ACADEMIC PRESS

Molecular Phylogenetics and Evolution 24 (2002) 374–378

MOLECULAR PHYLOGENETICS AND EVOLUTION

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Is the evolution of *Cnox*-2 Hox/ParaHox genes "multicolored" and "polygenealogical?"

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Received 26 November 2001

Abstract

Understanding the evolution of metazoan bauplans is linked to understanding the evolution of Hox and ParaHox genes. At the base of metazoan radiation we see in both cases a quite confusing picture yet. Here *Cnox*-2 is one of the best studied diploblast Hox genes. Homologs of this gene are known from Placozoa and several Cnidaria. In those cases where full length gene sequences, or at least full length homeobox sequences, are available the relationship to Hox genes from triploblastic animals as well as the classification to Hox or ParaHox genes can be controversially discussed. The existing data on possible gene functions also reveal a quite heterogeneous picture. It seems conceivable that part of the "multicolored" picture relates to a "polygenealogical" origin of the *Cnox*-2 gene. © 2002 Elsevier Science (USA). All rights reserved.

Hox genes are a key tool for unraveling the roots and pathways of the metazoan radiation on a macroevolutionary scale (Plaza et al., 2001). The possession of these key regulatory genes is believed to be a synapomorphy for the Metazoa, and the role of Hox genes in axis formation is an important aspect in the development of the overall pattern organizing the metazoan embryo (Ferrier and Holland, 2001; Ferrier and Holland; Martindale et al., this volume). The discovery of Hox genes in cnidarians (Schierwater et al., 1991), one of the most basal metazoan groups, has sparked intriguing discussions on the origin of the anterior–posterior body axis in metazoans (cf. Galliot, 2000). Up to five Hox genes have been found in a single cnidarian (Finnerty and Martindale, 1999; Kuhn et al., 1996, 1999). Comparative sequence analyses and expression studies have subsequently fueled the formulation of a number of conflicting hypotheses concerning the role of Hox genes in axis formation (for review see Galliot, 2000; Schierwater and DeSalle, 2001). One reason for these conflicting views is due to the sparse sampling of taxa or full length gene sequences used to generate the comparative data. Another reason is the different—and to some degree subjective—evaluation and interpretation of characters available for comparative analyses.

Commonly the approach taken is to compare sequence similarity and to assign homology of putative Hox genes with sufficient similarity. Likewise, similar expression patterns of Hox genes assigned to the same homology group are interpreted as the result of conserved function for the development of homologous developmental patterns or body parts. This approach may lead to a *circulus vitiosus* (circular reasoning) and the misinterpretation of homology. As an example, we cite the case of *Cnox*-2, which has been suggested to have homologs in the genomes of Placozoa, Anthozoa, Hydrozoa, Scyphozoa, and Ctenophora (for refs. see: Galliot, 2000; Schierwater and DeSalle, 2001). In addition, in the opinion of many researchers, homologs of *Cnox*-2 also exist in the genomes of all triploblastic animals and here the putative homolog is called Gsx. *Cnox*-2 would then belong to the ParaHox gene family (Ferrier and Holland, 2001). Conflicting views on the homology assignment of *Cnox*-2 and Gsx illustrate the non-triviality of assessing homology based on sequence data analyses. Ongoing attempts to unravel the cellular

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PII: S1055-7903(02)00238-5

and molecular functions of *Cnox*-2 genes in diploblastic animals will clearly add to this discussion, but are themselves prone to the *circulus vitiosus* problem in the process of assigning homology of these genes and their subsequent morphological manifestation.

1. Current approaches

At present, the most widespread approach to clarifying the role of Hox genes in animal evolution follows these three assumptions (a) Hox gene evolution correlates with body plan innovations in metazoan animals, (b) cnidarians/diploblasts—because of their basal position in animal phylogeny—represent a key group for understanding Hox gene evolution, and (c) both Hox and ParaHox genes are present in diploblastic animals (Ferrier and Holland, 2001).

To examine the validity of these assumptions we need to (1) characterize a sufficient number of Hox genes from a sufficient number of taxa; (2) unravel phylogenetic relationships between animal phyla; and (3) reliably assign gene homologies (or orthologies in case of intragenomic duplications). The first point boils down to a more or less straightforward work of isolating Hox genes from a larger number and broader phylogenetic representation of the tree of life. A fair amount of this work has already been done with respect to this goal (Galliot, 2000). The second point, the inference of phylogenetic relationships in our opinion eagerly waits for developmental data to be included in character analyses (Bang et al., 2000). The third point, to assign homology to Hox genes, while seemingly simple, is not. We suggest that in the case of diploblast/triploblast Hox genes the assignment of homology is not necessarily straightforward as evidenced by the *Cnox*-2 gene example.

