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Expression and evolutionary conservation of *nanos*-related genes in *Hydra*

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Abstract The *Drosophila* gene *nanos* encodes two particular zinc finger motifs which are also found in germline-associated factors from nematodes to vertebrates. We cloned two *nanos* (*nos*)-related genes, *Cnnos1* and *Cnnos2* from *Hydra magnipapillata*. Using whole-mount in situ hybridization, the expression of *Cnnos1* and *Cnnos2* was examined. *Cnnos1* was specifically expressed in multipotent stem cells and germline cells, but not in somatic cells. *Cnnos2* was weakly expressed in germline cells and more specifically in the endoderm of the hypostome where it appears to be involved in head morphogenesis. In addition to structural conservation in the zinc finger domain of *nanos*-related genes, functional conservation of *Cnnos1* was also demonstrated by the finding that a *Cnnos1* transgene can partially rescue the nos^{RC} phenotype that is defective in the egg production of *Drosophila*. Thus, the function of *nanos*-related genes in the germline appears to be well conserved from primitive to highly evolved metazoans.

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Introduction

In sexual reproduction, genetic information is transmitted from one generation to the next through germ cells. In higher metazoans, germ cells are segregated from somatic cells during early embryogenesis. In *Drosophila*, for example, pole cells are produced in the posterior end of the embryo where they are separated from the rest of the somatic cells. Later, they migrate into the gonad and eventually become germ cells. In contrast, in asexual reproduction which many lower metazoans undergo, there needs to be a mechanism to ensure the transmission of germ cells into asexual offspring. This is achieved by the presence of totipotent cells or multipotent stem cells which can give rise to both germ cells and somatic cells. For example, the neoblasts in planaria are totipotent stem cells which can differentiate to any other cells (Wolff and Dubois 1948; Baguña et al. 1989). *Hydra* has multipotent interstitial stem cells which undergo self-renewal to maintain their own population and produce germ cells as well as three types of somatic cells, nematocytes, neurons and gland cells (David and Gierer 1974; David and Murphy 1977; Bode et al. 1987; Bosch and David 1987). In addition to multipotent stem cells, *Hydra* also have egg- or sperm-restricted stem cells (germline stem cells) (Littlefield 1985, 1991; Nishimiya-Fujisawa and Sugiyama 1993, 1995). The latter cells are considered to be derived from multipotent stem cells, although no direct evidence is available. It is important to examine the differentiation of multipotent stem cells and germline stem cells, as well as their origin, in embryogenesis in *Hydra*. However, experimental analyses have been limited by the lack of appropriate markers specific to these cells. Thus, one of the aims of the present study was to obtain such markers. Possible candidates are some of the components of germ plasm.

In some animals, germ cell formation is associated with a cytoplasmic determinant(s) called germ plasm (pole plasm for insects). In early development of *Drosophila*, the posterior pole plasm is incorporated into pole cells, the germline progenitor cells. Furthermore, the transplantation of pole plasm into the anterior pole of eggs can induce ectopic germ cell formation, strongly suggesting the presence of determinants for germline fate (Illmensee and Mahowald 1974; Okada et al. 1974). Similar cytoplasmic localization of germ plasm is observed in *Caenorhabditis elegans* (Strome and Wood 1983; Wolf et al. 1983) and *Xenopus* (Czolowska 1969; Whitington and Dixon 1975). In lower invertebrates, the presence of a germ plasm-like entity has also been reported. In *Hydra*, both multipotent stem cells and germ cells contain dense bodies, but their mass decreases as somatic cell differentiation proceeds (Noda and Kanai 1977). Likewise in planaria, neoblasts and germ cells contain chromatoid bodies (Coward 1974; Hori 1982).

The germ plasm is characterized by electron-dense structures, called germinal (polar) granules which are rich in RNA and proteins. In *Drosophila*, several components involved in polar granule formation and pole cell formation are known. For example, Oskar, Tudor and Vasa proteins are required for polar granule formation (Lehmann and Nüsslein-Volhardt 1986; Ephrussi and Lehmann 1992; St. Johnston 1993), mitochondrial large ribosomal RNA (mtlrRNA) and germ cell-less protein are necessary for pole cell formation (Kobayashi and Okada 1989; Jongens et al. 1992, 1994; Iida and Kobayashi 1998; Robertson et al. 1999), while Nanos protein (Nos) is necessary for pole cell differentiation (Kobayashi et al. 1996; Forbes and Lehmann 1998) as well as abdominal formation (Lehmann and Nüsslein-Volhardt 1991). The ultrastructural similarity of germ plasms among different animal phyla led to the search for the *Drosophila* homologs in various species. As a result, *vasa*-related genes have been found in the mouse, rat, frog, zebrafish, silkworm, nematode and planaria (Roussell and Bennett 1993; Fujiwara et al. 1994; Komiya et al. 1994; Komiya and Tanigawa 1995; Gruidl et al. 1996; Ikenishi et al. 1996; Olsen et al. 1997; Yoon et al. 1997; Nakao 1999; Shibata et al. 1999), mtlrRNAs in the frog (Kobayashi et al. 1998), and *nanos*-related genes in the frog, leech and nematode (Mosquera et al. 1993; Pilon and Weisblat 1997; Kraemer et al. 1999; Subramaniam and Seydoux 1999). Here we focus on *nanos*-related genes.

It is well known that Nanos together with Pumillio repress the translation of the maternal *hunchback* (hb) mRNA in the posterior of the embryo, thereby directing normal abdominal segmentation (Tautz 1988; Wang and Lehmann 1991; Wharton and Struhl 1991; Barker et al. 1992; Murata and Wharton 1995; Wreden et al. 1997). The interaction of Nanos and Pumillio is also required for the migration of pole cells to the gonads and for regulation of gene expression in the migrating pole cells (Asaoka-Taguchi et al. 1999). Furthermore, *nanos* appears to be required for the maintenance of stem cells (Bhat 1999). In mutants with loss of function alleles of *nanos*, only a few eggs are produced (Gavis and Lehmann 1992; Wang et al. 1994; Curtis et al. 1995). The egg-laying defect in the absence of Nanos activity appears to be due to degeneration of the germline stem cells (Bhat 1999).

In *C. elegans*, three *nanos* homologs, *nanos-1*, *nanos-2* and *nanos-3* have been found (Kraemer et al. 1999; Subramaniam and Seydoux 1999). Although their cytoplasmic localization has not been firmly established, they are all required for germ cell survival. NANOS-3 has been shown to form a complex with FBF, a Pumillio relative in *C. elegans*, that controls the sperm-oocyte switch in hermaphrodites (Kraemer et al. 1999). In *Xenopus*, the mRNA of a *nanos*-related gene, *Xcat2* is localized to the germ plasm, although its function is as yet unknown (Zhou and King 1996). All the deduced proteins from these *nanos*-related genes have little sequence similarity except for the C-terminal region which contains two presumptive zinc finger motifs which are probably required for RNA-protein or protein-protein interaction (Curtis et al. 1997).

