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Original Article

A homeobox gene of the *orthodenticle* family is involved in antero-posterior patterning of regenerating planarians

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ABSTRACT We studied the expression of *DtOtx*, a homeobox gene of the freshwater planarian *Dugesia tigrina* closely related to the *Drosophila orthodenticle (otd)* and vertebrate *Otx* genes, which are known to control head development in both fruit flies and vertebrates. *DtOtx* was not significantly expressed in adult planarians but it was activated within one hour in regenerating tissues with a clearly asymmetric pattern. Animals sectioned transversally, either between the head and the pharynx, or caudal to the pharynx, give rise to a head-containing fragment regenerating a tail region and to a tail-containing fragment regenerating a head region. *DtOtx* was found to be activated in both regeneration blastemas but its transcripts were much more abundant in the head-regenerating tissues than in the tail-regenerating tissues. The same asymmetric distribution of *DtOtx* transcripts was observed in central portions of the body regenerating both head and tail structures and in animals laterally regenerating after a longitudinal cut. These data suggest a role of this gene in patterning the body axis of these primitive bilateria, at least during regeneration.

KEY WORDS: *homeobox, head, regeneration, patterning, evolution planarian*

Introduction

Planarians are flatworms, relatively simple triploblastic metazoans generally considered to represent the first organisms to exhibit bilateral symmetry and cephalization (Brusca and Brusca, 1990). Some species have been used as model systems to study regeneration (Slack, 1980). In fact, they can easily regenerate along any body axis: anteriorly (head regeneration), bidirectionally (head and tail regeneration), posteriorly (tail regeneration) and laterally (left or right side regeneration) (Brøndsted, 1969). This regeneration occurs by a mixed epimorphic and morphallactic mechanism (Muñoz-Mármol *et al.*, 1998), requires cell proliferation to produce new tissues (Saló and Baguñà, 1984) and does not imply cell dedifferentiation (Saló and Baguñà, 1989). In fact, in the adult body there are undifferentiated self-renewing stem cells, termed neoblasts (Baguñà, 1981), capable of forming the regenerating blastema. A number of homeobox genes, including most *Hox* genes, have been isolated in the freshwater species *Dugesia tigrina* (Garcia-Fernandez *et al.*, 1991,1993; Tarabykin *et al.*, 1995; Bayascas *et al.*, 1997), as well as in related ones (Balavoine and Telford, 1995; Balavoine, 1996). We looked for possible planarian homologs of homeobox genes of the *otd/Otx* family (Finkelstein and Boncinelli, 1994). These genes are known to control head development in both fruit flies and vertebrates. *orthodenticle* mutant larvae are characterized by deletions of wide head regions (Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991) and mouse embryos homozygous for *Otx2* null mutations lack head and all cerebral structures anterior to rhombomere r3 in rostral hindbrain (Acampora *et al.*, 1995; Matsuo *et al.*, 1995; Ang *et al.*, 1996). Here we show that at least one gene of this family is present in the genome of *Dugesia* and that it is promptly activated during regeneration with a strong preference for head-regenerating regions.

Results

Cloning of DtOtx

We amplified DNA extracted from *Dugesia tigrina* by PCR methodology using two primers corresponding to two of the most conserved peptide sequences between the *Otx* and the *otd* homeodomains (see Materials and Methods) and found a 94bp fragment, representing part of a homeobox. We subsequently used this fragment to screen a genomic library and obtained a 10 kb clone containing the genomic region corresponding to a portion of the coding sequence of a gene related to the *otd/Otx* family. Figure 1a shows the presumptive peptide sequence of the cloned region of the corresponding homeoprotein, including the homeodomain (boxed). This homeodomain contains a lysine residue in position 50. Alignment with other homeodomains of this

