

Cngsc*, a homologue of *goosecoid*, participates in the patterning of the head, and is expressed in the organizer region of *Hydra

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SUMMARY

We have isolated *Cngsc*, a hydra homologue of *goosecoid* gene. The homeodomain of *Cngsc* is identical to the vertebrate (65-72%) and *Drosophila* (70%) orthologues. When injected into the ventral side of an early *Xenopus* embryo, *Cngsc* induces a partial secondary axis. During head formation, *Cngsc* expression appears prior to, and directly above, the zone where the tentacles will emerge, but is not observed nearby when the single apical tentacle is formed. This observation indicates that the expression of the gene is not necessary for the formation of a tentacle per se. Rather, it may be involved in defining the border between the hypostome and the tentacle zone. When *Cngsc*⁺ tip of an early bud is grafted into the body

column, it induces a secondary axis, while the adjacent *Cngsc*⁻ region has much weaker inductive capacities. Thus, *Cngsc* is expressed in a tissue that acts as an organizer. *Cngsc* is also expressed in the sensory neurons of the tip of the hypostome and in the epithelial endodermal cells of the upper part of the body column. The plausible roles of *Cngsc* in organizer function, head formation and anterior neuron differentiation are similar to roles *goosecoid* plays in vertebrates and *Drosophila*. It suggests widespread evolutionary conservation of the function of the gene.

Key words: Goosecoid, Hydra, Head formation, Spatial patterning

INTRODUCTION

A major issue in development concerns axis formation and axial patterning. Accumulating evidence indicates that the genes regulating parts of these developmental processes are common in a variety of animals. For example, Hox genes are involved in the patterning of the anteroposterior axis in vertebrates as well as insects (Regulski et al., 1987; Watrin and Wolgemuth, 1993). The expression patterns of several genes isolated from hydra suggest they might be involved in axial patterning as are their homologues in more complex organisms. These include a homologue of a Hox gene (Shenk et al., 1993a,b; Endl et al., 1999) as well as homologues of *Hnf3β* (Martinez et al., 1997), *brachyury* (Technau and Bode, 1999) and *orthodenticle* (Smith et al., 1999) genes. Further, an *emx* homologue isolated from hydractinia, a close relative of hydra, also appears to have a role in axial patterning in that organism (Mokady et al., 1998). Since hydra and hydractinia are members of the Cnidaria, the most primitive group of animals with a defined body plan, these results suggest mechanisms governing axial patterning may have arisen early and been conserved throughout metazoan evolution.

In many organisms, axis formation is initiated in an organizer region such as the dorsal lip of the blastopore in frog embryos and Henson's node in chick embryos. Similarly, in hydra, the hypostome, the apical part of the head or the

developing hypostome in a bud act as organizer region. Transplantation of part of the hypostome to the body column of a host animal results in the formation of a second axis in which the hypostomal tissue induces the host tissue to form parts of the head and a body column (Browne, 1909; Yao 1945). Grafting experiments have shown that signals generated in the head and transmitted to the body column set up a gradient of head formation capacity as well as a gradient of head inhibition, which organize and pattern the single axis of the animal (e.g. MacWilliams, 1983a,b). These results have been explained in terms of a reaction-diffusion model (e.g. Meinhardt and Gierer, 1974). More recently data acquired using molecular markers (Bode et al., 1988; Technau and Holstein, 1995; Mitgutsch et al., 1999) have provided support for an updated version of the model (Meinhardt, 1993).

Thus, it is of interest to determine if genes known to be involved in organizer activity in more complex organisms exist in hydra and to what extent their function has been conserved. One family of such genes are the *goosecoid* genes. In vertebrates, *gsc* homologues are expressed in the organizer region of an embryo (Blumberg et al., 1991; Blum et al., 1992; Izpisua-Belmonte et al., 1993; Stachel et al., 1993), and play a well-known role in initiation of axis formation in *Xenopus* (Cho et al., 1991). Further, Goosecoid, a transcription factor, has a similar DNA-binding specificity as does Bicoid, the maternal morphogen responsible for anterior patterning in the

Drosophila embryo (Struhl et al., 1989). *gooseoid* genes are also involved in patterning parts of the head in vertebrates (Gaunt et al., 1993; Izpisua-Belmonte et al., 1993; Thisse et al., 1994) as well as in *Drosophila* (Goriely et al., 1996; Hahn and Jackle, 1996).

We have isolated and characterized *Cngsc*, a hydra member of the family of *gooseoid* genes. The structural and functional homology of *Cngsc* with *Xgsc* indicates that this gene family appeared early in metazoan evolution. The complexity of *Cngsc* expression pattern suggests the gene has several functions. It appears to have roles in delineating the regions of the head and in the development of an anterior part of the nervous system, and is associated with the organizer region. Further, the expression patterns of hydra homologues of *gooseoid*, *brachyury* and *Hnf3 β* suggests an evolutionary conservation of an interaction of these genes involved in axial patterning.

MATERIALS AND METHODS

Animals and culture conditions

Strains of two different species of hydra were used: the Basel, L2 and Zurich strains of *Hydra vulgaris* (provided by T. Holstein) and the 105 strain of *Hydra magnipapillata* (Martinez et al., 1997). Hydra were fed and maintained as described previously (Martinez et al., 1997).

Isolation and characterization of a Hydra homologue of *gooseoid*

The touch-down PCR method (Schunck et al., 1995) was used to isolate a fragment of the hydra *gooseoid* gene. Single-stranded cDNA transcribed from whole animal mRNA of the Basel strain of *H. vulgaris* was used as a template. The PCR reaction was carried out using degenerate oligonucleotide primers corresponding to the amino acid sequences of the conserved regions of the homeodomain of vertebrate *gooseoid* homologues [sense: AT(A/C/T)TT(C/T)ACNGA(C/T)GA(A/G)CA; antisense: (A/G)TT(C/T)TT(A/G)AACCANAC(C/T)TCNAC; antisense for nested PCR: NAC(C/T)TCNAC(C/T)TT(C/T)TC(C/T)TC]. The reaction was carried out using *Taq* polymerase (Fisher) under the recommended conditions with the addition of 2.5 mM (NH₄)₂SO₄. The reaction cycles were (95°C, 30 seconds; 52°-42°C, 2 minutes; 72°C, 1 minute) \times 4 cycles for each T_{ann}. 5'RACE (Frohman, 1993) was performed using the following antisense primers: GTCAGGGTAGTGC GTT and CAATCTTTCTAATACGTTTAA (for nested PCR).

