DISTOX GENES: A MILESTONE IN THE EVOLUTION OF HOX CLUSTERS?

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Developmental evolution

Many biologists are interested in finding common patterns on life. Their ultimate hope is to ascertain the basic rules that make living things alive. On the other hand, living things are different; hence, mechanisms that underlay evolutionary change may be envisaged to explain distinct patterns. To explain evolution, both points of view should be taken into account: what is maintained, and what is or may be changed. Here is where evolutionary and developmental biologists meet.

Embryonic development has been rarely considered as an evolutionary character, but developmental processes evolve just like other aspects of organisms. Furthermore, many embryogical studies have usually not recognised the potential contribution that evolutionary biology can make to the understanding of development. Only the integration of developmental and evolutionary biology can explain the mechanisms at the basis of the wide array of body plans that characterise and differentiate phyla.

One of the reasons for the estrangement between development and evolution may have been the difficulty of analysing embryogenesis in a broad range of animals. The most important findings in development have been achieved using very few "animal model systems". Many of these models were chosen in the last century or in the past decades by technical and, even, budgeting reasons. However, few animal species have been chosen by its evolutionary importance.

Research on development-evolution had its heyday in the late XIX- early XX century. In the last 10-15 years, the introduction of powerful molecular techniques, has reopened comparative approach to developmental processes. The interest of the evolution of development is now at the centre of research, when genes of divergent animal phyla are cloned and the functions for a large number of them seem to be conserved across great phylogenetic distances.

Hox genes and evolution of body plans

One of the gene families playing a central role in the race to understand the conservation of developmental processes is the homeobox family (Duboule, 1994a). Homeobox genes comprise a diverse multigenic family encoding transcription factors, many of which are key control genes in embryonic development (Bürglin, 1994). A subfamily of homeobox genes, the HOX genes, is particularly interesting from an evolutionary perspective: most metazoan have Hox genes. Organisation into gene clusters is a characteristic feature of Hox genes. Insects and nematodes posses a single Hox gene cluster; in mammals, there are (at least) 38 Hox genes in four clusters, derived by duplication from a single cluster present in the closest relative to the vertebrates (Garcia-Fernàndez & Holland, 1994). A colinear relationship between chromosomal position, activation time, and anterior expression limit of *Hox* genes suggest that clustering may be important for precise spatio-temporal gene regulation and consequently, embryonic patterning (Duboule 1994b, van der Hoeven et al., 1996).

Sometime in metazoan evolution, an ancestral Hox gene was tandemly duplicated, originating the most primitive Hox cluster. The aim of our work is to analyse the evolution of Hox clusters in the lower metazoans. More precisely, we wish to analyse the presence, genomic organisation, and function of Hox genes in an important point during metazoan evolution: if the primordial function of the Hox cluster was to define regionalisation along the antero-posterior body axis, then the timing of piecing together Hox genes to work co-ordinately in defining anterior-positerior axis would coincide with the evolutionary origin of the oldest metazoan with clear antero-posterior polarity.

Polyclad, an archetypal flatworm?

Following this reasoning, we have decided to analyse Hox genes in the most primitive phyla with clear cephalization, bilateral symmetry, and antero-posterior polarity: the Phylum Platyhelmintes. Several species of this phylum have been analysed in search for Hox genes (Oliver et al.,1992, Bartels et al. 1993, Saló et al.,1995). The most extensive analysis has been done in the freshwater planarian *Dugesia tigrina* (Tricladida), with the isolation of several Hox genes (Bayascas et al. 1996). Shortly, actual data on Hox genes in flatworms is based mostly in Tricladida and parasitic flatworms. Both have a derived embryonic development; thus, they may not be a good representative of the "archetype" Platyhelmint. (Baguñà & Boyer, 1990)

Taking into account the above discussion, we have chosen an animal that may be a good biological model for Platyhelmint development. The species chosen was *Discocelis tigrina*. *Discocelis* (Cl. Turbellaria, O. Polycladida) are marine flatworms, hermaphroditic, with cross-sexual reproduction, and characterised for laying hundreds of endolecithic eggs. The initial development of *Discocelis* is of the quartet spiral type, with equal clevage (Kato 1939). This type of development is considered archetypal among the Spiralia, placing *Discocelis* in a good position of ancestrallity. One major difference among polyclads is that *Discocelis* does not pass through a larval stage, developing directly from the egg to a miniature adult, with all axes and structures perfectly developed. While some authors consider the presence of a larval stage an indicator of primivitivity (Baguñà & Boyer 1990, Boyer 1995), as Hox genes are expressed late in development, and have clear roles in antero-posterior patterning, we thought that an animal with direct development would give more hints for studying the evolution of Hox clusters.

