes have been found to be involved in nervous system development in mouse and *Drosophila*. To date, no members of these genes are known in molluscs. We cloned orthologs of *orthodenticle*/*otx* and *orthopedia*/*otp* from the gastropod *Patella vulgata*, and designated them *Pv-otx* and *Pv-otp* respectively. Our analysis of the spatio-temporal expression pattern of *otx* and *otp* orthologs during *P. vulgata* embryogenesis leads to the following conclusions. First, *Pv-otx* is expressed in and around the stomodaeum and our analysis thus supports the previously suggested conservation of the protostome and deuterostome larval mouth regions. Second, we find that *Pv-otp* is involved in the development of the larval apical sensory organ, suggesting a conserved role for this gene family in nervous system development. A similar conserved role in nervous system development has been proposed for *orthodenticle/otx* genes and we suggest that part of the cells expressing *Pv-otx* are involved in the development of the anterior nervous system. Last, we postulate that *otx* genes were ancestrally involved in the development of ciliary bands in bilaterians.

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Introduction

Comparative analysis of genes involved in development of multicellular organisms has revealed conserved sets of transcription factors that are deployed by numerous species. One of these is the class of homeodomain transcription factors, characterised by a specific 60-amino-acid DNA-binding domain. Many members of this class can be found throughout the Metazoa. The best-known example is the Hom/Hox cluster that is involved in anterior/posterior patterning in various species (Ferrier and Holland 2001). Two other examples of such conserved homeodomain transcription factors are the *orthodenticle*/*otx* and *orthopedia*/*otp* gene families. Members of these gene families have been identified in all three major classes of bilaterians: deuterostomes (such as vertebrates), lophotrochozoans (such as molluscs and annelids) and ecdysozoans (such as arthropods and nematodes).

The *orthodenticle* gene was first found in the arthropod insect *Drosophila* and is expressed in the anterior part of the embryo (but not in the anterior-most region) where it plays a role in patterning the rostral brain (Cohen and Jurgens 1990; Finkelstein et al. 1990). Null mutations in the gene cause absence of the forebrain in the fly embryo (Hirth et al. 1995). A second domain of *orthodenticle* expression is a stripe of cells next to the ventral midline that will become part of the ventral nerve cord (Cohen and Jurgens 1990; Finkelstein et al. 1990). Orthologs of *orthodenticle* have been found in many other organisms. Vertebrates (deuterostomes) have two copies, *otx1* and *otx2* (Simeone et al. 1992). *Otx2* is involved in the induction of the anterior neural plate. Both *otx1* and *otx2* are important for the development of the brain from the midbrain/hindbrain boundary up to the forebrain, but not for the most anterior part of the brain (Acampora et al. 2000). A role for *otx* genes in development of anterior neural structures has subsequently been

discovered in other deuterostomes, viz cephalochordates (Williams and Holland 1998; Tomsa and Langeland 1999), hemichordates (Harada et al. 2000), ascidians (Wada et al. 1996; Wada and Saiga 1999) and in lophotrochozoans, viz annelids (Bruce and Shankland 1998). Another commonality between members of the *orthodenticle*/*otx* gene family in deuterostomes as well as protostomes is their involvement in the development of the larval mouth (Shoguchi et al. 2000; Arendt et al. 2001), which has led to the suggestion that larval foregut and mouth regions are homologous among bilaterians (Arendt et al. 2001).

The *orthopedia* (*otp*) gene was discovered in *Drosophila* and mouse simultaneously (Simeone et al. 1994). Its homeobox sequence showed similarity to that of *orthodenticle/otx* genes, as well as *antennapedia/ant* genes. Despite differences in the expression patterns of *otp* genes in the fly and in the mouse, expression in the nervous system was found in both species. In *Drosophila*, *otp* was found in a metameric pattern along the brain and ventral nerve cords (Simeone et al. 1994). In the mouse expression was found mainly in restricted regions of the diencephalon, hindbrain and spinal cord (Simeone et al. 1994). In the mouse, the most essential role of *otp* is in development of the hypothalamus (Acampora et al. 1999; Wang and Lufkin 2000). Other orthologs of *otp* have so far only been found in echinoderms (deuterostomes), where *otp* appeared to have a role in skeletal growth (Di Bernardo et al. 1999), and in planarians (platyhelminths, belonging to the lophotrochozoans), where expression was found in the adult brain (Umesono et al. 1997).

We are interested in determining the level of conservation of gene function between ecdysozoans, deuterostomes and lophotrochozoans. To date, however, no data on the expression or function of *otp* genes during early development are available for lophotrochozoans and *otx* genes have only been studied in the annelids. We thus analysed the expression of orthologs of *otx* and *otp* genes in the gastropod mollusc *Patella vulgata*, a lophotrochozoan. We describe here the cloning, characterisation and spatio-temporal expression pattern of *Patella* orthologs of *orthodenticle* and *orthopedia*. We have found conserved and possible novel roles for these genes.

