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Evolution

Homeobox genes in the ribbonworm *Lineus sanguineus*: Evolutionary implications

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ABSTRACT **From our current understanding of the genetic basis of development and pattern formation in *Drosophila* and vertebrates it is commonly thought that clusters of *Hox* genes sculpt the morphology of animals in specific body regions. Based on *Hox* gene conservation throughout the animal kingdom it is proposed that these genes and their role in pattern formation evolved early during the evolution of metazoans. Knowledge of the history of *Hox* genes will lead to a better understanding of the role of *Hox* genes in the evolution of animal body plans. To infer *Hox* gene evolution, reliable data on lower chordates and invertebrates are crucial. Among the lower triploblasts, the body plan of the ribbonworm *Lineus* (nemertini) appears to be close to the common ancestral condition of protostomes and deuterostomes. In this paper we present the isolation and identification of *Hox* genes in *Lineus sanguineus*. We find that the *Lineus* genome contains a single cluster of at least six *Hox* genes: two anterior-class genes, three middle-class genes, and one posterior-class gene. Each of the genes can be definitely assigned to an ortholog group on the basis of its homeobox and its flanking sequences. The most closely related homeodomain sequences are invariably found among the mouse or Amphioxus orthologs, rather than *Drosophila* and other invertebrates. This suggests that the ribbonworms have diverged relatively little from the last common ancestors of protostomes and deuterostomes, the urbilateria.**

The genetic basis of development of vertebrates and arthropods has been extensively studied during the last decade. It has become clear that although these organisms have strikingly different body architectures, many of the regulatory genes they use to establish their body plan are conserved (1). Most prominent among them are the homeobox genes that encode gene regulatory proteins containing a DNA-binding homeodomain. Initially identified in *Drosophila*, they are present in many, if not all, eukaryotic organisms (2). *Hox* genes, a subfamily of homeobox genes, are involved in the specification of body structures along the anterior–posterior (A-P) body axis in all animals analyzed so far, including arthropods and chordates (3–6). In arthropods, nematodes, and chordates, these genes are organized in clusters (7). During development, their physical linear order is reflected in their respective region-specific expression domains along the A-P axis (8). Thus, it has been suggested that the physical linear order of the ancestral *Hox* complex is mechanistically linked to the spatial and temporal order of their expression. This is referred to as spatiotemporal colinearity (9). It has been proposed that different body patterns may evolve through changes in number, regulation, or function of *Hox* genes (10). Knowledge of the evolutionary history of *Hox* genes will allow us to compare different body plans and thus will provide insights into how new body plans have evolved. Until recently, extensive studies of *Hox* genes have been performed mainly on flies and vertebrates. To enable us to draw a reliable scenario about how *Hox* genes have evolved, studies of *Hox* genes have to be broadened to include representatives of other phyla, in particular different protostomes.

Based on the original hypothesis of Lewis (11) that the Bithorax-Complex of *Drosophila* arose by unequal crossing-over, leading to tandem duplications of the *Hox* genes, we have proposed that the primordial *Hox* cluster may have arisen by a series of consecutive tandem duplications from unequal crossing-over (12). The first step is thought to have led from the original prototype gene (Ur-*Hox* gene) to the two terminal genes of the cluster, which evolved into an anterior and posterior gene, respectively, and, therefore, had the longest time to diverge. Subsequently a series of unequal crossings-over presumably generated the interior genes. Because of these multiple recombination events, the internal genes are mosaics, combining segments of their ancestral genes, and their sequences become homogenized. Because the interior genes were generated later in evolution and were homogenized, they have diverged less from the Ur-*Hox* gene than the external genes. This hypothesis is consistent with the finding that the homeodomain sequences diverge progressively from the consensus sequence, going from the center toward the termini (12). It is also supported by the fact that the terminal genes share an intron at the same position in the homeodomain (amino acids 44/45) (12). The progressive divergence of the homeobox sequences from the center toward the termini of the clusters is also apparent in a distance matrix based on pairwise sequence comparisons in all possible combinations to quantify sequence similarities (13), as well as in phylogenetic trees that are rooted on the *Antennapedia (Antp)* gene, that corresponds most closely to the consensus sequence and may have conserved the sequence of the Ur-*Hox* gene best (12). A quantitative phylogenetic analysis using the neighbor-joining method basically supports the above model (14). The phylogenetic tree indicates that the Ur-*Hox* gene first duplicated and diverged into an anterior and a posterior gene. In vertebrate phylogeny, the anterior gene, through a series of duplications, gave rise to eight anterior cognate group genes, whereas the posterior gene duplicated repeatedly and diverged into five posterior group genes. The sequence and time of these duplications can be roughly estimated (14). To test the predictions of the unequal crossing-over model and the phylogenetic distance tree, we have to obtain information about evolutionary intermediates (if they still exist) that did not undergo all of the gene duplication events.

