Compartmentalized Nervous System in Hydra and the Mechanisms of its Development

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Summary

The last common ancestor of bilateria and cnidaria is considered to be the first animal to obtain nervous system over 700 million years ago. After that, animals have developed their own nervous systems that are seen now. During a long course of evolution traits of nervous systems in these animals are vastly diverged and different from each other. Some of the traits, however, seem to be shared between higher animals (vertebrates and/or arthropods) and cnidaria. These seemingly similar traits can be classified into two groups: analogous traits and homologous traits. The analogous traits are defined as traits currently shared by these animals but might have been different in the past or may be different in the future. The homologous traits are long conserved among animals during their evolution. Therefore, they should shed light on what prototypical nervous system was like. The question is how to distinguish them. Generally to say, the homologous traits share common underlying mechanisms to realize them.

This study and previous studies demonstrated interesting new aspects of *Hydra* nervous system. First, the nerve net of *Hydra* is divided into subpopulations. Second, each neuron subset expresses specific combination of neuropeptide genes. Third, each neuron subset is localized in a restricted region(s) along the oral-aboral axis. Fourth, some neighboring subsets of neurons are separated from each other with clear boundaries between them. And finally one of the possible functions of neuron subsets is local action by a localized neural neurotransmitter(s). The last two aspects are pointed out for the first time in this study. All these features imply that the neuron subsets in *Hydra* are neural compartments and they behave as sort of functional units like those of

higher organisms. Are these seemingly similar traits analogous or homologous? In order to address this issue, I attempted to elucidate the mechanisms for generating neuron subsets of *Hydra* and compared them to that of neural compartments of higher animals.

A compartment is generally defined as a subdivided tissue that consists of lineage-restricted non-intermingling sets of cells between neighboring compartments. According to this definition, neuron subsets in *Hydra* may not be the neural compartments equivalent to higher organisms, because there is no lineage-restriction in the formation of neuron subsets in *Hydra*. The tissue displacement in *Hydra* continuously moves neurons in a subset into a neighboring subset. Despite this, however, each neuron subset keeps its location and size of population constant, maintaining clear boundaries between subsets.

There are two possible mechanisms to supply neurons for balancing a loss of neurons caused by the tissue displacement: new neuron differentiation from precursors and phenotypic conversion of preexisting neurons. By comparing tissue displacement rate and labeling kinetics of BrdU in Hym-176A+ neurons in the lower peduncle of adult *Hydra*, I estimated that about 70% of neuron turnover in the neuron subset was accounted for by new differentiation and the remaining 30% appeared to be accounted for by phenotypic conversion. I also found another example for phenotypic conversion in the middle of this neuron subset. These results suggest that both of the two mechanisms are involved in the formation of neuron subsets.

New differentiation always occurred near the upper boundary of the neuron subset although neuron precursors could penetrate further down in the subset. When the situation was created where no preexisting neurons were present such as during foot regeneration or replacement of the normal foot with the nerve-free epithelial foot,

essentially all the neurons produced were newly differentiated ones and distributed within the subset, not restricted at its upper boundary. These results suggest that new differentiation appears to be regulated by lateral inhibition of preexisting Hym-176A+ neurons and that more rapid new differentiation prevails in case of emergency in which new neurons are required. At the moment, the fate of neurons is not known when they leave the subset by tissue displacement. Cell death might be involved in addition to phenotypic conversion. Taken together, in *Hydra* although lineage-restriction may not be involved in maintaining clear boundaries and keeping the size of subsets constant, these are regulated positively by both of new differentiation and phenotypic conversion, and negatively by lateral inhibition and possibly cell death.

Next, I addressed the issue as to what determine the position of neuron subsets in *Hydra*. Prepattern genes, pairs of mutually repressing homeobox genes, such as Otx, Pax and Hox, determine the region where neural compartments are formed in higher animals. These genes are regulated by a few secreting molecules, such as Wnts, FGFs and retinoic acid. In *Hydra*, counterparts for some of these molecules are identified but only Wnts appear to be involved in axis formation. In this study I have shown that activation of the Wnt signaling pathway with LiCl and/or ALP, both of which inhibit GSK-3 β as their common target, altered positional information and therefore localization of neuron subsets in *Hydra*. This suggests that the Wnt signaling pathway is conserved between neural compartments and neuron subsets in determining their localization.

How does this positional information direct region-specific differentiation of neuron subsets in *Hydra*? There must be a transcriptional control involved in it. I compared 5'-flanking genomic regions among Hym-176 paralogous genes. Some of them

are expressed in different neuron subsets located in different axial regions while the others are in the same axial region. It is expected that the same or similar region-specific cis-regulatory elements may be shared by genes expressed in subsets of neurons located in the same axial region. I have found several conserved motifs. One of them was similar to the STATx binding motif that was shared by subsets of paralogues expressed in the lower peduncle. Although this one might be conserved between neuron subsets and neural compartments, others showed no homology to known motifs. These results seem to indicate that the mechanism of region-specific gene regulation in neuron subsets and neural compartments is not well conserved. In other words, cnidarians might have invented cis-regulatory elements of their own.

Comparison of the involved mechanisms between neuron subsets in *Hydra* and neural compartments in higher animals showed both conservation and divergence. This may be taken for granted, because it has been enormously long time since they were separated from the last common ancestor. However, a crucial point was that one of the most fundamental signaling systems all through the animal evolution, the Wnt pathway was conserved as one of the underlying mechanisms to determine the position of compartments between *Hydra* and higher organisms. Downstream genes activated by the Wnt pathway appear to be different. More evidence should be accumulated. But I would like to temporarily conclude that it is too early to give up the idea that neuron subsets in *Hydra* and neural compartments in higher animals are homologous structure reminiscent of the ancient nervous system. I would further pursue my studies along this line.

Introduction

1. *Hydra* as a model organism for studying evolution of nervous system

1.1. Phylogenetic position

Hydra is a fresh water animal, belonging to the Phylum Cnidaria, one of the three pre-bilaterian animal phyla (cnidarians, ctenophores and poriferans). Recent molecular phylogenetic studies that are based on both large and small subunit ribosomal DNA revealed that cnidarians are the true sister group to the Bilateria and that ctenophores are basal to cnidarians and bilaterians [1]. The phylum Cnidaria is further divided into four classes (Anthozoa, Hydrozoa, Scyphozoa and Cubozoa) and *Hydra* belongs to the class Hydrozoa. Among these four classes the Anthozoa are now generally regarded as basal, because all metazoans other than Hydrozoa, Scyphozoa and Cubozoa have a circular mitochondrial DNA (mtDNA), while these three classes of cnidaria have a linear type of mtDNA, suggesting they lost the circular type after diverging from the common ancestor with the Anthozoa [2]. This notion is further supported by some phylogenetic studies [3][4][5]. These studies revealed that the Anthozoa is basal to the other classes and Hydrozoa is basal to the Scyphozoa and the Cubozoa.

1.2. Body plan

1.2.1. Single axis

Hydra has several characteristic features in their body architecture (**Fig. 1**). First, Hydra has only a single body axis that runs from the oral end to the aboral end. The body is basically a tube radially symmetric around the oral-aboral axis. The body consists of a head at the oral end, the gastric region that include budding region and a foot at the aboral end. The head refers to hypostome with a mouth at the tip, several tentacles and their base. The tentacles are used for capturing prey and locomotion. The gastric region covers most of the body column. The budding region is located about 2/3 of the body column from the oral end. Budding is a way of asexual reproduction of Hydra. The foot refers to the tissue below the budding region and consists of peduncle (a stalk) and basal disk. The peduncle is considered to be a pump to circulate the gastric fluid throughout the cavity. The basal disk is used for attaching to the substrate.

1.2.2. Diploblastic animal

Hydra is diploblastic, that is, its body consists of only two cell layers, ectoderm and endoderm but lacks mesoderm (**Fig. 1**). Between the two cell layers Hydra has the extracellular matrix called mesoglea. The epithelial cells and mesoglea make up and maintain the body architecture.

1.2.3. Three stem cell lineages

Hydra consists of three stem cell lineages, ectodermal epithelial cell, endodermal epithelial cell and interstitial cell (I-cell) as shown in **Fig. 2**. No interchange among

these three lineages occurs. Both ectodermal and endodermal epithelial cells are muscles cells. Despite of the differentiated state, the epithelial cells in the body column undergo self-renewal with average cell cycle time of about 3 days. The ectodermal epithelial cells go through final S-phase in the tentacle bases, subhypostomal region and near the basal disk and respectively differentiate into tentacle battery cells, hypostomal epithelial cells and basal disk specific glandular (or mucous) cells. At least battery cells and glandular cells are terminally differentiated cells and arrested in G2 phase [6]. Endodermal epithelial cells in the body column are also continuously in the mitotic cycle and become non-dividing in the tentacles, hypostome and basal disk. Unlike stem cells of epithelia of higher organisms, which only proliferate and differentiate, the epithelial stem cells in hydra also have some physiological functions. The ectodermal epithelial cells have the protective function of skin, and their large cytoplasmic vacuoles are involved in osmoregulation [7]. Endodermal epithelial cells are involved in the digestion of food in the gastric cavity. In addition, epithelial cells of both layers participate in the movement of the animal [8]. Muscle processes of ectodermal epithelial cells run longitudinary along the body axis and that of endodermal epithelial cells run circumferentially, perpendicular to the axis.

All cell types other than those mentioned above are derived from the I-cells, which are morphologically homogeneous, but functionally heterogenous. The I-cell population is known to consist of multipotent stem cells and cells committed to particular differentiation pathways. The multipotent stem cells are located in the interstitial spaces between ectodermal epithelial cells (**Fig. 1**) and are distributed evenly in the body except for head and basal disk. They are continuously in the mitotic cycle with an average cell cycle time of 24 hours [9]. The committed cells undergo one or

more cell divisions depending on the pathways, thereby amplifying the number of differentiated cells. For example, when a stem cell is committed to the nematocyte pathway, it undergoes a series of synchronous cell divisions up to 4 (**Fig. 2**). Since cytokinesis is incomplete, daughter cells are connected to each other by cytoplasmic bridges to form a cluster. The same type of nematocytes is produced from a single cluster. Following maturation of nematocytes, the cluster breaks up and individual nematocytes migrate mainly to the tentacles. When a stem cell is committed to the neuron pathway, it usually undergoes a single round of cell division to differentiate into two neurons. The interstitial stem cells also have a capacity to produce gland cells and germline cells. Gland cells are localized in the endoderm and produce digestive enzymes. Germline stem cells also localized in the space between ecotdermal epithelail cells produce either eggs or sperm depending on their sex [10][11][12]. However, since both gland cells and germline stem cells undergo continuous mitosis, it is not known to what extent these cells are supplied from I-cells by new differentiation under a normal condition.

1.2.4. Organized tissue

In spite of the relatively simple body plan, *Hydra* has two highly organized tissues, nervous and gastric systems, both of which are absent in Porifera, the most basal metazoan phyla. Thus *Hydra* (or cnidarians in general) is the first organism in animal evolution that has developed highly organized tissue systems in the relatively simple body plan. The nervous system of Hydra will be described in more detail later. Gastric system of Hydra is very similar to digestive tracts in higher animals that consist of outer muscles running longitudinally and inner muscles running circumferentially. Both undergo peristalsis which is under control of a single layer of nerve net in *Hydra*

but multiple layers of nerve plexus in higher animals [13].

1.2.5. Tissue displacement

Hydra can reproduce asexually and regenerate strongly. These two features are beyond the scope of this thesis, and I will not mention them in any more details. However, I should note here a characteristic feature of *Hydra* tissue dynamics, called tissue displacement, which might be responsible for asexual reproduction and strong regenerative capacity of *Hydra*. All three stem cells of the body column in *Hydra* are constantly in the mitotic cycle [14]. Thus the epithelial tissue is continuously growing, while the adult animal keeps a constant size. This indicates that tissue is lost at the same rate as it is produced [15]. Most of the loss is due to displacement of tissue into developing buds, which eventually detach from the parental body. The tissue near the head is displaced upward to tentacles and hypostome, and is sloughed off from the extremities, while the rest of tissue is displaced downward. The tissue that is not incorporated by developing buds is further displaced and sloughed off from the tip (center) of the basal disk. Thus, essentially all epithelial cells are constantly moving upward or downward, respectively and changing their axial location [16]. This continuous tissue turnover also can be seen in epithelia of higher organisms, such as the epidermis or the intestinal epithelium. What is specific to *Hydra* is that other cell types including neurons are also displaced together with epithelial cells, indicating there are no static tissues in *Hydra*.

2. Nervous system in *Hydra*

2.1. Overview of its structure

The nervous system of *Hydra* consists of a net-like structure extending throughout the body but unevenly with higher concentrations in the head and foot than the body column. The nerve net is not only displaced together with epithelial cells, but also maintained constant by balancing a loss of neurons from the body column and a supply of neurons from I-cells by differentiation [17]. This dynamic feature of nerve net is unique to Hydra, which enables us to study neurogenesis in adult animals, and is quite different from the nervous system in higher organisms, in which neurons are generated only during embryogenesis.

In adult *Hydra*, I-cells are constantly proliferating. Once committed to neuron differentiation, the neuron precursors generally divide once to produce two neurons. In the gastric region about a half of the precursors divide and differentiate in situ, while a remaining half migrate individually either to the head or foot at a speed of about 1mm/hr. The migrated neuron precursors divide once within 24 hours to produce a pair of neurons [18].

2.2. Complexity

A large number of previous studies, such as morphological [19][20], histological [21] and ultrastructural [22] studies revealed that the nervous system of *Hydra* is not a simple nerve net, but consists of multiple subsets of neurons. Up to now, many molecular markers specific to neurons in *Hydra* have been obtained and used to visualize the nerve net. These studies further support the idea of the complex nervous

system of *Hydra*.

In 1980's, antibodies were used to visualize neurons. For example, antisera against neuropeptides of other animals (anti-gastrin/CCK-like [23], anti-substance P-like [24], anti-neurotensin-like [25], anti-bombesin-like [26], anti-oxytocin/vasopressin-like [27], anti-(FM)RFamide-like [28][29]) or monoclonal antibodies against unknown antigens in *Hydra* neurons (JD1 [30], RC9 [31], TS26 [32], TS33[32]) were employed. Although these immunohistochemical approaches could visualize antigen-specific subsets of neurons and their discrete distribution, all the neuropeptides other than RFamide peptides are not actually identified in *Hydra*. Thus, these early studies of neural markers left an unsolved issue as to what they were visualizing.

In late 1990's, some of neuropeptide genes were identified in *Hydra* and characterized in terms of their expression patterns as summarized in **Table 1**. These results gave us more specific configuration of nerve net than the previous immunohistochemical methods had lent. Neuropeptide genes are respectively expressed in discrete subsets of neurons and more interestingly, the subsets are distributed in axially different regions. Hym-176 is one of those neuropeptides that are expressed in region-specific manners [33]. It is highly expressed in peduncle neurons and lowly in gastric neurons. Some other subsets expressing their own specific neuropeptide genes were shown to overlap each other or to be mutually exclusive in the same axial region. Although these studies demonstrated the axially regionalized nervous system of *Hydra*, there were no studies to address the issue as to the boundaries of two neighboring subsets. Are they separated from each other with an exclusive boundary, or partially overlapped regionally but exclusive in a cellular level, or forming a new subset

expressing both genes at their overlapped boundary?

The other unsolved problem is how each neuron subset is maintained. The location and the size of each neuron subset are kept constant in the continuous flow of tissue displacement. What is the mechanism for enabling this?

