Genomic organization and expression of the planarian homeobox genes

Dth-1 and Dth-2

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SUMMARY

We have characterized the genomic organization of Dth-1 and Dth-2, planarian homeobox-containing genes, previously described at the cDNA level (J. Garcia-Fernàn dez, J. Baguñà and E. Saló (1991), Proc. Natl. Acad. Sci. USA, 88, 7338-7342). Genomic analysis shows that Dth-1 and Dth-2 genes encode proteins of 533 and 363 amino acids respectively. The open reading frame of Dth-1 is interrupted by two large introns of 8 kb and 12 kb. Dth-2 also shows two introns, but these are short (42 bp and 44 bp) and the second interrupts helix III at position 44-45, as is the case with other homeobox genes from such divergent animals as Drosophila, honeybee, C. elegans, ascidians, and mouse, which suggests an ancient evolutionary relationship between these genes.

The spatial distribution of transcripts in adult tissues, determined by in situ hybridization, demonstrates that Dth-1 is expressed at a high level in the gastrodermal cells, while Dth-2 is expressed in the peripheral parenchyma, at higher levels in the dorsal than the ventral regions. Their specific spatial distribution suggests a possible role for these homeobox genes in determination and/or differentiation of specific cell types. The expression pattern of both genes is more or less continuous, but in Dth-1 clustered discontinuous labelling in areas surrounding the gastrodermis may indicate a specific expression of this gene in groups of undifferentiated cells (neoblasts) already committed or determined to gastrodermal cell fates.

In situ hybridization analysis during early regeneration shows expression only in the postblastema (stump) differentiated areas while no expression has been detected in the undifferentiated blastema, indicating that neither gene has a role in pattern formation mechanisms known to occur at the early stages of regeneration (0-3 days). Hence, Dth-1 and Dth-2 are planarian homeobox genes presumably involved in specific cell or tissue determination and/or differentiation.

Key words: planarian, homeobox, gene expression, in situ hybridization

INTRODUCTION

The homeobox is a short and ancient DNA sequence widely distributed on the phylogenetic tree, from plants to vertebrates (Gehring, 1987; Vollbrecht et al., 1991; Shashikant et al., 1991). Such a wide distribution of homeboxes and their extraordinary degree of sequence conservation suggests a fundamental biological function (Gehring, 1985). From Drosophila to mammals most of the homeobox genes have key roles in development; the best known are the Antennapedia (Antp) type genes, which are involved in the formation of axes and body segments (Affolter et al., 1990; De Robertis et al., 1991; McGinnis and Krumlauf, 1992). Some homeodomains that are highly divergent from the Antp-type have also been isolated and act as tissue-specific transcription factors that define a cell type (Lazzaro et al., 1991).

We have recently isolated the cDNA of two planarian homeobox-containing genes, Dth-1 and Dth-2 (Garcia-Fernàndez et al., 1991). Both contain a novel homeodomain closely related to that of NK-2 of Drosophila (Kim and Niremberg, 1989), TTF-1 of rat (Guazzi et al., 1991), Nkx-2, 3 and 4 of mouse (Price et al., 1992), and Eghbx-3 of the cestode Echinococcus granulosus (Oliver et al., 1992), which may define a new homeobox class.

Since planarians belong to the phylum Platyhelminthes, class Turbellaria, considered to be the sister group of all the other Bilateria (Field et al., 1988; Riutort et al., 1992; Adoutte and Philippe, 1993), the structure of the homeobox genes of planarians should shed some light on the origin and evolution of homeobox genes. Furthermore, to gain understanding of the role of homeobox genes in planarians, two research approaches have been followed. First, gene structure has been determined to define the regulatory sequences that allow comparative intra- and interspecies studies. Second, spatiotemporal expression patterns have been studied to provide insights into their function.

