Evolution of Antp-class genes and differential expression of *Hydra Hox/paraHox* genes in anterior patterning

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The conservation of developmental functions exerted by Antp-class homeoproteins in protostomes and deuterostomes suggested that homologs with related functions are present in diploblastic animals. Our phylogenetic analyses showed that Antp-class homeodomains belong either to non-Hox or to Hox/paraHox families. Among the 13 non-Hox families, 9 have diploblastic homologs, *Msx*, *Emx*, *Barx*, *Evx*, *Tlx*, *NK-2*, and *Prh/Hex*, *Not*, and *Dlx*, reported here. Among the Hox/paraHox, poriferan sequences were not found, and the cnidarian sequences formed at least five distinct cnox families. Two are significantly related to the paraHox Gsx (cnox-2) and the mox (cnox-5) sequences, whereas three display some relatedness to the Hox paralog groups 1 (cnox-1), 9/10 (cnox-3) and the paraHox cdx (cnox-4). Intermediate *Hox/paraHox* genes (PG 3 to 8 and *lox*) did not have clear cnidarian counterparts. In *Hydra*, *cnox-1*, *cnox-2*, and *cnox-3* were not found chromosomally linked within a 150-kb range and displayed specific expression patterns in the adult head. During regeneration, *cnox-1* was expressed as an early gene whatever the polarity, whereas *cnox-2* was up-regulated later during head but not foot regeneration. Finally, *cnox-3* expression was reestablished in the adult head once it was fully formed. These results suggest that the *Hydra* genes related to anterior *Hox/paraHox* genes are involved at different stages of apical differentiation. However, the positional information defining the oral/aboral axis in *Hydra* cannot be correlated strictly to that characterizing the anterior–posterior axis in vertebrates or arthropods.

The discovery of structural and functional homologies between regulatory genes used by *Drosophila* and vertebrates during their development led to the hypothesis that animals would share a common set of genes for defining the head, trunk, and posterior regions at early developmental stages (1–6). The proposed genes were homeobox genes belonging either to the Antp class, like *empty-spiracle* (*emx*), *even-skipped* (*evx*), *Hox* genes, or to the Prd class, like *orthodenticle* (*Otx*), *goosecoid*. Phylogenetic analyses performed on a vast amount of Hox homeodomain (HD) sequences, including representatives from all classes of homeobox genes from animals, protozoa, fungi, and plants, confirmed the monophyly of the Antp class as well as its position as a sister group to the Paired class (7). Within the Antp class, the *Hox* gene organization is distinctive and enigmatic: the genes map in clusters, and the order of individual genes within a cluster correlates with their temporospatial expression pattern along the anterior–posterior body axis during development (8). Recently, it was proposed that the common bilaterian ancestor of protostomes and deuterostomes had at least seven *Hox* genes (9). However, the question of the composition of the ancestral HOX cluster remains open. Analysis of *Hox* homeobox sequences (10) suggested that the conserved HOX cluster emerged early in the evolution of metazoans from an original cluster harboring three ancestral genes, one located at the 5′ end related to the *AbdB*/paralog group 9 (PG-9), a central one, precursor for the *Dfd*-like and the *Antp*-like genes (PG-4/6), and a 3′ located gene ancestral for the *pb*- and *lab*-like genes (PG-1/2). A similar organization of the evolutionary sister of the HOX cluster, the paraHOX cluster, was actually observed in amphioxus (11). However, analysis of a more complete set of HD sequences led to the hypothesis that an original and ancient split, rather, occurred between the anterior and posterior *Hox* genes, which later on duplicated separately (12).

Because the Cnidaria can be regarded as the sister group to the Bilateria, analysis of cnidarian genomes will likely provide insights into the structure and function of ancestral Antp-class genes. Within the last years, a large number of diploblastic Antp-related genes have been isolated (13–31). Among those, cnidarian sequences related to *Hox* and *paraHox* genes were found (15, 17–19, 22, 23, 25, 28, 29, 31), but the characterization of diploblastic Hox families, as well as their possible relatedness to triploblastic families, remains unclear in several cases (26, 32). Moreover, although the expression analyses suggested that several cnidarian Antp-class genes are involved in patterning (15, 19, 24, 27, 29), the developmental role of the cnidarian *Hox*-related genes at the time the oral/aboral axis is defined remains confused. In this paper, we have reconsidered the phylogeny of the whole Antp class of homeobox genes in light of three *Hydra* genes we have identified, related to *distal-less* (*Dlx Hv*) (*Hv*, *Hydra vulgaris*) not (*Cnot Hv*), and *Prh/Hex* (*CnHex Hv*). In addition, we have investigated the chromosomal clustering of three *Hydra Hox/paraHox* genes and their differential temporospatial regulation during budding and regeneration.