Abbreviation: A-P, anterior–posterior.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. P81192, P81193, and Y16570–Y16575).

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Lineus, a marine ribbonworm, may represent such an intermediary stage of *Hox* gene evolution. The phylogenetic position of the ribbonworms (nemertini) has been much debated (15–19). They belong to the triploblasts. *Lineus* is bilaterally symmetrical and has a rather sophisticated organization along the anterior–posterior (A-P) body axis. These different A-P body regions are defined according to specific morphological characteristics such as eyes in region 1 (R1), brain in R2, mouth in R5, and anus in R9 (Fig. 1). Nemerteans have a lateral nervous system, rather than a ventral one as found in most other invertebrate phyla. They share spiral cleavage with platyhelminths, annelids, and mollusks. They have a body cavity with an eversible proboscis (rhynchocoel) and blood vessels that have been interpreted as coelomic cavities (20). In earlier studies, it has been suggested that living nemerteans might be similar to the last common ancestor between invertebrates and vertebrates (16–19). These last common ancestors have been termed Urbilateria and presumably represent primitive coelomates (21). Animals possessing a coelomic cavity have been subdivided into two fundamental groups: the protostomes, such as arthropods, annelids, and mollusks, and the deuterostomes, such as echinoderms and chordates (chordates include ascidians, Amphioxus, and vertebrates). In protostomes the blastopore gives rise to mouth, whereas in deuterostomes the mouth is formed secondarily by a perforation of the ectoderm, and the anus arises at or close to the site of the original blastopore (22). However, the embryological distinction between protostomes and deuterostomes is not as clear as it would seem, especially in the case of nemerteans. In polychaetes the blastopore is elongated along the anteroposterior axis and the lateral blastopore lips fuse along the midline, leaving two openings, an anterior one, which will form the mouth, and a posterior one giving rise to the anus (23). The snail *Viviparus* and the polychaete *Eunice* show deuterostomy (23), even though they are classified as protostomes.

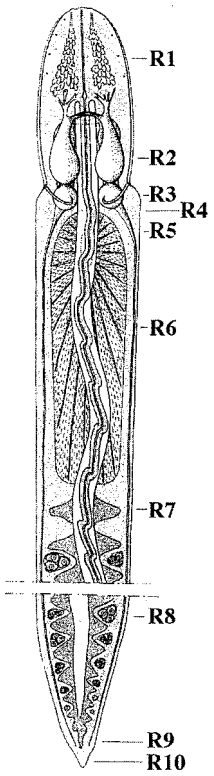

FIG. 1. Scheme of *Lineus* body organization. The different body regions (R) along the A-P axis are numbered starting from the anterior end and are characterized by the following structures: R1, cephalic glands, rhynchodeum, and eyes; R2, cerebral ganglia; R3, sensory cerebral organs; R4, postcerebral, preesophageal connective tissue; R5, mouth; R6, posterior esophagus and nephridia; R7, anterior intestine (gonads absent); R8, middle and posterior intestine plus gonads; R9, anus; R10, caudal end.