H. vulgaris cDNA library (Sarras et al., 1994) was screened under high-stringency conditions (Sambrook et al., 1989). Clones were sequenced using the 'Thermo Sequenase radiolabeled terminator cycle sequencing kit' (Amersham).

Tissue manipulations and in situ hybridization

Several kinds of tissue manipulations were coupled with an in situ hybridization analysis of *Cngsc* expression on whole mounts (Martinez et al., 1997). The antisense RNA probe (0.4 ng/ml) included the part of *Cngsc* gene upstream of the homeodomain.

For regeneration experiments, animals of the L2 strain of *H. vulgaris* were bisected either directly beneath the tentacles, or half way down the body column and incubated at 18°C. Periodically samples of both lower and upper halves were analyzed.

To induce ectopic tentacle formation in the body column, animals of the 105 strain of *H. magnipapillata* were treated with 0.1 mM DAG (1,2-dioctanoyl-*sn*-glycerol; Sigma) (Müller, 1989). Animals were treated once daily over a period of 2 -10 days. Thirty animals were incubated with 3 ml DAG solution in 60 mm Petri dish with rotation

for 30 minutes on the first day and for 2 hours on the following day, and then washed five times for 5 minutes with hydra medium. Animals were fed daily throughout the treatment. DAG solution was freshly prepared in hydra medium and sonicated for 30 seconds prior to use.

Hydroxyurea (HU) treatment (Bode et al., 1976) was used to reduce the population size of cells of the interstitial cell lineage. Animals of the L2 strain of *H. vulgaris* were cultured in 10 mM HU in hydra medium for 4-5 days. This HU solution was renewed daily. The reduction of the interstitial cell population was monitored using toluidine blue staining (Diehl and Burnett, 1964).

Phylogenetic analysis

The evolutionary relationships among 19 paired-like genes were analyzed using the 64 amino-acid sequence of the homeodomain. Genes were chosen from a wide range of metazoan phyla. Phylogenetic trees were generated using PAUP 3.1.1 (Swofford, 1993). *Drosophila Prd* gene (Frigerio et al., 1986) was chosen as an outgroup. A heuristic search was performed with tree bisection and reconstruction branch swapping (100 replicates, random addition).

Xenopus embryos and mRNA microinjections

Eggs were obtained from *Xenopus* females injected with 600 units of human chorionic gonadotropin, fertilized in vitro and cultured in 0.1 \times MMR (Newport and Kirschner, 1982). Embryonic stages were determined according to Nieuwkoop and Faber (1967). The full-length clone of *Cngsc* was generated by ligation of the largest *Cngsc* clone obtained from cDNA library and product of 5'RACE. Capped synthetic RNAs were generated by in vitro transcription of plasmids containing *Cngsc* using mMessage mMachine kit (Ambion). For microinjections, embryos were placed in a dish containing 3% Ficoll, 0.5 \times MMR. 1.5 ng of either *Cngsc* mRNA or *Cngsc* mRNA missing the homeobox was injected into a single ventral blastomere at the 4- to 8-cell stage. Embryonic morphology was assessed at stages 39-40.

RESULTS

Cngsc is a hydra homologue of *gooseoid*

Isolation and characterization of *Cngsc*

Using two pairs of degenerate primers encoding conserved regions of the *gooseoid* homeodomain [TIFTDEQ, VEVWFKN and TIFTDEQ, EEKVEV], a fragment of a hydra *gooseoid* homologue, named *Cngsc*, was isolated with nested PCR. 5'RACE was used to obtain the 5' portion of the coding region, and the 5'RACE fragment was used to screen a hydra cDNA library (Sarras et al., 1994). The sequence of the entire coding region based on the largest isolated clone and the fragment obtained with 5'RACE predicts a protein of 205 amino acids (GenBank accession number AF183398).

Gooseoid proteins share two conserved regions, the homeobox and a GEH domain at the N terminus. The amino acid sequence of the *Cngsc* homeodomain show 65-72% identity with other Gooseoid homeodomains (Fig. 1A). The lysine at the position 50 (K50) of the *Cngsc* homeodomain is typical for some paired-like homeoproteins including Gooseoid (Wilson et al., 1993). Members of five classes of homeoproteins (Engrailed, Msh, NK1, NK2 and Gooseoid) share seven amino acid sequence at the N terminus of the protein termed the GEH (Gooseoid Engrailed Homology) region, also referred as eh-1 domain. *Cngsc* shares three (1, 6 and 7) of the four residues (1, 3, 6 and 7) of this heptapeptide that are most conserved among the several classes of homeobox genes containing this domain (Fig. 1B).

A phylogenetic analysis (Fig. 2) encompassing members of

A.

Cngsc	Hv	KRRHRTIFSD	EQLNVLRLRF	NKTHYPDIV	REEVAGIINL	TEEKVEVWFK	NRRARWRKQK	% identical
Dgsc	Dm	-----TE	---EQ--AT-	D-----VL	--QL-LKVD-	K--R-----	----K----	70
Zgsc	Dr	-----T-	---EA--N--	QE-K---GT	--QL-RRVH-	R-----	----K----	72
Xgsc	Xl	-----T-	---EA--N--	QE-K---GT	--QL-RRVH-	R-----	----K----	72
gsc	Gg	-----T-	---EA--N--	QE-K---GT	--QL-RKVH-	R-----	----K--R--	70
gsx	Gg	T-----TE	E--QA--T--	HQNQ----T	--HL-NR-H-	K--R-----	----K--H--	65
gsc1MM	Mm	T-----E	---QA--A--	VQNQ----GT	--RL-VR-R-	R--R-----	----K--H--	66
dharm	Dr	RGQI--V-T-	N-TEQ-----	AV-D--T-ET	-A-L-QNTG-	S--T-R----	----RKR-T	52

B.

consensus	FSIDNLI
Cngsc	Hv -LME---
Dgsc	Dm -T--S---
Xgsc	Xl -----
Cnmsx	Hv ----YL-
msh	Dm --VASL-
msx-1	Mm --V-ALM
en	Dm ---S---
en	Ce -G--R--
CnNK-2	Hv --V--D--
NK2	Dm --H--SD--

