Molecular evidence for deep evolutionary roots of bilaterality in animal development

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Edited by John B. Gurdon, University of Cambridge, Cambridge, United Kingdom, and approved May 26, 2006 (received for review February 23, 2006)

Nearly all metazoans show signs of bilaterality, yet it is believed the bilaterians arose from radially symmetric forms hundreds of millions of years ago. Cnidarians (corals, sea anemones, and “jellyfish”) diverged from other animals before the radiation of the Bilateria. They are diploblastic and are often characterized as being radially symmetrical around their longitudinal (oral–aboral) axis. We have studied the development of orthologs of a number of family members of developmental regulatory genes that are expressed asymmetrically during bilaterian embryogenesis from the sea anemone, Nematostella vectensis. The secreted TGF-β genes Nv-dpp, Nv-BMP5–8, six TGF-β antagonists (Nv-Chordin, Nv-Noggin1, Nv-Noggin2, Nv-Chordin, and Nv-Chordin-like), the homeodomain proteins Nv-Goosecoid (Nv-Gsc) and Nv-Gbx, and the secreted guidance factor, Nv-Netrin, were studied. Nv-dpp, Nv-Chordin, Nv-Noggin1, Nv-Gsc, and Nv-Gbx are expressed asymmetrically along the axis perpendicular to the oral–aboral axis, the directive axis. Furthermore, Nv-Gbx and Nv-Chordin are expressed in restricted domains on the left and right sides of the body, suggesting that the directive axis is homologous with the bilaterian dorsal–ventral axis. The asymmetric expression of Nv-Noggin1 and Nv-Gsc appear to be maintained by the canonical Wnt signaling pathway. The asymmetric expression of Nv-Noggin1 and Nv-Gsc, and Hox orthologs Nv-Anthox7, Nv-Anthox8, Nv-Anthox1a, and Nv-Anthox6, in conjunction with the observation that Nv-Noggin1 is able to induce a secondary axis in Xenopus embryos argues that N. vectensis could possess antecedents of the organization of the bilaterian central nervous system.

Cnidarians, ctenophores, and placozoans are animal groups thought to have diverged from the rest of the Metazoa, the Bilateria, before the origins of triploblasty and bilateral symmetry (1–5). Although examples of bilaterality have been described at a morphological level in many cnidarians (6–8), it has been difficult to homologize relationships between cnidarian body axes with those of bilaterian metazoans. Anthozoans such as Nematostella vectensis have been described as being bilaterally symmetrical (7, 8) because of the anatomy of internal mesenteries and the position of a ciliated groove in the animal’s pharynx (the sipohonoglyph). The plane of bilateral symmetry that runs perpendicular to and includes the oral–aboral axis is called the “directive axis” (Fig. 1 B–D). Previous assertions of the homology of the directive axis to any bilaterian axis rest on the expression of a single N. vectensis gene, Nv-dpp (9) (an ortholog of dpp/BMP2/BMP4), which, along with its antagonist, sog/Chordin, is causally involved with establishing the dorsal–ventral (D–V) axis of Drosophila/paramecium and vertebrates (10, 11). Although differences exist in the initial symmetry-breaking events in different bilaterians, e.g., regionalized dorsal in Drosophila (12), and β-catenin in vertebrates (11, 13), at least one conserved aspect of the patterning of the D–V axis in both protostomes and deuterostomes appears to be the antagonistic interaction of diffusible extracellular ligands of the TGF-β superfamilly (BMP2/BMP4/dpp) and their antagonists (e.g., Chordin/sog). With sampling mostly from within the Ecdysozoa (Drosophila and Caenorhabditis elegans) and within the deuterostomes (echinoderms and chordates), it appears that deuterostomes have diversified both the number of interacting ligands (nodals, bone morphogenetic proteins (BMPs), growth differentiation factors, and TGF-β) and antagonists (noggin, follistatin, gremlin, and cerberus). Many of these genes in vertebrates are expressed asymmetrically in the dorsal lip of the blastopore during gastrulation (i.e., the Spemann Organizer) and have been shown to be causally involved in the elaboration of D-V features (11). Other genes, including the homeodomain transcription factors goosecoid (Gsc) and gastrulation brain homeodomain (Gbx), are also expressed either in the dorsal lip during gastrulation (Gsc) or involved in patterning the brain (Gbx) (14–17).