Embryological studies on *Lineus ruber* indicate that after gastrulation the blastopore closes almost completely and that the mouth forms as a secondary invagination of the ectoderm, close to the site of the closed blastopore (24). Furthermore, the axis of the larva (and adult) relative to the polarity of the egg may change as a function of foregut morphogenesis in the embryo (25). During foregut formation the mouth moves to various degrees from the vegetal pole to the ventral side of the body, e.g., in *Lineus torquatus* the stomodaeum moves toward the opposite side of the ptilidium larva (25). This suggests that nemerteans may be in an evolutionary transition zone between proto- and deuterostomes, a point that is worth reexamination. The sequence data of 18S ribosomal RNA give a relatively clear picture; the nemertean *Cerebratulus lacteus* falls within the protostome coelomate clade (26). These data have been confirmed and extended by Carranza *et al.* (27) by comparing the complete 18S rRNA sequences of 35 species from platyhelminths and various other phyla, including the nemertean *Prostoma eilhardi*, which is most closely related to annelids and mollusks within the protostomes and more closely related to deuterostomes (Fig. 2) than to arthropods and platyhelminths. In a similar study of 18S rRNA sequences the nemertean *Lineus sp*. was also found to be closely related to mollusks and annelids (28).

We have analyzed the *Hox* genes of *Lineus sanguineus* and found them to be clustered in a relatively small segment of DNA that can be resolved by pulse-field electrophoresis. The cluster contains at least six *Hox* genes, corresponding to the vertebrate homologs *Hox*1 and 3 (anterior class), *Hox* 4, 6, and 7 (middle class), and *Hox* 9 (posterior class). Because at least one member (*Hox5*) present in vertebrates and Amphioxus seems to be missing in *Lineus*, the *Hox* cluster of nemerteans may be an early evolutionary intermediate, unless the missing gene has been lost secondarily. Interestingly, the homeodomain sequences of *Lineus* are more closely related to those of vertebrates and Amphioxus, rather than to those of *Drosophila* and other invertebrates.

MATERIALS AND METHODS

DNA Extraction. To isolate genomic DNA we followed the procedure described by Hempstead *et al.* (29) with few modifications. All steps were performed at 4°C, and the SDS concentration in the extraction buffer was increased to 5%.

Cloning Homeobox Fragments and *Hox* Genes from Genomic DNA. Homeobox fragments were amplified from genomic DNA as described by Murtha and Ruddle (30) and PCR products were cloned into the Bluescript vector.

The *L. sanguineus* genomic library used is a λ Fix II library prepared with *Mbo*I partially digested DNA (31). High-stringency screening of the library was performed as described by Sambrook *et al.* (32). For the low stringency screens, hybridization temperatures were chosen to allow cross-hybridization between related homeobox sequences. Using a hybridization buffer containing 50% formamide, we reduced the hybridization temperature to 37°C.

Pulse-Field Gel Electrophoresis. *Preparation of embedded DNA.* Worms were flash-frozen in liquid nitrogen and ground to fine powder with a liquid nitrogen-chilled mortar and pestle. The powder was immediately mixed to 1% low-melting agarose. The embedded worm powder was then treated with proteinase K (33). For endonuclease digestion, a liquid digestion procedure was used (33). Complete digestion was obtained after overnight incubation at 37°C whereas 2-hr digestion was performed for partial digestion.