Fig. 1. Amino acid sequence alignments of (A) the homeodomains of Goosecoid proteins, and (B) GEH-domains of Goosecoid and other proteins. Dashes indicate residues identical with Cngsc in A and with the consensus sequence in B. (A) Homeodomains of hydra (Cngsc), *Drosophila* (D-gsc) (Gorieli et al., 1996), zebrafish (Zgsc) (Stachel et al., 1993), *Xenopus* (Xgsc) (Blumberg et al., 1991) and chicken (gsc and gsx) (Izpisua-Belmonte et al., 1993; Lemaire et al., 1997) goosecoid proteins, and zebrafish (dharm) (Yamanaka et al., 1998) and mouse (gsc1MM) (Galili et al., 1997) goosecoid-like proteins. The diagnostic lysine residue (K50) is in bold. (B) GEH-domains of goosecoid proteins of hydra (Cngsc), *Drosophila* (Dgsc) and *Xenopus* (Xgsc); msh proteins of hydra (Cnmsx) (McCord and H. R. B., unpublished data), *Drosophila* (msh) (Isshiki et al., 1997) and *M. musculus* (msx-1) (Kuzuoka et al., 1994); engrailed proteins of *Drosophila* (Poole et al., 1985) and *C. elegans* (Wilson et al., 1994); and NK-2 proteins of hydra (Grens et al., 1996) and *Drosophila* (Nirenberg et al., 1995).

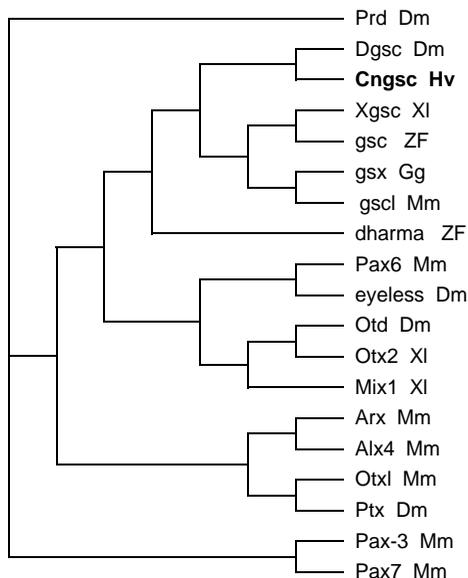


Fig. 2. Phylogenetic analysis of paired-like class proteins using parsimony. See Materials and Methods for details. The Genbank accession numbers for the sequences are as follows: Dgsc DM (U52968); Xgsc XL (M81481); gsc ZF (L03394); gsx Gg (Y09850); gsc1 MM (U70231); dharm ZF (AB010103); Arx MM (AB006103); Alx4 MM (AF001465); Pax6 MM (X63963), eyeless DM (X79493); Otd DM (X58983); Otx2 XL (Z46972); Mix1 XL (1170320); Otx1 MM (U80036); Ptx DM (AJ001519); Pax3 MM (AF014366); Pax7 MM (1352721), Prd DM (M14548).

the paired-like family of homeobox genes indicates that *Cngsc* clusters together with *goosecoid* homologues and not with other members of the family.

Southern analysis showed that the hydra genome contains a single copy of the *Cngsc* gene (data not shown), which produces a single 1.2 kb transcript, as revealed by northern analysis (data not shown).

Evolutionary conservation of the function of *Cngsc*

Since the structure of *Cngsc* resembles that of other goosecoid proteins, it was of interest to determine if it was sufficiently



Fig. 3. Embryos injected with *Cngsc* mRNA into a single ventrovegetal blastomere at a 4-8 cell stage. The induced secondary axes are indicated with the arrows. A control embryo is shown at the bottom of the picture.

conserved to carry out the function of goosecoid in another organism. Since *Xgsc* has been shown to induce a secondary axis in *Xenopus* embryos (Cho et al., 1991), we tested whether *Cngsc* has the same activity. 1.5 ng of in vitro synthesized mRNAs encoding either the full-length *Cngsc* or *Cngsc* with the homeodomain deleted were injected into a single ventrovegetal blastomere of a 4- to 8-cell *Xenopus* embryo. In three independent experiments ($n=68$), injection of full-length *Cngsc* mRNA resulted in the induction of a secondary axis in 20-50% of the injected embryos (Fig. 3). The secondary axes contained morphologically visible somites and neural tubes enriched with pigmented melanocytes. Occasionally hindbrain structures formed, but no eyes or cement glands were detected. Uninjected embryos ($n>100$) and embryos injected with the same amount of *Cngsc* mRNA with a deletion of the sequence encoding the homeodomain ($n=70$) did not develop secondary axes. Thus, axis-inducing activity of *Cngsc* is similar to the activity of injected *Xgsc* mRNA.

In sum, the structural and functional data indicate that *Cngsc* is clearly a homologue of *gooseoid*.

The pattern of *gooseoid* expression in adult hydra

Hydra has a simple body plan consisting of a head, body column and a foot ordered along a single axis. The body wall consists of two epithelial cell layers, the ectoderm and endoderm, separated by the mesoglea, a typical basement membrane. The tissue dynamics of an adult hydra (Campbell, 1967; David and Campbell, 1972) require that patterning, cell differentiation and morphogenesis be continuously active to maintain the form of the animal. Hence, genes regulating these processes can be examined in adult hydra.

In an adult hydra, *Cngsc* is expressed in three locations: two in the head and one in the body column (Fig. 4A,B). The head of hydra consists of two parts: the apical half, the hypostome, which is a dome containing the mouth, and the lower half which is the tentacle zone from which the tentacles emerge (Fig. 4D). One domain of expression of *Cngsc* expression is in the ectoderm at the very tip of the hypostome. (Fig. 4C). The only cells in the ectoderm of this region are epithelial cells and neurons (Bode et al., 1973). The dotted staining pattern in this region suggests that *Cngsc* is expressed in the nerve cells which are dispersed among the epithelial cells (Koizumi et al., 1988).

The second domain of *Cngsc* expression in the head is in a ring in the endoderm at the border between the hypostome and the tentacle zone. It is confined to the very basal part of the hypostome and extends into the tentacle zone forming a scalloped pattern around the apical portion of each tentacle (Fig. 4C,D), but is not expressed in the tentacles themselves. At the base of the hypostome, the endoderm has the shape of several wedges facing the middle (Fig. 4F) with epithelial cells at the base of each wedge and gland cells or mucous cells on the edge of each wedge. *Cngsc* expression is confined to the epithelial cells as it is expressed in wedge shapes near the base of the ectoderm as shown in the transverse section in Fig. 4E. The level of *Cngsc* expression in this border region is not consistent as it varies from very high to very low.

The third expression domain of *Cngsc* is in the epithelial cells of the endoderm in the body column (Fig. 4A,B). Expression is not detected in the most apical part of the body column near the head/body column border, but it is strong in the gastric region between the head and the budding zone. In the peduncle, the gene is expressed at a lower level than in the gastric region and expression is absent in the foot.

