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Hox and ParaHox Genes in Flatworms: Characterization and Expression[1]

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SYNOPSIS. Flatworms (phylum Platyhelminthes) are favourite organisms in Developmental Biology and Zoology because of their extraordinary powers of regeneration and because they may hold a pivotal place in the origin and evolution of the Bilateria. Hox genes play key roles in both processes: setting up the new anteroposterior pattern in the former, and as qualitative markers of phylogenetic affinities among bilaterian phyla in the latter. We have searched for Hox and ParaHox genes in several flatworm groups spanning from freshwater triclads to marine polyclads and, more recently, in the acoels, the likely earliest extant bilaterian. We have isolated and sequenced eight Hox genes from the freshwater triclad *Girardia tigrina* and three Hox and two ParaHox genes from the polyclad *Discocelis tigrina.* Data from the acoels *Paratomella rubra* and *Convoluta roscoffensis* is also reported. Flatworm Hox sequences and 18S rDNA sequence data support clear affinities of Platyhelminthes to spiralian lophotrochozoans. The basal position of acoel flatworms supported from recent 18S rDNA data, remains still uncertain. Expression of Hox genes in intact and regenerating adult organisms show nested patterns with graded anterior expression boundaries, or ubiquitous expression. New approaches to study the function of Hox genes in flatworms, such as RNA interference are briefly discussed.

INTRODUCTION

The almost endless capacity of flatworms to regenerate have made them one of the favourite organisms for studying the cellular and molecular mechanisms leading to pattern restoration. Flatworms, namely freshwater planarians, can regenerate along any body axis, replace any missing structure by intercalation whenever any axial discontinuity is experimentally produced, and generate additional or supernumerary structures after various types of cuts and grafts (Brønsted, 1969; Baguñà *et al.*, 1994; Baguñà, 1998). There is hardly in nature anything matching the amazing feat of a tiny planarian tail piece, which lacks eyes, sense organs, brain ganglia, pharynx, digestive system and reproductive structures, giving rise in a matter of days to a whole body bearing such structures. Flatworms are the sole invertebrate regenerating epimorphically (*e.g.*, making a blastema) for which a main role for undifferentiated stem-cells or neoblasts have been demonstrated (Baguñà *et al.*, 1989; reviewed in Baguñà *et al.*, 1994 and Baguñà, 1998). Moreover, most flatworms show a great plasticity both in the growth of an individual and in its final size. They can stand long periods of starvation during which they shrink from the adult size to, and sometimes beyond, their initial size at hatching. When feeding is resumed, they grow again to adult size.

In regeneration and growth/degrowth, cells face either dramatic (regeneration) or smooth but continuous and reversible (growth/degrowth) changes in positional identity, namely along the anteroposterior (AP) axis. The markers for the anterior-posterior (AP) axis are the clustered Hox genes (Krumlauf, 1994; Lawrence and Morata, 1994). They have been found in all metazoan examined, including cnidarians, are organized in conserved genomic clusters and are generally arranged along the chro-

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mosome in the same order as they are expressed along the AP axis (Duboule, 1994). Their main function is to give positional identity within a homogeneous field of cells and transform them into a series of unique pattern elements along the AP body axis. In planarian regeneration, they could be instrumental to transforming the homogeneous blastema and postblastema fields into discrete patterned regions corresponding to the lost elements. In growth/degrowth, their AP boundaries of expression should be finely tuned to fit or to drive the allometrically changing body proportions of the growing or degrowing organisms.

Phylogenetic background

The study of Hox genes in flatworms had until very recently an additional evolutionary interest. For the last 50 yr, the prevalent view as to how triploblast bilaterian organisms evolved from radially symmetrical diploblasts was from a gastrula-like planuloid ancestor (represented by the planula larva of cnidarians) to a simple acoeloid descendant. These basal triploblasts were assumed to be acoelomate worms represented by todays platyhelminths, with no segments and coelom and bearing a blind gut (no anus) (reviewed in Willmer, 1990). Were it so, the study of the number, variety and order of the Hox genes in Platyhelminthes hold the promise to be a key element to understand the origin and evolution of the Hox cluster. The alternative to this planuloid-acoeloid hypothesis considers that the tree might lead from diploblastic organisms to a rather complex bilaterian ancestor with coelom and segments (archicoelomate hypothesis; Remane, 1963), leaving acoelomate (*e.g.*, Platyhelminthes) and pseudocoelomate (*e.g.*, Nematoda) organisms as derived from coelomates by simplification. Hence, flatworms had secondarily lost segments, coelom and the anus.

In the last five years, molecular based animal phylogenies have partially solved this controversy. First, the bulk of the Platyhelminthes (the Rhabditophora, *sensu* Ehlers, 1985) was shown not to be the sister group to the rest of the Bilateria but to fall within the protostomes (Carranza *et al.*, 1997; Balavoine, 1997; Littlewood *et al.*, 1999). Second, the most recently published trees of the animal kingdom based on 18S rDNA gene sequences and different ''signature'' Hox genes resolves into three principal clades (Aguinaldo *et al.*, 1997; Balavoine, 1998; de Rosa *et al.*, 1999). One, leads to the traditional deuterostomes, whereas the other two split the traditional protostomes into two novel branches. Of these, one groups the moulting animals (including arthropods and nematodes) into the new clade Ecdysozoa, and the other groups the Eutrochozoa (phyla with trochophore larvae) and the lophophorates into the Lophotrochozoa. In all analyses, Platyhelminthes (at least the Rhabditophora) fall within the Lophotrochozoa. However, most relationships within and between these three new clades are unclear. Prominent among them is the uncertain position of the Platyhelminthes among the rest of the lophotrochozoan taxa.