In this report, we describe the genomic sequence including the 5′ and 3′ flanking sequences of two planarian homeobox genes: Dth-1 and Dth-2. Both homeobox genes show tissue-specific expression in the adult and during regeneration. Dth-1 is detected in the intestinal epithelium, while Dth-2 is expressed in the peripheral parenchyma, with higher values in dorsal than in ventral areas.
MATERIALS AND METHODS

Species

Planarians used in this study belong to the species Dugesia (G) tigrina (Girard) and were collected near Barcelona. They were maintained in spring water. Two-week-starved organisms were used in all experiments.

Regenerating organisms

Planarians, 9-10 mm long, were cut postpharyngeally according to Saló and Baguñà (1984) and left to regenerate in Petri dishes with spring water in the dark at 17°C.

Genomic library construction and screening

Standard protocols to extract DNA were not suitable for planarian tissues because of its high nuclease and mucopolysaccharide content. Thus, we had to adapt the guanidine isothiocyanate method initially described for RNA extraction (Chirgwin et al., 1979) and modify it as follows: planarians were homogenized in guanidine thiocyanate, phenol/chloroform treated, and dialized against several changes of 10 mM Tris-HCl pH 8.0, 50 mM EDTA, pH 8.0, 100 mM NaCl. After digestion of RNAs (1 hour at 37°C with 100 µg/ml RNase A), deproteination (3 hours at 50°C with 200 µg/ml proteinase K in 1% SDS, 0.1 M NaCl) and phenol/chloroform treatment, CsCl was added to a final density of 1.55 g/ml. Samples were then ultracentrifuged for 72 hours at 168,000 g (rotor Kontron TFT50), and 0.5 ml fractions of the gradient were tested for the presence of DNA. Positive fractions were finally dialized against TE and kept at 4°C.

High relative molecular mass DNA was partially digested with Sau3A, then fractionated in a sucrose gradient to obtain DNA fragments of 15-20 kb, which were then dephosphorylated. The genomic library was constructed by cloning this DNA into the BamHI site of λ-Charon35 vector (Wilhelmine and Blattner, 1983). The total number of recombinant clones obtained was approximately 8×10⁶. This library was amplified and 2.5×10⁶ recombinant clones were screened for sequences homologous to Dth-1 and Dth-2 cDNAs (Garcia-Fernàndez et al., 1991) using the method of Benton and Davies (1977). Probes (complete cDNAs) were 32P-labelled by random priming and hybridized to phage DNA on nitrocellulose filters (Schleicher & Schuell) at high stringency conditions, only the hybridization temperature and formamide concentrations were raised to 40°C, and 46% (vol/vol). Middle-stringency hybridized filters were washed 2×15 minutes at 65°C in 1× SSC, 0.1% SDS.

In situ hybridization

In situ hybridizations were carried out according to Ingham et al. (1985), with several modifications.

Tissue preparation

Intact and regenerating planarians were fixed in 4% paraformaldehyde in PBS (10 mM phosphate buffer pH 7.4, 150 mM NaCl) or 2% paraformaldehyde plus 0.1% glutaraldehyde in PBS for 1 hour at 4°C, and washed twice with PBS for 15 minutes prior to storage in 70% ethanol at 4°C. They were embedded in Paraplast plus according to Langdale et al. (1987). 8 µm sagittal and transverse sections were cut in an Anglia Scientific 500 microtome and dried on poly-D-lysine precoated slides at 42°C. To obtain frozen sections, planarians were fixed in 4% paraformaldehyde in PBS for 45 minutes at 4°C, washed for 30 minutes in PBS, cryoprotected by incubation in 20% succharose in PBS for 12 hours and embedded in OCT (Miles Laboratories) at −70°C. 10 µm sections were cut on a Reichert-Jung 2.800 Frigocut E microtome and post-fixed in 4% paraformaldehyde.

Probes

The Dth-1 and Dth-2 cDNA clones (Garcia-Fernàndez et al., 1991) were cloned in pBluescript SK+ (Stratagene) and used as templates for synthesis of sense and antisense RNA probes following the suppliers instructions (RNA transcription kit, Stratagene), using uridine 5′-(α-[35S]thio)triphosphate (1000 Ci/mmol, Amersham) diluted 1:2 with nonlabelled rUTP. Probe length was reduced to an average of 200 nucleotides by limited alkaline hydrolysis, as described by Cox et al. (1984). Final specific activity was approximately 7×10⁶ cts/minute per µg.

