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## Expression of developmental genes during early embryogenesis of *Hydra*

Received: 7 April 2003 / Accepted: 16 June 2003 / Published online: 16 July 2003  
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**Abstract** *Hydra* is a classical model to study key features of embryogenesis such as axial patterning and stem cell differentiation. In contrast to other organisms where these mechanisms are active only during embryonic development, in *Hydra* they can be studied in adults. The underlying assumption is that the machinery governing adult patterning mimics regulatory mechanisms which are also active during early embryogenesis. Whether, however, *Hydra* embryogenesis is governed by the same mechanisms which are controlling adult patterning, remains to be shown. In this paper, in precisely staged *Hydra* embryos, we examined the expression pattern of 15 regulatory genes shown previously to play a role in adult patterning and cell differentiation. RT-PCR revealed that most of the genes examined were expressed in rather late embryonic stages. In situ hybridization, nuclear run-on experiments, and staining of nucleolar organizer region-associated proteins indicated that genes expressed in early embryos are transcribed in the engulfed “nurse cells” (endocytes). This is the first direct evidence that endocytes in *Hydra* not only provide nutrients to the developing oocyte but also produce maternal factors critical for embryogenesis. Our findings are an initial step towards understanding the molecular machinery controlling embryogenesis of a key group of basal metazoans and raise the possibility that in *Hydra* there are differences in the

mechanisms controlling embryogenesis and adult patterning.

**Keywords** *Hydra* · Embryogenesis · Nurse cells · Endocytes · Pattern formation

### Introduction

*Hydra* has a long history as a model system in developmental biology. In the last few years many of the molecular components involved in the development of the adult form have become known (for recent reviews see Steele 2002; Bosch and Khalturin 2002; Bosch 2003). Axis formation in *Hydra* is controlled by a head organizer located in the hypostome (Broun and Bode 2002). Genes expressed in the hypostome include the *Brachyury* homolog *HyBral* which correlates with changes of the head activation level (Technau and Bode 1999) and, therefore, may be an essential part of the organizer activity (Technau et al. 2000). Another conserved gene which plays a critical role in head development and axis formation in *Hydra* is *budhead*, a *fork head / HNF-3 $\beta$*  ortholog expressed in the lower part of the hypostome (Martinez et al. 1997). Genes of the WNT signaling pathway such as *HyWnt*, *Hy $\beta$ -cat*, and *HyTcf* (Hobmayer et al. 2000) are also expressed early during head formation and, therefore, were suggested to play a similar role in *Hydra* cell-fate-specification events as in other invertebrates and vertebrates. Systematic screening for molecules involved in determining the body axes resulted in isolation of several peptides, which appear to be part of the positional information system along the apical-basal body axis in *Hydra* (Takahashi et al. 1997; reviewed in Bosch and Fujisawa 2001). Among the peptides which are able to induce gastric cells to change their differentiation pathway, the 12-amino-acid peptide HEADY was shown to be a potent inducer of apical fate and also sufficient for head induction (Lohmann and Bosch 2000). Several homeobox genes appear to be involved in translating positional information along the body axis into the precise

Edited by D. Tautz

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spatial and temporal expression of key regulatory genes. Among these is homeodomain factor *CnNK-2* which is sensitive to the positional value gradient at the basal end of the body axis (Grens et al. 1996), as well as *Cnox-2*, an ortholog of the ParaHox *Gsx* gene, which appears to prevent body column tissue from forming a head (Shenk et al. 1993a, 1993b; Endl et al. 1999). Homeobox genes with a spatially restricted expression pattern include the *Hydra* *gooseoid* ortholog, *Cngsc*, which in adult polyps is expressed at the border between the hypostome and tentacle zone (Broun et al. 1999), as well as the *Hydra* ortholog of *aristaleless*, *CnAlx*, which is expressed in tentacles (Smith et al. 2000). *CnOtx*, an *Otx* gene in *Hydra*, is expressed at high levels in developing buds and aggregates, where it was proposed (Smith et al. 1999) to have a role in cell movements that are involved in the formation of new axes. In addition to axial patterning, control of interstitial stem cell differentiation also appears to be based mostly on orthologs of bilaterian genes. For example, *Cnnos1*, a *nanos*-related gene, is specifically expressed in *Hydra* in multipotent stem cells as well as in germ line cells, but not in somatic cells, suggesting that *Cnnos1* similar to *nanos* in *Drosophila* and *Caenorhabditis elegans* is required for maintenance of stem cells and germ cell formation (Mochizuki et al. 2000).

Due to the continuously active signalling and patterning processes, *Hydra* generally is regarded as an “embryonic model system” in which processes active in bilaterian animals only during certain embryonic stages can be studied in adults (Gierer 1974; Bode and Bode 1984). The underlying assumption is that the machinery governing adult patterning, i.e. bud formation and regeneration, mimics mechanisms which are active during early embryogenesis. Whether, however, the molecular mechanisms used to establish positional information, cell types and tissues in embryos are also used to maintain them in adults, is not known.

Little is known about the molecules governing embryogenesis in *Hydra*. Oocytes differentiate from clusters of interstitial cells committed to the egg lineage. On the basis of gene expression, it has been suggested that *CnOtx* is involved in determination of the oocyte by defining a subset of cells in the egg field (Miller et al. 2000). Within each cluster, one of the cells develops into an oocyte, while the other interstitial cells, often referred to as nurse cells, endocytes, shrunken cells, or pseudocells, are phagocytosed and become incorporated into the cytoplasm of the developing oocyte (Tardent 1985; Honnegger et al. 1989; Martin et al. 1997). These condensed endocytes constitute the bulk of the ooplasm and persist throughout embryogenesis. Due to a high content of glycogen particles and lipid droplets, they provide nutrients for the developing oocyte (Tardent 1985; Honnegger et al. 1989). In the absence of an oocyte, endocytes enter the apoptotic program and are digested (Miller et al. 2000). Following fertilization, oocytes develop by a holoblastic, radial cleavage pattern into a coeloblastula (Martin et al. 1997). Embryos gastrulate by multipolar immigration. Gastrulation results in the defi-

inition of the two germ layers, ectoderm and endoderm, a key developmental process that distinguishes adult patterning from embryonic development. During gastrulation, cells of the two germ layers are separated, yet only the ectodermal epithelium is being formed while immigrated cells form clusters in the blastocoel. Unlike most other cnidarians, gastrulation in *Hydra* is followed by a cuticle stage (see Fig. 1). During this stage, surrounded by a thick protective outer layer that is also commonly referred to as embryotheca (Martin et al. 1997), the embryos persist in diapause. The second developmental process that distinguishes adult patterning from embryonic development, i.e. the determination of the three cell lineages and in particular the appearance of interstitial stem cells as well as the formation of the endodermal epithelium, takes place at late cuticle stage just before hatching. Finally, young *Hydra* directly hatch from the cuticle stage, thereby omitting the typical planula larva stage (Martin et al. 1997).