We have cloned a number of developmental genes responsible for generating bilaterality in other systems, including TGF-β family members, their antagonists, and other classical “organizer” genes (11, 18), a feat facilitated by the recent sequencing of the N. vectensis genome (by the Department of Energy Joint Genome Institute, Walnut Creek, CA). Genome coverage is currently at about five times, which indicates that only 0.6% has not yet been sequenced (19). In addition to previously reported Nv-dpp (BMP2/BMP4) and Nv-GDF5-like (9), orthologs to four other TGF-β ligands have been recovered (20, 21). However, no orthologs of the TGF-β family members nodal, a gene required for mesendoderm formation in chordates (22, 23), or lefty/antivin, a gene involved in chordate left-right asymmetry (22), have been detected in cnidarians, suggesting that these genes arose after the cnidarian–bilaterian divergence or have been lost in the Cnidaria. We identified orthologs to several BMP antagonists that are asymmetrically expressed in the dorsal lip of the vertebrate embryonic blastopore (Fig. 1 A4): chordin/sog (Nv-Chordin), two noggin (Nv-Noggin1 and Nv-Noggin2), two follistatins (Nv-Follistatin1 and Nv-Follistatin-like), and Nv-Gremlin (all of which bind TGF-β ligands extracellularly) (11), but an ortholog of Cerberus (another potent TGF-β inhibitor) does not appear to be present in N. vectensis. We also recovered the homeodomain genes Gsc (Nv-Gsc) and Gbx (Nv-Gbx). Thus, cnidarians have many, but not all, of the genes involved in vertebrate D-V patterning and substantially more than ecdysozoan protostomes. No protoctist taxon to date has been shown to possess all four orthologs for BMP antagonists: noggin, chordin, follistatin, and gremlin (24), consistent with other studies showing that the lower diversity of some gene families in the model ecdysozoans is not caused by the expansion of these families in the deuterostomes, but by reduction in the ecdysozoans (20, 25, 26).

Nv-dpp and Nv-BMP5–8 are the only TGF-β ligands expressed during gastrulation in N. vectensis (21). Along with their antagonist Nv-Chordin, all three are initially coexpressed asymmetrically at the onset of gastrulation (9, 21). Their main function at gastrulation appears to regulate germ-layer segregation and/or early epithelial patterning rather than D-V polarity (21). Additionally, because morphological asymmetries do not appear until later in develop-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BMP, bone morphogenetic protein; D-V, dorsal-ventral.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ517920–DQ517928).

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ment in N. vectensis, it is unknown how the initial asymmetric expression of these genes at gastrulation relates to the adult body plan. We have studied the deployment of candidate bilaterian D-V patterning genes to determine whether any are expressed asymmetrically during the development of this anthozoan.

Results

TGF-β Antagonist Expression. After gastrulation NvChordin is expressed around the entire oral pole (21), but its expression becomes restricted to a “W”-shaped ectodermal pattern on one side of the mouth during early planula stages (Fig. 2B and Fig. 8A, which is published as supporting information on the PNAS web site). Double-label in situ hybridizations show that this asymmetric ectodermal NvChordin expression is on the opposite side of longitudinal body wall endodermal Hox gene expression (NvAnthox8; Fig. 2B and see Fig. 7A), but on the same side as NvDpp expression in pharyngeal ectoderm (9) (Fig. 8E). NvChordin and NvDpp expression are down-regulated in oral/pharyngeal ectoderm by midplanula stages. Therefore, at early planula stages, the transient ectodermal NvChordin and NvDpp expression associated with the pharynx define a single pole of the directive axis of the embryo (see Fig. 7).