3. Aims of this study

First, I will show a new set of neuron subsets distributing in axially different regions. I analyzed with whole-mount *in situ* hybridization the expression patterns of Hym-176 gene together with other four Hym-176-related genes found in *Hydra* ESTs. I will describe the subdivided nerve net in *Hydra*, especially the boundaries between neighboring subsets in the peduncle region. Second, I will describe possible underlying mechanisms to form and maintain neuron subsets. This involves issues as to how region-specific neurons are formed, how the number of neurons constituting each subset is kept constant, what control the region specificity and how it can be interpreted by neuron precursors. Finally, taking the subset of *Hym-176* expressing neurons in the lower peduncle as an example, I will describe the possible function of neuron subsets.

Based on these observed results, I will discuss compartmentalization of *Hydra* nervous system and its relative complexity to that of higher organisms. I hope that this study will help us understand the evolution of nervous system and what the prototypical nervous system was like.

Materials and Methods

1. Animal culture

Most of experiments were carried out by using the wild type strain 105 of *Hydra magnipapillata,* which was cultured as described previously [34]. Epithelial *Hydra*, which lack all I-cell lineages except for gland cells, was produced from strain 105 by colchicine treatment [35] and has been maintained in this laboratory for several years as described [11]. Epithelial polyps were used for grafting experiments. The AEP strain of *Hydra carnea* obtained from H.R. Bode [36] are used to obtain embryos and to generate transgenic *Hydra*. Gametogenesis is constitutively induced in this strain by restricting feeding (2-3 times a week). The culture solution was changed a few hours after feeding.

2. Splinkerette-PCR

Fragments of the 5'-flanking genomic region of Hym-176 paralogues were isolated with splinkerette-PCR [37]. Briefly, 2 μ g of genomic DNA were digested with 20 U of restriction enzyme for 1hr and then heated at 65 oC for 10 min. Two restriction enzymes (HindIII and EcoRI) were used separately. The splinkerette linker was prepared as follows. The mixture containing 150 ug/ml splinktop oligonucleotide, 150 ug/ml splink oligonucleotide, 10 mM Tris-HCl (pH 8) and 5 mM MgCl2 was heated at 90 oC for 30 seconds and then annealed at room temperature for 20 minutes. Two splink

oligonucleotides were used, corresponding to each restriction enzyme (splinkHin for HindIII and splinikEco for EcoRI). These oligonucleotides sequences were listed below. The 10% of the digested DNA solution was mixed with 30% of the splinkerette linker reaction to ligate them with 5 U of T4 DNA ligase (Takara) for overnight at 14 oC. In this ligation reaction, the splinkerette linker should correspond to the restriction enzyme that was use for digestion of genomic DNA.

For splinkerette linker:

splinktop,

5'-CGAATCGTAACCGTTCGTACGAGAATTCGTACGAGAATCGCTGTCCTCTCCAACG AGCCAAGA-3'

splinkHin, 5'-AGCTTCTTGGCTCGTTTTTTTTTGCAAAAA-3'

splinkEco, 5'-AATTTCTTGGCTCGTTTTTTTTTGCAAAAA-3'

The 1/40 volume of the ligated reaction was subjected to nested PCR. The primary PCR was carried out with touchdown [38] and hot star to decrease non-specific amplification. In touchdown, annealing temperature was shifted stepwise from 72 oC to 70 oC. For each PCR reaction, linker-specific primer and Hym-176 paralogue specific primer were used as a forward and reverse primer set, respectively. The restriction enzymes and primers used for each Hym-176 paralogues were as follows.

Enzyme used: HindIII for Hym-176A, B, C and E; EcoRI for Hym-176D

For primary PCR:

linker, 5'-CGAATCGTAACCGTTCGTACGAGAA-3'

Hym-176A, 5'-CCATCGATTTCGTTATCGGTATCTTCATCGTC-3'

Hym-176B, 5'-CCGTCAACTTCGCTACCAGTTTCTTCGTC-3'

Hym-176C, 5'-CGCTACTAATTTTGTCATCAGTGTCTTCGTTATCG-3'

Hym-176D, 5'-CTTCATCATCACGAAGTGGCAGTGAGTTG-3'

Hym-176E, 5'-CAGTGTCTTCATCATCGCGAAGTGGTAGTG-3'

For secondary PCR:

linker, 5'-TCGTACGAGAATCGCTGTCCTCTCC-3'

Hym-176A, 5'-CAATGAGTTGACAGACAAAACCAC-3'

Hym-176B, 5'-AAATCAGAAAGATGTATAGAACCAG-3'

Hym-176C, 5'-AAAGGCATTGAGTTGACTGAC-3'

Hym-176D, 5'-TAACTTGATTAGATGTTTCTGGTTG-3'

Hym-176E, 5'-AGTATTGCATTAAACACGTACCAC-3'

3. The 5'- and 3'-RACE

The 3'-RACE were carried out only for *Hym-176D* to make sure that its 3'-end in Hmp15428 is correct, by using SMART RACE cDNA Amplification Kit (CLONTECH) according to its instruction manual. The primers used were as follows: 5'-GGCGTGATGTCGATTTTC-3' for the primary PCR and 5'-AACAAAATTGGAAAATCGACTC-3' for the secondary PCR. The 5'-RACE was carried out to determine the transcription initiation site by using GeneRacer kit (Invitrogen) according to its instruction manual. All primers used were as follows:

For reverse transcription:

Hym-176A, random primer

Hym-176B, 5'-TCATTTTCTGATTCACTCATGTTAC-3'

Hym-176C, 5'-TCGTTAATCTGACTGCTTTGAG-3'

Hym-176D, 5'-TCTAATTCGGTTATGTCACCATC-3'

Hym-176E, 5'-GTGTCGCCATAGATTCCG-3'

For primary PCR:

Hym-176A, 5'-CAATGAGTTGACAGACAAAACCAC-3'

Hym-176B, 5'-CCGTCAACTTCGCTACCAGTTTCTTCGTC-3'

Hym-176C, 5'-CGCTACTAATTTTGTCATCAGTGTCTTCGTTATCG-3'

Hym-176D, 5'-CTTCATCATCACGAAGTGGCAGTGAGTTG-3'

Hym-176E, 5'-CAGTGTCTTCATCATCGCGAAGTGGTAGTG-3'

For secondary PCR:

Hym-176A, 5'-TAAAACCAGTAATGCATAGAACACG-3'

Hym-176B, Not performed

Hym-176C, 5'-AAAGGCATTGAGTTGACTGAC-3'

Hym-176D, 5'-ACAGCCAAAATCACAAAGATG-3'

Hym-176E, 5'-AGTATTGCATTAAACACGTACCAC-3'

4. Whole-mount in situ hybridization

In situ hybridization analysis on whole mounts using DIG- or fluorescein-labeled RNA probes was carried out essentially as described previously [39]. The antisense RNA probes for Hym-176 paralogues were prepared from EST clones; Hmp10082 (DDBJ Acc# AB018544) for *Hym-176A*, Hm02822 (Acc# BP515965) for *Hym-176B*, Hm00388 for *Hym-176C*, Hmp15432 (Acc# BP518502) for *Hym-176D* and Hmp21432 for *Hym-176E*. All clones contained full length of coding regions except for Hm00388 which was 4 bases shorter at its 5'-end. The antisense RNA probes for *Hym-301* [40] and *Hym-355* [41] were prepared from plasmids produced by who found them in our laboratory. The

antisense RNA probes for *CnNK-2* [39] was prepared from the full length of cDNA obtained by conventional PCR techniques with primers designed according to the reported sequences as follows:

CnNK-2 forward, 5'-CTTAATGGATTTTAGCATCTTACCG-3';

CnNK-2 reverse, 5'-GTAAAACGGCCAAGAATTTGGG-3'.

The template plasmid for the full lengh of *HyBMP5-8b* [42] was donated from H. Bode. Each RNA probe was used at the concentration of approximately 0.4 ng/μl. For the second staining in double in situ hybridization, samples that finished the first staining, usually with BCIP/NTB were incubated in 4% paraformaldehyde in PBST (PBS containing 0.1% Tween20) overnight at 4 oC. After washing 6 times for 5 min each, they were incubated in anti-DIG or -fluorescein antibody. Following procedure is the same as the first staining.

5. LiCl and alsterpaullone treatments

Either newly detached polyps or adult animals of the *H. magnipapillata* 105 strain were treated with 2 mM LiCl in *Hydra* medium for 7 days with daily feeding, but in some cases mentioned in text, newly detached polyps were treated for 3 days without feeding. Adult animals of 105 strain were treated with 5 μM alsterpaullone for 3 days without feeding. Periodically, samples were taken and analyzed for gene expression by in situ hybridization.

6. BrdU labeling and detection

Animals of the *H. magnipapillata* 105 strain were treated with 2 mM BrdU (bromodeoxyuridine) in *Hydra* medium with feeding daily. Samples were periodically analyzed for gene expression by in situ hybridization followed by BrdU detection as follows. Animals processed for in situ hybridization were incubated in 3N HCl for 1 hr at room temperature. Then they were washed 3 times for 10 min each with PBT (PBS containing 0.1% Tween20). After that, they were incubated in blocking solution (PBT containing 0.5% BSA) for 1 hr, then with 4% anti-BrdU mouse IgG (BD) in blocking solution for 1 hr, followed by washing twice for 30 min each with PBT. Then they were incubated with 0.5% biotinylated anti-mouse IgG antibody (Amersham) in blocking solution for 1 hr, followed by washing twice for 30 min each with PBT. Then they were incubated with 1% Alexa488-streptavidin conjugate (Invitrogen) in PBT for 1hr, followed by washing twice for 30 min each with PBT.

7. Transgenic *Hydra*

Transgenic *Hydra* was generated by a modified method of Wittlieb et al. [43]. AEP females were cultured until eggs matured. Then egg-bearing females were mated with males to fertilize eggs. After fertilized, embryos were removed from females at the stages to be injected. The 1- to 2-cell stage of embryos were microinjected manually with a glass capillary needle, which is allowed for continuous flowing of the DNA solution by pressured mineral oil. Thus precise volume to be injected is uncontrollable, but we estimate it around 1-10 nl for each injection. The DNA solution contained the hoTG

construct (**Fig. 17A**) donated from A. Boettger (originally came from the Bosch laboratory). The plasmid DNA of hoTG was prepared by using the Qiagen (Valencia, CA) Mini Prep Kit and resuspended in water. This purified DNA was further diluted to 1-10 ng/ul with water containing 0.5% (w/v) blue dextran for visual assistance. During injection, embryos were immersed in the culture medium containing 0.5% (w/v) of Ficoll 2000 to prevent the cytoplasm of embryos from leaking out when pulling out the injection needle. Embryos were hold with a micropipette tip connected to an orally sucking device when they were injected. Glass capillary needles for microinjection were produced by using a pipette puller (NARISHIGE, PP-83) at the setting of 82-85 in heater 1 and 80 in heater 2. After injection, embryos were further kept in the same solution containing Ficoll for 3 days, then cultured in the solution replaced to Ficoll-free normal medium for 2 weeks. This 2-week culture at 12 oC increases the hatching rate. After then, embryos were returned to 18 °C until they hatched.

8. Peptide treatment

Epithelial *Hydra* starved for 24 hours were used to test the activity of a neurppeptide Hym-176. Before the assay polyps were transferred to a 24 well-plate and kept still for 1 hour before the peptide application. Synthetic Hym-176 dissolved in water (10^{-3}M) was added gently to the well containing polyps to the final concentration of 10^{-6}M. Polyps were periodically photographed.

Results

1. Neural subdivision in *Hydra*

1.1. Isolation of neuropeptide Hym-176/Hym-357 precursor gene paralogues

The precursor gene that encodes two neuropeptides, Hym-176 and Hym-357 was isolated by Yum et al [33]. Both peptides were identified through the *Hydra* peptide project that this laboratory has been undertaking to systematically identify all the signaling peptides in *Hydra*. The two peptides evoke muscle contraction as neurotransmitter and neuromodulator, respectively [44]. Using this precursor gene as a query, I searched *Hydra* EST database and found five EST clones (Hmp10082, Hm02822, Hm00388, Hmp15428 and Hmp21432) that are quite similar to the original Hym-176/357 precursor gene.

The DNA sequence of Hmp10082 was identical to the original Hym-176/357 precursor gene (AB018544) except one base difference in the 5' untranslated region (UTR). Thus I used Hmp10082 as a gene for the neuropeptide Hym-176/357 and called it here *Hym-176A*. Hm02822 and Hmp21432 also contained complete open reading frames (ORFs) and were named as *Hym-176B* and *E*, respectively. Hm00388 lacked an expected initiation codon and Hmp15428 had an unexpected stop codon, resulting in shorter ORF. To obtain complete ORF of Hm00388 or to make sure that the unexpected stop codon in Hmp15428 is not an artifact, 5'- and 3'-RACE were carried out for respective clones. A fragment of 71bp upstream from the 5'-end of Hm00388 was obtained by the 5'-RACE. This fragment contained an in-frame initiation codon that

was not included in Hm00388. This complete cDNA was named as *Hym-176C*. The 3'-RACE of Hmp15428 clarified that the unexpected stop codon in this clone was not an artifact but rather thought to produce a truncated ORF. We named this clone as *Hym-176D*.

The alignment of DNA sequences for the coding regions of these five Hym-176 related genes are shown in **Fig. 3**. Over all DNA sequences of these genes were quite similar to each other. Recently, the *Hydra* genomic database becomes available, although it is still incomplete. According to this database, all these five Hym-176 related genes have no introns. More interestingly all four genes but *Hym-176A* are positioned within a 100kb of successive genomic region as shown in **Fig. 5**. Because the entire sequence of this region has not been determined, it is still unknown whether *Hym-176A* is also located in their neighborhood. These data suggest that the *Hym-176B, C, D* and *E* are all paralogues to *Hym-176A*, the original Hym-176/357 precursor gene and may be generated by gene duplication.

Comparison of the deduced precursor sequences revealed well conservation in the N-terminal regions of all five paralogues (**Fig. 4**). This may be due to that these regions contain signal peptides necessary for proper targeting to endoplasmic reticulumn and Golgi. As illustrated in **Fig. 4**, conservation of peptide regions varied from one paralogue to the other. None of four paralogues contained peptides completely identical to Hym-176 peptide encoded by *Hym-176A*. *Hym-176B* seemed to produce totally different peptide in the corresponding region. The *Hym-176E* precursor did not even have an obvious peptide sequence which is flanked by processing sites, particularly glycine-basic amino acid residue(s) at the C-terminus that serves as an amidation signal. Only *Hym-176C* and *D* precursors appeared to contain peptides whose C-terminal tripeptides

(PKV) were shared by that of Hym-176.

Concerning the C-terminus peptides in the precursors, *Hym-176B* was the only one that had a peptide identical to Hym-357(**Fig. 4**). The *Hym-176C* precursor also had a similar peptide with only two amino acids altered (known as Hym-690-4-2). The *Hym-176D* precursor was truncated before the C-terminus peptide. The *Hym-176E* precursor seemed to produce a peptide somewhat related to Hym-357.

1.2. Gene expression

To determine the expression patterns of these five genes, *in situ* hybridization was carried out on whole mounts of *Hydra*. All of these genes were expressed only in neurons, but in discrete subsets, each of which distributed in different regions along the body axis (**Fig. 6**). The *Hym-176A* was expressed strongly in the neurons of the lower peduncle, weakly in gastric region, upper peduncle and around the mouth (**Fig. 6**-A,F,I,K). The *Hym-176A*+ neurons in the peduncle has a characteristic morphology with thick but short processes, while those in the gastric region have long thin processes [33]. This expression pattern is consistent with the results of the original Hym-176/357 precursor gene which were described previously [33]. The *Hym-176B* was expressed throughout the gastric region, the upper peduncle and around the mouth (**Fig. 6**-B,G,J). No signals were detected in the neurons of the lower peduncle. Thus both of the *Hym-176A* and *B* were expressed in gastric region, upper peduncle and around mouth. To determine whether these two genes were expressed in the same neurons, double staining of whole-mount in situ hybridization was carried out (**Fig. 7**).