In an effort to understand the molecules controlling embryogenesis in *Hydra* we used two approaches. The first is directed toward isolation of embryo-specific genes while the second one focuses on the expression of genes known to play a major role in adult patterning and cell differentiation. We report here results from the latter approach. The expression patterns observed are intriguing for two reasons. First, some genes thought to play key roles during adult morphogenesis are expressed before fertilization and during early embryonic stages in incorporated endocytes. This is the first direct evidence that endocytes in *Hydra* not only provide nutrients for oocyte growth but in addition serve the distinct purpose of transmitting maternal factors that are critical for embryogenesis and patterning. Second, some regulatory genes known to play a major role in adult patterning were found to be expressed only in middle and late embryonic stages, indicating that regulatory mechanisms used to generate pattern in early embryos may not be identical to those used in adult polyps.

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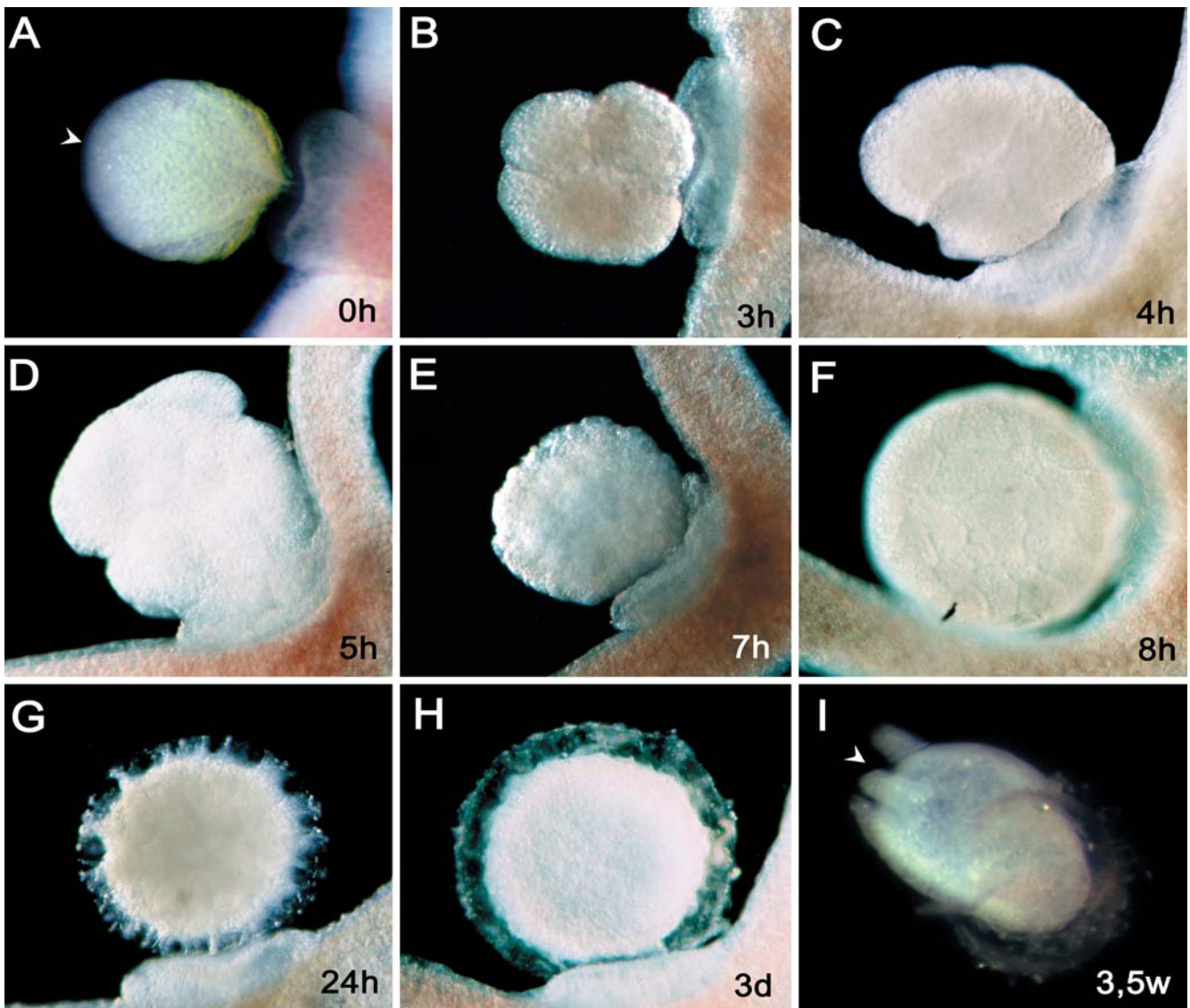
## Materials and methods

### Animals, culture conditions, and staging of embryos

Experiments were carried out with animals of the AEP strain belonging to the *Hydra vulgaris* group. This strain is derived from male and female strains described previously (Martin et al. 1997). The animals were cultured according to standard procedures at 18°C. For induction of gametogenesis, polyps were fed daily for at least 3 weeks before starving for 4 days followed by feeding every third day.

### PCR amplification of developmental genes

RNA was isolated from embryos with peqGOLD RNAPure (Peqlab) according to the manufacturers instructions and purified by DNase-treatment for 30 min at 37°C followed by phenol-chloroform extraction. The template used for RT-PCR was cDNA transcribed from total RNA with a first-strand-cDNA synthesis kit from Amersham-Pharmacia-Biotech with Not(dT)<sub>18</sub>-primer. PCR



**Fig. 1A–I** Embryogenesis of *Hydra vulgaris*. **A** oocyte; **B** 4-cell stage; **C** 8-cell embryo; **D** 16-cell stage; **E** late cleavage; **F** coeloblastula; **G** postgastrula embryo generating spikes; **H** cuticle-

stage embryo; **I** hatching polyp. *Arrowheads* in **A** and **I** mark the oral end of the body axis corresponding to the sperm entry point

reactions were carried out using Taq polymerase and standard reaction conditions. Samples of different developmental stages were equilibrated by PCR with primers for the *Hydra* actin gene which served as a control. cDNA corresponding to one embryo was used as a template in 20- $\mu$ l PCR. PCR conditions were: 5 min at 94°C (1 cycle), 30 s at 94°C, 45 s at annealing temperature (given below), 45 s at 72°C (cycle number given below), followed by 5 min at 72°C. Annealing temperatures and cycle numbers used were: actin: 56°C, 18 cycles; HyAlx: 52°C, 33 cycles; Cnox2: 62°C, 36 cycles; CnOtx: 64°C, 29 cycles; Cngsc: 57°C, 41 cycles; budhead 54°C, 28 cycles; CnNK-2 54°C, 31 cycles; HyTcf: 58°C, 29 cycles;  $\beta$ -catenin: 57°C, 36 cycles; HyBral:56°C, 32 cycles; Heady: 52°C, 40 cycles; Hym301:54°C, 28 cycles; HyWnt: 62°C, 39 cycles; Frizzled: 56°C, 31 cycles; Hyp1: 52°C, 39 cycles; Cnnos1: 56°C, 29 cycles. For each gene, one primer set has been selected (Table 1). Each PCR was repeated at least three times from three independent RNA samples.

