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Planarian Hox genes: novel patterns of expression during regeneration

J. R. Bayascas, E. Castillo, A. M. Muñoz-Mármol and E. Saló*

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071 Barcelona, Spain

*Author for correspondence (e-mail: esalo@porthos.bio.ub.es)

SUMMARY

Platyhelminthes are widely considered to be the sister group of coelomates (Philippe, H., Chenuil, A. and Adoutte, A. (1994) *Development* 1994 Supplement, 15-24) and the first organisms to show bilateral symmetry and cephalization. Within this phylum, the freshwater planarians (Turbellaria, Tricladida) have been used as model systems for studying bidirectional regeneration (Slack, J. M. W. (1980) *J. Theor. Biol.* 82, 105-140). We have been attempting to identify potential pattern-control genes involved in the regeneration of planarian heads and tails after amputation. Since Hox cluster genes determine positional identity along the anteroposterior axis in a wide range of animals (Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993) *Nature* 361, 490-492), we performed an extensive search for Hox-related genes in the planarian *Dugesia(G)tigrina*. Sequence analyses of seven planarian *Dthox* genes (*Dthox-A* to *Dthox-G*) reveal high similarities with the homeodomain region of the Hox cluster genes, allowing us to assign planarian *Dthox* genes to anterior and medial Hox cluster paralogous groups. Whole-mount in situ hybridization studies in regenerating adults showed very early, synchronous and colocalized activation of *Dthox-D, Dthox-A, Dthox-C, Dthox-E, Dthox-G* and *Dthox-F*. After one hour of regeneration a clear expression was observed in all *Dthox* genes studied. In addition, all seemed to be expressed in the same regenerative tissue, although in the last stages of regeneration (9 to 15 days) a differential timing of deactivation was observed. The same *Dthox* genes were also expressed synchronously and were colocalized during intercalary regeneration, although their expression was delayed. Terminal regeneration showed identical *Dthox* gene expression in anterior and posterior blastemas, which may prevent these genes from directing the distinction between head and tail. Finally, continuous expression along the whole lateral blastema in sagittal regenerates reflected a ubiquitous *Dthox* response in all types of regeneration that was not related specifically with the anteroposterior polarity.

Key words: planarian, homeobox, Hox cluster genes, regeneration

INTRODUCTION

There are two biological processes in which anteroposterior positional values are specified: embryonic development and regeneration. In development, the Hox cluster genes determine these values in a wide range of metazoa, from nematodes to vertebrates (Keynon, 1994; Lawrence and Morata, 1994; Akam 1995; Krumlauf, 1994). This functional resemblance revealed that the genomic organization of Hox cluster genes is colinear with their expression in the various body regions (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 proximodistal determination. Planarians can regenerate along any body axis: anteriorly (head regeneration), bidirectionally (head and tail regeneration) and posteriorly (tail regeneration) (Fig. 1 numbers 1 to 3); bilaterally (left to right and right to left) (Fig. 1 number 4); or intercalary (between head and tail) (Fig. 1 number 5) (Brondsted, 1969; Slack, 1980). This gives us the opportunity to study the function of Hox cluster genes in novel patterning events that do not occur in development or in amphibian regeneration.

Planarian regeneration is a classic example of an epimorphic process that depends on cell proliferation to produce new tissue (Saló and Baguñà, 1984). This process does not require cell dedifferentiation (Saló and Baguñà, 1989), since in the adult there are undifferentiated totipotent proliferating cells called neoblasts (Baguñà, 1981). Wounds heal 30 minutes after amputation and, from the first hour of regeneration, there is a strong mitotic response of neoblasts close to the wound (Saló and Baguñà, 1984). Below the wound epithelium, a new undifferentiated tissue, the blastema, is produced by the basal addition and aggregation of groups of neoblasts, which divide in the stump and stop dividing inside the blastema. Thus, the blastema grows by the addition of neoblasts produced in the postblastema, a 500 μm region underlying the blastema (Saló and Baguñà, 1985a). Between 3 and 5 days after wounding, differentiation begins within the blastema and postblastema, and normal body proportions are finally attained after 4 weeks. Since planarians show clear anteroposterior (head-to-tail) polarity, we reasoned that *Dthox* genes may be instrumental in defining this polarity, as they do in more evolved organisms. According to several models (Keynon, 1994; Lawrence and Morata, 1994; Akam 1995; Krumlauf, 1994), we therefore expected nested colinear expression of the *Dthox* genes along the anteroposterior axis with a differential pattern in both edges (Saló et al., 1995), but no expression along the lateral axis during bilateral regeneration. To test these predictions, we report

the isolation and sequence comparison of seven Hox cluster-related genes (*Dthox*) isolated from the planarian *Dugesia(G)tigrina*. We also analyzed by whole-mount in situ hybridization the expression of these genes during different stages and modalities of regeneration. Most genes were expressed very early in the regenerative stump, concurrent with the early mitotic response. Their initial expression was synchronous and coincident and was maintained until late stages of regeneration, when their deactivation was differential. Their pattern of expression was identical in anterior, posterior and lateral regeneration, but clearly delayed in intercalary regeneration.

MATERIALS AND METHODS

Species

Planarians *Dugesia (G) tigrina* (Girard) were collected in Calders river (Barcelona, Spain). They were maintained in spring water. 2-week-starved organisms were used in all experiments.

Regenerating organisms

Planarians, 9-10 mm long, were cut transversally at different anteroposterior levels (Saló and Baguñà, 1984) and sagittally. They were left regenerating in Petri dishes with spring water in the dark at 17°C.

Isolation and sequencing of planarian *Dthox* genes

All genes were first isolated by degenerate PCR on either genomic DNA, cDNA libraries from different stages of regeneration or cDNA from regenerative blastemas. The PCR primers used were SO1 (5′-GARYTNGARAARGARTT-3′) and SO2 (5′CKNCKRTTYTGRAACCA-3′), corresponding to homeodomain residues 15-20 and 48-53 of Hox class genes. Cycling conditions and cloning strategies were as described (Holland, 1993), with annealing temperatures of 50°C for 40 cycles. Two genomic libraries in λCharon-35 and λFixII (Stratagene), and four cDNA libraries constructed in λgt10 and λZAP (Stratagene) were screened at high stringency (50% formamide, 42°C) with the degenerate PCR-derived probes, or with oligonucleotides corresponding to the PCR-amplified central specific sequences of *Dthox-A, Dthox-B, Dthox-C, Dthox-D, Dthox-E, Dthox-F* and *Dthox-G* under high stringency conditions (hybridization at 65°C; washes 6× SSC-0.05%sodium pyrophosphate 50°C). Multiple genomic clones of all these planarian Hox genes, except *Dthox-B*, were isolated. In addition, several cDNA clones from *Dthox-C, Dthox-D, Dthox-E* and *Dthox-F* were isolated. Positive clones were restriction-mapped, the hybridizing bands were cloned into plasmid vectors, and homeoboxes and flanking regions were sequenced. Sequence data were analyzed using the GCG sequence Analysis Software Package.