Among all Hox or ParaHox genes from diploblastic animals, *Cnox*-2 is the gene for which we have the most information (Cartwright et al., 1999; Cartwright and Buss, 1999; Finnerty and Martindale, 1999; Gauchat et al., 2000; Hayward et al., 2001; Kuhn et al., 1996; Kuhn et al., 1999; Masuda-Nakagawa et al., 2000; Schierwater et al., 1991; Schummer et al., 1992; Shenk et al., 1993a; Shenk et al., 1993b; Yanze et al., 2001). One way to assess homology of a *Cnox*-2 with other Hox genes is to look at the sequence similarity (and apply the "specific quality/structure criterion"), compare the expression patterns ("function criterion"), and compare the genomic organization of the gene ("anatomical environment") criterion (Schierwater and Kuhn, 1998). These comparisons can be used to assess the initial quality of the homology of *Cnox*-2 and other Hox genes and phylogenetic affinity of the genes then becomes the final arbiter of homology assessment (cf. Sarkar et al., this volume). We have some data to start comparing sequences and expression patterns within the diploblastic animals. If we assume that *Cnox*-2 is a Gsx (ParaHox gene) homolog we can extend the comparisons to metazoans in general.

2. *Cnox*-2 gene sequence and gene structure

Sequence similarity within the homeobox/homeodomain has been used as a straightforward arbiter of homology between Hox sequences from different diploblasts (cf. Schierwater and Kuhn, 1998). Furthermore, the gross *Cnox-2* genomic intron/exon structure also seems to be similar in comparisons among different organisms. Data from three hydrozoans (*Eleutheria dichotoma*, *Hydractinia echinata*, and *Tubularia spec.*), two scyphozoans (*Cassiopeia xamachana* and *Aurelia spec.*), and the placozoan *Trichoplax adhaerens* reveal that the *Cnox*-2 gene harbors a single short intron flanked by two short exons (Fig. 1A; Ender and Schierwater, unpublished data). Thus, the gross genomic intron/exon organization of *Cnox*-2 genes seems to be conserved. However, the size of the intron and exon-1 regions, which ranges from 172 to 490 bp and 379 to 537 bp, respectively, does not readily support homology for the 5′ region of the gene. Neither does the DNA sequence similarity in these regions readily suggest the assignment of homology if different classes or phyla are compared. For example, sequence similarity between the placozoan *Trox*-2, the scyphozoan *Scox*-2, and the hydrozoan *Cnox*-2 declines to less than 35% in the intron and appears to be random in the exon-1 region. On the other hand, within the Hydrozoa, exon-1 sequences seem to be conserved; Fig. 1B. One can think of two mutually exclusive alternatives for this observation: (a) the whole gene has evolved as a unit, i.e., all regions of the gene are homologous between classes and phyla, but relaxed selection pressures within the 5′ region have allowed sequences to diverge to a level approximating random similarity; (b) the 5′ regions of the *Cnox*-2 gene from hydrozoans is not homologous to the corresponding region from the scyphozoan and placozoan *Cnox*-2 genes. In the latter case the *Cnox*-2 gene would have evolved as a genetic chimera harboring modules from different genes and consequently the evolution of *Cnox*-2 genes would be "polygenealogical".

3. Expression and functional studies

Expression studies of Hox genes in diploblastic animals are very limited. If we look at the sparse expression data available, we find more of a "multicolored picture" of the role of these genes in development. For example, Hox gene expression may be oral or aboral, and may be ectodermal or entodermal, depending on the species and developmental stage (Fig. 2): All but aboral ectodermal