#### In situ hybridization on whole-mounts and tissue sections

Fragments obtained by PCR were purified with QiaQuick gel-extraction kits (Qiagen) and cloned in pGEM t-vectors (Promega). Sense and antisense digoxigenin-labeled RNA probes of *HyTcf* and *Cnnos1* were synthesized using the RNA digoxigenin-labeling kit from Roche. *HyTcf* whole-mount in situ hybridization was performed as previously described (Grens et al. 1996) with minor modifications. Embryos were fixed in fresh 4% paraformaldehyde in culture medium at 4°C overnight. Proteinase K treatment (10  $\mu$ g/ml) was for 30 min at 37°C. The final probe concentration used for hybridization was approximately 0.3 ng/ $\mu$ l DIG-labeled RNA probe; hybridization was carried out for 18 h overnight. For *Cnnos1* whole-mount in situ hybridization we used the method described by Corbo et al. (1997). Hybridization was for 60 h. For in situ hybridization on tissue sections, embryos were fixed overnight in freshly prepared 4% paraformaldehyde at 4°C. After fixation the embryos were washed twice with pure methanol and transferred into absolute ethanol via 50%, 70% and 90% ethanol washes, for 10 min each. After treating with absolute ethanol (2 $\times$ 10 min), the

**Table 1** Oligonucleotide primer sequences

Primer name	Primer sequence 5'-3'
Actin	AAG CTC TTC CCT CGA AGA ATC
Actin	CCA AAA TAG ATC CTC CGA TCC
HyAlx-forward	gca tgc ata tga tac ttg g
HyAlx-reverse	GAA ATG GGG AGA GTT ATT CG
Cnox2-forward	GTA TAA GAT ACG GCA GCA GCC TG
Cnox2-reverse	GAA GAG GTA CCT TCA CCT GGT TC
CnOtx-forward:	CCG ATA GCC CCT TGG ATA GTC C
CnOtx-reverse	GGG TAA GAG TGT CCA GTG CCT C
CnGsc-forward	CCG AGG TTA GAG TTT CAA TGC
CnGsc-reverse	TGA CTA CTT TGA CGC CGT AGC
budhead-forward	GCC AAA CCG CCT TAC AGY TAY ATH TC
budhead-reverse	TAA CAT CCG TTT TCR AAC ATR TTB CC
CnNK-2-forward	AAC TGC GAA AAC CGA ATA CC
CnNK-2-reverse	TAC GGT GGA GGT CTT GTA CG
HyTcf-forward	GGC GAA GCT ACA GAA GTT CC
HyTcf-reverse	CGT CGA GTG CCT TTC GTC
$\beta$ -catenin-forward	GAA TGC TTG AAG ATC GTC GG
$\beta$ -catenin-reverse	ACA TTC CTG TTC TAG CAC GC
HyBra1-forward	GAC ATT GAT GGA GTT GCG C
HyBra1-reverse	CCG AGC TAG TGG TAG CTG G
Heady-forward	AGT CAG CCA CAG CAA CAT AGG
Heady-reverse	AGT CAG CCA CTC GGG GAG C
Hym301-forward	TGA ATG ATC CTT CAT TTG GG
Hym301-reverse	GAA CAA TAA CCA TTC AAG TAG C
HyWnt-forward	TGG ATG GCG CTT GGG ACG C
HyWnt-reverse	CAG CGC CAA CCT TCG TCC G
Frizzled-forward	CAT CGA AAC CCT GAA GAA GC
Frizzled-reverse	ATG CAC AAC CAG GTT TAC CC
Hyp1-forward	TTA AAG GAC ATC AAC AAC GC
Hyp1-reverse	CAG TTA GCT CTT GCA TTG C
Cnnos1-forward	GAT CCA GAT GAG ACG AGT G
Cnnos1-reverse	CAT GTG ACT TGC AGA GTG G

embryos were washed with methylbenzoate (2x15 min), then with benzol (2x10 min), incubated for 10 min in benzol/Paraplast (50:50, 37°C), then impregnated with Paraplast (2x30 min, 56°C) and embedded into Paraplast blocks. Sections of 12  $\mu$ m were cut and spread on polylysine-coated slides. The sections were deparaffinated with xylene (2x10 min), washed with 99% ethanol (2x3 min) and rehydrated by successive washes in 75% ethanol (2x3 min), 50% ethanol/50% PBT (2x3 min), 25% ethanol/75% PBT (2x3 min) and PBT (3x5 min). Afterwards the sections were treated with 10  $\mu$ g/ml proteinase K in PBT at room temperature. Digestion was stopped with two 5-min long washes with 4 mg/ml glycine/PBT. The sections were then washed with PBT (2x5 min), and treated with 0.1 M triethanolamine (pH 7.8, 2x3 min). The triethanolamine solution was then exchanged and 2.5  $\mu$ l/ml acetic anhydride was added into it (2x5 min). Samples were washed with PBT (2x5 min) and refixed in 4% paraformaldehyde/PBT (15 min, room temperature). After washing with PBT (5x5 min), the sections were treated with 50% hybridization solution (HS)/50% PBT (5 min) and prehybridized in 100% HS (1 h, 55°C, humid chamber). HS ingredients were 5x SSC, 50% formamide, 1x Denhardt's, 100  $\mu$ g/ml yeast tRNA, 100  $\mu$ g/ml heparin, 0.1% CHAPS, and 0.1% Tween 20. Hybridization with digoxigenin-labeled riboprobe was carried out overnight at 55°C in a humid chamber. After hybridization the sections were washed (humid chamber, 55°C, 10 min each wash) with HS, 75% HS/25% 2x SSC, 50% HS/50% 2x SSC, 25% HS/75% 2x SSC, and twice for 20 min with 2x SSC/0.1% CHAPS. The sections were then washed at room temperature for 2x5 min with MAB (0.1 M maleic acid, 150 mM NaCl, pH 7.4), then for 20 min with MAB/1% BSA (MAB-B), and 40 min with 80% MAB-B/20% heat-inactivated sheep serum at 4°C. Alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche) were added into this blocking solution and the samples were incubated overnight at 4°C. After washing with MAB (8x15 min), the sections were washed twice with NTMT

(100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl<sub>2</sub>, 0.1% Tween 20) and stained with BM-purple (Roche). The substrate reaction was stopped by washing with DEPC-treated water. After that the sections were treated overnight with methanol and embedded into Euparal.

#### Nuclear run-on transcription assay

The transcription of genes during the earliest stages of embryogenesis was studied by nuclear run-on analysis. Nuclei were isolated from 120 fertilized eggs, 2- and 4-blastomere-stages and allowed to finish transcription in the presence of [ $\alpha$ -<sup>32</sup>P]UTP as described previously (Gellner et al. 1992). The <sup>32</sup>P-labeled RNA was extracted with peqGOLD RNAPure (Peqlab) and hybridized at 42°C in 30% STARKS (30% formamide, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 5x SSC and 1% SDS) for 72 h to PCR-fragments immobilized on nylon membrane. Afterwards, hybridization filters were washed twice with 2x SSC containing 0.1% SDS at 37°C for 20 min and exposed to X-ray film for 4 days. DNA fragments were generated by PCR and purified from a 1.5% agarose gel using a QiaQuick gel-extraction kit (Qiagen). Thereafter, DNA was denatured by incubation with 0.1 mM NaOH for 10 min at 94°C. The DNA was spotted onto a nylon membrane using the Schleicher and Schüll slot-blot apparatus; 5  $\mu$ g DNA/slot was applied.