Preparation of digoxigenin-labelled RNA probes

Digoxigenin-labelled probes with sizes between 400 and 1240 bp for whole-mount in situ hybridization were synthesized according to the manufacturer's protocol (Boehringer).

Whole-mount in situ hybridization

2-week-starved organisms were sectioned at different levels and left to regenerate at 17°C for specified times. Before fixation, the organisms were treated for 2 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 minutes each). The planarians were fixed at 4°C overnight in 4% paraformaldehyde in PBS, washed in PBS, dehydrated and stored at –20°C in 70% ethanol. After rehydration, they were washed in PTw (PBS with 0.1% Tween-20) three times (5 minutes each), and the organisms were treated with proteinase K (10 μg/ml) in PTw for 15 minutes at room temperature. Digestion was stopped by two washes in 2 mg/ml glycine in PTw (5 minutes each). Planarians were then acetylated in 0.1 M triethanolamine (pH 7.8) supplemented with 0.5% acetic anhydride for 10 minutes and rinsed with PTw, postfixed in 4% paraformaldehyde in PBS for 20 minutes and rinsed in PTw five times (5 minutes each). Planarians were prehybridized for 1 hour at 55°C in hybridization solution (50% formamide, 5× SSC, 1 mg/ml yeast RNA, 50 μg/ml heparin(Sigma H-3125), 0.1% Tween-20 (Sigma P-1379)). The digoxygenin-labelled antisense or sense probe (10 mg/ml in hybridization solution) was heated to 80°C for 2 minutes, diluted to 0.5 μg/ml in hybridization solution and added to samples for hybrid-

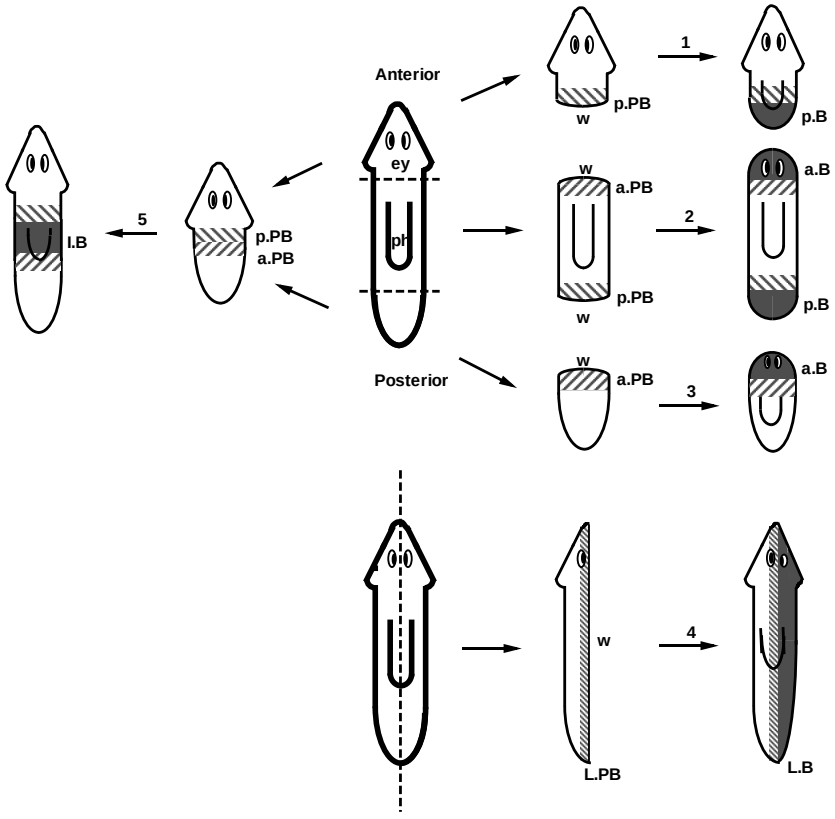

Fig. 1. Diagrammatic dorsal view of *Dugesia(G)tigrina* cut at different body levels (---) to visualize the different types of regeneration: (1) posterior regeneration; (2) bidirectional regeneration; (3) anterior regeneration; (4) lateral regeneration and (5) intercalary regeneration. Arrows indicate the direction of the different regenerative processes. Hatching areas define the different postblastema tissues. The postblastema is the old tissue close to the wound that by continuous cell (neoblast) proliferation and movement to the wound produces the new regenerative tissue (blastema). Stippled areas define the different blastema tissues. The blastema initially is an undifferentiated tissue that growth from its base by addition of postblastema neoblasts, lately initiates their differentiation in the missing structures. a.PB, anterior postblastema; p.PB, posterior postblastema; L.PB, lateral postblastema; a.B, anterior blastema; p.B, posterior blastema; L.B, lateral blastema; I.B, intercalary blastema; ey, eye; ph, pharnyx; w, wound.

ization at 55°C for 72 hours. Following hybridization, the planarians were washed in 100%, 75%, 50%, 25% hybridization solution in 2× SSC, twice in 2× SSC and twice in 0.2× SSC (30 minutes each), all at 55°C. The organisms were rinsed twice in PTw and then incubated for 1 hour in blocking solution (1% Boehringer blocking reagent, 5% heat-inactivated calf serum, 2 mg/ml BSA). After blocking, the organisms were incubated overnight at 4°C with 1:2000 alkaline-phosphatase (AP)-conjugated anti-digoxigenin antibody (Boehringer), which had been preabsorbed with 8 mg/ml planarian powder in the above blocking buffer overnight at 4°C. The organisms were rinsed eight times in PTw (15 minutes each), and three times in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 5 mM levamisole) (5 minutes each). Signal was detected following incubation of the organisms 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 (2 to 4 hours), the organisms were washed twice in PTw, postfixed for 20 minutes in 4% paraformaldehyde, cleared 10 minutes in methanol and stored in glycerol at 4°C. Sense riboprobes were hybridized and developed in parallel with antisense riboprobes and served as a negative controls. Photography was performed using a Zeiss axiophot connected by Sony video camera to a Macintosh Centris computer running Adobe Photoshop software.