These three patterns of *Cngsc* expression were found in all four species examined, which included the three strains of *Hydra vulgaris* (Basel, L2 (not shown) and Zurich) and the 105 strain of *Hydra magnipapillata* (not shown).

Expression of *Cngsc* during axis formation in adult hydra

Xgsc plays an essential role in axis formation in *Xenopus* embryo (Cho et al., 1991). Later studies revealed that *gooseoid* orthologues participate in formation of anterior structures in both vertebrates (Izpisua-Belmonte et al., 1993; Gaunt et al., 1993) and *Drosophila* (Hahn and Jackle, 1996). Since *Cngsc* is expressed in the head, which plays an organizing role in hydra patterning (Webster, 1966; MacWilliams, 1983a,b), we examined the expression of this gene in situations where head structures, a complete head, or a new body axis is formed.

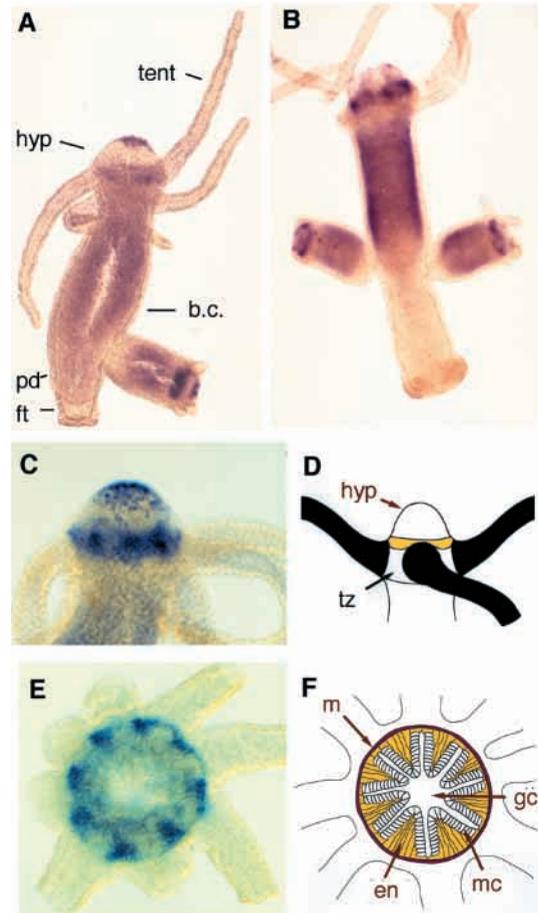


Fig. 4. Expression pattern of *Cngsc* in adult animals of the (A) Basel and (B) Zurich strains of *H. vulgaris* and in the head of animals of Zurich strain (C,E). Diagrams of the major expression pattern in the head (C) and a transverse section through the lower hypostome (E) are shown in D and F, correspondingly. Tent, tentacle; hyp, hypostome; tz, tentacle zone; m, mesoglea; gc, gastric cavity; en, endodermal epithelial cells; mc, mucous cells; b.c., body column; pd, peduncle; ft, foot.

Bud formation

Budding is the asexual form of reproduction in hydra. Bud formation occurs in the budding zone located two thirds of the distance down the body column from the head. The first visible sign of bud formation is the appearance of a circular placode in the ectoderm. Then both tissue layers evaginate forming a cylindrical protrusion that eventually develops in a complete animal with a head at the distal end and a foot at the proximal end (Otto and Campbell, 1977).

Changes in *Cngsc* expression began with the inception of budding. As the placode formed (stage 1), *Cngsc* expression was lost from this region (Fig. 5A). Then as the bud began to evaginate (stage 2), this loss of expression spread back into the budding zone covering most of the presumptive bud tissue, or bud field (Fig. 5B). By stage 3, expression had reappeared occasionally as a spot covering most of the endoderm in the protrusion (Fig. 5C), but more frequently as a ring covering all but the distal tip of the endoderm (Fig. 5D). By stage 4 (Fig. 5E), the expression was clearly a ring below the distal tip and,

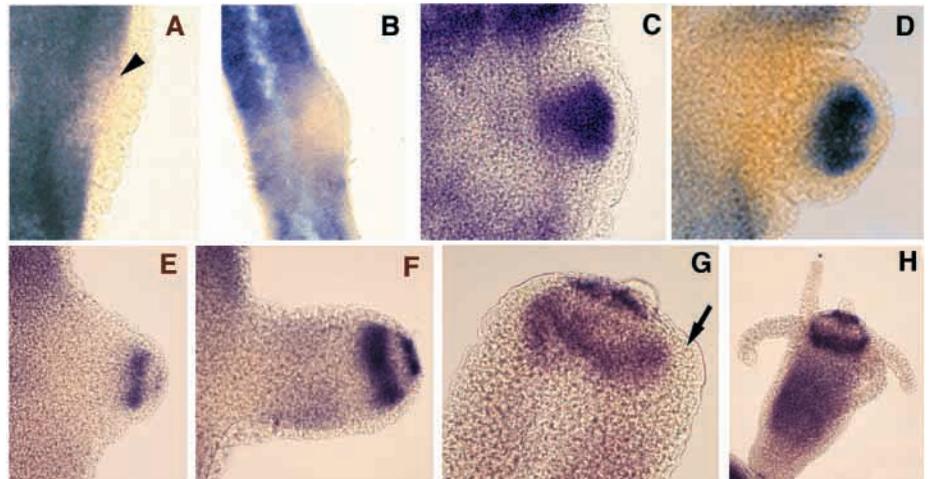


Fig. 5. Changes in *Cngsc* expression pattern during bud formation in *H. vulgaris* (Zurich strain). The stage corresponding to each figure is described in the text. Arrowhead in A indicates the reduction of *Cngsc* expression in the endoderm. Arrow in G indicates on the location of an emerging tentacle.

by stage 6 (Fig. 5G), tentacles began to emerge proximal to the ring. This region of expression of *Cngsc* is maintained throughout the remainder of the development of the bud (Fig. 5H, bud before detaching) and continues in the adult stage. However, the level of expression changed throughout the budding process. During the early bud stages (stages 3-6), expression was very strong decreasing slightly during later stages of budding, reaching a still lower level in the adult hypostome.