Since the expression of *Hym-176A* in the gastric region was weak, it was not easy

to determine under the bright field if the neurons were expressing both *Hym-176A* (in blue) and *B* (in red) genes at the same time (**Fig. 7**-A). However, when the stained cells were seen through a red-pass filter, only neurons stained blue could be seen (**Fig. 7**-B), suggesting they express *Hym-176A*. All the stained cells found under the bright field emitted fluorescence under dark field (**Fig. 7**-C), indicating they express *Hym-176B*, because the red chromophore (Vector Red) used here are fluorescent. These results suggest that a subset of cells expressing *Hym-176B* also express *Hym-176A*. This is also supported by the result that the number of *Hym-176B* expressing cells was larger than that of *Hym-176A* in gastric region (**Fig. 6**-I and J).

Next I examined *Hym-176A* and B expressing neurons near the boundary between the upper and lower peduncle. In this case, *Hym-176B*+ neurons were stained blue and *Hym-176A*+ neurons red (**Fig. 7**-D). It should be pointed out that if *Hym-176A*+ neurons were stained red with Vector Red, no neurons expressing this gene could be detected in gastric region nor in upper peduncle, because of its low expression in these regions as compared to its expression in lower peduncle. Only neurons expressing *Hym-176A* strongly in the lower peduncle could be seen in red. The blue neurons and the red neurons were intermingling at the boundary between them. However, this was not always the case. In other samples, there was a room without both neurons between the regions expressing them (data not shown). Since, the blue staining cover up the red staining under bright field, it is not possible to see if *Hym-176B*+ neurons are also expressing *Hym-176A*. To avoid this problem, the stained samples were observed under a fluorescent microscope. The blue neurons in **Fig. 7**-D did not emit florescence in **Fig. 7**-E. Thus these neurons did not express *Hym-176A*. Taken together, these results indicated that the *Hym-176B*+ neuron subset in upper peduncle

intermingled with the *Hym-176A*+ neuron subset in lower peduncle at their boundary but there are no subsets of neurons expressing both of these two genes between the two subsets.

The *Hym-176C* and *D* were expressed only in lower peduncle neurons (**Fig. 6**-C, L and D, M, respectively). In order to compare the expression of these genes with that of *Hym-176A* in the peduncle, a combinatorial double in situ hybridization was carried out. **Fig. 7**-G shows the result of *Hym-176A* and *Hym-176D* expression under bright field. *Hym-176D* (blue) appeared to be expressed in an upper half and *Hym-176A* (red) in a lower half. However, under dark field, it was shown that *Hym-176A* was expressed also in neurons of an upper half and moreover in the same neurons expressing *Hym-176D* (**Fig. 7**-H). This suggested that a subset of *Hym-176A*+ neurons was further subdivided into two groups; in the upper group both *Hym-176A* and *Hym*-176*D* were expressed whereas in the lower group expressed only *Hym-176A*. Similar results were obtained with neurons expressing Hym-176D and C (**Fig. 7**-I, J). A subset of *Hym-176C*+ neurons also expressed *Hym-176D* in an upper half, while in a lower half *Hym-176D* expression was lost. The Hym-176E was only expressed in tentacle neurons (**Fig. 6**-E, H).

All these results described here are summarized in **Fig. 8**. A subset of tentacle neurons expressed the *Hym-176E* gene. A subset of neurons around mouth and in gastric region expressed *Hym-176B*. Some of these neurons also expressed *Hym-176A*. A subset of neurons in an upper half of lower peduncle region expressed all of *Hym-176A*, *C* and *D* at the same time. Neurons in a lower half of lower peduncle probably expressed *Hym-176A* and *C* at the same time, because they were expressed in the same neurons in an upper half. However, a final answer should be obtained by double in situ hybridization with probes from *Hym176A* and *Hym176C*. None of these five paralogues

was expressed in the basal disk.

These results also demonstrated that there are clear boundaries between the subset expressing *Hym-176B* and the subset expressing all of *Hym-176A*, *C* and *D* (boundary A), and between this subset and the subset expressing *Hym-176A* and *C* (boundary B).

2. Mechanisms involved in formation of neural subsets

2.1. How to maintain the neuron subsets?

2.1.1. New differentiation or phenotypic conversion?

The axial position and the population size of a neuron subset are kept constant in spite of the continuous flow of tissue displacement. There are two possible mechanisms to supply neurons for balancing a loss of neurons and maintaining a neuron subset: new differentiation and phenotypic conversion. In new differentiation, neurons in a neuron subset are supplied by differentiation from neuron precursor cells. They migrate to the place where they are supposed to differentiate, perform their last mitosis, and then differentiate to neurons. In phenotypic conversion, neurons in a subset are supplied by phenotypic conversion from neurons of a neighboring subset. They move to the place where they are supposed to differentiate by tissue displacement and change their phenotype. In order to discriminate this two mechanisms, *Hydra* was labeled with the thymidine analogue, BrdU (bromodeoxyuridine) and analyzed the appearance of BrdU-incorporated *Hym-176A*+ neurons. Since neuron precursors generally undergo a single round of cell cycle before overt differentiation, BrdU is incorporated in their

nuclei. In contrast, phenotypic conversion does not involve mitosis and therefore, neurons that undergo the conversion will not be labeled with BrdU.

Fig. 9 shows the results of continuous labeling in the peduncle. BrdU labeled nuclei were stained green while *Hym-176A*+ neurons were red. Most of the BrdU-labeled *Hym-176A*+ neurons first appeared near the upper boundary (boundary A in **Fig. 8**) of the neuron subset, although a small number of the double positive neurons were detected further down (3 days of labeling; **Fig. 9**A). The double-labeled neurons increased gradually in number and spread downward in time (5 and 7 days of labeling; **Fig. 9**B,C). The kinetics of labeling index of *Hym-176A*+ neurons was examined and shown in **Fig. 10**. Assumed that a whole population of *Hym-176A*+ neurons in the neuron subset is replenished by new differentiation, the turnover time of *Hym-176A*+ neurons was estimated to be 22 days. Since neurons are displaced toward the aboral end together with epithelial cells, the tissue displacement time was measured. Epithelial cells in the mid-peduncle position was marked with India ink according to Campbel [16] and traced. It took about 15 days until the dye entered the basal disk. This indicates that *Hym-176A*+ neurons at the upper boundary of the neuron subset traverse for 15 days before reaching the lower boundary. The results indicated that new differentiation account for 70% of the turnover of *Hym-176A*+ neurons in the neuron subset. The rest might be explained by phenotypic conversion.

This discrimination of newly differentiated neurons and neurons arose by phenotypic conversion is only possible for a certain duration after labeling, because a certain fraction of newly differentiated neurons labeled with BrdU above the neuron subset might be phenotypically converted to *Hym-176A*+ neurons in long term. My results indicated that, even if phenotypic conversion was involved in part, its effect was

not obvious during first 7 days, because the increase of labeling index was linear and showed no sign of acceleration which might be expected if influx of BrdU labeled *Hym-176A*+ neurons by phonotypic conversion occurred.

On the other hand, there were no accumulation of BrdU+/*Hym-176A*+ neurons in the boundary between *Hym-176A+D+* subset and *Hym-176A+D-* subset (boundary B in **Fig. 8**). This suggests that neurons in the latter subset are generated by phenotypic conversion and it is likely that they are from neurons in the former subset by loosing the expression of *Hym-176D*.

2.1.2. Lateral Inhibition

Next, I addressed why new differentiation took place only in the upper boundary of the neuron subset. One possibility is that migration of neuron precursors might be hindered by densely packed neurons of several types in the lower peduncle region and they are forced to differentiate there. This possibility, however, unlikely because BrdU-labeled neurons expressing a neuropeptide Hym-355 gene were detected anywhere in lower peduncle on day-3 of labeling, not restricted in its upper boundary (**Fig. 9**-D). This indicated that neuron precursors could penetrate into the lower peduncle and differentiated another neuron types. In other word, the boundary-restricted new differentiation is specific to a *Hym-176A*+ neuron subtype.

This restricted differentiation raised another question if they differentiate only near the boundary or if they can potentially differentiate everywhere within the region but are unable to do so because of some inhibitory mechanisms. To test which one is correct, it was examined how new differentiation would be affected if the preexisting *Hym-176A*+ neurons were absent. In the first experiment the peduncle was removed

from a BrdU labeled polyp at the lower end of budding region and the remaining upper tissue was allowed to regenerate peduncle (**Fig. 11**A). During reestablishment of a whole subset, newly produced *Hym-176A*+ neurons appeared everywhere in their proper region and were not restricted to the upper boundary (**Fig. 11**B-E). Furthermore, essentially all the newly produced *Hym-176A*+ neurons were labeled with BrdU indicating new differentiation (**Fig. 11**F). The results are consistent with the 2nd possibility that new differentiation can occur everywhere within its region but usually suppressed by some mechanisms.

In peduncle regeneration, positional information that determines a whole subset may not recover all at once and this might change the differentiation pattern of *Hym-176A*+ neurons. To exclude this possibility, a lower half of a BrdU labeled polyp was replaced with that of a non-labeled epithelial (nerve-free) polyp by grafting (**Fig. 12**A) and the differentiation pattern of *Hym-176A*+ neurons was examined. As shown in **Fig. 12**B-D, newly produced neurons were distributed evenly in the lower peduncle. Essentially all of these neurons were labeled with BrdU. These results indicate that new differentiation can occur everywhere within the region, not restricted at the boundary of the region in the absence of preexisting *Hym-176A*+ neurons. Thus, all the results support the idea that new differentiation near the upper boundary of the *Hym-176A*+ region is due to inhibition from preexisting *Hym-176A*+ neurons.

In both regeneration and grafting experiments, more than 90% of the newly produced *Hym-176A*+ neurons were labeled with BrdU. This is in contrast to the results obtained in the normal adult polyps that continuously budded. The apparent difference may be explained by the situations that neuron precursors are involved. In both regeneration and grafting, a large number of neurons are required to differentiate in a

short period of time. This was further confirmed when continuous labeling of BrdU was carried out on the newly detached polyps, in which tissue was expanding. The total number of *Hym-176A*+ neurons increased from 270 to 430 in 5 days, while the increase of BrdU labeled *Hym-176A*+ neurons in the same period of time was about 160. Thus, the net increase of the total neurons was accounted for by new differentiation.

2.2. Specification of the regions where neural subdivision takes place

To become a particular type of neurons at a certain position along the body axis, cells probably has to respond to the positional information and differentiate accordingly. Subsets of neurons that expressed one or more of Hym-176 paralogues were distributed in different regions along the oral-aboral axis (**Fig. 8**). This axial patterning implies that the positional information along oral-aboral axis may be involved in determination of the region where a particular subset of neurons should be formed. To test this possibility, the positional information was altered with lithium chloride (LiCl) and alsterpaullone (ALP), both of which are known as an inhibitor of GSK-3 β (Glycogen Synthetase Kinase 3 β), and then the distribution of neuron subsets was examined.

In *Hydra,* LiCl is known to have two alternative actions to the positional value with depending on its concentration and species used. In *Hydra vulgaris*, treatment with 0.5 mM LiCl decreases the level of positional value, causing cells of the body column to behave as if they were located closer to the foot (foot activation) [45][46][47]. On the other hand treatment with 2 mM LiCl increases the level of positional value, causing cells of the body column to behave as if they were located closer to the head (head activation) [47]. In *Hydra magnipapillata*, the species we use, however, the response to LiCl was found to be different from that of *Hydra vulgaris*. Moreover,

developmental stages of polyps affected the response to the drug.

When young polyps detached from their parental body within last 24 hours were treated with 2mM LiCl, a head-specific marker gene, epitheliopeptide Hym-301 gene, gradually expanded its expression toward the aboral direction (**Fig. 13**-A, B, C). The treatment also caused the expansion of tentacle zone. The treatment with 0.5mM LiCl had no obvious effects and 4mM LiCl had severe effects on the animals, indicating the toxicity (data not shown). This result suggested that 2mM LiCl treatment increased the positional value in the body resulting in head activation. With this treatment, however, the morphology of a lower half of the body column appeared to turn into foot. Also, the expression of the foot-specific marker gene *HyBMP5-8b* [42], *Hydra* counterpart of BMP was increased up to a half of the body (**Fig. 13**-I). This suggested that foot activation also occurred in this part, although this is preliminary indication because *HyBMP5-8b* was expressed up to slightly upper region of the foot even in the absence of this treatment(**Fig. 13**-H). Taken together, treatment with 2 mM LiCl seemed to increase the level of positional value in the upper half of the body and decrease it in a lower half simultaneously.

Under these circumstances, the expression patterns of *Hym-176A* and *B* remarkably changed. On day-3, a considerable numbers of neurons strongly expressing *Hym-176A* appeared in lower gastric region (**Fig. 13**-B vs A). However, prolonged treatments (5 days) at the same concentration did not further increase the number of *Hym-176A*+ neurons in lower gastric region (**Fig. 13**-C).

The expression of *Hym-176B*, which was observed throughout the gastric region in day-5 control polyps (**Fig. 13**-D), decrease from both ends of the gastric region by the 2 mM LiCl treatment for 3 days(**Fig. 13**-E). This tendency continued in prolonged

treatment. After 5 days of treatment, the total number of *Hym-176B*+ neurons also appeared to decrease (**Fig. 13**-F) and finally the neurons were confined to the middle part of the gastric region on day 7 (**Fig. 13**-G). The neurons around the mouth also disappeared on day 7. These results again suggests that LiCl raised a level of positional value in an upper half of the body column and lowered in a lower half. Accordingly, *Hym-176A*+ neurons expanded throughout the lower half of the body column and *Hym-176B*+ neurons were squeezed to the middle part of the body column.

The treatment with 2mM LiCl on adult polyps was also effective, but resulted in slightly different observations. Adult polyps used here were more than a week old after detachment and bore one or two buds. In these animals, expression of *Hym-176A* expanded upward from its original peduncle expression by the treatment as was found in young polyps (**Fig. 14**-A-D). In contrast to the young polyps, the *Hym-176B*+ neurons were not confined in the middle of the gastric region in adult polyps. Instead, the expression gradually disappeared from the lower gastric region and appeared to form a gradient up to the tentacle region (**Fig. 14**-E-H). These results suggested that 2 mM LiCl increased the foot activation up to the middle of the gastric region but not head activation in adult polyps. This was confirmed by the expression of marker genes. The expression of *Hym-301* showed the slight expansion of tentacle zone on day 3, but decreased thereafter (**Fig. 14**-A-D), while a foot marker gene, CnNK2, expanded up to the middle of the gastric region (**Fig. 14**-I, J).

Head activation can be specifically increased by alsterpaullone (ALP)[48]. Adult polyps were treated with 4 μM of ALP for 3 days. With this treatment the head marker, *Hym-301* turned to express all over the body (**Fig. 15**-A). The neurons expressing *Hym-176A* in lower peduncle did not change their location but decreased in number (**Fig.**

15-B). The same treatments affected the *Hym-176B* expression more dramatically. The expression was confined only to a lower half of the peduncle, where *Hym-176A*+ neurons were observed (**Fig. 15**-C). The expression of Hym-176E was also affected by ALP. After 3 days treatment with 4 μM of ALP, *Hym-176E* expression disappeared from the basal part of the tentacles (**Fig. 15**-D-F). These results suggest that the head activation also occurred by the ALP teatment and that neurons expressing *Hym-176B* responded to the head activation, resulting in confinement of the expression in the peduncle. The behavior of the *Hym-176E* expression suggested that expanded head potential reached into tentacles, pushing out the *Hym-176E* expression to more distal position in tetacles, but these behaviors did not occur by LiCl-induced head activation (data not shown).

