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Developmental Expression Pattern

Posterior expression of *nanos* orthologs during embryonic and larval development of the anthozoan *Nematostella vectensis*

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ABSTRACT Cnidarians are primitive animals located in a basal position in the phylogenetic tree of the Animal Kingdom, as an outgroup of the Bilaterians. Therefore, studies on cnidarian developmental biology may illustrate how fundamental developmental processes have originated and changed during animal evolution. A particular example of this is the establishment of polarity along the body axes, which is under the control of a number of developmental genes, most of them conserved in evolution and playing similar roles in diverged species. Concerning the anterior-posterior axis, genetic and molecular studies on *Drosophila* have shown that the *nanos* gene plays an essential role in defining posterior structures during early embryonic development. Here we report the isolation of two *nanos* orthologs in the anthozoan *Nematostella vectensis*. We show that *nanos* mRNA is asymmetrically distributed in the fertilized egg and this asymmetry is maintained during embryonic development. At gastrula and planula larva stages, *nanos* expression is permanently associated with posterior body regions. These results, together with our previous analysis in the hydrozoan *Podocoryne carnea*, indicate that posterior *nanos* expression during development is a conserved feature among cnidarians. Therefore, the potential role of cnidarian *nanos* in defining axial polarity as a posterior determinant would represent an ancestral trait in the Animal Kingdom.

KEY WORDS: *Cnidaria, planula, anterior-posterior axis, axial patterning, evolution*

A major topic in developmental biology is the study of the origin and evolution of the genetic mechanisms responsible for the specification of animal body axes. This process has been deeply investigated in *Drosophila*, where a number of genes responsible for anterior-posterior (A-P) patterning have been identified (reviewed in Gilbert, 2003). Essentially, maternal gene products that appear differentially localized in the early embryo regulate the expression of zygotic segmentation genes, which, ultimately, define the domains of *Hox* gene expression. *Hox* products confer positional identity to the different embryonic and adult territories along the A-P axis (reviewed in Gilbert, 2003). An example of an early-acting gene that participates in the specification of A-P polarity is *nanos*, whose maternal product is a CCHC-type Zn-finger protein localized to the posterior region of the early *Drosophila* embryo (Wang and Lehmann, 1991). *nanos* is essential for the appropriate formation of posterior structures, namely the abdomen (Wang and Lehmann, 1991). Additionally, *nanos* plays a role during the differentiation of the *Drosophila* germline (Kobayashi *et al.*, 1996).

Most of these *Drosophila* axial patterning genes are conserved and play related functions in different animal groups, so they can be studied to trace the evolution of the process throughout the phylogenetic tree of the Animal Kingdom. In this tree, the phylum Cnidaria appears located at a basal position, as outgroup of the Bilateria (Adoutte *et al.*, 2000), so its study might provide significant insights on how basic animal body plans have originated and evolved at the base of the tree.

In this regard, we recently found that in the hydrozoan *Podocoryne carnea*, *nanos* orthologs are transiently expressed at the posterior pole of the early embryo (Torras *et al.*, 2004), raising the possibility that they are involved in the control of axial patterning, in a similar way as in *Drosophila*. To determine if this is a conserved feature among cnidarians, we decided to analyze *nanos* expression during development of the anthozoan *Nematostella vectensis*.

The starlet sea anemone *Nematostella vectensis* (Hand and

Abbreviations used in this paper: AP, anterior-posterior.