Hybridization

After acid treatment (20 minutes in 0.2 M HCl) and neutralization (10 minutes in 2× SSC), sections were deproteinized for 30 minutes at 37°C in 1 µg/ml proteinase K, postfixed in 4% paraformaldehyde, treated for 10 minutes with freshly prepared 0.1% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0), dehydrated in ethanol, and air dried. Approximately 8×10⁶ cpm of 35S-labelled probe was applied in 40 µl of hybridization buffer (50% formamide, 1 µg/ml yeast tRNA, 50 mM DTT, 500 µg/ml polyadenylic acid, 10% dextran sulphate, 0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM phosphate buffer pH 6.8, 5 mM EDTA). Hybridization was carried out at 50°C for 12 hours, and the slides were then washed for 5 hours in buffer (50% formamide, 10 mM DTT, 0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM phosphate buffer pH 6.8, 5 mM EDTA), rinsed for 5 minutes at 37°C in NTE solution (0.5 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 10 mM DTT), treated for 30 minutes at 37°C with 20 µg/ml
RESULTS

Isolation of genomic sequences

An amplified genomic library of Dugesia (Girardia) tigrina was screened at high stringency with completed Dth-1 and Dth-2 cDNAs as probes (Garcia-Fernández et al., 1991). The structure and restriction map of five overlapping lambda DNA clones spanning more than 45 kb for Dth-1, and one clone spanning more than 15 kb for Dth-2 are shown in Fig. 1 and Fig. 2 respectively. We have confirmed that these genomic clones exactly match cDNA sequences previously reported (data not shown).

A Southern blot of D. tigrina genomic DNA digested with EcoRI or HindIII was probed under high stringency conditions with a Dth-1 cDNA fragment (Dth-1 5' probes, nucleotides 1-995 of the cDNA), and with the whole cDNA of Dth-2. As shown in Fig. 3A, three strong hybridizing bands were detected in each digestion using Dth-1 5' as a probe (6 kb, 5.5 kb and 4 kb bands in the EcoRI digestion, and 10 kb, 9 kb and 6 kb bands in the HindIII digestion), corresponding to the three exons deduced in the genomic map. A single 5.5 kb band in the EcoRI digestion, and 20 kb, 3.5 kb and 2.2 kb bands in the HindIII digestion were detected in the hybridization with the Dth-2 cDNA probe (Fig. 3B). The weak extra bands of 7 kb (EcoRI) and 20 kb (HindIII) observed in the Dth-1 hybridization (Fig. 3A) were considered genomic partial digestions or polymorphic sites. For Dth-2, the HindIII bands of 20 kb and 3.5 kb (Fig. 3B) both cover the 5' end of the Dth-2 gene, and are due to a polymorphic HindIII site produced by the insertion of a mariner-like transposon (work in progress) found in some but not all the genomic clones isolated from this region. We conclude that Dth-1 and Dth-2 are single copy genes in the D. tigrina genome.

To determine whether the D. tigrina genome contains other genes possessing a similar homeobox to Dth-1 and Dth-2, Southern genomic hybridization was carried out under middle stringency conditions using the Dth-1 cDNA homebox sequence (hs probe Fig. 4). Only the 5.5 kb in the EcoRI digestion and the 9.0 kb in HindIII digestion corresponding to the third exon, deduced in the genomic map, was detected (Fig. 3C), the small weak bands are artefactual, as deduced from the control hybridization with plasmid DNA (results not shown). No extra band corresponding to the Dth-1 or Dth-2 homebox was detected. These results suggest that it is unlikely that a gene containing a similar homeobox to that of Dth-1 or Dth-2 is present in the D. tigrina genome. The same experiment performed with another planarian species Dugesia (Schmidtia) mediterranea at middle stringency shows weak bands of 7 and 5 kb (EcoRI) and 5, 3 and 2.5 kb (HindIII; Fig. 3C) which suggests the presence of similar homebox genes in D. mediterranea.