RESULTS

Isolation and characterization of *Dthox* gene

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

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Antennapedia                                                RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN

                % Identity

Dthox-B         DthoxB  DthoxG                                      NI-K--C-P---DL-NR-Q--------
Dthox-G         56.0                    ENVYNYNDDACCTVSSTT  S--C-SA--NN-LV-------Y-N--A-G--T-LSKQ-L-----V----------Q---K  KEIEKSRNQ
Amphihox-3      66.7    70.0              GTTEPGESPGLGGAA  G--A-TA--SA-LV-----------C-P--V-M-AM-N----------------Y---Q  KVKGGGSGGG
m a-3           70.4    71.7                    …GPPGQAS  S----TA---P-LV-----------M-P--V-M-NL-N----------------Y--DQ  KGKGML…
m b-3           66.7    68.3           EGCG16SSG10DKSPPGSAA  S--A-TA--SA-LV-----------C-P--V-M-NL-N-S--------------Y--DQ  KAKGLASSSG
m d-3           70.4    70.0               ENCEDKSPPGP.A  S--V-T---SA-LV-----------C-P--V-M-NL-N----------------Y--DQ  KAKGILHSPA
Amphihox-2      55.0    61.7               NADFLTSPTDQVNS  SR-L-TVF-NT-L--------Y-K-VCKP--K---SY-D-N---V---------RQ-RRD  TKGRSEIGTD
m a-2           63.0    66.7                  SLEIADGSGGG  SR-L-TA--NT-L----------K--C-P--V---AL-D-----V-V--------H-RQT  QCKENQNSEG
m b-2           63.0    63.7                  GPGLPECGGSG  SR-L-TA--NT-L----------K--C-P--V---AL-D-----V-V--------H-RQT  E…
d pb            66.7    65.0                    EFVPENGL  PR-L-TA--NT-L----------K--C-P------AS-D-----V-V--------H-RQT  LSKTDDEDHK
d zen1          63.0    68.3           CVELNVEAAPTATTRSSEK  S--S-TAFSSL-LI---R---L-K--A-T-----SQR-A-----V----------L--ST  NRKGAIGALT

Dthox -A        DthoxA  DthoxD                      YGQLILSD.  S--N-TA---Q-R--V-------K----K------QS-Q-S-S-V------------DH  HLPGNKQRLS
Dthox-D         70.0                NFPAFPWMQKSNMRKNKSDKSVE....  Y--C--A-S-Y---------YY-Q----------NSV--S-------------Y--DV  VLKTNEFPS
Amphihox-4      80.0    75.0                    SNT..STSY.NGQD.  T--S-TA---Q-V----------------------S-G----------------D-  RLPNTKTRSS
m b-4           78.0    78.3           EPVVYPWMRKVHVST..VNPNYAGGE.  P--S-TA---Q-V--------Y---------V--------S----------------DH  KLPNTKIRSG
m c-4           82.0    81.6           QPIVYPWMKKIHVST..VNPNYNGGE.  P--S-TA---Q-V--------Y--------------S---S----------------DH  RLPNTKVRSA
m d-4           82.0    78.3           PAVVYPWMKKVHVNS..ANPNYTGGE.  P--S-TA---Q-V----------------------T---P----------------DH  KLPNTKGRSS
d Dfd           76.6    76.6           ERIIYPWMKKIHVAGV.ANGSYQPGME  P--Q-TA---H-I--------Y-------------T-V-S----------------D-  KLPNTKNVRK
CTs-Dfd         80.0    75.0                                        ----------------------------------------H  KLPNTKTRLS
Hr-Lox6         75.0    72.5                                        ------NG-------------------------------D-  RLPNSKSGKI
Amphihox-5      75.0    75.0                        AGTGD...  N--T-TA-------------------------------------------------  KLKSLSQCQQ
m a-5           78.0    78.3           QPQIYPWMRKLHISH..DNIGGPE...  G--A-TA---------------------------------S----------------D-  KLKSMSMAAA
m b-5           78.0    78.3           TPQIFPWMRKLHIS...HDMTGPD...  G--A-TA---------------------------------S----------------D-  KLKSMSLATA
d Scr           75.0    71.6           PPQIYPWMKRVHLGTSTVNANGE....  T--Q-TS-------------------------------------------------H  KMASMNIVPY
CTs-Lox5        75.0    78.0                                        -Y---------------G-S------------------  NLAKLTGPNG
Hr-Lox5         72.5    80.0                                        YS-------------S-A-S------------------  NVQKLTGPGG
m a-7           73.3    75.0           SFRIYPWMRSGPD.............  ----------------------------------------------------H  KDESQAPTAA
m b-7           70.0    76.6           NFRIYPWMRSGPD.............  --------------------Y-------------T----------------------  KTSGPGTTGQ