Materials and methods

Cloning of *Pv-otx* and *Pv-otp*

To obtain a fragment of the homeodomain of a putative *orthodenticle/otx* ortholog, degenerate PCR primers based on conserved domains of *otx* genes were designed. First, a PCR with primers otx-fw1 (sequence TTYGGIAARACIMGITAYCC) and otx-rv1 (sequence YTGIARYTGYTGICKRCAYTT) was performed on genomic *P. vulgata* DNA under standard PCR conditions. Nested PCR was performed on 1 µl of this reaction under the same conditions with primers otx-fw2 (sequence GITAYCCIGAYATHTTYATG) and otx-rv2 (sequence RTTYTTRAACCAIACYTGIAC). Next, nested 5′ RACE PCR on a 40-cell-stage λZap-cDNA library was performed. The first PCR was performed with primers pBS-A (vector specific, sequence CTATGACCATGATTACGCCAAG) and otx-rv1; in the nested PCR, primers T3 (vector specific, sequence AATTAACCCTCATAAAGGG) and otx-rv2 were used.

Based on the resulting 561-bp 5′ fragment, new, specific primers were designed. Primer otx-fw3 (CCACATTTACCCGAGCTCCAGC) was used to obtain the 3′ part of this gene by 3′ RACE on a 16-h trochophore λZap-cDNA library with T7 (vector specific, sequence GTAATACGACTCACTATAGGGC) as the reverse primer. This resulted in a 2.2-kb 3′ PCR fragment. From this fragment, by standard PCR using primers otx-fw4 (sequence ATTACAGGAATGAATGGTTTGATG) and otx-rv3 (AAATCAAGTTACCAATACTTGTATTA), we obtained a 1,257-bp fragment that was used as a probe to screen the 16-h trochophore λZap-cDNA library according to Sambrook (1989). Both strands of the resulting 2.5-kb cDNA were completely sequenced.

We also performed 3′ RACE PCR on trochophore stage cDNA; the first PCR was performed using primers Otx-fw5 (TTTCAGAAAACTCGCTATCC) and pBS-E; in the nested PCR primers Otx-fw6 (GCTATCCCGATATCTTTATG) and T7 were used. We obtained a 694-bp PCR fragment that contained an open reading frame (ORF) more similar to *otp* genes, than to *otx* genes. This can be explained by the fact that the primers were designed for a region where the sequences of the two genes are very similar (in the middle of the homeobox). A larger part of this gene was obtained by performing PCR with primers Otp-rv1 (GAATCCAGTTAAACACGCTG) and pBS-A on the trochophore cDNA library, which gave a 794-bp fragment. From this fragment, by standard PCR using primers Otp-fw1 (AGATTTGTCGGATAAGGATAGAAA) and Otp-rv2 (CACGACATTTGTGACGTAGTAA), we obtained a 645-bp fragment that was used as a probe to screen the trochophore cDNA library according to Sambrook (1989). The resulting cDNA was completely sequenced on both strands.

Fertilization and embryo rearing

Mature *P. vulgata* were collected at the Atlantic coast near the Station Biologique at Roscoff, France and were kept in running natural seawater. Embryos were obtained as described previously (van den Biggelaar 1977).

Embryo fixation and in situ hybridisation

Embryos were fixed and stored and in situ hybridisation was performed as described in Nederbragt et al. (2002). Digoxigenin-labelled RNA probes were synthesised from PCR products using the DIG RNA labelling kit (Roche). For *Pv-otx*, the Pv-otxFw2/Pv-otxRv2 PCR fragment was used. For *Pv-otp*, the 794-bp PCR fragment was used.

Results

Cloning of *Pv-otx* and *Pv-otp*

An 84-bp fragment of the homeodomain of a putative *otx* ortholog was obtained by performing PCR on genomic DNA of *P. vulgata* with degenerate primers based on the consensus sequence of the homeobox of known *otx* genes. A longer PCR fragment of this gene was obtained by performing 5′ RACE PCR on a 40-cell-stage cDNA library with the same degenerate primers, and with primers specific for the vector. This resulted in a 561-bp PCR fragment, whose sequence contained an ORF, encoding part of a homeobox sequence closely similar to that of *otx* genes of other animals (not shown). Based on the sequence of this fragment, new sequence-specific PCR primers were designed in order to obtain the 3′ part of the gene. PCR with these primers resulted in a 2.2-kb 3′ RACE PCR