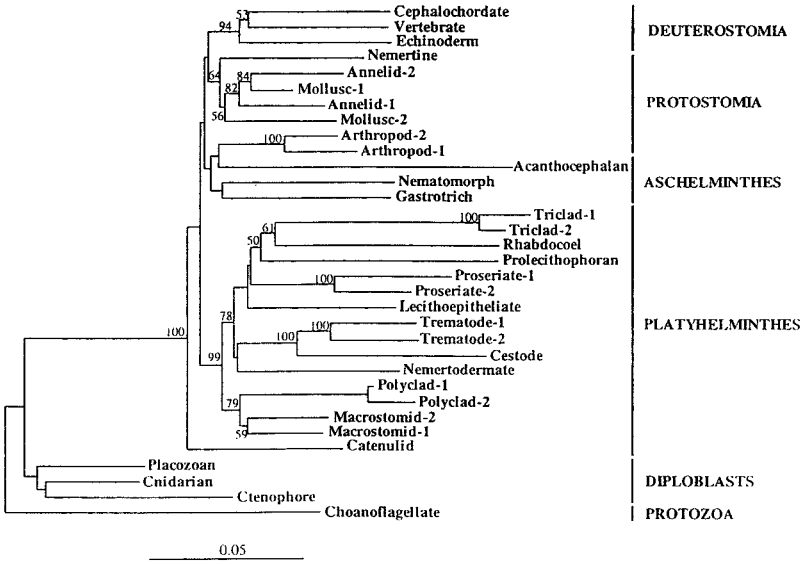

FIG. 2. Evolutionary distance tree. Based on the neighbor-joining method including the complete 18S ribosomal RNA sequences of 33 species belonging to 16 different phyla, with special emphasis on platyhelminths. The nemertean listed is *Prostoma eilhardi*. The numbers at the nodes are percentages of 1,000 boot trap replicates that support the branch, with only values over 50% being represented. All branch lengths are drawn to scale. The choanoflagellate *Sphaeroeca volvox* is used as outgroup. The Fitch–Margoliash method gives the same topology. (Reproduced with permission from ref. 27.)

Pulse-field gel electrophoresis conditions. Gel (0.8%) was run with fields of 6 V/cm by using 120° reorientation and a switch interval of 1.5 sec. The run was performed during 12 hr at 14°C with 0.5× TAE running buffer (Tris/acetate/EDTA). We have set up these conditions for the Chef Mapper apparatus (Bio-Rad).

Alkaline capillary transfer was performed for 48 hr, and the hybridization procedure was the same as described above.

RESULTS AND DISCUSSION

Amplification of Homeobox Sequences by PCR. To identify *L. sanguineus* homeobox genes, we have performed a PCR screen on genomic DNA. For this purpose we have used degenerate primers derived from the consensus coding sequences of the first and third α helices of the homeodomain (30). Analysis of the PCR products by agarose gel electrophoresis showed the presence of a single band. DNA from this band was subcloned. Of 50 clones sequenced, we found five different homeobox sequences. Based on both nucleotide and amino acid sequence analysis, one has been classified as a *caudal*-type homeobox and the four others as *Hox*-type homeoboxes. Sequence data obtained from these short DNA fragments were not sufficient to assign these four *Hox*-type homeoboxes to defined *Hox*-class genes. Therefore, we screened a genomic library to clone and analyze more coding sequences of these genes.

Isolation and Sequence Analysis of *L. sanguineus* Hox/HOM-Type Genes. We used the isolated PCR fragments as probes to screen a *L. sanguineus* genomic library under high-stringency conditions. For each homeobox probe, we isolated multiple clones that were restriction-endonuclease mapped and partially sequenced. Sequences of these genes revealed that we isolated the corresponding gene for each homeobox probe, thus confirming the authenticity of all five homeoboxes amplified by PCR. To extend our screen to different *Hox* genes we have performed a second round of screening in which reduced stringency conditions were chosen to allow cross-hybridization between related *Hox* sequences. From this screen we have isolated 36 phages. Sequencing of phages with different restriction-endonuclease maps led to the identification of three new homeobox genes. Sequence analysis of the homeobox plus adjacent sequences allowed us to unambiguously classify each gene (the deduced amino acid sequences are shown in Fig. 3). Among the eight homeobox genes identified, six are *Hox*-type genes, one is a *caudal*-type gene (*Lscdx*), and one is a *NK-1*-type gene (*LsNK*). When the complete homeodomains with some flanking sequences are used for comparison, the *Lineus* sequences can be assigned unequivocally to their orthologs in vertebrates or Amphioxus. Therefore, we decided to discuss homology and assignment in terms of membership to an ortholog group according to the classification of vertebrate *Hox* genes into 13 paralog groups. Because each *Lineus Hox* gene can be assigned to a different ortholog group, we named these genes *LsHox1, LsHox3, LsHox4, LsHox6, LsHox7*, and *LsHox9,* according to the ortholog group to which they belong.

Sequence comparison with the known *Hox* genes gave an intriguing result because for each *LsHox* gene the highest sequence identity was found invariably with mouse and Amphioxus *Hox* genes. For each of the *LsHox* homeodomains (except *LsHox9*) the sequence identities with their chordate orthologs vary between 88 and 100%. One hundred percent of sequence identity was found between the *LsHox7* and *Amphi-Hox7* homeodomains. In the case of the *LsHox9* homeodomain the highest percentage of sequence identity was found to be 69% only with the mouse *Hoxc-9* homeodomain, but *LsHox9* is clearly a posterior gene like *Hoxc-9*.