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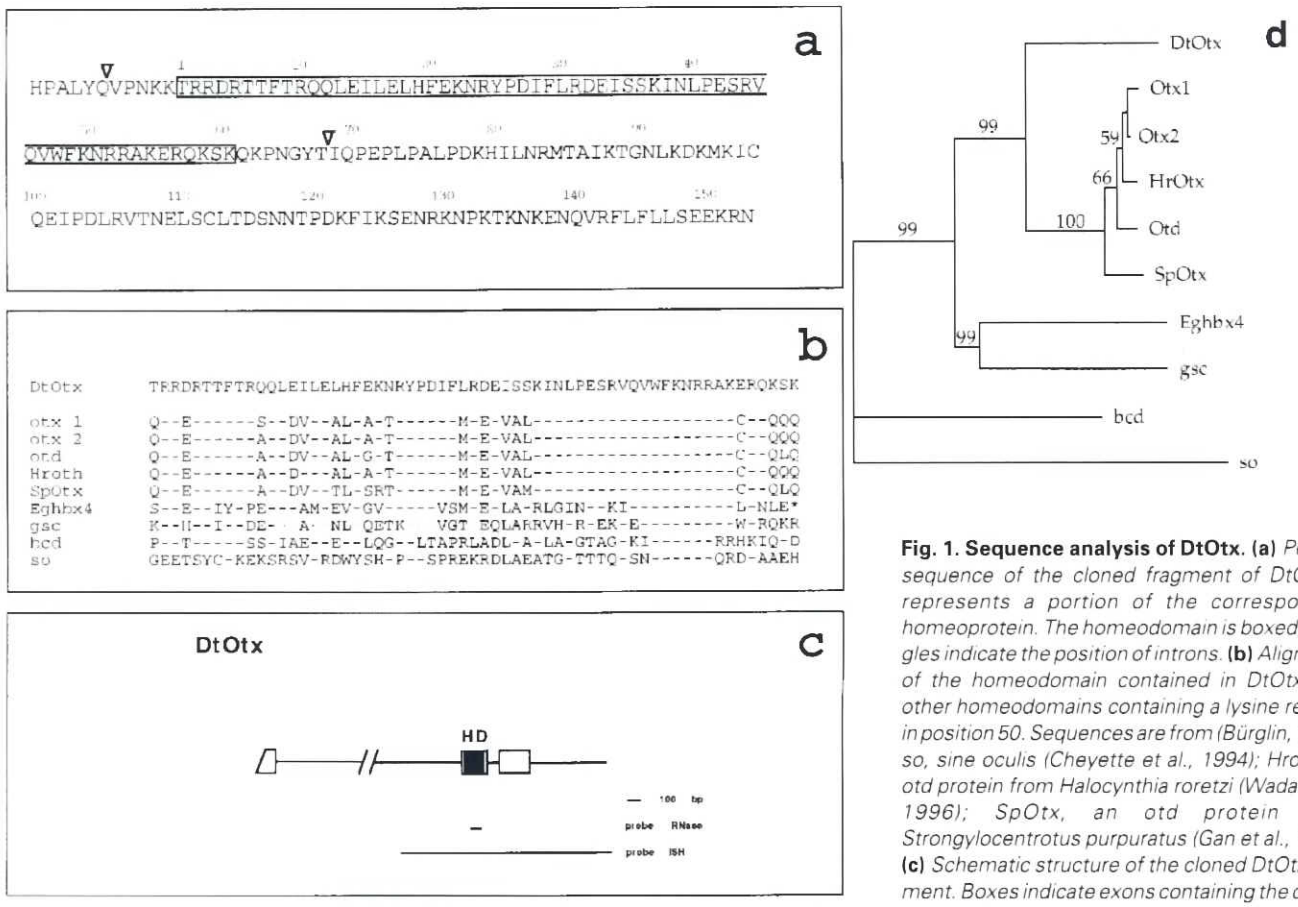

Fig. 1. Sequence analysis of DtOtx. (a) *Peptide sequence of the cloned fragment of DtOtx. It represents a portion of the corresponding homeoprotein. The homeodomain is boxed; triangles indicate the position of introns.* **(b)** *Alignment of the homeodomain contained in DtOtx with other homeodomains containing a lysine residue in position 50. Sequences are from (Bürglin, 1994); so, sine oculis (Cheyette et al., 1994); Hroth, an otd protein from Halocynthia roretzi (Wada et al., 1996); SpOtx, an otd protein from Strongylocentrotus purpuratus (Gan et al., 1995).* **(c)** *Schematic structure of the cloned DtOtx fragment. Boxes indicate exons containing the coding region and a solid box indicates the homeobox. Fragments used as probes are also indicated.* **(d)** *Neighbor-joining phylogram with the amino acid sequences corresponding to the homeodomain region, of the genes aligned in Figure 1c. All branch lengths are proportional to the distances between sequences. The majority rule consensus tree of the 15 most parsimonious trees gives essentially the same topology (not shown). The bootstrap values are shown over the corresponding nodes for the neighbor-joining algorithm (1000 replicas). The tree was rooted with the midpoint rooting option.*