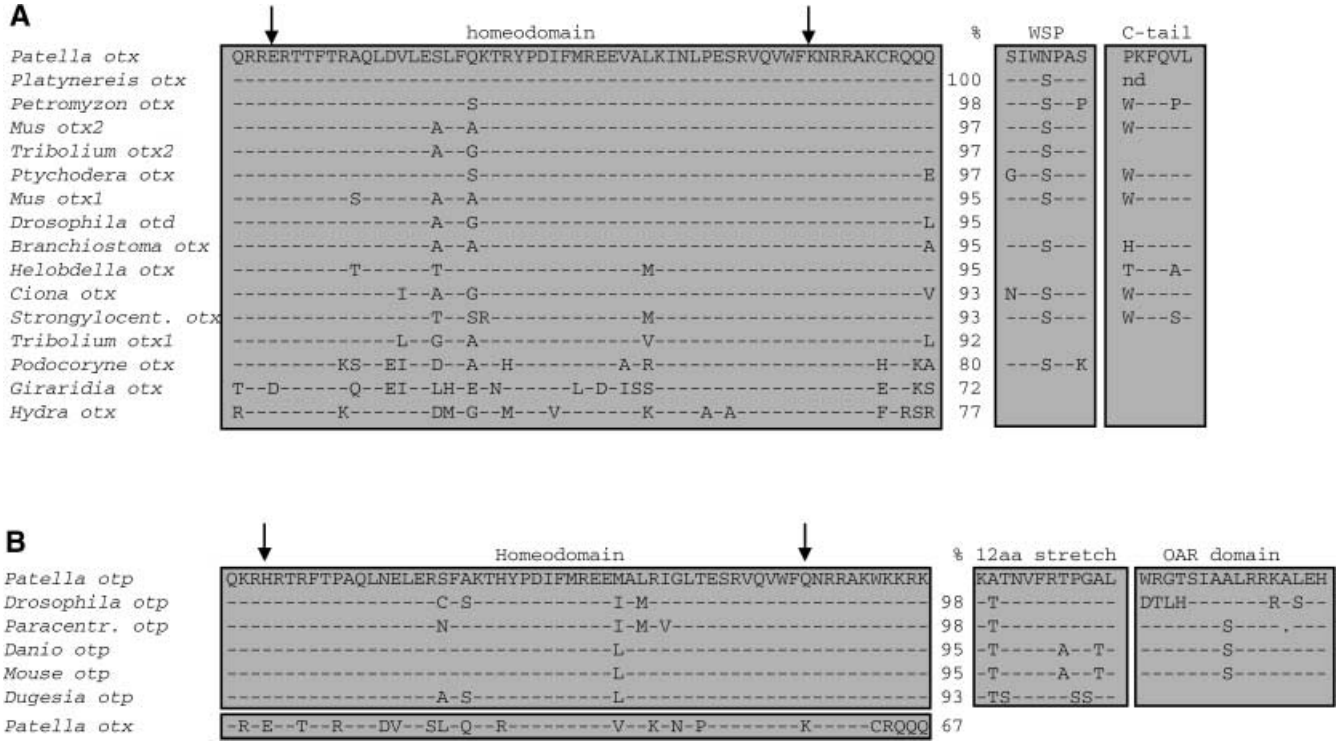

Fig. 1A, B *Patella vulgata* orthologs of *orthodenticle/otx* and *orthopedia/otp*. Comparison of the *Pv-otx* (**A**) and *Pv-otp* (**B**) conserved domain sequences with those of other orthodenticle and orthopedia proteins (*nd* not determined). A *dash* indicates an amino acid residue identical to the corresponding *Patella* residue, a *dot* indicates a gap introduced to optimise the alignment. *Arrows* indicate two positions in the homeobox that can be used to discriminate *otx* from *otp* class homeoboxes (see text). The percentage sequence identities (%) of the homeobox sequences are indicated. For *Pv-otx*, alignments of the WSP domain and the conserved C-terminal tail sequences, if present, are also shown. For the alignment of the *Pv-otp* homeobox the *Pv-otx* sequence is included for comparison. Alignments of the 12 amino acids directly downstream of the *Pv-otp* homeobox and the OAR domain sequences, if present, are also shown. Accession numbers of the sequences used are available on request (*Strongylocent. Strongylocentrotus*, *Paracentr. Paracentrotus*)

fragment. This fragment was used as a probe to obtain a full-length cDNA of the *otx* ortholog from a trochophore-stage cDNA library. This cDNA was sequenced completely on both strands and based on the close similarity with other *otx* genes (see below) we designated this gene *Pv-otx*. The sequence of *Pv-otx* has been deposited in GenBank under accession number AF440098.