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Uhlinger, 1992) is progressively emerging as a useful model system to approach questions related to evolutionary developmental biology. Among the cnidarians, anthozoans are considered primitive, since they only have one of both cnidarian adult body forms, namely the polyp, while the medusa is missing (reviewed in Brusca and Brusca, 2003). Also, molecular phylogenetic analyses suggest that anthozoans are basal cnidarians (Bridge *et al.*, 1995). In *Nematostella*, both sexes are separated and adult polyps release either male or female gametes to the water. The fertilized egg undergoes a variable pattern of cleavage that results into a hollow blastula. Next, during the process of gastrulation, cells located at the posterior pole of the blastula invaginate and the blastopore forms. These invaginating cells finally fill up the internal cavity and give rise to the endoderm, whereas the external cells become ciliated and form the ectoderm (Hand and Uhlinger, 1992). This bi-layered developmental stage corresponds to the canonical cnidarian planula larva (Brusca and Brusca, 2003). The *Nematostella* planula swims actively, always with the same pole to the front. This pole, referred to as anterior, is easily recognizable by an apical sensory tuft of large cilia. The posterior pole of the planula corresponds to the region where the blastopore was located at the gastrula stage (Hand and Uhlinger, 1992). A few days later, the planula settles and transforms into a juvenile polyp by formation of four major structures, i. e.: 1) a gastrovascular cavity, called coelenteron, that opens in the endoderm, 2) a mouth with a pharynx formed by invagination of the posterior ectodermal region, 3) a number of endoderm projections, called mesenteries, that connect with the pharynx and 4) four tentacles surrounding the oral opening (Hand and Uhlinger, 1992; Martindale *et al.*, 2004; Finnerty *et al.*, 2004). Therefore, with respect to the polarity of the body axis, the posterior region of the planula gives rise to the mouth of the polyp. Since this significant feature has been also observed in hydrozoans (Freeman, 1981; Schwoerer-Böhning *et al.*, 1990), the correspondence between the larval A-P axis and the aboral-oral axis in the adult polyp is considered a general feature of cnidarian development. Remarkably, the juvenile *Nematostella* polyp moves in the opposite direction than the planula (Hand and Uhlinger, 1992; our observations), that is, with its mouth to the front. In other words, during the transition from planula to polyp there is an inversion of the swimming direction.

Here we provide evidence that *nanos* expression in *Nematostella* is restricted to posterior precursors throughout embryonic and larval development. First, we show that maternal *nanos* mRNA is localized to one side of the fertilized egg. During early embryogenesis, *nanos* expression remains restricted to approximately half of the blastomers located at one side of the cleaving embryo. In the gastrula, only cells located in the anterior pole and those that invaginate do not show *nanos* expression. In the planula larva, *nanos* mRNA accumulates preferentially in the