In most vertebrate embryos goosecoid, noggin, and follistatin are typically expressed asymmetrically along with chordin at gastrulation in the dorsal blastopore lip (Fig. 1D) (11, 17, 27, 28). In N. vectensis, however, this is not the case. NvFollistatin-like is expressed throughout the endoderm after its invagination at gastrulation (Fig. 9G–I, which is published as supporting information on the PNAS web site), but orthologs to the two noggin genes (NvNoggin1 and NvNoggin2) and follistatin (NvFollistatin) are not yet expressed. At early planula stages NvNoggin1 (Fig. 2D and E) and NvGsc (Fig. 3A–D) are expressed in a highly asymmetric manner along the directive axis, whereas NvFollistatin is expressed in a ring of pharyngeal endoderm (Figs. 2C and 9) and NvNoggin2 is expressed weakly throughout the endoderm (Fig. 10, which is published as supporting information on the PNAS web site). NvNoggin1 is expressed strongly in endoderm on only one side of the pharynx above the position where the ciliated siphonoglyph will form (Fig. 2D and E) from midplanula stages through adult polyp formation and in the endodermal base and tips of the developing tentacles (Fig. 2D). Double-label in situ staining experiments show that NvNoggin1 (Fig. 2F and G), NvDpp (9), and NvChordin (Fig. 2B) are expressed on the same pole of the directive axis, whereas the Hox genes NvAnthox8 (Fig. 2A), NvAnthox7, NvAnthox1a (Fig. 3E), and NvGDF5-like (9) are expressed along the opposite pole (see Fig. 7). NvNoggin1 is also coordinately expressed with the TGF-β member, NvActivin, in the endoderm of developing tentacle buds and around the oral pole (Fig. 11, which is published as supporting information on the PNAS web site) and may be involved in epithelial growth or morphogenesis (29).

Although no ortholog to Cerebros, a BMP antagonist expressed in the Spemann Organizer (30), was recovered, we did isolate an ortholog to a related DAN-family BMP antagonist, NvGremlin (Fig. 12, which is published as supporting information on the PNAS web site). NvGremlin is not expressed during gastrulation, but is only expressed in an endodermal ring around the mouth during polyp formation (Fig. 2H). In vertebrates, gremlin orthologs are BMP2/
BMP4/BMP7 inhibitors, and in N. vectensis, NvDpp and NvBMP5–8 are expressed pan-endodermally during polyp stages, indicating that circumoral inhibition of BMP signaling may be occurring.

**Goosecoid Is Expressed Asymmetrically Along the Directive Axis.** In several bilaterians surveyed (14, 31–34) the homeodomain gene Gsc has been characterized as a blastopore and anterior foregut marker; however, Gsc expression has not been characterized during embryogenesis outside of the Bilateria, although it has been shown to function as a head patterning gene in adult Hydra (35). In N. vectensis, NvGsc is not expressed during gastrulation, but is initially expressed transiently in diametrically opposed domains of body wall endoderm and pharyngeal endoderm and ectoderm during early planula stages (Fig. 3 A and B). Later, NvGsc expression is restricted to pharyngeal endoderm at each pole of the directive axis but is expressed more strongly along the Hox-expressing pole (Fig. 3 C and D) perhaps similar to asymmetric gsc expression in bilaterian foreguts (31, 32, 34).