In the present study, we will describe the cloning and expression of two *nanos*-related genes of *Hydra*: *Cnnos1* and *Cnnos2*. *Cnnos1* mRNA was expressed in both multipotent stem cells and germline cells, while *Cnnos2* mRNA was distinctly expressed in the endoderm of the hypostome, the apical tip of the body column and also weakly in germline cells. We will also present the evolutionary conservation of *Cnnos1*, whose transgene can rescue the egg-laying defect caused by mutation of *nanos* in *Drosophila*. Finally, the relationship and evolutionary conservation of the *Hydra Cnnos* genes and other *nanos*-related genes will be discussed.

Materials and methods

Hydra and culture conditions

Five types of *Hydra* derived from a strain nem-1 of *Hydra magnipapillata* (Sugiyama and Fujisawa 1977; Nishimiya-Fujisawa and Sugiyama 1993, 1995) were used. They were normal male, normal female, pseudo-epithelial male, pseudo-epithelial female and epithelial *Hydra*. Pseudo-epithelial animals contain only male germline cells (germline stem cells and possibly their early differentiation intermediates) if they are male, or female germline cells if female (Nishimiya-Fujisawa and Sugiyama 1993, 1995). Epithelial *Hydra* is essentially composed of epithelial cells and contains no interstitial cell lineage (Marcum and Campbell 1978; Sugiyama and Fujisawa 1978) except for gland cells.

Normal animals were cultured with daily feeding according to Sugiyama and Fujisawa (1977). Pseudo-epithelial *Hydra* and epithelial *Hydra* were cultured by hand-feeding according to the method described by Nishimiya-Fujisawa and Sugiyama (1993).

Sexual induction

Sexual differentiation was induced by combining gentle aeration and starvation as described by Sugiyama and Fujisawa (1977).

A

CnNOS1

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  1 MALSLCKTKD LFSSREGLNL DKFSFNYKQD SQFSVFRDYL GLIRLILPEN
 51 RIEMSKLHSP TESVDNVDFF FNYPNIISSN RHRIDSLDPD ETSESSSSEG
101 RGDNEVIYEN LSSSEEYSPY RQSKVNSIHS NVLAQTYAQT LESLIANQSV
151 VSQQIHNHVQ QQIQSKALKN LSKTSVCVFC RNNGESREFY SSHTLKDNEG
201 NTMCPILRAY TCPLCKSHGN QSHTIKYCPK YTPKPKTDKL LGISMPLL
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CnNOS2

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  1 MTMTDVYMQN FVLKNIFETD DELDLWSSSD SSNSSECSPT FFSRSAKGIN
 51 DFKLYKDYSG LSNLLTNVNI SDDNPLVDGD HRLSFLPRNQ IYNKLSNDPE
101 GSDVDVYSPT RDLALRSRDN NNNFPDVKQL PPSTRLSKSD RDMIISGAFQ
151 QQLSFINKHS DRSQIKMNPP LQAPPSPALV QPQQTLYNSH DSLTLRASSN
201 VCVFCRNNGE SENVYASHVL KDTDGRTSCP ILRAYTCPIC KANGDNSHTI
251 KYCPMNQNAR SASTFNGLSL PPSVNMAPRN TFPQPVRGNF RSPFPVNLTP
301 RINLGAKIR
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B

Fig. 1A, B Deduced amino acid sequences of CnNOS1 and CnNOS2 and their relationship with other Nanos-related proteins in the region of CCHC double zinc finger motifs. **A** Deduced amino acid sequences of CnNOS1 and CnNOS2. The positions of CCHC in the zinc finger motifs are shown in bold letters. **B** Alignment of the conserved putative zinc finger regions in Nanos-related proteins. Two CCHCs are marked with *dots*. CnNOS1 and CnNOS2 were from *Hydra magnipapillata* (*Hm*), HRO-NOS from *Helobdella robusta* (*Hr*; Pilon and Weisblat 1997), Nanos from *Drosophila melanogaster* (*Dm*; Wang and Lehmann 1991), XCAT-2 from *Xenopus laevis* (*Xl*; Mosquera et al. 1993), NANOS-1 and NANOS-2 from *Caenorhabditis elegans* (*Ce*; Subramaniam and Seydoux 1999), and NANOS-3 from *C. elegans* (*Ce*; Kraemer et al. 1999)

Isolation of *Hydra nanos*-related genes

Hydra nanos-related cDNA was isolated by a series of RT-PCRs. Standard molecular techniques (Sambrook et al. 1989) were used throughout the study, unless otherwise specified.

Total RNA was extracted from normal female polyps starved for 8 days by an AGPC method (Chomczynski and Sacchi 1987). First-strand cDNA was synthesized using total RNA as the template, random hexamer primers (Pharmacia), and SuperScriptII reverse transcriptase (Life Tech). For PCR, the following degenerate primers were designed from the amino acid sequences (in parentheses) of two different regions within the well-conserved zinc finger domain among Nanos-related proteins (Fig. 1B):

nosFW(CVFCENN); CGG AAT TCC GTG (CT)GT ITT (CT)TG (CT)(ACG)(AG) IAA (CT)AA
nosRV(HTIKYCP); CGG GAT CCC GGG (AG)CA (AG)TA (CT)TT IA(CT) IGT (AG)TG

where restriction sites for *Eco*RI and *Bam*HI are underlined, respectively, and I stands for inosine. nosFW and nosRV were designed from Nanos (*D. melanogaster*), XCAT-2 (*Xenopus laevis*) and HRO-NOS (*Helobdella robusta*) (Fig. 1B). PCR was carried out to obtain a fragment of the zinc finger domain by using first-strand cDNA as the template, nosFW and nosRV as upstream and downstream primers, respectively, and Taq DNA polymerase (Roche), with 40 cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 60 s. The amplified DNA was separated on a 1% agarose gel, recovered and cloned directly into the pCR2.1 plasmid with the Original TA Cloning Kit (Invitrogen), or digested with *Eco*RI and *Bam*HI and then cloned into the pBluescript-SK(+) plasmid (Stratagene). After sequencing the inserts, *Cnnos1* was obtained. The *Cnnos2* fragment was cloned into the plasmid as described above after digesting the amplified DNA with *Hin*fI, which digested the *Cnnos1* fragment.