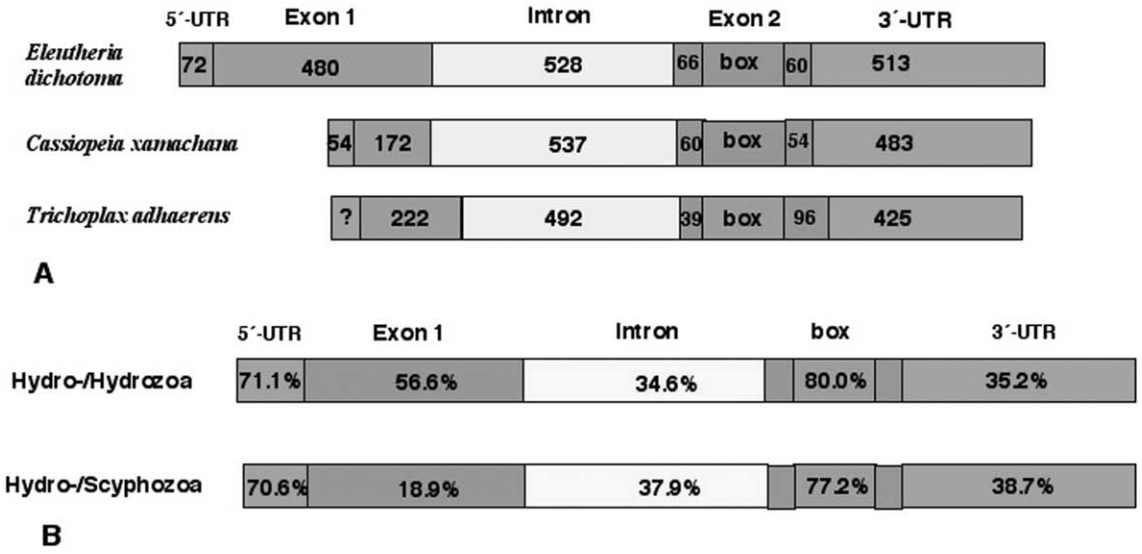

Fig. 1. Genomic structure of the diploblast *Cnox*-2 gene. (A) The principal organization with one short intron separating two short exons is identical in Hydrozoa (here shown for *Eleutheria dichotoma*), Scyphozoa (here shown for *Cassiopeia xamachana*), and Placozoa (*Trichoplax adhaerens*). Striking differences, however, are found in the length of exon-1, which differs dramatically between classes and phyla. (B) DNA sequences within the exon-2 region are conserved within different hydrozoans (here shown for *Eleutheria* and *Tubularia*) but decline to a level of randomness if representatives of different cnidarian classes (here for *Eleutheria* and *Cassiopeia)* are compared (Ender and Schierwater, unpublished data).

expression has been seen in embryos of the anthozoan *Acropora* (Hayward et al., 2001). In contrast, aboral or anterior entodermal expression has been found in two-day-old embryos and planula larvae of the hydroid *Podocoryne* (Yanze et al., 2001). In two other hydroids, *Hydractinia* and *Hydra*, overall but oral ectodermal expression patterns have been seen in adult polyps or regenerates (no information is available on embryonic or larval stages; Cartwright et al., 1999; Cartwright and Buss, 1999; Gauchat et al., 2000). Although some kind of role in axis formation is generally discussed, the paucity of data at present hardly allows any general conclusions on the developmental roles of *Cnox*-2 in cnidaria. One may or may not assume that similarities in the differentiation of the embryonic anterior entoderm—so far found in *Podocoryne* larvae only—resembles a conserved function between the diploblast *Cnox*-2 and the triploblast Gsx gene. One may as well assume (i) that *Cnox*-2 genes are involved in a diversity of developmental programs throughout all stages of ontogeny, (ii)