Here, I summarize the results obtained with LiCl and ALP treatments. First, ALP can induce head activation in adult polyps while LiCl can do this only in young polyps. Second, head activation caused by LiCl and ALP affected some genes equally but not all genes. *Hym-176B* was affected by both of LiCl-induced and ALP-induced head activation, while *Hym-176E* is only affected by ALP-induced head activation. Third, *Hym-176A* also can be affected by head activation caused by both reagents, but not so extensively as *Hym-176B*. This might be because the *Hym-176A* expressing region is far from head. These data supported that the region where a certain neuron subset occur is determined by the positional value, in which GSK3, a common target of both LiCl and ALP is involved.

2.3. How do genes respond to the region specific signals?

2.3.1. Analysis of 5'-flanking genomic regions

The region specific expression of Hym-176 paraloues along the oral-aboral axis suggests that these genes may have position-specific cis-regulatory elements in their upstream, which might be conserved among genes that are expressed in the same region. The assumption is based on the idea that these upstream regions as well as their coding regions may have evolved divergently from a single ancestral gene.

For this analysis, 5'-flanking genomic region of each gene were obtained by the splinkerette method [37]. The nucleotide length obtained for each gene is as follows: 1113 bp upstream of the cording region for *Hym-176A*, 1469 bp for *Hym-176B*, 1007 bp for *Hym-176C*, 860 bp for *Hym-176D*, and 1987 bp for *Hym-176E*. Then the transcription start sites (TCSs) were determined by the oligo-capping 5'-RACE method. There were multiple TCSs for a single gene, but main TCSs were found in 80 bp upstream of the coding region for *Hym-176A*, 96 bp for *Hym-176B*, -12 bp for *Hym-176C*, 121bp for *Hym-176D*, 120 bp for *Hym-176E*. The major transcripts of the gene *Hym-176C* started from the inside of the coding region (**Fig. 16**). This would explain the reason why the original EST clone (Hm00388) lacked the 1st ATG. No introns were found in the 5' untranslated region (5'-UTR) of all genes.

To find the known cis-regulatory elements shared between paralogues in their upstream, a conventional transcription factor binding motif search program such as TFSEARCH was executed. The search revealed the presence of a large numbers of putative cis-elements for known transcription factors in the obtained 5'-flanking sequences. As shown in **Fig. 16**, multiple alignment showed that the STATx(signal

transducers and activators of transcription)-like consensus sequence, which is shaded in yellow was completely conserved among three of five Hym-176 paralogues (*Hym-176A, C,* and *D*). These three genes were expressed in the same subset of neurons that was located in an upper half of the lower peduncle (**Fig. 7**-G, I). This suggests that a STATx related transcription factor may be involved in expressional regulation of these genes in the lower peduncle.

Next, unidentified cis-elements shared between paralogues in their 5' upstream regions were searched. Although the 5'-flanking sequences were less conserved than their cording regions, there were some sequence motifs shared between paralogues expressed in the same region. The motifs box-shaded in green are shared by *Hym-176A* and *B* and are potential cis-elements involved in the specific expression of these genes in the gastric region. The motifs box-shaded in blue shared by *Hym-176A* and *C* are potential cis-elements involved in the specific expression of these genes in the lower half of the lower peduncle, because *Hym-176D*, which is expressed in upper peduncle does not have these motifs at all in its upstream.

2.3.2. Transgenic *Hydra* as a reporter system

To examine if the regulatory motifs described above are active in vivo, a reporter assay system is indispensable. The technology was not available in *Hydra* until quite recently. A German group succeeded in producing complete transgenic Hydra for the first time by microinjection a GFP construct into early embryos [43]. Based on this report, I have started to generate transgenic *Hydra*. The construct used (hoTG) is shown in **Fig. 17**-A. It is basically the same as the one the German group used. Green fluorescence protein (GFP) gene is driven by *Hydra* β-actin promoter and followed by

its 3'-genomic region including the termination/ polyadenylation signal. I also made constructs, in which GFP is driven by the promoters of Hym-301, Hym-176A, B and CnNOS (*Hydra* Nanos) gene with some variations of linker regions. However, none of them have expressed GFP in embryo so far. Therefore I will describe here only the results with the hoTG construct.

Injection was carried out with 1-cell or 2-cell stage of embryos as shown in materials and methods and **Fig. 17**-B-D. Some of these embryos started hatching about 3 weeks later at earliest but usually it took 6 months for half of them to finish hatching. I obtained four founders that showed stable but mosaic expression of GFP out of 81 injected embryos (**Fig. 17**-E, F).

It is specific to *Hydra* that asexual budding stochastically gave rise to descendants that expressed GFP only in one of three stem cell lineages; ectodermal epithelium, endodermal epithelium and I- cells. By selecting budding offsprings we have obtained animals illuminated in almost all cells of either ectodermal or endodermal epithelium. It is more difficult to have animals with almost all cells in the I-cell lineages expressing GFP, but ideally possible. So far, no evidence for germline transmission of transgene was obtained. Thus, I examined whether it could in effect occur.

The four founders were propagated and crossed with a parental AEP. AEP strain often changes its sex from female to male and therefore both sexes are available all the time. More than 30 out of 130 F1 progenies expressed GFP stably but in mosaic at first. Then they gradually expressed GFP all over the body in the same animals (**Fig. 17**-H). The reason why they did not express GFP in whole body from the beginning is not known. Some of these fluorescent progenies expressed GFP ubiquitously in both of ectodermal and endodermal layers, but the others did only in ectodermal layers. None of

them was expressed only in endodermal layers (**Fig. 17**-I-K). To test whether GFP is expressed also in I-cell lineages, the oral half of this animal was grafted to the aboral half of epithelial *Hydra*, which consisted of only epithelial cells and lacked all the cells in the I-cell lineage except for gland cells (**Fig. 18**A). During grafting, I-cells, nerve precursors and nematocytes migrate from a normal half to an epithelial half [49][50].

The aboral half was isolated and allowed to regenerate to examine if it contained any cells in the I-cell lineage. As shown in **Fig. 18**B, only neuron precursors (small interstitial cells) and neurons were GFP positive. Since the number of neurons was not large even two weeks after isolation, only a small fraction of neurons appeared to express GFP. No other cells labeled with GFP were detected. The results indicate that in this F1 strain all the ectodermal and endodermal epithelial cells and a limited number of neurons and neuron precursors were labeled with GFP.

Although the gene expression of *Hydra* β-actin has not been reported so far, it would be expected that the gene is expressed in all kinds of cells, if its promoter functions properly. In contrast, the F1 progenies showed no ubiquitous expression in all three cell lineages, suggesting the promoter region used in the construct was not sufficient for the expression level of the endogenous β-actin gene. Or there might be some suppression effects on transgenes that depend on their integration sites of the genome [51].

To make sure that the GFP expression can be further transmitted stably through a germline, we obtained backcross F2 progenies by mating F1 progenies with original AEP *Hydra* (**Fig. 17**-G). Nearly half of F2 progenies expressed GFP in the same manner as F1 progenies, in mosaic at first and ubiquitously after then. This suggests that the transgene is stably transmitted after a few passages through germline in *Hydra*,

although the transmission may be a little more frequent than that of expected by Mendelian segregation.

3. Function of a neuron subset

The function of neuron subsets revealed by the expression of peptide genes can be assumed at least to localize neurotransmitters (or neuromodulaters) so that their local actions could be secured. To examine this hypothesis, the *Hym-176A*+ neuron subset was taken as an example and the action of a neurotransmitter Hym-176 was analyzed. It is reported that Hym-176 evoked contraction of the body column at the concentration of 10-5M [44]. I examined its effect at lower concentrations. At 10-6M, the peptide specifically induced peduncle contraction of epithelial polyps but had little effect on the gastric region (**Fig. 19**). At 10-7M, the effect was marginal (data not shown). The results is in good agreement with the hypothesis of local action by a localized neurotransmitter(s).

Discussion

1. Origin of nervous system

Is *Hydra* (or cnidarians in general) the first organism that has developed the nervous system in animal evolution? Ctenophores, one of the three pre-bilaterian animal phyla are known to have a kind of centralized nervous structure, called apical organ that is located at the aboral pole and functions as a primitive brain controlling their locomotion by integrating sensory information [52]. With this and other morphological features, ctenophores have been thought to be the sister group to bilaterally symmetrical animals. However, recent molecular phylogenetic studies that are based on both large and small subunit ribosomal DNA tell us that cnidarians are the true sister group to the Bilateria and that ctenophores are basal to cnidarians and bilaterians [1]. This indicates that ctenophores are the first organisms to have developed the nervous system in animal evolution.

However, this may not indicate that the common ancestor of ctenophores and cnidarians is the origin of our type of nervous system. Because there are some lines of evidence that the ctenophore apical organ doesn't have any immunoreactive serotonergic neurons [53] while a species of cnidaria, *Phialidium gregarium* has [54], or that the only isolated brain marker gene in ctenophores, a forkhead type of nuclear factor gene, Brain Factor 1, which is commonly expressed in anterior neural structure of higher bilaterians, is not expressed in the ctenophore apical organ [55]. These facts suggest that the ctenophore brain-like structure is not homologous to bilaterian brains and has evolved independently after diverging from the common ancestor of cnidarians

and bilaterians. Thus it might be possible that the true brain can be traced back to the bilaterian-cnidarian common ancestor. Further, it may be possible that *Hydra* is the first organism to have the nervous system with characteristics in common with bilaterians. What are these shared characteristics and if any, how closely the nervous systems are related, homologous or analogous, between *Hydra* and bilaterians is the subject I will discuss in this thesis.

2. Compartmentalized nervous system in *Hydra*

Recent molecular biological studies have presented bunch of evidence that the nervous system in *Hydra* consists of many neural subpopulations, each one of which expresses different neural marker genes. So far six neuropeptide genes and one transcription factor gene (CnASH; cnidarian achaete-scute homolog) have been reported their expression in neuron [56]. In addition to them, we have identified in this study four more genes as paralogues of the one previously reported (Hym-176) [33]. Expression patterns of all these neuropeptide genes are summarized in **Table 1**. Expression of Hym-176 paralogues is also summarized in **Fig. 8**. These results have presented a new insight of complicated nervous system in *Hydra*, although this is preliminary and more supportive evidence remain to be shown for elucidating a complete view of the nervous system.

As shown in **Table 1**, the nervous system of *Hydra* was divided into 13 neuron subsets (A-M) (the lower panel of

Table 1). Since coexpression has not been studied in all combinations of genes listed, the number of subsets could become lower. On the other hand, more neuron-specific markers are expected to be obtained, the final number of subsets would be higher. In summary, this study and previous studies demonstrated an interesting new aspect of *Hydra* nervous system. First, the nerve net of *Hydra* consists of at present 13 subpopulations. Second, each subset of neurons expresses specific combination of neuropeptide genes. Third, each subset of neurons is localized in a restricted region(s) along the oral-aboral axis. Finally, this aspect is proposed for the first time in this study: Some neighboring subsets of neurons are separated from each other with clear boundaries between them (boundary A/B in **Fig. 8**). These features and our demonstration of function of Hym-176A+ neuron subset (**Fig. 19**) imply that these neuron subsets in *Hydra* are neural compartments and they behave as a sort of functional unit like that of higher organisms.

3. Comparison to higher organisms

These characteristic distributions of neuron subsets in *Hydra* remind us of compartmentalized nervous system of higher animals. Compartment is defined as a subdivided tissue that consists of lineage-restricted non-intermingling sets of cells between neighbouring compartments [57]. The central nervous system of vertebrates is subdivided along the anterior-posterior (AP) axis into several compartments; forebrain, midbrain, hindbrain and spinal cord. Their boundaries are regulated by so-called prepattern genes, pairs of mutually repressing homeobox genes. The forebrain-midbrain

boundary arises from negative interactions between anterior Pax6 and posterior Pax2. The midbrain-hindbrain boundary does so between anterior Otx2 and posterior Gbx2. Thus each compartment is characterized by the combination of the specific prepattern gene expression: Pax6 and Otx2 for forebrain, Pax2 and Otx2 for midbrain and Pax2 and Gbx2 for hindbrain. The hindbrain and spinal cord is patterned by Hox genes, which are expressed in different anteroposterior domains.

Neural compartments are also found in arthropods with underlying molecular mechanisms being widely shared with vertebrates. The central nervous systems of arthropods are subdivided into several compartments; protocerebrum, deutocerebrum, tritocerebrum, subesophageal ganglion and ventral nerve cord. The Pax2/5/8 genes expressed in the midbrain-hindbrain boundary (MHB) of vertebrates are also expressed in deutocerebrum-tritocerebrum boundary (DTB) of arthropods [58]. The otd/Otx2 and Hox genes are expressed in the region anterior and posterior to DTB/MHB, respectively in commom between these taxa. Furthermore Otx and Hox genes are functionally indispensable for proper neural development. Functional substitution between taxa is also available for these genes [59]. Therefore it can be said that the Otx - Pax - Hox subdivision (tripartite brain) of the vertebrate brain is homologous to the similar subdivision in arthropods and maybe was present in urbilaterians, the last common ancestor of proto- and deuterostomes.

This tripartite ground plan itself, however, cannot be directly traced back to the last common ancestor of bilaterians and cnidarians. Because the cnidarian counterparts of Otx [60] and Hox [61] are expressed in axially somewhat different regions but only in epithelial cells, neither in neurons nor in other cells of the I-cell lineage. In addition, CnASH, achaete-scute homolog of *Hydra*, is the only gene that is reported to be

expressed in neurons of *Hydra* except for neuropeptide genes. Thus it still remains unknown whether the subdivided nervous system of *Hydra* is homologous or analogous or totally unrelated to the neural compartments of bilaterians. In the next section I will discuss underlying mechanisms in formation and maintenance of neuron subsets in hydra, comparing to those of neural compartments in bilaterians and address if these two characteristic neural structures are analogous or homologous to each other.

4. Analogy or homology?

4.1. Maintenance of compartments

A compartment is generally defined as a subdivided tissue that consists of lineage-restricted non-intermingling sets of cells between neighboring compartments. According to this definition, neuron subsets in *Hydra* may not be the neural compartments equivalent to higher organisms, because of the two features specific to the nervous system of *Hydra*: Neurons are full-time supplied from the pool of continuously mitotic multipotent stem cells; and every single cell of *Hydra* is continuously displaced along the body axis. Thus there is no static and no lineage-restricted neuron subset in *Hydra*. Despite this, however, each neuron subset keeps its location and size of population constant, maintaining clear boundaries between subsets.

There are two possible mechanisms to supply neurons for balancing a loss of neurons caused by the tissue displacement: new neuron differentiation from precursors and phenotypic conversion of preexisting neurons. By comparing tissue displacement rate and labeling kinetics of BrdU in *Hym-176A*+ neurons in the peduncle of adult *Hydra*, I estimated that 70% the *Hym-176A*+ neuron subset occurred by new

differentiation from neuron precursors and the remaining 30% by phenotypic conversion from another type of neurons coming from above the upper boundary by tissue displacement (**Fig. 10**).

I have also found a strong evidence for phenotypic conversion within a compartment of *Hym-176A*+ neurons in the lower peduncle. As shown in **Fig. 7**-F, G and also summarized in **Table 1**, neurons in an upper half of the compartment expressed both *Hym176A* (gene# 4) and *Hym-176D* (gene#5) lost *Hym-176D* expression when they were displaced into a lower half. Since new differentiation of *Hym176A+* neurons was not observed in the middle of the compartment of *Hym-176A*+ neurons (**Fig. 9**-A), phenotypic conversion must have occurred during displacement of the upper half tissue into the lower half.