Full-length sequences of *Cnnos1* and *Cnnos2* were obtained by 5′- and 3′-rapid amplification of cDNA end (RACE) methods (Frohman et al. 1988). Primers used in the experiments were as follows:

CDS-pr; CTA AGC AGT GGT AAC AAC GCA GAG $T_{(28)}$-(ACG)(ACGT)
RT-pr; TAC GGC TGC GAG AAG ACG ACA GAA GGG
5′-PCR; TAC GGC TGC GAG AAG ACG ACA GAA
3′-PCR-1; CTA AGC AGT GGT AAC AAC GCA GA
3′-PCR-2; AAG CAG TGG TAA CAA CGC AGA GT
nos1FW1; GCT CGC ACA CTT TAA AAG ATA ACG A
nos1FW2; CAC TCT GCA AGT CAC ATG GTA ACC
nos1RV1; TTG CAG AGT GGG CAT GTG TAG
nos1RV2; CCT TCG TTA TCT TTT AAA GTG TGC GA
nos2FW1; AAA AGA TAC TGA TGG CCG AAC A
nos2FW2; CTT GTC CTA TTC TTC GCG CA
nos2RV1; CAA ATA GGA CAT GTA TAT GCG CG
nos2RV2; AAT AGG ACA AGA TGT TCG GCC

The cDNA template for RACE was synthesized with the SMART PCR cDNA Synthesis Kit (Clontech) using total RNA, CDS-pr and RT-pr primers, and SuperScriptII reverse transcriptase (Life Tech).

The 5′ region of *Cnnos1* or *Cnnos2* cDNA was amplified by nested PCR. The first PCR was carried out as described above us-

ing the cDNA template, RT-pr as an upstream primer, nos1RV1 or nos2RV1 as a downstream primer, and Taq DNA polymerase (Roche). The second amplification was performed similarly except that 1/50 of the first PCR mixture was used as the template and 5′-PCR and nos1RV2 or nos2RV2 were used as upstream and downstream primers, respectively. The amplified DNA was cloned into the pCR2.1 plasmid (Invitrogen) and the 5′ sequences both for *Cnnos1* and *Cnnos2* cDNA were determined.

The 3′ sequence of *Cnnos1* or *Cnnos2* cDNA was determined in a similar fashion to the 5′ sequence, except that different sets of primers were used. In the first PCR, nos1FW1 or nos2FW1 and 3′-PCR-1 were used as upstream and downstream primers, respectively. In the second PCR, nos1FW2 or nos2FW2 was used as an upstream primer and 3′-PCR-2 as a downstream primer.

DNA sequencing

DNA sequences were determined for both strands using the Dye Terminator Cycle Sequencing Kit (PE-Applied Biosystems) and the ABI PRISM 377 DNA Sequencing System (PE-Applied Biosystems).

In situ hybridization

Digoxygenin (DIG)-labeled RNA probes were prepared using the DIG-RNA Labeling Kit (Roche). The *Cnnos1* cRNA probe corresponded to the first 696 bp of the cDNA, which partially included the first zinc finger motif (Fig. 1A), and the *Cnnos2* probe corresponded to the full-length cDNA. In situ hybridization on whole-mounts of *Hydra* was carried out as described by Grens et al. (1995, 1996).

For simultaneous visualization of the *Cnnos1* and *CnASH* mRNA expression, the *CnASH* cRNA probe which corresponded to the *CnASH* cDNA sequence from 50 bp to 815 bp (Grens et al. 1995; amplified using RT-PCR from *H. magnipapillata* total RNA) was also prepared by labeling with fluorescein using Fluorescein Labeling Mix (Roche). Hybridization was carried out with 1 µg/ml of DIG-labeled *Cnnos1* cRNA probe and 2 µg/ml of fluorescein-labeled *CnASH* cRNA probe. After post-hybridization washes, samples were incubated overnight in a 2,000-fold diluted alkaline phosphatase (AP)-conjugated anti-fluorescein antibody solution (Roche). Color reaction was performed by using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Lab.). After examining the intensity of staining under a microscope, the enzyme was denatured by heating at 65°C for 30 min. The samples were fixed overnight with 4% paraformaldehyde and subjected to the second color reaction to detect the DIG-labeled probe. The color reaction was carried out using NBT/BCIP Mix Solution according to the manufacturer's protocol (Roche). The stained samples were fixed in 4% paraformaldehyde in PBS, dehydrated and mounted in Permount (Fisher).

Construction of a *Cnnos1* vector for transformation and transformed *Drosophila* lines

A full-length coding sequence of *Cnnos1* (747 bp) was amplified by PCR under the conditions described in RACE (see above), using the following primers which carry an *Nde*I cutting site at the 5′ end (underlined): 5′-CCC ATA TGG CGT TAT CTT TAT GTA AAA C (upstream primer) and 5′-CCC ATA TGC TAA AGT AAA GGC ATG CTG A (downstream primer). The amplified product was purified, digested with *Nde*I at 37°C and cloned into an *Nde*I site of a pBS-KS vector which contains the *nanos* promoter [P*nos* and *NOS* 3′-UTR (*nos* 3′UT)] regions (a gift from E. Gavis; Asaoka-Taguchi et al. 1999). The P*nos-Cnnos1-nos* 3′UT fragment was excised by *Hin*dIII digestion from the vector and sub-cloned into the pCaSper 4 P-element vector (Thummel and Pirrotta 1992). This construct was co-injected into *yw* embryos with a helper plasmid to obtain transformants (Spradling 1986). Three independent w^+ transformants were inbred to establish homozygous *Cnnos1/Cnnos1* lines (designated as *Cnnos1*–4, –19 and –20). Flies from these transformant lines were crossed to nos^{RC} mutants (Wang et al. 1994) and tested for complementation of *nanos* mutant phenotypes. Flies homozygous for the *Cnnos1* transgene and nos^{RC} and control flies homozygous and heterozygous for nos^{RC} were allowed to lay eggs for a further 7 days after eclosion. The number of eggs laid each day was scored and summed up. All flies were maintained and crossed under standard conditions at 25°C.

Results

Hydra have two *nanos*-related genes

Two *nanos*-related genes called *Cnnos1* and *Cnnos2* were obtained by a series of RT-PCRs from *H. magnipapillata* (see Materials and methods). The complete nucleotide sequences of these two genes can be obtained from DDBJ (accession numbers AB037080 for *Cnnos1* and AB037081 for *Cnnos2*). From northern blot analysis, at least *Cnnos1* mRNA was considered to be full-length (data not shown). Figure 1A shows the deduced amino acid sequences of CnNOS1 and CnNOS2. The putative CnNOS1 contains 248 amino acid residues, while CnNOS2 contains 309. In both sequences, two CCHC (bold letters) zinc finger motifs were highly conserved. In fact, alignment of Nanos-related proteins from various animals showed that only the regions of zinc finger motifs are well conserved (Fig. 1B). The identity of CnNOS1 in this region to CnNOS2, XCAT-2 (*X. laevis*), Nanos (*D. melanogaster*), HRO-NOS (*Helobdella robusta*, leech) NANOS-1, NANOS-2 and NANOS-3 (*C. elegans*) was 72.2, 57.4, 50.0, 50.0, 32.1, 34.8 and 28.8%, respectively (Fig. 1B). CnNOS1 and CnNOS2 are closer to each other than to any other Nanos-related proteins.

