GENETIC REGULATORY NETWORKS in Embryogenesis and Evolution

Proceedings
of a workshop
sponsored by
THE CENTER FOR ADVANCED STUDIES
IN THE SPACE LIFE SCIENCES
AT THE MBL

11–14 June 1997

Marine Biological Laboratory,
Woods Hole, Massachusetts

Funded by
THE NATIONAL AERONAUTICS
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CONTENTS

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Introduction

The participants in the workshop "Genetic Regulatory Networks in Embryogenesis and Evolution" gathered to consider the challenging and provocative problem of the evolutionary mechanisms that led to the appearance and diversification of animal body plans. They share the conviction that the developmental regulatory circuitry encoded in the genomes of modern invertebrate animals holds the keys to understanding metazoan evolution.

Detailed analyses of gene regulation in a wide variety of invertebrates, considered from a comparative perspective, occupied the attention of the participants. Michael Akam's group (Cambridge University) has established that, in *Drosophila,* it is the *cis*-regulatory system of the *Ubx* gene that determines exactly where the gene will be expressed. Michael Levine and his group (UC Berkeley) described recent extensions of their elegant analyses of the *cis*-regulatory mechanisms that are required to set the boundaries of expression domains in early *Drosophila* embryos. The first developmental *cis*-regulatory analysis ever carried out in a molluscan embryo, reported by Andre van Loon (Utrecht), revealed that negative controls are required to confine expression of a tubulin gene to the trochoblast lineage. Gary Ruvkun and his group (Harvard Medical School) reported progress in one of the most essential, but generally still unsolved problems in unraveling gene regulatory networks: *viz.,* how to find the downstream target genes of given transcription factors. Work from the Davidson group on sea urchins and the Levine group on *Drosophila* and ascidians has, along with other studies in vertebrates, led to the emerging general view that metazoan *cis*-regulatory regions are organized into modules wherein local interactions between negative and positive elements occur. In many cases, negative interactions are required to set boundaries. This view, in turn, provides a major insight into the evolutionary generation of novel developmental processes, by exchange, translocation, addition, or subtraction of *cis*-regulatory modules.

Among the most exciting themes of the workshop was phyletic homology in patterns of gene expression. An extensive search for genes that are expressed in the ascidian embryo and that also have homologs in other chordates and related invertebrates was described by Nori Satoh (Kyoto). Prominent among these are the transcription factors, *forkhead* and *Brachyury.* The Levine group reported that the transcriptional activation of *Brachyury* in ascidian embryos is a direct zygotic event in the specification of notochord cells. Satoh showed that *Brachyury* is activated late in embryogenesis at the anterior end of the gut in embryos of *Ptychodera,* an indirectly developing hemichordate; it is also expressed in the secondary mesenchyme that delaminates from the anterior end of the gut in sea urchin embryos.

Homologous cellular processes underlying embryogenesis in various taxa was another major theme of the workshop. Recent work on annelids from Marty Shankland's group (UT, Austin) and studies of nemerteans and flatworms by Jonathan Henry (University of Illinois) and Mark Martindale (University of Chicago) have illuminated the homologies—found throughout the Spiralia—that relate the contributions of the micromere quartet to axial symmetry. Martindale extended this comparative view of early embryonic development to the radially organized animals, cnidarians and ctenophores. The morphogenetic processes of later embryogenesis, invagination and ingression, are rapidly being characterized in molecular terms to the sea urchin embryo by Dave McClay's group (Duke University). The observation, from Chuck Ettensohn (Carnegie-Mellon), that secondary mesenchyme cells can replace the normal skeletogenic lineage of the sea urchin embryo suggests that embryonic skeletogenesis may really be a character of an adult body plan that has been, to different extents, heterochronically inserted into embryogenesis.

Participants in this workshop also focused on the general mechanism used by most invertebrate bilaterian groups to specify the fate of blastomeres during cleavage, largely in response to short-range signals from adjacent blastomeres. Dave McClay's group showed that beta-

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catenin is localized, in sea urchin embryos, to the nuclei of exactly those vegetal plate cells that express the vegetal plate marker *Endo16*. Joel Rothman (UC Santa Barbara) described the successive utilization of the *Notch* signaling pathway that specifies all eight sublineages descendant from the AB blastomere in *Caenorhabditis elegans*.

The experimental proceedings of the workshop were interlaced with theories, arguments, and speculations, which provided an unusual intellectual quality to the proceedings. Davidson, Cameron, and Peterson had previously proposed that the relatively rapid elaboration of the body plans of the large animals that have populated the Earth since before the Cambrian boundary was due to the appearance of populations of undifferentiated cells, set aside from the job of forming the embryo, and equipped with the prerequisite, regional specification capacity to pattern the adult. Davidson and colleagues now inferred further that the *Hox* gene cluster is not utilized in the development of the embryo or larva of a modern indirectly developing species, but is used at the stage when the adult body plan is generated from the larval imaginal rudiment; *i.e.*, from set-aside cells. Pedro Martinez and Cesar Arenas in Davidson's laboratory recently characterized the *Hox* gene cluster of *Strongylocentrotus purpuratus*, and measured the expression of these genes. None of the "anterior" *Hox* genes, nor some others, are expressed at all until the adult rudiment forms. Are these larvae indeed representative of the earliest metazoans in their regulatory characteristics? The flexible schedule, which left time for extensive discussion, provided the participants in this workshop with the luxury of being able to think in an evolutionary sense about discoveries in both gene regulation and the control of development.

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Evolution of Cleavage Programs in Relationship to Axial Specification and Body Plan Evolution

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We examine egg organization and the role of the early cleavage program in establishing the axial properties of larval and adult body plans. Here we review our own work and that of others on various invertebrate metazoans, including cnidarians, ctenophores, polyclad flatworms, and some protostome spiralians—nemerteans, molluscs, and polychaete annelids.

The Ctenophores

Most metazoan body plans can be characterized as having, either elements of radial symmetry (such as the Cnidaria and Ctenophora) or bilateral symmetry (*e.g.*, protostomes and deuterostomes). Cnidarians are considered to be radially symmetrical around their longitudinal body axis, called the oral-aboral axis, whereas ctenophores display "biradial symmetry" around their oral-aboral axis. Although their phylogenetic relationship to other metazoans remains controversial, ctenophores may represent the sister-group to the Bilateria. The transition from radial to bilateral symmetry can be viewed as one of the most important events in body plan evolution, and the study of the extant cnidarians and ctenophores may therefore help us to understand the evolution of the anterior-posterior and dorso-ventral axes of bilaterians.

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Virtually all bilaterian metazoan phyla undergo a stereotyped, species-specific cleavage program, but some basal metazoans (*i.e.*, sponges and most cnidarians) do not. This raises the question of how and why stereotypical cleavage programs evolved. One distinct feature of ctenophores, which sets them apart from other radially symmetrical forms, is their phylum-specific, highly stereotyped mode of development (see 1–3 for reviews). Previous work has demonstrated that the cleavage program is causally involved with the establishment of cell fates in the ctenophore (4–6), and that the capacity to replace structures derived from missing blastomeres ("mosaic development") is lacking. For example, previous chalk-particle marking experiments indicated that the eight rows of comb plates in ctenophores are derived from the four e_1 micromeres of the 16-cell embryo (7), and deletion of these four micromeres results in the absence of ctene rows and their associated endodermal canal system (8–10).