#### Silver-staining of nucleolar organizer region-associated proteins

To investigate whether *Hydra* nurse cells are metabolically active, nucleolar organizer regions were examined for argyrophilic proteins. Nucleolar organizer regions (NORs) are segments of DNA, encoding for ribosomal RNA. They are associated with argyrophilic proteins and, thus, they can be localized through silver staining. Silver staining of nucleolar organizer region-associated proteins (AgNORs) is a standard assay for cellular and nucleolar activity (Öfner et al. 1995). *Hydra* embryos were fixed in 4% paraformaldehyde in culture medium overnight at 4°C. Subsequently they were washed and incubated in borate buffer, pH 9.44, for 20 min at room temperature and incubated in 1 g/ml silver nitrate solution at 37°C. The reaction was stopped by washing thoroughly with distilled water when the samples appeared to be light brown, usually after 45 min. Samples were then mounted and pressed slightly to release nurse cells from the cytoplasm of the blastomeres.

#### Histology and electron microscopy

For semi-thin sections embryos were fixed and processed as for electron microscopy. Semi-thin sections of 1  $\mu$ m were taken with an LKB ultratome and stained as described previously (Kuznetsov et al. 2002). For TEM analysis, embryos were fixed in 3.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 18 h at 4°C. After rinsing with 0.075 M cacodylate buffer for 30 min, the samples were postfixed in 1% OsO<sub>4</sub> in 0.075 M cacodylate buffer for 2 h at 4°C, washed again for 30 min, and dehydrated in a sequential ethanol series. After incubation in 1,2-propylenoxide twice for 30 min each time, the fixed tissues were embedded in Agar 100 resin (Agar Scientific, Essex) and sectioned using a Ultratome Ultratuc E (Leica). The ultra-thin sections were stained with 2.5% uranylacetate for 5 min, rinsed with distilled water and then treated with lead citrate solution for 2 min. The ultrastructure was examined under electron microscopes CM10 and EM208S (Philips).

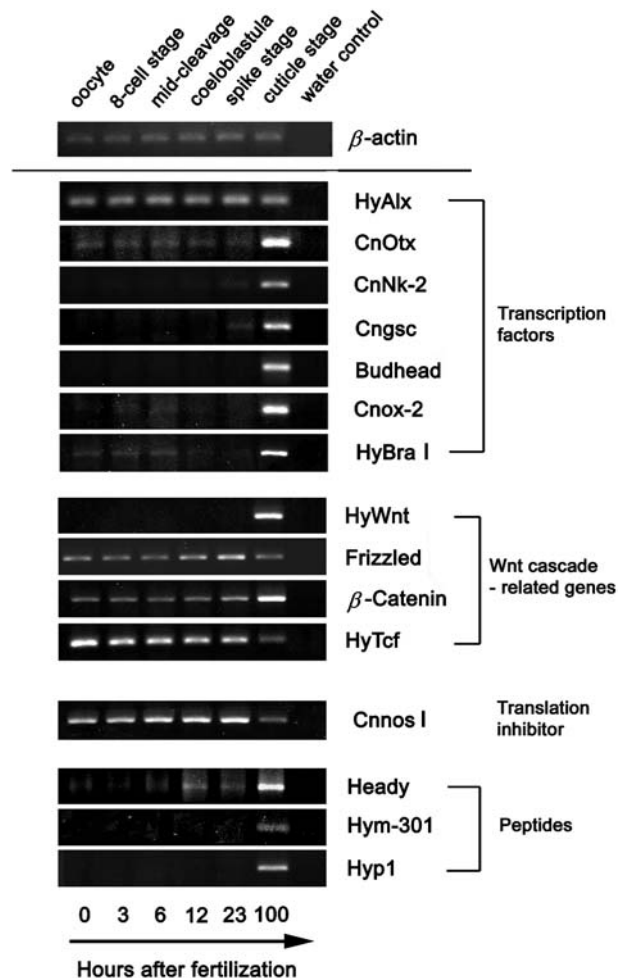
## Results

### Morphological aspects of *Hydra* embryogenesis and staging

In a study of embryogenesis of *Hydra vulgaris*, Martin et al. (1997) have introduced a system of stages, based on morphological criteria that represent major developmental steps. For the following gene expression analysis we have employed this system. As indicated in Fig. 1A, initial asymmetry which determines the oral end of the future body axis is established by the sperm entry point at the distal end of the oocyte which is also the future head of the polyp. Embryonic development of *Hydra* begins with radial cleavages (Fig. 1 B–E). About 8 h post-fertilization a coeloblastula is formed (Fig. 1F). Subsequently, gastrulation occurs by multipolar immigration. At the end of gastrulation, the blastocoel is filled with prospective endodermal cells, while the cells remaining on the surface form an epithelial layer. About 24 h post-fertilization cells of the outer layer develop filopodia and secrete cuticular material which is continuously deposited over the surface of the embryo forming a multilayered protective structure (Fig. 1G–H). The specification of the three cell lineages (ectodermal epithelial cells, endodermal epithelial cells and interstitial cells) occurs late during the cuticle stage (Martin et al. 1997). After a variable period of time, ranging from 2 to 24 weeks, the small polyp hatches (Fig. 1I), the head end of the hatchling coming out first.

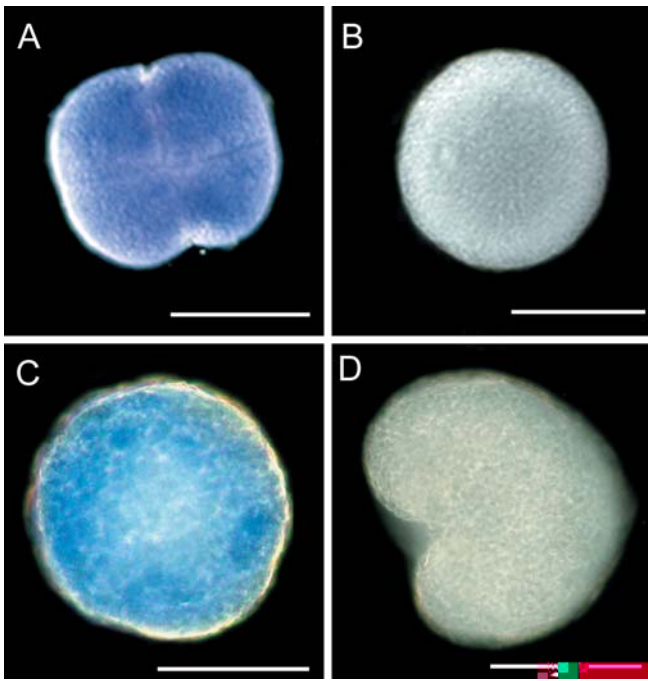
### Spatial and temporal expression analysis of developmental genes in early embryos