**Other Genes Supporting the Bilateral Body Plan Organization.** NvGbx is expressed in two parallel longitudinal stripes in body wall endoderm during early planula stages (Fig. 4A). Later in development, transcripts disappear but new expression appears in two symmetric domains in pharyngeal endoderm (Fig. 4A–C). Double-label in situ show that unlike all other described genes that are expressed along one or both poles of the directive axis, NvGbx expression is bilateral, flanking the directive axis on the left and right sides (Fig. 4 D and E). In vertebrates Gbx shows bilateral expression domains in mesendoderm flanking the neural tube and in neuroectoderm (16). NvChordin is also expressed along the left-right axis, in two bilateral patches in pharyngeal endoderm during later polyp stages, flanking NvNoggin1 expression at the base of the first two mesenteries (Fig. 4F). Thus, N. vectensis expresses gene products in left and right domains, perpendicular to the directive axis (see Fig. 7). We have not yet detected any evidence for left-right asymmetry, which in deuterostomes may have evolved under the influence of nodal-related genes and their regulators (e.g., lefty/antivin, Cerberus, and caronte; ref. 22).

**The Guidance Molecule Netrin Is Expressed Asymmetrically Along the Directive Axis.** Netrins are a family of secreted guidance factors, which can attract or repulse migrating neurons and neuronal growth cones depending on the context of the signal and the particular receptor binding the netrin ligand (36) in a diverse array of bilaterians (36–41). In protostomes, netrin is expressed at the ventral midline, and in the chordates, netrin is expressed in the ventral region of the dorsal nerve cord (the embryonic dorsal pole) (36–41), which has led some authors to argue for D-V inversion of body plan organization in these groups (42). The N. vectensis ortholog of bilaterian netrin, NvNetrin (Fig. 13, which is published as supporting information on the PNAS web site), is expressed asymmetrically in the developing pharynx and body wall endoderm, during early planula development (Fig. 5 A and B), and in a group of ectodermal cells forming the apical tuft, a sensory structure that also expresses other neural genes such as NvCOE (43). Double-label in situ hybridizations show that NvNetrin is coexpressed asymmetrically in the body wall endoderm with a number of Hox genes (Fig. 5 C–F). During late planula and polyp development, NvNetrin shifts from an asymmetric expression pattern to a circumoral endodermal ring and to the endodermal tips of the growing tentacles, the latter staining coincident with NvNoggin1 expression (Fig. 5 G and H).

**Lithium Chloride Treatment Radializes Organizer Gene Expression.** Lithium chloride has been used as a teratological agent to affect embryological development for well over a century (e.g., ref. 44). In N. vectensis, chronic lithium chloride treatment radializes the normally asymmetric pharyngeal expression of NvNoggin1 (Fig. 6 A and B) and NvGsc (Fig. 6C). The radializing affect of lithium chloride appears to be conserved in other metazoans such as sea urchins (45) and in amphipathians (46, 47). Lithium chloride affects the canonical Wnt signaling pathway that may be involved in maintaining asymmetric gene expression during early N. vectensis development defines a third body axis. (A–C) NvGbx is expressed bilaterally in body wall endoderm (b.end) (A and B) and pharyngeal endoderm (p.end) (C). (D and E) Double-label in situ hybridization shows that NvGbx (purple) is expressed along the left-right axis, bilaterally, flanking NvAnthox8 expression in planula-stage embryos. (F) Double-label in situ hybridization showing the latest expression of NvChordin in polyp-stage embryos. NvChordin is expressed in two bilateral patches of cells to the left and right of the siphonoglyph NvNoggin1 expression. The asterisk denotes the mouth. All embryo views are lateral with anterior to the left, except B and E, which are oral views. (Magnifications: x100.)

**Fig. 3.** Gsc is asymmetrically expressed along the directive axis during development. (A and B) NvGoosecoid (gsc) is expressed initially in body wall endoderm (b.end) (A) and pharyngeal endoderm (p.end) and ectoderm (p.ect) (B). (C and D) During planula stages, NvGsc is expressed in pharyngeal endoderm, asymmetrically along the directive axis, and in the endoderm below the apical tuft (at) (C). (E) NvAnthox1a is expressed in body wall endoderm on one pole of the directive axis. (F) Double-label in situ hybridization showing that the larger domain of NvGsc pharyngeal endoderm expression is on the same pole of the directive axis as Hox expression (NvAnthox1a). The asterisk denotes the site of the blastopore and mouth. All embryo views are oral, except A and C, which are lateral views, anterior to the left. (Magnifications: x100.)