EVOLUTION

SPECIAL FEATURE

Methods

Culture of Animals and Regeneration Experiments. The multiheaded mutant *Chlorohydra viridissima (Cv)* and the *Hv* (Irvine strain) species were cultured as previously described (33). For regeneration experiments, bisection was performed at midgastric position on budless *Hydra* after a 2-day starvation period.

Cloning of Homeobox-Containing Genes. The *cnox-2* (1,031 bp), *Cnhex* (593 bp), *Cnot* (1,031 bp), and *msh* (525 bp) cDNAs were isolated by screening a *Hv* λgt10 cDNA library with the end-labeled 50-mer oligonucleotide as described in refs. 15 and 34. The 125-bp *Dlx hv* cDNA fragment was obtained by reverse transcription–PCR (RT-PCR) by using degenerate primers (forward: GIMGI-

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Abbreviations: *Cv, Chlorohydra viridissima*; *Hv, Hydra vulgaris*; HD, homeodomain; ML, maximum likelihood; NJ, neighbor joining; PFGE, pulse field gel electrophoresis.

The sequences reported in this paper have been deposited in the GenBank database (accession nos. *cnox-1 Hv*, AJ252181; *cnox-2 Hv*, AJ277388; *cnox-3 Hv*, AJ252182; *dlx Hv*, AJ252183; *cnot Hv*, AJ252184; *cnHex Hv*, AJ252185; *msh Hv*, AJ271008).

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TAYMGWACWGCWTTYWC; reverse: CKWCKRTTYTGRAACCAWATYTT) and further extended up to 381 bp by the 3′ rapid amplification of cDNA ends procedure by using the specific primer CAGAACTGGCAGAAACCCT. The *cnox-1 Hv* gene was obtained as a 847-bp fragment after inverted PCR performed on XmnI self-ligated genomic DNA by using the GACGACGATCACGAGTTAAATACCT (reverse) and TCTGGGACTCACTAAGGTCAAGA (forward) primers. Subsequently, the primers CCAAATAGACCAATAATTGCAAAGTCTC (forward) and GCGAAACCAAGGTGAATCATCGTCT (reverse) were used to isolate the corresponding cDNA by RT-PCR. The *cnox-3 Hv* gene was isolated from genomic DNA as a 392-bp PCR fragment by using primers derived from the *cnox-1 Hm* sequence (18), forward CTAATGAGTCARATTCARACWAARCA and reverse CATGATTAAAAATCGTT-CAATATGTTCAAGG.

Phylogenetic Sequence Analyses. HD sequences were collected on databases by using the NETBLAST search (GCG Wisconsin Package, Ver. 9.1) and aligned by using the PILEUP (GCG) and GENETIC DATA ENVIRONMENT software, Ver. 2.2 (35). The evolutionary tree was inferred from 200 HDs sequences (60 residues) by using the neighbor-joining (NJ) method (36) applied to Dayhoff's PAM distance matrix (37). The reliability of internal branches was assessed by using 200 bootstrap replicates (38). The PHYLO-WIN program (39) was used for distance computations and NJ tree-building and bootstrapping. In complement, a subset of 67 sequences was analyzed with both the NJ method, applied as above with 1,000 bootstrap replicates, and the maximum-likelihood (ML) method by using the quartet puzzling algorithm (40) that automatically assigns estimations of support to each internal branch. Dayhoff's percent accepted mutations substitution model was used, and the chosen model of rate heterogeneity was a discrete Gamma distribution with eight categories, all necessary parameters being estimated automatically from the dataset. The quartet puzzling search was conducted by using PUZZLE software, Ver. 4.02, with 25,000 puzzling steps.