By the end of stage 4, the second domain of *Cngsc* expression appeared in the ectoderm of the bud tip (Fig. 5E), where the mouth will form. Initially a few spots, presumably neurons expressing *Cngsc* were observed. Subsequently, the number of cells expressing *Cngsc* increased as the bud developed (Fig. 5F-H), but the intensity of *Cngsc* expression did not change. To determine if these spots were neurons, interstitial cells, which give rise to neurons, were eliminated by treatment with hydroxyurea (Sack and Davis, 1979). Animals devoid of interstitial cells continue to bud, but the speckled expression pattern in the tip of the developing bud never appeared, indicating the spots are neurons expressing *Cngsc* (data not shown).

Finally, expression of *Cngsc* in the endoderm of the developing body column began to reappear during stage 5 (Fig. 5F). Unlike the endodermal ring, where the level of expression declines with development, and the ectoderm of the bud tip, where the level per cell is constant, the level of expression in the developing body column continues to increase throughout bud formation reaching an adult level shortly after detachment.

Head and foot regeneration

Following bisection of a hydra, the basal part will regenerate a normal head. Animals were bisected in the upper quarter of the body column. Within 3 hours, the epithelial layers stretched across and closed the wound. *Cngsc* expression in the apical tip remained constant until 4 hours after bisection, and thereafter began dropping rapidly so that, by 12 hours, the tip was devoid of expression of the gene (Fig. 6A). After that the dynamics of *Cngsc* expression is similar to that observed during bud development. By 24 hours, the gene was expressed in a spot or narrow ring in the endoderm of a regenerating tip (Fig. 6B). Thereafter, a ring of high level *Cngsc* expression developed below the tip and tentacles began to emerge just below this ring (Fig. 6C). At about the same time or a little later, the transcript showed up in the ectoderm of the regenerating tip in the speckled pattern (Fig. 6C,D).

When animals were bisected midway through the body column, the changes in *Cngsc* expression were the same as following decapitation, although the reappearance of expression at the apical tip was delayed (Fig. 7). This is consistent with the known decrease in the rate of head regeneration the further down the body column that bisection occurs (Webster and Wolpert, 1966; MacWilliams, 1983a,b).

Bisection of the body column results in the regeneration of a foot at the basal tip of an apical half. *Cngsc* expression in the regenerating basal tip vanishes 9 hours after bisection but, unlike head regeneration, it is not restored later (data not shown). Thus, *Cngsc* expression is related to head regeneration specifically.

In sum, the data suggest that expression of *Cngsc* is most likely

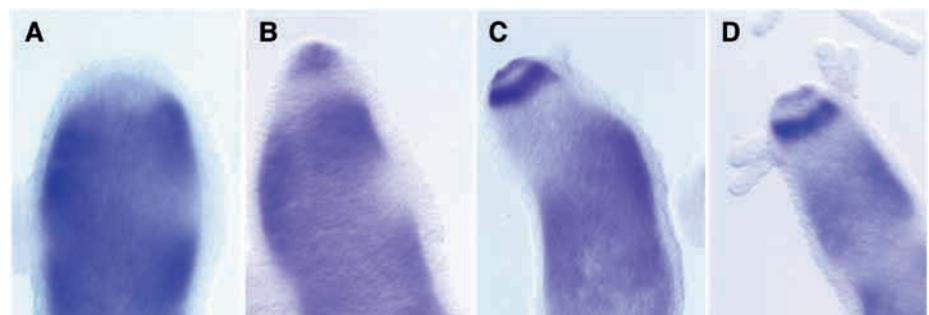


Fig. 6. *Cngsc* expression during head regeneration following decapitation in *Hydra vulgaris* (Zurich strain). (A) 12 hours, (B) 24 hours, (C) 48 hours, and (D) 72 hours after decapitation.

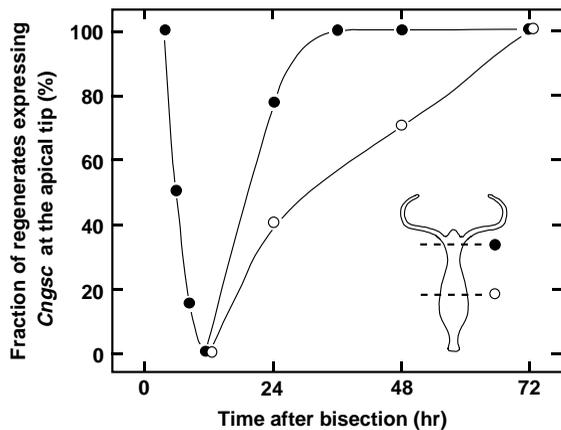


Fig. 7. Appearance of *Cngsc* expression at the apical tip during head regeneration in *Hydra vulgaris* (Zurich strain) after bisection. Animals were bisected either directly below the tentacle zone (filled circles) or in the middle of the gastric region (open circles).

associated with the patterning and/or morphogenesis of the head and body column, but not with the initiation of a new axis.

***Cngsc* expression is not required for tentacle formation**

The localization of *Cngsc* expression at the upper edge of the tentacle zone in an adult animal as well as during head formation could mean that the gene is involved in the patterning or morphogenesis of tentacles. Several observations suggest this is not the case.

Sometimes during head regeneration a single tentacle forms at the apical tip instead of the usual hypostome at the tip surrounded by a ring of tentacles below in the tentacle zone (Fig. 8A). Morphologically this apical tentacle is the same as a normal one and it stains with the tentacle-specific antibody TS19 (Bode et al., 1988) (data not shown). However, the ring of *Cngsc* that normally forms in a regenerating head does not appear and the region at the base of the apical tentacle remains devoid of *Cngsc* transcripts (Fig. 8A). Whenever a hypostome forms during head regeneration, the normal ring of *Cngsc* expression appeared. This occurred when the usual 5-7 tentacles formed (Fig. 6B), or if only two tentacles formed (Fig. 8B), or if a single tentacle emerged in the normal radial direction (Fig. 8D).

Prolonged treatment of animals with diacylglycerol (DAG) raises the gradient of positional value and causes the formation of ectopic heads and individual ectopic tentacles along the body column (Muller, 1989). *Hydra magnipapillata* were treated with DAG and 5 days after start of treatment ectopic tentacles began to emerge in the middle of the animal. There was no sign of increased *Cngsc* expression near or at the base of ectopic tentacles. At the same time, expression of *Cngsc* in the developing bud was normal (Fig. 8C).

These results indicate that *Cngsc* is not directly involved in the morphogenesis of the tentacle. Rather, the fact that expression of *Cngsc* always precedes the formation of a hypostome and a ring of tentacles during head formation indicates that the gene could be involved in the spatial programming within hydra head.