Structure of the planarian homebox genes Dth-1 and Dth-2

Figs 1 and 2 show the genomic nucleotide sequences of 7.2 kb and 3 kb of the Dth-1 and Dth-2 genes, respectively. Taking into account that Dth-1 and Dth-2 are the first planarian genes in which the genomic sequences have been characterized, an analysis of the region upstream of the cap site was performed to identify possible promoter and regulatory elements in the DNA. The putative TATA-boxes are not completely adjusted to the consensus (Breathnach and Chambon, 1981) although they are quite similar in the relative position with respect to the startpoint of transcription (Fig. 4A). The CAAT-boxes perfectly match the consensus (Latchman, 1991), the homology between Dth-1 and Dth-2 sequences increasing in some extranucleotides (Fig. 4B). The transcriptional startpoint determined by primer extension showed a high degree of conservation compared to the consensus (Breathnach and Chambon, 1981; Hultmark et al., 1986; Fig. 4C). Finally, the exon/intron boundary sequence conservation (Watson et al., 1992) is quite similar. The donor splice junction sequences are extremely conserved, principally in the exon region before the GT consensus, only the short introns show a substitution of G for A at position −1. The acceptor splice junction sequence is conserved, though the sequences immediately preceding this junction are extremely rich in AT rather than CT, and position −3 corresponds to a T (Fig. 4D). Dth-1 and Dth-2 genes have a single long open reading frame consisting of 533 and 363 amino acid residues respectively, including a highly diverged homeodomain similar to the Drosophila NK-2 homeobox.

The Dth-1 genomic sequence is organized in three exons of 478, 406 and 970 nucleotides in length and two introns of 8 and 12 kb in length located at positions 473 and 800 (nucleotides) of the mRNA sequence respectively. Primer extension analysis using the OD2 antisense oligonucleotide (Fig. 5A) gave one band of 171 nucleotides in length that defines the transcriptional origin, 178 nucleotides upstream from the origin of the longest cDNA described (Garcia-Fernández et al., 1991), and a final size of 1.8 kb for the Dth-1 cDNA.

The Dth-2 genomic sequence is organized in three exons of 404, 305 and 537 nucleotides in length and two small introns of 42 and 44 nucleotides in length located at positions 405 and 752 (nucleotides) of the transcribed sequence. The second is located after position 44, inside helix III of the homeodomain. Such intron localization has already been described in several other homebox genes (Mlodzik et al., 1988; Cribs et al., 1992; Celniker et al., 1989; Cohen et al., 1989; Allen et al., 1991; Kim and Niremberg, 1989; Walldorf et al., 1989; Saiga et al., 1991; Burglin et al., 1989; Hawkins and McGhee, 1990; Schaller et al., 1990). Primer extension analysis using the ES4 antisense oligonucleotide shows two bands of 160 and 164 nucleotides in length which define two closer transcriptional origins, with more frequent use for the short one (Fig. 5B).

The homeodomain of Dth-1 is located in the third exon, while the Dth-2 homeodomain is located between exons...
Fig. 1
two and three. Homeodomain comparisons of Dth-1 and Dth-2 with the different sequences previously described shows the highest similarity with the homeodomains of Drosophila NK-2 (80-82%; Kim and Niremberg, 1989), the rat TTF-1 (77-87%; Guazzi et al., 1990) the mouse Nkx-2.2 (83-82%), Nkx-2.3 (67-78%) Nkx-2.4 (77-88%; Price et al., 1992) and the platyhelminth cestode Echinococcus granulosus Eghx-3 (65-62%; Oliver et al., 1992). All (Fig. 6) show the same helix III with some unusual positions in all three helices of the homeodomain (Garcia-Fernàndez et al., 1991). In addition, the sequence identity also goes beyond the homeobox. In the amino acid sequences, downstream of the different members of the NK-2 homeobox family, the identity extends to three additional amino acids, two acidic (positions 62 and 66) and one basic (position 63). Moreover, downstream of Dth-1 and TTF-1 home-domain the identity extends to five additional amino acids (Fig. 6A). At the other end of the homeodomain, the similarity predicted between Dth-1 and the Nkx-2.2 amino acid sequence extends for a further five amino acids (Fig. 6A). All these results taken together would define them as members of a new class of homeobox containing genes that could be named NK-2, according to the first gene isolated.