Using intracellular cell lineage techniques on embryos of the lobate ctenophore *Mnemiopsis leidyi,* however, we showed that the m_1 micromeres (Fig. 1A) also contribute to comb plate formation during normal development. Thus, if comb plates do not form after e_1 micromere removal, then some blastomere fates in the early embryo must not be precociously specified at the time of their birth, as previously argued. Rather, inductive interactions by the descendants of the e_1 micromeres organize (Fig. 1B) development in adjacent ectodermal and endodermal lineages (11, 12). We suggest that stereotypic cleavage programs arose during metazoan evolution as a reliable means of segregating factors to distinct embryonic lineages, some of which serve as inductively active "signaling centers." These signaling centers or-

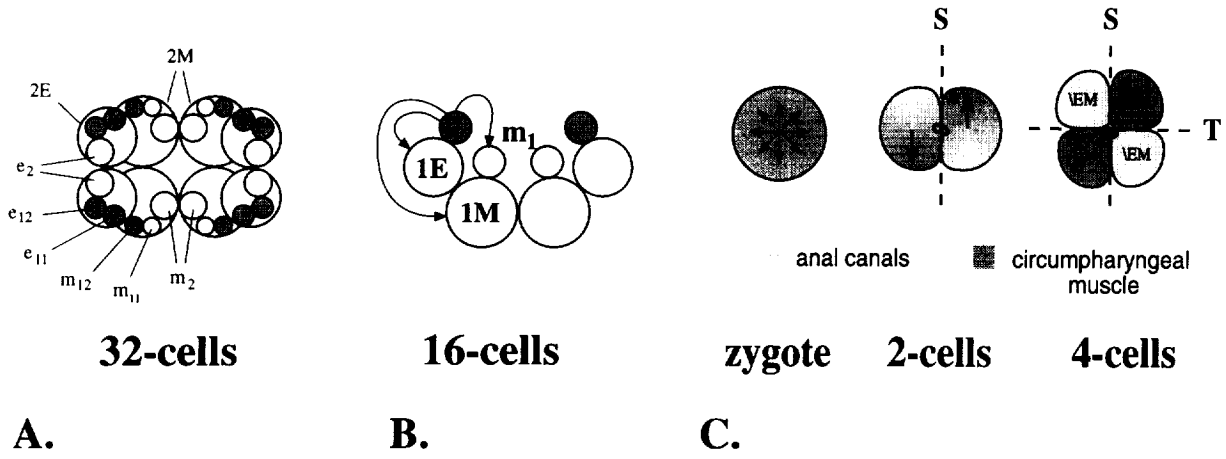

Figure 1. Views of the ctenophore embryo at various stages of development. (A) Aboral view of a 32-cell stage embryo. The shaded cells indicate the blastomeres which normally give rise to comb plates. Note that only one of the two daughter cells of the m_1 micromere makes comb plates (m_{12}). Previous work failed to detect any contribution from the 'M' lineage. (B) Lateral view of a 16-cell stage embryo. The arrows indicate that the e_1 micromeres (shaded) are required to induce comb plates from m_1 micromere lineages (unshaded) and for endodermal drivatives from the 1E and 1M macromeres. (C) Oral views of the changing axial properties. Following fertilization the embryo is radially symmetrical around the preumptive oral-aboral axis. By the 4-cell stage two distinctly different types of blastomeres are present. Only one pair, the \EM blastomeres, will give rise to the endodermally derived anal canals, while the other pair, /EM, will give rise to muscle cells around the pharynx. This means that these blastomeres become polarized in opposite orientations sometime before the end of second cleavage.

ganize subsequent development in adjacent lineages. Stereotypic cleavage patterns are a means of reliably positioning these organizing centers and the cells that respond to their signals. Other cells may be determined by autonomous mechanisms. These strategies for early patterning are prevalent in many metazoan embryos (13, 14). Although the molecular nature of inductive signals in ctenophores is unknown, several known pathways, such as those involving wg/β-catenin, are reasonable places to start looking.

Most authorities believe that bilaterally symmetrical organisms evolved from a radially symmetrical ancestor. Little is known about how this transition occurred. For example, no agreement about the relationship between the oral-aboral axes of cnidarians and ctenophores and the anterior-posterior axis of bilaterians has been reached. The apparent conservation in the molecular mechanisms leading to the establishment of the dorsoventral axis in the common ancestor of protostomes and deuterostomes (the *dpp/sog* orthologs) adds to speculation that an existing axis was co-opted for the dorso-ventral axis. On the other hand, this axis may have arisen *de novo* (15). We have shown in ctenophores that some mesodermal and endodermal lineages are organized in a pattern that is diagonally opposed to the first and second cleavage planes (Fig. 1C). Because these lineages give rise to the oral-anal plane, this organization could reflect a transition from a radial to a bilaterally symmetrical body plan (16). We propose different scenarios for generating these changes in body plan organization based on the expression of highly conserved developmental regulatory genes.

The Spiralians

The highly stereotypic cleavage pattern referred to as "spiral cleavage" occurs in most of the extant invertebrate phyla, including the molluscs, annelids, vestimentiferans, pogonophorans, echiurans, sipunculids, nemerteans, gnathostomulids, mesozoans, and polyclad turbellarians. Cell lineage analyses, mainly conducted on annelidan and molluscan embryos, suggested that the ultimate fates of blastomeres are tremendously conserved.

More recently, we have been examining the development of representatives from a number of different spiralian phyla to determine the extent of homologies in the spiralian developmental program. For instance, our work in collaboration with Barbara Boyer has confirmed earlier reports that the polyclad flatworms display a cell lineage fate map similar to that of the annelids and molluscs (17, 18, 19). Early investigators suggested that the acoelomate platyhelminthes (flatworms) are basal to the bilaterian metazoans, but more recent phylogenetic analyses place

them as basal members of the protostome spiralians (20, 21). In either case, the developmental pattern exhibited by the polyclads should be more closely representative of the basal condition within the Spiralia.

We have also demonstrated that the Nemertea, a coelomate invertebrate phylum, also exhibits strong homologies to the basic spiralian cleavage program. In addition to possessing cell quadrant identities similar to those found in other spiralians (*i.e.*, A, B, C, and D quadrants), the embryos also give rise to both ecto- and endomesoderm (22, 23). Although the general spiralian developmental program is highly conserved in this group, it does exhibit some modifications in the form of what Lillie referred to as "adaptations in cleavage" (23). These include the formation of a first quartet of micromeres of greatly increased size that generates the majority of the larval ectoderm. Other changes have occurred in the sublineages that give rise to certain structures, such as the ciliated band (derived from first, second, and third quartet derivatives) and the ectomesoderm (derived entirely from 3a and 3b).

The most significant modifications in Nemertea of the spiralian developmental program seem to have affected the mechanisms involved in cell fate and axis determination. Those employed by the nemerteans appear to be distinct from those utilized by equal-cleaving molluscs (24, 25), and differences are encountered between different nemertean species. Our research indicates that larval nemertean axial properties are actually specified before cleavage begins, a condition that does not appear to take place in the embryos of equal-cleaving molluscs (24). Furthermore, the embryos of the indirect-developing nemertean *Cerebratulus lacteus* appear to exhibit a great deal of regulation, while those of a direct-developing species, *Nemertopsis bivittata,* do not (25). We believe that nemerteans exhibit a derived developmental condition, and agree with previous reports that the ancestral spiralian developmental condition was one in which equal, quartet spiral cleavage occurred, and quadrant fates and axial properties were established epigenetically *via* inductive interactions (26).

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Changes in Cell Lineage Specification Elucidate Evolutionary Relations in Spiralia

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Introduction

Comparative embryology of the various body plans, and an understanding of the molecular regulation of the establishment of these body plans, are powerful tools that can help us reconstruct the evolutionary relations between the animal phyla. The first Metazoa were undoubtedly radially symmetrical animals with two germ layers: ectoderm and endoderm. Examples of these diploblastic creatures can still be found today, in marine and fresh waters throughout the world. The evolution of a third germ layer, the mesoderm, led, in part, to the great Precambrian radiation of the animal kingdom. Elucidation of the developmental mechanisms underlying the formation of mesoderm could shed light on the evolutionary relations among different phyla. Whether this third germ layer evolved once or developed convergently in a number of ancestral diploblastic forms remains to be demonstrated.