Cnnos1 is expressed in germline cells

The expression pattern of *Cnnos1* in polyps was analyzed by whole-mount in situ hybridization. For the analysis, normal, pseudo-epithelial and epithelial polyps were used (see Materials and methods). These differ with regard to the cell composition of the interstitial cell lineage (Fig. 2A). Fig. 2B–F show the results. In an epithelial polyp, no stained cells were observed (Fig. 2B). In contrast, both in male and female pseudo-epithelial polyps, only large interstitial cells were stained (Fig. 2C–F). Since all the interstitial cells in pseudo-epithelial *Hydra* are germline stem cells and possibly their early differentiation intermediates, and since they are all large in size, the stained cells represent germline cells. In male pseudo-epithelial *Hydra*, most of the stained cells occurred as strings of cells lined up along the body axis (Fig. 2C, D). This is a property typical of male germline cells (Littlefield 1985; Littlefield et al. 1985; Nishimiya-Fujisawa and Sugiyama 1993). In female pseudo-epithelial *Hydra*, the stained cells occurred as clumps of cells (Fig. 2E, F), which is typical of female germline cells (Littlefield

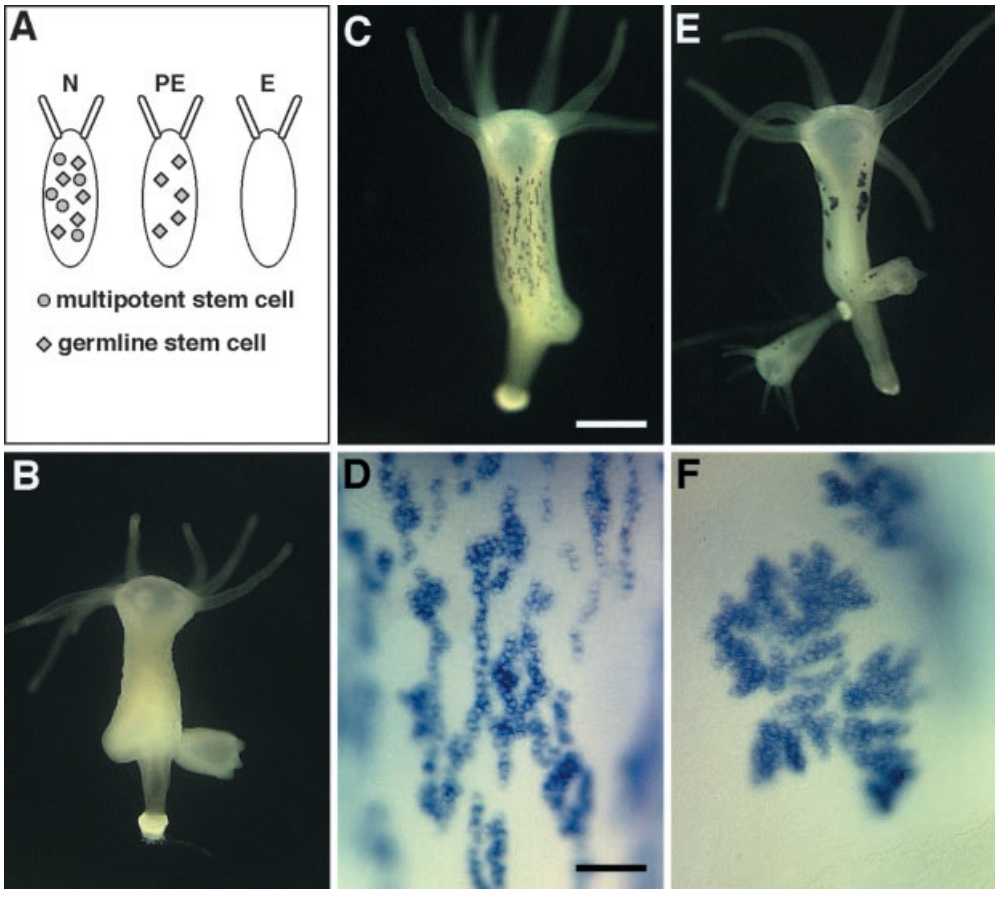

Fig. 2A–F Expression of *Cnnos1* mRNA in epithelial and pseudo-epithelial *Hydra* analyzed with whole-mount in situ hybridization. **A** Schematic illustrations of 3 types of *Hydra* which have different cell compositions in the interstitial cell lineage; normal (*N*), pseudo-epithelial (*PE*) and epithelial (*E*) polyps. **B** Epithelial polyp. **C, D** Low and high magnifications of a male pseudo-epithelial polyp. **E, F** Low and high magnifications of a female pseudo-epithelial polyp. **B,C,E** *Scale bar* 500 µm; **D, F** *scale bar* 50 µm

1991; Nishimiya-Fujisawa and Sugiyama 1995). Thus, *Cnnos1* appeared to be expressed in germline cells irrespective of sex. At present, we are unable to quantify the precise fraction of stained germline cells in pseudo-epithelial *Hydra*. However, the number of stained cells per polyp appeared to roughly match the total number of germline cells detected by interstitial cell-specific monoclonal antibody C41 (David et al. 1991; Nishimiya-Fujisawa and Sugiyama 1993, 1995).

Cnnos1 is also expressed in multipotent stem cells

Expression of *Cnnos1* mRNA in normal polyps was also examined by whole-mount in situ hybridization. In male polyps, strings of cells typically found in male pseudo-epithelial *Hydra* (Fig. 2C, D) were also detected (Fig. 3A, B). Likewise in female polyps, clumps of cells found in female pseudo-epithelial *Hydra* (Fig. 2E, F) were stained (Fig. 3C, D). These cells are considered to be germline cells. In addition to these darkly stained cells, weakly stained large interstitial cells which mostly occurred as single cells or pairs of cells were observed (Fig. 3B, D). They were scattered throughout the body column except for two extremities, the head and foot, where only very few were found (Fig. 3A, C). Multipotent stem cells in *Hydra* form a subpopulation of large interstitial cells which occur singly or in pairs and represent about 4% of the total cells (David and Gierer 1974). Furthermore, their concentration is uniform in the gastric region but about 20-fold lower in both the head and foot (David and Plotnik 1980). These features closely match the weakly stained cells observed here. Thus, these cells can be assumed to be multipotent stem cells and possibly early committed cells to the nematocyte or nerve cell pathway.