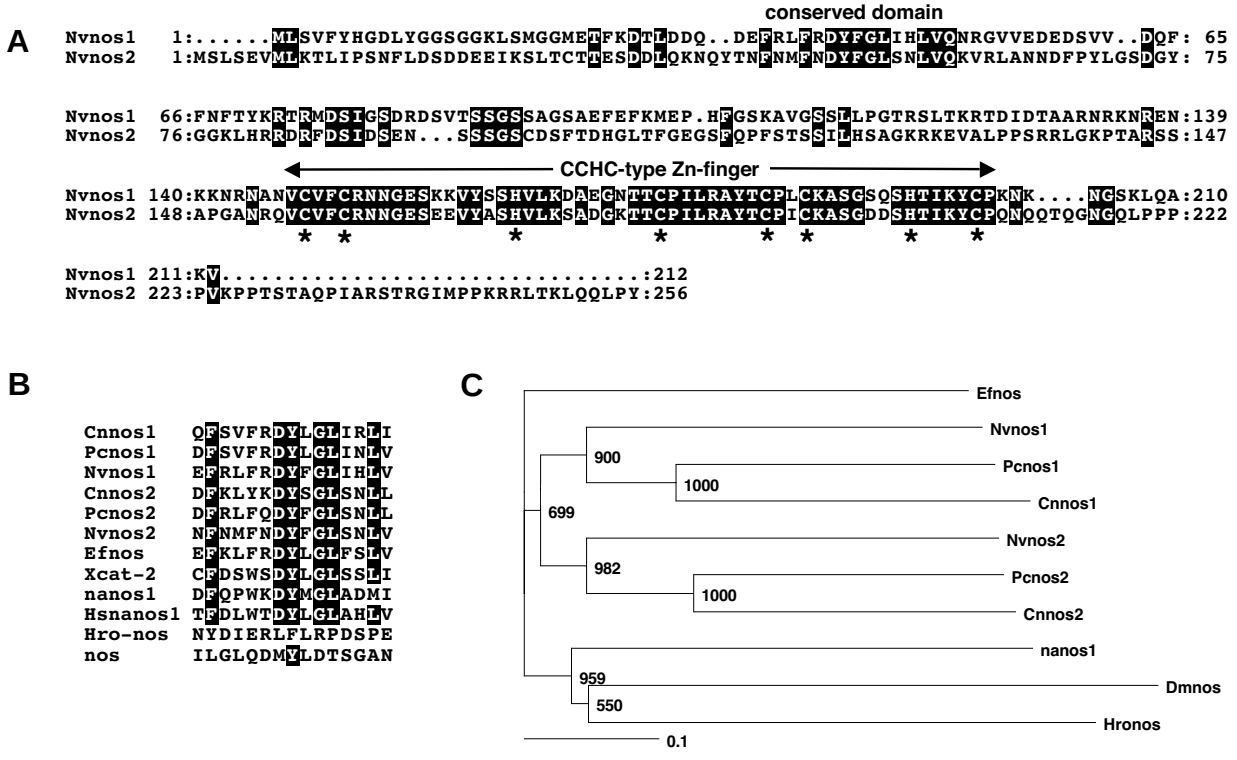

Fig. 1. *Nvnos1* and *Nvnos2* sequence analysis. (A) *Deduced amino acid sequence of* Nematostella nanos *cDNAs (*Nvnos1 *and* Nvnos2*) aligned to each other. The CCHC-type Zn-finger domain and the upstream conserved domain are indicated. Conserved Cs and Hs are indicated by stars.* **(B)** *Presence of the upstream conserved domain in non-protostome animals. Sequences are from the cnidarians* Hydra *(*Cnnos1 *and* Cnnos2*),* Podocoryne *(*Pcnos1 *and* Pcnos2*) and* Nematostella *(*Nvnos1 *and* Nvnos2*), the sponge* Ephydatia fluviatilis *(*Efnos*), the deuterostomes* Xenopus *(*Xcat-2*), zebrafish (*nanos1*), human (*Hsnanos1*) and the protostomes* Helobdella robusta *(*Hro-nos*) and* Drosophila *(*nos*) (refs. in Torras* et al.*, 2004).* **(C)** *Phylogenetic analysis of full-length Nanos proteins. Gene abbreviations as in B. Numbers in the branches correspond to bootstrap replicates out of 1000 supporting the topology shown. Efnos sequences were defined as outgroup and used to root the tree.*