Our recent research has focused on the development of two cell lines typical of the Spiralia (*i.e.*, phyla with spiral cleavage): (1) the stem cell of the mesodermal bands (the mesentoblast) and (2) the trochoblasts. The mesentoblast was chosen because the origin of mesoderm is consistently similar in different Spiralia. The mesentoblast is formed from a single primary endodermal cell that is induced to follow a developmental program different from the other endodermal cells. After induction, the mesentoblast divides and gives rise to the stem cell of the left and right mesodermal bands. A comparative study of mesentoblast formation may be used to elucidate the evolutionary relations within the Spiralia.

Trochoblasts were analyzed because this cell line can be found in a number of Spiralia. These cells are ectodermally derived and form the prototroch—the larval locomotory organ typical of such spiralians as molluscs and annelids but absent in other spiralians like the nemerteans and flatworms.

In this paper, we discuss the molecular and developmental aspects of trochoblast and mesentoblast formation and their significance to the analysis of the phyletic relations between spiralian phyla.

Trochoblasts

The trochoblasts constitute the first fully specified cell line in a number of spiralian embryos (1). Detailed knowledge of trochoblast specification, however, is limited to *Patella vulgata*, the common limpet. Specification in *Patella* requires that the third cleavage is executed correctly (2); if this cleavage is inhibited, no trochoblast-specific gene expression will occur. Trochoblast specification is completed after the fourth cleavage; thereafter the trochoblasts divide only twice more and then differentiate into ciliated cells. From the fourth cleavage onward, specification is autonomous: *i.e.*, cells isolated from the 16-cell embryo go through two cleavages, enter a division arrest, and become ciliated, just as in the intact embryo.

To investigate the molecular mechanism of trochoblast specification, we first focused on genes encoding tubulin as part of this process. Trochoblasts bear a large number of cilia which, in turn, are mainly composed of tubulin. *In situ* hybridization revealed that tubulin genes are expressed one cell cycle before the last division of the trochoblasts (3). One of the tubulin genes that we cloned from the *Patella* genome appeared to be trochoblast spe-

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cific. The promoter of this gene was coupled to the *Lac-Z*-reporter gene, and the construct was injected into 2-cell embryos. Expression of the reporter gene appeared to be limited to the trochal cells, and began about 30 minutes after the appearance of tubulin mRNA (3). Extensive mapping in the promoter showed that only a small region, between −108 and −1 with respect to the transcription start, is absolutely required for correct expression (4). In this region, two elements—located between −108 and −68 and between −52 and −42—serve different functions in establishing correct spatiotemporal gene expression. Mutation of the −108/−68 element results in expression of the reporter gene in non-trochoblasts. Mutation in the −52/−42 region completely abolishes expression of the reporter gene. In addition to these two elements, two others located in the regions −418/−108 and +1/+487, are required for correct expression; these latter elements can be located either before or after the −108/−1 region. We therefore consider the region −108/−1 to be the core of the promoter.

Nuclear proteins from different stages of development were isolated and a southwestern blot performed; the core region was used as a probe. Each stage shows a specific array of proteins binding to this core region (A. H. E. M. Klerkx and A. E. van Loon, unpub. data). We therefore assume that at different times in development, different proteins bind to the core region.

As trochoblasts are not exclusively formed in gastropods, but also in other molluscan classes, the *Patella* tubulin promoter was coupled to the *Lac-Z* gene and injected into embryos of representatives of other classes of molluscs. Embryos of a polyplacophoran (*Acanthochiton*) and a scaphopod (*Dentalium*) showed an expression pattern completely comparable with that in *Patella* (A. H. E. M. Klerkx, W. G. M. Damen, A. E. van Loon, and J. A. M. van den Biggelaar, unpub. data). Thus, the molecular mechanism for the regulation of a trochoblast-specific gene is conserved in representatives of different molluscan classes.

The spiralian taxon Annelida is presumed to include the closest relatives of the molluscs, and these worms form trochoblasts that originate from the same cell line as in the molluscs. The tubulin promoter gene construct therefore was injected into embryos of the polychaete annelid *Platynereis*. Seven of the resulting embryos survived to the trochophore stage. One of these showed expression, and that expression was limited to trochoblasts. Injections of the construct into a large number of embryos of another polychaete annelid, *Nereis*, have not resulted in *Lac-Z*-expression.

Nemerteans do not develop into larvae with a prototroch, but are supposed to be ancestral to the molluscs and annelids. We therefore examined the expression of the *Patella* tubulin promoter construct in nemertean embryos (*Cerebratulus lacteus*). Expression was found, but was not restricted to a specific domain of the 24-h larvae. Similarly, embryos of another spiralian taxon, the flatworms, do not develop trochophore larvae. A small number of embryos of the polyclad flatworm *Hoploplana* were injected with the construct, and no expression was found.

The molecular aspects of trochoblast-specific gene expression in molluscs have been conserved in the Polyplacophora, Scaphopoda, and Gastropoda. As the trochoblasts arise from the same cells in molluscs and annelids, we conclude that they are spiralian phyla with a close evolutionary relationship. The conservation, in molluscs and annelids, of the molecular mechanism regulating the expression of a trochoblast-specific gene needs further support. On the other hand, nemerteans and flatworms do not share the formation of trochoblasts, but nemerteans seem to have the molecular mechanism that is required for a cell-specific expression of the *Patella* trochoblast-specific gene. We therefore consider nemerteans, as well as flatworms, to be more distantly related to molluscs and annelids.

Mesentoblast

In many Spiralia, the most important contribution to the mesoderm derives from the mesentoblast, which produces the two mesodermal bands. In ancestral molluscs, the mesentoblast arises from a primary endodermal cell after an inductive interaction with micromeres in the animal hemisphere (5). This induction also establishes the plane of bilateral symmetry and dorsoventral polarity.

In embryos of *Patella,* the endodermal macromeres 3A–3D extend in the animal direction and make contact with the ectodermal micromeres of the opposite animal pole. Of these macromeres, only one maintains these contacts. This macromere becomes the mesentoblast precursor cell (5). Previous work on the development of the dorsoventral axis and mesentoblast formation in a number of gastropod families has shown that gastropod evolution has been accompanied by a heterochronic shift in mesentoblast formation (6, 7, Figure 1). In a series of gastropods, from Archeogastropoda to Pulmonata, mesentoblast formation is shifted from late cleavage stages to much earlier developmental stages.

In annelid embryos a similar extension of the macromeres 3A–3D occurs during the interval between the fifth and the sixth cleavage. But no single macromere is centralized. Despite the uniform contact that these cells have with the micromeres, one is induced to produce the mesentoblast. This inductive event also establishes the dorsoventral axis. A heterochronic shift in the specification of the dorsal quadrant, comparable to that found in gastropods, is also found in annelids (8).

Specification of the dorsoventral axis and mesentoblast

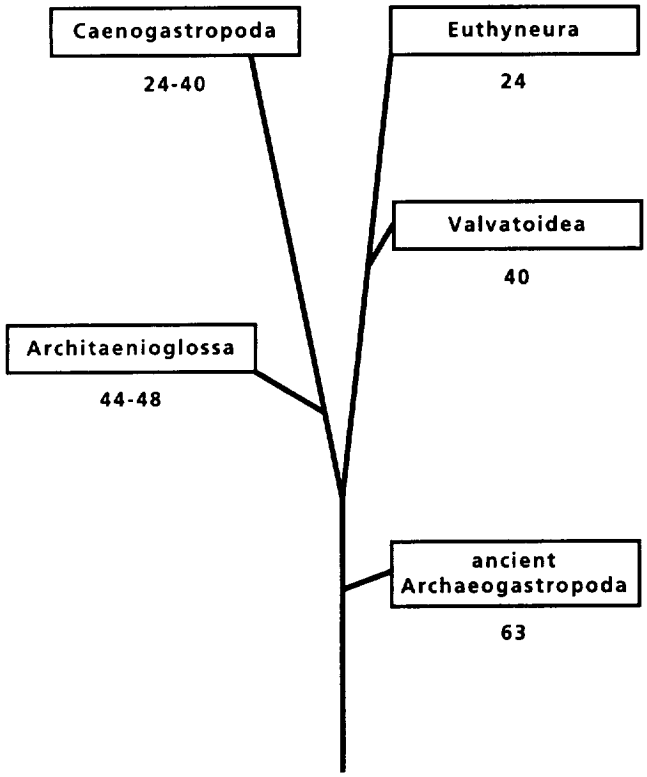

Figure 1. Phylogenetic relations among different gastropod taxa based upon the number of cells in the embryo at the time of the mesentoblast (3D) division (the number of cells is written under the boxed taxon name). The mesentoblast divides into the mesodermal stem cell (4d) and an endoderm precursor (4D). Caenogastropods, together with the Architaenioglossa, probably constitute a separate group, as they form polar lobes during at least the first two cleavages. (After van den Biggelaar and Haszprunar, 1996, ©Allen Press, used with permission)