To obtain information about the genes controlling early embryogenesis, we analyzed the expression of a number of *Hydra* genes shown previously to be involved in axial patterning and cell differentiation. Temporal profiles of individual genes were obtained by RT-PCR analysis from precisely staged embryos. The analysis commences with the oocyte (Fig. 1A) and extends into the cuticle stage (Fig. 1G–H), spanning the transition from maternal to embryonic control of development, and including the determination of the two germ layers. Cuticle stage embryos were harvested 3 days after fertilization. The data are shown in Fig. 2. We first analyzed the expression of a number of transcription factors. Transcripts for homeobox genes *HyAlx* and *CnOtx* as well as the T-box gene *HyBral* are already found at a low level in oocytes and throughout embryogenesis. Expression of these genes substantially increases at cuticle stage. The expression in oocytes indicates that these genes are maternally supplied and, therefore, required to mediate specification steps prior to zygotic expression. In contrast, transcripts for the NK2 group homeobox gene *CnNk-2*, the homeobox gene *Cngsc*, forkhead gene *Budhead*, and ParaHox homolog *Cnox2* are not detectable in oocytes and only found in late embryos about 24 h post-fertilization. We next analyzed the



**Fig. 2** Temporal expression of developmental genes during embryogenesis

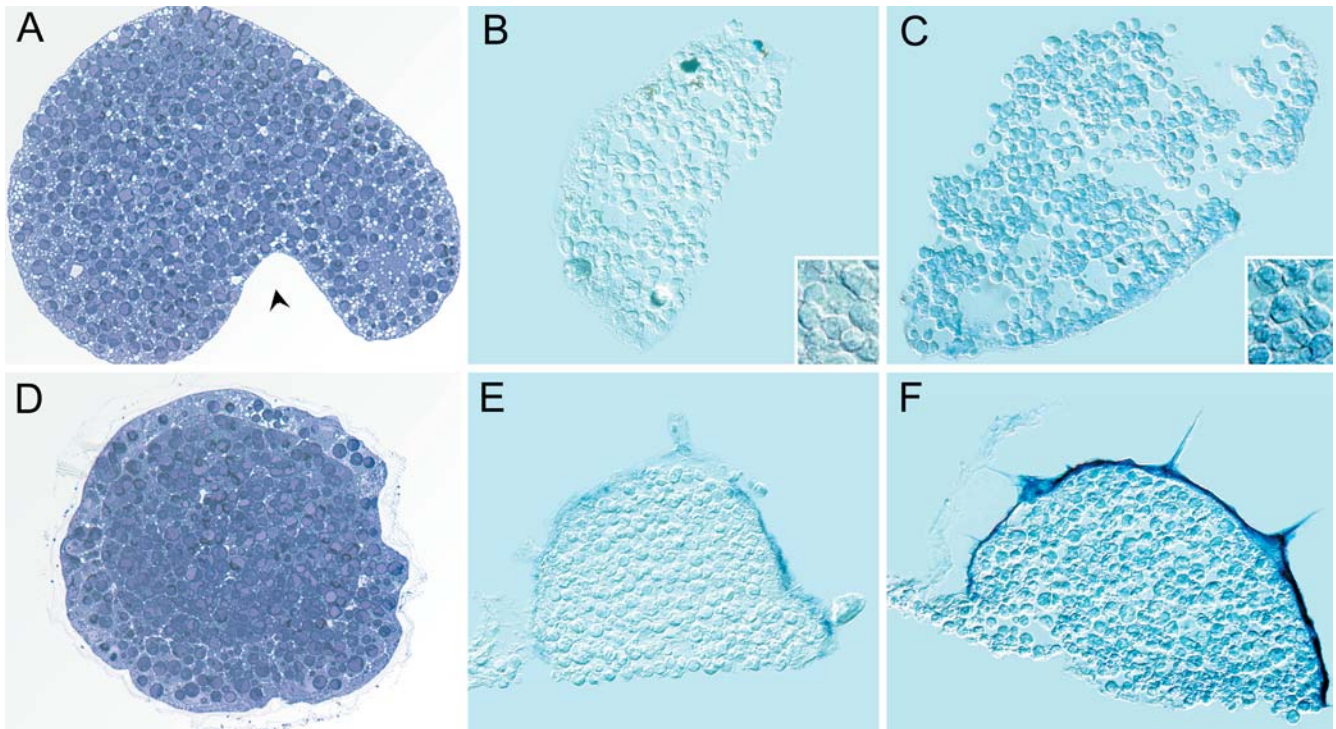
expression of *Cnnos1* which in adult polyps appears to be involved in germ cell differentiation (Mochizuki et al. 2000). Figure 2 indicates that during embryogenesis *Cnnos1* is strongly expressed in oocytes and early embryos at a time when no cell differentiation is yet initiated. Interestingly, the level of *Cnnos1* transcripts appears to be downregulated at the cuticle stage at the time when specification of the interstitial cell lineage occurs. Genes of the Wnt signaling cascade (*Wnt*, *Frizzled*,  $\beta$ -*catenin*, *Tcf*) are expressed in *Hydra* during adult patterning and have been proposed to be required for axial patterning (Hobmayer et al. 2000; Minobe et al. 2000). As shown in Fig. 2, *Frizzled*,  $\beta$ -*catenin* and *Tcf* are expressed in oocytes and throughout embryogenesis. However, all attempts to detect transcripts for the reported *Wnt* homolog (Hobmayer et al. 2000) in early embryos failed. Onset of *Wnt* expression is not detected before the cuticle stage, at approximately 3 days after fertilization. This was unexpected since this *Wnt* gene was proposed previously (Hobmayer et al. 2000) to be involved in early events of adult axial patterning. Similar to *Wnt*, transcripts for the genes encoding signal peptide Heady as well as peptide Hym-301 are not found in



**Fig. 3** Spatial expression pattern of *HyTcf* (A) and *Cnnos1* (C) in *Hydra* embryos. Whole-mount in situ hybridization using 4-cell stage (A) and late gastrula stage (C) about 12 h post-fertilization. Transcripts are distributed in all blastomeres. Sense control is shown in B and D. Bars 1 mm