**Spatial localization of Dth-1 and Dth-2 messenger by in situ hybridization**

As an aid to understanding the tissue sections of D. tigrina, we have included a brief morphological and histological description of intact and regenerating planarian. A more detailed description can be found elsewhere (Baguñà et al., 1988; Rieger et al., 1991).
Fig. 2. DNA sequence of the Dth-2 gene and corresponding amino acid sequence of the putative Dth-2 genomic and cDNA clones. The three exons are boxed and the homeobox is hatched. Exon junctions in the cDNA are marked. Restriction sites are coded as E, EcoRI; H, HindIII; P, PvuII; and T, PstI. The different probes used in this study, the region sequenced, and the positions of the TATA box are indicated. (B) Complete genomic sequence of Dth-2, and predicted amino acid sequence, deduced by sequence comparison of cDNA and genomic clones illustrated in A. The boundaries of a 42 and a 44 bp intron are indicated by arrowed brackets. The putative CAAT and TATA boxes are boxed and the homeobox sequences are in bold. The polyadenylation signal (AATAGA) and the position of the ES4 oligonucleotide used for primer extension experiments are underlined. The position of the two transcription startpoints (trs); the poly(A)* site and the 5' end of the longest cDNA are indicated. Dth-2 genomic DNA sequence has been deposited in the GenBank/EMBL database under the accession number X69202.
Planarians (Platyhelminthes, Turbellaria, Tricladida) are characterized by being triploblastic, acoelomates, unsegmented and bilaterally symmetrical organisms and by the lack of circulatory, respiratory and skeletal structures. The digestive system consists of a pharynx and a blind, three-branched and diverticulated gut lacking an anus (Fig. 7). The gut (intestine) is made up of two different cell types: the columnar or digestive cells and the secretory goblet cells. A solid mass of tissue, the parenchyma, fills the space between the cellular monostratified ciliated epidermis and the gut, and surrounds the internal organs. The parenchyma consists of several non-proliferating differentiated cell types and a particular class of undifferentiated mitotic cells usually called neoblasts that give rise to all the differentiated cell types of the adult (Baguñà, 1981; Ehlers, 1985; Baguñà et al., 1990). Regeneration involves an early stage (0-2 days) of accumulation of undifferentiated cells (neoblasts) beneath the wound epithelium due to a combination of cell proliferation and cell migration (Saló and Baguñà, 1984, 1989). At 2 days of regeneration this accumulation of neoblasts is visible externally, being called the regenerative blastema. It is during this early period that regeneration involves an early stage (0-2 days) of accumulation of undifferentiated cells (neoblasts) beneath the wound epithelium due to a combination of cell proliferation and cell migration (Saló and Baguñà, 1984, 1989). At 2 days of regeneration this accumulation of neoblasts is visible externally, being called the regenerative blastema. It is during this early period that regeneration involves an early stage (0-2 days) of accumulation of undifferentiated cells (neoblasts) beneath the wound epithelium due to a combination of cell proliferation and cell migration (Saló and Baguñà, 1984, 1989). At 2 days of regeneration this accumulation of neoblasts is visible externally, being called the regenerative blastema. It is during this early period that

**Fig. 3.** Genomic Southern blots of *Dugesia* (*G*) *tigrina* DNA digested with *Eco*RI(*E*) or *Hind*III(*H*) and hybridized under high stringency conditions to (A) *Dth*-1 5′ probe (1-995 nucleotides) and (B) *Dth*-2 whole cDNA clone. (C) Genomic Southern blots of *Dugesia* (*G*) *tigrina* and *Dugesia* (*S*) *mediterranea* digested with the same restriction enzymes as in A and hybridized to a *Dth*-1 homeobox-containing fragment of 150 nucleotides (hb probe), under middle stringency conditions.