class (Fig. 1b) clearly shows its similarity with the homeodomains of the *otd/Otx* family (Finkelstein and Boncinelli, 1994): it is 70% similar to both the murine *Otx1* and *Otx2* homeodomains. Accordingly, we termed the gene *DtOtx*. The transcriptional organization of the cloned region, obtained through the analysis of 5' and 3' RACE products, is shown in Figure 1c. In contrast to both the otd and Otx homeodomains (Simeone *et al.*, 1992; Vandendries *et al.*, 1996) this gene lacks the intron within the homeobox, but contains a conserved intron just upstream from the homeobox. The comparative analysis of Otx sequences show that the DtOtx protein groups with the Otx family are the most divergent (Fig. 1d), which is in agreement with the phylogenetic position of platyhelminths at the base of protostomates.

Expression pattern of DtOtx

We used a 1800bp fragment containing the two exons of the carboxy-terminal region (Fig. 1c) to study the expression of the gene. Whole-mount *in situ* hybridization experiments did not detect any expression of this gene in intact adults, whereas a strong activation was detectable in regenerating fragments within 1 h after amputation (Figs. 2,3). Figure 2a shows the expression of *DtOtx* in regenerating fragments after pre-pharyngeal cuts. Tail-containing fragments regenerating a head (H) showed a strong hybridization signal whereas head-containing fragments regenerating a tail (T) showed a significantly fainter signal. The branched brown signal observed in the cephalic gut region in a') was due to some residual endogenous alkaline phosphatase activity and cannot be confused with the violet diffuse hybridization signal (see Fig. 3a''). The higher expression intensity of *DtOtx* in head-regenerating regions than in tail-regenerating regions was also observable in regenerating fragments after transverse post-pharyngeal cuts (Fig. 2b) in all 16 animals examined both at 3 days (b') and 5 days (b'') after the cut. The same asymmetric *DtOtx* activation was also observed in regenerating fragments after double, i.e., pre- and post-pharyngeal, cuts (Fig. 3a). In all fragments examined head-regenerating regions show higher expression of *DtOtx* than tail-regenerating ones. Conversely, similar experiments with some *Hox* genes (Bayascas *et al.*, 1997) (see also Fig. 3a''') revealed a pattern of expression of uniform intensity in head-regenerating and tail-regenerating fragments. In all cases the hybridization signal appears to be in both blastema and postblastema. We also analyzed laterally regenerating animals after a longitudinal cut (Fig. 3b) and found a higher expression in anterior regions as compared with medial and posterior regions (Fig. 3b'). Similar