Cnnos1 is not expressed in somatic cells

As described above, darkly stained clusters of interstitial cells are considered to be germline cells. However, we cannot completely exclude the possibility that some of them may be clusters of nematoblasts. Also, weakly stained interstitial cells could be early committed cells to the nematocyte pathway. In order to examine these possibilities, we performed double in situ hybridization using *Cnnos1* and *CnASH* cRNA probes. *CnASH*, a cnidarian *achaete-scute* homolog, is expressed in the nematocyte pathway just after commitment to maturing stages in *Hydra* (Grens et al. 1995). Fig. 4 shows the staining in a normal polyp. Red and purple stains represent *CnASH* and *Cnnos1* expression, respectively. No double-stained cells were detected. Although no exact quantification has been made, we roughly estimate that the *CnASH*-positive cells represent 50%–60% of the total cell population in the nematocyte pathway, at least in *H. magnipapillata*

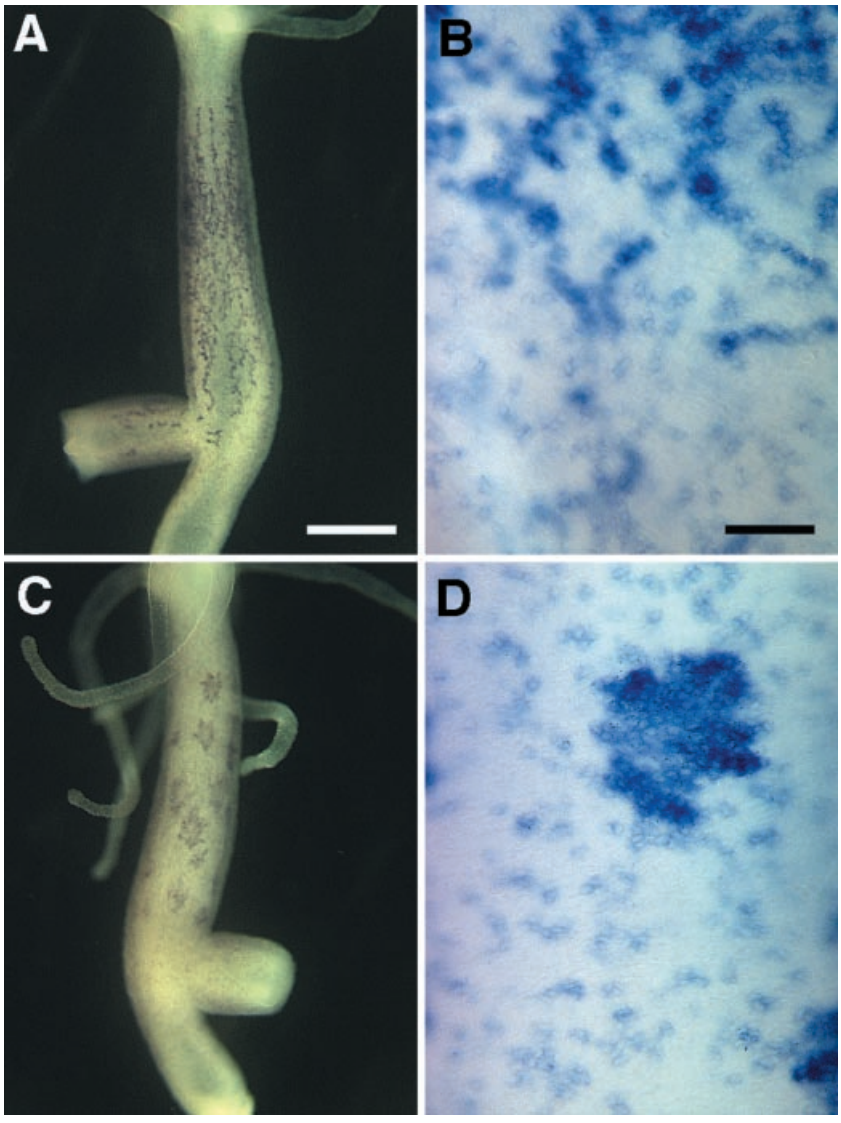

Fig. 3A–D Expression of *Cnnos1* mRNA in normal polyps analyzed with whole-mount in situ hybridization. **A, B** Low and high magnifications of a male polyp. **C, D** Low and high magnifications of a female polyp. **A, C** *Scale bar* 500 µm; **B, D** *scale bar* 50 µm

(K. Mochizuki. and T. Fujisawa, unpublished). Nevertheless, no evidence was found that *Cnnos1* mRNA is expressed in cells in the nematocyte pathway.

The neuron precursors are considered to be small interstitial cells which occur as single cells or pairs of cells (Heimfeld and Bode 1984; Bode et al. 1990). These small interstitial cells (5 µm–7 µm in diameter in whole-mount polyps used in the present study) were not observed among the *Cnnos1* mRNA-positive cells.

Thus, *Cnnos1* expression appears to be confined to multipotent stem cells and germline cells. The expression probably ceases immediately after multipotent stem cells are committed at least to the nematocyte pathway and possibly to the nerve cell pathway as well.

Cnnos1 expression in sexually induced polyps

The results presented above indicate that *Cnnos1* expression is low in multipotent stem cells but high in germline stem cells and early committed germline cells. Here, we examined its expression during gametogenesis in sexually induced polyps. Figure 5 shows the results. In male polyps, germline cells increase in number and migrate toward several sites on the body (Fig. 5A). Aggregated cells proliferate further to lift up the ectodermal cell layer. This mound of cells is called the testis, which is by no means comparable to the testis of higher organisms. The cells around the testis undergo spermatogenesis and mature sperm accumulate in the center of the testis. *Cnnos1* expression was very strong in germ cells which migrated massively toward the developing testes (Fig. 5B). However, as spermatogenesis proceeded, *Cnnos1* expression declined and was completely lost in mature sperm (see the empty regions in the center of the testes in Fig. 5A, C). Thus, *Cnnos1* expression ceased halfway through spermatogenesis.

During oogenesis, germline cells increase in number and form massive aggregates between the ectoderm and mesoglea and lift up the ectoderm. During this stage a

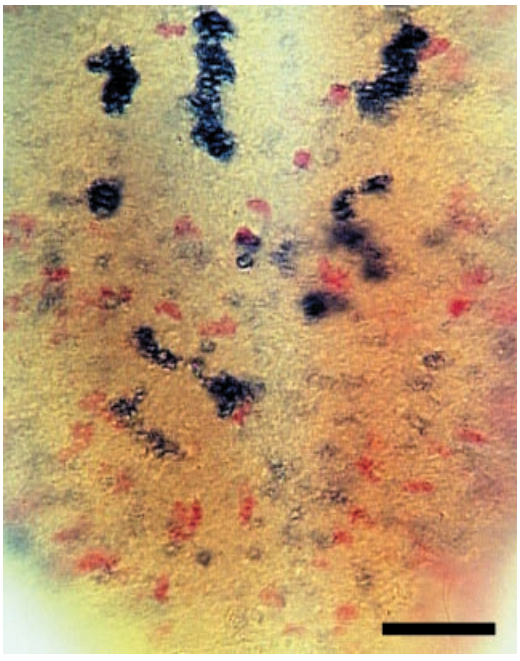

Fig. 4 Expression of *CnASH* and *Cnnos1* mRNA in a normal polyp analyzed with whole-mount double in situ hybridization. *CnASH* expression is shown in *red* and *Cnnos1* in *purple*. No overlapping staining was observed. *Scale bar* 50 µm

single oocyte is produced within an aggregate and the rest of the germ cells become nurse cells which are eventually phagocytosed by the oocyte (Honegger et al. 1989). Figure 5D and E depicts the oocyte and nurse cells strongly expressing *Cnnos1*. The oocyte grows further and forms a large rounded protrusion at the body column. During oogenesis, the level of *Cnnos1* expression and the number of cells expressing the gene kept on increasing and the message accumulated to a high level in unfertilized eggs (Fig. 5F). This suggests that *Cnnos1* is expressed both in oocyte and nurse cells, and presumably bears some maternal activity during embryogenesis.