**Fig. 4.** Comparison of the consensus sequences of the promoter components and the donor and acceptor splice sites of *Dth*-1 and *Dth*-2 with the consensus sequences (Breathnach and Chambon, 1981; Latchman, 1991; Hultmark et al., 1986; Watson et al., 1992). (A) TATA box; (B) CAAT box; (C) transcription startpoint, and (D) exon/intron boundary sequence.

**Fig. 5.** Mapping the sites of transcription initiation by primer extension. (A) *Dth*-1 primer extension using the OD2 oligonucleotide, lane 1: 0.5 µg of poly(A); lane C: control with 25 µg of yeast tRNA. A single band of 171 nucleotides can be observed in the lane 1. (B) *Dth*-2 primer extension, lane 1: 25 µg of total RNA and 0.3 pmol of ES4 oligonucleotide; lane 2: 25 µg of total RNA and 0.5 pmol of ES4 oligonucleotide; lane C: control with 25 µg of yeast tRNA and 0.3 pmol of ES4 oligonucleotide. Two bands of 164 and 160 nucleotides not present in the control can be observed. Lane M: sequencing reactions as size markers.
**Alignment and percentage homologies of the amino acid sequences of Dth-1 and Dth-2 with the different members of the NK-2 family.**

-alignments show abun-
ding silver grains in the intestinal cells, different numbers of intestinal branches being present, depending on the anteroposterior position of the transverse sections (Fig. 7). Sagittal sections show a clear increase in label in the basal region of the intestinal cells (Fig. 9B). No signal was observed in any other tissue or region, even in the pharyngeal epithelium (Figs 7, 9B). A more accurate analysis of grain distribution (Fig. 10A,C) shows a homogeneous expression of Dth-1 in all intestinal cells and in some clusters of cells surrounding the gut. These clusters of cells could be neoblasts committed or determined to become intestinal cells.

In situ hybridizations to transverse sections, using the antisense strand of the whole cDNA of Dth-1, showed abundant silver grains in the intestinal cells, different numbers of intestinal branches being present, depending on the anteroposterior axis takes place (Saló, 1984; Baguñà et al., 1990). Later on (5-7 days), new structures are differentiated within the blastema and postblastema areas, and the regeneration is complete after 3-4 weeks.

In situ hybridization analysis performed with both probes in sagittal sections of organisms at early stages of regeneration (Fig. 11) did not show any expression within the blastema, while clear expression was observed in the old differentiated tissues. These results suggest that neither gene has an important role in the process of early pattern formation during regeneration.

To illustrate the specificity of our probes, Figs 8 and 9E show transverse and longitudinal sections hybridized with sense RNA. All the tissues, even the gut and the peripheral parenchyma, display a low level of hybridization. Hence, the control probes do not show any specific binding.

**DISCUSSION**

We present an analysis of the genomic organization of Dth-1 and Dth-2 homeobox genes in the planarian D. tigrina. We have also established, for the first time in planarians, a working in situ hybridization technique sensitive enough to monitor the expression of its developmental control genes.

**Sequence analysis**

The analysis of Dth-1 and Dth-2 homeobox-containing genes, the first genomic sequences reported in planarians, confirm the high degree of conservation of the different promoter components and exon/intron boundary sequences. The genomic sequences of the two genes, however, show a striking differences in the size of their introns:
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two long introns for Dth-1 and two short ones for Dth-2. No clear difference is observed in the consensus sequences between short and long, in contrast to what was found in C. elegans (Fields, 1990). The second intron of Dth-2 is located in helix III (after amino acid codon 44 of the homeodomain). This intron location, already described in several other homeobox genes of Drosophila, honeybee, C. elegans, ascidians and mouse, has never been described in lower organisms. This finding may mean that all these genes have maintained this characteristic intron position from a common ancestor and suggests a common ancient origin from an old branch of the homeobox gene evolutionary tree.