New 18S rDNA suggests that one order of platyhelminths, the Acoela, not included in the previous analyses, are in fact not flatworms but the most basal bilaterian animals (Ruiz-Trillo *et al.*, 1999). In addition, new 18S rDNA data on another order of basal platyhelminths, the Nemertodermatida, suggest they cluster together with the acoels at the base of the bilaterians (unpublished data, J.B.). Recently, Berney *et al.* (2000) directly contradicted the basal position of the Acoela based on analyses of elongation factor 1-alpha (EF1a), placing them within the turbellarian rhabditophorans (allied either to the triclads or to the polyclads). However, new EF1a sequences from additional species of Acoela, rhabditophoran flatworms, and other Metazoa do not support the acoel-rhabditophoran clustering (Littlewood *et al.*, 2001) refuting the conclusions of Berney *et al.* (2000). Altogether, this bring us back to the planuloid-acoeloid hypothesis of the origin of the triploblastic bilaterians. However, to give a final answer to the question of the position of the acoels (and nemertodermatids) an attractive approach is to look for the presence or absence in them of signature Hox genes typical of the lophotrochozoans or of the protostomes as a whole.

Hox genes may, therefore, be instrumental to get a better understanding of these

two basic problems: 1) how the anterior-posterior pattern is reset in a fast process such as regeneration as well as in a slow process such as growth and degrowth; and 2) as qualitative markers of phylogenetic affinities to sort out the origin of bilaterians and the relationships among their main clades. This warrants the study of the Hox genes in a phylum, such as the Platyhelminthes, with extensive powers of regeneration and growth/degrowth and holding a privileged position either as early bilaterians or as a group of basal lophotrochozoans.

HOX AND PARAHOX GENES

Hox genes

To date, several Hox genes and gene fragments have been detected, cloned and sequenced in platyhelminths (for recent comprehensive reviews, see Balavoine, 1998; Bayascas *et al.*, 1997, 1998, and Orii *et al.*, 1999). PCR amplification of genomic DNA or cDNA from regenerating blastemas, or of planarian cDNA libraries, has been the most succesful technique employed. In *Girardia tigrina,* this screening yielded seven Hox genes, named *Dthox-A* to *Dthox-G* (Bayascas *et al.*, 1997). Sequence analysis and comparison to other Hox genes obtained in another planarian species (*Polycelis nigra,* Balavoine and Telford, 1995) suggested the existence of a cluster with at least seven genes. A recent survey of 44 Hox gene/gene fragments from nine species of Platyhelminthes, including three parasitic species (Orii *et al.*, 1999) resolved into seven groups (named Plox1 to Plox*7,* after *pl*anarian HOM/H*OX* homeobox genes) corresponding to the *Drosophila* paralogous groups lab, zen, Dfd, Scr, Antp, and Ubx/abdA. As recently pointed out (Telford, 2000), Lox 5 in Annelida and Plox5/6 in Platyhelminthes are orthologous to *Drosophila ftz* instead of *Antp*; hence, this paralogous group should be ftz/Antp. Although no *proboscipedia* (*pb*) orthologous gene was considered by Orii *et al.* (1999), the presence of a *bona fidae* pb ortholog in *G. tigrina* (the gene *DthoxB*; Bayascas *et al.*, 1997) indicates that orthologs to the *Drosophila* Hox genes lab, pb, zen, Dfd, Scr, Antp/ftz, Ubx/abdA exist in platyhelminths. To that list, we should add an Abd-B related gene (*GtAbd-Bb*), found in our lab (unpublished data, E.S.). Figure 1A summarizes the equivalences between the Hox genes from the most thoroughly studied species of freshwater triclads (*G. tigrina*: *DthoxA-G* and *GtAbd-Bb*; *P. nigra*: *Pnox1–8,* and *Dugesia japonica*: *Djhox#0004, 1020, 1051, 1053* and *2007,* as well as other lesser studied species*).* We propose an improvement to Orii's *et al.* (1999) nomenclature, here only applied to freshwater triclads, using the most encompassing name *pl*atyhelminth HOM/*HOX* homeobox genes (Plhox1 through Plhox9), including *DthoxB* as Plhox2, Ubx/abdA related genes as Plhox7/8 and the recent *GtAbd-Bb* as Plhox9. This would ease to compare extant and new platyhelminth Hox genes to the accepted nine paralogous groups of the lophotrochozoan Annelida and the ecdysozoan *Drosophila* (lab to Abd-B), despite their actual orthology remains to be proved. Because orthology between the deuterostome paralogous groups PG6–13 and their counterparts in lophotrochozoans and ecdysozoans are still debatable, deuterostomes are not included in Figure 1A.