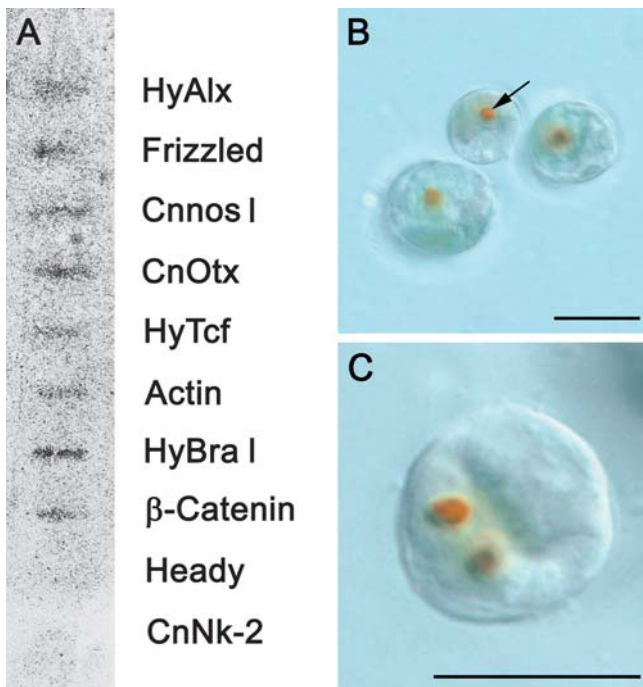
oocytes and early embryos. *Heady* appears to be expressed from the coeloblastula stage on. Expression of the peptide Hym-301 and polypeptide Hyp-1 is observed from the cuticle stage on. In adult polyps, expression of both peptide genes is sensitive to changes in positional signals along the body column (Takahashi et al. 1997; Hermans-Borgmeyer et al. 1996). Overall, the RT-PCR analysis in Fig. 2 shows two major temporal expression patterns: first, there are maternal genes expressed in oocytes and then throughout embryogenesis. Examples include *Frizzled*,  $\beta$ -*catenin*, *Tcf*, *HyAlx*, *CnOtx*, *HyBra-1*, and *Cnnos1*. *Cnnos1* in contrast to the other maternal genes seems to be down-regulated at the cuticle stage. The second group of genes (*Wnt*, *CnNk2*, *Cngsc*, *Budhead*, *Cnox2*, *Hym-301*, and *Hyp-1*) is not expressed before cuticle formation, about 24 h post-fertilization.

To independently confirm these expression data and to localize the transcripts within embryos, we performed whole-mount in situ hybridization using various embryonic stages. As shown in Fig. 3, mRNA for transcription factor *Tcf* is found in all four blastomeres of a 4-cell stage embryo. Similarly, mRNA for *Cnnos1*, which is also expressed throughout embryogenesis, was detected in all blastomeres of the gastrulating embryo shown in Fig. 3C. A very similar expression pattern was observed for the early genes  $\beta$ -*catenin* and *HyAlx* (data not shown). We note that none of the genes analyzed so far is expressed in



**Fig. 4A–C** Section of a *Hydra* zygote during the first cleavage. A Semi-thin section demonstrates that the cell is filled with nurse cells. The site of furrow initiation is marked with an *arrowhead*. B, C Expression pattern of *Cnnos1* using Dig-labeled sense (B) and antisense (C) probe. D–F) Section of a cuticle-stage embryo. D

Semi-thin section shows that the majority of the endocytes is now restricted to the endoderm. In situ hybridization to *Cnnos1* sense (E) and antisense (F) riboprobes. *Cnnos1* transcripts are present in nurse cells. Staining of the cuticle is unspecific and occurs both with sense and antisense probes



**Fig. 5** Evidence for transcriptional (A) and translational (B, C) activity of nurse cells. A Nuclear run-on experiment detects newly synthesized transcripts for early but not for late developmental genes. B, C Staining of argyrophilic nucleoli proteins in nurse cells of 2-cell stage embryos. Bars 10  $\mu$ m

only a region or subset of blastomeres of the developing embryo.

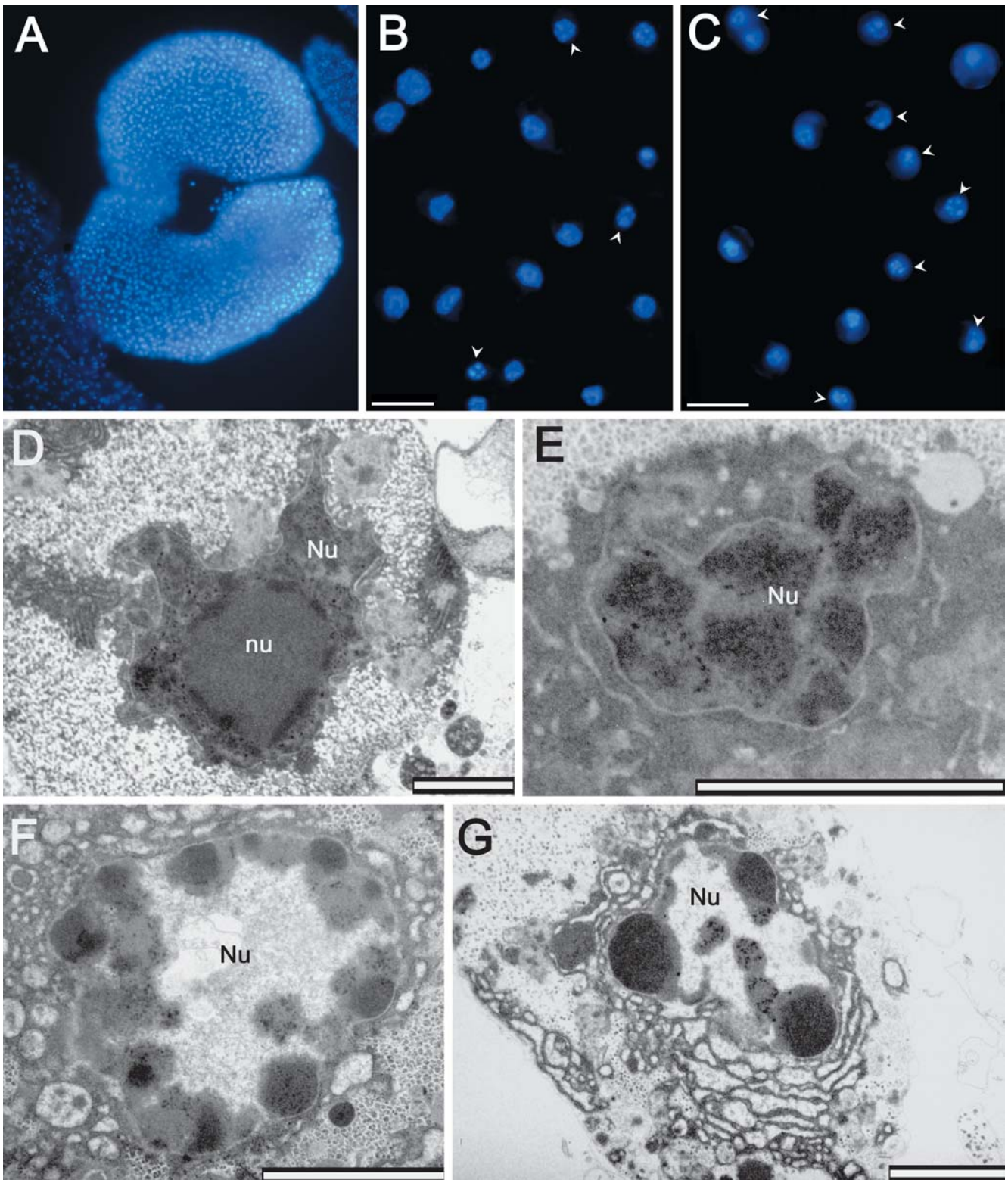
Do engulfed endocytes contribute to transcription of regulatory genes?