**Fig. 4.** Left/right expression of genes during Nemastostella development defines a third body axis. (A–C) NvGbx is expressed bilaterally in body wall endoderm (b.end) (A and B) and pharyngeal endoderm (p.end) (C). (D and E) Double-label in situ hybridization shows that NvGbx (purple) is expressed along the left-right axis, bilaterally, flanking NvAnthox8 expression in planula-stage embryos. (F) Double-label in situ hybridization showing the latest expression of NvChordin in polyp-stage embryos. NvChordin is expressed in two bilateral patches of cells to the left and right of the siphonoglyph NvNoggin expression. The asterisk denotes the mouth. All embryo views are lateral with anterior to the left, except B and E, which are oral views. (Magnifications: x100.)
N. vectensis Genes Have Conserved Biological Activity. The developmental regulatory genes studied here were identified by sequence similarity, and their orthology assignments were supported by phylogenetic analysis (Figs. 12–16, which are published as supporting information on the PNAS web site). One would anticipate that these genes would have similar activity as their vertebrate counterparts. TGF-β antagonists have no known function other than to inhibit TGF-β signaling (i.e., they are not ligands for their own signal-transducing receptors), and they are considered to be proneural in all organisms studied (11, 18, 48). As a test of whether N. vectensis orthologs we expressed NvNoggin1 in Xenopus laevis embryos. Injections of synthetic mRNA for NvNoggin1 into the ventral marginal zone of the early blastula generated an ectopic dorsal axis identical to ones induced by ectopic expression of Xenopus Noggin (Fig. 17, which is published as supporting information on the PNAS web site), a result characteristic of BMP inhibition. We continued to test other components of the N. vectensis TGF-β system for homologous function in Xenopus assays, but the results indicate that the components and function of the TGF-β agonist-antagonist system were well diversified in the bilaterian–cnidarian ancestor and the differential regulation of these conserved genes could play important patterning roles during development (49).

Discussion

Evolutionary Predecessor of the D-V Axis? The asymmetric expression of such a large number of both transcription factor and secreted gene families in multiple tissues along the N. vectensis directive axis indicates that these animals have a great deal of spatial complexity that is not readily apparent at the morphological level (Fig. 7). The fact that NvChordin, NvNoggin1, NvGsc, and NvDpp all are expressed asymmetrically along the directive axis at some point in the development of the juvenile body suggests that the cnidarian directive axis might be homologous to the bilaterian D-V axis (Fig. 7). In bilaterians, TGF-β ligands and antagonists are expressed at opposite poles of the D-V axis (11), whereas in N. vectensis, TGF-β ligand and antagonist asymmetric gene expression is confined to domains within the pharynx, at the ciliated siphoglyph pole of the directive axis. In vertebrates, chordin, noggin, follistatin, and goosecoid are coexpressed asymmetrically in dorsal mesoderm during early stages of gastrulation (Fig. 1D) (11, 17, 28), but N. vectensis does not have definitive mesoderm, and NvGsc, NvNoggin1, and NvFollistatin are not expressed until after gastrulation is complete. The earliest expression of NvChordin is confined to oral ectoderm and may be playing a role in germ-layer segregation (21). In vertebrates, all three secreted TGF-β inhibitors (noggin, chordin, and follistatin) are required coincidently in the dorsal lip to function as a dorsal organizer (48). This requirement indicates that a heterochronic shift in the deployment of TGF-β antagonists and other organizer genes during gastrulation may have occurred in bilaterians and that in cnidarians these genes may function initially in establishing germ-layer identity (21) and later in development play a role in axial patterning. NvFollistatin-like (Fig. 9 G–I), and NvNoggin2 (a cnidian-specific gene duplication; Fig. 8) are expressed uniformly in the endoderm, and during gastrulation NvChordin is expressed topographically in an area separating ectoderm from endoderm (21). Arguments have been made that mesoderm evolved from a bifunctional mesendoderm, so it might be that regulation of TGF-β signaling (e.g., via nodal-related genes (22, 23)) was involved with the evolution of a definitive mesodermal germ layer (50, 51) and additional genes were recruited for mesodermal patterning at later stages of metazoan radiations. However, an alternative hypothesis would be that some of these genes played a role in D-V patterning in the common ancestor of cnidarians and bilaterians, and that this role has been reduced in some cnidarians. The radializing affects of lithium chloride on NvNoggin and NvGsc (Fig. 6), both asymmetrically deployed in the pharynx along the directive axis during normal development (Fig. 7), suggests that the secondary radialization from a bilaterally symmetrical body plan in some forms may be related to changes in the canonical Wnt signaling pathway.