Dthox-C         DthoxC  DthoxE  DthoxF                                  -----K-----------T-I----------V--------H  NIAKLTGPGS
Dthox-E         93.0                    DMVVYPWMNPKMNNSESSSD..  H--S-------------------K-------------S----------V--------DH  NIPKLNGPGT
Dthox-F         83.0    78.0                              VQ  KR------S-H---------Q--H------------N---S-------------L---R  QQIRELNDEI
Amphihox-6      90.5    88.0    81.6    TPPVFPWMRKGSS..QTAMGE.  K----------------------K----K-------L-G----------------------  KIPSLNATTI
m b-6           88.1    86.6    83.3    STPVYPWMQRMNSCNSSSFGPS  GR-------------------Y-----------------------------------S  KLLSASQLSA
m c-6           88.1    83.3    85.0    SIQIYPWMQRMNS.HSVGYGAD  -R----I-S--------------------------N----------------------S  NLTSTLSGGG
d Antp          90.5    90.0    83.3    PSPLYPWMDSQFGKCQE.....  ----------------------------------------------------------  KTKGEPGSGG
d Ubx           83.3    83.3    85.0                      TNGL  -R-------------------T-H---------M----------------------L---L  QAIKELNEQE
d Abd-A         90.5    85.0    88.3    ...RYPWMTLTDWMGSPFER()  ----------F-----------------------------------------------L  RAVKEINEQA
Amphihox-7      92.8    91.6    83.3                        PE  ----------------------K-----------------------------------  KLESLKQQPA
m a-7           92.8    91.6    83.3    SFRIYPWMRSGPD.........  ----------------------------------------------------------H  KDESQAPTAA
m b-7           90.5    86.6    81.6    NFRIYPWMRSGPD.........  --------------------Y-------------T----------------------  KTSGPGTTGQ
Amphihox-8      90.5    86.6    86.6                        PE  -R------S--------------K--------------G----------------L---A  AMLCPPKAET
m b-8           78.6    78.3    78.3    SPTQLFPWMRPQAAAG.......  -R------S-----------L--P----K----VS---G-----V--------------  NKDKFPSSKC
m c-8           78.6    76.6    76.6    SPSLMFPWMRPHAPG........  -RS-----S-----------L--P----K----VS---G-----V--------------  NKDKLPGARD
m d-8           80.9    75.0    78.3    SPSQMFPWMRPQAAPG.......  -R------S-F---------L--P----K----VS-T-A-----V--------------  NKDKFPASRP
CTsx-2          80.0    80.0    98.0                          Q--H----K-------N---S--------------L---R  QQIKDLNEAV
Hm Lox4         82.5    78.3    87.0                  PNSSQ  -R------S-Y---------Q-------K-------C----------------V---K  QQIKELNE
Hm Lox2         82.5    78.3    80.0                  PNSNQ  -R--------Y---------K------------LS-T-Y----------------E---V  QAIRELNEIE
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Fig. 2. Alignments between deduced homeodomain sequences of freshwater planarian Dthox genes and those with higher similarity encoded by mouse, amphioxus, *Drosophila* cluster genes (Bürglin, 1994, 1995; Garcia-Fernàndez et al., 1994) and polychaete annelid *CTsx-2, CTs-Lox5* and *CTs-Dfd*, (Dick and Buss, 1994) and leeches *Hm Lox-2, HmLox-4, HrLox-5* and *HrLox-6* (Wong et al., 1995; Shankland et al., 1991; Wysocka-Diller et al., 1989). Dashes indicate amino-acid identity to Antennapedia in the homeodomain only; dots indicate gaps introduced to optimize alignments. Also shown are the partial C-terminal and N-terminal flanking regions of the homeodomain including, in some cases, the hexapeptide. Percentage identities, with respect to *Dthox* homeodomains, cover the homeodomain only. Arrowheads indicate intron positions. Sequences have been deposited with EMBL/GenBank databases and assigned the accession numbers: (X95411-X95417).

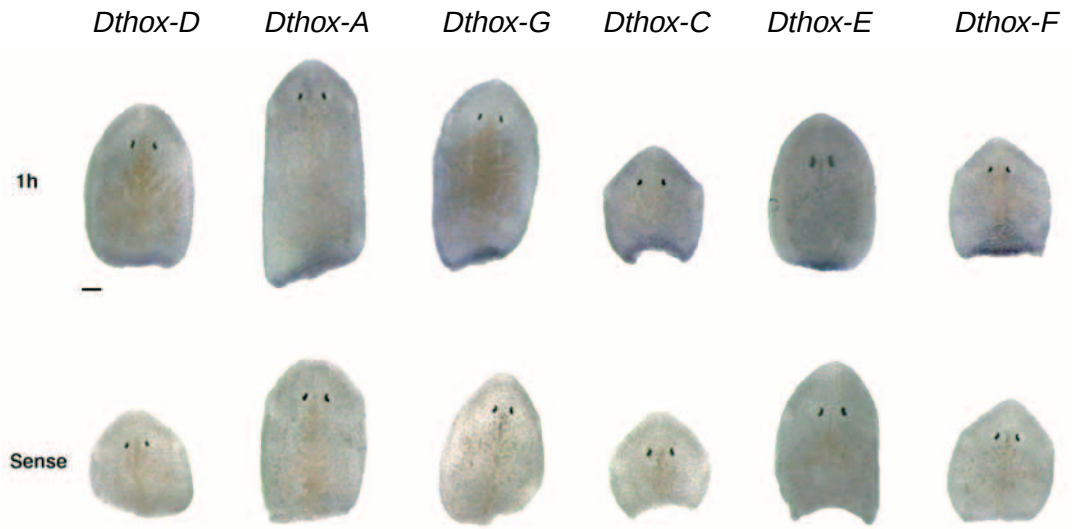

Fig. 3. *Dthox-D, Dthox-A, Dthox-G, Dthox-C, Dthox-E and Dthox-F* gene expression at 1 hour of posterior terminal regeneration in *Dugesia(G)tigrina* as visualized by whole-mount in situ hybridization. No expression was detected for *Dthox-B*, probably due to the small size of the riboprobe (data not shown). Animals from the same experiment and similar cut level, hybridized with the different riboprobes are shown from the dorsal surface. Scale bar 400 μm. The regenerating planarians illustrated were collected at 1 hour after cutting. An early and synchronous expression for *Dthox-A*, to *Dthox-G* was observed at 1 hour of regeneration in a region close to the wound (the postblastema). The second line shows the hybridization with sense probes at 1 hour of posterior terminal regeneration. The brown signal observed in the gut of most organisms is due to some remainder endogenous alkaline phosphatase activity.

to isolate seven different *Dugesia(G)tigrina* Hox genes, which we call *Dthox-A* through *Dthox-G*. Sequence analyses and comparison with the homeodomain and flanking regions of Hox cluster genes (Fig. 2) permitted us to order the planarian *Dthox* genes in three main groups. *Dthox-B* and *Dthox-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 homeodomain and in the flanking regions related to PG 2 and PG 3 were conserved. The low percentage of similarity between both genes (56%) allowed us to consider them as independent. *Dthox-A* and *Dthox-D* were most similar to PG 4 and PG 5 with 82% similarity in the homeodomain, sharing some specific residues of these PGs. The percentage of similarity (70%) and different intron positions between both genes suggests an independent or old common origin. The last group (*Dthox-C*, *Dthox-E* and *Dthox-F*) was similar to different medial PGs (6 to 8): *Dthox-C* and *Dthox-E* shared a high similarity in their homeodomain (93%) and two new intron positions in their homeoboxes, which permits us to consider them as recent duplicates. Moreover, they were most similar to amphioxus PG7 (93-92%) and to the *Drosophila Antp* gene (90%). On this basis, *Dthox-C* and *Dthox-E* were considered as putative orthologues of *Antp*. 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) (Fig. 2). In the homeodomain, the identity is between 85 and 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