LsHox1 is assigned to ortholog group 1 based on its 90% sequence identity to *Amphi Hox1* and 88% identity to mouse *Hoxa1* and *Drosophila labial*. Among the 17 aa characteristic for the *Hox1* group, 11 are shared by *LsHox1*. In the C-terminal flanking sequences 4 aa are shared with *Hoxa1* and *labial* and 2 are shared with *Amphi Hox1*. In the N-terminal flanking sequences 3 aa are shared with *Hoxa1*.

LsHox3 shows 91% sequence identity with murine and human *Hoxb3* and murine *Hoxd3*, whereas the *Amphi Hox3* and *Drosophila proposcipedia* show 88 and 73% sequence identity, respectively. Ten of 13 characteristic amino acids are shared with the *Hox3* group, whereas none of the 9 aa that are typical for *Hox2* are found in *LsHox3*. In addition, two lysine residues are highly conserved at positions +1 and +3 of the C-terminal flanking sequences, which allows us to assign *LsHox3* to ortholog group 3.

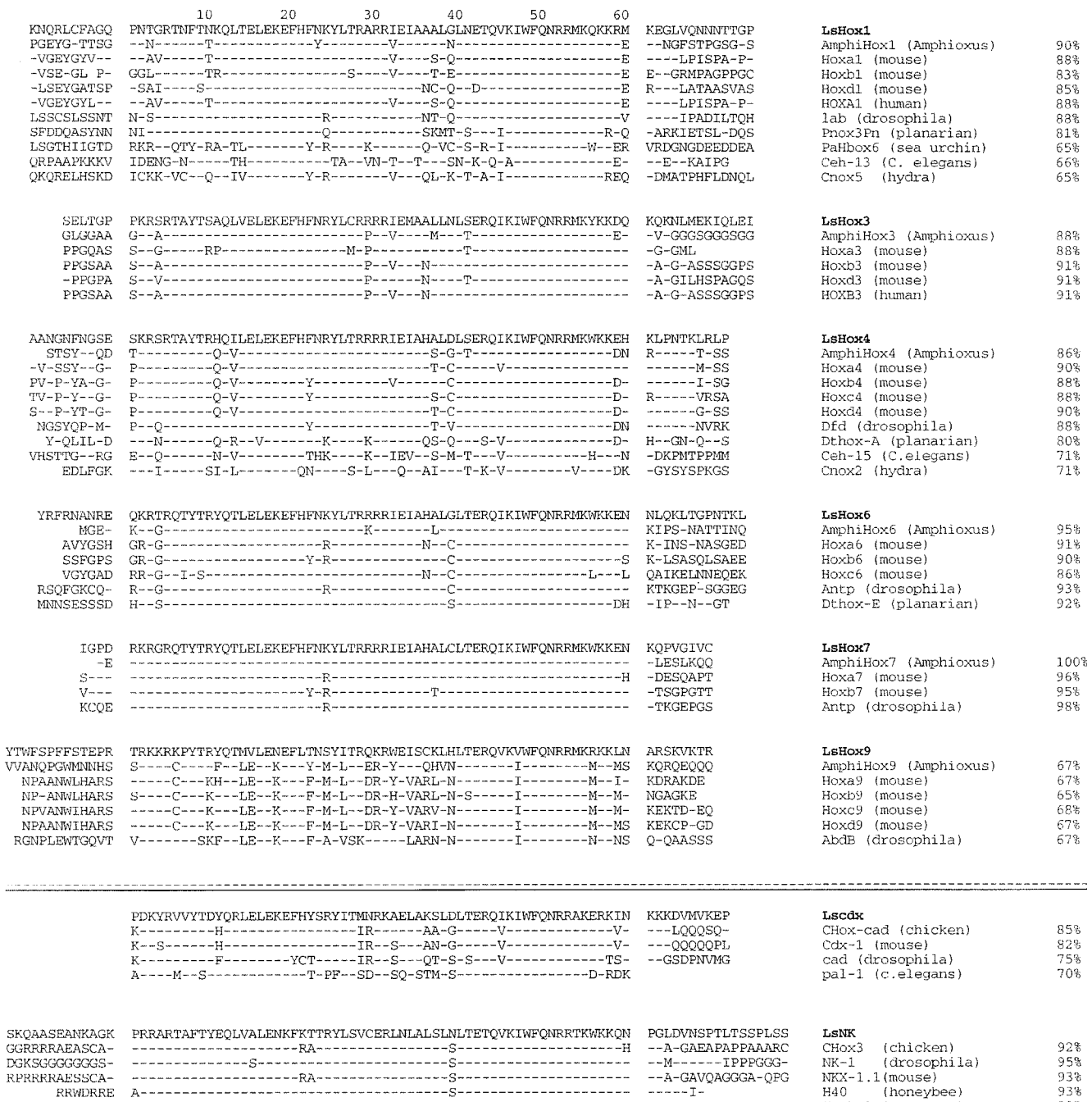

FIG. 3. Sequence alignments of homeodomains and flanking sequences encoded by *LsHox* genes (*Upper*) plus *caudal/Cdx* and *NK-1* orthologs (*Lower*). Dashes indicate amino acid identity between domains encoded by *L. sanguineus* homeobox genes and the most similar sequences from other phyla. Percentages indicated correspond to sequence identities within the homeodomain between *L. sanguineus* genes and the sequences from other phyla. The sequence comparison is limited to the small number of homeodomains known in protostomes for which complete sequence data are available.

LsHox4 clearly belongs to ortholog group 4 because the 8 aa downstream to the homeodomain have 90% sequence identity with those of the mouse genes *Hoxa4*, *Hoxb4*, and *Hoxd4* and also the upstream flanking sequences share 55% identity with *Hoxa4*, *Hoxb4*, *Hoxc4*, and *Hoxd4*. Again the closest similarity within the homeodomain is with a mammalian gene—90% with murine *Hoxa4* and *Hoxd4*—whereas *Drosophila Deformed* and *Amphi Hox4* show 88 and 86%, respectively.