A number of genes are associated with the development of the anterior end of bilaterian animals, in particular with anterior neural and foregut structures. NvGbx expression in bilateral patches along body wall endoderm (Fig. 4A) is reminiscent of the bilateral endomesodermal expression in the head region of vertebrates (16). NvGbx disappears in body wall endoderm (as does endomesodermal expression in vertebrates) (16) and reappears in two bilateral patches of the pharynx (Fig. 4C). In vertebrates, gbx staining persists...
in the hindbrain where it interacts with otx, FGF, and wt8 genes to establish the midbrain–hindbrain boundary (16, 52). The N. vectensis ortholog of Wnt8, NwWnt8, is expressed in endoderm surrounding the pharynx (26), and Otx genes are expressed at the oral end of the pharynx (and in the tentacle buds) (K.P. and M.Q.M., unpublished work), suggesting that the oral pole of anthozoans is homologous to the bilaterian anterior (not posterior or ventral) pole (9, 53). Because the mouth, the single opening to the gut, forms at the end of the pharynx (and in the tentacle buds) (K.P. and M.Q.M., unpublished work), this implies homology of the oral/aboral axis to both germ layers. The asymmetric expression of NvChordin, NvDpp, NvNoggin, and NvAnthox8 in the pharynx on the opposite side of the expression of NvNetrin, three Hox genes, the larger domain of NvGsc, and NvGDF5-like, suggests homology of the directive axis to the D-V axis of bilaterians. The bilateral expression of NvGbx and NvChordin marks, the left–right axis.

**Function of Asymmetrically Expressed Genes.** In both flies and vertebrates, inhibitors of TGF-β signaling (e.g., noggin, chordin, follistatin, and gremlin) promote neural differentiation (11). In N. vectensis embryos the TGF-β inhibitors NvNoggin1 and NvChordin are expressed asymmetrically in the pharynx throughout late planula and polyp stages (Figs. 2 D and E and 4F). At least one of these genes, NvNoggin1 has BMP antagonistic activity when tested in the amphibian ectopic dorsalization assay (Fig. 17) just as its ortholog does in vertebrates (54, 55). Among metazoan model organisms, BMP antagonists have been shown to affect body pattern by affecting graded BMP activities. These antagonists also act synergistically with mitogen-activated protein (MAP) kinase signals to specify primary neural cell fate, with the MAP kinase signals provided by secreted FGF ligands in vertebrates (11, 56–60) and EGF ligands in Drosophila (61–63). In urochordates, FGF signals on their own may act to induce neural tissue (64). In N. vectensis FGF ligands are expressed in both the pharynx and apical sensory tuft (D.O.M., G.H.T., and M.O.M., unpublished work), suggesting that neural tissue could be induced where the FGF signals intersect with the BMP inhibitors. This notion is supported by expression of various genes implicated in neural fate specification in the planula apical tuft, a sensory structure at the anterior end of the swimming stage. The apical tuft expresses NvDpp and NvBMP5–8, and their inhibitor (NvNoggin1), and a variety of genes associated with neural tissue including NvGsc, NvNetrin, NvCOE (43), NvAnthox1 (9), NvSoxB1, and NvFoxD1 (65), some of which are also expressed in the pharynx (NvGsc, NvDpp, NvBMP5–8, NvNoggin1) (Fig. 7). Although we have no direct evidence yet, it is tempting to speculate that the asymmetric expression of genes described in this article has the potential to determine the distribution of presumptive nervous elements.