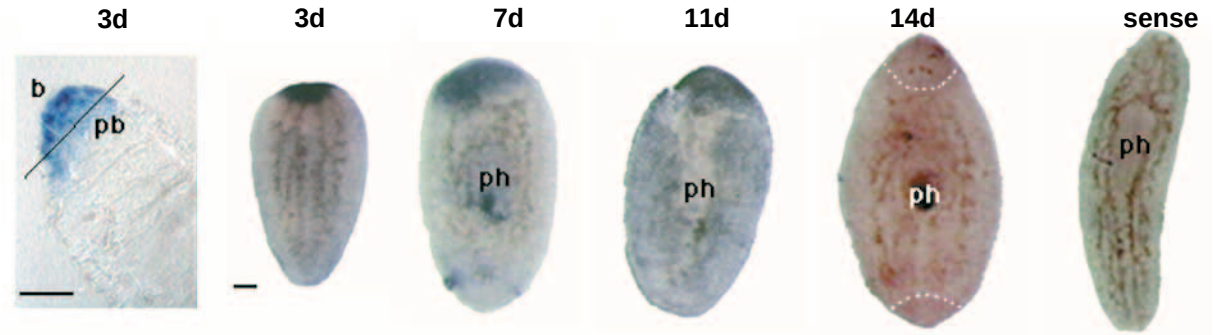

Fig. 4. Expression of *Dthox-F* during different stages of terminal regeneration. The planarians illustrated represent progressively later stages of anterior regeneration: 3 days (3d) in sagittal paraffin section and whole mount, 7 days (7d), 11 days (11d) and bidirectional regeneration at 14 days (14d) after cutting. As a control a sense *Dthox-F* probe at 3 days of anterior regeneration is shown. *Dthox* gene expression spreads along with the blastemal growth including the 14 days stage with a weak and homogeneous signal in both anterior and posterior blastemas, the dotted line demarks the base of both blastemas. Similar patterns of expression were observed with the other *Dthox* genes except with a differential deactivation (Table 1). Scale bars 400 μm. b, blastema; pb, postblastema; ph, pharynx.

Fig. 5. *Dthox-E* expression at 6 days of bidirectional regeneration of planarians cut at different anteroposterior levels. Anterior blastema to the top and posterior blastema to the bottom. Symmetrical expression was observed in both terminal blastemas independently of the cut level. Posterior blastemas show smaller size and expression than anterior ones. Similar results were observed with the different *Dthox* genes. g, gut branches; ph, pharynx. Scale bar 400 μm.

substitution and four specific residues with *CTsx-2*. The high degree of similarity allowed us to consider *Dthox-F* as a putative orthologue of *Ubx/Abd-A*. This particular subclass of Hox genes is a widely shared characteristic of the protostomes and is apparently absent in deuterostomes (Akam, 1995).

Current data from Platyhelminthes (Oliver et al., 1992; Webster and Mansour, 1992; Bartels et al., 1993; Balavoine and Telford, 1995; Tarabykin et al., 1995; Balavoine, 1996) show a high number of anterior and medial Hox genes in this phylum: at least five. Taken together, these results suggest a putative ancestral cluster defined by at least two representatives of the anterior group and three representatives of the medial group. The apparent absence of the more posterior genes (*Abd-B*-like) in the Platyhelminthes and Cnidarians (Schummer et al., 1992; Shenk et al., 1993) probably indicates that we were unable to isolate them because they are highly divergent. Recent data on evolutionary distances among different amino acid sequences of Hox genes suggest that the divergence between medial and posterior Hox genes occurred before the divergence of acoelomates and

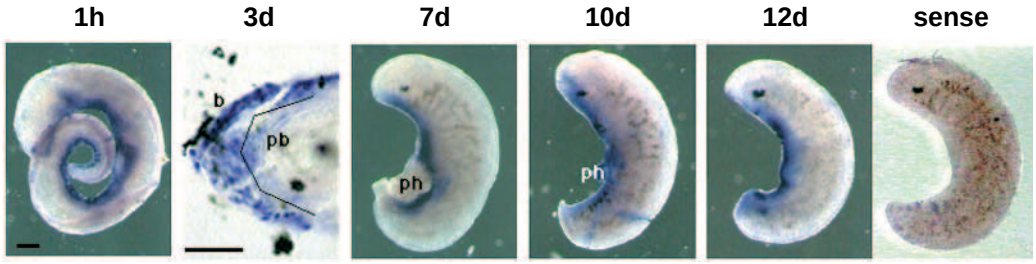

Fig. 6. *Dthox-F* expression at different stages: 1 hour (1h), 3 days (3d), 7 days (7d), 10 days (10d) and 12 days (12d) of lateral regeneration viewed from the dorsal side and visualized by whole-mount in situ hybridization. An early expression was observed at 1 hour of regeneration. The 3 days picture shows a sagittal paraffin section of a whole-mount hybridized organism with an even expression in the parenchyma of blastema (b) and postblastema (pb). The maximal expression came at 3-5 days and was maintained up to 12 days. 10 days laterally regenerating organisms where hybridized with a sense *Dthox-F* probe as a control. Scale bars 400 μm. Similar patterns of expression were observed with the other *Dthox* genes except with a clear differential deactivation.

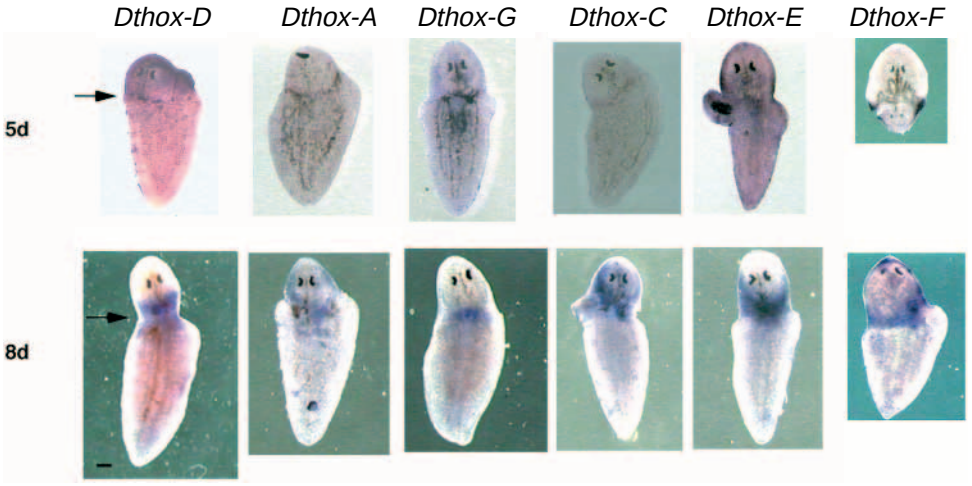

Fig. 7. *Dthox* gene expression at 5 days (5d) and 8 days (8d) of intercalary regeneration produced by planarian head-tail grafts (Saló and Baguñà, 1985b) viewed from the dorsal side and visualized by whole-mount in situ hybridization**.** As a control non-intercalary 8 days regenerating head-head grafts hybridized with *Dthox-F* can be observed in the first line right. The intercalary blastema between the two grafts is shown by arrowheads. Scale bar 400 μm. At 5 days of regeneration, there are no expression, it can be only observed a line of high density of pigmented cells accumulated along the edge between the two grafts due to the absence of wound healing. While, at 8 days of regeneration *Dthox-C* to *Dthox-G* shows clear expression in the intercalary blastema and the two postblastemas. No expression was observed in the control. The lateral expression present in some of the head grafts, even at 5 days, are due to the lateral regeneration produced by the transplantation protocol.