Before onset of embryogenesis, the *Hydra* oocyte phagocytoses several thousand endocytes which are maintained throughout embryogenesis. Stimulated by the observation (Fig. 2) that a number of regulatory genes appear to be maternally transcribed, we asked whether endocytes are involved in providing those maternal transcripts or factors in addition to nutrients. The activity and potential contribution of endocytes was examined by four methodologically different approaches.

1. To determine whether transcripts are localized within the engulfed endocytes, we performed in situ hybridization on tissue sections using *Cnnos1* as marker gene. Figure 4A–C shows a section through a fertilized egg which is just starting the first cleavage. Figure 4D–F shows a section through a *Hydra* embryo at cuticle stage. Both embryos are packed with endocytes. In situ hybridization shows that *Cnnos1* transcripts are localized within phagocytosed endocytes in both early (zygotic, Fig. 4C) and late (cuticle stage, Fig. 4F) embryos. Similar observations could be made with the early genes *Tcf* and *HyAlx* (data not shown) and

indicate that endocytes obviously not only provide nutrients as previously suggested but in addition contain transcripts for developmental genes. Are the nuclei of these engulfed cells transcriptionally active?

2. To determine whether oocytes and early embryos are capable of transcribing developmental genes, we performed nuclear run-on experiments. Nuclei were isolated from oocytes and early embryos up to the 4-cell stage and incubated with  $\alpha$ - $^{32}$ P UTP. Such nuclear preparations contained thousands of nuclei from engulfed endocytes per every oocyte nucleus.  $^{32}$ P-labeled RNA was hybridized to DNA of genes which were shown by RT PCR (Fig. 2) and by in situ hybridization (Figs. 3, 4) to be maternally encoded and expressed in early embryos. As control, the  $^{32}$ P-labeled RNA was hybridized to genes which were shown to be expressed only at late stages of embryogenesis. As shown in Fig. 5A, nuclei taken from oocytes and early embryos actively transcribe all the early genes. No hybridization signal could be obtained to genes such as *CnNk2* and *Heady* which are expressed rather late in embryogenesis. The embryos taken for this experiment were of 1- to 4-cell stage and, therefore, contained few zygotic nuclei and a vast number of endocyte nuclei. Since for newly synthesized transcripts to be detected in nuclear run-on experiments a large number of active nuclei is required (Gellner et al. 1992), the signal observed in Fig. 5A must depend at least partially on the activity of the endocyte nuclei.
3. Other workers have reported that nurse cells are dying cells displaying properties of apoptotic cells (Tardent 1985; Honegger et al. 1989). One hallmark of apoptosis is the shut-off of nucleolar activity and degradation of the nucleoli. Thus, to assess further the contribution of nurse cells, we used silver staining of nucleolar organizer region-associated proteins (Ag-NORs) as a marker of cellular and nucleolar activity. Argyrophilic nucleolar proteins were visualized by silver staining. As indicated in Fig. 5B and C, in early embryos the majority of nurse cell nucleoli were found to be AgNO<sub>3</sub>-positive. It, therefore, seems reasonable to assume that most of the endocyte nuclei in early embryos are metabolically active. In addition, staining of active mitochondria with rhodamine-123 indicated that the mitochondria of these endocytes are functioning normally as neither hyperpolarized nor depolarized mitochondria could be found (unpublished data).
4. We re-examined endocyte morphology in 2-cell stage and cuticle stage embryos at light and electron microscopy level. Staining of early embryos at the 2-cell stage with Hoechst 33342 (Fig. 6A, B) indicated that the majority of nuclei of engulfed endocytes have the typical interphase morphology. Some of them, however, due to their condensed and fragmented chromatin, clearly could be classified as apoptotic nuclei. In later stages of *Hydra* embryogenesis, the number of apoptotic nuclei increases substantially (Fig. 6C). Figure 6D–G shows ultrastructural features of four different endocyte nuclei from embryos at the



**Fig. 6A–G** Nurse cells undergo apoptosis during embryogenesis. **A, B**) Hoechst 33342 staining of 2-cell stage embryo. Magnification in **B** indicates that in early embryos only some of the endocyte nuclei (*arrowheads*) are showing clear apoptotic morphology. **C** Hoechst 33342 staining of a cuticle-stage embryo indicates that numerous endocyte nuclei (*arrowheads*) are showing apoptotic

morphology. **D–G** Ultrastructural evidence for nurse cells undergoing apoptosis in cuticle-stage embryos. **D** Normal endocyte nucleus (*Nu*) with large nucleolus (*nu*). **E–G** Successive stages of apoptosis with disappearing nucleoli, chromatin condensation and fragmentation, and vacuolarization of intracellular membranes. *Bars 2  $\mu$ m*

cuticle stage. While in Fig. 6D the nucleus contains an intact nucleolus, nuclei of nurse cells shown in Fig. 6E–G show different steps of apoptosis with chromatin condensation, fragmentation, disappearance of nucleoli, and vesicularization of intracellular membranes. Taken together, these studies show that in young embryos the majority of endocytes appear to be undegraded, metabolically active, and loaded with transcripts for developmental genes. In older embryos, consistent with previous observations, more and more nurse cells appear to undergo apoptosis and degradation.

## Discussion

*Hydra* is a classical model to study how patterns are made and maintained. The complex cellular and molecular processes involved are studied in adult polyps using *Hydra*'s remarkable capacity to regenerate, to proliferate asexually by budding, and to form a pattern de novo from aggregates. The literature of pattern formation in *Hydra* is pervaded by the concept that these patterning steps rely on factors and molecules which are also used in patterning the embryo. When describing how a polyp is regenerating from a cohesive aggregate of individual cells, Gierer (1974) states that "the structure resembles an intermediate stage of the developing hydra embryo ...". Similarly, "the embryonic quality of the body tissue" has been proposed to be responsible for *Hydra*'s regeneration capability (Bode and Bode 1984). But, is the machinery used to establish patterns in embryos also used to maintain them in adult polyps?

### Comparing developmental decisions and gene expression in embryos and adult *Hydra* polyps

In embryos as well as in adults, distinct positional values are generated which specify the location of cells along the body axis and contribute to the formation of prepatterns preceding the development of visible patterns. Key developmental processes that in *Hydra* characterize embryonic development are those associated with the establishment of polarity along the future body axis, the definition of the two germ layers and the determination of the three cell lineages. In adult polyps patterning processes are used for maintenance of positional information along the body axis and for formation of buds. When analyzing the expression of genes, which have been shown previously to be involved in adult patterning, in developing embryos we made a number of interesting observations.