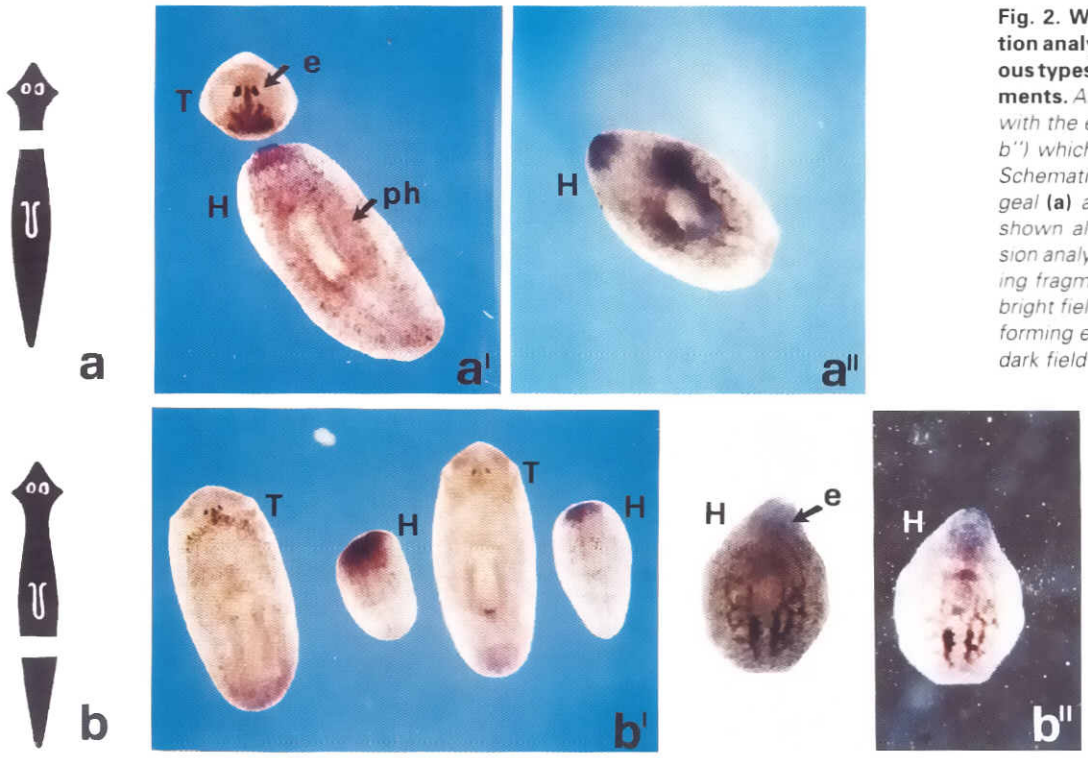

Fig. 2. Whole-mount *in situ* hybridization analysis of *DtOtx* expression in various types of regenerating planarian fragments. *Analysis was at 3 days after the cut, with the exception of fragments shown in b'') which have been analyzed at 5 days. Schematic representations of pre-pharyngeal* **(a)** *and post-pharyngeal* **(b)** *cuts are shown along with corresponding expression analysis.* **b'')** *shows a head-regenerating fragment at 5 days. On the left side a bright field image is displayed to show the forming eyes (e), and on the right side the dark field to show the hybridization signal. Hybridization with sense probe gave no signal (not shown). The branched brown signal observed in the cephalic gut region in* **a')** *and in posterior gut regions in* **b'')** *was due to some residual endogenous alkaline phosphatase activity. H indicates head-regenerating regions and T indicates tail-regenerating regions. e, eye; ph, pharynx.*

experiments with some *Hox* genes (Bayascas *et al.*, 1997) revealed expression patterns of uniform intensity along the entire body axis.

RNase protection experiments confirm that DtOtx is expressed differentially in anterior and posterior blastema

In order to confirm and quantitatively evaluate the difference between the levels of *DtOtx* transcripts in head-regenerating versus tail-regenerating regions, we performed RNase protection experiments on RNAs extracted from fragments at various times after amputation using a probe obtained from within the homeobox (Fig. 1c) and giving a protected region of 70bp. Figure 4 shows examples at 1 h or 5 days. This analysis revealed that head-regenerating fragments expressed *DtOtx* at least 10 times more intensely than tail-regenerating fragments. Activation of *DtOtx* was already detectable after 1 h, reached peak expression at about 3-4 days and was barely detectable starting from 10-11 days, when regeneration was almost completed (not shown).

Discussion

Some freshwater planarians, such as *Dugesia tigrina* strain used, do not reproduce sexually and do not undergo embryogenesis, unlike most metazoans. Conversely, they are characterized by two related phenomena; continual growth/degrowth and extensive regeneration (Baguñà *et al.*, 1994). Planarians are subject to continual cell turnover and any body part is replaced through proliferation and differentiation of a distinct cell population constituted by undifferentiated self-renewing neoblasts (Baguñà, 1981). These are usually distributed throughout the mesenchyme of the worm, especially in regions adjacent to the brain and ventral nerve cords. In an adult worm they look inactive but participate in the continual replacement of cells in all body tissues typical of an adult animal (Brøndsted, 1969). Regeneration is by no means an exceptional event in these animals, but in fact entails an ordinary, genetically programmed series of events. In a wounded animal, neoblasts closest to the wound begin to migrate to the site of the damage where they actively proliferate. Below the wound epithelium a small bulge, the regeneration blastema, is formed through the accumulation of the incoming neoblasts. The blastema grows by the addition of new neoblasts originated by active proliferation in the postblastema, a 500 μm region underlying the blastema (Salò and Baguñà, 1985). Within the blastema, incoming neoblasts stop dividing and begin to differentiate. After a few days the first regenerated structures become apparent and in a couple of weeks regeneration of missing parts is completed, even if the resulting animal does not attain its normal, proportioned, shape until approximately 4 weeks. Planarians are Bilateria showing a clear antero-posterior polarity and regeneration retains the antero-posterior polarity of the amputated animal. This phenomenon is usually attributed to the presence of an antero-posterior gradient of some sort operating throughout the body. This gradient may be maintained either through the differential accumulation of extracellular molecules or through the persistence of differentially determined cell states (Brøndsted, 1969; Bayascas *et al.*, 1997).