Table 1. Planarian Hox deactivation

	Dthox genes					
	Dthox-D	Dthox-A	Dthox-G	Dthox-C	Dthox-E	Dthox-F
Percentage of blastema* with signal above background at different days of regeneration.						
7 days	100%	100%	100%	100%	100%	100%
9 days	57%	100%	100%	100%	100%	100%
11 days	10%	75%	88%	65%	81%	100%
13 days	0%	40%	75%	50%	60%	100%
15 days	0%	0%	0%	0%	0%	80%

*Percent values for each group were calculated out of 15-20 anterior and posterior blastemas each. All the whole mounts were performed in the same experiment.

coelomates (1000 million years ago) (Zhang and Nei, 1996). No data are yet available about the genomic organization of these *Dthox* genes; the large number of repetitive DNA elements in the genome of this planarian (Garcia-Fernandez et al., 1993, 1995; Robertson, 1996) has prevented chromosome walking. The high number of orthologous planarian Hox genes suggests that the cluster organization should be maintained.

Whole-mount in situ hybridization of *Dthox* genes in planarian regeneration

Whole-mount in situ hybridization studies were performed to study the patterns of *Dthox* expression from the beginning (1 hour) to the end (15 days) of regeneration.

No expression was detected in non-regenerating adults by whole-mount in situ hybridization, but some *Dthox* cDNA fragments were amplified by RT-PCR, providing evidence for their expression in intact adults. Probably their low level of expression makes undetectable by whole-mount in situ hybridization.

The expression of *Dthox-D*, *Dthox-A*, *Dthox-C*, *Dthox-E*, *Dthox-G* and *Dthox-F* genes took place rapidly after cutting. At 1 hour of terminal regeneration, there was a high level of transcription, which was synchronous and spatially coincident in the postblastema (Fig. 3), rather than colinear (Duboule, 1994a, 1995). Paraffin sections of whole-mount in situ hybridized organisms showed an even expression of *Dthox* genes in all parenchymal cells of the blastema and postblastema (Fig. 4). Expression remained high over the next 3-5 days and expanded as the blastema grew (Fig. 4). In later stages of regeneration, when mitosis approached basal values and differentiation finished, expression decreased (10-15 days). The same pattern of expression was observed independently of the amputation level along the anteroposterior axis (Fig. 5). As controls for specific expression in whole-mount in situ hybridization, sense probes of all *Dthox* genes (Figs 3, 4) were utilized without any expression in the blastema.

Dthox genes ceased their expression at different times of regeneration independently of the level of amputation (Table 1), with a slight delay in lateral regeneration compared with terminal regeneration. We observe three periods of expression: a short one defined by *Dthox-D* with 9 or 11 days of expression in terminal and lateral regeneration, respectively; a medium one defined by *Dthox-A*, *Dthox-C*, *Dthox-E* and *Dthox-G* with 13 or 14 days; and the longest, defined by *Dthox-F* with almost 15 days in both types of regeneration.

Surprisingly, the spatial and temporal pattern of *Dthox* expression was identical in anterior (head) regeneration and posterior (tail) regeneration (Fig. 5). In both anterior and posterior blastemas, *Dthox* genes were activated equally and their expression was maintained similarly. At late stages of regeneration (up to 5 days) and independently of their anteroposterior position, the anterior blastemas were slightly larger than the posterior and showed broad *Dthox* expression throughout (Fig. 5).

Bilateral regeneration also showed an early expression of *Dthox* genes. After 1 hour of regeneration, there was a high level of transcription (Fig. 6), which was synchronous and spatially coincident in the lateral postblastema. The pattern of expression was similar to that observed in terminal regeneration and followed the same temporal pattern of each *Dthox* gene observed in terminal regeneration. Only a delay of 24-48 hours in their deactivation can be observed due to the larger process of lateral regeneration compared to the terminal one. Lateral blastemas showed a clear, continuous *Dthox* expression throughout their length, covering the whole anteroposterior axis. Paraffin sections confirmed this expression in all lateral blastema and postblastema cells (Fig. 6).

Intercalary regeneration produced by the juxtaposition of anterior and posterior terminal tissues (Saló and Baguñà, 1985b) induced extremely delayed but coincident *Dthox* expression, which was not detectable until 7-8 days of regeneration (Fig. 7). Grafted tissues with the same axial values do not induce intercalary blastema nor *Dthox* expression (Fig. 7). The expression of all *Dthox* genes in intercalary regeneration was roughly similar.

DISCUSSION

A novel mechanism of Hox regulation during regeneration

After wounding, regenerative tissue is quickly formed (Baguñá et al., 1994), accompanied by cell proliferation and *Dthox* expression. These processes are not necessarily related, since regions far from the wound show an increase in mitosis without *Dthox* expression and the blastema, which does not contain mitotic cells, expresses *Dthox* genes. A clear, synchronous expression was observed within the first hour of regeneration, in roughly the same strip of postblastema cells for *Dthox-D*, *Dthox-A*, *Dthox-C*, *Dthox-E*, *Dthox-G* and *Dthox-F* genes. This fast, synchronous *Dthox* expression during early regeneration

is apparently inconsistent with the general principle of temporal and spatial colinearity described in vertebrate development (Duboule, 1994a).

This early *Dthox* colocalization is maintained throughout the whole regenerative process, independently of the site of amputation. Sequential deactivation was observed in late regenerative stages, suggesting a new mechanism for *Dthox* regulation. These observations are consistent with descriptions of axolotl limb regeneration (Gardiner et al., 1995), where *HoxA13* and *HoxA9* expression are initally synchronous colocalized, whereas they later acquire different spatial domains of expression, similar to those observed in development. We suggest that regeneration is initiated by generalized activation of Hox gene expression, which produces new cells ready to acquire positional information, and, later, they sequentially switch off Hox expression. A model of differential Hox expression depending on the number of cell divisions in vertebrate development (Duboule, 1995) cannot be applied for *Dthox* deactivation processes in planarian regeneration since the regenerative cells (neoblasts) do not divide inside the blastema. An alternative model based on differential cellular memory of gene activation, as in the case of *omb* and *spalt* in *Drosophila* wing imaginal discs (Smith, 1996; Lecuit et al., 1996) can be considered. According to this model, the neoblasts in the postblastema region will activate *Dthox* expression and, as the regeneration proceeds, the neoblasts will become part of the blastema and will deactivate differentially their *Dthox* genes depending on their particular activating memory. We have no data regarding protein distribution, so this model does not consider possible post-transcriptional regulation. Nevertheless, transcriptional regulational has been shown to be an important factor in Hox gene activity (Pick et al., 1990; Duboule, 1994b).