Pv-otx is 2574 bp long, contains a putative start methionine at position 403 followed by a deduced ORF of 287 amino acids, a stop codon and a 1,291-bp 3′ untranslated region (UTR) with a consensus polyadenylation signal close to the 3′ end. The 5′ UTR is 402 bp long; there is an in-frame stop codon 5′ of the ORF (position 208), indicating that the cDNA most likely contains the entire coding region of *Pv-otx*. The deduced protein contains a homeodomain sequence (Fig. 1A), a so-called WSP domain [named after the central three conserved amino acids, tryptophane(W)-serine(S)-proline(P), cf. Muller et al. 1999; in *Patella*, the sequence is actually WNP] and a conserved C-terminal 6-amino-acid sequence (Muller et al. 1999) found in many *otx* genes (Fig. 1A). The homeodomain of *Pv-otx* has a glutamate (E) at the 4th position, and lysine (K) at the 50th position, characteristic of the homeobox domain of the *otx* class (Galliot et al. 1999).

Figure 1A shows a comparison of the conserved *Pv-otx* domains with the corresponding domains of other *otx* genes. The homeobox of *Pv-otx* is 100% identical to that of *Platynereis*, and has a high identity (92–98%) with those of most species, with the exception of the *otx* homeoboxes of the platyhelminth *Girardia* (72%) and the cnidarians *Podocoryne* (80%) and *Hydra* (77%; Fig. 1A).

The cloning of the *orthopedia* ortholog of *P. vulgata* was facilitated by the fact that a 3′ RACE PCR with nested PCR primers based on the 560-bp 5′ RACE *otx* fragment resulted in the cloning of a 637-bp PCR fragment which coded for an ORF that was more similar to *otp* genes than to *otx* genes. Both *otx* and *otp* genes are very similar in their homeobox sequences, therefore the PCR primers based on the *Patella otx* sequence were able to also recognise the *Patella otp* sequence. A larger fragment of the *otp* ortholog was obtained by 3′ RACE PCR, and the trochophore stage cDNA library was screened with this larger fragment as a probe. The resulting cDNA was sequenced completely on both strands and based on the close similarity with other *otp* genes (see below) we called this gene *Pv-otp*. The sequence of *Pv-otp* has been deposited in GenBank under accession number AF440099. The *Pv-otp* cDNA is 787 bp long with an open reading frame of 254 amino acids. There is no apparent in-frame stop codon at the 5′ side, nor can a start methionine be detected, indicating that this cDNA most likely is part of a longer tran-