The deduced amino acid sequences of Dth-1 and Dth-2 homeodomains are very similar to the Drosophila NK-2 (Kim and Niremberg, 1989), the rodent TTF-1 (Guazzi et al., 1990), the mouse Nkx2.2, Nkx2.3, Nkx2.4 (Price et al., 1992) and the cestode Echinococcus granulosus Eghbx-3 (Oliver et al., 1992). The high degree of conservation between the different components of this group, despite the phylogenetic distance between planarians and vertebrates
Fig. 9. Localization of the planarian *Dth*-1 (A, B) and *Dth*-2 (C, D) transcripts in sagittal sections of adult planarians. (A) Bright-field and (B) dark-field sagittal sections hybridized to *Dth*-1 antisense RNA probe. (C) Bright-field and (D) dark-field sagittal sections hybridized to *Dth*-2 antisense RNA probe. (E) Section equivalent to B hybridized to the sense RNA probe as a control. pr, parenchyma; id, intestine diverticula; a-p, anteroposterior axis; d-v, dorsoventral axis. For other abbreviations, see legend of Fig. 7.

Fig. 10. Dark-field images at high magnification of: (A) transverse sections hybridized to *Dth*-1 antisense probe. Clusters of hybridization in areas surrounding the gastrodermis are indicated by arrowheads. (C) Sagittal sections hybridized to *Dth*-1 antisense probe. Stronger expression in the basal portion of the intestinal cells is indicated by an arrowhead. (B) Transverse and (D) sagittal sections hybridized to *Dth*-2 antisense probe, the lack of hybridization of *Dth*-2 probe to the longitudinal nerve cord is indicated by an arrowhead. g, gut; gl, gut lumen. Bar, 50 μm.
Planarian homeobox genes and the low similarity to the other homeobox classes including Antennapedia, suggests that they may be considered as a new class of ancient homeobox-containing genes, the NK-2 class. In addition, the members of this new class share an identical recognition helix, with the same residue in position 50 and some specific replacements scattered throughout the homeodomain, which may confer a specific consensus recognition sequence other than the Antennapedia, as has been demonstrated for TTF-1, Nkx-2.2, and Nkx-2.3 (Price et al., 1992).

Temporal expression and tissue specificity of Dth-1 and Dth-2 transcripts

Large amounts of Dth-1 mRNA are found throughout the gastrodermis, in intact adult planarians. These results suggest that Dth-1 expression is associated with differentiation of the endodermal tissues. Several homeobox-containing genes have been described, whose pattern of expression in the embryo, or in some cases in the adult, involves cells or tissues of endodermal origin (Mlodzik et al., 1985; Mlodzik et al., 1988; Saiga et al., 1992; Wang et al., 1990; Rosa, 1989; Wright et al., 1988; Frumkin et al., 1991; Duprey et al., 1988). None shows any clear sequence similarity to the Dth-1 homeobox. Furthermore, no member of the new NK-2 family shows any specific expression in the endoderm derivatives, and Dth-1 is thus the only component of the NK-2 family with endoderm-specific expression.

Additional studies also show the expression of Dth-1 in clusters of undifferentiated parenchyma cells close to the gut in the adult. We postulate that this expression could be associated with determination or commitment of these cells to intestinal cells. Indeed, intestinal cells are short-lived cells (approx. 40-60 days; Baguñà and Romero, 1981) and are continuously being replaced by differentiating neoblasts, as has been extensively documented by light and electron microscopy (Hori, 1986).