Immunohistochemical studies have shown a great deal of molecular complexity in neuropetide expression in Hydra (66, 67), and gene expression studies with an increasing broad range of molecular markers with potential roles in neural patterning have highlighted distinct domains of the N. vectensis body plan. NvNetrin is expressed in the same longitudinal stripe of body wall where several Hox genes are expressed. The circumpharangeal expression of N. vectensis homologs of genes such as NvNetrin, NvGremlin, NvNotch1, NvNotch2, and NvDelta (H.M., D.Q.M., and M.O.M., unpublished work), correspond to the location of the nerve ring surrounding the mouth of cnidarians (68). This circumpharangeal concentration of nerves has been suggested to represent a precursor to the bilaterian CNS (69). Asymmetric pharyngeal expression of NvNoggin1 and NvAnthox8 could correspond with a dorsal supraesophageal ganglion, whereas a lateral NvGbx domain, and the longitudinal NvAnthox7, NvAnthox8, NvAnthox1a, and NvNetrin expression confined to a defined strip along the opposite (i.e., ventral) body wall, would then correspond to the layout of the typical protostome CNS (Fig. 7).

Unfortunately, the nervous system of N. vectensis is still poorly characterized. Cnidarians have both ectodermal and endodermal “nerv nets” that in hydrozoans are derived from endodermally derived stem cells (70). Most of the asymmetric genes in the pharynx (except for NvDpp) are expressed in the pharyngeal (NvAnthox6, NvAnthox8, NvNoggin1, NvGsc, NvGbx) or body wall (NvAnthox7, NvAnthox8, NvAnthox1a) endoderm. One possibility is that Hox and other neural patterning genes were originally involved in endodermal nervous system patterning and were recruited to generate an ectodermally derived definitive CNS in bilaterian descendants. We predict that continued molecular analyses will demonstrate a highly patterned nervous system in N. vectensis organized along a bilaterally symmetrical system that belies the animal’s simple morphological appearance.

**Conclusions**

It is surprising that such a large number of molecular asymmetries exist in an animal that has so little overt morphological complexity. The observation that many of the orthologs of genes expressed asymmetrically in both deuterostomes and protostomes are also asymmetrically expressed in N. vectensis suggests that the axial properties of N. vectensis could represent features present in the common ancestor of cnidarians and bilaterians. Some of the genes that we have shown to be expressed asymmetrically in N. vectensis (NvGsc, NvBMP5–8) are expressed in radial patterns in radially symmetric hydrozoan cnidarians (36, 71). There is great diversity in the morphological symmetry properties of the Cnidaria. Although some taxa are radially symmetric, many appear bilaterally symmetric or even directionally asymmetric (72). We suggest that the ancestor of cnidarians was originally bilaterally symmetrical, with a clear D-V polarity (along the directive axis) and an anterior–posterior polarity in which the mouth/anus of cnidarians is equivalent to the anterior end of bilaterians (9). Although one might not expect all bilaterian features to be present in an animal derived from...
phylogenetic analyses are available in Supporting Text, which is published as supporting information on the PNAS web site.

In Situ Hybridization. In situ hybridizations using 1- to 3-kb digoxigenin and fluorescein-labeled antisense ribonucleotide probes were performed as described (21). Additional details are available in Supporting Text.