The presence of duplicated genes for some Hox groups in *P. nigra* and *G. tigrina* are intriguing features. The duplicated Hox genes (*Pnox1a/1b, Pnox2/3,* and *DthoxC/E*; see Fig. 1A) may result from tetraploidy followed, or not, by diploidization and in some species, mixoploidy. The asexual race of *G. tigrina* spread all over Europe is a diploidized tetraploid being also a frequent mixoploid (n = 8, with 2n and 3n cells; Ribas *et al.*, 1989). A similar situation may hold for *D. japonica* (Orii *et al.*, 1999). Mixoploidy has not been reported for *Polycelis nigra,* but examples from triploidy to hexaploidy and hyperploidy are frequent (reviewed in Benazzi and Benazzi-Lentati, 1976).

Hox genes in polyclads (marine flatworms of the Order Polycladida) were searched by PCR amplification from genomic DNA of *Discocelis tigrina* with ''universal'' Hox primers named SO-1 and SO-2 (Bayascas *et al.*, 1997). We sequenced 43

A	Hox genes							
Drosophila	lab	pb	zen	Dfd	Scr	ftz/Antp	Ubx/abdA	AbdB
Annelids	Lox7/ Nvi-lab	Nvi-pb	Nvi-Hox3	Lox6/ Nvi-Dfd	Lox20/ Nvi-Scr	Lox5/ Nvi-Lox5	Lox2/Lox4 Nvi-Lox2/ Nvi-Lox4	Nvi-Post1 Nvi-Post2
Orii's et al (1999)	Plox1	-------	Plox2	Plox3	Plox4	Plox5/6	Plox7	------
New Proposal	Plhox1	Plhox2	Plhox3	Plhox4	Plhox5	Plhox6	Plhox7/8	Plhox9
Triclads	Dlox2 Pfox2 Pfox3 **Pnox2** **Pnox3** PWoxA	DthoxB	Djhox1020 Dlox4 DthoxG Pnox4 PWoxE	Djhox1051 DthoxA PWoxB	Djhox0004 DthoxD Pnox8	Djhox1053 Djhox2007 **DthoxC** **DthoxE** Pnox7	Dlox1 DthoxF Pfox1 **Pnox1a** **Pnox1b** PWoxC PWoxD	GtAbd-Bb
Special signatures						Spiralian peptide	UbdA peptide	

B	Hox genes			ParaHox genes	
	lab/Plhox1	Dfd/Plhox4	Ubx/abdA/ Plhox7	Xlox	cad/Cdx
Policlad: *Discocelis tigrina*	Distox-A	Distox-D	Distox-F	DistXlox	Distcad
Identity	85% Dm lab 83% Amphihox1 80% Pnox3 80% M Hox1	80% DthoxA 80% Amphihox4 78% M Hox4 77% Dm Dfd	87% Hm Lox4 83% DthoxF 80% Dm Ubx 80% Dm abdA	92,6% M IPF-1 81,5%AmphiXlox 77,7% Htr A2	70,3% Pr Cad 70% Human-Cdx1 66% Dm cad 61% AmphiCdx
Special signatures			UbdA peptide		

C	Hox genes		ParaHox genes
	lab/Plhox1	ftz/Antp/ Plhox6	cad/Cdx
Acoel	CrLab	PrAntp/CrAntp	Prcad
Identity	87,0%Dm lab 82,6%Pnox3 73,9%Amphihox1	81,5%Dm Antp 77,7%Dthox-C 77,7%Amphihox8	70,3% M Cdx4 66,6% Dm cad 62,9%AmphiCdx
Special signatures		?	?

FIG. 1. Hox and ParaHox genes from Platyhelminthes with their homeodomain identities to another Hox gene and presence of specific signatures. **A,** Hox genes from freshwater triclads (Platyhelminthes, Tricladida) with homologies to Hox genes from *Drosophila* and Annelida and a new proposal for Platyhelminth Hox nomenclature (Plhox1–9). The comparison to Orii's (*et al.* 1999) nomenclature, and molecular signatures from homeobox flanking peptides is highlighted. Presumptive duplicated genes, in bold. Sequence data from the GenBank/EMBL databases and references: Bartels *et al.*, 1993; Balavoine and Telford, 1995; Bayascas *et al.*, 1997; Orii *et al.*, 1999. For further details, see text. **B,** Hox and ParaHox genes isolated from the polyclad flatworm *Discocelis tigrina* (Platyhelminthes, Polycladida). Molecular signatures from homeobox flanking peptides are highlighted. Complete homeodomain identities to other Hox and ParaHox genes from different vertebrates and invertebrates are indicated. **C,** Hox and ParaHox genes isolated from the acoel flatworms *Paratomella rubra* and *Convoluta roscoffensis* (Platyhelminthes, Acoela). Partial homeodomain identities to other Hox and ParaHox genes from different vertebrates and invertebrates are indicated. Abbreviations: Amphi, *Branchiostoma floridae*; Cr, *Convoluta roscoffensis*; Dj, *Dugesia japonica*; Dl *Dendrocoelum lacteum*; Dm, *Drosophila melanogaster*; Dist, *Discocelis tigrina*; Dt, Gt, *Girardia tigrina*; Hm, *Hirudo medicinalis*; Htr, *Helobdella triserialis*; M, Mouse, Nvi, *Nereis viridescens*; Pf, *Polycelis felina*; Pn, *Polycelis nigra*; Pr, *Paratomella rubra*; PW, *Phagocata woodworthi*. GenBank accession numbers are: *Dugesia japonica*: Djhox: AB024406- AB024410. *Dendrocoelum lacteum*: Dlox1: L41858; Dlox2: L41860; Dlox4: L41859. *Girardia tigrina*: Dthox-A to Dthox-G: X95411-X95417. *Polycelis felina*: Pfox1 to Pfox3: L41855-L41857. *Polycelis nigra*: Pnox1a: L41845; Pnox1b: L41846; Pnox2: L41847; Pnox3: L41848; Pnox4: L41850; Pnox8: L41851; Pnox7: L41854. *Phagocata woodworthi*: PWox-A: L19217; PWox-B: L19179; PWox-C: L19176; PWox-D: L19178; PWox-E: L19177. *Discocelis tigrina*: Distox-A: AJ300660; Distox-D: AJ300661; Distox-F: AJ300662; DistCad: AJ300663.