LsHox6 is very similar to *Amphi Hox6* (95% identity), but only 2 aa are shared in the flanking sequences. However, the 2 aa at positions 6 and 7 (Q and T) indicate that this gene belongs to ortholog group 6 and not to group 5, which has T and (S or A) at these positions. These amino acids have been shown to be functionally important in the distinction between *Antp* and *Scr* (34). *LsHox6* shows 92% sequence identity and 5 of 10 identical amino acids in the C-terminal flanking sequences to the planarian *Dthox-E*.

LsHox7 can be assigned to ortholog group 7 because it shares 100% sequence identity with *Amphi Hox7*. There are also three characteristic amino acids in the N-terminal flanking and a lysin at position +1. This gene is more closely related to *Antennapedia* (98%) than to *abdominal A* (95%), and it lacks the sequences at the C terminus that encode the *Ubd*-A peptide (28). *LsHox7* is more closely related to *Antennapedia* than *LsHox6*, especially in the N-terminal arm.

The only homeodomain that is more difficult to assign is *LsHox9* because the maximum sequence identity shared with any known homeodomain is 68% for *Hoxc9* and 67% for *Hoxa9*, *Amphi Hox9*, and *Abdominal B* of *Drosophila*. It lacks the *Ubd*-A peptide or other striking similarities in the flanking sequences. Nevertheless, the characteristic amino acids near the N terminus (T1, K3, P7, T12, and L21) together with the overall similarity identify *LsHox9* clearly as a member of the posterior group.

The *LsHox1* and *LsHox7* also have a conserved motif in the C-terminal region, as do their respective vertebrate orthologs. *LsHox1* shares a serine-rich domain with the vertebrate paralog group 1 genes, whereas *LsHox7* has a glutamate stretch near the C terminus in common with the vertebrate paralog group 7 genes (Fig. 4) (34).