There are some other examples of phenotypic conversion in *Hydra*, such as FMRFamide-like immunoreactivity positive (FLI+) neuron subset [29], vasopressin-like immunoreactivity positive (VLI+) neuron subset [62], TS26+ ganglion to TS33+ sensory conversion [32] and RC9+ ganglion neuron subset [31]. Phenotypic conversion also explains the fate of neurons when they leave the compartment by tissue displacement. It is, however, also possible cell death occurs when neurons cross the boundary of compartment. At present no data are available to show the cell death.

Contrary to phenotypic conversion, there are examples that show new differentiation is the major source for neuron differentiation. For example, a JD1+ sensory neuron subset derives only by new differentiation [31]; Essentially all of the L-96+ (recognized by a monoclonal antibody raised against unknown epitope) neurons in the peduncle are derived from BrdU labeled precursors [63]. In this study, during foot regeneration (**Fig. 11**), in grafted epithelial foot (**Fig. 12**) and also in the peduncle of a

young polyp, more than 90% of newly produced neurons were by new differentiation. In all my cases, new neurons are acutely required and under these circumstances, the rate of new differentiation appears to increase.

Phenotypic conversion of neurons indicates that the phenotype of neurons in *Hydra* is not irreversibly determined unlike those of higher animals. Whether this plasticity of neuron gained in *Hydra* still persists or lost in higher animals is another interesting issue in evolution of nervous system.

Another interesting aspect I found in this study is apparent lateral inhibition of new differentiation by preexisting neurons. New differentiation of *Hym-176A*+ neurons occurred only near the upper boundary of the compartment (**Fig. 9**A). However, neuron precursors could penetrate deep into the compartment and became *Hym-355*+ neurons, not indicating uneven distribution of neuron precursors near the boundary. When preexisting *Hym-176A*+ neurons were removed, new differentiation occurred anywhere in the compartment. This suggests that lateral inhibition is involved in new differentiation of at least *Hym-176A*+ neurons. This is reasonable because newly produced neurons at the upper boundary traverse for many days in a compartment during which they can function. If the neurons are produced near the lower boundary, they soon move out the compartment and lose their function. This would be economically unjustified. Notch signaling might be involved in this process as in *Drosophila* neurons [64].

4.2. Prepattern

In higher animals, underlying positional information called neural prepattern in an undifferentiated neuroepithelium determines where neural differentiation can take

place [65]. Not only vertebrates but also arthropods have three neural prepattern genes, such as Otx, Pax, Hox in common, and they are required to form anterior-posterior (AP) neural compartments in these organisms. Expression of these prepattern genes is regulated by the FGF and Wnt families, and retinoic acid (RA) in vertebrate. All of these factors are secreted by caudal tissue.

On the other hand, *Hydra* counterparts of Otx (CnOtx) [60] and Hox (Cnox1, 2 and 3, respectively for PG1, Gsx and Mox in vertebrates) [61] are expressed all over the body except for both extremities in some cases. These genes are expressed in ectodermal epithelial cells, but not in I-cell lineages. *Hydra* counterpart of Pax is isolated but not yet determined its expression. Nevertheless, currently obtained data suggest that Otx, Pax and Hox seem not to be involved in neural subdivision in *Hydra*.

Wnt signaling pathways are conserved also in *Hydra*. Five components of this pathway, Wnt, GSK3b, b-catenin, Tcf/Lef and DKK have their *Hydra* counterparts. They are all expressed in the head region and also in the presumptive head region during budding and regeneration. However, their precise expression patterns are slightly different within the head. For example, HyWnt is expressed in the tip of hypostome, which is considered to be a head organizer in *Hydra*. These data suggest that Wnt signaling is involved in axis formation, especially in head formation in *Hydra* [66][67].

Wnt signaling is known to be up-regulated by LiCl, which inhibits GSK-3, a suppressive component of Wnt signaling. Activation of Wnt3A by LiCl posteriorizes their neural compartments in vertebrate [68]. LiCl is also effective in *Hydra*, but in a rather complicated manner. It is known to bring about head activation or foot activation depending on its concentration and *Hydra* species used.

I observed for the first time in this study that the same concentration of LiCl

evoked different responses depending on the species and developmental stages of *Hydra*. In *H. vulgaris*, 0.5 mM of LiCl induces foot activation (aboralization), while 2mM induces head activation (oralization) [45-47]. In *H. magnipapillata*, the effective range of concentration appeared to be narrow: 0.5mM had no effect and 4 mM was toxic to the animal. Expression of positional marker genes (*Hym-301*, *CnNK-2* and *HyBMP5-8b*) and morphological observation clearly showed that both head and foot activation was evoked at the same time by 2mM of LiCl in young polyps, while only foot activation was evoked in adult polyps by the same concentration of LiCl (**Fig. 13** and **Fig. 14**). Further investigations are required for complete understanding how LiCl evoked these different actions.

More interestingly, the axial position of neural compartments was also changed according to this alteration of positional value. Hym-176A+ neurons spread upward but seemingly up to the boundary of expanded foot region (**Fig. 13**-A-C). Hym-176B+ neurons were confined in the boundary between expanded head and foot tissues (**Fig. 13**-D-G). In adult polyps, Hym-176A+ neurons spread upward but were not suppressed from the above. Hym-176B+ neurons receded also upward but not confined. This is because only foot activation occurred in this case (**Fig. 14**). These results demonstrated that the axial position of neural compartments is regulated by a signaling system affected by LiCl in *Hydra*. There reported another example, in which L96 (monoclonal antibody, epitope unknown) positive neuron subsets were expanded by LiCl treatment [63].

Although there was no direct evidence that Wnt signaling pathway activated by LiCl induced head activation in my experiments and also in others', many lines of indirect evidence support this view. In addition, I demonstrated here that a more

specific GSK-3 inhibitor, ALP also induced head activation and accordingly altered localization of neuron subsets (**Fig. 15**). Since ALP is also known to inhibit CDK2(cyclin-dependent protein kinase 2)/cyclin A with equivalent IC_{50} values [69], the present results might be interpreted by the change in cell cycle parameters. In either case, my results imply that positional information governed in part by Wnt signaling pathway determines axial position of neuron subsets.

On the other hand, the foot activation mechanism by LiCl is totally unknown. Almost all 9 Wnt orthologues identified in *Hydra* genome sequences are expressed in hypostome (personal communication from Holstein, T.W.). Thus it is unlikely that Wnt molecules in the head regulates foot activation and that additional Wnt molecules are present in the foot. Some other molecules should be involved in foot activation. Preliminary experiments involving foot-activating peptides, Hym-330 and Hym346 showed no obvious alterations in locations of Hym-176A+ neurons.

Although it is widely accepted that LiCl inhibits GSK-3, the targets of LiCl widely vary from other kinase such as MAPKAP-K2 (mitogen activated protein kinase activated protein kinase 2), PRAK (p38-regulated/activated kinase) and CK2 (casein kinase 2) to other enzymes such as inositol monophosphatase and histone deacetylase [70]. Moreover the target of GSK-3 is not only the β-catenin in the Wnt pathway but also the glycogen synthase in the insulin pathway, CREB in the PKA pathway, elF2B in translation initiation, and c-JUN in cell cycle control [71]. All of these signaling pathways are possibly involved in LiCl-induced foot activation in *Hydra*. I have preliminary data that food intake affects foot activation evoked by LiCl. Therefore, I think that insulin and PKA pathways are the first two candidates to be examined.

In summary, neural prepattern genes are not conserved functionally between

bilaterians and *Hydra*, while Wnt signaling pathway, which is considered to regulate neural prepattern genes is functionally conserved between them. This implies that the Wnt signaling pathway may regulate neural differentiation from oral extremity (head organizer), while unknown signaling pathway may do so from aboral extremity. The balance of these two activities may determine the position of neuron subsets by way of unknown neural prepattern genes, or directly by some secreted molecules.

4.3. Transcriptional control

In order to initiate differentiation, neuron precursors are required to respond to the signals coming externally, in case of *Hydra* conceivably from ectodermal epitherial cells [72], which tell them where to differentiate. Once they receive these signals, signaling cascades eventually activate their most downstream effecter molecules, usually transcription factors, which function by binding to specific DNA sequence upstream of their target genes. Thus neuron subsets localized in the same axial position are expected to share some DNA sequence motifs in the upstream of the different neuropeptide genes expressed in the subset. *In silico* analysis revealed several position-specific motifs that may regulate position-specific expression of Hym-176 paralogues (**Fig. 16**). None of these motifs showed similarity to known cis-regulatory elements, except for the one that was similar to the STATx binding motif shared by palalogues expressed in lower peduncle. STAT proteins were originally identified as factors binding to the promoter element of IFN-α (interferon-α) induced genes [73]. Further studies have revealed that they are involved in the signal transduction not only of IFN, but also of various cytokines or growth factors. Some of these factors may be

involved in position-specific neuron differentiation in the peduncle.

These position-specific motifs should be tested for their function by reporter assays. We generated transgenic *Hydra* by a modified procedure of Wittlieb et.al. Although several different constructs, in which different promoters including those of Hym-176 paralogues drive GFP, were injected, no founders except for those expressing GFP driven by *Hydra* β-actin were obtained. The reason for this is unknown, but it is most reasonable to think that these promoters that gave negative results are not strong enough to express GFP, as compared to the β-actin promoter. Because some animals, which were obtained through the transgenic procedure but expressing no GFP were confirmed that they still have the injected construct a few weeks later by conventional PCR method (data not shown). Thus, for promoter assays some methods to enhance promoter activity of Hym-176 paralogues must be attained.

I demonstrated for the first time germline transmission of transgene by back-crossing four founders expressing GFP driven by β-actin promoter. In spite of the expectation of ubiquitous expression of β-actin, most of the transgenic F1 or F2 progenies showed mosaic expression of GFP shortly after birth. These unexpected events also occur in other transgenic animals. Although the exact reason for it is not known, one possibility is that the integration site of the transgene in the host genome may be responsible for it. Endogenous regulatory elements in the vicinity of the integration site might have some suppressive effects. These suppressive effects sometimes can be overcome by using long sequence of native genomic DNA instead of cDNA, because these sequences probably contain insulators, which inhibit nonspecific stimulation by neighbour stimulators and prevent the silencing of the transgene. The mosaic expression turned into ubiquitous as *Hydra* grew for unknown reason. This

seems to be specific to *Hydra*. Important thing is that this recovery of expression is specific to epithelial cell lineages but not to I-cell lineage. This may be a result of lineage specific gene regulation of unknown mechanisms.

5. Conclusion

Comparison of the involved mechanisms between neuron subsets in *Hydra* and neural compartments in higher animals showed both conservation and divergence. This may be taken for granted, because it has been enormously long time since they were separated from the last common ancestor. However, a crucial point was that one of the most fundamental signaling systems all through the animal evolution, the Wnt pathway was conserved as one of the underlying mechanisms to determine the position of compartments between *Hydra* and higher organisms. Downstream genes activated by the Wnt pathway appear to be different. More evidence should be accumulated. But I would like to temporarily conclude that it is too early to give up the idea that neuron subsets in *Hydra* and neural compartments in higher animals are homologous structure reminiscent of the ancient nervous system. I would further pursue my studies along this line.

Acknowledgement

I thank Hanz Bode and Angelika Boettger for providing plasmid constructs. I am very grateful to all the lab staffs for doing everything that supported this work.

Tables

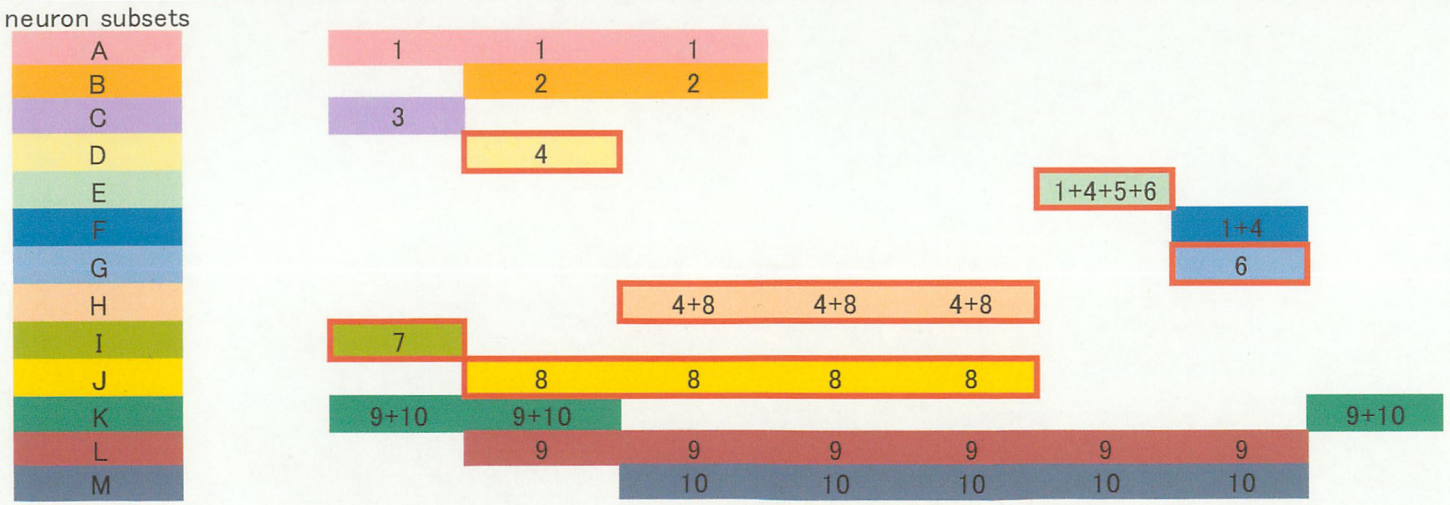
Table 1. Neuropepitide genes and their expression in different regions along the body axis. All the reported results along with the present resuls are summarized. Relative levels of expression of each gene in different regions are shown in High and Low. Blue and red colors indicate that coexpression of these genes in particlular regions have been studied. The same color in a given region indicates coexpression. When coexpression has not been analyzed, high or low is shown in black.

In the lower half panel, expression of different neuron subsets is shown along the body axis. Each box shows subset of neurons. The same neuron subset is shown in the same colored box. The number in the box shows the gene# expressed in the neuron subset. The neuron subsets identified in this study is fringed in red. For example, neuron subset E (light green) expresses genes 1, 4, 5 and 6 at the same time only in an upper half of the lower peduncle.

This neuron subset map is automatically generated from the gene expression data in Table 1 according to some simple rules. Those genes are picked up which are expressed in each subdivided region of animal body. If there is an evidence showing co-expression in the same neuron, these genes are grouped in a same neuron subset. If there is no data for co-expression, they are grouped in different subsets. Finally throughout the body, neuron subsets expressing the same combination of genes are defined as the same neuron subsets even if they are located far from each other.

Ref. Darmer **[74]**, Mitgutsch **[75]**, Hansen **[76, 77]**, Yum **[33]**, Liviev **[78]**, Takahashi **[41]**

Gene#	Peptide type encoded	Gene name	Expression								Reference
			Tentacle	Hypostome	Gastric region		Peduncle			Basal disk	
					upper	lower	upper	lower			
								upper	lower		
1	RFa	Prepro A	High	High	Low			High	High		Darmer (1998)
2		Prepro B		High	Low						Mitgutsch (1999)
3		Prepro C	High								Hansen (2000, 2002)
4	PKVa/YKPa	Hym-176A		Low	Low	Low	Low	High	High		Yum (1998)
5		Hym-176D						High			this study
6		Hym-176C						High	High		this study
7		Hym-176E	High								this study
8		Hym-176B		High	**High**	**High**	**High**				this study
9	LWa		High	**High**	Low	Low	Low	Low	Low	High	Leviev (1997)
10	PRGa	Hym-355	High	High	Low	Low	Low	Low	Low	High	Takahashi (2000)



Figures

Fig. 1. Basic body plan of *Hydra*.