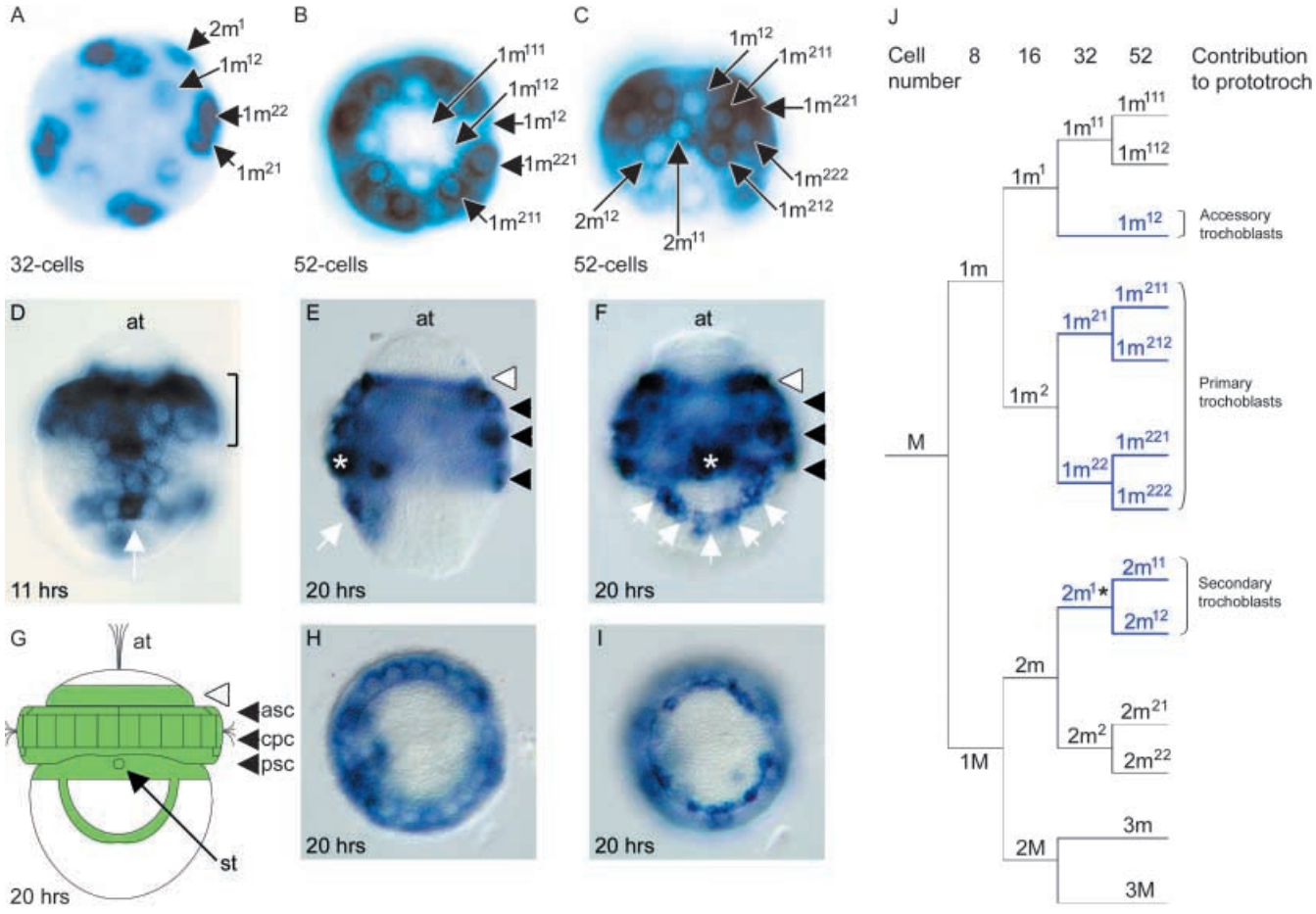

Fig. 2A–J Expression of *Pv-otx* in the embryo and larva. Optical sections (with the aid of DIC/Nomarski) of whole-mount embryos in situ hybridised for *Pv-otx*. **A**–**C** The individual cells of one quadrant (indicated *m*) are named according to the nomenclature for spiralian embryos (Wilson 1892). **A** Apical view of a 32-cell-stage embryo showing expression in the $1m^{12}$, $1m^{21}$ and $1m^{22}$ cells and in one quadrant in the $2m^{1}$ cell. **B** Apical view and **C** lateral view of the same 52-cell-stage embryo showing expression in the $1m^{12}$, $1m^{211}$, $1m^{212}$, $1m^{221}$, $1m^{222}$, $2m^{11}$ and $2m^{12}$ cells. No expression in seen in the $1m^{111}$ and $1m^{112}$ cells and fainter expression in other cells. **D** Lateral, probably ventral, view of an 11-h early trochophore larva. Expression is seen in the prototroch (*bracket*) and in cells on the probably future ventral side (*arrow*). **E** Right and **F** ventral view of a 20-h-old trochophore larva. Expression in the prototroch: in the anterior supporting cells, the ciliated prototroch cells and the posterior supporting cells (*black arrowheads*). Expression was also detected in a ring of cells anterior to the prototroch (*white arrowhead*), in the stomodaeum (*asterisk*) and cells surrounding it, and in two half circles of cells that run on the ventral side and meet in the ventral midline (*arrows*). **G** Schematic representation, ventral view, of the 20-h-old trochophore larva showing the *Pv-otx* expression in *green*. **H**, **I** Anterior view of the same embryo as in **E**, **F**, optical section at the level of the prototroch (**H**) and of the anterior ring of stained cells (**I**). **J** Cell-lineage diagram (for one quadrant) of *P. vulgata* with contributions of the cells to the prototroch indicated. Cells expressing high levels of *Pv-otx* are in *blue*. (*asterisk* only in one quadrant, *asc* anterior supporting cells, *at* apical tuft, *cpc* ciliated prototroch cells, *psc* posterior supporting cells, *st* stomodaeum)

script. An in-frame 3′ stop codon and a short (5 bp) 3′ UTR are also present in this cDNA. The deduced protein contains a homeodomain sequence (Fig. 1B), a conserved stretch of 12 amino acids directly downstream of the homeodomain (Umesono et al. 1997; Galliot et al. 1999) and a so-called OAR (*otp*/*glx*/*rx*) domain (Galliot et al. 1999) found in other *otp* genes (Fig. 1B). The homeodomain of *Pv-otp* has a histidine (H) at the 4th position, and a glutamine (Q) at the 50th position, characteristic of the homeobox domain of the *otp* class (Galliot et al. 1999).

Figure 1B shows a comparison of the conserved *Pv-otp* domains with the corresponding domains of other *otp* genes. The *Pv-otp* homeobox shows a high identity with that of other species (93–98%, Fig. 1B). For comparison, the homeodomain of *Pv-otx* is included in the alignment (Fig. 1B). Based on the different conserved domains discussed above, and on the discriminating 4th and 50th residue of the homeobox, the two cDNAs could be assigned with certainty to the *otx* class and *otp* class of homeobox genes, respectively.