Cnnos2 is expressed both in the hypostomal endoderm and germ cells

Expression of *Cnnos2* was examined in normal polyps and also in pseudo-epithelial polyps. Surprisingly, substantial expression of *Cnnos2* was detected in the hypostomal endoderm in normal (Fig. 6A, B), pseudo-epithelial and epithelial polyps (data not shown). The endodermal epithelial cells in the hypostome are folded in such a way that five protrusions can be seen when viewed from the top (Fig. 6B). Mucous cells are lined up along the outer edge of each protrusion and they do not express *Cnnos2*, leaving the area empty (Fig. 6B). This localized expression of the gene prompted us to examine its expression during head formation in budding and regeneration. Figure 6C–E shows the results of whole-mount in situ hybridization during budding. Expression was first detected in the endoderm of bud protrusion (stage 3 bud; Otto and Campbell 1977). The area of expression became confined to the tip of the bud as its development proceeded. A similar expression pattern was observed during head regeneration; however no expression was detected during foot regeneration (data not shown).

Cnnos2 expression in germline cells was somewhat erratic. In some polyps, normal or pseudo-epithelial, male or female, only a few clusters of germline cells expressed *Cnnos2*. Figure 6F and G shows the stained germline cells in male and female pseudo-epithelial *Hydra*, respectively. In others, no expression was observed. The reason for this is not known, but some possibilities are offered in the Discussion.

Table 1 Effects of the *Cnnos1* transgene on the egg-laying defect of *nos*^{RC} females

Genotype of females	Average no. of eggs laid by a single female[a]	Significance[b]
+/*nos*^{RC}	185.0	–
nos^{RC}/*nos*^{RC}	6.6	–
Cnnos1-4/*Cnnos1*-4; *nos*^{RC}/*nos*^{RC}	61.5	<0.002
Cnnos1-19/*Cnnos1*-19; *nos*^{RC}/*nos*^{RC}	42.0	<0.003
Cnnos1-20/*Cnnos1*-20; *nos*^{RC}/*nos*^{RC}	49.0	<0.001

[a] From 14 to 24 females from each line were allowed to lay eggs during 7 days after eclosion
[b] Probability was calculated by a Student's or Welch's *t*-test and compared with *nos*^{RC}/*nos*^{RC}

Cnnos1 rescued the defect in egg formation in a *Drosophila nanos* mutant

A high degree of conservation of two zinc finger motifs among *nanos*-related genes (Fig. 1B) raised the possibility that *Cnnos1* may be able to act in germline formation in *Drosophila*. To test this possibility, we examined whether transformed *Cnnos1* can rescue *nanos* mutant phenotypes in *Drosophila*. Females homozygous for a strong allele, *nos*^{RC}, produce only a few eggs (Wang et al. 1994). Thus, we compared the number of eggs laid by females carrying homozygous *Cnnos1* and *nos*^{RC} with the wild type and the mutant. The results are shown in Table 1. A control wild type female carrying heterozygous *nos*^{RC} (+/*nos*^{RC}) produced an average of 185.0 eggs in a week, whereas a female of *nos*^{RC} homozygous mutant produced 6.6 eggs. A female carrying homozygous *Cnnos1* and *nos*^{RC} produced 42.0 eggs–61.5 eggs during the same period of time. Thus, all three independent lines carrying the *Cnnos1* transgene rescued, although partially, the phenotype of the *nos*^{RC} mutant. However, the *Cnnos1* transgene failed to rescue the abdominal defect of the *nos*^{RC} or *nos*^{BN} mutation (Wang et al. 1994; data not shown).

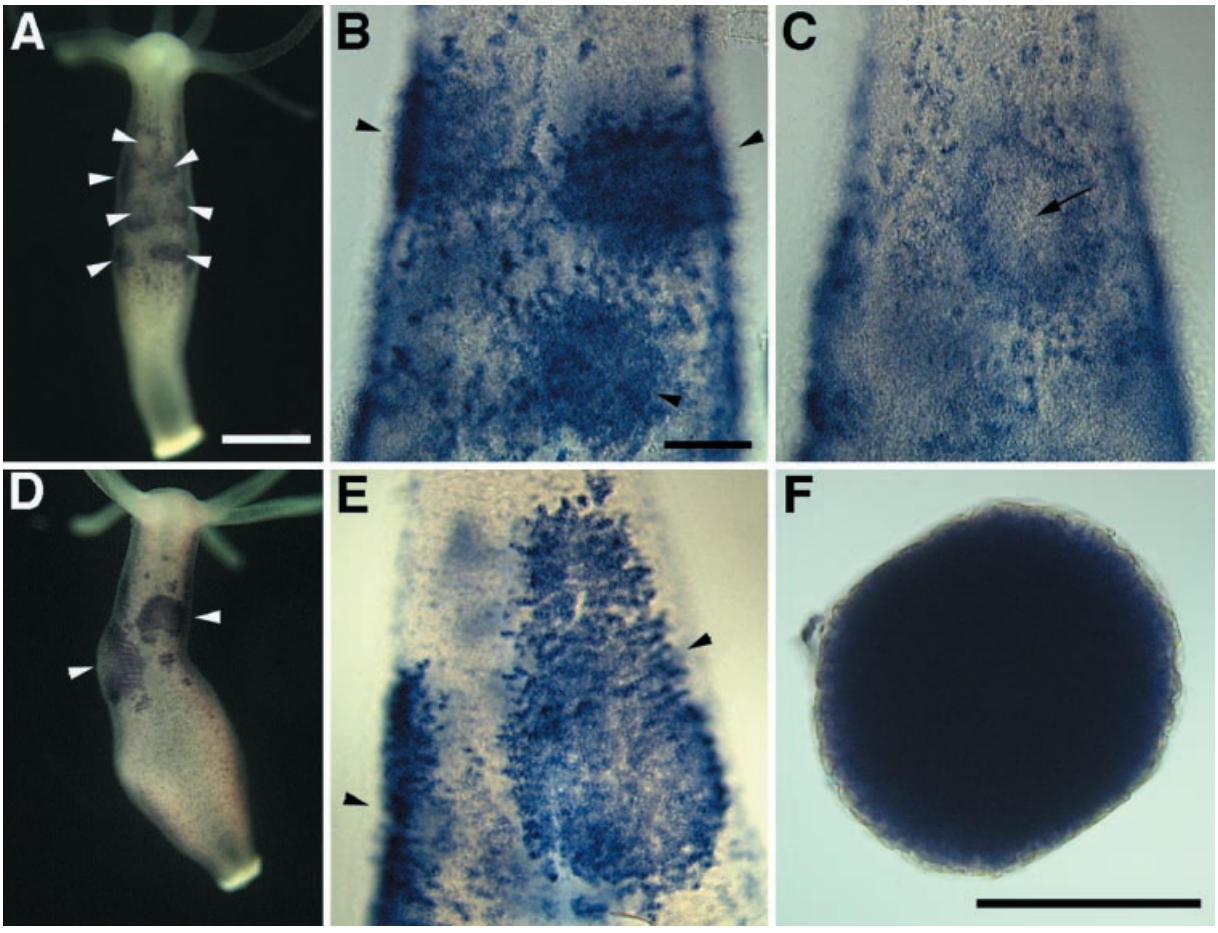

Fig. 5A–F Expression of *Cnnos1* mRNA in sexually induced polyps analyzed with whole-mount in situ hybridization. **A** Low magnification of a male polyp showing testes at late stages of formation. **B** High magnification of testes at early stages. **C** High magnification of the polyp shown in **A**. **D, E** Low and high magnification of a female polyp showing massive aggregation of germ cells. **F** Unfertilized egg. **A, D, F** *Scale bar* 500 µm; **B, C, E** *scale bar* 100 µm