Evolution of the *Hox* Gene Cluster. To date, the genome organization of *Hox* genes has been investigated in humans, mouse, puffer fish, Amphioxus, *Drosophila*, Tribolium, and *Caenorhabditis* (35, 36). Amphioxus and the three invertebrate species have a single *Hox* cluster (37–40) whereas vertebrates possess multiple clusters, four copies in humans, mice (35), and puffer fish (36), and probably three in the most primitive jawless vertebrates (lampreys) (41), suggesting that several duplications of the entire cluster have occurred during vertebrate evolution. The cluster of *Caenorhabditis* is fairly diverged, but in the other species the colinear organization is strongly conserved. None of the *Lineus Hox* genes identified belong to the same ortholog group, which implies that *Lineus* also contains a single *Hox* cluster. This is supported by our preliminary analysis of the *Lineus* genome by pulse-field electrophoresis and Southern blotting (Fig. 5). If genomic DNA is cut with *Not*I and probed successively with *LsHox3* and *LsHox7*, identical patterns of hybridization are observed with a single band in the range of 200–300 kb being radioactively labeled. A similar result is obtained if the DNA is partially digested with *Sac*II. To test whether the pattern of hybridization is specific for each probe, we chose *Pst*I digestion as an internal control, because only the *LsHox3* probe contains a *Pst*I site. As expected the *LsHox3* probe yields two bands of hybridization when the DNA is cut with *Pst*I, whereas a single band is observed when the DNA is probed with *LsHox7* (Fig. 5). These data are consistent with the assumption of a single *Hox* cluster in *Lineus*, but the entire cluster has to be analyzed in detail or sequenced to confirm this hypothesis.

Recently it has been proposed that a common ancestor of protostomes and deuterostomes might have had a rather sophisticated body morphology elaborated under *Hox* and *Pax* gene control (42). To understand the evolution of animal architecture we have to understand the evolution of *Hox* genes and their role in the specification of body plans. Because the body plans of most phyla were already established at the Cambrian "explosion," we have to study the more primitive phyla like cnidarians and "lower" triploblasts. Several hypotheses for *Hox* gene evolution have been put forward (12–14, 39–44). It has been proposed that even before cnidarian divergence there was at least one anterior-class (*labial* type), one middle-class (*Antp* type), and one posterior-class *(Abdominal-B* type) gene that arose from the duplication of a unique ancestral gene. So far, anterior- and middle-class *Hox* genes have been found in most protostomes studied. However, posterior class genes have only been identified in some arthropods (43, 44) and in *Caenorhabditis* (45). Thus, the existence of an ancestral representative of the posterior class is more questionable. However, this could be due to technical reasons, because the standard primers that are used do not detect posterior-class genes easily. In *Lineus* we have identified an *Abdominal-B* type gene, which shows significant sequence conservation compared with both arthropod and chordate ortholog group 9 genes. This gene was not detected by PCR methodology but only when the library was screened by hybridization at reduced stringency, indicating that it could easily have been missed. The identification of *Abdominal-B* type genes in *Lineus*, *Caenorhabditis*, *Drosophila*, and Amphioxus indicate that the posterior-class genes arose early in metazoan evolution. This is consistent with our hypothesis that *Hox* genes arose by repeated unequal crossing-over from a Ur-*Hox* gene, which first duplicated to form the terminal anterior- and posterior-class genes (12). The middle-class genes then would have been added progressively in between these two most ancient genes, which have most extensively diverged from the Ur-*Hox* gene. This hypothesis predicts that primitive *Hox* clusters should lack middle-class genes but contain the anterior (*labial* type) and posterior *(Abdominal-B* type) genes. Our findings in *Lineus* so far are consistent with this hypothesis. The *Lineus* cluster contains at least six genes (*LsHox 1*, *3*, *4*, *6*, *7*, and *9)*, including a representative of the

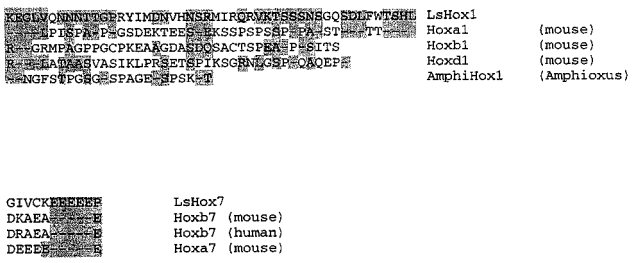

FIG. 4. Sequence alignments of the C-terminal domains encoded by the *LsHox1* and *LsHox7* genes with chordate OG-1 genes (*Upper*) and vertebrate PG-7 genes (*Lower*), respectively. Dashes indicate identical amino acids. Conservative changes and identical residues are boxed in.