We cloned *DtOtx*, a flatworm homeobox gene of the *otd/Otx* family and observed its activation within one hour after amputation in the regenerating region, as previously reported for the homeobox genes of the *Hox* family (Bayascas *et al.*, 1997,1998). The presence of *DtOtx* transcripts was detectable throughout the process of

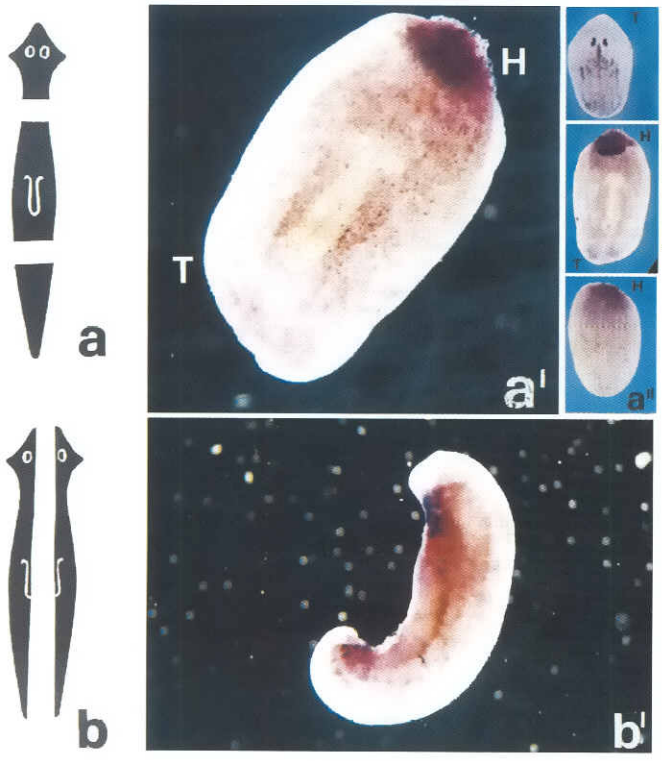

Fig. 3. Whole-mount *in situ* hybridization analysis of *DtOtx* expression in various types of regenerating planarian fragments 3 days after the cut. *Schematic representations of both pre- and post-pharyngeal* **(a)** *and longitudinal* **(b)** *cuts with corresponding expression analysis.* **a')** *shows a central portion, whereas* **a'')** *shows the three portions of a regenerating animal after a pre-pharyngeal and post-pharyngeal cut.* **a''')** *shows hybridization of a central portion with the Hox gene Dthox-F (Bayascas et al., 1997).* **b')** *shows a dorsal view of a laterally regenerating animal. A careful examination revealed that the expression level is minimal around the pharynx, a region that is known to show minimal regeneration capacity (Brøndsted, 1969). H, head-regenerating region; T, tail-regenerating region.*

regeneration with an abundance progressively declining after approximately day 10 of regeneration. After two weeks no expression was detectable in whole-mount *in situ* hybridization experiments as was the case for normal, non regenerating, adult animals. The observed abundance of these transcripts was significantly higher in blastemas and postblastemas of head-regenerating fragments than in those of tail-regenerating fragments. This is in sharp contrast with the behavior of some ubiquitous *Hox* genes which appear to be activated at the same level in the two types of regenerating regions (Bayascas *et al.*, 1997). Recently it has been reported that *Dthox-D* and *Dthox.C*, have nested expression along the antero-posterior axis in the tail region (Bayascas *et al.*, 1998). Both types of homeobox genes appear to be required to initiate the regeneration process, but they may play quite different roles. Recently it has been reported that a similar homeobox gene, namely *Djotp*, a planarian *orthopedia* homolog, is expressed in the branch region of both the mature and regenerating brain (Umesono *et al.*, 1997).

