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

ANNA STORNAIUOLO¹, JOSÉ R. BAYASCAS², EMILI SALÒ² and EDOARDO BONCINELLI¹,3

¹DIBIT, Istituto Scientifico H S Raffaele, Milan, Italy, ²Departament de Genética, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain and ³Center for Molecular and Cellular Pharmacology, CNR, Milan, Italy

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) (Brondsted, 1969). This regeneration occurs by a mixed epimorphic and morphallactic mechanism (Muñoz-Marmol 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* (García-Fernández 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 3 in rostral hindbrain (Acampora et al., 1993; 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 10kb 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
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 (Srinivasan, 1994); so, sine oculis (Cheyette et al., 1994); HrOtx, an otd protein from Halocynthia roretzi (Wada et al., 1996); SpOtx, an otd protein from Strongylocentrotus purpuratus (Gain et al., 1995). (c) Schematic structure of the cloned DtOtx fragment. Boxes indicate exons containing the coding region and a solid box indicates the homeodomain.

Expression pattern of DtOtx

We used a 1600bp 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
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., 1984). 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 (Brondsted, 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 (Salb 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 (Brondsted, 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
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 DTox 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 DTox 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 DTox 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 DTox along the body axis, is consistent with the observed DTox activation pattern after a longitudinal cut. A role for homeobox genes of the Otx/otf 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; Salo and Baguñà, 1998). 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 Culders 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 (Saló and Baguñà, 1988) and sagittally. They were left regenerating in Petri dishes with spring water at 17°C in the dark.

Materials and Methods

Species

Freshwater planarians of the species Dugesia (Girardia) tigrina (Platyhelminthes, Turbellaria, Tricladida) were collected in Culders 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 (Saló and Baguñà, 1988) and sagittally. They were left regenerating in Petri dishes with spring water at 17°C in the dark.
Cloning of the DIOtx gene

PCR with degenerate hemi-nested primers was performed in order to clone the DIOtx homeobox. The first two primers correspond to the most conserved part of the homeobox: oligo TFF, 5'(AC)GIGA(AG)AC(GI)GIAC-NCTT(CT)ACG3, corresponding to peptide REHTTI (Fig. 1b) and oligo PES: 5'(AC)TG1ACIC(TG)CG(TT)(CT)TCNNG3, corresponding to peptide PERSVQ.

The third oligo: DIF: 5'(AC)CTC(CT)TCIC(TG)CAT(AG)AA(TAG)AT(AG)TC3', corresponding to peptide DIFMREE was used with the oligo TFF in order to amplify a 94bp fragment, representing part of the DIOtx homeobox. It 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 dyeoxy method (Sanger et al., 1977). The 94bp fragment was used as a probe to screen a genomic planarian library in fos 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-Marmol et al., 1997).

Whole-mount in situ hybridization

Digoxigenin-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 dehydration 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 0.1 M 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 digoxigenin-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 1:2500 alkaline-phosphatase (AP)-conjugated anti-digoxigenin antibody (Boehringer) which had been pre-absorbed 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, 100 mM NaCl, 50 mM MgCl2, 5 mM evamisole) (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-fixed 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.

Acknowledgments

We are indebted to Antonio Faicella for a number of helpful comments and suggestions. Dave Ferrier for checking the English. This work was supported by grants from the DGICYT (Ministerio de Educación y Ciencia, Spain, PB92-0551 and PB95-0579) to E.S. and from EC BIOTECH Programme, the Telethon-Italia Programme and the Italian Association for Cancer Research (AIRC) to EB.

References


CHEVERTE, B.N., GREEN, P.J., MARTIN, K., GARREN, H., HARTENSTEIN, V. and ZIPURSKY, S.L. (1994). The sine oculis locus codes a homeodomain-containing...


Received: May 1998
Accepted for publication: July 1998