Hox fragments, corresponding to five different genes, named *Distox-A, B, D, E,* and *F* (from *Dis* cocelis *t*igrina HOM/H*OX* homeobox genes) which could be adscribed to particular Hox paralogous groups. Further rescreenings and DNA sequencing reduced the number to three *bona fidae* Hox genes: *Distox-A, Distox-D,* and *Distox-F,* orthologs to lab, Dfd and Ubx/abdA from *Drosophila* (Fig. 1B). Interestingly, the *Distox-F* gene bears the UbdA flanking peptide of the lophotrochozoans further proving the adscription of Platyhelminthes (or at least the Rhabditophora) to the Lophotrochozoa. Hox sequences from *D. tigrina* show no introns in the homeobox.

To test the suggested basal position of acoels as indicated from 18S rDNA sequences (Ruiz-Trillo *et al.,* 1999), we looked for Hox genes and, namely, for signature Hox flanking sequences (*e.g.,* the UbdA peptide and the spiralian peptide). PCR amplification from genomic DNA of the 18S rDNA slow evolving species *Paratomella rubra* has yielded so far a short *Antennapedia* related Hox gene sequence and a *caudal*-related ParaHox gene sequence (see Fig. 1C and below). A similar strategy applied to another acoel, *Convoluta roscoffensis,* yielded two short PCR fragments corresponding to *labial* and *Antennapedia* orthologs (Fig. 1C). Comparison of the 27 amino acid stretch to *Antennapedia* and other related genes (Ubx and abdA from protostomes, including other flatworms, and PG6–8 from deuterostomates) show a uniform and moderate degree of identity to all of them. Besides, 5 out of 27 residues appear specific to acoels. 3′ primer extension using rapid amplification of cDNA ends (Smart RACE, Clontech) has, so far, been unsuccesful to test the presence of the putative spiralian peptide. In addition, the *labial* fragment shows a similar degree of identity when compared to representatives of the main bilaterian clades, only 1 out of 27 residues being specific to acoels. Whether Hox genes in any of the flatworm studied are organized in cluster still remains to be proved.

ParaHox genes

Anterior-posterior axis patterning in all bilaterians is know to be mediated by the Hox cluster. In addition, the ancestral bilaterian is also believed to have possessed a second cluster, the ParaHox cluster, made of three clustered genes involved in patterning endodermal tissues (Brooke *et al.,* 1998). Phylogenetic evidence suggests that Hox and ParaHox cluster are sister clusters, both being descendants from an ancestral ProtoHox cluster (Brooke *et al.,* 1998; Finnerty and Martindale, 1999). Genes belonging to the three ParaHox classes (Gsh, Xlox and Cdx) have been reported in arthropods, annelids, nematodes, echinoderms, cephalochordates and chordates (see Brooke *et al.,* 1998, for references) and, more recently, in cnidarians (Finnerty and Martindale, 1999). The last finding means that the origin of Hox genes and ParaHox genes occurred prior to the evolutionary split between the Cnidaria and the Bilateria and predated the evolution of the anterior-posterior axis of bilaterians. Hence, ParaHox genes should also exist in Platyhelminthes.

Despite several attempts, no ParaHox genes have so far been found in triclads. To detect and isolate ParaHox genes in polyclads, primers SO-1 (Bayascas *et al.,* 1997) and Xlox-2 (5′RTTYTgRAACCA-DATYTTDATRTg3′), directed to ParaHox class homeoboxes were used to amplify them by PCR using as templates genomic and cDNA from different embryonic stages of *D. tigrina.* A small PCR clone of a gene was found belonging to the Xlox class and named, accordingly, *DistXlox* (from *Discocelis tigrina Xlox* class). Sequence comparison of *DistXlox* to other Xlox genes from annelids, cephalochordates and chordates show a rather high degree of identity, particularly with the *IPF-1* gene from the mouse (Fig. 1B). Using as probes the Hox and ParaHox genes already found we screened, at low stringency, a cDNA library pooled from most embryonic stages of *D. tigrina.* A large clone was isolated which contained most of the cDNA of the cad/Cdx class genes: the *Distcad* (from *Dis*cocelis *t*igrina *cau*dal class). Sequence comparisons of *Distcad* to other cad/Cdx class genes show the presence in the former of specific residues that define it as a *bona fidae* cad/Cdx class gene (Tauler, 2000). The percentage of identity at the homeodomain, be-

tween 60–70% (Fig. 1B), is lower than that of *DistXlox* to the other Xlox class genes. With the exception of a few scattered residues at the aminoterminal region flanking the homeodomain, no other areas of similarity between polyclad ParaHox genes and other ParaHoxs were found.