The differential expression of *DtOtx* in anterior versus posterior regenerating regions was detectable in all experiments, regardless of the site of amputation, whether pre-pharyngeal or post-pharyngeal. These observations are best explained as the results of removing anterior factors normally inhibiting head-regeneration, possibly through *DtOtx* activation. This activation is a relatively quick event, essentially paralleling the initial strong mitotic response of neoblasts close to the wound (Saló and Baguñà, 1984). It is conceivable that the differential activation of *DtOtx* contributes to the restoration of the appropriate body polarity in the regenerating animals. The presence of a preexisting antero-posterior gradient, of relatively diffusible molecules or of tissue competence, able to differentially activate *DtOtx* along the body axis, is consistent with the observed *DtOtx* activation pattern after a longitudinal cut. A role for homeobox genes of the *Otx/otd* family in establishing the body plan and in particular the identity of anterior body regions was already proposed in both flies and vertebrates (Finkelstein and Boncinelli, 1994; Boncinelli and Mallamaci, 1995; Bally-Cuif and Boncinelli, 1997). Interestingly, at least one gene of this family is present in bilaterally symmetrical taxa as distant as flatworms, insects and vertebrates and it is reasonable to hypothesize a similar role in head specification in the various systems. Further experiments are clearly required to elucidate the specific function of these genes in regenerating planarians.

Materials and Methods

Species

Freshwater planarians of the species *Dugesia (Girardia) tigrina (Platyhelminthes, Turbellaria, Tricladida)* were collected in Calders river (Barcelona, Spain). They were maintained in spring water at 17°C in the dark.

Production of regenerating organisms

Two-week-starved planarians were used in all experiments. Planarians 9-10 mm long were cut transversally at the pre-pharyngeal or post-pharyngeal level (Salò and Baguñà, 1985) and sagittally. They were left regenerating in Petri dishes with spring water at 17°C in the dark.

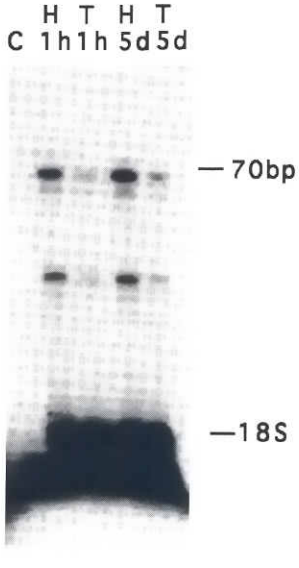

Fig. 4. RNase protection analysis of *DtOtx* transcripts in RNAs extracted from regenerating fragments. *Head-regenerating (H) and tail-regenerating (T) tissues are shown, 1 hour (1h) and 5 days (5d) after the cut. The size of the protected fragment is indicated as well as the position of the protected fragment of the 18S gene. C indicates a tRNA control lane.*

Cloning of the DtOtx gene

PCR with degenerate hemi-nested primers was performed in order to clone the *DtOtx* homeobox. The first two primers correspond to the most conserved part of the homeobox: oligo TFT: 5'(AC)GIGA(AG)(AC)GIACIA-CNTT(CT)AC3', corresponding to peptide RERTTFT (Fig. 1b) and oligo PES: 5'AC(CT)TGIACIC(TG)I(CG)(TA)(CT)TCNGG3', corresponding to peptide PESRVQ.

The third oligo, DIF: 5'AC(CT)TC(CT)TCIC(TG)CAT(AG)AA(TAG)-AT(AG)TC3', corresponding to peptide DIFMREEV was used with the oligo TFT in order to amplify a 94bp fragment, representing part of the *DtOtx* homeobox. I is inosine and N any nucleotide. PCR cycles were run as follows: 5 min at 98°C; (1 min at 98°C, 1 min at 48°C, 1 min at 72°C) 5 times; (1 min at 94°C, 1 min at 48°C, 1 min at 72°C) 35 times and finally 5 min at 72°C. The DNA band corresponding to the expected size was excised from agarose gel, electroeluted before cloning in pGEM3 vector (Promega) and subjected to sequencing by the dideoxy method (Sanger *et al.*, 1977). The 94bp fragment was used as a probe to screen a genomic planarian library in fix II. In order to clone a more extended cDNA fragment, 5' and 3' RACE was performed according to the Marathon cDNA Amplification Kit (Clontech).