Dthox genes do not distinguish head from tail during regeneration

The extreme regenerative capacity of planarians allowed us to analyze the expression of Hox-related genes during respecification along either anteroposterior or lateral body axes, which would be difficult to observe in development and regeneration of other systems. One of these is terminal regeneration: in both anterior and posterior regeneration, *Dthox* genes show an equal expression independently of the axial position of the section and the structure to be regenerated. This means that *Dthox* genes cannot distinguish head from tail during regeneration. This can be considered the first exception to the generally accepted model of Hox cluster genes respecting directionality of the body axis and questions the conserved function attributed to the Hox cluster genes in anteroposterior positional information (Slack et al., 1993).

Dthox genes during lateral regeneration

Lateral regeneration produced by sagittal cut of the whole planaria produces a long blastema throughout the entire anterior-posterior axis. This lateral regeneration allowed us to test the function of *Dthox* genes in new axis restitution. The unexpected homogeneous expression of *Dthox* genes along the anteroposterior blastema produced during lateral regeneration suggests three possible hypotheses related to the function of these genes in this primitive phylum: (1) that the ancestral function of Hox cluster genes was simply to specify positional information in any axis, whereas later in evolution, these genes became fixed to define anteroposterior position; (2) following the zootype hypothesis (Slack et al., 1993), the ancestral function of Hox cluster genes was to specify positional identity along the anteroposterior axis, whereas in the regenerative process they were co-opted to define any type of axis similarly to the way in which vertebrates re-use the Hox cluster genes to define the new axis of the limbs; or (3) that *Dthox* genes could not be related in axis respecification and are simply reflecting activation of cell proliferation and differentiation or, may reflect the 'opening' of the chromatin in totipotent cells (neoblasts) ready to acquire new positional values. We expect that this issue will be resolved by extending our present study to Platyhelminth development and by using more sensitive methods to detect *Dthox* expression in adult non-regenerating planarians (unpublished data).

Terminal versus intercalary regeneration

Intercalary regeneration is produced when surfaces of different positional value along the anteroposterior axis are opposed. In planarians, both pieces (stumps) contribute equally to the building of the regenerate (Saló and Baguñà, 1985b). This process prevents the epithelial-mesenchymal interaction produced in the other types of regeneration (terminal, bidirectional, lateral) associated with wound healing and fast, early *Dthox* expression, suggesting an active role of the epithelial-mesenchymal interaction in *Dthox* gene activation. In contrast, during intercalary regeneration, there is no epidermal wound healing since the two pieces fuse together with their parenchyma. Thus, instead of an epithelial-mesenchymal interaction there is a mesenchymal-mesenchymal interaction, perhaps leading to the delayed *Dthox* expression observed in this type of regeneration. Considering that some of the early responding genes in tissue induction of vertebrate appendage development and regeneration are *msx* genes (Davidson, 1995; Akimenko et al., 1995), we are planning to isolate the homologous genes from planaria and check their activation during these two types of regeneration (unpublished data).

Initiation of regeneration and determination of polarity

Our results from intercalary regeneration support the requirement of a discontinuity between tissues to initiate a regeneration process. The rapid induction and polarity determination of regenerative tissue in the healing region could be produced by a discontinuity of positional values between the healing region that defines the most distal positional values and the old tissue close to the wound that will become the source of the new blastema (postblastema) (Baguñá et al., 1994). Initiation of the regenerative process and its polarity would initially be determined by differential epidermal interaction throughout the old parenchyma (Chandebois, 1979) or by a differential parenchymal response depending on the position that the remaining uninjured polarized tissue originally occupied in the worm (Slack, 1980, 1982). This would define the most distal positional values that would be intercalated to the stump values by neoblast proliferation.

In conclusion, we have shown that *Dthox* expression bears not obvious relation to axial polarity during planarian regeneration and can be considered one of the first indications that question the generalized model of Hox genes respecting body axes.

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REFERENCES

Akam, M. (1995). Hox genes and the evolution of diverse body plans. *Phil. Trans. R. Soc. Lond. B* **349**, 313-319.

Akimenko, M. A., Johson, S. L., Westerfield, M. and Ekker, M. (1995). Differential induction of four *msx* homeobox genes during fin development and regeneration in zebrafish. *Development* **121**, 347-357.

Baguñà, J. (1981). Planarian neoblasts. *Nature* **290**, 14-15.

Baguñà, J., Saló, E., Romero, R., Garcia-Fernàndez, J., Bueno, D., Muñoz-Mármol, A. M., Bayascas-Ramírez, J. R. and Casali, A. (1994). Regeneration and pattern formation in planarians: cells, molecules and genes. *Zoological Science* **11**, 781-795.

Balavoine, G. and Telford, M. J. (1995). Identification of planarian homeobox sequences indicates the antiquity of most Hox/homeotic gene subclasses. *Proc. Natn. Acad. Sci. USA* **92**, 7227-7231.

Balavoine, G. (1996). Identification of members of several homeobox genes in a planarian using a ligation-mediated polymerase chain reaction technique. *Nucleic Acids Res.* **24** No. 8, 1547-1553.

Bartels, J. L., Murtha, M. T. and Duddle, F. H. (1993). Multiple Hox/HOM-class homeoboxes in Platyhelminthes. *Molecular Phylogenetics and Evolution* **2**, 143-151.

Brønsdted, H. V. (1969). *Planarian Regeneration*. Oxford: Pergamon Press.

Bürglin, T. R. (1994). A comprehensive classification of Homeobox genes. In *Guidebook to the Homeobox Genes* vol. (ed. D. Duboule), pp. Oxford, UK: Oxford University Press.

Bürglin, T. R. (1995). The evolution of homeobox genes. In *Biodiversity and Evolution* vol. (ed. R. Arai, M. Kato and Y. Doi), pp. Tokyo: The National Science Museum Foundation.

Chandebois, R. (1979). The dinamics of wound clousure and its role in the programing of planarian regenerationI. Blastema emergence. *Develop. Growth Different.* **21**, 195-204.