A final evidence that helped us in choosing *Discocelis* as a model is based in molecular phylogenetic studies. Polyclads are not fast clocks in terms of sequence evolution. Ribosomal 18S sequence data shows that policlads are among the most primitive flatworms (Carranza, Riutort and Baguñà, personal comunication). Hence, in terms of phylogeny, policlad worms may be a good model system for studying the evolution of development

Discocelis HOX PCR survey

For an initial survey of Hox genes in *Discocelis tigrina* we have used PCR for amplification from genomic DNA with "universal" Hox primers. These primers (SO1, 5'-GARYTNGARAARGARTT-3' and SO2, 5'-CKNCKRTTYTGRAACCA-3', corresponding to homeodomain residues 15-20 and 48-53) should amplify most Hox genes, giving a heterogeneous band of 117 bp from which 27 informative aminoacid can be obtained from each single clone. Using such approach, and adapting the PCR conditions we sequenced 43 Hox fragments, corresponding to five different genes, named Distox-A, B, D, E, and F (figure 1).

Sequence comparison allows the genes to be ascribed to the particular Hox paralogous groups as shown in figure 1. Although the survey is not exhaustive, and the short sequence could lead to misasignments, several conclusions can be drawn:

1) A broad variation of Hox genes may be present in *Discocelis*, suggesting a rather complex or complete Hox cluster. Data from other flatworms (Balavoine and Telford, 1995, Bayascas et al., 1996) points to the existence of 6 to 8 Hox genes in the phylum. Thus, a limited survey (only 43 clones sequenced) gave us a good selection of Hox genes. The clone corresponding to Distox-A was by far the most amplified; this may be due to the high C/G content of this gene (92 %), that may be favoured in the annealing step of the PCR reaction. This biased amplification may preclude the amplification of the other genes, suggesting that other Hox genes do exist in *Discocelis*, and were not amplified in this initial survey.

2) Only a single member of a given paralogous group has been amplified. This is a good indication that *Discocelis*, if any, has only one Hox cluster; in other words, that not secondary duplication (e.g., by tetraploidization) took place in the *Discocelis* lineage.

3) Even from short sequences, clear paralogies may be established. Surveys in other animals revealed a lot of uncertainty (Bartles et al.,1993, Balavoine and Telford, 1995, Saló et al.,1995) and even misasignments in the ascriptions. These data together with the very high similarity to some *Drosophila* and vertebrate (represented by amphioxus) Hox genes (fig. 1), suggest that *Discocelis* may have "canonical" Hox genes, that is, with very low secondary divergence in the lineage leading to its particular corner of evolution. The argument extends to the primer sequences: amplification with SO1 and SO2 implies that the particular residues encompassing these primers are present in the genes amplified. In other animals (DiGregorio et al.1994), amplification was precluded due to sequence divergence in the primer area, and the full homeobox sequences showed a overall sequence divergence.

4) Five Hox genes have been amplified from genomic DNA. The situation of the primers reveals that there are not introns in this area in any of the *Discocelis* Hox genes. Introns in the homeobox may interfere some way with the high level structure and function of the Hox complex. Moreover, clusters with none or few temporal colinearity may be more permissive to disorganisation of the complex. If introns in the homeobox are a "disturbing" element of Hox clusters for the colinear regulation, then the lack of introns in *Discocelis* points to a "canonical" Hox cluster. In fact, canonical clusters, as the amphioxus or mammal, seem to have few or none introns in the homeobox, while less canonical complexes (very big, fragmented, with repetitive elements, extra non-Hox genes, and with high sequence divergence) often have introns in the homeobox (e.g., Drosophila, ascidians, other flatworms).

	Distox A	Distox B	Distox D	Distox E	DistoxF
Hox Paralogous group	Hox 1 lab	Hox 2 pb	Hox 4 Dfd	Hox 5 Scr	Hox 6-8 Ubx/AbdA
number of clones	22	1	6	3	11
% identity to *Drosophila*	81	100	74	67	78
% identity to *Apnhioxus*	78	70	85	63	74

Figure 1. PCR survey of Dicocelis tigrina Hox genes, using degenerate primers corresponding to the most conserved region of the Hox-class homeodomain. An heterogeneus PCR band was cloned, and several clones sequenced (number of clones for each genes are inicated below the gene names). Percent identity refer to the Discocelis sequence amplified (positions 21 to 47 of the homeodomain) compared to the amphioxus (as representative of vertebrates) and Drosophila Hox genes.

Discussion

We report a preliminary data focusing in the origin and evolution of HOX clusters in lower metazoans. We discuss the reasons underlying the election of *Discocelis tigrina* as a model for the lowest bilateral animals. We believe that the phylogenetic position and apparent archetypal development, turn *Discocelis* into a good representative of a key group in the evolution of metazoan. The insights that Distox and other genes, in terms of sequence conservation, gene duplications, genomic organisation, and expression, may give into the mechanisms of developmental evolution are still a black box, but the initial data based solely in a PCR survey, are encouraging, and several characteristics of the clones point to the existence of a canonical HOX cluster in *Discocelis*. We are currently screening new genomic libraries looking for a possible Hox genomic linkage that has not been reported below the nematodes. On the other hand, the machinery for studying the expression of Distox genes is on the way, with laboratory facilities to obtain eggs and embryos, that will be used for in situ hybridization experiments.

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