The ring of *Cngsc* expression is localized in the organizer region

Transplantation of a piece of the hypostome into the body column of a host animal leads to the formation of a second axis with a head at the distal end (Browne, 1909; Webster and Wolpert, 1966). Part of the hypostome, the tentacles as well as the body column of the second axis are formed by tissues of the host (Browne, 1909). Transplantation of the distal part of a developing bud of hydra into a body column of a host also results in the induction of second axis (Li and Yao, 1945). In this case, the hypostome of the induced second axis is derived from the graft, while the tentacles and body column arise from the host tissue. In both cases, the hypostomal tissue of the adult and the presumptive hypostome of the bud have the characteristics of an organizer. In vertebrates, the early embryonic expression of gooseoid is localized within the organizer region. Thus, it was of interest to determine if the expression of *Cngsc* in the endodermal ring of developing hypostome of a bud was also within the region of high organizing capacity.

Cngsc is expressed in the distal part of a stage 4-5 bud as a ring in the endoderm and in an ectodermal neurons (Fig. 5E,F). To determine the ability of the distal (*Cngsc*⁺) and the proximal (*Cngsc*⁻) parts of the bud to induce secondary axes when grafted to the host, two types of grafts were carried out. One was a lateral graft in which either parts of a stage 4-5 bud was grafted into the body column of a host animal (Fig. 9A,B). In the other, the same regions of the bud were grafted axially into the body column of a stage 6-7 bud (Fig. 9C,D). 6 days after grafting the transplants were scored for the number that induced second axes as well as the number of tentacles induced in the host tissue. The results are presented in Table 1.

Lateral grafts of either the distal or the proximal part of a bud frequently led to the induction of a second axis (Table 1). When the distal part of the bud was used the tentacles and the body column were at least partially, and sometimes completely, derived from the host tissue (Fig. 9A; Table 1). Conversely, when proximal tissue was used the secondary axis was derived primarily from the grafted tissue, while induction of tentacles and body column in the host tissue occurred much less frequently (Fig. 9B; Table 1). Similar differences were found with the axial grafts. When the distal part of a bud was grafted axially into a developing bud, it induced the formation of a head on the body column of the host bud. The tentacles of such a head were partly derived from the transplant and in part induced tentacles were formed by the host tissue (Fig. 9C). The proximal part of a bud neither induced a second axis nor were any tentacles formed (Fig. 9D).

These results show that the distal domains of *Cngsc* expression in a stage 4-5 bud mark the region with a high capacity for induction of secondary structures.

DISCUSSION

***Cngsc* is a hydra homologue of gooseoid**

The *gooseoid* gene was originally isolated from *Xenopus* (Blumberg et al., 1991) and later homologues from other vertebrates (Stachel et al., 1993; Blum et al., 1992; Izpisua-Belmonte et al., 1993) as well as from *Drosophila* (Goriely et al., 1996) were identified. *Cngsc* appears to be a homologue as

Table 1. Comparison of the inductive properties of the distal and proximal regions of a stage 4-5 bud

Type of graft	Region of bud	Number of grafts	2nd axes formed		Tentacles formed on 2nd axes		
			Number	%	Total number	Derived from the host tissue	Number/2nd axis*
lateral	distal	30	26	87	64	34	1.3
lateral	proximal	43	27	63	81	10	0.4
axial	distal	13	10	77	22	10	1.0
axial	proximal	11	0	0	0	0	0

*N/2nd axis=number of tentacles derived from host/number of 2nd axis formed.

well, as it has several characteristics of this class of paired-type homeobox genes. The homeodomain is 65-72% identical with other Goosecoid homeodomains, and has a lysine at position 50 (K50) in the homeodomain, which is typical of the Goosecoid homologues. Also, a phylogenetic analysis clearly groups Cngsc with other Goosecoid homologues. Further, Cngsc shares GEH domain with other Goosecoid homologues at the N terminus.

Another measure of conservation is the ability of Cngsc to substitute for *goosecoid* in a functional assay. The injection of either Xgsc or Cngsc into the ventral side of a *Xenopus* embryo, a second axis is induced. Since hydra is a member of the Cnidaria, a phylum that arose early in metazoan evolution, the *goosecoid* gene also arose early in metazoan evolution and the parts of its structure known to be relevant to its function have been conserved.

Cngsc plays two roles in head formation

The several domains of Cngsc expression suggest the gene may have multiple functions in hydra. In both vertebrates and *Drosophila*, the early embryonic expression of *goosecoid* orthologues is associated with the regions that will form parts of the head or other anterior structures (Goriely et al., 1996; Cho et al., 1991; Blum et al., 1992; Izpisua-Belmonte et al., 1993; Thisse et al., 1994). Similarly, Cngsc is expressed in the developing head as well as the adult head. The gene exhibits two different expression patterns in different parts of the head indicating it may have two separate roles during head formation.

Formation of the border between the hypostome and the tentacle zone

Cngsc is expressed in the endoderm in a ring at the border between two regions of the head, hypostome and tentacle zone (Fig. 4A,B). This is true in the development of a head during bud formation as well as in the adult head where the tissues are in a steady state of production and loss (Campbell, 1967).

The gene does not appear to be required for the initial patterning of either region of the head. Two hypostome-specific genes, *HyBral* and *budhead*, appear earlier during bud formation and head regeneration (Martinez et al., 1997; Technau and Bode, 1999) than does Cngsc, indicating that patterning of the hypostome begins before Cngsc first appears, and, though the gene is expressed before tentacles appear, it is not necessary for their formation. When the result of head regeneration is a single apical tentacle, or when individual ectopic tentacles form on the body column following DAG treatment, there is no increase in Cngsc expression at the base of these tentacles.

However, the gene could play a role in setting up the border between the two regions of the head. During early stages of head regeneration both hypostome-specific (*HyBral*; *budhead*) and tentacle-specific (TS-19) markers are expressed in the regenerating tip (Technau and Bode, 1999; Martinez et al., 1997; Bode et al., 1988). Further, Mitgutsch et al. (1999) have shown that during the early stages of head regeneration, tentacle-specific and hypostome-specific neurons intermingle in the regenerating tip. This overlap of expression of both hypostomal and tentacle markers ceases only after the Cngsc ring appears in the endoderm when the tentacle-specific markers are restricted to the tentacle zone. During bud formation, the sequence of events is different. Hypostome markers (*HyBral*, *budhead*) appear very early in the presumptive hypostome followed by Cngsc, which initially is expressed broadly in the developing bud and overlaps with *HyBral*. The tentacle marker TS-19 appears thereafter (Technau and Holstein, 1995), but only after Cngsc expression has been reduced to a ring, and only on the proximal, or future basal, side of the Cngsc ring. In both forms of head formation, tentacle buds do not appear until the Cngsc ring has been established.