Davidson, D. (1995). The function and evolution of *Msx* genes: pointers and paradoxes. *Trends Genet.* **11** No. 10, 405-411.

Dick, M. H. and Buss, L. W. (1994). A PCR-based survey of homeobox genes in *Ctenodrilus serratus* (Annelida: Polychaeta). *Molecular Phylogenetics and Evolution* **3**, 146-158.

Duboule, D. and Dollé, P. (1989). The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. *EMBO J.* **8**, 1497-1505.

Duboule, D. (1994a). Temporal colinearity and the phylotipic progression: a basis for the stability of a vertebrate Bauplan and evolution of morphologies trough heterocrony. *Development* **1994 Supplement**, 135-142.

Duboule, D. (1994b). *Guidebook to the Homeobox Genes* vol. (ed. D. Duboule), pp. Oxford, UK: Oxford University Press.

Duboule, D. (1995). Vertebrate *Hox* genes and proliferation: an alternative pathway to homeosis? *Current Opinion in Genetics and Development* **5**, 525-528.

Garcia-Fernàndez, J., Marfany, G., Baguñà, J. and Saló, E. (1993). Infiltration of *mariner* elements. *Nature* **364**, 109-110.

Garcia-Fernàndez, J. and Holland, P. W. H. (1994). Archetypal organization of the amphioxus *Hox* gene cluster. *Nature* **370**, 563-566.

Garcia-Fernàndez, J., Bayascas-Ramírez, J. R., Marfany, G., Muñoz-Mármol, A. M., Casali, A., Baguñà, J. and Saló, E. (1995). High copy number of highly similar *mariner*-like transposons in planarian (Platyhelminthe): evidence for a trans-phyla horizontal transfer. *Molecular Biology and Evolution* **12**, 421-431.

Gardiner, D. M., Blumberg, B., Komine, Y. and Bryant, S. (1995). Regulation of *Hox-A* expression in developing and regenerating axolotl limbs. *Development* **121**, 1731-1741.

Holland, P. W. H. (1993). *Cloning Genes using the Polymerase Chain Reaction* (ed. Stern, C. D and Holland, P. W. H.) Oxford: Oxford University Press.

Kenyon, C. (1994). If birds can fly, why can´t we? Homeotic genes and evolution. *Cell* **78**, 175-180.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell* **78**, 181-189.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387-393.

Oliver, G., Vispo, M., Mailhos, A., Martinez, C., Fielitz, W. and Ehrlich, R. (1992). Homeobox-containing genes in the parasitic flatworm *Echinococcus granulosus*. *Gene* **121,** 337-342.

Philippe, H., Chenuil, A. and Adoutte, A. (1994). Can the Cambrian explosion be inferred through molecular phylogeny? *Development* **1994 Supplement**, 15-25.

Pick, L., Schier, A., Affolter, M., Schimdt-Glenewinkel, T. and Gehring, W. (1990). Analysis of the *ftz* upstream element: germ later-specific enhancers are independently autoregulated. *Genes Development* **4**, 1224-1239.

Robertson, H. M. (1996). Multiple *mariner* transposons in flatworms and hydras are related to those of insects. *J. Heredity* (in press).

Saló, E. and Baguñà, J. (1984). Regeneration and pattern formation in planarians I. The pattern of mitosis in anterior and posterior regeneration in *Dugesia (G) tigrina*, and a new proposal for blastema formation. *J. Embryol. Exp. Morph.* **83**, 63-80.

Saló, E. and Baguñà, J. (1985a). Cell movement in intact and regenerating planarians. Quantitation using chromosomal, nuclear and cytoplasmic markers. *J. Embryol. Exp. Morph.* **89**, 57-70.

Saló, E. and Baguñà, J. (1985b). Proximal and distal transformation during intercalary regeneration in the planarian *Dugesia(S)tigrina*. *Roux's Arch. Dev. Biol.* **194**, 364-368.

Saló, E. and Baguñà, J. (1989). Regeneration and pattern formation in planarians. II. Local origin and role of cell movements in blastema formation. *Development* **107**, 69-76.

Saló, E., Muñoz-Mármol, A. M., Bayascas-Ramirez, J. R., Garcia-Fernàndez, J., Miralles, A., Casali, A., Corominas, M. and Baguñà, J. (1995). The freshwater planarian *Dugesia (G) tigrina* contains a great diversity of homeobox genes. *Hydrobiologia* **305**, 269-275.

Schummer, M., Scheurlen, I., Schaller, C. and Galliot, B. (1992). HOM/HOX homeobox genes are present in hydra (*Chlorohydra viridissima*) and are differentially expressed during regeneration. *EMBO J.* **11**, 1815-1823.

Shankland, M., Martindale, M. Q., Nardelli-Haefliger, D., Baxter, E. and Price, D. J. (1991). Origin of segmental identity in the development of the leech nervous system. *Development* **1991 Supplement 2**, 29-38.

Shenk, M. A., Bode, H. R. and Steele, R. E. (1993a). Expression of *Cnox-2*, a HOM/HOX homeobox gene in hydra, is correlated with axial pattern formation. *Development* **117**, 657-667.

Slack, J. M. W. (1980). A serial threshold theory of regeneration. *J. Theor. Biol.* **82**, 105-140.

Slack, J. M. W. (1982). *Regeneration and the Second Anatomy of Animals*. (ed. D. Order) New York: Alan R. Liss, Inc.

Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993). The zootype and the phylotypic stage. *Nature* **361**, 490-492.

Smith, J. (1996). How to tell a cell where it is. *Nature* **381**, 367-368.

Tarabykin, V. S., Lukyanov, K. A., Potapov, V. K. and Lukyanov, S. A. (1995). Detection of planarian *Antennapedia*-like homeobox genes expressed during regeneration. *Gene* **158**, 197-202.

Webster, P. J. and Mansour, T. E. (1992). Conserved classes of homeodomains in *Schistosoma mansoni*, an early metazoan. *Mech. Dev.* **38**, 25-32.

Wong, V. Y., Aisemberg, G. O., Gan, W. and Macagno, E. R. (1995). The leech homeobox gene Lox-4 may determine segmental differentiation of identified neurons. *J. Neurosci.* **15**, 5551-5559.

Wysocka-Diller, J. W., Aisemberg, G. O., Baumgarten, M., Levine, M. and Macagno, E. R. (1989). Characterization of a homologue of bithorax-complex genes in the leech *Hirudo medicinalis*. *Nature* **341**, 760-763.

Zhang, J. and Nei, M. (1996). Evolution of Antennapedia-Class Homeobox genes. *Genetics* **142**, 295-303.

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