A similar approach using genomic and cDNA from *Paratomella rubra* (Acoela) as template and PCR amplification has yielded a ParaHox gene fragment from the cad/Cdx class: the *Prcad* (from *P*aratomella *r*ubra *cad*al class gene). Despite it shares some sequence stretches to other cad/Cdx genes from *D. tigrina*, *Drosophila*, amphioxus and the mouse, its degree of identity (between 60–70%) is rather low (Fig. 1C).

HOX GENE EXPRESSION

Expression of Hox genes in Platyhelminthes has recently been reported by Bayascas *et al.* (1998) in the freshwater planarian *G. tigrina* using RT-PCR, and by Orii *et al.* (1999) in *D. japonica* by whole mount *in situ* hybridization and RT-PCR methods. Analysis of ten different anterior-posterior body regions from adult intact *G. tigrina* by RT-PCR, using the axially ubiquitous homeobox containing gene *Dth-2* (Garcia-Fernàndez *et al.*, 1993) as standard, show two types of Hox expression pattern (Fig. 2, and see Fig. 1 for Hox nomenclature): 1) a nested expression along the AP axis for *Dthox-D* (Scr/Plhox5) and *Dthox-C* (Antp/ftz/Plhox6); and 2) an ubiquitous expression of *Dthox-G* (zen/Plhox3), *Dthox-A* (Dfd/Plhox4), *Dthox-E* (Antp/ftz/Plhox6) and *Dthox-F* (Ubx/abdA/Plhox7/8). Similar analyses in *D. japonica*, using the EF2 gene as standard, also show a regional expression of two genes: the *Djhox#1053* (Antp/ftz/Plhox6) and the *Djhox#0004* (Scr/Plhox5). The rest of genes were either undetectable or its expression were not reproducible.

G. tigrina Dthox-D (Plhox5) shows a more anterior expression boundary than *Dthox-C* (Plhox6) (Fig. 2). This agrees with current models of Hox expression (Duboule, 1994) which anticipate a nested expression of Hox genes along the AP axis. Even so, the anterior boundaries of expression of both genes are difficult to define due to the extreme sensitivity of the RT-PCR methods.

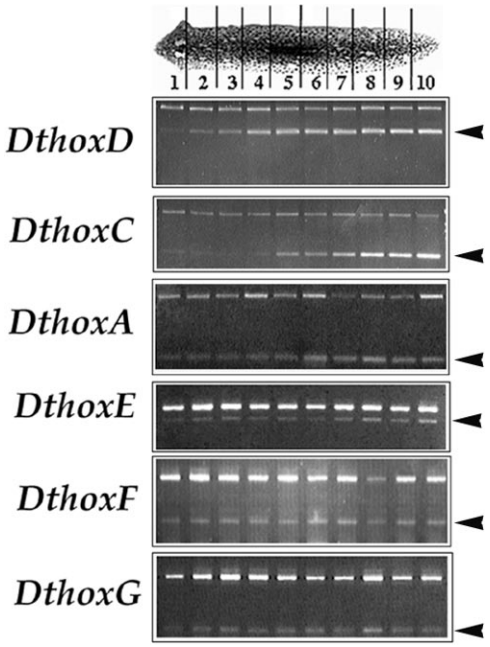

FIG. 2. Adult *Girardia tigrina* Hox gene expression at different antero-posterior (1 to 10) regions deduced by RT-PCR. As a control an ubiquitous axial transcript, the homeobox gene *Dth-2* (Garcia-Fernàndez *et al.*, 1993) was also amplified in each reaction (upper band). Double, control and specific Hox gene (head arrow), PCR amplification showing antero-posterior nested expression for *Dthox-D* (Plhox5), and *Dthox-C* (Plhox6) while *Dthox-A* (Plhox4), *Dthox-E* (Plhox6), *Dthox-F* (Plhox7/8), and *Dthox-G* (Plhox3), show an ubiquitous expression.

An intriguing feature is the increasing gradient of expression from the anterior boundary to more posterior regions. This may reflect either actual increasing levels of expression in more posterior cells or increasing densities of the still undisclosed cells expressing the Hox genes. Whole mount *in situ* hybridization of *DthoxD* in *G. tigrina* show a prepharyngeal anterior boundary (Fig. 3A, arrows) sharper than the one deduced after RT-PCR. A closer inspection of the tail area, shows groups of labelled cells which seems to lay within the parenchyma (Fig. 3B). Epidermal as well as gastrodermal cells are unlabeled. The nature of the labeled cells is unknown, though their spatial distribution (Baguñà and Romero, 1981) and clustering is reminescent of the undifferentiated totipotent stem-cells or neoblasts. In *D. japonica*, *Djhox#0004* (Scr/