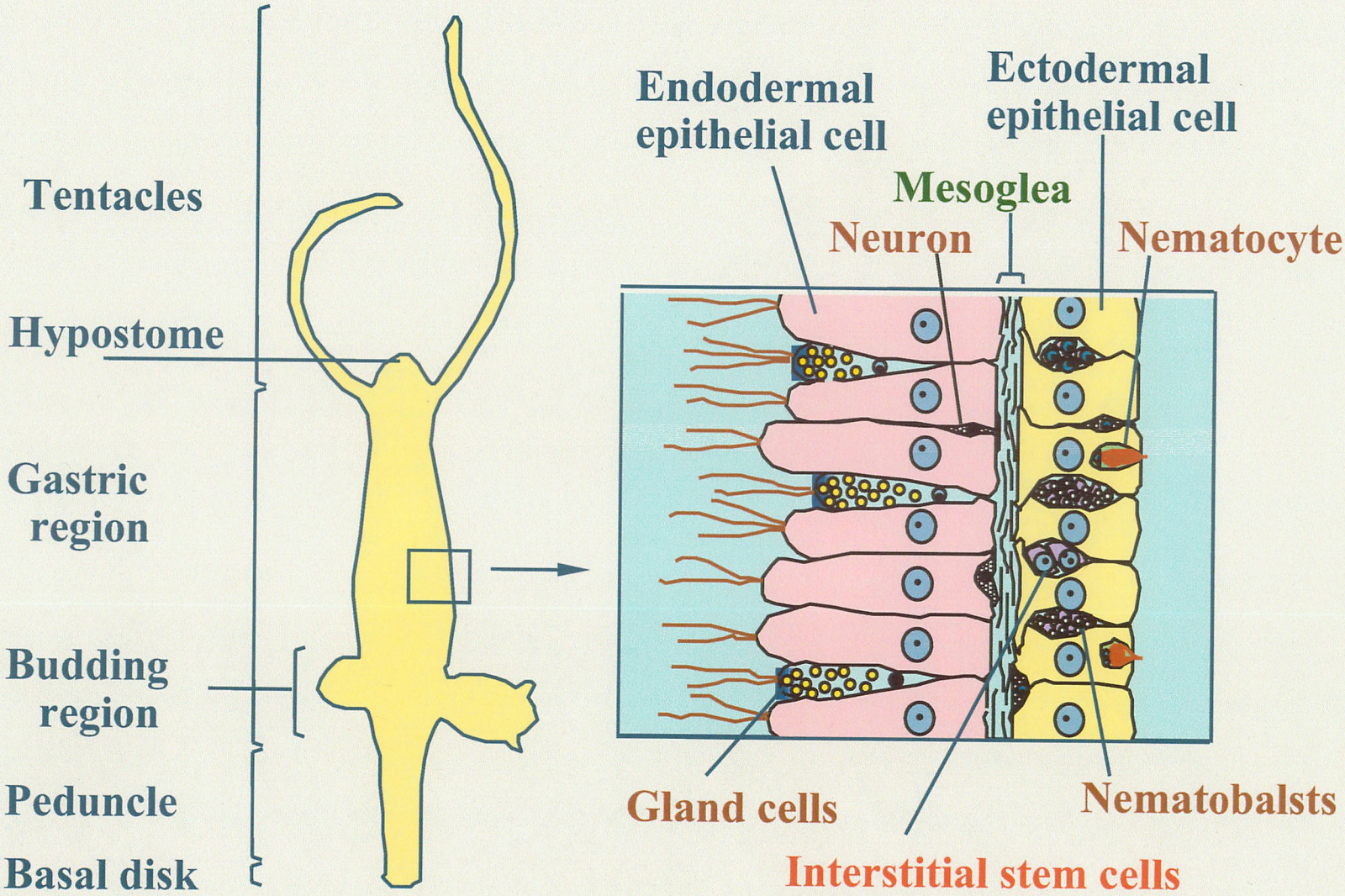

Tentacles
Hypostome
Gastric region
Budding region
Peduncle
Basal disk
Endodermal epithelial cell
Ectodermal epithelial cell
Mesoglea
Neuron
Nematocyte
Gland cells
Nematobalsts
Interstitial stem cells

Fig. 2. Three stem cell lineages of *Hydra*.

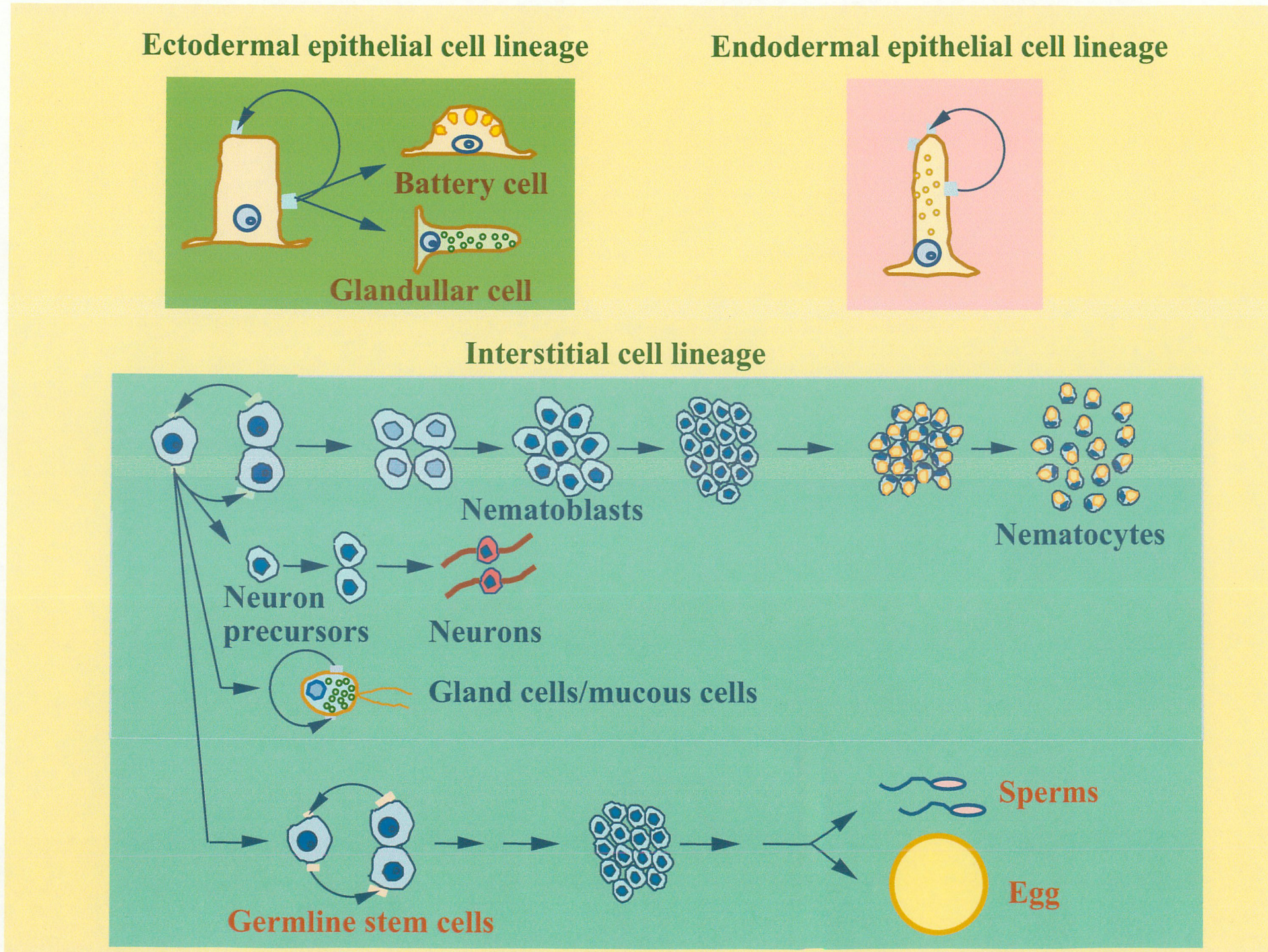
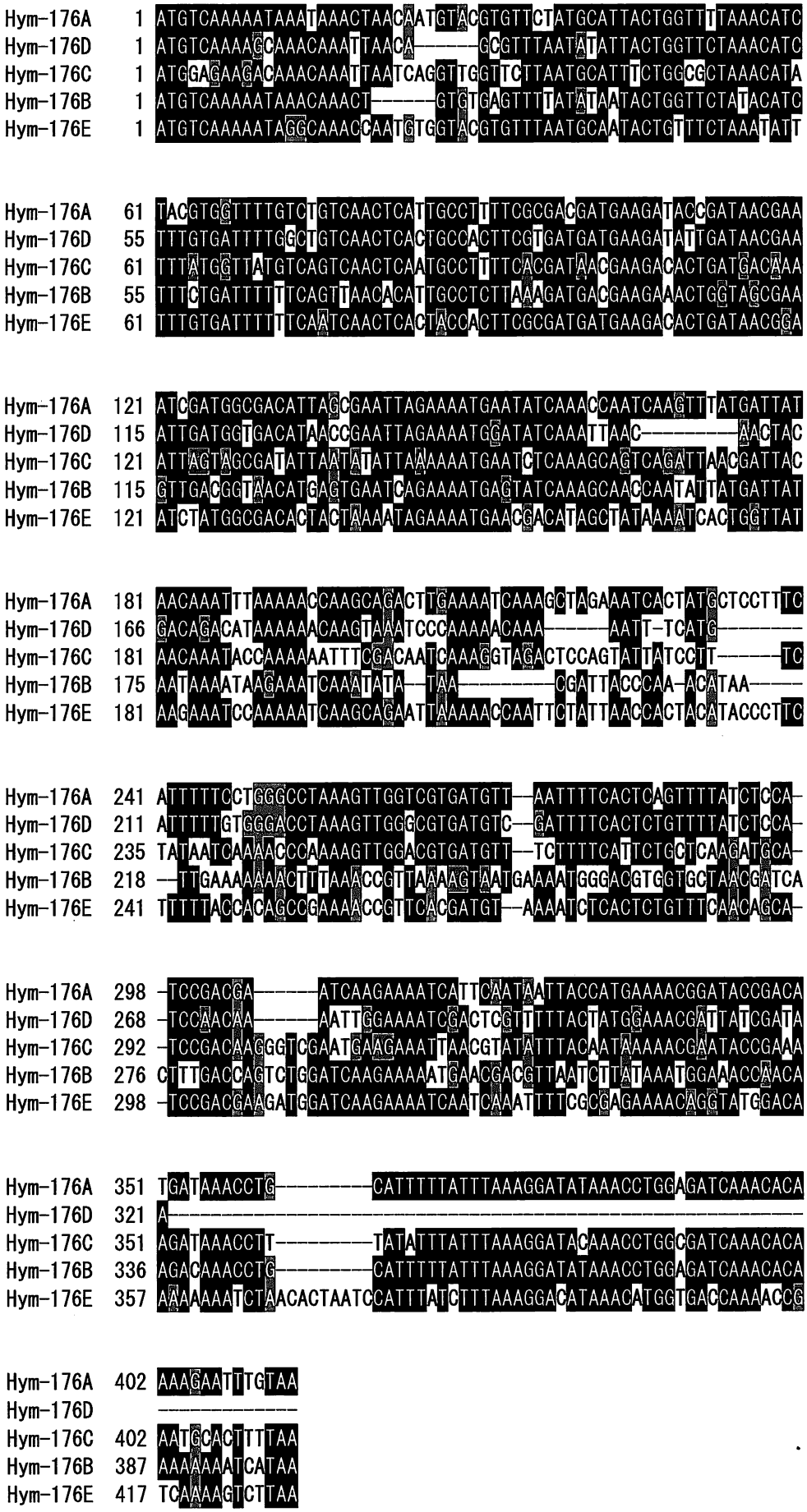

Ectodermal epithelial cell lineage
Battery cell
Glandullar cell
Endodermal epithelial cell lineage
Interstitial cell lineage
Nematoblasts
Nematocytes
Neuron precursors
Neurons
Gland cells/mucous cells
Sperms
Egg
Germline stem cells