Expression of *Pv-otx* and *Pv-otp* during *Patella* embryogenesis

Early development of *Patella* is characterised by a cleavage pattern called spiral cleavage. Spiral cleavage is typ-

ical for molluscs, as well as for a number of other lophotrochozoan phyla that collectively are called the Spiralia (Nielsen 2001). The cleavage pattern is highly regular with cleavages at oblique angles giving smaller animal blastomeres and larger vegetal blastomeres. As the angle of the cleavage plane shifts between cleavages, a "spiral" arrangement of blastomeres is formed. Since the cleavage pattern is invariant, it is possible to denominate individual blastomeres. The nomenclature we used is according to Wilson (1892).

At 19°C, the embryo of *Patella* hatches after 10–12 h of development and a free-swimming, so-called trochophore larva develops. This larva is characterised by the prototroch, that includes a band of ciliated cells used for locomotion, and an apical tuft, cells with long cilia at the anterior end of the larva (Damen and Dictus 1994). We studied the spatio-temporal expression of *Pv-otx* and *Pv-otp* during embryonic development with whole-mount in situ hybridisation using digoxigenin-labelled riboprobes. Figure 2 shows the expression of *Pv-otx*. The first expression was detected at the 32-cell stage in the $1m^{21}$ and $1m^{22}$ cells (Fig. 2A; nomenclature of cells after Wilson 1892). At the 52-cell stage their daughter cells $1m^{211}$, $1m^{212}$, $1m^{221}$ and $1m^{222}$ strongly expressed the *Pv-otx* gene (Fig. 2B, C). These cells are the precursor cells of the primary trochoblasts (Damen and Dictus 1994). Staining was also observed in the $1m^{12}$ cells (Fig. 2A–C), which are the precursor cells of the accessory trochoblasts (Damen and Dictus 1994). At 32 cells, expression was observed in the $2m^{1}$ cell in one quadrant (Fig. 2A) and at the 52-cell stage in all $2m^{1}$ daughter cells, the $2m^{11}$ and $2m^{12}$ cells (Fig. 2C), which are the precursors of the secondary trochoblasts (Damen and Dictus 1994). Thus, all cells that will later form the prototroch expressed *Pv-otx* (summarised in Fig. 2J). At the 52-cell stage, much weaker staining was observed in other cells, whereas the $1m^{111}$ and $1m^{112}$ cells (located most animally) did not show any staining (Fig. 2B, C). At 11 h of development (Fig. 2D), the early trochophore larva stage, the prototroch and cells anterior and posterior to it were stained (bracket in Fig. 2D), and cells on one side lying posttrochally (arrow in Fig. 2D). At the 20-h trochophore stage, the last time point studied, staining (schematically represented in Fig. 2G) was detected in the prototroch (black arrowheads in Fig. 2E–G): in the ciliated prototroch cells (cpc, Fig. 2H), the cells anterior to them, the anterior supporting cells (asc) and the cells posterior to them, the posterior supporting cells (psc). One row of small cells anterior to the anterior supporting cells was also stained (white arrowhead in Fig. 2E–G, I). In the ventral part the staining extends more towards the posterior side (Fig. 2E–G): the stomodaeum (asterisk in Fig. 2E, F) and a half circular band of ectodermal cells were also stained (white arrow(s) in Fig. 2E, F). The expression pattern is summarised in Fig. 2G.

The expression pattern of *Pv-otp* is shown in Fig. 3. At 5.5 h of development (1 h after 64-cell stage), two cells, lying most anteriorly, were stained for *Pv-otp* (Fig. 3A, B). These cells belong to the $1m^{111}$ cells, but we have not been able to determine which two of the four $1m^{111}$ cells ($1a^{111}$–$1d^{111}$) are the cells expressing *Pv-otp*. We studied the expression of *Pv-otp* at the 40- to 64-cell stage, but the results varied between embryos and between experiments; however, expression was always in the $1m^{11}$ or $1m^{111}$ cells. We never detected expression before the 40-cell stage. These cells continued to express *Pv-otp* throughout development (Fig. 3C, D). At 20 h of development, the last time point studied, expression was seen in a half circular (horseshoe-shaped) band of cells with the opening directed towards the dorsal side