Discussion

In the present study, we cloned two *nanos*-related genes, *Cnnos1* and *Cnnos2* from *H. magnipapillata*, and their expression was examined. *Cnnos1* is expressed in multipotent stem cells and germline cells, but not in somatic cells.

As summarized in Fig. 7, *Cnnos1* is expressed strongly in germline cells. This is supported by the following observations. In pseudo-epithelial *Hydra*, only interstitial cells expressed *Cnnos1* (Fig. 2). Since all the interstitial cells in pseudo-epithelial *Hydra* are germline cells (Littlefield 1985; Nishimiya-Fujisawa and Sugiyama 1993), *Cnnos1* is expressed only in this type of cell. Clusters of cells strongly expressing *Cnnos1* in normal male and female animals (Fig. 3) respectively showed the morphological characteristics typical for the male and female germline cells found in pseudo-epithelial *Hydra*. These cells did not express *CnASH* (Fig. 4), which is a specific marker gene for the nematocyte pathway (Grens et al. 1995).

Cnnos1 is weakly expressed in multipotent stem cells. This is supported by the following observations. In normal animals, the majority of the weakly stained cells were single or pairs of large interstitial cells (Fig. 3). They were distributed non-uniformly along the body column, being very scarce in both the head and the foot. These features are attributed to multipotent stem cells (David and Gierer 1974; David and Plotnik 1980). *CnASH* was not expressed in these *Cnnos1*-positive cells (Fig. 4). Furthermore, none of the *Cnnos1*-positive cells were single or pairs of small interstitial cells, which are believed to be neuron precursors (Heimfeld and Bode 1984; Bode et al. 1990). Thus, it would appear that *Cnnos1* is not expressed in somatic cells. However, some caution should be taken before drawing any final conclusion. Firstly, we do not know if all of the neuron precursors are small interstitial cells or not. Secondly, although *CnASH* is a good marker for the nematocyte pathway, about 50% of the cells in the nematocyte pathway appear to express the gene in *H. magnipapillata* (K. Mochizuki and T. Fujisawa, unpublished). Thirdly, the differentiation of gland cells from multipotent stem cells is poorly understood.

As summarized in Fig. 7, *Cnnos1* expression during spermatogenesis and oogenesis was different. In sperma-

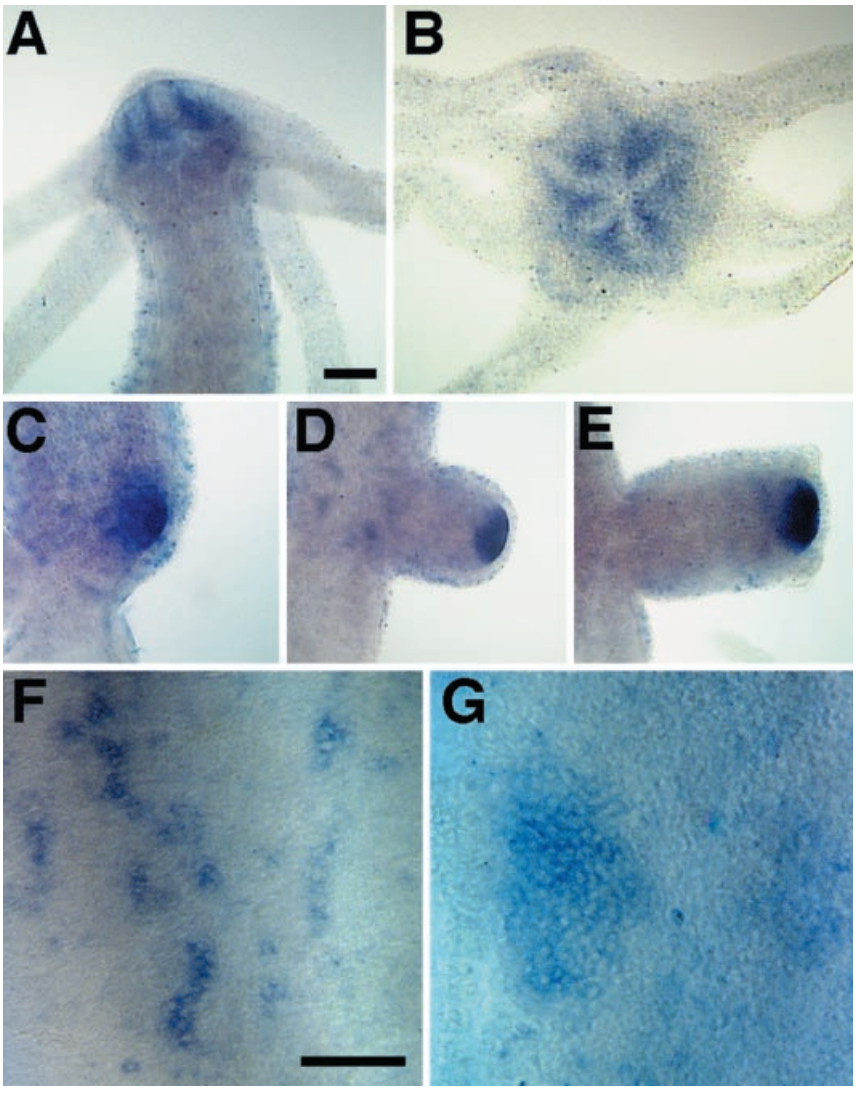

Fig. 6A–G Expression of *Cnnos2* mRNA analyzed with whole-mount in situ hybridization. **A** Expression in the endodermal epithelial cells in the hypostome of a normal polyp (*side view*). **B** *Top view*. **C–E** Expression at different stages of budding of normal polyps. **F** Germline cells in a male pseudo-epithelial polyp. **G** Germline cells in a female pseudo-epithelial polyp. **A–G** *Scale bar* 50 µm

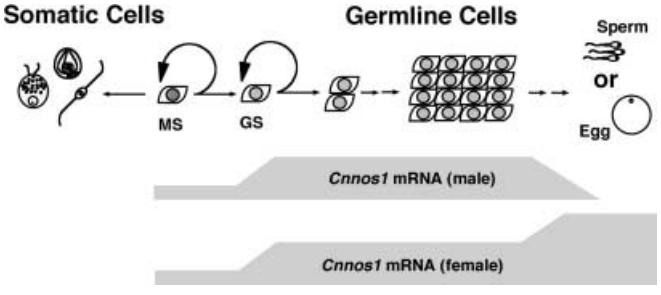

Fig. 7 Summarized expression patterns of *Cnnos1* mRNA in male and female interstitial cells (see text for details). *MS* Multipotent stem cells; *GS* germline stem cells

togenesis, its expression ceased halfway through, whereas strong expression persisted throughout oogenesis and the message was highly accumulated in the egg, suggesting the possibility of maternal activity (Fig. 5). Similar results were obtained with northern blot analyses (data not shown).