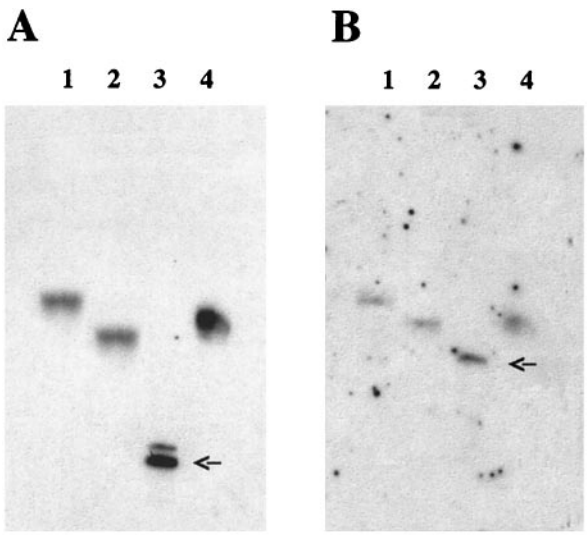

FIG. 5. Southern blot analysis of pulse-field gel. (*A*) Hybridization pattern obtained with the probe H3 (*LsHox3* subclone). (*B*) Hybridization pattern obtained with the probe H7 (*LsHox7* subclone). 1, uncut genomic DNA; 2, DNA digested with *Not*I; 3, DNA digested with *Pst*I; 4, DNA partially digested with *Sac*II. The same blot was hybridized successively with both probes. The blot was first hybridized with the probe H3. The autoradiogram was obtained after 12 hr of exposure. The blot was then dehybridized and hybridized again with the probe H7. The autoradiogram was obtained after 30 hr of exposure. The second hybridization has given signals of lower intensity and a higher background compared with the first hybridization. Both probes give the same hybridization pattern except in the case of DNA digested with *Pst*I. This enzyme was used as a control because we expected, in this case, a different hybridization pattern with the two probes. The results confirm first, that no signal was left after dehybridization and second, that the hybridization patterns obtained for each probe are specific.

anterior class (*LsHox1*) and the posterior class (*LsHox9*). We also have proposed that *empty spiracles* (and *orthodenticle*) and *caudal*, the two genes that share the YPWM motif with the Hox genes and are expressed at the extreme anterior and posterior ends of the embryo, respectively, might originally have been located at the ends of the cluster (12). Whether *LsCdx*, the *caudal* homolog, still forms part of the cluster (distal to *LsHox9)* remains to be determined. It is difficult to prove that a certain gene is absent from the cluster, but the available evidence would suggest that *LsHox5* may be missing from the cluster. *LsHox5* has not been identified in the screen, and a "chromosome walk" between *LsHox4* and *LsHox6* spanning 64 kb has not given any indication of a homeobox gene in between. However, all the *Hox* genes have to be mapped, and eventually the entire cluster will have to be sequenced to answer this point definitively. The possibility of a gene translocation also has to be considered. In fact, one additional *Hox* gene belonging to the *Ubd*-A (*Ubx/abdA*) group that is characteristic for the protostomes has been identified by using a different set of primers (A. Adoutte and G. Balavoine, personal communication). This gene would tentatively be assigned to *LsHox8*.

Because the branch point between nemerteans and chordates corresponds to the point of divergence between protostomes and deuterostomes, we propose that the last common ancestor of protostomes and deuterostomes, the urbilateria, had a *Hox* cluster with at least six or seven genes. The analysis of this cluster should provide further insights into the evolution of animal body plans. Even though *Lineus* is not easily amenable to genetic analysis, the extensive regeneration capacity and the development of grafting procedures (46) offer alternative possibilities for the study of *Hox* gene function in this animal.

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