Pulse Field Gel Electrophoresis (PFGE) Analysis. *Hydra (Cv)* were dissociated (41), and PFGE analysis was performed according to ref. 42 with a few modifications: ×0.25 PBS was used instead of PBS, and each block contained 2×10^6 cells. The gel was run for 43 h at 15°C by using the CHEF system at 150 V, 30″–300″. After migration, the DNA was depurinized for 20 min in 0.25 M HCl, transferred onto Hybond-N membrane (Amersham) under alkaline conditions (43), and hybridized successively to the different probes, as in ref. 15.

***In Situ* Hybridization.** *In situ* hybridization by using digoxigenin-labeled riboprobes was performed on whole *Hydra* following ref. 24, except that proteinase K digestion was replaced by radioimmunoprecipitation assay buffer permeabilization (44). The *cnox-1, cnox-2*, and *cnox-3* probes were 260, 835, and 392 bp long, respectively.

Results

Cloning of Not, Prh/Hex, and Distal-less *Hydra* Homologs. To perform an extensive analysis of Antp-class genes, we carried out cloning strategies (15) to obtain a complete set of *Hydra* representatives. We report here the cloning of three cnidarian Antp-class genes, homologous to the *not (Cnot Hv), Prh/Hex (CnHex Hv)*, and *distal-less (Dlx Hv)* genes. The putative HD encoded by these three genes showed a high degree of similarity with their triploblastic counterparts (73%, 78%, and 82%, respectively), and these *Hydra* sequences helped define a consensus sequence with family-specific residues (Fig. 1). Surprisingly, the *not* and *Prh/Hex* families do not yet have protostome counterparts.

ANTP-CLASS	**l26 / ST27 / Q44 / K46 / Q50**
PRD-Class	P26 / D27 /E32 / R44 / Q46 / A54
Non-Hox	**S27 / E30 / T43**
Emx	P1/I4/A7/P10/S11/**L14**/A19/E21/**N23**/H24/V27/G28/A29/k32/Q33/T54/Q59
Not	i7/e11/E14/**E19**/Q23/Q24/M26/V27/G28/t29/I54/W56/Q59/S60
Dlx	P4/I7/Y8/S10/N17/R18/R19/Q21/T23/Q24/A27/L28/P29/A32/G39/Q42/S54/f56/l59
Msx	N1/P4/P7/t10/**L14**/R18/K19/r21/K23/Q24/I28/A29/A32/F34/S37/A54/L59/Q60
Tlx	R1/AP4/R10/**K18**/k19/K24/S28/K39/T47/T54/W56/Q59/s60
Hex	Q5/V6/R7/t13/**K18**/Q23/K24/S27/P28/K32/k36/**R43**/T47/A54/W56/L59/K60
Barx	vA7/d10/MQ14/Q23/K24/TV28/PQ29/d30/QD42/T47/Y49/MT54/W56/TV60
NK2	r1/V6/L7/Q10/A11/Y14/R18/R19/k21/Q23/k24/A28/P29/e32/H33/P42/Y54
En	E1/P4/A7/R15/K17/R24/T27/E28/R30/Q32/E37/N41/K51/A54/I56
Evx	Y4/A7/R10/E11/R15/K18/E19/R22/E23/N24/R28/R30/C32/N39/T44/M54/R60
Hox/paraHox	**L14/ K18/ E19/ N23/ T27/ R28/ R30/ I32/ R43/ M54**
PG-2	L4/A7/N10/T11/H21/F22/K24/C27/P29/D39/H56/Q59/T60
Gsx	S1/i4/A7/S10/S27/L29/K43/V54
Cnox-1	K4/F8/QH10/r11/v14/y22/K24/d39/E59
PG-1	N7/F8/NT10/K11/T14/H21/F22/K24/A29/A36/N41/T43/Q56/R59
Mox	E4/A7/F8/K10/H23/N24/L29/Y32/V36/W56/R58/V59/K60
Cdx	K1/D2/Y4/V6/V7/D10/H11/R13/H21/R24/R29/SA32/G39/A54/E56/R57
PG-9	KC4/P7/k10/y11/T13/L21/SM24/Qd29/N39/N60
Cnox-3	K4/R10/E29

Fig. 1. Conserved residues at specific positions provide a common signature for all Antp-class HDs and thonon-Hox or the Hox/paraHox HDs (bold). In addition, a family-specific signature is given where identical or equivalent residues between diploblastic and bilaterian sequences are underlined.

