AMPHIOXUS HOX GENES: INSIGHTS INTO EVOLUTION AND DEVELOPMENT

Jordi GARCIA-FERNÀNDEZ[1] and Peter W. H. HOLLAND[2]
[1]*Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08071 Barcelona, Spain*
[2] *School of Animal and Microbial Sciences, The University of Reading, Whiteknights, RG6 6AJ, United Kingdom*

The homeobox in developmental evolution

A dozen of years ago, the discovery of vertebrate HOX genes related to the homeotic selector genes of *Drosophila* (HOM) caused great excitement to developmental biologists, raising hopes for finding common patterning mechanisms in diverse animal species. The homeobox, a 180 bp DNA sequence that codes for an helix-turn-helix DNA-binding domain, the homeodomain, was even thought to be the "Rosetta stone" of Developmental Biology (Slack, 1984). After twelve years of intense search, homeodomain proteins have been found in most eukaryotic organisms. The role of several homeobox subfamilies have been widely conserved through evolution, but there are detailed differences in homeobox gene number, genomic organisation, and gene expression between taxa. It has been proposed that changes in homeobox gene numbers and their expression have been at the base of body plan evolution (Holland and Garcia-Fernàndez, 1996)

A subfamily of homeobox genes, the HOX genes, is particularly interesting from an evolutionary perspective. Current evidence suggests that all, or almost all, multicellular animals possess Hox genes organised into one or more chromosomal clusters. Data in low invertebrates on number, type, and genomic organisation of Hox genes, however, is still fragmentary (e.g., Bayascas et al., 1996), precluding a prediction of the precise evolutionary time for the origin of the HOX cluster.

A colinear relationship between chromosomal position, activation time, and anterior expression limit of vertebrate *Hox* genes suggest that clustering may be important for precise spatio-temporal gene regulation and hence, embryonic patterning (Duboule, 1994). The regional expression of *Hox* genes at specific stages of development constitutes a major component of the "zootype", a developmental genetic character thought to be shared by all (or most) ánimal taxa (Slack et al., 1993).

The conservation in developmental control genes and their functions between divergent taxa is fascinating, and have captured the imagination of developmental biologists world-wide. This conservation points to the existence of very ancient, conserved, and not easily modifiable mechanisms for controlling body patterning in animals. However, more attention should be paid to the divergence between developmental control genes and mechanisms in different taxa, and their possible significance. In particular, we are interested in analysing the genetic changes that may have permitted innovations in body plan evolution. Gene duplication, followed by functional divergence, may be one class of mutations that permits major evolutionary changes.

The origin of vertebrates

The phylum Chordata (Vertebrata, Cephalochordata and Tunicata) provides some excellent examples for studying the evolution of embryonic development. All members of the phylum possess a notochord, a dorsal nerve cord, and segmented mesoderm. However, vertebrates differ from their closest relatives in having an elaborate craniofacial region, a clearly tripartite brain, paired sense organs, and extensive tissues derived from migratory neural crest cells. These differences seem to represent major evolutionary innovations, hence their origin may have required the evolution of new developmental programs in the ancestors of the vertebrates.

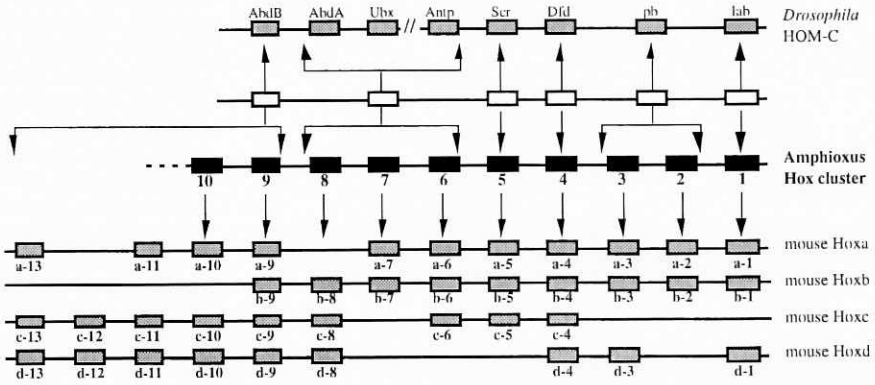

Figure 1. Proposed evolutionary relationships between mammalian, Drosophila, and amphioxus Hox gene clusters. The single amphioxus Hox cluster has an archetypal organisation in relation to vertebrate clusters. Reproduced from Garcia-Fernàndez and Holland (1994), with permission from *Nature*, copyright 1994, Macmillan Magazines Ltd.

The lancelet (amphioxus) belongs to the subphylum Cephalochordata, and historically occupied an important place in discussions of the origin of the vertebrates, generating a voluminous literature in the 19th century.

In the 20th century, however, amphioxus received little attention until very recently , when advances in molecular biology recalled amphioxus as a key milestone in vertebrate evolution. Amphioxus (Cephalochordata) are thought to be the closest living relative to the vertebrates: comparative embryology and anatomy suggest that they are living descendants from a critical intermediate stage in vertebrate evolution. Thus, amphioxus may be, morphologically and anatomically, an archetype of the vertebrates. Furthermore, the emerging consensus from molecular phylogeny reveals the Cephalocordata as the sister group of vertebrates (Wada and Satoh, 1994).