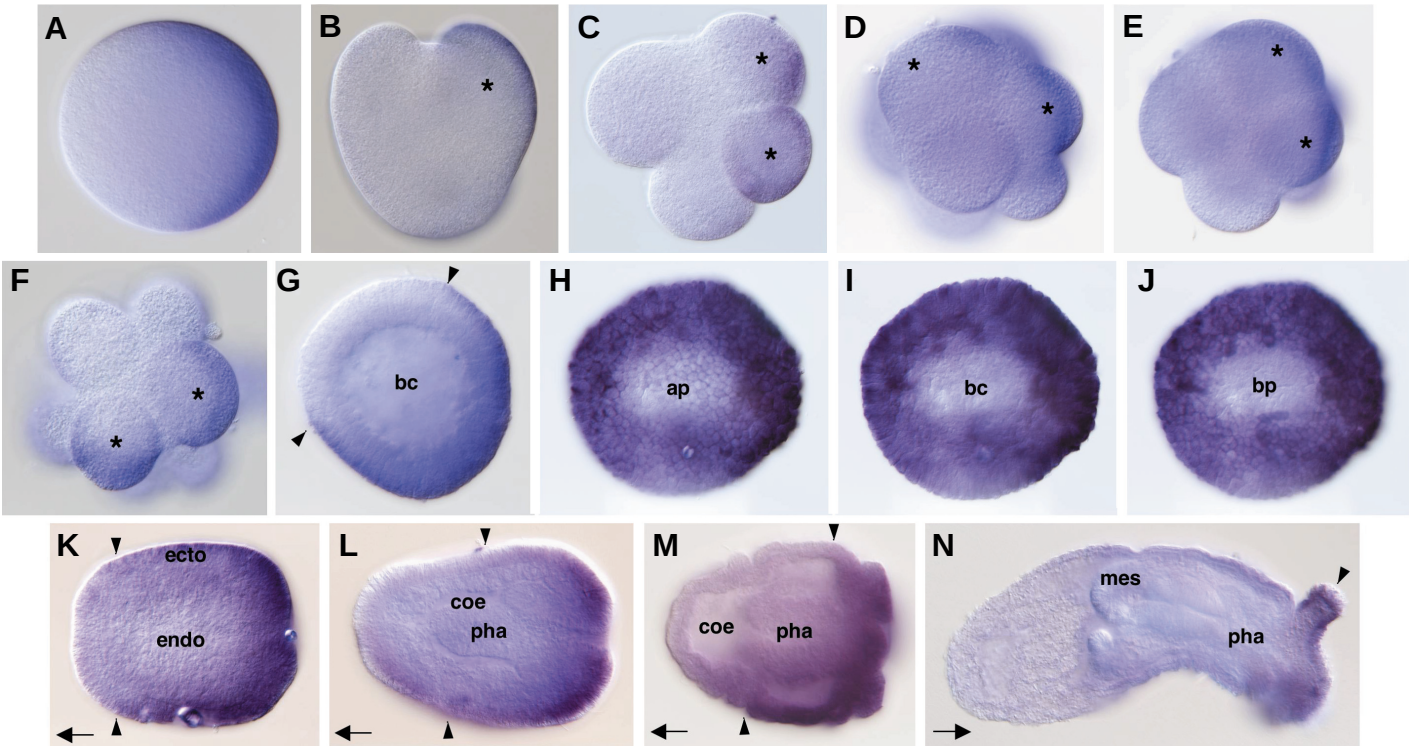

Fig. 2. *Nvnos1* expression pattern during *Nematostella* development. (A) *Fertilized oocyte stained for* Nvnos1 *mRNA. Staining forms a gradient with peak levels in one side that decrease towards the centre. This pattern is consistently observed in nearly all embryos examined. No staining is detected using* sense *riboprobes.* **(B-F)** *Cleaving embryos. Cells containing* Nvnos1 *mRNA are indicated by stars.* **(B)** *Beginning of the 2-cell stage.* Nvnos1 *expression is detected in one of the cells but not in the other.* **(C)** *4-cell stage. Only two adjacent cells contain* Nvnos1 *mRNA.* **(D,E)** *Same 8-cell stage embryo shown at two different focal planes. In both cases,* Nvnos1 *expression is localized in two adjacent cells.* **(F)** *12-cell embryo showing* Nvnos1 *expression.* **(G)** *Hollow blastula.* Nvnos1 *expression is localized to one side and extends to the limits indicated by arrowheads.* **(H-J)** *Same early gastrula stained for* Nvnos1 *shown at three optical focal planes.* **(H)** *View at the level of the anterior pole (ap). Note that anterior unstained cells appear at the same focal plane that surrounding stained cells.* **(I)** *Optical middle section. The empty hole corresponds to the blastocoele (bc).* **(J)** *View at the blastopore (bp) level. Note that unstained invaginating cells are slightly out of focus in relation to the surrounding stained cells.* **(K-M)** *Lateral views of planulae oriented with anterior to the left and posterior to the right. The anterior apical sensory tuft of large cilia is lost during the process of* in situ *hybridization. Arrowheads indicate the approximate anterior limits of gene expression.* **(K)** Nvnos1 *expression. Staining accumulates preferentially in the ectoderm of the posterior region.* **(L)** *Older planula showing* Nvnos1 *staining. Note the forming coelenteron and pharynx. Although* Nvnos1 *expression is still posteriorly localized, some asymmetry is observed.* **(M)** *Old planula showing sustained* Nvnos1 *expression in the posterior region.* **(N)** *Juvenile polyp.* Nvnos1 *expression is limited to the tentacle buds (arrowhead). Abbreviations: ap, anterior pole; bc, blastocoele; bp, blastopore; coe, coelenteron; ecto, ectoderm; endo, endoderm; mes, mesenteries; pha, pharynx. Swimming direction is indicated by an arrow at the bottom left of the figures.*