First, homeobox genes *HyAlx* and *CnOtx*, which in polyps are expressed during regeneration and budding, are also expressed in early embryos. Both genes are detected in unfertilized oocytes and, therefore, maternally encoded. *HyAlx* expression is maintained at about the same

level throughout embryogenesis while the *CnOtx* expression level increases drastically at the cuticle stage. Although future studies have to reveal whether these transcription factors are active at that early developmental stage and in which regulatory circuit they are involved, the data indicate that in *Hydra* pattern formation processes controlled by homeobox genes appear to be required for both embryonic and adult body plan formation. Second, genes such as *CnNk2*, *Cngsc*, *Budhead*, *Cnox2*, *Hym-301*, and *Hyp-1* are not expressed in early embryos. Since these genes in adult polyps are sensitive to changes in positional values and are activated during regeneration and budding, the observation may indicate that the machinery used to establish patterns in early embryos may not be identical to the machinery used for maintaining patterns in adults. If this view is correct, then the mechanisms controlling patterning in polyps may not reflect an embryonic feature. Third, in contrast to the expression in the putative organizer region of regenerating polyps, *Wnt* is not expressed in early embryos. Wnt proteins are secreted ligands that pattern insect and vertebrate embryos by directing the differentiation of different cell fates (reviewed in Wodarz and Nusse 1998). In *Hydra* polyps all members of the canonical Wnt signaling pathway (*Wnt*, *Frizzled*,  $\beta$ -*catenin*, *Tcf*) are expressed (Hobmayer et al. 2000; Minobe et al. 2000). The underlying assumption is that these molecules act together to regulate axial patterning. Since in embryos our RT-PCR and in situ data revealed transcripts for *Frizzled*,  $\beta$ -*catenin*, and *Tcf* but not for *Wnt*, this raises the question of whether these molecules act in one cascade as suggested or whether a not yet identified member of the Wnt family is interacting with *Frizzled* in *Hydra* embryos. Almost all components of the Wnt cascade have multiple homologs in the mammalian genome with the vertebrate genome encoding around 15 Wnt factors (Cadigan and Nusse 1997; Wodarz and Nusse 1998; Cadigan 2002). Which Wnt factors and which Wnt receptors are relevant for embryonic development in *Hydra*? Are different Wnt-receptor combinations active in polyps and embryos? Or is the Wnt cascade in *Hydra* embryos put together piece by piece? This in itself would be similar to the situation in *Xenopus* where in early embryos only the "cytoplasmic" members of the Wnt cascade (*Frizzled*, *disheveled*, *GSK-3*,  $\beta$ -*catenin*, and *Tcf*) are expressed and transcripts for *Wnt8*, the ligand to the *Frizzled* receptor, do not appear before gastrulation (Bachvarova et al. 2001). The fact (Fig. 2) that, in contrast to Wnt,  $\beta$ -*catenin* is expressed in early *Hydra* embryos may indicate that this gene plays a critical role in setting up the organizer similar to  $\beta$ -*catenin* in early frog embryos. In any case, the observation that developmental signals activate Wnt in polyps but not in early embryos, provides further evidence that patterning in polyps and embryos may not be based on an identical set of molecules. Fourth, analysis of *Cnnos1* gene expression suggests that this gene has different functions in polyps and embryos. In polyps, *Cnnos1*, due to its restricted expression in cells of the interstitial cell lineage, may play a role in interstitial and germ cell

control (Mochizuki et al. 2000). In embryos (Figs. 2, 3, 4, 5), *Cnnos1* is maternally encoded, expressed in early embryos long before cell lineage specifications, and downregulated at a time when interstitial cell determination occurs. Although the function of *Cnnos1* in *Hydra* embryos is not known, it may be involved in determining the body axis and providing a coordinate system for subsequent patterning similar to its recently described role in insects (Lall et al. 2003). The strong expression of *Cnnos1* in endocytes at the cuticle stage (Fig. 4F) may support this idea. Alternatively, since in early embryos all cells have stem cell properties, *Cnnos1* may be involved in maintaining stem cell fate. In this view, the observed decrease in the transcript level at the cuticle stage may be correlated with the appearance of a stem cell lineage and the lineage-restricted expression pattern.

Finally, another lingering question concerns the presence and function of embryo-specific factors in *Hydra* and for which post-fertilization events they are needed. Are there genes in *Hydra* embryos involved in the establishment of the initial oral-aboral asymmetry which are not expressed in adult polyps? Interestingly, by gene expression profiling we recently have identified a number of genes expressed exclusively during embryogenesis and not responding to adult patterning signals (Genikhovich, Kürn and Bosch, in preparation). We hope that their characterization will contribute to understand the oocyte-to-embryo transition and, as such, offer new insights into the initiation of embryonic development in *Hydra*.

#### The role of endocytes in maternal control of embryogenesis in *Hydra*

Oogenesis in *Hydra* has been described in detail (Zihler 1972; Honegger et al. 1989; Martin et al. 1997; Miller et al. 2000), proposed to be unique in the animal kingdom (Kleinenberg 1872; Tardent 1974), and interpreted as “one of the most primitive ways of building an egg” (Tardent 1974). Oocytes in *Hydra* feed on adjacent cells by phagocytosing them in large numbers and incorporating them within the ooplasm. The engulfed cells retain their membranes, nucleus and organelles throughout most stages of embryogenesis. Thus, the *Hydra* egg, in addition to its own nucleus contains hundred to thousands of additional nuclei. Much speculation on *Hydra* oogenesis has focused on the role of these cells. Their putative involvement in oocyte nutrition led to the term “nurse cells” (Martin et al. 1997). Other workers have termed them as endocytes, “shrunken cells” (Zihler 1972) or “pseudocells” (Kleinenberg 1872) because of their condensed cytoplasm and apoptotic features. The observation of transcripts of developmental genes located in the cytoplasm of these engulfed cells has inspired us to re-investigate their properties and role in early embryogenesis. Endocytes were not only observed to contain transcripts for a number of genes involved in patterning and cell differentiation but were also found to actively transcribe these genes. Moreover, within their nuclei

nucleolar organizer region-associated proteins could be visualized as a marker of nucleolar activity. It seems, therefore, reasonable to speculate that these cells not only provide nutrients but actively contribute to the molecular machinery essential for embryonic patterning. The data reported here also raise a number of new questions. How do the transcripts get transmitted from the endocytes to the oocyte? Are active transport mechanisms involved or are the molecules simply released into the cytoplasm of the embryo? Are the transcripts translated within the endocytes? If so, how are the proteins targeted across the membrane?

#### Conclusion

*Hydra* is a well established developmental model. Recent studies stress the high degree of conservation of genetic mechanisms controlling development in *Hydra* and bilaterian animals. However, in *Hydra*, all studies are carried out using adult polyps. In this paper we provide evidence that embryonic development and adult patterning processes in *Hydra* are at least partially based on different regulatory genes. We also show that endocytes (“nurse cells”) might play a much more active role in embryogenesis than previously thought.

**Acknowledgements** A.C.F. acknowledges Katrin Thamm for help with the in situ hybridizations on *Hydra* embryos. We would like to thank Antje Thomas for excellent technical assistance. We are grateful to Ulrich Technau for sharing unpublished observations on *Hydra* embryos and we thank him and Matthias Habetha for critical comments on the manuscript. Supported by grants from the Deutsche Forschungsgemeinschaft (to T.C.G.B.). G.G. is recipient of a Ph.D. fellowship from the Daimler-Benz foundation.

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