formation in nemertean and flatworm embryos show similarities and differences compared to molluscs and annelids and to each other. The nemertean embryo is not divided into dorsal, ventral, right, and left quadrants, but into two dorsolateral and two ventrolateral quadrants (9). Despite this alternative quadrant arrangement with respect to the first cleavage planes, bandlets of mesenchymal cells seem to be derived from the same endomesodermal cell (4d) as in annelids and molluscs (10). Like molluscs and annelids, flatworm embryos are also divided into dorsal, ventral, and two lateral quadrants; the specification of the dorsal quadrant, however, must be different. After the formation of the fourth quartet of micromeres, the micromeres 4a–4d extend in the animal direction, in contrast to the macromeres 3A–3D in molluscan and annelid embryos. Finally, it is the micromere of the ventral quadrant (4b) that maintains the contacts with the animal micromeres (van den Biggelaar, unpub. obs.). Micromere 4d of the opposite dorsal quadrant then develops the mesentoblast. These differences in mesentoblast formation between annelids, molluscs, and nemerteans on the one hand, and flatworms on the other hand, again demonstrate that molluscs, annelids and nemerteans are more close related than with flatworms. The differences between nemerteans and the other two phyla (annelids and molluscs) with respect to the dorsoventral axis specification would argue that annelids and molluscs are more closely related to each other than either is to the nemerteans.

Conclusion

Resemblances in mesentoblast specification and the conservation of the regulatory mechanisms of a trochoblast-specific gene in three different classes of molluscs are consistent with the idea of a monophyletic origin of the molluscs. Trochoblast and mesentoblast specification (coupled to dorsoventral axis formation) in molluscs and annelids strengthens the idea of a close phylogenetic relationship between these phyla. Nemerteans and flatworms have distinct modes of dorsoventral axis formation and do not have a trochoblast cell line, excluding a close evolutionary relation with annelids and molluscs as well as with each other.

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Axial Patterning in the Leech: Developmental Mechanisms and Evolutionary Implications

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The phylogenetic history of animal body plans, particularly those of the segmented protostomes (arthropods + annelids), is one of the most important topics in the current study of the evolution of developmental mechanisms. Genetic studies of the fruitfly *Drosophila* have uncovered a wealth of information about the molecular biology of development, but the degree to which other animals utilize similar or different mechanisms is not entirely clear, nor is it obvious how and when the mechanisms observed in *Drosophila* first arose during evolution.

Glossiphoniid leeches, such as *Helobdella*, offer numerous advantageous features for embryological research. *Helobdella* embryos are highly amenable to 'classical' embryology: the eggs are large, undergo stereotyped cell lineages, and single cells can be identified and manipulated during the developmental stages when segmentation is being established (1). The segmental body plan of the leech is generated through the iterative cell divisions of teloblastic stem cells situated in a posterior growth zone (2)—a process so outwardly different from what is known of segmentation in insects or vertebrates that a detailed comparison is likely to be informative. Comparing the cellular and molecular mechanisms that underlie pattern formation in different taxa can reveal those aspects of a process that are likely to be homologous or convergent, and can thereby yield significant insight into the evolutionary origin of patterning mechanisms such as segmentation.

This paper was originally presented at a workshop titled *Genetic Regulatory Networks in Embryogenesis and Evolution.* The workshop, which was held at the Marine Biological Laboratory, Woods Hole, Massachusetts, from 11 to 14 June 1997, was sponsored by the Center for Advanced Studies in the Space Life Sciences at MBL and funded by the National Aeronautics and Space Administration under Cooperative Agreement NCC 2-896.

The research in our laboratory has addressed various aspects of pattern formation in *Helobdella.* First, we have examined the establishment of symmetry properties in the unsegmented head and the segmented trunk of the leech. Consistent with the traditional view of spiralian development, the teloblastic stem cells of the posterior growth zone—which generate all of the segmented ectoderm and mesoderm—derive exclusively from the D quadrant of the early 4-cell embryo, with the eventual plane of bilateral symmetry bisecting the derivatives of that quadrant (2). In contrast, lineage tracer analysis of the first quartet of micromeres—progenitors of the unsegmented head ectoderm—reveals that the plane of right-left symmetry falls between bilaterally homologous A and D quadrant derivatives on the left of the embryo, and homologous B and C quadrant derivatives on the right (3). This disparity between the symmetry properties of the first quartet micromeres in the head and the D quadrant derivatives in the trunk is schematized in Figure 1. These results are, in fact, quite consistent with classical studies of polychaete annelids—overlooked by generations of subsequent reviewers—in which the symmetry of the embryonic quadrants was shown to alternate back and forth by 45° between the derivatives from each successive round of micromeres (4, 5).

A second area of interest is the genetic basis of the distinction between the head and the trunk. Studies of fruitflies and mice have shown that a certain group of genes (*otd* and *ems* in flies, and their vertebrate orthologues *Otx* and *Emx*) encode transcription factors that are expressed predominately in head structures—anterior to the boundary of Hox gene expression—in both of these disparate taxa (6). We have cloned and sequenced a *Helobdella* orthologue of *otd* (called *Lox22-Otx*) and, using *in situ* hybridization, have found that it is expressed in every major part of the unsegmented head, including