Phylogenetic Analyses of Antp-Class Sequences. See Fig. 7, to be published as supplemental data on the PNAS web site, www.pnas.org. The comparison of 230 Antp-class HD sequences revealed highly conserved residues at specific positions (L26 S/T27 Q44 K46 Q50) that are different or variable in the Prd-class HDs and provided thus a signature for the Antp class (Fig. 1). To clarify relationships between these sequences, phylogenetic analyses were applied to a dataset that included systematically Antp-class HDs from invertebrate organisms (Fig. 2). When rooted with a Prd-class sequence (Hbx4 Eg), the tree inferred from the NJ analysis showed the clear distribution of these sequences into either the non-Hox or the Hox/paraHox families. This distinction was confirmed by analysis of 67 sequences by both the NJ and ML methods. We tested successively a fixed set of Hox/paraHox sequences in the presence of different non-Hox sequences and obtained a significant monophyly of the Hox group when the long branches and the evx sequences were removed (Fig. 3 and data not shown). Given the limited number of sites, the criterion used to define a family was that grouping of HD sequences from at least two distinct species was supported by bootstrap values higher than 50%.

High Conservation of *non-Hox* Antp-Class Gene Families in Metazoans. The 13 non-Hox families share few specific residues (S27, E30, T43) in addition to the Antp-class ones and do not appear as a monophyletic group in the phylogenetic tree (Fig. 2). All of them, except Bar-H1 and Tlx/Hox11, were highly supported in our analysis. In addition, within most families a clear congruence between gene and species trees was observed. Of these non-Hox families, three displayed a poriferan cognate member (*Msx*, *Tlx/Hox11*, and *NK2*), and eight a cnidarian one (*Not*, *Emx*, *Barx*, *Dlx*, *Msx*, *Evx*, *Prh/Hex*, and *NK2*). Thus only four families have no diploblastic counterpart yet (*Bsh*, *En*, *Gbx*, and *Lbx*). In contrast to the other non-Hox families, the Evx HDs actually display five of the Hox-specific residues (Fig. 1). Thus, the position of the Evx family remains ambiguous.

Definition and Conservation of Cnidarian *Hox/paraHox* Families. The alignment of Hox HD sequences detected 10 Hox-specific residues, also present in most paraHox sequences (Fig. 1). Within the Hox/paraHox group, no poriferan sequence could be found. In addition, the upper part of the tree that contains sequences representative of PG-3 to PG-8 as well as the lox and Ftz sequences included no cnidarian sequences (Fig. 2). In fact, the 23 cnidarian HD sequences formed at least 5 distinct families distributed in the