These results are consistent with a view of the patterning events governing head formation that has been developed based on a large body of grafting and regeneration experiments. The principal process governing head formation is the head activation gradient, which is maximal in the hypostome and graded down the body column (MacWilliams, 1983a,b). This gradient has also been referred to as a gradient of source density (Meinhardt, 1993). Following decapitation, as observed with the tentacle-specific marker, TS19 (Bode et al., 1988), the head activation in the regenerating tip rises to the level necessary for tentacle formation, and then continues to the level for hypostome activation. The next step as postulated by Meinhardt (1993) is the inhibition of tentacle formation by a component(s) of the hypostome formation pathway. Thereafter, genetic pathways governing tentacle formation would be restricted to the lower part of the head. This process is illustrated in Fig. 10.

Because of the tissue dynamics in hydra, the patterning processes are continuously active in the adult and, thus, the pathway governing hypostome formation is also continuously active. To confine this latter process to the apical tip, Cngsc could be acting as a repressor of the hypostome formation process at the border between the hypostome and tentacle zone. As part of this process Cngsc could repress *HyBral*, the hydra *brachyury* homologue (Technau and Bode, 1999), thereby restricting expression of this gene to the hypostome. This would be consistent with the known interactions between *goosecoid* and *brachyury* in *Xenopus*. In the mesoderm of early *Xenopus* embryos, Xgsc and Xbra expression overlap, while later after gastrulation has been initiated they are expressed in neighboring non-overlapping regions. Further, Xgsc has been shown to inhibit transcription of the Xbra gene (Artinger et al., 1997). Since Cngsc can act as Xgsc in induction assay and, thus, can recognize the same target genes, as Xgsc does, it is plausible, that Cngsc inhibits *Hybrial*.

With this model one could explain the sorting out of the early overlap of the hypostomal and tentacle markers during head regeneration and their subsequent separate maintenance. Cngsc could play a similar role in head formation during budding,

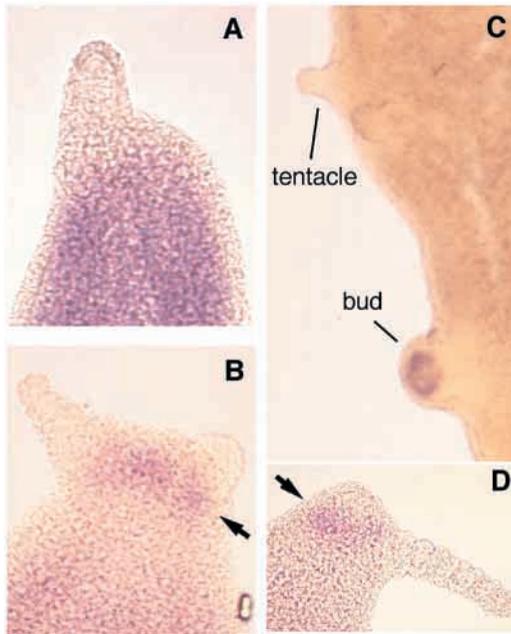


Fig. 8. The expression of *Cngsc* during (A) regeneration of a single apical tentacle, (B) two radial tentacles, (D) one radial tentacle, and (C) the formation of ectopic tentacles (arrowheads) after DAG treatment. Arrow in B indicates the appearance of the ring of *Cngsc* expression during regeneration of radial tentacles. *Hydra vulgaris* (Zurich strain) was used in A, B and D, and *Hydra magnipapillata* (105 strain) was used in C.

although the sequence of events is slightly different. As reflected in the timing of the appearance of region-specific markers, the hypostome is established first. Then the border is set up followed by the establishment of the tentacle zone. Tentacle markers and tentacles always appear after the *Cngsc* ring has been established. By establishing the border, the hypostomal process is restricted to the apical end. The results obtained with the several markers and their interpretations are consistent with the difference in the sequence of events during the two types of head formation as postulated by Meinhardt (1993), and as observed by Technau and Holstein (1995) using two region-specific antibodies.

Formation of apical neurons

gooseoid homologues play a role in the formation of parts of the anterior nervous system in vertebrates (Thisse et al., 1994; Gaunt

et al., 1993) and *Drosophila* (Goriely et al., 1996; Hahn and Jackle, 1996). *Cngsc* also appears to have such a role in hydra.

The nervous system of hydra is a net of neurons that extends throughout the body column with the individual neurons located among the epithelial cells (Bode et al., 1973). As neurons and epithelial cells are the only cell types in the ectoderm at the apex of the hypostome, the small dots of *Cngsc* expression in this region are most likely a subset of these neurons. This view is supported by the results of the hydroxyurea experiment. Berking (1980) showed that many of the new neurons at the distal end of a developing bud arise by migration of neuron precursors from the body column. After elimination of the neuron precursors with hydroxyurea, no *Cngsc*⁺ cells were observed in the distal ectoderm of developing buds.

Cngsc is expressed within the hydra organizer

In vertebrate embryos specific regions, termed organizers, have the ability to induce surrounding tissue to form a more-or-less complete second axis when transplanted to an ectopic site of the embryo. The dorsal lip of the frog embryo and Henson's node of the chick embryo are two examples. A similar phenomenon occurs in hydra. A piece of the hypostome transplanted into the body column induced the host tissue to form a second axis consisting of a complete head and body column (Browne, 1909; Yao, 1945). The tip of a developing bud, the presumptive hypostome, has the same inductive properties (Li and Yao, 1945).

In vertebrates, the early embryonic expression of *gooseoid* is localized within the organizer region (Blumberg et al., 1991; Blum et al., 1992; Izpisua-Belmonte et al., 1993; Stachel et al., 1993). The same is true in hydra as the both endodermal ring and ectodermal domain of *Cngsc* expression are within the developing hypostome. The apical part of a bud where *Cngsc* is expressed was able to induce body column tissue to form head and body column structures in transplantation experiments. In contrast, the neighboring proximal region, with a much lower-to-absent level of *Cngsc* could form a second axis, but at most induced the host to form part of the body column. Hence, the apical domains of *Cngsc* expression are localized within the organizer region in hydra. Whether the gene has a role in organizer function is not known.