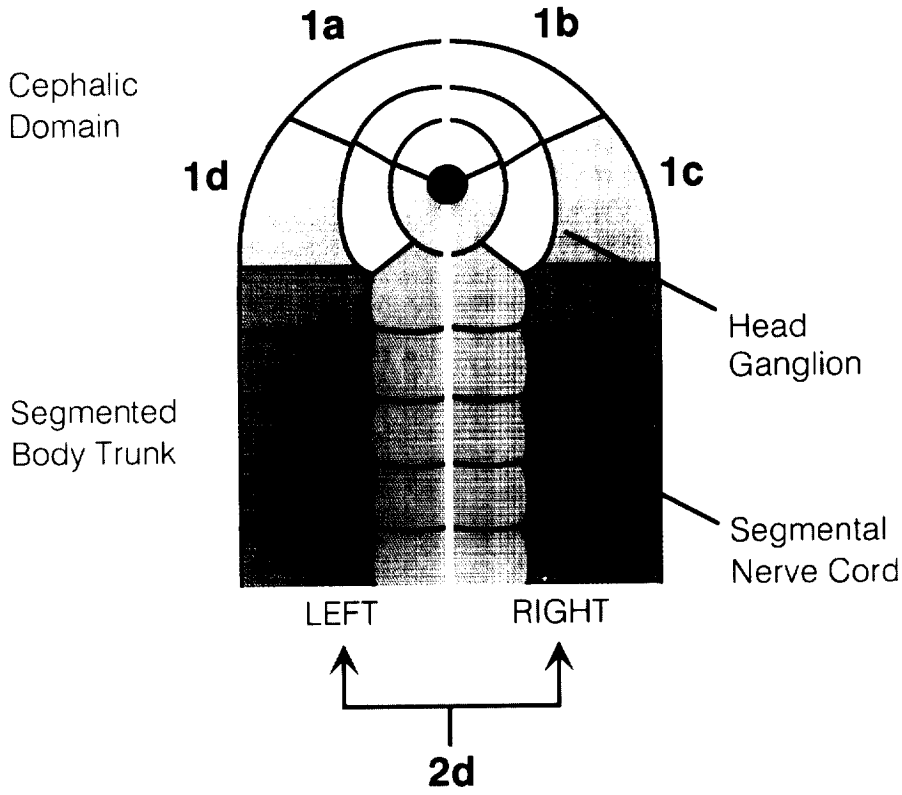

Figure 1. Differing symmetry properties in the progeny of different micromere quartets. Schematic diagram of clonal contributions to ectodermal tissues—body wall and central nervous system—at the head end of the *Helobdella* germinal plate, shown in dorsal view. Four primary micromere clones are shown in shades of orange, with the embryonic mouth or stomadeum situated at the point where the four clones meet. Note that the plane of bilateral symmetry falls between the 1a and 1d clones on the left and the 1b and 1c clones on the right. The definitive ectoderm of the segmented body trunk (turquoise) derives exclusively from the 'micromere' 2d, otherwise known in leeches as cell DNOPQ. In the trunk, the plane of bilateral symmetry thus bisects the derivatives of the D quadrant.

many derivatives of the first quartet of micromeres (7). In contrast, *Lox22-Otx* is not detectably expressed in the body wall of the segmented trunk, and is expressed in only two pairs of segmentally iterated CNS neurons, similar to what is seen in *Drosophila* embryos. This finding further supports the idea that head and trunk were genetically distinct body domains by an early stage in the bilaterian radiation (6).

One possible evolutionary scenario is that the *otd/otx* genes were originally involved in the pattern formation of a radially symmetric pre-bilaterian ancestor, in which they were very likely expressed in patterns concentric about the oral-aboral axis. In the Bilateria, the expression of these genes could then have been relegated to the head by the addition of a trunk domain whose developmental patterning relied on the cooptation or expansion of other genetic pathways. If this model is correct, then the radial organization of micromere cell lineages (3) and *Lox22-Otx* expression (7) that we find around the mouth of the *Helobdella* embryo could be interpreted as the remnants of a radially organized pre-bilaterian body plan, remnants that have not yet been obscured by the later addition and expansion of trunk-patterning mechanisms.

A third line of research focuses on the analysis—through cloning and expression—of *Helobdella* genes orthologous to known *Drosophila* genes involved in segmentation and segment identity. Most of our work to date has focused on the leech Hox genes (8–10). The leech, like many higher animals, has a number of Hox genes that show segmentally restricted patterns of expression; and the ordering of those expression domains along the anteroposterior body axis corresponds closely with the ordering of their orthologues in other species. The most anteriorly expressed leech Hox gene—the *labial* orthologue *Lox7*—is expressed in all segmental ganglia, but not in the unsegmented head region (10). The majority of leech Hox genes that have been characterized to date are expressed only during the later stages of development, primarily in neurons and mesodermal derivatives that are undergoing terminal differentiation. In addition, cell

transplantation studies have indicated that the segmental founder cells of the leech ('primary blast cells') already possess an intrinsic segment identity several days prior to the onset of Hox gene expression, and will express that original identity—including segment-specific Hox expression—even if their descendant clones are forced to develop in inappropriate segments (9, 11). These findings suggest that, in leeches, the expression of most Hox genes is involved only in the later stages of segmental diversification, and not in the initial establishment of segment identity. However, there are at least two leech Hox genes that do show earlier expression patterns, and their potential significance for segment identity specification is still under investigation.

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Hox Genes in Arthropod Development and Evolution

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The Hox genes have become a paradigm for the conservation of developmental mechanisms throughout the animal kingdom. They encode transcription factors that act as molecular markers for the position of cells along the major body axis (1). Individual Hox genes are activated at different positions in the early embryo, establishing a pattern that is maintained throughout much of development. This differential expression has been shown to control the development of region-specific structures in nematodes, arthropods, and chordates, and may be a shared characteristic of triploblastic metazoan animals.

Hox genes and the diversity of development within insects

The *Drosophila* Hox cluster also contains homeobox genes that have no close homologues in other species. These genes, *bicoid, zen,* and *fushi-tarazu* (*ftz*), serve roles in development different from those of the canonical Hox genes. All are involved in establishing the body plan during the early syncytial stage of *Drosophila* development.

Syncytial development is not universal within the insects. Many lower insects make much or all of their segment pattern after cellularization—a point made particularly clear by recent studies of the grasshopper, *Schistocera,* which show that the blastoderm becomes cellular even before the aggregation of cells to form the embryonic primordium (2). Yet more remarkable is the diversity of development among the parasitic Hymenoptera (3). Species within the same family may exhibit extreme differences in early embryogenesis: The Braconidae contain ectoparasitic species, laying yolky eggs that show syncytial development similar to that of *Drosophila,* and endoparasitic species that lay small, yolk-free eggs that undergo total cleavage and "short germ" patterning (M. Grbic and M. Strand, unpub. data).†

It is now clear that at least two of the atypical *Drosophila* Hox genes—*zen* and *ftz*—derive from ancient members of the Hox cluster that have evolved particularly rapidly within the insect lineage (4, 5). We suggest that the developmental role of these genes changed in the ancestors of the insects, with a loss of many of the functional constraints that act on the 'canonical' Hox genes.

Hox genes, homeosis, and the evolution of segment identity

It has long been suggested that mutation in the Hox genes may contribute to morphological diversity of the arthropods (6), but this idea has been criticized on the grounds that dramatic homeotic transformations could not possibly contribute to natural evolution because such changes in form would never be selectively advantageous. Recent changes in our understanding of the role and regulation of Hox genes provide a way out of this dilemma.

In the 1970s and 80s, genetic analysis suggested that the Hox genes served to give an identity to all cells in a segment. This was interpreted to mean that Hox genes were ubiquitously and uniformly expressed in whole segments, under 'monolithic regulation'. It is now clear that the regulation of the Hox genes is more complex (7). Whether or not a given gene will be active in a particular segment is defined in the early embryo, by signals that make certain of the Hox regulatory domains "open for business." However, each of these regulatory domains

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† See addition to Literature Cited.

has a complex modular structure, like that of other patterning genes. In later development, these modules are regulated independently in different cell types and stages of development, even within a single segment. Moreover, this detailed regulation is important for the specification of segment identities. A single Hox gene can specify the development of several different segment types, and our studies on the *Ultrabithorax* gene show that this is in large part dependent on the precise spatial and temporal regulation of the gene (8). Changing this pattern of *Ubx* regulation within segments can alter fine details of segment development (9). Recent work in my laboratory (D. Stern, unpub. data)‡ and elsewhere (10) suggests that allelic variants that affect *Ubx* function can also be observed in natural populations.

From this perspective, it is easy to understand how gradual changes in the regulatory elements of Hox genes may contribute to the evolution of segment morphology. Summed over time, such changes may lead to differences in Hox gene function between species that would be comparable to the effects of overt homeotic mutations—even though no such mutations need ever have been fixed.

To test whether such changes have occurred, we initiated a survey of Hox gene structure and expression in diverse arthropods. Orthologs of all the *Drosophila* Hox genes can be identified in Crustacea and Myriapods (11 and M. L. Smith, unpub.) Surprisingly, three genes that define the identity of diverse trunk segments in insects (*Antp, Ubx,* and *abd-A*) are all expressed throughout the thorax of the branchiopod crustacean *Artemia,* but are not expressed in the postgenital abdomen (12). If the Hox genes can be used as markers for homologous segments, these differences suggest a novel relationship between body regions in insects and Crustacea (13).