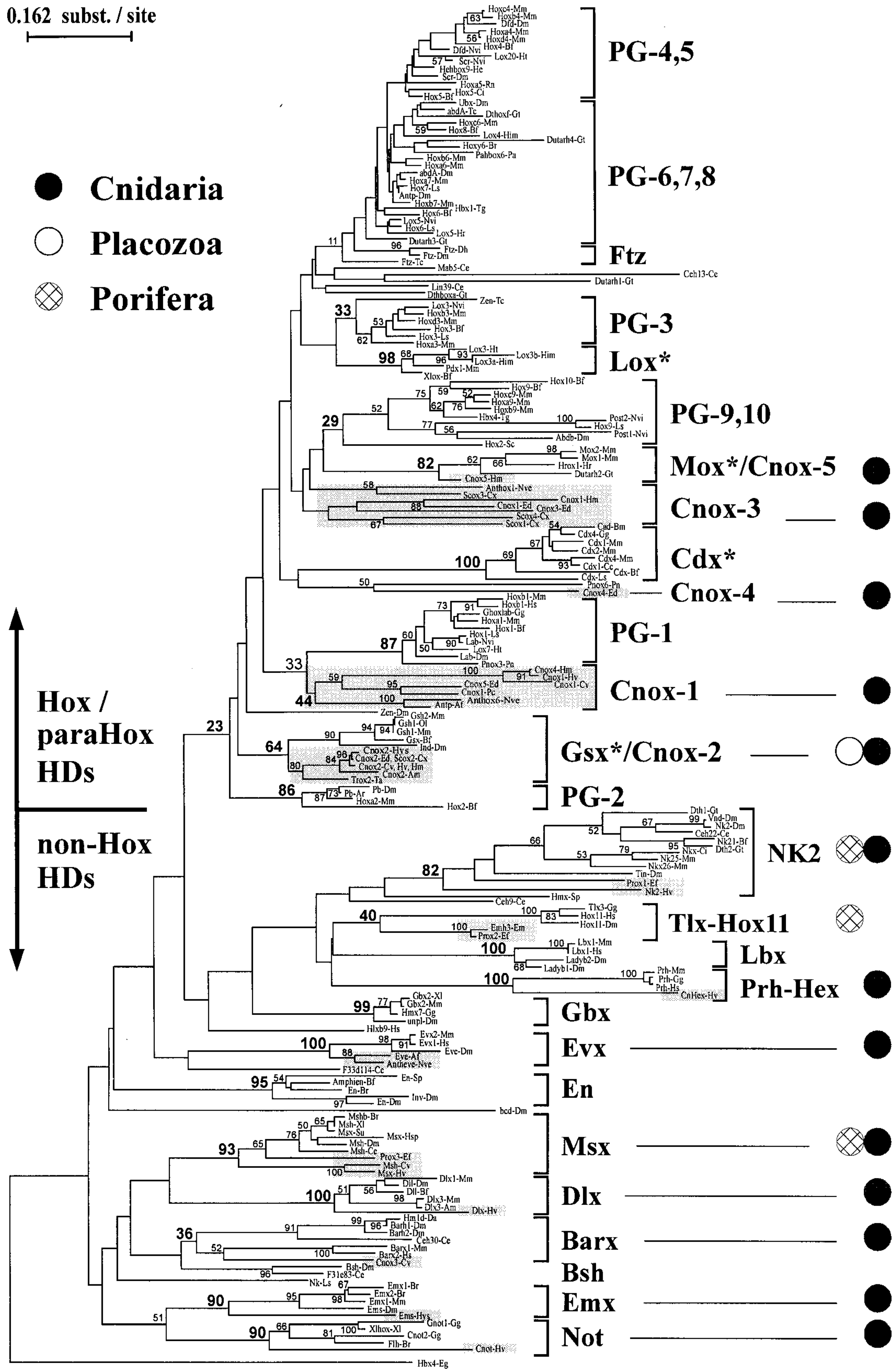

Fig. 2. Phylogenetic relationships between 200 Antp-class genes inferred by NJ analysis by using Dayhoff's PAM distance matrix. The tree was rooted with a Prd-class sequence. All branch lengths were drawn to scale. Numbers at nodes indicate percentages of 200 bootstrap replicates that support the branch; values under 50% are omitted, except for some significantly important nodes. Support values for each family defined on the right are marked in bold. Sequences from diploblasts are in shaded boxes.