Amphioxus Hox genes

Drosophila/vertebrate comparison of HOX clusters predicted that amphioxus would possess several Hox genes, clustered in the genome, and playing roles in regionalisation of the body plan during embryogenesis. In order to investigate the origin of the four vertebrate HOX clusters and the paralogous groups, we analysed the complexity and organisation of Hox genes in the amphioxus *Branchiostoma floridae*. Using the polymerase chain reaction (PCR), we cloned short fragments (112 bp) from nine amphioxus Hox genes (Garcia-Fernàndez & Holland, 1994). These PCR clones analyses were difficult to interpret, but let to our tentaive prediction of a single HOX gene cluster. Using the same techniques, other laboratory predicted two clusters (Pendleton et al. 1993). The

discrepancy was subsequently resolved by exhaustive genomic and cDNA screening, followed by genomic walking. This conclusively demonstrated that the amphioxus genome has a single HOX gene cluster (Garcia-Fernàndez & Holland, 1994). Thus, chromosome walking, although time consuming, and sometimes boring, was far more informative and conclusive than PCR. We finally demonstrated that the single HOX cluster of amphioxus contained at least 10 Hox genes (*AmphiHox-1* the most 3' or downstream gene, to *AmphiHox-10* the most 5' or upstream) in an array spanning 270 kb. Gene numbers relate to the position in the cluster of a given gene, but most importantly, they reflect the relationships between amphioxus and vertebrate Hox genes. Each amphioxus Hox gene could be assigned to a particular vertebrate paralogous group (see figure 1). For example, *Amphihox-1* is most similar to the three vertebrate Hox genes belonging to the paralogy group 1 (Hoxa-1. b-1 and d-1). One important implication of these similarities is that Hox cluster duplication in vertebrates must have occurred soon after the divergence of the amphioxus lineage, this is, just at the origin of vertebrates. Recent data from other taxa (reviewed in Holland and Garcia-Fernàndez, 1996) also supports that HOX cluster duplication took place close to the vertebrate origins

The amphioxus *Hox* gene cluster has an unprecedented organisation of particular interest from an evolutionary perspective. One of the most intriguing features of the amphioxus *Hox* cluster was the similarity between its organisation and that inferred for the last ancestor of the vertebrates to posses a single *Hox* cluster. The amphioxus genome has apparently retained the ancestral and archetypal pre-vertebrate, pre-duplication, HOX cluster organisation. The evolutionary relationships between the HOX genes clusters of mammals (mouse), insects (*Drosophila*), and amphioxus is shown in figure 1.

Tail flexibility

The above conclusions are based in the analyses of the amphioxus Hox cluster genes 1 to 10. However, the most 5' genes (the "tail" genes) in amphioxus were not analysed in previous works, due to the sequence divergence of the mammals paralogous groups 11 to 13. Recently, we have isolated two additional amphioxus Hox genes, *AmphiHox-11* and *AmphiHox-12*, linked to the 5' end of the published AmphiHOX cluster (unpublished data). In this case, the number reflects the position in the cluster, but not a clear relationship to mammal groups 11 or 12. This "terminal variability", opposed to the "anterior constraint" has been referred as "laxitas terminalis" by other authors, analysing the 5' end of the zebra fish clusters (van der Hoeven et al., 1996). However, we prefer to introduce the expression **"tail flexibility"** to explain the evolutionary permissiveness at the posterior end of the cluster, and of the body plan. In contrast, the 3´end, or the "head" end, of the Hox cluster is more restrictive to evolutionary changes.

Gene duplication at the origin of vertebrates

Hox genes in mouse and human form part of more extensive paralogy groups of related linked genes in different chromosomes. Thus, *Hox* cluster duplication coincided with the enlargement of several gene families. Data currently available in amphioxus (reviewed in Holland et al., 1994, Holland and Garcia-Fernàndez, 1996, Sharman and Holland, 1996) point to the same picture as *Hox* genes: single members of other vertebrate multigenic families are present in the amphioxus genome (see Holland, 1996 for a compilation of all amphioxus genes cloned). Hence, after divergence of the lineage leading to the amphioxus and the vertebrates, duplication of homeobox and other genes in the vertebrate lineage took place. Accumulating data on amphioxus and lower vertebrates, together with the presence of extensive paralogy groups within mammalian genomes, strongly suggest that a very extensive phase of gene duplication occurred close to vertebrate origins (Holland and Garcia-Fernàndez, 1996). Recent evidence and phylogenetic analyses of several gene families, analyzed by Sharman and Holland (1996), lead to the conclusion that two major phases of gene duplication took place close to vertebrate origins (figure 2): phase 1 occurred just at the origin of vertebrate, after the divergence of chepalochordates. This phase did not imply a tretaploidization of the genome, but many different genes became duplicated. Phase 2 occurred close to the gnathostome ("higher vertebrates") origin, this second event may have involved a full tetraploidization of the genome. The generation of these new genes may have played a vital role in vertebrate origins: redundant duplicated genes were subsequently permissive to phenotypic change, able to diverge and be recruited for new roles, becoming responsible for developmental innovations restricted to the vertebrate lineage.

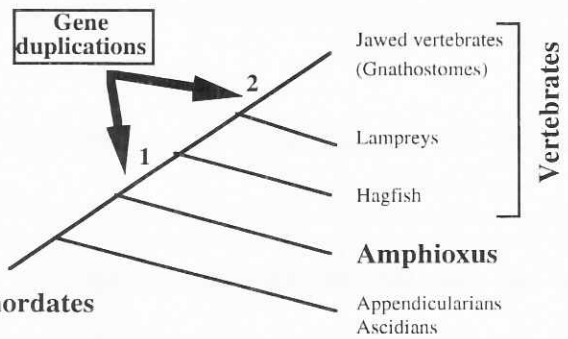

Figure 2 Phylogenetic relationships between chordates showing the timing of the proposed gene duplications (1 and 2). Modified from Holland et al., 1994, and Sharman and Holland, 1996.

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