posterior region. Finally, posterior *nanos* expression disappears when the swimming polarity reverses and the primary polyp forms. Altogether, these results indicate a conservation of posterior *nanos* expression during early cnidarian development and suggest an ancient involvement of *nanos* in axial patterning as a posterior determinant.

Results and Discussion

To study the ancestry of *nanos* expression in posterior embryonic regions, we have isolated corresponding orthologs in the anthozoan *Nematostella vectensis* and analyzed their expression patterns throughout development. We have isolated two *Nematostella nanos* orthologs, *Nvnos1* and *Nvnos2*, encoding proteins of 212 and 256 amino acid residues, respectively (Fig. 1A). Both proteins contain a domain with two CCHC-type Zn-fingers highly similar to those present in Nanos proteins from other species (not shown). Also, they show a short region close to the amino-terminus that is conserved in other animals with the exception of the protostomes (consensus FxxxxDYxGLxxL) (Fig. 1A, B). The functional significance of this region is presently unknown. We also performed phylogenetic analysis with the complete Nanos amino acid sequences from *Nematostella*, *Hydra*, *Podocoryne*, the sponge *Ephydatia*, the leech *Helobdella*, *Drosophila* and zebrafish (refs. in Torras *et al.*, 2004) using the neighbor-joining method. In the resulting tree (Fig. 1C), the cnidarian *type 1 nanos* genes group with themselves with preference to *type 2* genes from the same species, thus suggesting that both cnidarian *nanos* genes arose from an ancestral duplication that predated cnidarian diversification, as previously suggested by analysis of the Zn-finger domain (Torras *et al.*, 2004).

Next, we analyzed the expression of *Nvnos1* and *Nvnos2* during *Nematostella* development by RT-PCR (data not shown). We found that *Nvnos1* mRNA is already present in very early embryos and is probably of maternal origin. The levels of *Nvnos1*

mRNA remain fairly constant throughout embryonic and larval development. On the contrary, *Nvnos2* expression is firstly detected at gastrula stages, so there is no maternal contribution for this transcript. Both *Nvnos1* and *Nvnos2* showed similar levels of expression at gastrula and planula stages.

We also analyzed *Nvnos1* and *Nvnos2* expression patterns during *Nematostella* embryonic and larval development by whole-mount *in situ* hybridization. Contrary to ubiquitous *nanos* expression in *Podocoryne* early embryos (Torras *et al.*, 2004), *Nvnos1* mRNA of maternal origin appears localized to one side of the fertilized egg (Fig. 2A). This asymmetrical distribution is maintained during 2 and 4-cell stages, where *Nvnos1* mRNA is detected only in one half of the embryo (Fig. 2B, C). Although cleavage in *Nematostella* embryos does not follow a stereotyped pattern (Martindale *et al.*, 2004), we always detect *Nvnos1* mRNA in contiguous cells located at one side of the cleaving embryo (Fig. 2D-F). In the resulting hollow blastula, *Nvnos1* expression is present throughout approximately one half of the embryo (Fig. 2G). Due to the spherical shape of the blastula, it is difficult to determine precisely its orientation. Nevertheless, this localized *Nvnos1* expression is sustained in immediately following stages, which can unambiguously be oriented; since in these embryos *Nvnos1* is posteriorly localized (see below), we assume that its expression in the blastula corresponds to the presumptive posterior region.