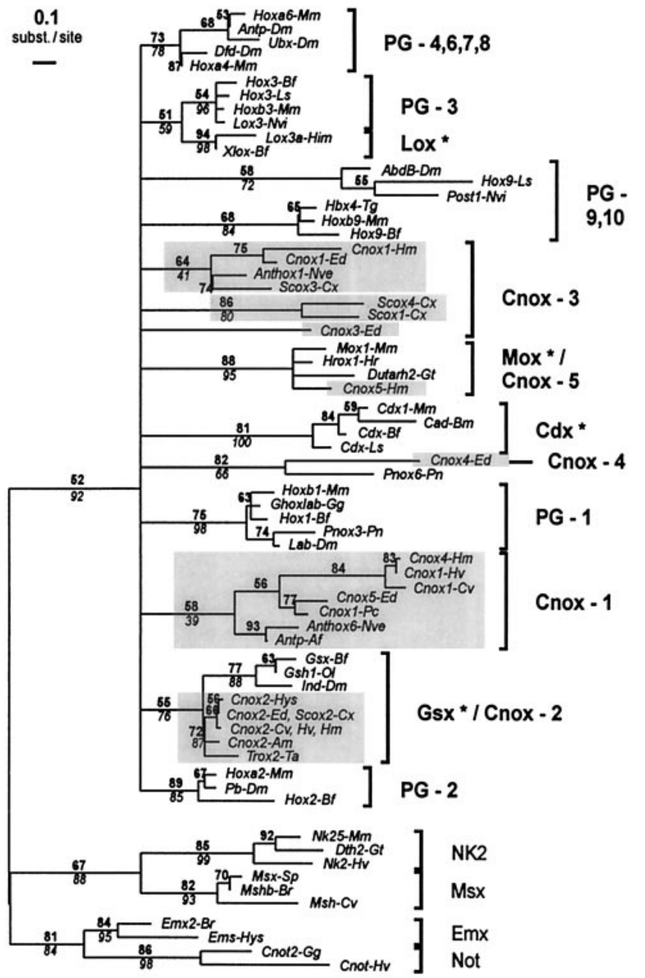

Fig. 3. Phylogenetic tree of 57 Hox/paraHox HD sequences inferred by ML analysis where 242,075 of the 677,040 possible quartets of sequences (35.8%) were unresolved, leading to a multifurcating tree with a Log likelihood value of −3,656.89. Numbers above the branches correspond to the quartet puzzling support values; numbers under branches indicate percentages of 1,000 bootstrap replicates for NJ analysis of the same dataset. Ten sequences from non-Hox families (NK2, Msx, Not, and Emx) were used as an outgroup. Shaded boxes as in Fig. 2.

vicinity of anterior (PG-1/2, Gsx) and posterior (PG-9/10, cdx) Hox/paraHox sequences from bilaterian species. The *cnox-1* sequences isolated from anthozoan (17, 31) and hydrozoan (15, 18, 22, 23) species appeared as a monophyletic family in both NJ and ML trees (Figs. 2 and 3). This family, whose several members display the PG1-specific F8, N/Q10, R/K11, Q56 residues, is related to the PG-1 HDs only in the NJ tree and with a low bootstrap value (Fig. 2). The cnox-2 HDs, present in anthozoan (D. Miller, personal communication), scyphozoan (28), hydrozoan (15, 18, 19, 23, 29), and placozoan (26) species, share 8 specific residues with the Gsx HDs. Moreover, in both the NJ and ML analyses, cnox-2 branches together clearly with the paraHox Gsx sequences. Similarly, the *cnox-5* gene, isolated so far only from *Hydra* (18), appeared as the diploblastic *Mox* counterpart. In contrast, the cnox-3 sequences, which were isolated from scyphozoan (28) and hydrozoan (18, 23) species and as multiple copies in two species (23, 28), are more heterogeneous than other cnox families. In all analyses, they form three groups that may come together depending on the type of analyses and selection of sequences (Figs. 2 and 3 and data not shown). In the 67-sequence NJ tree, the cnox-3 HDs that share the K4, R/K10, and E/D29 with the PG-9/10 sequences, cluster together with those (data not shown). Finally, the cnox-4 sequence was characterized only once from hydrozoan (23) and found related to the planarian Pnox-6 HD (45). Cnox-4 harbors 5 Cdx-specific residues (Fig. 1), suggesting that, in agreement with its position in the NJ tree, cnox-4 might share some common ancestor with the cdx family. If one considers *cnox-1* and *cnox-3* as representatives of *proto-Hox* genes, their chromosomal linkage would be expected. For this reason, we hybridized *Cv* genomic DNA submitted to PFGE with the *cnox-1 Cv*, *cnox-2 Cv*, and *cnox-3 Hv* sequences but could detect no chromosomal linkage between these three genes in the range of 150 kb (Fig. 4).