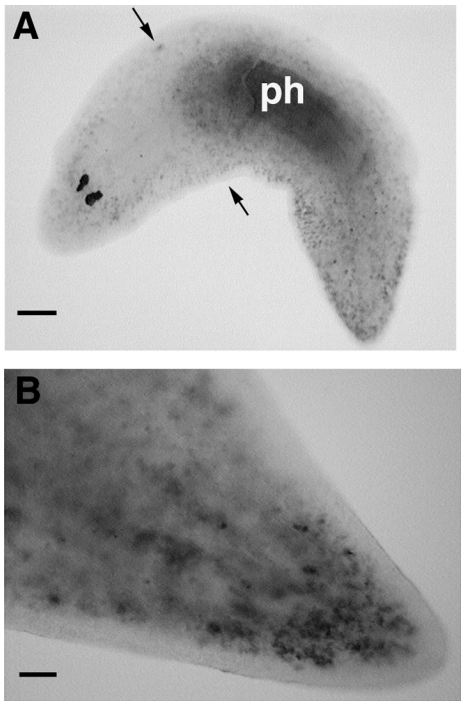

FIG. 3. Expression of *DthoxD* (Plhox5) gene in an intact *Girardia tigrina.* A, Whole-mount *in situ* hybridization. Expression of *DthoxD* transcript is seen along the antero-posterior axis with an anterior boundary at the prepharyngeal region (arrows). The strong labeling seen in the pharynx and pharynx cavity (ph) is background noise produced after the long incubations needed to enhance the signal. B, Higher magnification of the tail region showing groups of labelled cells scattered within the parenchyma. Scale bars, A: 0.5 mm; B: 0.1 mm.

Plhox5, Fig. 1A) and *Djhox#1053* (Antp/ftz/Plhox6, Fig. 1A) are also increasingly expressed along a spatial gradient in the posterior region with particularly high levels at the posterior tip (Orii *et al.*, 1999).

The finding of a large number of Hox genes ubiquitously expressed along the AP axis of intact organisms after RT-PCR is puzzling. They may either result from artefactual unspecific amplification, from axially homogenous leaking transcriptional activity, or because such genes have lost the canonical Hox function of being colinearly activated along the AP axis. We should also bear in mind, however, that Hox genes in adult organisms have functions other than those related to AP axial specification: *e.g.*, cell differentiation. In any case, this remains an interesting feature worth further investigation.

In regenerating *G. tigrina,* RT-PCR shows down-regulation of *Dthox-C* and *Dthox-D* in the blastema (head-forming area) of regenerating tail fragments, and their sequential activation (*Dthox-D* expressed earlier than *Dthox-C*) in the blastema (tail-forming area) of regenerating head fragments as early as at 1 day of regeneration (Bayascas *et al.*, 1998). Similar data has been produced for the *Djhox#1053* gene of *D. japonica* (Orii *et al.*, 1999) both by RT-PCR and whole mount *in situ* hybridization. In trunk pieces regenerating both heads and tails, weak expression is detected only in the posterior (tail forming) distalmost parts one day after cutting when the blastema is barely visible. On the second day, expression appears within the blastema and postblastema tail forming area. Expression in the anterior (head forming) blastema was never observed. Interestingly, laterally regenerating organisms produced by longitudinal saggital cuts expressed *Djhox#1053* only in the posterior half of the organism. Furthermore, X-ray irradiation causes a depletion of neoblasts in intact organisms and an inability to regenerate (Wolff and Dubois, 1948). The expression of *Djhox#1053* in X-ray irradiated planarians cut two days after irradiation, was tested by whole mount *in situ* hybridization (Orii *et al.*, 1999). Irradiated head pieces neither regenerate nor show any signs of expression of this gene. Irradiated tail pieces did not regenerate either and the expression of *Djhox#1053* faded away in parallel to the loss of neoblasts. This is a further hint that neoblasts may be the main source of cells expressing Hox genes.

Altogether, these data indicate that planarian Hox genes are either activated or inhibited at early stages of regeneration. Moreover, patterns of Hox gene expression, albeit poorly known, suggest that whereas in anterior regeneration (tails regenerating heads) distal areas may be determined first with patterning proceeding in a distal to proximal direction (*i.e.*, from anterior to posterior), in posterior regeneration (heads regenerating tails) patterning proceeds from proximal to distal (which is, again, from an-

terior to posterior). If this turns out to be true for the rest of Hox genes yet to be studied, it would indicate that the colinear model of AP activation of Hox genes (Duboule, 1994) also holds during regeneration in planarians.

TESTING THE FUNCTION OF HOX GENES IN FLATWORMS

The best, and classical, way to test the function of any gene in any organism is to generate loss-of-function mutants for that particular gene. In other words, to perform a genetic analysis. To undertake it, organisms must be small, easy to culture, short-lived, with several generations per year, leaving as many offsprings as possible, and having a small genome. Moreover, methods to induce, detect and recover individuals bearing induced mutations should be at hand. Almost none of these requirements are met by any species of flatworms. However, this non-amenability of flatworms to perform the classical genetic analysis has recently been overcome in the freshwater planarian *Schmidtea mediterranea* (Sanchez and Newmark, 1999) using the genetic interference method caused by injected double-stranded (ds) RNA (RNAi) first described in *Caenorhabditis elegans* (Fire *et al.*, 1998). Body-wall myosin dsRNA and opsin dsRNA injected into intact and regenerating organisms depleted the internal pool of mRNAs and inhibited their activation during regeneration (Sanchez and Newmark, 1999). A similar study aimed to unravel the network of genes regulating the formation of eyes in *G. tigrina* gave equivalent results (Pineda *et al.*, 2000). Thus, when *G. tigrina sine oculis* (*so*) dsRNA was injected to head regenerating organisms neither *so* mRNA nor *opsin* mRNA were activated and eyes never appeared despite that a new, complete, and well proportioned head forms.