Despite some reservations, we conclude here that *Cnnos1* is expressed weakly in multipotent stem cells, strongly in germline cells, but not at all in somatic cells. *Cnnos1* is, therefore, the first good marker gene which is specific for germline cells and multipotent stem cells, and accordingly it can be used to distinguish these cell types.

Cnnos2 is expressed in the hypostomal endoderm in addition to germline cells

Cnnos2 expression was detected in germline cells (Fig. 6F, G). However, the number of clusters expressing it per polyp was small and the fraction of animals expressing it was also small. There are two possible explanations for this. First, the level of *Cnnos2* expression is low, bordering on the detection threshold. Second, *Cnnos2* is expressed only in a subset of germline cells. Unless higher sensitivity for detecting the signal is attained, it will be difficult to decide which of these explanations is most appropriate.

The most striking fact is that *Cnnos2* is expressed in the endodermal epithelial cells in the hypostome (Fig. 6A, B). Its expression was also detected during budding (Fig. 6C–E) and head regeneration (but not during foot regeneration; data not shown) in the presumptive hypostomal region well before the head structure was formed. This strongly suggests the involvement of *Cnnos2* in hypostome morphogenesis. A very similar expression pattern has been reported with *HyBra1*, a *Hydra Brachyury* homolog (Technau and Bode 1999). The gene is assumed to be involved in hypostome formation. *HyBra1*, however, appears to be expressed earlier during budding and head regeneration than *Cnnos2*. (K. Mochizuki and T. Fujisawa, unpublished). Therefore, it is possible that *Cnnos2* is a downstream gene of *HyBra1*. The relationship between these genes will be examined in the near future.

Evolutionary conservation of *nanos*-related genes in the activity of germline stem cells

The similarity among Nanos-related proteins from animals in various phyla is restricted to the putative CCHC double zinc finger motifs (Fig. 1B). This structural conservation of Nanos-related proteins suggested functional conservation. This was shown by a rescue experiment of the *nanos* phenotype in *Drosophila* by the *Cnnos1* transgene (Table 1). Females homozygous for nos^{RC} produce only a few eggs, although it is not known which stage of oogenesis the mutation affects (Wang et al. 1994). The presence of homozygous *Cnnos1* partially rescued the phenotype of the mutant. This strongly suggests that *Cnnos1* activity in germline stem cells is conserved during evolution. However, the transgene of *Cnnos1* was unable to rescue the abdominal defect of a nos^{RC} or nos^{BN} mutant (Wang et al. 1994; data not shown). For abdominal formation, Nanos and Pumillio are required (Wharton and Struhl 1991; Murata and Wharton 1995), although no direct interaction between these two proteins has been reported. Asaoka-Taguchi et al. (1999) have reported that Nanos and Pumillio are also involved in the migration of pole cells and in gene expression in migrating pole cells. By contrast, in *C. elegans*, FBF, a Pumillio homolog, directly binds to the N-terminal region of NANOS-3 and the complex regulates the translation of *fem* RNA, thereby achieving the switch from spermatogenesis to oogenesis in hermaphrodites (Kraemer et al. 1999). Recently, a region C-terminal to the zinc finger domain has also been shown to be necessary for abdominal formation and germ cell migration (Arrizabalaga and Lehmann 1999). Considering these facts, the failure of *Cnnos1* to rescue the abdominal defect may be attributed to poor conservation in both the region which is N-terminal and the region which is C-terminal to the zinc finger motifs (Fig. 1B).

Although the rescue experiment in *Drosophila* suggests that *Cnnos1* is involved in the activity of germline cells, its function in *Hydra* is not known. Expression of *Cnnos1* mRNA in multipotent stem cells and germline cells and the accumulation of messages in eggs (Fig. 7) are consistent with the idea that *nanos*-related genes are required for various aspects of germline development. The observation that *Cnnos1* expression ceased when the multipotent stem cells enter the somatic pathways (Figs. 4 and 7) is also consistent with the Nanos function of translational repression: CnNOS1 may be involved in repressing the translation of somatic genes, thereby maintaining the stem cell properties.

Two *nanos*-related genes were cloned from *Hydra* (Fig. 1A). We have also cloned both *Cnnos1* and *Cnnos2* from other cnidarians including Hydrozoans (*Hydractinia echinata*, *Tima formosa*, *Eirene spp.* and *Craspedacusta sowerbyi*), Scyphozoans (*Aurelia aurita* and *Sanderia malayensis*) and an Anthozoan (*Acropora digitifera*) (K. Mochizuki et al., unpublished). Thus, *Cnnos1* and *Cnnos2* are conserved in all three classes of Cnidaria. Furthermore, since other animals in different phyla have 1–3 *nanos*-related genes which cannot be classified to either one of the *Cnnos* genes, a *Cnnos* was possibly duplicated during cnidarian evolution. In this respect, it is worth examining *nanos*-related genes in even lower metazoans, such as sponges.

The final point we wish to make is with regard to *Cnnos2*. *Cnnos2* is expressed in the hypostome (Fig. 6A, B) and appears to be involved in hypostome formation (Fig. 6C–E). If this is the case, the question arises as to whether axis formation of *nanos*-related genes is also a function which was conserved during evolution. At present, we do not believe this to be the case for the following reasons:

1. No *nanos*-related genes in *C. elegans* are involved in morphogenesis.
2. The amino acid sequences in the regions both N-terminal and C-terminal to the zinc finger domain are not conserved among different phyla. This is consistent with the failure of *Cnnos1* to rescue the abdominal defect of *nanos* mutants.
3. *Cnnos1* and *Cnnos2* appear to have diverged during cnidarian evolution. Since both genes are expressed in germline cells, but only *Cnnos2* is expressed in the hypostome, any possible morphogenetic function was probably acquired after their divergence. Kraemer et al. (1999) have proposed that since *nanos* genes in *C. elegans* and *Drosophila* control germline survival, the ancestral function of the *nanos* genes may have been protection against germline death, and that the sperm-oocyte switch in nematode and axis formation in *Drosophila* may have been added later in evolution. Our present study appears to support this view.

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