Differential Expression of *Hydra Hox/paraHox*-Related Genes During Head Regeneration. We have examined the expression patterns of three *Hydra Hox/paraHox*-related genes, *cnox-1, cnox-2*, and *cnox-3*, by whole-mount *in situ* hybridization (Fig. 5). In adult polyps, all three genes were restricted to the ectodermal layer; *cnox-1* was expressed at low levels in the body column, slightly higher in the hypostome, and at high levels in the future head region of developing buds from stage 2. *Cnox-2* transcripts were found along the body column and in a specific subset of head epithelial cells. *Cnox-2* was turned on in evaginating buds from stage 3. Finally, *cnox-3* was detected exclusively in few cells of the head region. None of them were expressed in the basal region of the animal. During regeneration, *cnox-1* expression was turned on early, 2 h after bisection, regardless of the polarity in ectodermal cells of the regenerating tip. Subsequently, its expression became head specific. In contrast, *cnox-2* was turned on at a later stage, 24 h after cutting but specifically in the head-regenerating stump. This early–late *cnox-2* expression that became detectable several hours before tentacle rudiments emerged persisted in the developing head for 24 h before

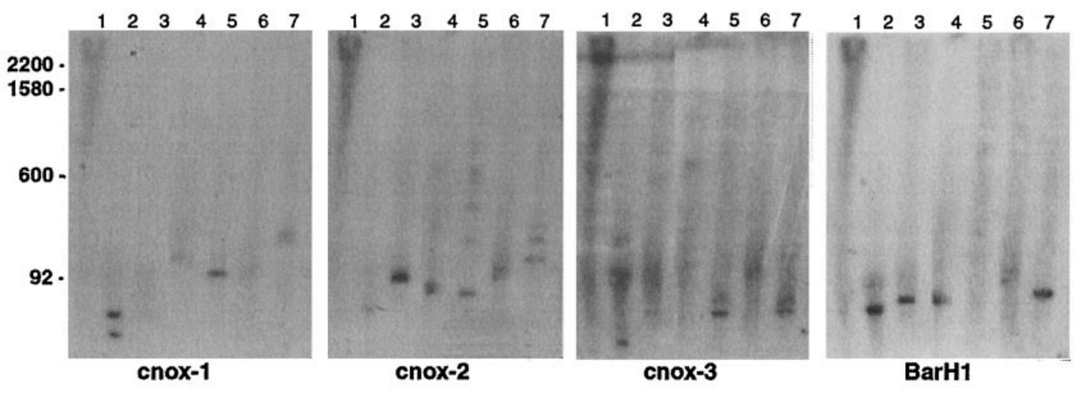

Fig. 4. PFGE analysis of *Cv* genomic DNA predigested with *Cla*I (2), NruI (3), MluI (4), NarI (5), *Pvu*I (6), *Sma*I (7), or undigested (1) and hybridized to *cnox-1 Cv*, *cnox-2 Cv*, *cnox-3 Hv*, and *Bar-H1 Cv* [initially named *cnox-3 Cv* (15)] probes.

Fig. 5. Expression pattern of the *Hv Hox/paraHox* genes, *cnox-1* (*Top*), *cnox-2* (*Middle*), and *cnox-3* (*Bottom*) in adult (*Left*) and regenerating *Hydra*. Time points after cutting are given; arrowheads and arrows indicate foot- and head-regenerating stumps, respectively. st, budding stage.

the adult pattern was reestablished (Fig. 5). The *cnox-2* expression detected in the adult body column persisted during regeneration but at a reduced level. Finally, *cnox-3* displayed no modulation during either head or foot regeneration.

Discussion

Early Evolution of Antp-Class HDs. The phylogenetic analysis performed on a representative set of Antp-class HDs sequences showed the conservation of seven distinct non-Hox and three Hox/paraHox families from diploblasts to bilaterians. These families can be identified through their residue-specific signature. According to their position within the helix structure, most of these residues are not participating in the DNA-binding function and are thus supposed to be involved in protein–protein interactions, specific for a given family (46). *Non-Hox* genes are likely the most ancestral ones, some of them being identified in both Porifera and Cnidaria, whereas *Hox/paraHox* genes would be more recent, so far isolated only from Placozoa and Cnidarians among diploblasts. According to the ambiguous position of the Evx HD sequence and its chromosomal linkage to the *Hox* gene(s) in coral (17) and mammals (47), one might propose that *Hox* genes derived from an Evx-like ancestor gene that duplicated before the cnidarians diverged. Moreover, both *Hox* and *paraHox* genes were identified in cnidarians, thus the duplication of an ancestral minimal cluster of two or three *proto-Hox* genes predating the Cnidaria divergence is a plausible scenario (Fig. 6). The absence of chromosomal linkage between *cnox-1* and *cnox-3* in *Hydra* could be explained by the rather phylogenetically derived position of *Hydra* within the Cnidaria phylum and the loss of clustering along evolution. If true, this means that clustering is not required for the developmental function of the *Hydra Hox*-related genes. Interestingly, as previously mentioned (48), none of the *Hox/paraHox* central genes, PG-3 to PG-8 and lox, were found in cnidarians, which thus probably emerged independently after the divergence of the Cnidaria phylum or alternatively disappeared during the evolution of this phylum. We cannot rule out the possibility that some of the cnox-3 sequences represent intermediate proto-Hox genes.