Both sets of results provide strong evidence for the efectiveness and usefulness of this technique to assay any planarian gene. To test the function of Hox genes in anteroposterior axis specification in flatworms, single, double and triple injections of dsRNA of *Dthox-D* and *-C* and *GtAbd-Bb* in intact and regenerating planarians are presently being studied.

ARE PLATYHELMINTHES BASAL OR DERIVED LOPHOTROCHOZOANS? AN ASSESSMENT AND FURTHER WAYS TO TEST IT

From 18S rDNA sequences (Carranza *et al.*, 1997; Aguinaldo *et al.*, 1997; Balavoine, 1997; Ruiz-Trillo *et al.*, 1999) and from what is known on their set of Hox genes, Platyhelminthes, or at least the Rhabditophora (*sensu* Ehlers, 1985), seem to belong to the Lophotrochozoa. In other words, they are not primitive bilaterians as the long held planuloid-acoeloid hypothesis states. Evidences for are: (1) the clustering of most Platyhelminthes, acoels being the only exception so far (Ruiz-Trillo *et al.*, 1999), within the lophotrochozoan clade in all recently published trees drawn from 18S rDNA sequences; (2) the presence of the typical ''spiralian'' peptide in the Lox5 ortholog Plhox 6 (see Fig. 1A). This peptide appears to be a reliable diagnostic character for spiralians (Bayascas *et al.*, 1998; de Rosa *et al.*, 1999); (3) the presence of the UbdA peptide containing gene (see Fig. 1A, B) which which could be considered a diagnostic feature for protostomes (ecdysozoa + lophotrochozoa); (4) the presence, as in all other lophotrochozoans studied so far, of a full set of Hox genes (anterior, central and posterior); and (5) the presence of quartet-type spiral cleavage in all Platyhelminthes (the only exception being the duet-type of acoels), which bear strong similarities to other quartet cleavers belonging to the Lophotrochozoa (*e.g.*, annelids, molluscs, nemerteans, . . . ; Boyer *et al.*, 1996, 1998).

All phyla included within the three main branches of the now accepted tree of metazoans (deuterostomes, ecdysozoans and lophotrochozoans) have a one-way through gut. Moreover, most have coelom, and several bear segments. Despite being lophotrochozoans, Platyhelminthes have a blind gut (no anus) and lack coelom and segments. Comparative molecular embryology claim that segments and coelom are homologous structures (that is, derived by descent from a common ancestor) across phyla (Kimmel, 1996; Holland *et al.*, 1997). Such premises led to two main propositions. First, the bi-

laterian ancestor was a rather complex organism with one-way through gut, coelom, segments and, very likely, some sort of appendages (De Robertis, 1997). Second, because Platyhelminthes lack these features, they must be secondarily derived lophotrochozoans. In other words, they may be rooted well within the lophotrochozoan branch and not at its base (Balavoine, 1997, 1998). To explain how organisms (Platyhelminthes) lacking such advantageous structures are at the crown and not at the base of the lophotrochozoans, progenesis (attainment of sexual maturity in larval forms) was advanced as the most likely mechanism (Rieger, 1985). It was postulated (Balavoine, 1997, 1998) that a heterochronic transformation in a coelomate ancestor with a trochophore-like larva and complete metamorphosis, such as nemertines or annelids, lead to an acoelomate descendant with a modified larvae with incomplete metamorphosis, such as the Müller larva of polyclads. More specifically, development of particular adult characters were thought to be transferred before metamorphosis; hence, sexual maturity was attained by an individual retaining a ''larval'' morphology (*i.e.,* lacking anus, coelom, segments, and the circulatory system). Nemertines were further postulated as likely intermediates between complex annelids and simplified flatworms (Balavoine, 1998).