Therefore, the restriction of *nanos* expression to posterior regions of the cleaved embryo is essentially conserved in both cnidarian species *Podocoryne* (hydrozoan) and *Nematostella* (anthozoan). The distribution of maternal *Nvnos1* mRNA in the *Nematostella* zygote (Fig. 2A) is closely reminiscent of *nanos* expression in the *Drosophila* freshly laid egg (Wang and Lehmann, 1991). In *Drosophila*, untranslated sequences present in maternal *nanos* mRNA are responsible for its anchoring to the embryonic posterior pole (Gavis *et al.*, 1996). It is possible that a conserved mechanism could mediate *Nvnos1* mRNA localization in *Nematostella* embryos, although we have not detected any particular sequence similarity in the untranslated regions. In *Podocoryne*, the restriction of *nanos* mRNA to posterior regions is a later event, firstly detected in the blastula (Torras *et al.*, 2004).

At the early gastrula stage, the embryonic A-P axis can be morphologically recognized for the first time, since the blastopore, which forms at the posterior pole, is easily visible. By this stage, *Nvnos1* is highly expressed throughout most of the embryo (Fig. 2I), but not in the anterior pole (Fig. 2H). Also, invaginating cells at the blastopore region do not show *Nvnos1* expression (Fig. 2J). This result indicates that the expression observed at the blastula (Fig. 2G) (and probably at previous stages) corresponds to presumptive posterior regions. Again, this pattern is well conserved when compared to *nanos* expression in the *Podocoryne* gastrula, which remains restricted to the posterior region (Torras *et al.*, 2004). Moreover, in both species, invaginating cells do not show *nanos* expression (Fig. 2J; Torras *et al.*, 2004). However, in *Nematostella* the posterior domain of expression is largely expanded when compared to *Podocoryne*, thus leaving a relatively small anterior region without *nanos* expression (Fig. 2H).

During development of the planula larva, *Nvnos1* expression remains preferentially localized to posterior regions of the ectoderm (Fig. 2K). A similar expression pattern is observed for *Nvnos2* at this stage (data not shown). Contrary to *Nvnos1*, *Nvnos2* expression is firstly detected at early gastrula stages both by RT-PCR and *in situ* hybridization (data not shown), so its origin is exclusively zygotic. A significant difference between *Nematostella* and *Podocoryne* is the persistence of *Nvnos1* and *Nvnos2* expression in planula stages, because in *Podocoryne* both orthologs are totally silent (Torras *et al.*, 2004).

At the time when the coelenteron, the pharynx and the mesenteries form, *Nvnos1* expression is maintained at the posterior ectoderm with a certain asymmetry (Fig. 2L, M). Finally, when the juvenile polyp forms and the swimming direction is reversed, *nanos* expression disappears from the epidermal body trunk; we only observe *Nvnos1* staining in the developing tentacles (Fig. 2N). Similar results were obtained for *Nvnos2* (data not shown). The fact that both *Nvnos1* and *Nvnos2* show similar expression patterns throughout development raises the possibility of functional redundancy. Alternatively, since both genes have been maintained after such an early duplication, it is conceivable that they may play independent functions.

In the juveniles, no *nanos* expression is detected in the mesenteries, where the gonads will develop in the adult animal. Since *nanos* expression in developing germ cells is well conserved throughout the Animal Kingdom, including the cnidarians (Mochizuki *et al.*, 2000; Torras *et al.*, 2004), we suggest that *Nematostella* germline differentiation takes place in later stages of development, as it is the case for *Podocoryne* (Torras *et al.*, 2004). In this regard, we detected *Nvnos1* and *Nvnos2* expression in both aboral and oral regions of the adult polyp by RT-PCR (data not shown), probably associated to the developing germline cells. The fact that, in addition to *nanos*, cnidarian developing germ cells show conserved expression of other germline-specific genes such as *vasa* (Mochizuki *et al.*, 2001) and *Piwi* (Seipel *et al.*, 2004), as in Bilateria, suggests that germline development in Metazoa is a homologous process.