Apical Differentiation and *Hox/paraHox* Gene Expression. In *Hydra*, *cnox-1* expression was observed in the regenerating stump at the time head organizer was establishing (49). However, in the marine hydrozoan *Podocorynae carnae*, *cnox1* was not found expressed

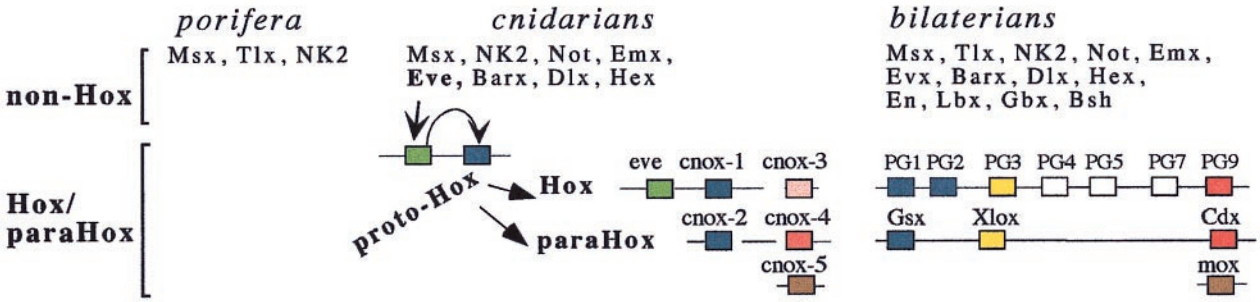

Fig. 6. Scheme describing a plausible scenario of the evolution of Antp-class genes from diploblastic to bilaterian animals.

during developmental stages but mostly in striated muscular cells at the medusae stage (22). This suggests that the role of *cnox-1* in specifying positional information is not ubiquitously conserved in cnidarians. *Cnox-2*, whose expression in adult polyps was similar to that found in *Hydractinia* (29), behaves as an early–late gene during head regeneration. In addition, we could detect no *cnox-2* expression during foot regeneration. This temporal regulation of *cnox-1* and *cnox-2* expressions during head regeneration is in agreement with our previous reverse transcription–PCR analysis of *cnox-1* and *cnox-2* expression during *Cv* regeneration (15) but contradicts that found by Shenk *et al.* (19, 50). By using immunocytochemistry, these authors found Cnox-2 expression suppressed during head regeneration but enhanced during basal regeneration, and, in adult polyps, at low levels in the apex contrasting with high levels in the body column and the basal disk. Because in *Hydra* cells migrate obligatorily from the body column toward the extremities, this cnox-2 antibody likely did not detect cnox-2-expressing cells located in the head region or during head regeneration.

The spatial regulation of *cnox-1* and *cnox-2* in the adult head together with their temporal regulation during head regeneration suggest a developmental function in differentiation and maintenance of the apical pole in *Hydra*. In bilaterians, *PG-1* and *Gsx* genes are involved in the differentiation of anterior embryonic regions (8, 11). Thus this anterior function might have been retained from diploblasts to triploblastics. Similarly, the role of the *emx* Antp-class gene (27) and the *prdl-a* paired-class gene (33) during head patterning might be evolutionarily conserved. In contrast, the *cnox-3* gene, which displays some relatedness with the posterior *Hox* genes, was not found involved either in basal or in apical differentiation. These results suggest that head formation but not axis differentiation can be traced back in the cnidarians (51), which is partially in agreement with the zootype hypothesis (6). Antp-class as well as Prd-class genes were not found outside the metazoan animals, and their emergence with new developmental functions in diploblasts could have favored the evolution of highly adapted and more complex structures.

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