Evolutionary comparisons

Hydra is a member of Cnidaria, the first group that appeared

Fig. 9. Design and the results of (A,B) lateral and (C,D) axial grafts. Host tissues were labeled with India ink; donor tissues were unlabeled. Arrows indicate ink-labeled tentacles formed from the host tissue. Arrowhead indicates an apical tentacle formed from donor tissue. (C) The induced second axis is emerging from the body column of the developing bud. Unlabeled donor tissue formed the hypostome and two of the tentacles. (D) The unlabeled donor tissue formed part of the body column of the bud that detached from the parent.

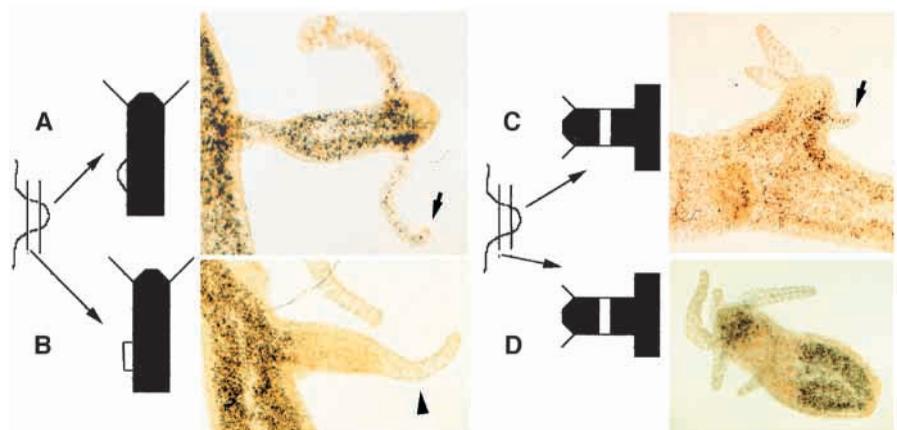
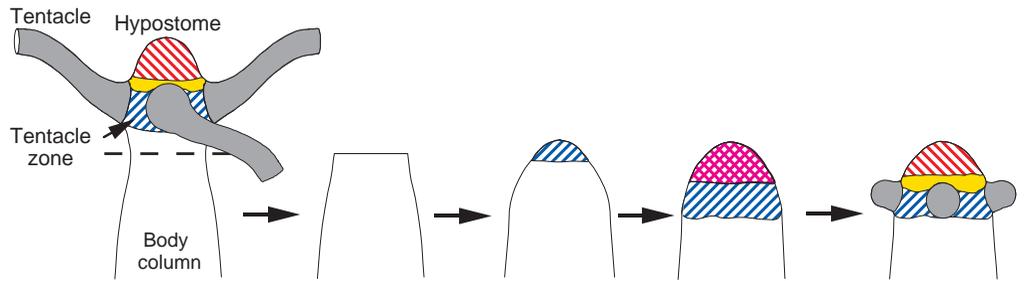


Fig. 10. Diagram of the changes that occur during head regeneration leading to the partition of the developing head into hypostome and tentacle zone tissue. ▨, tentacle tissue; ▩, hypostome tissue; ▧, overlap of hypostome and tentacle tissue; ■, *Cngsc* expression at the hypostome/tentacle zone border.



during metazoan evolution that has a defined body plan. The presence of a *goosecoid* homologue in hydra shows that this gene family appeared early in metazoan evolution. The fact that *Cngsc* can substitute for *Xgsc* and induce a secondary axis when injected into a *Xenopus* embryo indicates that the structure of the *Cngsc* protein has been sufficiently conserved to recognize the same target genes recognized by *Xgsc*. In turn, it is of interest to know to what extent the functions of the gene are similar across the metazoan phyla. There appear to be several similarities.

goosecoid homologues play a role in the development of the anterior nervous system. The gene is expressed in tissue that will form the brain hemispheres in *Drosophila* (Goriely et al., 1996) as well as in the developing forebrain of vertebrate embryos (Gaunt et al., 1993; Thisse et al., 1994). The hydra homologue is expressed in a subset of neurons at the anterior end of the head.

goosecoid also plays other roles in head development. It is expressed in the anterior foregut and ring gland in *Drosophila* embryos (Goriely et al., 1996; Hahn and Jäckle, 1996) while, in vertebrate embryos, homologues of the gene are expressed in the anterior foregut and prechordal mesoderm, which has roles in head formation (Blum et al., 1992; Izpisua-Belmonte et al., 1993; Thisse et al., 1994). Correspondingly, in hydra, *Cngsc* is expressed in the anterior gut and plays a role in head formation plausibly by establishing and maintaining the border between the two head regions. Of interest in this region are the complementary expression patterns of *Cngsc* and *HyBral* in the adult and the changes in these patterns during head development. As described above, the patterns and their changes are reminiscent of those seen during early embryonic development in *Xenopus* suggesting a similar interaction among the two genes; namely, that *Goosecoid* represses expression of *Brachyury*.

Similar to *goosecoid* homologues in vertebrates (Blumberg et al., 1991, Blum et al., 1992; Izpisua-Belmonte et al., 1993; Stachel et al., 1993), *Cngsc* is expressed in a region that has organizer activity. In this region expression domain of *Cngsc* overlap with one of *budhead*, hydra homologue of *Hnf3 β* (Martinez et al., 1997). The vertebrate homologues of these genes share expression domains in anterior mesoderm too. The interaction between these genes regulates the expression of signal molecules as *Shh* and *FGF8* and is required for normal dorsoventral patterning of the neural tube in mouse (Filosa et al., 1997). This gene interaction also might have a role in patterning and functioning of organizing tissue in hydra. Whether *Cngsc* has a direct role in organizer function in hydra is not known.

Finally, *Cngsc* is strongly expressed in the endoderm of the body column. As will be described elsewhere this expression could reflect a role of the *goosecoid* gene in the coordinated organized movement of cells in a sheet. Evidence for such a role

has been obtained in *Xenopus* (Niehrs et al., 1993), and changes in expression patterns in hydra after specific manipulations that cause cell rearrangement in the endoderm suggest a similar role.

In sum, there are a number of parallels in terms of plausible function between *Cngsc* and *goosecoid* homologues in organisms that arose later in evolution. Thus, not only has the structure been conserved, but it is likely that a number of the functions of the gene have been conserved throughout metazoan evolution. The comparison of expression patterns of hydra homologues of *goosecoid*, *Hnf3 β* and *brachyury* suggests that interactions between these genes are parts of the mechanism of axial patterning, that also have been conserved in evolution.

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