Within the Crustacea, a wider taxonomic survey of a single class of Hox gene products reveals a striking correlation between the boundaries of Hox gene expression and the diversity of segment types (14). Many basal crustacean lineages have an array of similar thoracic segments, and *Ubx/Abd-A* class Hox genes are typically expressed in all of these segments. In other, more derived crustacean lineages, the most anterior thoracic segments are transformed into maxillipeds—supplementary feeding appendages that resemble in part the gnathal segments. In these segments, Ubx/abd-A proteins are not expressed. It seems that the alteration in segment organization has been achieved by shifting the domains of Hox gene expression relative to a conserved array of segments.

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Heterochronic Genes in Development and Evolution

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Heterochrony is an evolutionary term that describes the comparatively common phylogenetic variation between species in the relative timing of developmental events. Heterochronic variation has also been induced by mutation to identify genes that regulate the timing of developmental events (1, 2). Genes that control the temporal dimension of development, heterochronic genes, can be thought of as the temporal analogs of the homeotic genes, which regulate spatial dimensions (*e.g.*, anterior-posterior, dorsal-ventral axes) during development of metazoans. These pathways generate graded or binary levels of regulatory factors that pattern one axis of the developing animal. Heterochronic genes may be the target of mutations that cause heterochronic change in phylogeny. In the nematode *Caenorhabditis elegans,* heterochronic genes mediate the temporal pattern of stage-specific expression of cell fates. Correct timing of many stage-specific developmental events depends on the time-dependent decrease of the LIN-14 and LIN-28 proteins, two key regulatory factors that promote early larval fates (3, 4, 5). Their decrease is thought to be the result of the time-dependent increase in the LIN-4 RNA, which binds to the mRNAs of both *lin-14* and *lin-28* and somehow inhibits their translation (6, 7). LIN-14 [a novel protein (3)] and LIN-28 [an RNA binding protein (5)] function at the first (L1) and second (L2) larval stages respectively, to prevent the premature activation of LIN-29 (8). LIN-29 is a transcription factor induced at the L4 stage that is required for adulthood. Null mutations in *lin-14,* for example, result in the activation of LIN-29 one stage too early, with the result that certain adult features are precociously expressed in larval stages. Additional, as yet unknown, heterochronic genes are postulated to function in the genetic pathway between LIN-14/28 and LIN-29. Hormonal control of developmental timing is a common theme throughout phylogeny. For example, heterochronic mutations that involve hormonal signaling have been identified in vertebrates as well as *C. elegans* (9).

The level of LIN-14 protein forms a temporal gradient that specifies stage-specific cell lineages during development of *C. elegans.* Mutations that perturb this level perturb the temporal sequence of cell lineages. LIN-14 is a nuclear protein, but is not homologous to any known protein. To experimentally establish how graded LIN-14 levels act to specify stage-specific cell fates (including the mechanism used by *lin-14* to control downstream genes, *i.e.*, transcription, splicing, etc.) we are identifying factors that mediate *lin-14* action. We expect these to include targets of *lin-14,* as well as factors that act in combination with LIN-14. We have used a genetic analysis to identify genes that function downstream of *lin-14* in the heterochronic pathway. We have isolated suppressors of two heterochronic mutants that respectively result in opposite heterochronic phenotypes, precocious *lin-14(lf)* mutants and retarded *lin-4(lf),* mutants. Some of these suppressor mutations define new heterochronic genes. We have also succeeded in using epistasis analysis to order various other known heterochronic genes into the heterochronic pathway.

In addition, we have identified mutations in the *let-7* gene that result in a retarded heterochronic phenotype and partially suppress the precocious phenotypes of *lin-14(lf)* mutations. We have also identified mutations in the gene *lin-41* which display a precocious phenotype and partially suppress the retarded phenotypes of *let-7* mutations. We have mapped these genes and are currently attempting to clone them via transformation rescue, as well as to order

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them into the heterochronic pathway using epistasis analysis. This molecular analysis should reveal additional aspects of this pathway, and may hint at the molecular function of LIN-14.

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A Common Theme for LIM Homeobox Gene Function Across Phylogeny?

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The identification of the molecular components of the developmental neurogenic programs in different organisms has revealed an astounding degree of conservation across phylogeny, suggesting that the basic mechanisms of neural development have also been conserved in evolution. One class of conserved neural regulatory genes, the LIM homeobox genes, encode transcription factors with two Zn-finger-like LIM domains and a DNA-binding homeodomain (1). Vertebrate members of this class have been implicated in neurogenesis by correlative expression evidence; *e.g.*, the combinatorial expression of LIM homeobox genes in the vertebrate spinal cord suggested a "LIM-code" for specific motorneuronal targeting choices (2). Genetic analysis in *Drosophila* also demonstrated their essential role in axon pathfinding and the determination of neurotransmitter identity (3, 4).

The genome of the nematode *Caenorhabditis elegans* is almost completely sequenced, thus allowing the analysis of complete gene families in a metazoan organism. *C. elegans* contains seven LIM homeobox genes. Almost all *C. elegans* LIM homeobox genes fall into subclasses that are defined by the presence of similar genes from arthropods and vertebrates, suggesting a common origin for different subclasses of LIM homeobox genes (Fig. 1; *C. elegans* proteins are underlined).

This paper was originally presented at a workshop titled *Genetic Regulatory Networks in Embryogenesis and Evolution.* The workshop, which was held at the Marine Biological Laboratory, Woods Hole, Massachusetts, from 11 to 14 June 1997, was sponsored by the Center for Advanced Studies in the Space Life Sciences at MBL and funded by the National Aeronautics and Space Administration under Cooperative Agreement NCC 2-896.

Function of the *C. elegans ttx-3* and *lin-11* homeobox genes

We recently described the function of two *C. elegans* LIM homeobox genes, *ttx-3* and *lin-11*, in a neural circuit subserving thermoregulatory behavior (5, 6, 7). The neural pathway for thermotaxis includes the sensory neuron AFD and the connected interneurons AIY and AIZ (Ref. 5; see Fig. 2). The *ttx-3* null mutation causes the same behavioral defect as laser ablation of AIY, implying that AIY does not signal in this mutant (5). A *ttx-3*-GFP reporter construct shows that *ttx-3* is expressed exclusively in the AIY interneuron pair (6). AIY is generated in *ttx-3* mutants, arguing that no fundamental changes in cell fate have taken place. However, AIY exhibits abnormal axonal projections, manifested mainly by the outgrowth of additional small neurites. These defects could be due to misregulation of *ttx-3* downstream target genes involved directly in axonal pathfinding, or they could be due to misregulation of *ttx-3* downstream target genes involved in synaptic signaling, which could, as a secondary consequence, cause axonal sprouting defects.

ttx-3 is continuously expressed in AIY from mid-embryogenesis throughout adulthood and is required to maintain its own expression, suggesting that *ttx-3* may also act in a neural maintenance pathway for AIY. Considering that thermotactic behavior manifests a simple learning and memory task, AIY represents a prime candidate for an interneuron that integrates and memorizes sensory inputs, for example by variable patterns of synaptic connections. We consider the possibility that *ttx-3* is part of an autoregulatory loop that regulates the initial expression of downstream target genes involved in neural

signaling and that may also modulate downstream gene expression in behavioral plasticity (6).

We have identified a second LIM homeobox gene, *lin-11*, that is expressed and functions in the opposing interneuron of the thermoregulatory circuit, AIZ (7). *lin-11* null mutant animals display cryophilic defects that phenocopy laser ablation of the AIZ interneuron. Although the *lin-11* expressing neurons, including AIZ, are formed in *lin-11* null mutant animals, they display neuroanatomical defects, comparable to those neural defects observed in *ttx-3* mutant animals. Like *ttx-3*, *lin-11* expression is also maintained in postmitotic neurons throughout adulthood. Thus, distinct LIM homeobox genes specify two functionally related antagonistic interneurons within a neural network dedicated for thermoregulatory processes (see Fig. 2).