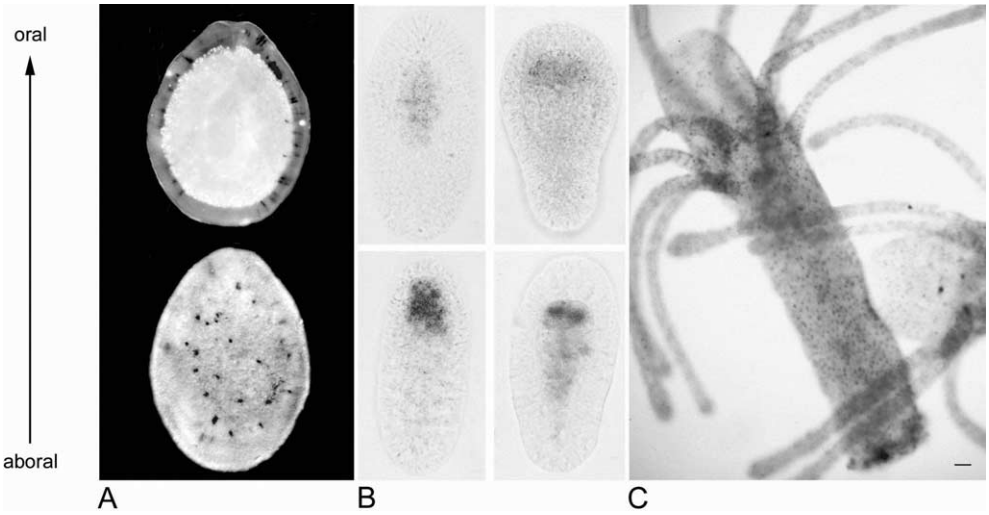

Fig. 2. Examples for *Cnox*-2 expression patterns in the anthozoan *Acropora* (A), and in two hydrozoans, *Podocoryne* (B) and *Hydractinia* (C). The anthozoan embryos show all but aboral ectodermal expression (Hayward et al., 2001, modified), while the hydrozoan embryo mainly shows aboral or anterior entodermal expression (Yanze et al., 2001, modified). In adult hydrozoan polyps ectodermal expression throughout the body column but with low expression in the head region is found (here shown for feeding polyps of *Hydractinia*; Cartwright et al., 1999, modified).

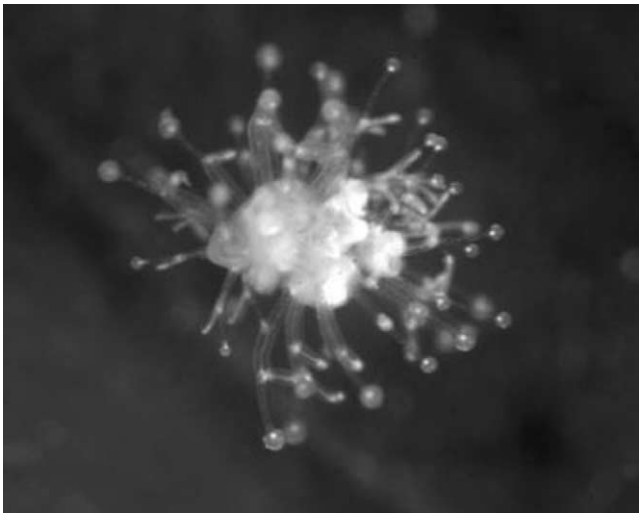

Fig. 3. Multiple oral pole phenoptype of vegetative medusae of the hydrozoan *Eleutheria dichotoma* as the result of inhibition of the *Cnox-2* gene by antisense oligos (Grimm and Jakob, unpublished data).

that the developmental roles of these genes are different in distant taxa, and (iii) that changes of function of these genes have occurred throughout the history of animal evolution.

No studies have yet been published on approaches for exploring gene function by means of gene inhibition via antisense oligos or RNAi. Unpublished data from one of our labs (B.S.; Grimm and Jakob, personal communication) suggest multiple functions of the *Cnox*-2 gene in the hydrozoan *E. dichotoma*. In these experiments, RNAi treatment results in quite striking features including additional bifurcations of the tentacles and the formation of multiple oral–aboral body axes in vegetatively reproducing (budding) medusae. *Cnox*-2 antisense oligos regularly produce phenotypes in which the daughter medusae are not released from the parent, resulting in individuals with multiple oral poles and tentacle rings (Fig. 3). Only among other interpretations one may interpret these findings as a hint that *Cnox*-2 is involved in axis formation in *E. dichotoma*. Finally it should be noted that if *Cnox*-2 was shown to be functionally involved in axis formation, the question would still remain open as to whether the cnidarian oral–aboral axis is homologous to the a–p axis of triploblastic animals.

4. Conclusions

The ''polygenealogical'' nature of the different *Cnox*-2 gene regions in different phyla and the ''multicolored'' pictures of *Cnox*-2 expression/function within the cnidaria do not readily support the hypothesis of a simple evolution by descent of this gene in the basal diploblasts and the more derived triploblasts. Yet the limited data do equally well support the idea that *Cnox*-2 evolution has been chimaeric and combines modules from different genes.

Acknowledgments

We acknowledge support from the Deutsche Forschungsgemeinschaft (DFB Schi 277/10-2) and the Human Frontier Science Program Organization (HFSP RGP0221/2001/-M).

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