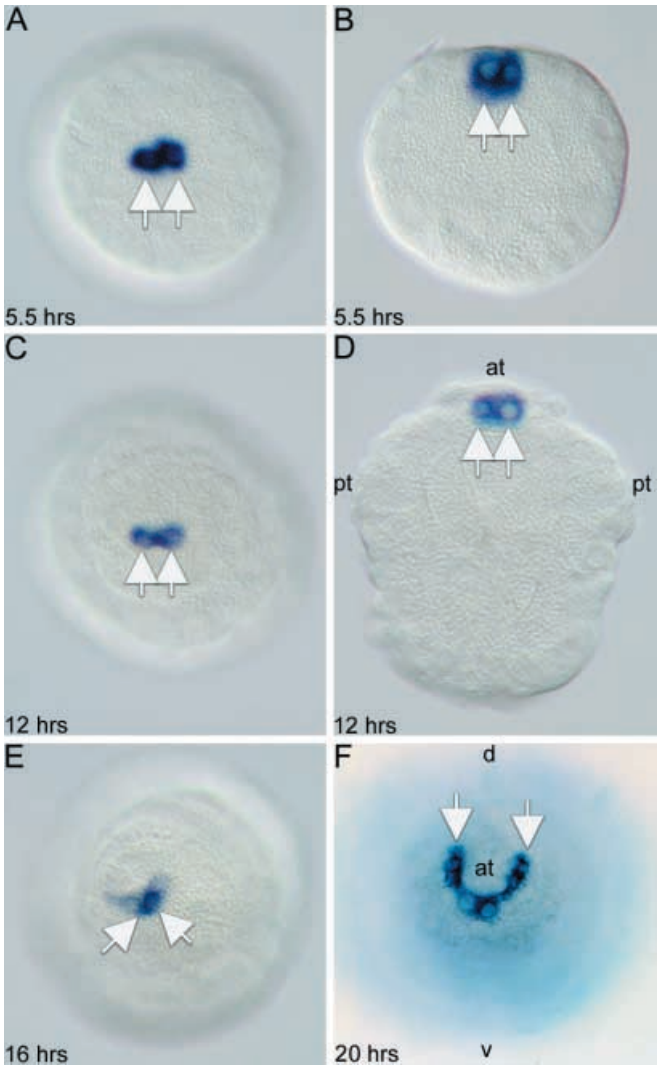

Fig. 3A–F Expression of *Pv-otp* in the embryo and larva. Optical sections (with the aid of DIC/Nomarski) of whole-mount embryos in situ hybridised for *Pv-otp*. **A** Apical and **B** lateral view, apical side up, of a 5.5-h-old embryo. Expression is seen in two cells lying apically in the centre of the embryo (*arrows*). **C** Apical/anterior and **D** lateral view of a 12-h early trochophore larva. Expression is seen in two cells at the anterior side (*arrows*). **E** Anterior view of a 16-h-old trochophore showing the expression domain (*arrows*) that is more expanded relative to the expression at 12 h. **F** Anterior view, dorsal side up, of a 20-h-old trochophore. The expression domain is now a horseshoe shape (*arrows*) around the apical tuft (*at*) with the opening towards the dorsal side (*d* dorsal, *pt* prototroch, *v* ventral)

(Fig. 3E, F). These cells embrace the apical tuft. We do not know whether the cells expressing *Pv-otp* in the 20-h larva are the clonal descendants of the two cells expressing the gene at earlier stages, or alternatively, whether at later stages additional cells start expressing *Pv-otp*.

Discussion

We cloned two cDNAs from the gastropod mollusc *P. vulgata* that could be assigned to the *otx* and *otp* class of homeobox genes, respectively. *Pv-otx* and *Pv-otp* are the first cDNAs for *otx* and *otp* genes described in molluscs.

Pv-otx expression in the posterior ventral ectoderm

The closest relatives of the molluscs, in an evolutionary sense, where the expression of an *otx* ortholog has been studied are the annelids. A recent analysis of genes expressed in the polychaete *Platynereis* showed that the *Platynereis otx* gene is expressed around and in the stomodaeum of the trochophore larva (Arendt et al. 2001). The *Pv-otx* expression around and in the stomodaeum we found in *Patella* resembles the expression in this annelid. Arendt et al. (2001) used the annelid *otx* expression around and in the stomodaeum as an argument for their hypothesis that larval mouth regions of protostomes and deuterostomes are homologous. With the expression of *Pv-otx* in the stomodaeum of *P. vulgata*, we extend the argument for this homology with our data on molluscs. However, whereas Arendt et al. (2001) did not detect expression posterior of the stomodaeum, we do show ectodermal expression of *Pv-otx* extending posterior at the ventral side of the trochophore larva. The fate of the cells expressing *Pv-otx* in this region is unknown.

Otx and *otp* genes and development of the nervous system

A second domain of *Pv-otx* expression was the ring of small cells anterior to the prototroch, i.e. anterior to the anterior supporting cells. From the literature it is well known that *otx* genes are involved in nervous system development across bilaterians (see Introduction). Furthermore, in two other lophotrochozoans, the annelids *Platynereis* and *Helobdella*, the expression of *otx* genes has been shown to be associated with nervous cells in the head region of the developing animal (Bruce and Shankland 1998; Arendt et al. 2001). Although we do not know the exact fate of the anterior-most cells that express *Pv-otx*, it is very possible that these cells will obtain a neural fate.

