Planarian Hox genes: novel patterns of expression during regeneration

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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 developmental values in a wide range of metazoa, from nematodes to vertebrates (Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993) Nature 361, 490-492). 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

(Saló and Baguñà, 1984). This process does not require cell de-differentiation (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

Planimarians 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

Planimarians, 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’-GARYTNGARAARGATT-3’) and SO2 (5’CKNCKRTTYGTRAACCA-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 AZAP (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 digoxigenin-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 hybridization solution) was heated to 80°C for 2 minutes, diluted to 0.5 µg/ml in hybridization solution and added to samples for hybridization.

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, pharynx; w, wound.

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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 TW and then incubated for 1 hour in blocking solution (1% BSA blocking 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.

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 regenerable blastemas or libraries. Subsequent screening of genomic and cDNA libraries with the PCR-amplified sequences allowed us to isolate and characterize Dthox, a member of the Hox gene cluster, which is expressed in the planarian CNS and is orthologous to Antennapedia.

Fig. 2. Alignments between deduced homeodomain sequences of freshwater planarian Dthox genes and those with higher similarity encoded by mouse, amphibious, Drosophila cluster genes (Bürglin, 1994, 1995; García-Fernández et al., 1994) and polychaete annelid cDNA libraries with the PCR-amplified sequences allowed us to isolate and characterize Dthox, a member of the Hox gene cluster, which is expressed in the planarian CNS and is orthologous to Antennapedia.
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. 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.

**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 homogenous 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.
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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...
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 Baguñá, 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.

Table 1. Planarian Hox deactivation

<table>
<thead>
<tr>
<th>Dthox genes</th>
<th>Dthox-D</th>
<th>Dthox-A</th>
<th>Dthox-C</th>
<th>Dthox-E</th>
<th>Dthox-G</th>
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<td>Percentage of blastema* with signal above background at different days of regeneration.</td>
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<tr>
<td>7 days</td>
<td>100%</td>
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<td>9 days</td>
<td>90%</td>
<td>100%</td>
<td>100%</td>
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<td>100%</td>
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<tr>
<td>11 days</td>
<td>0%</td>
<td>50%</td>
<td>80%</td>
<td>65%</td>
<td>81%</td>
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<tr>
<td>13 days</td>
<td>0%</td>
<td>0%</td>
<td>50%</td>
<td>60%</td>
<td>100%</td>
</tr>
<tr>
<td>15 days</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>80%</td>
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*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.
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 initially 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 mnx 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) (Baguñà 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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