Fig. 3. Alignment of nucleotide sequences of Hym-176 related genes. Only coding regions are shown. Nucleotides identical to the consensus, in this case the consensus is shared among 3 out of 5 genes, were shaded in black and nucleotides not identical but similar to the consensus is shaded in gray.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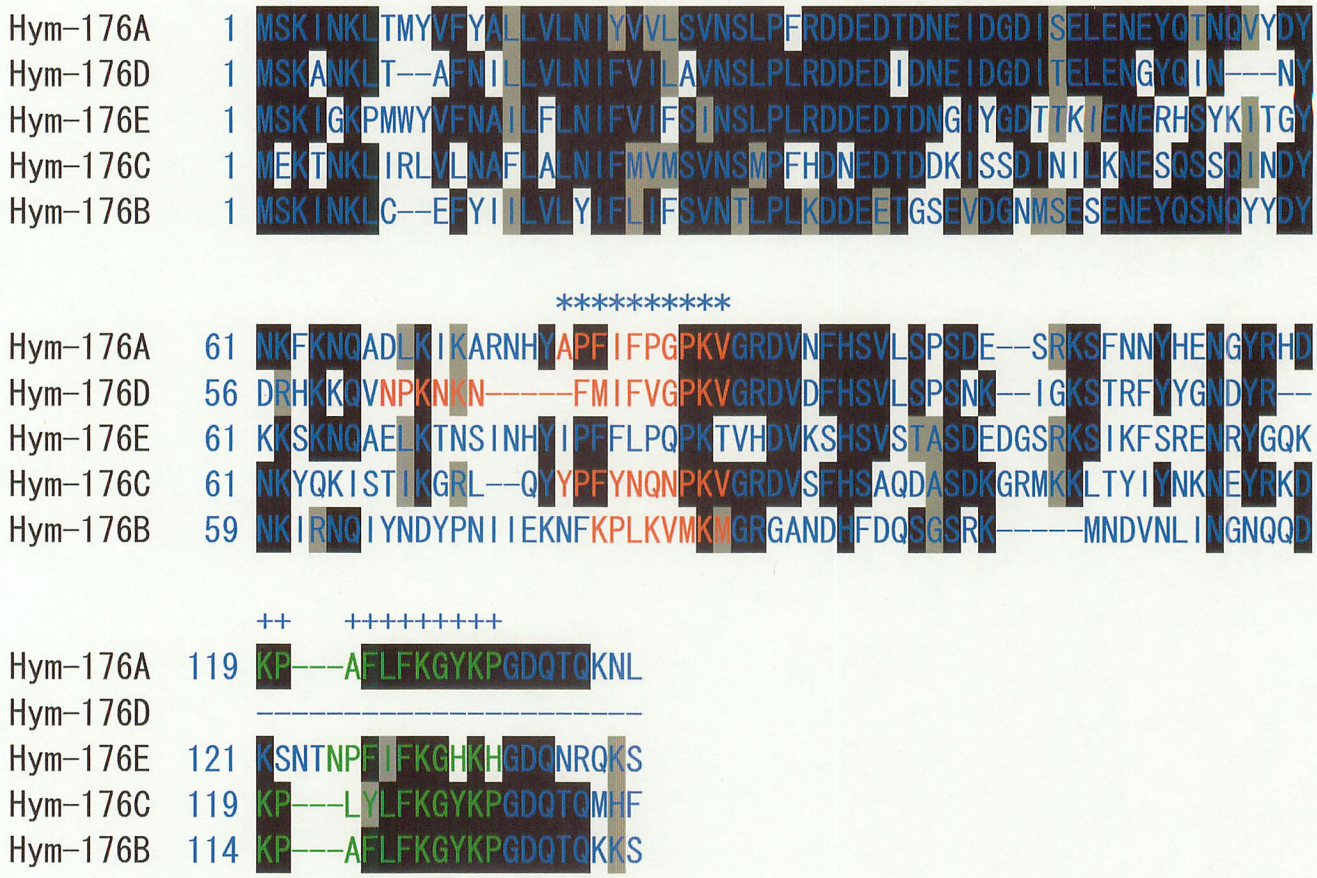
Hym-176C 1 ATGGAGAAGACAAACAAATTAATCAGGTTGGTTCTTAATGCATTTCTGGCGCTAAACATA
Hym-176B 1 ATGTCAAAAATAAACAAACT-------GTGTGAGTTTTATATAATACTGGTTCTATACATC
Hym-176E 1 ATGTCAAAAATAGGCAAACCAATGTGGTACGTGTTTAATGCAATACTGTTTCTAAATATT
Hym-176A 61 TACGTGGTTTTGTCTGTCAACTCATTGCCTTTTCGCGACGATGAAGATACCGATAACGAA
Hym-176D 55 TTTGTGATTTTGGCTGTCAACTCACTGCCACTTCGTGATGATGAAGATATTGATAACGAA
Hym-176C 61 TTTATGGTTATGTCAGTCAACTCAATGCCTTTTCACGATAACGAAGACACTGATGACAAA
Hym-176B 55 TTTCTGATTTTTTCAGTTAACACATTGCCTCTTAAAGATGACGAAGAAACTGGTAGCGAA
Hym-176E 61 TTTGTGATTTTTTCAATCAACTCACTACCACTTCGCGATGATGAAGACACTGATAACGGA
Hym-176A 121 ATCGATGGCGACATTAGCGAATTAGAAAATGAATATCAAACCAATCAAGTTTATGATTAT
Hym-176D 115 ATTGATGGTGACATAACCGAATTAGAAAATGGATATCAAATTAAC---------AACTAC
Hym-176C 121 ATTAGTAGCGATATTAATATATTATAAAAATGAATCTCAAAGCAGTCAGATTAACGATTAC
Hym-176B 115 GTTGACGGTAACATGAGTGAATCAGAAAATGAGTATCAAAGCAACCAATATTATGATTAT
Hym-176E 121 ATCTATGGCGACACTACTAAAATAGAAAATGAACGACATAGCTATAAAATCACTGGTTAT
Hym-176A 181 AACAAATTTAAAAACCAAGCAGACTTGAAAATCAAAGCTAGAAATCACTATGCTCCTTTC
Hym-176D 166 GACAGACATAAAAAACAAGTAAATCCCAAAAACAAA-------AATT-TCATG--------
Hym-176C 181 AACAAATACCAAAAAATTTCGACAATCAAAGGTAGACTCCAGTATTATCCTT-------TC
Hym-176B 175 AATAAAATAAGAAATCAAATATA---TAA---------CGATTACCCAA-ACATAA-----
Hym-176E 181 AAGAAATCCAAAAATCAAGCAGAATTAAAAACCAATTCTATTAACCACTACATACCCTTC
Hym-176A 241 ATTTTTCCTGGGCCTAAAGTTGGTCGTGATGTT---AATTTTCACTCAGTTTTATCTCCA-
Hym-176D 211 ATTTTTGTGGGACCTAAAGTTGGGCGTGATGTC---GATTTTCACTCTGTTTTATCTCCA-
Hym-176C 235 TATAATCAAAACCCAAAAGTTGGACGTGATGTT---TCTTTTCATTCTGCTCAAGATGCA-
Hym-176B 218 ---TTGAAAAAAACTTTAAACCGTTAAAAGTAATGAAAATGGGACGTGGTGCTAACGATCA
Hym-176E 241 TTTTTACCACAGCCGAAAACCGTTCACGATGT---AAAATCTCACTCTGTTTCAACAGCA-
Hym-176A 298 -TCCGACGA------ATCAAGAAAATCATTCAATAATTACCATGAAAACGGATACCGACA
Hym-176D 268 -TCCAACAA------AATTGGAAAATCGACTCGTTTTTACTATGGAAACGATTATCGATA
Hym-176C 292 -TCCGACAAGGGTCGAATGAAGAAATTAACGTATATTTACAATAAAAACGAATACCGAAA
Hym-176B 276 CTTTGACCAGTCTGGATCAAGAAAAATGAACGACGTTAATCTTATAAATGGAAACCAACA
Hym-176E 298 -TCCGACGAAGATGGATCAAGAAAATCAATCAAATTTCGCGAGAAAACAGGTATGGACA
Hym-176A 351 TGATAAACCTG---------CATTTTTATTTAAAGGATATAAACCTGGAGATCAAACACA
Hym-176D 321 A----------
Hym-176C 351 AGATAAACCTT---------TATATTTATTTAAAGGATACAAACCTGGCGATCAAACACA
Hym-176B 336 AGACAAACCTG---------CATTTTTATTTAAAGGATATAAACCTGGAGATCAAACACA
Hym-176E 357 AAAAAAATCTAACACTAATCCATTTATCTTTAAAGGACATAAACATGGTGACCAAAACCG
Hym-176A 402 AAAGAATTTGTAA
Hym-176D ----------
Hym-176C 402 AATGCACTTTTAA
Hym-176B 387 AAAAAAATCATAA
Hym-176E 417 TCAAAAGTCTTAA

Fig. 4. Alignment of amino acid sequences deduced from the nucleotide sequences of Hym-176 related genes. Amino acids conserved among 3 out of 5 sequences are shaded in black. Amino acids derived from a single neucleotide change are shadede in gray. Peptides or presumptive peptides encoded are shown in colored letters. The region of Hym-176 and Hym-357 peptide are shown with **** and ++++, respectively.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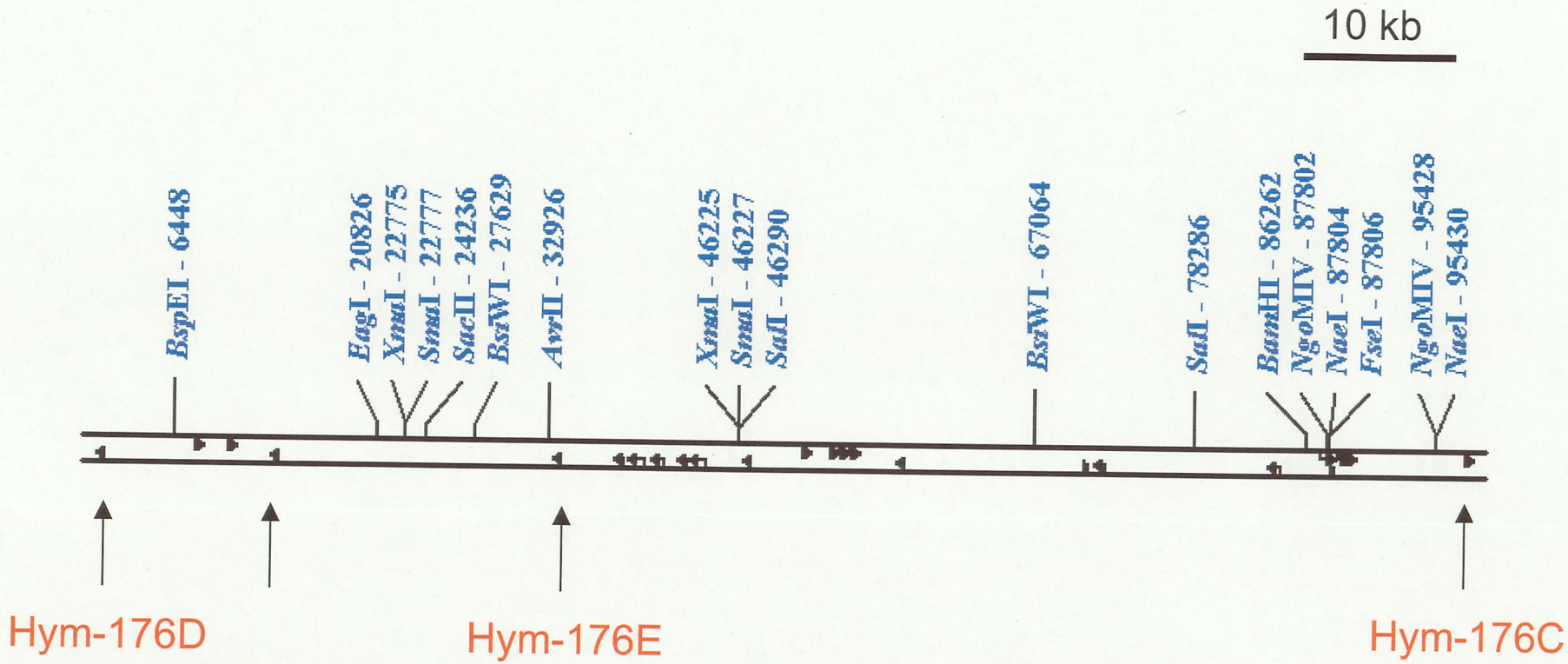
Hym-176E 1 MSKIGKPMWYVFNAILFLNIFVIFSINSLPLRDDEDTDNGIYGDTTKIENERHSYKITGY
Hym-176C 1 MEKTNKLIRLVLNAFLALNIFMVMSVNSMPFHDNEDTDDKISSDINILKNESQSSQINDY
Hym-176B 1 MSKINKLC--EFYIILVLYIFLIFSVNTLPLKDDEETGSEVDGNMSESENEYQSNQYYDY
****
Hym-176A 61 NKFKNQADLKIKARNHYAPFIFPGPKVGRDVNFHSVLSPSDE--SRKSFNNYHENGYRHD
Hym-176D 56 DRHKKQVNPKNKN-----FMIFVGPKVGRDVDFHSVLSPSNK--IGKSTRFYYGNDYR--
Hym-176E 61 KKSKNQAELKTNSINHYIPFFLPQPKTVHDVKSHSVSTASDEDGSRKSIKFSRENRYGQK
Hym-176C 61 NKYQKISTIKGRL--QYYPFYNQNPKVGRDVSFHSAQDASDKGRMKKLTYIYNKNEYRKD
Hym-176B 59 NKIRNQIYNDYPNIIEKNFKPLKVMKMGRGANDHFDQSGSRK-----MNDVNLINGNQQD
++ +++++++++
Hym-176A 119 KP---AFLFKGYKPGDQTQKNL
Hym-176D ----------
Hym-176E 121 KSNTNPFIFKGHKHGDQNRQKS
Hym-176C 119 KP---LYLFKGYKPGDQTQMHF
Hym-176B 114 KP---AFLFKGYKPGDQTQKKS

Fig. 5. Genomic organization of Hym-176 paralogues. A 100 kb genomic scaffold is shown. Although this region still contains unsequenced part, it contained four of five paralogues of Hym-176. The arrowheads show the ORFs and the direction of transcription.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EagI - 20826
XmaI - 22775
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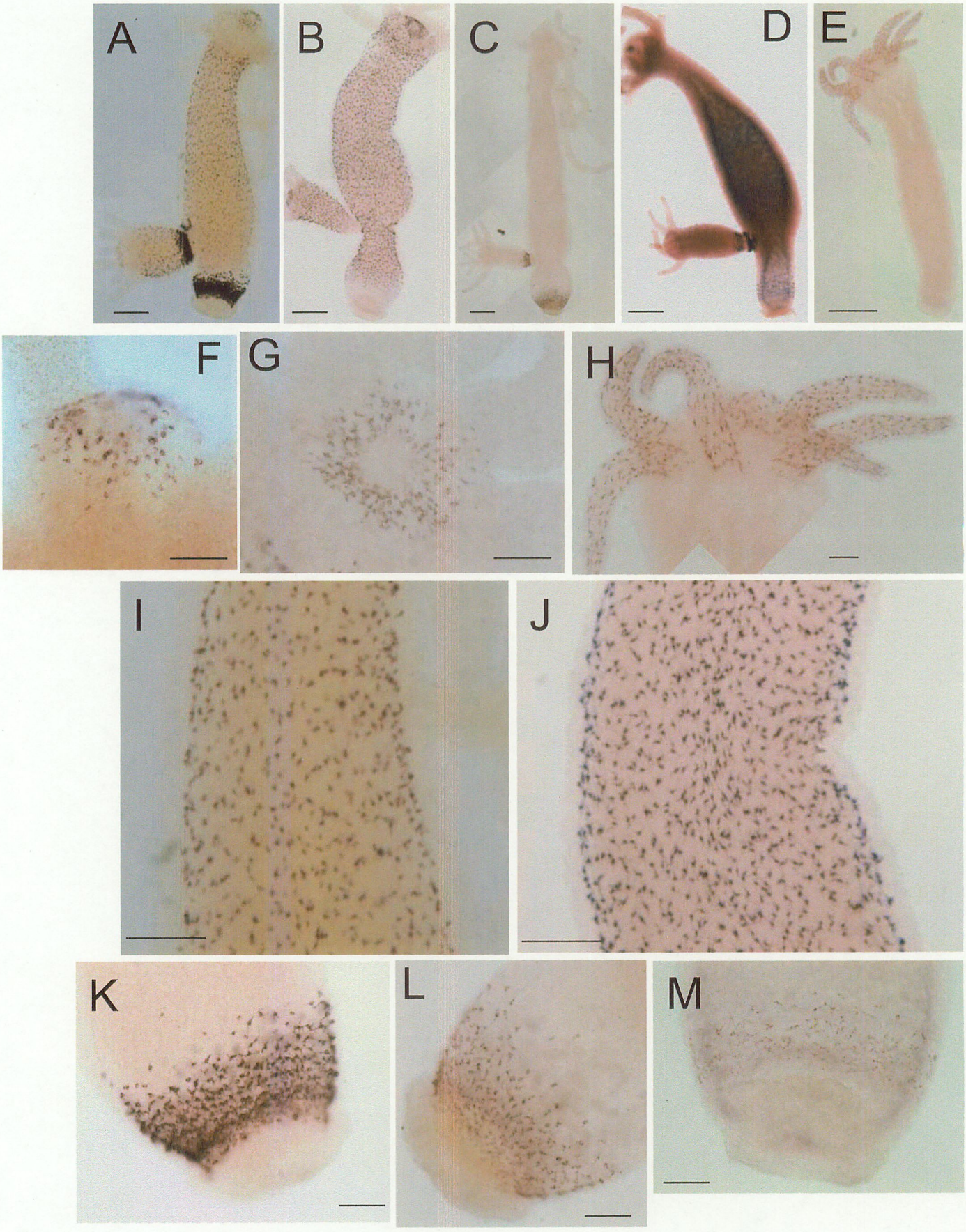
SmaI - 46227
SalI - 46290
BstWI - 67064
SalI - 78286
BamHI - 86262
NgoMIV - 87802
NaeI - 87804
FseI - 87806
NgoMIV - 95428
NaeI - 95430
Hym-176D
Hym-176B
Hym-176E
Hym-176C

Fig. 6. Whole mount in situ hybridization showing the expression of Hym-176 paralogues. A,F,I,K. Hym-176A expression in a whole body, hypostome, gastric region, and lower peduncle, respectively. B, G, J. Hym-176B expression in a whole body, hypostome and gastric region, respectively. C, L. Hym-176C expression in a whole body and lower peduncle, respectively. D, M. Hym-176D expression in a whole body and lower peduncle. E, H. Hym-176E expression in a whole body and tentacles, respectively. The bar indicates 200 um in A-E and 100 um in F-M.