How are thermoregulatory neural centers organized in more complex organisms? And is there any evidence for a conserved role for *ttx-3* and *lin-11* in the control of these neural centers? In fact, the organization of the *C. elegans* thermoregulatory network into two parallel, warm- and cold-processing pathways is remarkably simi-

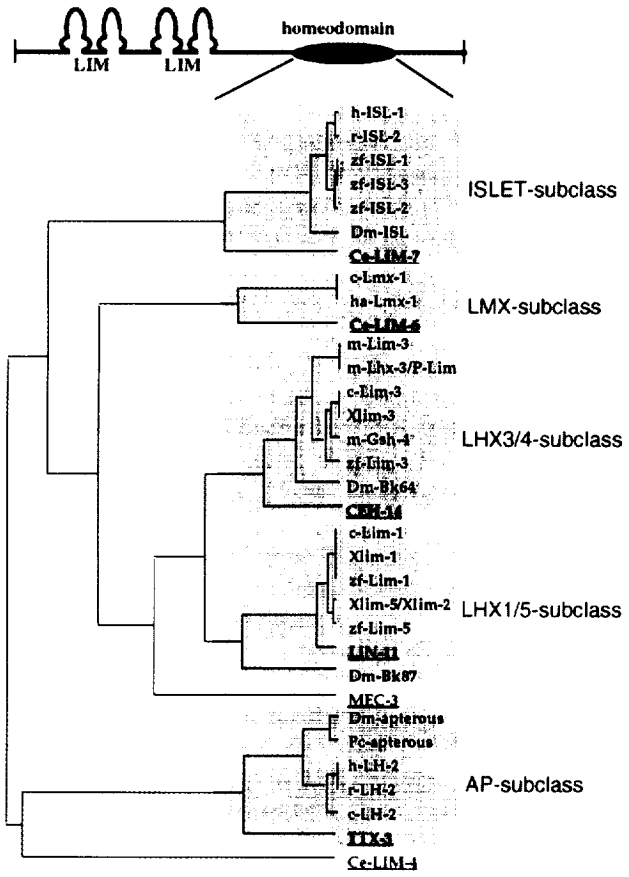

Figure 1. Dendrogram of LIM homeodomain proteins.

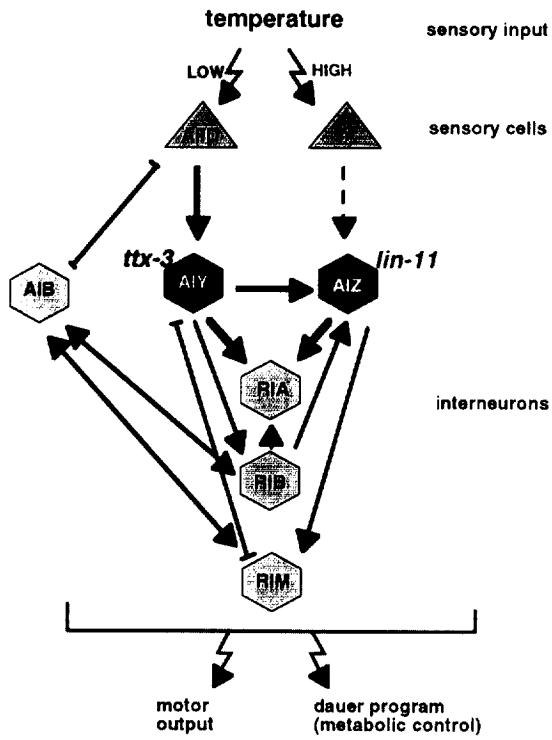

Figure 2. The neural thermoregulatory pathway in *Caenorhabditis elegans*.

lar to thermocontrol in vertebrates. The major thermoregulatory organ of vertebrates, the hypothalamus, contains distinguishable warm- and cold-sensing temperature processing units (8) that may be homologous to the antagonistic high and low temperature sensing pathways of the *C. elegans* thermotactic response pathway (Fig. 2). The vertebrate *ttx-3* homolog *Lhx2* and the *lin-11* homolog *Lhx1* are indeed expressed in the diencephalon, which gives rise to the thermoregulatory hypothalamus (9, 10). *lin-11* and *ttx-3* in *C. elegans*, and their homologs in mammals, may thus play a similar role in the development of two components of these related thermal processing networks.

Apart from their suggested role in the hypothalamus, the vertebrate *lin-11* and *ttx-3* homologs *Lhx1* and *Lhx2* are expressed in several additional places in the nervous system (9, 10). The additional roles of the vertebrate genes might parallel the function of the nematode homologs, making additional cases for a conservation of function throughout evolution. For example, *lin-11* is expressed and functions in the ventral nerve cord of *C. elegans*, where it is required for correct axon bundle fasciculation (7); vertebrate *Lhx1* is similarly expressed in motor neurons of the spinal cord. Additionally, *Lhx1* expression can be observed in sensory structures in the

brain (9), which correlates with *lin-11* expression in *C. elegans* head sensory neurons (7). In contrast, the comparison of expression and functions of nematode *lin-11* and vertebrate *Lhx1* also makes a very strong point for the acquisition of *additional* functions for a regulatory gene (or, alternatively, the loss of a function): While *Lhx1* is involved early in embryogenesis in neural induction (11), no such embryonic role exists for *lin-11* (7). Similarly, as *C. elegans* has no appendages, the function of *apterous,* the *Drosophila* homolog of *C. elegans ttx-3* in wing patterning, represents a clear case of co-option of a regulatory gene to a new developmental process.

Is there a common theme for LIM homeobox gene function in *C. elegans?* The functional analysis of the LIM homeodomain-encoding *ttx-3, lin-11,* and *mec-3* genes, all of which act late in neural development, demonstrated their role in determining the differentiated neural phenotype (6, 7, 12). To learn whether the other *C. elegans* LIM homeobox genes might share a similar role, we examined their expression pattern using GFP reporter gene fusion. We found *lim-4, lim-6,* and *lim-7* to be expressed in a non-overlapping subset of neuronal cells. While the expression of the *isl*-homolog *lim-7* is very dynamic and not confined to the nervous system, we found *lim-4* and *lim-6* to be exclusively expressed in a non-overlapping set of head sensory-, inter- and motorneurons. Note that, like *mec-3, ttx-3* and *lin-11, lim-4* and *lim-6* are also expressed in neurons after their final division and continue to be expressed throughout adulthood, suggesting that they might be involved in neuronal maintenance. We speculate that a common theme of *C. elegans* LIM homeobox genes is to determine a specific neural phenotype, as manifested perhaps by a specific neural connectivity or neurotransmitter choice. Our findings suggest that this is the phylogenically conserved function of LIM homeobox genes, and that some of the functions of the genes in *C. elegans*—such as the role of *lin-11* in vulval development—represent a later recruitment of these genes into additional cellular processes.

A comparison of expression characteristics of the *C. elegans* LIM homeobox genes leads to another interesting point: the expression of most, if not all of these genes is maintained in neural tissues throughout adulthood. This suggests a nontransient, but constitutive requirement for these genes throughout the life of the neuron, *e.g.,* in the maintenance of specific neural features.

We further propose that LIM homeobox gene function in neural development represents a function of these genes that has been conserved across phylogeny. This hypothesis is based on the functioning of *Drosophila* LIM homeobox genes in axon pathfinding and determination of neurotransmitter identity (3, 4), as well as the maintained neural expression of vertebrate LIM homeobox genes in postmitotic neurons (1). To our knowledge, LIM homeobox genes have so far been found exclusively in organisms that contain a nervous system, which provides some circumstantial evidence that LIM homeobox genes might have co-evolved with neural structures, whose complexity obviously requires the use of new classes of regulatory genes.

