## HOX GENES DISOBEY COLINEARITY AND DO NOT DISTINGUISH HEAD FROM TAIL DURING PLANARIAN REGENERATION.

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The Hox cluster genes encode a family of transcriptions factors that contain the homeodomain and determine the anteroposterior positional values in a wide range of metazoan, from nematodes to vertebrates. The genomic organisation of these genes is colinear with their expression domains in development (Duboule and Dollé, 1989). In contrast, during axolotl limb regeneration (Gardiner et al.1995) Hox genes are re-expressed non-colinearly, and seem to play an active function in proximo-distal determination. Planarians can regenerate along any body axis: anteriorly (head regeneration), posteriorly (tail regeneration), bilaterally (left to right and right to left) and intercalary (between head and tail). Since planarians show clear anteroposterior (head-to-tail) polarity, we reasoned that *Dthox* genes may be instrumental in defining this polarity in novel patterning events that do not occur in development or in amphibian regeneration.

We used two sets of degenerate oligonucleotides complementary to the conserved first and third helix of the homeobox to amplify, by PCR, genomic DNA or cDNA from regenerative blastemas or libraries. Subsequent screening of genomic and cDNA libraries with the PCR-amplified sequences allowed us to isolate seven different *Dugesia(G)tigrina* Hox genes, which we call *Dthox-A* through *Dthox-G*. Whole-mount in situ hybridisation studies were performed to study the patterns of *Dthox* expression from the beginning (1 hour) to the end (15 days) of different types of regeneration. Negative controls were performed with sense probes (Figure 2A).

Sequence analysis and comparison with the homeodomain and flanking regions of Hox cluster genes allowed us to order the planarian *Dthox* genes in three main groups. *Dthox-B* and *-G* show the highest similarity at the amino acid level (70%) with representatives of the paralogous groups(PG) 2 and 3. Some specific residues scattered through the homéodomain and in the flanking regions related to PG's 2 and 3 were conserved. The low percentage of similarity between both genes (56%) allowed us to consider them as independent. The global similarity of Dthox-G with the PG2/3 representatives is low, suggesting that the orthology of *Dthox-G* with PG2/3 is extremely speculative, so this gene could be considered as unclassified. Similar results have been obtained in the *Polycelis nigra* homologous gene *Pnox-4* (Balavoine and Telford, 1995). *Dthox-A* and *-D* are most similar to PG 4 and 5 respectively, with 82% in the homeodomain and a clear conservation in the flanking region immediately downstream. The percentage of similarity (70%) and different intron positions between both genes suggest an independent or old common origin.