Phylogenetic analysis

Homeodomain sequences of different Otx representatives and other homeodomains with K in position 50 were aligned. The resulting data matrix, was used to create a gene phylogeny with both distance and parsimony reconstruction methods, as previously described (Muñoz-Màrmol *et al.*, 1997).

Whole-mount in situ hybridization

Digoxygenin-labeled probe with a size of 1800bp corresponding to the region shown in Fig. 1c, was synthesized according to the manufacturer's protocol (Boehringer) and used in whole-mount experiments.

Two-week starved organism were sectioned with pre-pharyngeal, post-pharyngeal and lateral cuts and left to regenerate at 17°C for specified times. Before fixation, the organisms were treated for two minutes with a solution of 2% Cysteine-HCl (Serva) in mineral water (pH 4), to destroy the mucus, and were washed 4 times in mineral water (5 min each). The planarians were fixed at 4°C overnight in 4% paraformaldehyde in PBS, washed in PBS, dehydrated and stored at -20°C in 70% ethanol. After rehydration they were washed in PTw (PBS with 0.1% Tween-20) three times (5 min each), and the organisms were treated with proteinase K (10 μg/ml) in PTw for 15 min at room temperature. Digestion was stopped by two washes in 2 mg/ml glycine in PTw (5 min each). Planarians were then acetylated in 0.1M triethanolamine (pH 7.8) supplemented with 0.5% acetic anhydride for 10 min and rinsed with PTw, post- fixed in 4% paraformaldehyde in PBS for 20 min and rinsed with PTw five times (5 min each). Planarians were prehybridized for 1 h at 55°C in hybridization solution (50% formamide, 5xSSC, 1 mg/ml yeast RNA, 50 μg/ml heparin (Sigma H-3125), 0.1% Tween-20 (SIGMA P-1379). The digoxygenin-labeled antisense probes were heated to 80°C for 2 min and added to samples (1 μg/ml final concentration) for hybridization at 55°C for 72 h. Following hybridization the planarians were washed in 100%, 75%, 50%, 25%, hybridization solution in 2xSSC (5 min each), twice in 2xSSC (30 min each) and twice in 0.2xSSC (30 min each), all at 55°C. The organisms were rinsed twice in PTw and then incubated for 1 h in blocking solution (1% Boehringer blocking reagent, 20% heat-inactivated calf serum, 2 mg/ml BSA). After blocking the reaction, the organisms were incubated overnight at 4°C with 1:2000 alkaline-phosphatase (AP)-conjugated anti-digoxygenin antibody (Boehringer) which had been pre-adsorbed with 8 mg/ml planarian powder in the above mentioned blocking buffer overnight at 4°C. The organisms were rinsed eight times in PTw (15 min each), and three times in AP buffer (100 mM Tris pH 9.5, 100mM NaCl, 50 mM MgCl2, 5 mM levamisole) (5 min each). Signal was detected following incubation of the organism in AP buffer with 340 μg/ml NBT and 175 μg/ml BCIP (Boehringer) or BM purple (Boehringer 1442074). When the chromogenic reaction was complete (6 h to overnight), the organisms were washed twice in PTw, post-fixated for 20 min in 4% paraformaldehyde, cleared 10 min in methanol, and stored in glycerol at 4°C. Sense riboprobe was hybridized and developed in parallel with antisense riboprobe and utilized as a negative control. Photography was performed using a Zeiss axiophot microscope.

RNase protection experiment

Total RNA was extracted by the single-step RNA isolation technique Chomczynski and Sacchi (1987) from anterior fragments regenerating tails, at 1 h and 5 days of regeneration, and from posterior fragments regenerating heads at 1 h and 5 days of regeneration; all the fragments were obtained with post-pharyngeal cuts. A 70bp probe located in the homeobox was subcloned in pGEM 3. Antisense strand RNA probe was synthesized with T7 polymerase and hybridized to 50 μg RNA at 55 °C. RNase digestion and electrophoresis on 7% urea-polyacrylamide gels were carried out as previously described (Melton *et al.*, 1984). Expression of 18S ribosomal RNA was used as an internal control.

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