Lithium Chloride Experiments. Dejured embryos were cultured in a 25 mM solution of LiCl in one-third strength timed filtered sea water and fixed for in situ hybridization (51) at different time points during development.

Xenopus Methods. Synthetic capped mRNA was generated by in vitro transcription of linearized plasmids by using appropriate RNA polymerase kits as indicated (Message Machine; Ambion, Austin, TX). Plasmid-encoded vector (pGEM-7). Xenopus noggin [pSP5-Noggin-Myc; gift of Yoshiki Sasai, Center for Developmental Biology, The Institute of Physical and Chemical Research (RIKEN), Hyogo, Japan], or N. vectensis Noggin1 (pGEMT-Easy-NvNog1) were used. A volume of 5–10 nl was injected into 16-cells embryos obtained by in vitro fertilization.

We thank Craig Magie (University of Hawaii) for providing the congenital hypothyroid fish. G. H. T. thanks W. Lennarz for sabbatical leave from Stony Brook University and members of Kewalo Marine Laboratory for their generosity during his sabbatical. This work was supported by grants from the National Science Foundation and National Aeronautics and Space Administration (to M.Q.M.) and National Institutes of Health Grant HD032429 (to G.H.T.).

2. TBLASTN searches of the National Center for Biotechnology Information (NCBI) trace archive of the N. vectensis genome (data generated by the Joint Genome Institute) were performed by using metazoon orthologs of bilaterian patterning genes. The traces were then compiled into contigs by using ASSEMBLYLIGN (Accelrys, San Diego, CA) and SEQUENCER (Gene Codes, Ann Arbor, MI), and ORFs were determined based on BLASTX searches against the nr database at NCBI. Gene-specific primers were then designed for 5' and 3' RACE with annealing temperatures between 65°C and 70°C. RACE was performed by using the Smart Race cDNA amplification kit (BD Biosciences Clontech). RACE products were cloned into a plasmid vector (P-Gem T easy, Promega) and sequenced at Gene Gateway (Hayward, CA). Overlapping 5' and 3' RACE fragments were aligned and submitted to GenBank as composite transscripts.

Phylogenetic Analyses. Phylogenetic analysis of the TGF-β antagonists (noggin, follistatin, and gremlin) and homeodomain genes were performed to determine orthology. Bayesian and neighboring-joining analyses were performed. Additional details concerning phylogenetic analyses are available in Supporting Text, which is published as supporting information on the PNAS web site.

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2. TBLASTN searches of the National Center for Biotechnology Information (NCBI) trace archive of the N. vectensis genome (data generated by the Joint Genome Institute) were performed by using metazoon orthologs of bilaterian patterning genes. The traces were then compiled into contigs by using ASSEMBLYLIGN (Accelrys, San Diego, CA) and SEQUENCER (Gene Codes, Ann Arbor, MI), and ORFs were determined based on BLASTX searches against the nr database at NCBI. Gene-specific primers were then designed for 5' and 3' RACE with annealing temperatures between 65°C and 70°C. RACE was performed by using the Smart Race cDNA amplification kit (BD Biosciences Clontech). RACE products were cloned into a plasmid vector (P-Gem T easy, Promega) and sequenced at Gene Gateway (Hayward, CA). Overlapping 5' and 3' RACE fragments were aligned and submitted to GenBank as composite transscripts.

Phylogenetic Analyses. Phylogenetic analysis of the TGF-β antagonists (noggin, follistatin, and gremlin) and homeodomain genes were performed to determine orthology. Bayesian and neighboring-joining analyses were performed. Additional details concerning phylogenetic analyses are available in Supporting Text, which is published as supporting information on the PNAS web site.

In Situ Hybridization. In situ hybridizations using 1- to 3-kb digoxigenin and fluorescein-labeled antisense ribonucleotide probes were performed as described (21). Additional details are available in Supporting Text.

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