LIM homeobox genes presumably arose in evolution by a recombination event of homeodomain and LIM domain coding exons. This event probably happened only once, since (1) all LIM homeobox genes contain a very similar architecture, with two LIM domains at the N-terminus and one homeodomain at the C-terminus, and since (2) the first LIM domain of LIM homeodomain proteins is usually more similar to the first LIM domain of other LIM homeodomain protein than to their own second LIM domain (1). Gene duplications of a single common ancestor conceivably created the different subclasses of LIM homeodomain proteins; these duplication must have happened before the divergence of nematodes, arthropods, and chordates. This common ancestor, which contained multiple LIM homeobox genes, might have already contained a simple nervous system in which LIM homeodomain protein were employed to define specific neural features.

As mentioned above, LIM homeobox genes have obviously been recruited to function in additional non-neural processes, such as vulval patterning, limb development, and neural induction during gastrulation. These additional and relatively specialized functions of LIM homeobox genes in organs and processes specific for distinct phylogenetic branches presumably have been co-opted by specific phyla at later stages of evolution.

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Mechanisms of Specification in Ascidian Embryos

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Ascidians (subphylum Urochordata, class Ascidiacea) are ubiquitous marine animals. Since the work of Chabry (1) in 1887, which described the first blastomere destruction experiments in the history of embryology, ascidian eggs and embryos have served as an experimental system in developmental biology. The fertilized egg develops quickly into a tadpole larva (about 2600 component cells) consisting of a small number of tissues including epidermis, central nervous system with two sensory organs, nerve cord, endoderm, mesenchyme, notochord, and muscle (2). The lineage of these embryonic cells is described almost completely. In addition, the recent isolation of cDNA clones of various tissue-specific genes by our laboratory provides molecular probes with which to monitor the differentiation of each type of tissue (3). The advantageous features of the embryo, together with tissue-specific molecular probes, allow us to study the mechanisms underlying the specification and subsequent differentiation of embryonic cells (2, 3).

Maternal genes with localized mRNA

Fate restriction in ascidian embryos takes place relatively early; *i.e.*, most of the blastomeres in the 64-cell stage are already restricted to generating one type of tissue. Reflecting such an early fate restriction, the ascidian embryo shows a highly determinate mode of development, which may be dependent on prelocalized egg cytoplasmic determinants. Recently we isolated cDNA clones for several maternal genes with localized mRNA (4, 5). Because all of these mRNAs are localized in the posterior-vegetal cytoplasm of the egg and they later mark the posterior end of developing embryos, we named the genes *posterior end mark (pem)* (4). Thus far we have obtained cDNA clones for six *pem*s: *pem, pem-2, pem-3, pem-4, pem-5,* and *pem-6. pem-3* encodes a polypeptide with the KH domain and RING finger (a possible homolog of *C. elegans* MEX-3 that may function as an RNA-binding protein), whereas *pem-4* encodes a polypeptide with the C2H2-type zinc finger motif.

Muscle differentiation

The B-line presumptive muscle cells of ascidian embryos have extensive potential for self-differentiation dependent on determinants prelocalized in the myoplasm of fertilized eggs (2). Ascidian larval muscle cells therefore provide an experimental system with which to explore an intrinsic genetic program for autonomous specification of embryonic cells. Experiments with egg fragments suggested that maternal mRNAs are one of the components of muscle determinants (6). Expression of larval muscle actin genes (*HrMA4*) begins as early as the 32-cell stage, prior to the developmental fate restriction of the cells (7). The initiation of actin gene expression begins a few hours before the expression of an ascidian homolog of vertebrate MyoD (8). In addition, mutations in the proximal E-box of the 5′ flanking region of *HrMA4* did not alter the promoter activity for muscle-specific expression of a reporter gene (9). These results, together with the effects of deleting constructs of fusion genes, suggest that muscle determinants regulate directly—or indirectly *via* regulatory factors other than MyoD—the transcription of muscle-specific structural genes leading to the terminal differentiation. We have characterized *cis*-elements responsible for muscle-specific expression of *HrMA4,* and have identified two elements within about 100 bp upstream of the transcription initiation site (9).

This paper was originally presented at a workshop titled *Genetic Regulatory Networks in Embryogenesis and Evolution.* The workshop, which was held at the Marine Biological Laboratory, Woods Hole, Massachusetts, from 11 to 14 June 1997, was sponsored by the Center for Advanced Studies in the Space Life Sciences at MBL and funded by the National Aeronautics and Space Administration under Cooperative Agreement NCC 2-896.

Specification of notochord

Recent studies have revealed an important role for cell-cell interactions in the specification of certain types of ascidian embryonic tissue. Differentiation of ascidian notochord cells is induced during the early phase of the 32-cell stage by interaction of the presumptive notochord blastomeres with adjacent endoderm cells, as well as with neighboring presumptive notochord blastomeres (10). Immediately after the induction, an ascidian homolog (*As-T*) of mouse *Brachyury* gene is expressed only in the primordial notochord cells (11). Microinjection of synthetic *As-T* mRNA into fertilized eggs resulted in notochord differentiation in the presumptive notochord blastomeres with no induction process (12). The notochord differentiation was assessed by the appearance of a specific antigen (Not-1) and morphological features (an elongated cell with a large vacuole). In addition, *As-T* mRNA injection promotes notochord differentiation in lineages of nerve cord and endoderm, suggesting that this gene exerts a master control upon notochord differentiation (12).

Evolutionary aspects

Halocynthia roretzi has at least four T-domain genes. Although *As-T* is expressed exclusively in differentiating notochord cells, *As-T2* is expressed in differentiating muscle and the tip of the tail of the embryo (13). In addition, an ascidian homolog of *omb* is expressed in the nervous system, and a maternal *As-mT* mRNA is present in unfertilized eggs.

Because the notochord is one of the characteristic features shared by chordates, and because the *Brachyury* gene is responsible for notochord formation, comparative studies of *Brachyury* gene expression and function between primitive chordates and other deuterostomes (echinoderms and hemichordates) may give us some insight into the developmental mechanisms underlying the appearance of the chordates about 550 million years ago (14). We therefore compared the patterns of *Brachyury* gene expression among the deuterostome groups.

The amphioxus *Branchiostoma belcheri* contains two *Brachyury* genes. These genes are initially expressed in the involuting mesoderm of the gastrula, then in the differentiating somites of the neurula, followed by the differentiating notochord and finally in the tail bud of the 10-somite stage embryo (15). This pattern of expression of the amphioxus *Brachyury* resembles that of the vertebrate *Brachyury*.

The sea urchin *Brachyury* gene (*HpTa*) is transiently expressed in the lineage of secondary mesenchyme cells: first in the vegetal plate of the mesenchyme blastula, extending to the tip of the invaginating archenteron, and finally in the secondary mesenchyme cells at the late-gastrula stage (16). This result suggests that the present function of the *Brachyury* gene in the notochord of chordates originated prior to the branching of the lineage leading to chordates from that leading to echinoderms; it also suggests that, during sea urchin development, *Brachyury* is likely to specify embryonic cells to the secondary mesenchyme.

We also isolated a cDNA clone encoding a hemichordate (*Ptychodera flava*) homolog (*PfBra*) of the *Brachyury* gene and examined its expression pattern during embryogenesis (17). The *PfBra* is first expressed in the vegetal plate of the early gastrula. However, the expression is not detected in the extending tip of the invaginating archenteron, but remains at the blastopore region. In addition, the gene expression is evident in the ectodermal cells of the stomodium, and this pattern is retained in the 3-day-old tornaria larva. The stomochord, which was once thought to be homologous to chordate notochord (18), is formed in the basal region of the proboscis during metamorphosis. The *PfBra* expression should be determined during this later stage of embryogenesis.

Conclusion

The ascidian tadpole larva is regarded as a prototype of the ancestral chordate. Therefore, studies of developmental mechanisms involved in the formation of ascidian tadpoles provide new insights, not only into the ontogeny, but also into the phylogeny of chordates. Further analyses, particularly of *Brachyury* target genes in ascidians and sea urchins, may facilitate our understanding of the genetic circuitry underlying the origin and evolution of chordates.

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