The expression of *Pv-otp* is clearly associated with the developing nervous system of the larva. It has been shown that during later development of *Tectura scutum*, a gastropod mollusc that is a close relative of *Patella*, serotonergic cells develop in the middle of the apical region in a half circular band of cells with the opening to the dorsal side (Page 2002). These cells belong to the apical sensory organ of the trochophore larva. This nervous structure is involved in relaying the sensory input of the apical tuft, ciliated cells implied in sensory perception (Kempf et al. 1997; Hadfield et al. 2000). We found *Pv-otp* expression in a similar position and shape in *Patella* as the serotonin-positive cells in the *Tectura* trochophore larva. At this stage, however, no serotonin expression could be detected in the *Patella* larva (our unpublished observations). We suggest that *Pv-otp*-expressing cells constitute the anlage of the larval sensory organ and imply a role for *Pv-otp* in the development of this neural organ.

Otx genes are always associated with anterior nervous system development, but not with the most anterior regions of developing brains (Hirth et al. 1995; Wada et al. 1996; Bruce and Shankland 1998; Williams and Holland 1998; Wada and Saiga 1999; Tomsa and Langeland 1999; Acampora et al. 2000; Harada et al. 2000). The expression we find in *Patella* for *Pv-otx* is not in the most anterior part of the head region. In contrast, *Pv-otp* is found in the most anterior nervous structure of the trochophore larva, i.e. the apical organ. A comparable situation exists in the adult brain of another lophotrochozoan, the platyhelminth *Dugesia* (Umesono et al. 1999); unfortunately, no embryonic expression data are available for this species. In the adult *Dugesia* brain, an *otp* ortholog is expressed in the most anterior part of the adult brain, in the so-called brain branches, but is absent from the distal-most part of the branches, which are believed to form sensory structures (Umesono et al. 1997). This situation we find in the *Patella* trochophore as well: *Pv-otp* expression is found in the apical organ, i.e. in the nervous structure around the anterior sensory structure, but is not found in cells of the apical tuft, i.e. in the actual anterior sensory structure. Furthermore, two *Dugesia otx* orthologs are expressed in non-overlapping domains just posterior of the branches expressing an *otp* ortholog (Umesono et al. 1999) and *Pv-otx* expression is similarly also found posterior of the *Pv-otp*-positive domain. It remains to be seen whether this situation, with *otp*-expressed in cells around anterior sensory structures and *otx* expressed posterior to them, is common to all lophotrochozoans.

Otx genes and the development of ciliary bands

Our analysis of the expression of *Pv-otx*, suggests that this gene is involved in the development of the prototroch. In the larva, the prototroch consists of three rows of cells that surround the embryo: (1) the band of ciliated prototroch cells (cpc), (2) the band of anterior supporting cells (asc) that form a ring anterior to the ciliated cells and (3) a half ring of posterior supporting cells (psc) that is located posterior to the ciliated cells at the dorsal side (Damen and Dictus 1994). The prototroch develops from three sources of presumptive trochal cells, viz the primary trochoblasts, the accessory trochoblasts and the sec-

ondary trochoblasts (van den Biggelaar 1977; Damen and Dictus 1994). The primary trochoblasts are the first cells of the embryo that show overt differentiation in the form of ciliation (van den Biggelaar 1977). Initially, all trochal cells develop cilia; they subsequently become deciliated differentially, so that in the larva only the middle row of prototroch cells is ciliated. At the 32-cell to 52-cell stage, all prototroch precursor cells expressed *Pv-otx*. Later in development, in the trochophore larva, *Pv-otx* transcripts were found in the ciliated prototroch cells as well as in the anterior and posterior supporting cells. All cells contributing to the prototroch are thus characterised by the expression of *Pv-otx*. This could indicate a role for *Pv-otx* in the differentiation of the prototroch cells, suggesting that the Pv-otx homeobox transcription factor protein binds to promoters of downstream target genes that are involved in prototroch differentiation.

Expression of *otx* genes in ciliary bands has been found in larvae of four other invertebrates. In the annelid *Platynereis* an *otx* gene was found to be expressed in the preoral prototroch and the postoral metatroch (Arendt et al. 2001). In the echinoderm *Stichopus japonicus*, a sea cucumber, an *otx* gene was implicated in the early development of the ciliary bands and expression was found in the preoral and postoral ciliary bands of the auricularia larva (Shoguchi et al. 2000). Also, ciliated bands of the doliolaria larva of another sea cucumber, *Psolus chitinoides*, showed expression of an *otx* gene (Lowe and Wray 1997). In the hemichordate *Ptychodera flava*, ciliary bands, primarily of the oral ectoderm, express an *otx* ortholog (Harada et al. 2000). Thus, although not all of their ciliary bands express *otx* genes, in the larvae of two lophotrochozoans, the mollusc *Patella* (this study) and the annelid *Platynereis*, and of three deuterostomes, the echinoderms *Stichopus* and *Psolus* and the hemichordate *Ptychodera*, ciliary bands express this gene. These data lead us to postulate that participating in the development of ciliary bands is an ancestral function of *otx* genes in bilaterians.

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