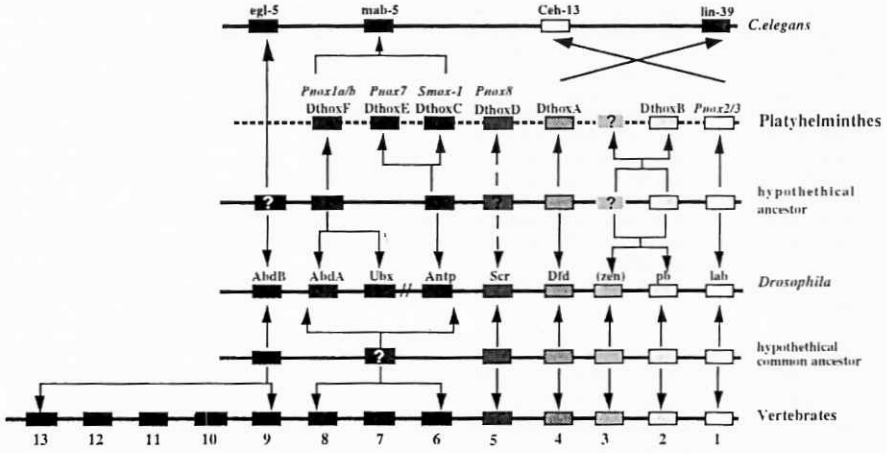


**Figure 1:** Organisation of the vertebrate, *Drosophila*,and the nematode *Caenorhabditis elegans* Hox clusters. Planarians may represent the organisation of the ancestor precluding the divergence of Arthropoda/Vertebrate/Nematoda. Paralogous relationships are indicated by arrowheads and grey colours code. Dashed line indicates no data about genomic organisation. Interrogation symbol means no obvious relation, and open boxes in paralogous group 3 indicates the possibility of *DthoxG* as a representative of this group. Also indicated de relationships with *Pnox* genes from *Policelis nigra* (Balavoine et al.; 1995) and *Smox-1* from *Schistosoma mansoni*. (Webster and Mansour, 1992).*Dugesia(G) tigrina* lacks representatives of labial and AbdominalB groups: This may be due to a technical problem, as the outer genes of the clusters are more diverged and could escape the initial PCR screen.

Partial homeobox sequence of *Pnox-8*, which is classified as orthologous to *Dfd*/PG-4 (Balavoine and Telford., 1995) defines this homology with *Dthox-D*. The last group (*Dthox -C -E* and *-F*) is similar to different medial PGs (6 to 8): *Dthox C* and *E* share a high similarity in their homeodomain (93%) and two new intron positions in their homeoboxes; this is one of the firsts indications that *Dugesia tigrina* could be a tetraploid diploidizated organism with two putative clusters. Moreover, they are most similar to amphioxus PG7 (93-92%) and to *Drosophila Antp* gene (90%); two downstream flanking positions are conserved with the *Antp* sequence and some other positions show conservative changes. On this basis *Dthox-C* and *-E* are considered as putative orthologues of *Antp* as well as *Pnox-7* from *Polycelis nigra* (Balavoine and Telford, 1995), and *Smox-1* from *Schistosoma mansoni* (Webster and Mansour, 1993). Finally, *Dthox-F* presented the highest similarity in the homeodomain and flanking sequences with *Ubx* , *Abd-A* and the Annelida genes *CTsx-2*, *Lox-2* and *Lox-4* (Dick and Buss, 1994; Wong et al., 1995). Partial homeodomain sequences of *Polycelis nigra* genes *Pnox-1a,b* define a clear homology to *Dthox-F*. In the homeodomain the identity is between 85-88%, sharing three specific positions (R:2, H:24, L:56) with *Ubx* and *Abd-A*. The downstream two-thirds of the homeodomain share near 100% homology with only one conservative substitution and four specific residues with *CTsx-2*. A high conservation in the downstream region was also observed with *Ubx*, allowing us to define a charged motif defined by Q-I(R/K)(E/D)LNE. The high degree of similarity allowed us to consider *Dthox-F* as a putative orthologue of *Ubx/Abd-A*.

Current data from Platyhelminthes (Balavoine, 1995;1996) show a high number of anterior and medial Hox genes in this phylum. Taken together, these results suggest a putative ancestral cluster defined by at least six genes (Figure 1). Nevertheless, no data are yet available about the genomic organisation of these *Dthox* genes; the large number of repetitive DNA elements in the genome of this planarian (Garcia-Fernandez, 1993; 1995) has prevented chromosome walking. In any case, the high number of orthologous planarian Hox genes suggests that the cluster organisation should be maintained.