Expression of Dth-2 is restricted to the peripheral parenchyma of the adults with more in the dorsal than the ventral areas. These results suggest that Dth-2 expression may be associated with differentiation of some parenchymal cell type (or types), widely distributed in the subepidermal region of the whole organism with a higher density in dorsal regions and dorsoventral edges than in ventral regions. Despite several studies at the light and electron microscope level (reviewed by Rieger et al., 1991), parenchyma’s cellular components and basic architecture are poorly understood since it is a loosely organized tissue composed of several cell types with numerous interdigitating cell processes that blur cell limits. In addition, monoclonal antibody staining of parenchyma cells in D. tigrina show a pattern that is bewildering in its complexity (Romero et al., 1991), which may reflect an unexpected functional heterogeneity among parenchymal cells or transient different functional stages in single cell types. Although identification of the cells that express Dth-2 must await the development of polyclonal antibodies, the general, discontinuous, patchy, distribution of Dth-2 expression suggests that more than one cell type may be labelled. If this is so, Dth-2 would participate in the determination and differentiation of a variety of mesodermal cell lineages.

Do Dth-1 and Dth-2 have any role during regeneration?

In situ hybridization studies at early stages of regeneration (0-3 days; Fig. 11) revealed that Dth-1 and Dth-2 are not
expressed in the blastema. Since new structures are determined along the anteroposterior axis during this period (Saló, 1984), we can tentatively conclude that neither gene is involved in early patterning processes. Besides, northern blot analyses also show that at 1-2 days of regeneration Dth-1 and Dth-2 have similar levels of expression to those of intact control organisms (García-Fernández et al., 1991).

Light and electron microscope studies of gut formation during regeneration (Hori, 1986) indicate that at 3-4 days small clusters of neoblasts gather close to the basal surface of the healed gastrodermis and begin differentiation. Between 5 and 7 days the process is at its maximum, and by 11-15 days the regenerating gastrodermis has already been formed within blastema and postblastema (stump) areas. Northern blot analyses at 5-7 days (García-Fernández et al., 1991) show a slight, though significant, increase in the expression of both genes. However, the actual variation in expression should be higher, as poly(A)+ RNA used for northern blots was obtained from the whole regenerant and not from the 1-2 mm area embracing the blastema and postblastema where most processes occurs. Indeed, in situ hybridizations of late regenerants (7-13 days) revealed the presence of Dth-1-negative gastrodermal cells and Dth-2-positive peripheral parenchyma cells in both blastema and postblastema areas (unpublished data).

These results strongly suggest that during regeneration neither gene is involved in early patterning events, although both are involved in late processes of cell determination and differentiation.

Implications of Dth-1 expression for growth/regrowth and embryonic development

Intact adult planarians are widely known for their ability to grow and degrow in body length and volume depending on temperature and feeding conditions (Baguñà and Romero, 1981; reviewed by Baguñà et al., 1990). These processes, fairly well understood at the kinetic level, may alternate back and forth without apparent impairment of the individual. One of the main consequences of growth and degrowth is that the number of iterated structures along the anteroposterior axis, such as the nerve ganglia, commissures and lateral nerves in the central nervous system, as well as the gut diverticula, increase and decrease respectively. How these changes are brought about has never been studied in detail, and competing hypothesis are entertained (see Baguñà et al., 1990, for a discussion on mechanisms). Light microscopy data suggest that during embryonic development, gut diverticula do not arise by bulging from the simple, saccular, embryonic endodermal epithelium but from the clustering of embryonic undifferentiated cells, similar to neoblasts, which later connect to the gut through differential growth and migration (Le Moigne, 1963).

Whether or not a similar mechanism operates during adult growth is an open question. In this context, the endodermal (gut)-specific expression of Dth-1 may be highly relevant. Dth-1 probes or antibodies may be used to monitor their expression in the embryonic gut and diverticula and validate the model suggested. They can also be used to analyse how new diverticula arise during growth and, even, how they regress during degrowth. Moreover, although embryonic development in freshwater planarians is highly modified (reviewed by Baguñà and Boyer, 1990), Dth-1 antibodies could also be used to detect the earliest stages and tissues in which this gene is expressed. Finally, Dth-1 could be used as a probe to isolate the homologous gene in other turbellarian orders, such as Polycladida, Macrostomida, Acoela and Catenulida, which have a canonical spiralian development. The expression of this gene in these organisms and meaningful molecular embryonic comparisons could be determined.

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