Together with our previous studies in *Podocoryne* (Torras *et al.*, 2004), these results indicate that the expression of *nanos* in developing posterior precursors is a conserved feature in the phylum Cnidaria. Due to the basal phylogenetic position of this phylum, a possible function of *nanos* as a posterior determinant in *Nematostella* and *Podocoryne* would represent an ancestral trait in the Animal Kingdom. Cnidarian *nanos* genes could regulate the formation of posterior/oral structures, such as the mouth or the tentacles.

If *nanos* plays a role as a posterior determinant in cnidarians, its expression domain should be in agreement with the distribution of the different cnidarian *Hox* genes along the A-P axis. Unfortunately, there is no obvious conservation between the reported *Hox* expression domains for *Nematostella* (Finnerty *et al.*, 2004) and *Podocoryne* (Yanze *et al.*, 2001). For example, in *Nematostella*, the expression of *anthox1* (an ortholog of the *Drosophila* "posterior" *Hox* gene *AbdB* (Finnerty *et al.*, 2004)) is localized to the anterior pole during blastula and gastrula stages, in a domain complementary to *nanos* expression. However, in *Podocoryne* the anterior pole is the domain of expression of *Cnox1-Pc*, an ortholog of the *Drosophila* "anterior" *Hox* gene *labial* (Yanze *et al.*, 2001).

In spite of the lack of conservation of *Hox* expression between *Nematostella* and *Podocoryne*, posterior *nanos* expression is well conserved in both cnidarian species. In this scenario it is conceivable that *Hox* genes may be dispensable for the formation

of the A-P axis during early cnidarian development, as it has been suggested in *C. elegans* (Wang *et al.*, 1993), the leech (Nardelli-Haefliger *et al.*, 1994) and the sea urchin (Arenas-Mena *et al.*, 1998). At early stages, *Nematostella* and *Podocoryne nanos* could be responsible for posterior patterning. Later, when the planula develops into a juvenile polyp and *nanos* expression disappears from the posterior epidermis, *Hox* genes could exert their role in regulating axial polarity. Then, for example, the aboral tip could be patterned under *anthox1* regulation, thus acquiring a "posterior" identity coincident with the time of the switch in the swimming direction.

Experimental Procedures

Animal culture

Nematostella vectensis animals were provided by Dr. Ulrich Technau (Sars Centre for Marine Molecular Biology, Norway). They were kept in filtered 33% artificial seawater at 17ºC and fed with freshly hatched *Artemia* nauplius larvae three times per week. The medium was changed once a week. Spawning was induced by a feeding/starvation regime, heat and light, as described (Fritzenwanker and Technau, 2002). Fertilized eggs, embryos and planula larvae were collected for *in situ* hybridization.

Polymerase chain reaction and cDNA cloning

RT-PCR fragments encoding the *nos* Zn-finger domain were obtained from RNA from *Nematostella* polyps using the oligonucleotides 5'-CGGAATTCCGTGYGTNTTYTGYVRNAAYAA-3' and 5'-CGGGATCCCGGGRCARTAYTTNAYNGTRTG-3' in 30 cycles at 43ºC as annealing temperature. Corresponding full-length cDNAs were isolated from a cDNA library provided by Dr. John Finnerty (Boston University) by PCR using specific oligonucleotides from the Zn-finger region and vector sequences. Both DNA strands were sequenced by using the ABI 377 analyzer (Perkin Elmer) and Big Dye (Applied Biosystems) terminator chemistry.

Phylogenetic analysis

Sequence alignments and neighbour-joining analysis were done using the Clustal X and Tree-View programs. Similar results were obtained using maximum parsimony and maximum likelihood methods with the PAUP and Tree-Puzzle programs, respectively (data not shown).

Whole-mount in situ *hybridization*

Fertilized eggs, embryos and planula larvae were processed for whole-mount *in situ* hybridization as described (Scholz and Technau, 2003).

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Note added in Proof:

Nvnos 1 and Nvnos2 sequences have been deposited in GenBank under the accession numbers DQ066724 and DQ066725, respectively.

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