Such a ''nice story'' (Balavoine, 1998) may be critizised on several grounds. Firstly, in all published comprehensive 18S rDNA trees (Carranza *et al.,* 1997; Littlewood *et al.,* 1999; Ruiz-Trillo *et al.,* 1999), Platyhelminthes (or at least Catenulida + Rhabditophora) appear as a monophyletic lophotrochozoan group outside the main lophotrochozoan clades (annelids, molluscs, and nemerteans). Were Platyhelminthes derived from any of them, they should appear buried within them. Secondly, the progenetic argument rests on considering the indirect mode of development with a larval stage as ancestral for Platyhelminthes. Actually, the reverse may be true. Larvae occur in polyclads and nowhere else and, even in them, only in some families. The more basal Platyhelminthes, the acoels, nemertodermatids, catenulids and macrostomids (Ehlers, 1985; Carranza *et al.,* 1997; Littlewood *et al.,* 1999) are, mostly marine or freshwater, with direct development. Thirdly, the progenetic scenario asumes homology between the trocophora larvae of higher spiralians and the Müller larvae of polyclad Platyhelminthes. This is also unconvincing. The only apomorphic character for the Trochozoa appears to be the presence of a prototroch (Rouse, 1999) defining a less inclusive taxon which does not include the spiralian Platyhelminthes. Fourthly, despite cell lineage of polyclads and the higher spiralians show striking similarities (Boyer *et al.,* 1996, 1998) they also show interesting differences (*e.g.,* in polyclads, mesentoblasts derives from 4d not from 3D, that is one cell cycle later than in higher spiralians; there are no trochal cells and no typical trochophore larvae; and mesectoblasts derives only from 2b and not from second and third quartet cells as in other spiralians; van den Biggelaar *et al.,* 1997; Boyer *et al.,* 1998), which may be considered likely symplesiomorphic characters for spiralians. Finally, most lophotrochozoans surveyed so far as regards Hox genes (*e.g.,* annelids, molluscs, and brachiopods) show distinct orthologs for Ubx and abdA (de Rosa *et al.,* 1999), which likely derived by duplication from an ancestral gene, the UbdA gene, characterized by the so-called UbdA flanking peptide (Balavoine, 1998). Despite extensive surveys, only a single UbdA gene representative has been found in Platyhelminthes. Unless the ''Ubx'' or the ''Abd-A'' representative has been secondarily lost in planarians, the most parsimonious explanation is that Platyhelminthes retain a single copy of the ancestral UbdA gene.

Altogether, this suggest Platyhelminthes are basal lophotrochozoans or, at least, basal spiralians, and not an acoelomate group derived by simplification from any of the extant groups of coelomate lophotrochozoans. This raises the fascinating question of what the last common ancestor of the lophotrochozoans looked like. Were it coelomate, segmented and bearing a one-way through gut, it requires, however, the rather unparsimonious assumption of the later loss of the segmented (and likely the coelomic) state in many unsegmented phyla, Platyel-

minthes among them. Were it acoelomate/pseducoelomate and unsegmented, did it have a blind gut as the platyhelminthes or a one-way through gut? Because all deuterostomes and ecdysozoans as well as all lophotrochozoans besides Platyhelminthes, bear one-way through guts, it is more parsimonious to assume that Platyhelminthes lost it. Then, it well may be that Platyhelminthes derived through a progenetic mechanism, not involving larval stages, from the last common ancestor of the lophotrochozoans or from the last common ancestor of the spiralians.

A final answer to this riddle requires additional data. First, and foremost, the correct cladistic structure of the Bilateria should be determined. Whereas 18SrDNA and Hox genes have been instrumental to define the three main bilaterian clades and to assign specific phyla to any of them (Balavoine, 1997; de Rosa *et al.*, 1999), they are unable to sort out these very old, and likely fast, cladogenic events. To break the impasse, new genes are needed. Recently, another old puzzle, the origin of the Angiosperms, has been solved through a combined phylogenetic analyses of five mitochondrial, plastid and nuclear genes from a very broad sampling of angiosperms (Qiu *et al.*, 1999; Soltis *et al.*, 1999) as well as by using duplicate phytochrome genes (Mathews and Donoghue, 1999). It is time for such approaches, which use extensive characters (genes) and taxa, to be applied, together with finding new molecular synapomorphies, to the origin and radiation of the Bilateria.

Second, if platyhelminthes lost segments, coelom and the anus from a more complex ancestor, developmental genes controlling such features, albeit modified, may still be present in their genome. Homologues to core segmentation genes (*engrailed, wingless*, some pair-rule genes), coelom/mesoderm genes (*e.g., Tinman/Nkx 2.5, DMEF2,* . . .), and hindgut/anal(posterior mesoderm genes (*e.g., Brachyury, fork head/HNF-3ß, cad/Cdx*) may be worth looking at in basal platyhelminthes and their expression, if any, analyzed. Some of these genes have already been found in freshwater planarians, polyclads and acoels, but their expression is still unknown. However, a word of caution is needed here. Arthur *et al.* (1999) and Davis and Patel (1999) have recently warned about excessive emphasis given to similarities in the expression of segmentation genes across phyla, which carries the risk of overlooking clear differences and mistaking independent evolution for common ancestry. Indeed, some of these genes (*e.g., engrailed*) are expressed before (*Drosophila, Amphioxus*) or after (*e.g.,* leeches) segmentation, whereas others (*e.g., hairy/her1*) are expressed very differently among insects and among chordates.

Finally, the recent findings of exceptionally well preserved animals and embryos from pre-Cambrian sediments (Bengtson and Zhao, 1997; Xiao *et al.*, 1998) provides funded hope that some ancestral bilaterian or lophotrochozoan form bearing primitive or transitional structures may be uncovered in a not too distant future. Whenever it occurs, long standing disputes such as the complex *versus* simple structure of the bilaterian ancestor (see references above), the direct *versus* indirect (larva bearing) lifecycle of the first bilaterian (Wolpert, 1999; Peterson *et al.*, 2000) and the homology *versus* homoplasic nature of key features such as segmentation and coelom formation (Arthur *et al.*, 1999; Davis and Patel, 1999), will be close to an end.

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