Whole-mount *in situ* hybridisation studies showed several unexpected features. First, expression of *Dthox-D, -C, -E, -G* and *-F* genes takes place rapidly: after 1 hour of regeneration there is high level of transcription which is temporally synchronous and spatially coincident, rather than collinear. The fast and synchronous *Dthox* expression during early regeneration is inconsistent with the principle of temporal and spatial colinearity described in vertebrate development (Duboule, 1994). The fact that the same type of Hox activation has been observed in axolotl limb regeneration suggest a new and specific mode of Hox activation in regeneration. In contrast, *Dthox* genes switch off their expression at different times of regeneration. This will create differential spatial expression of *Dthox* genes, without use of spatial or temporal colinearity during activation of the genes.Second, the spatial and temporal pattern of *Dthox* expression is identical in anterior (head) regeneration and posterior (tail) regeneration (Fig. 2B). This unexpected symmetrical expression means that *Dthox* genes can't tell head from tail, and rules out the conserved function attributed to the Hox cluster genes in antero-posterior positional information (Slack et al, 1993). Even in bilateral regeneration all *Dthox* genes shows, again, a clear and continuous expression along the whole lateral blastema and postblastema that covers the complete antero-posterior axis (Fig. 2C). Perhaps these results suggest that the ancestral function of Hox cluster genes was simply to specify positional information in any axis, that only later became fixed into anterior-posterior information in higher animals. Another hypothesis is to consider that in the regenerative process Hox cluster genes were co-opted to define any type of axis. Finally, considering that in the regenerative tissue (blastema) the undifferenciated cells (neoblasts) have to acquire new positional values, the ubiquitous *Dthox* expression can be considered as a proof that this cells have their genome open, ready to acquire new positional values. This issue could be resolved by extending the studies to Platyhelmintes development (Tauler et al, 1996, this issue).

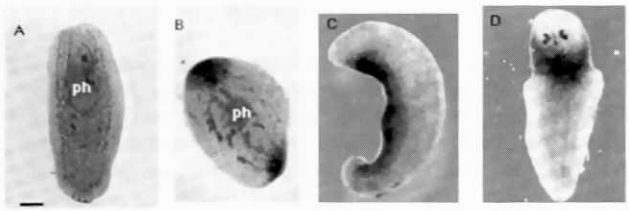


**Figure 2:** *Dthox* gene expression as visualised by whole-mount in situ hybridisation. Animals are shown from de dorsal surface, with anterior to the top. Scale bars 400 µm. A) 3 days anterior regenerating organism hybridised with DthoxF sense riboprobe as a negative control: no signal is detected. B) 5 days double regenerating organism hybridised with DthoxF antisense riboprobe: similar signal is detected in both the anterior and posterior blastemas. C) Expression of DthoxF in a 10 days lateral regenerating organism: a strong and homogeneous staining along the whole anteroposterior axis is showed. D) 8 days intercalary regeneration hybridised with DthoxE antisense riboprobe: the intercalary blastema between the two grafted pieces shows a high level of expression, while 5 days regenerating organisms do not share any signal (data not shown). Similar patterns of expression were obtained with the others *Dthox* genes in any type of regeneration, except with a clear differential deactivation. ph: pharnyx

Finally, intercalary regeneration produced by the juxtaposition of anterior and posterior terminal tissues (Saló and Baguñà, 1985), induces a synchronous, colocalized, but extremely delayed *Dthox* expression, which is not detectable until 7-8 days of regeneration (Fig. 4). In contrast to terminal regeneration, intercalary regeneration do not show a wound healing nor epithelial mesenquimal interactions. These data suggest an epithelial-mesenchimal interaction mediating *Dthox* early activation,

In conclusion, we have shown that Platyhelmintes bear an intricate complement of Hox genes, an that their expression doesn´t show obvious relation to axial polarity during planarian regeneration. Last can be considered one of the first indications that question the generalised model of Hox genes respecting body axes.

ACKNOWLEDGEMENTS. We thank J. Baguñà, for his support and guidance in the whole long period needed to obtain these results, J. Garcia-Fernàndez for helpful discussions and technical advice. This work was supported by grants from the DGICYT to E.S. and FPI fellowship to JR.B., A.MM. and E.C. from Ministerio de Educación y Ciencia, España; Comissió Interdepartamental de Recerca i Innovació Tecnològica (Generalitat de Catalunya) and AECI-Mutis, Ministerio de Asuntos Exteriores, respectively.

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