A
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L
M

Fig. 7. Double whole mount in situ hybridization of Hym-176 paralogues. A. Neurons in the gastric region expressing both or one of Hym176A (blue) and Hym-176B (red). B. The same image as A seen through red pass filter to detect neurons expressing Hym-176A (dark spots). C. The same image as A, but neurons expressing Hym-176B were detected with red fluorescence of the red chromophore (Vector Red). D. Neurons in peduncle expressing Hym-176A (red) and/or Hym-176B (blue). E. The same images as D, but neurons expressing Hym-176A were detected with red fluorescence of the red chromophore (Vector Red). F. Neurons in the lower peduncle expressing Hym-176A (red) and /or Hym-176D (blue). G. The same image as F, but neurons expressing Hym-176A were seen in dark field. H. Neurons in the lower peduncle expressing Hym-176C (red) and /or Hym-176D (blue). I. The same image as H but neurons expressing Hym-176C were seen in dark field. The bar indicates 50 um.

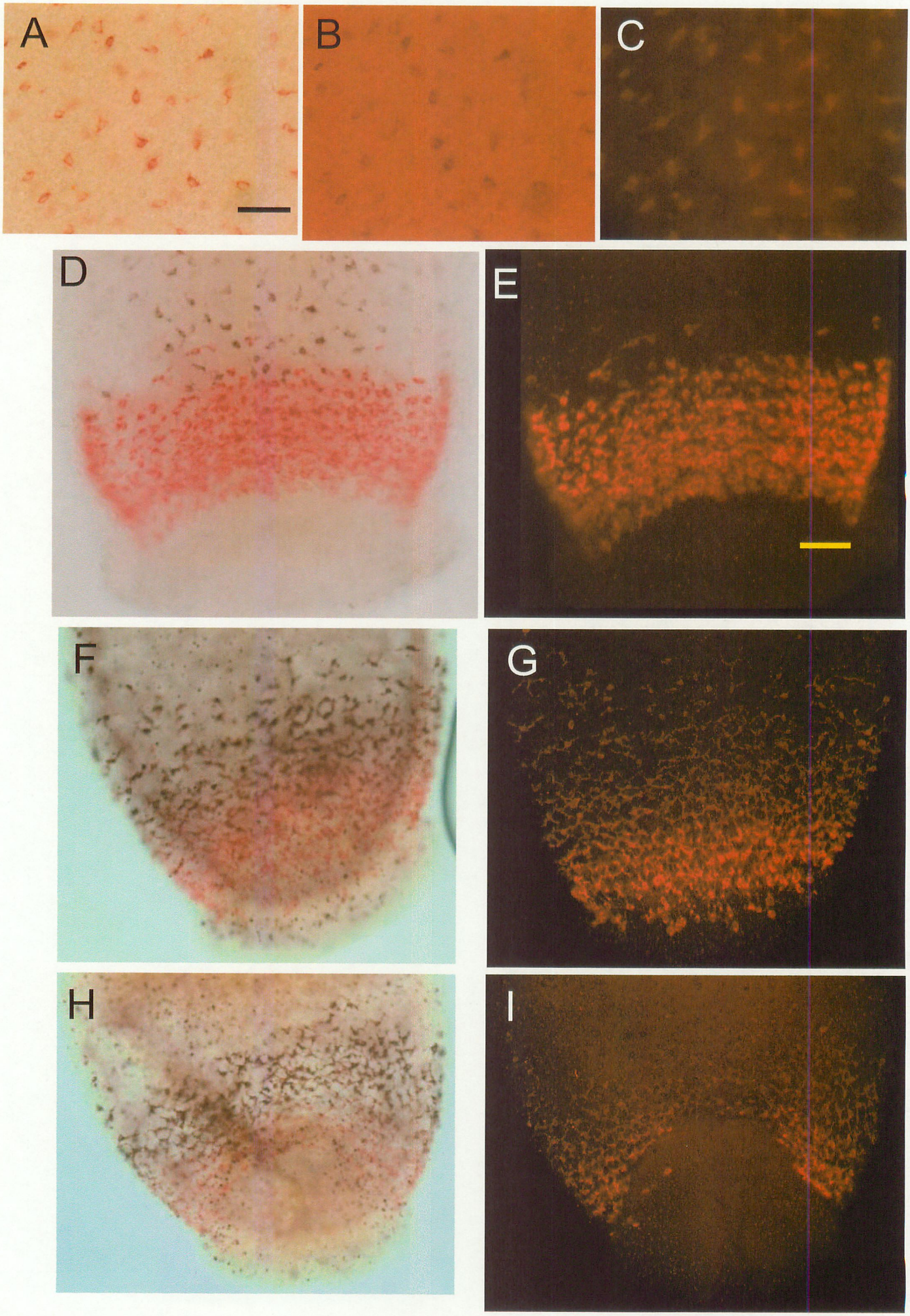

A
B
C
D
E
F
G
H
I

Fig. 8. Summary of expression patterns of Hym-176 paralogues. Vertical colored lines indicate the expression of Hym-176 paralogues (A to E) in different regions along the body axis. The dotted line indicates relatively low expression comparing to the other regions. Coexpression of the paralogues in the same neuron subsets is shown as multicolored circles.

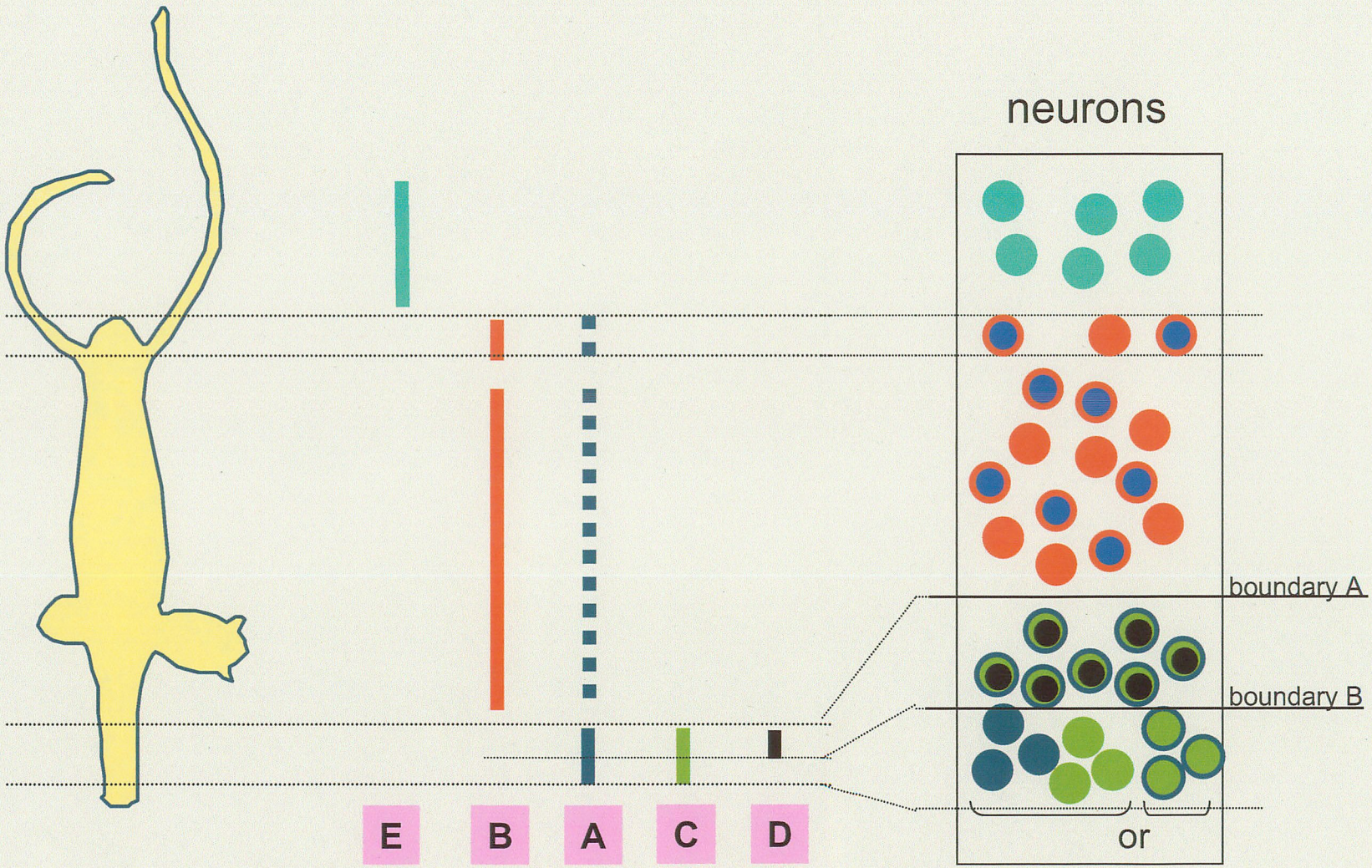

neurons
boundary A
boundary B
E
B
A
C
D
or

Fig. 9. Detection of BrdU labeled Hym-176A expressing neurons in the lower peduncle region of adult polyps. A. Continuous BrdU labeling for 3 days. B. Continuous BrdU labeling for 5 days. C. Continuous BrdU labeling for 7 days. D. Continuous BrdU labeling for 3 days but neurons were detected by a Hym-355 probe. Nuclei were stained in green and neurons expressing Hym-176A or Hym-355 were in red. Double positive neurons are marked by yellow circles. Bar, 100 um.

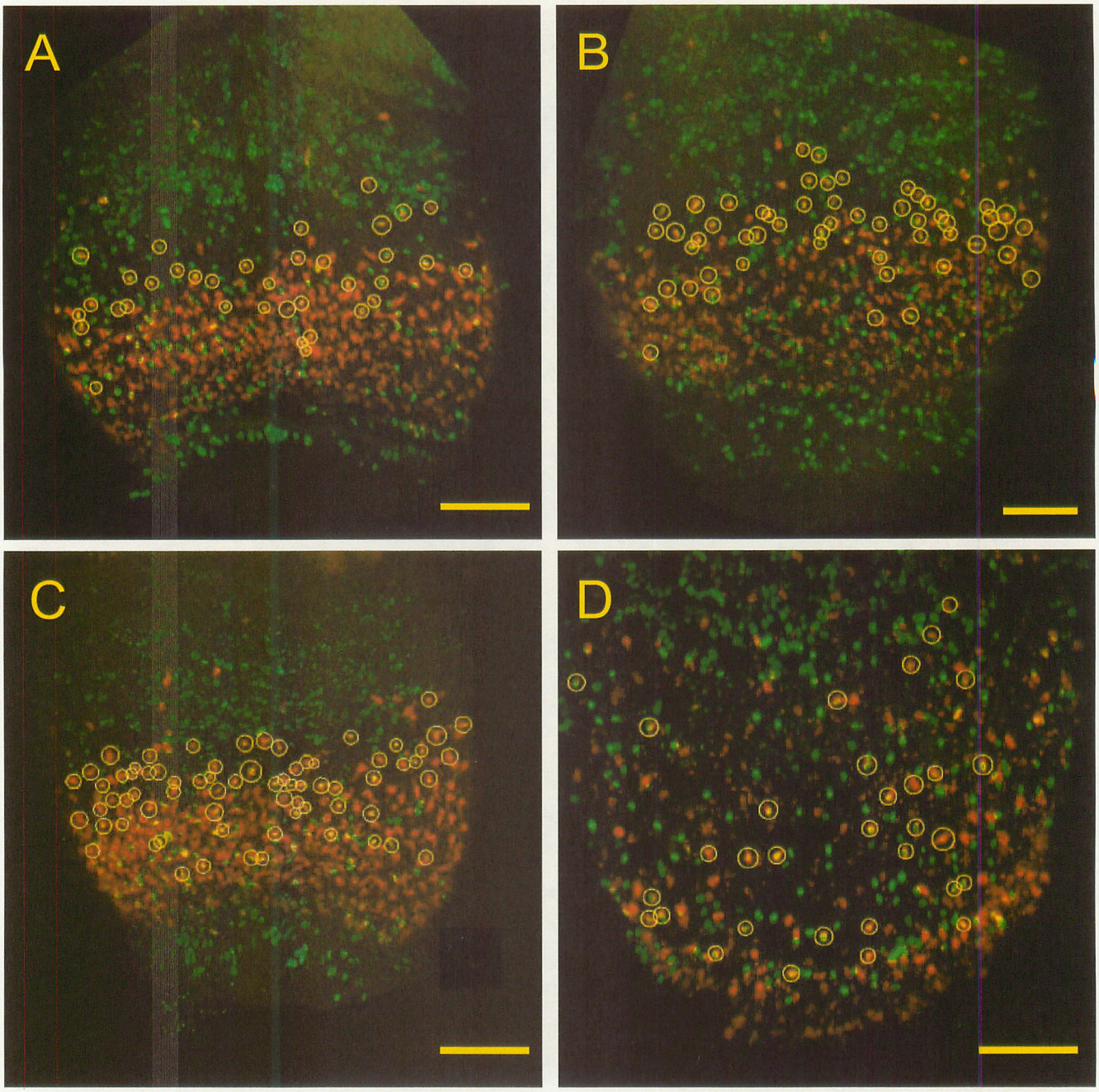

A
B
C
D

Fig. 10. Kinetics of BrdU labeling index of *Hym-176A* expressing neurons. Continuous BrdU labeling was carried out up to 7 days. Three animals were sampled on each day of 3, 5 and 7. Red points indicate average values.

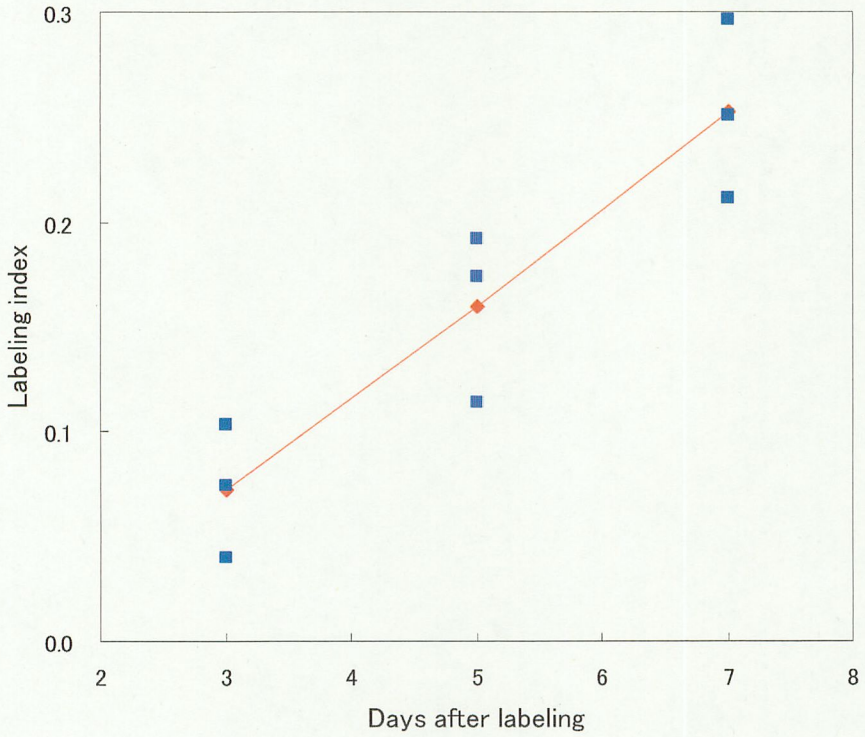

0.3
0.2
0.1
0.0
Labeling index
2
3
4
5
6
7
8
Days after labeling

Fig. 11. Appearance of BrdU labeled Hym-176A+ neurons during foot regeneration. A. Experimental scheme. Animals were labeled with BrdU for 2 days before bisection of foot. B. Two days of foot regeneration. C. Three days of foot regeneration D. Four days of foot regeneration. E. Five days of foot regeneration. F. Higher magnification of E. Hym-176A+ neurons in red and BrdU labeled nuclei in green. Bar, 50 um

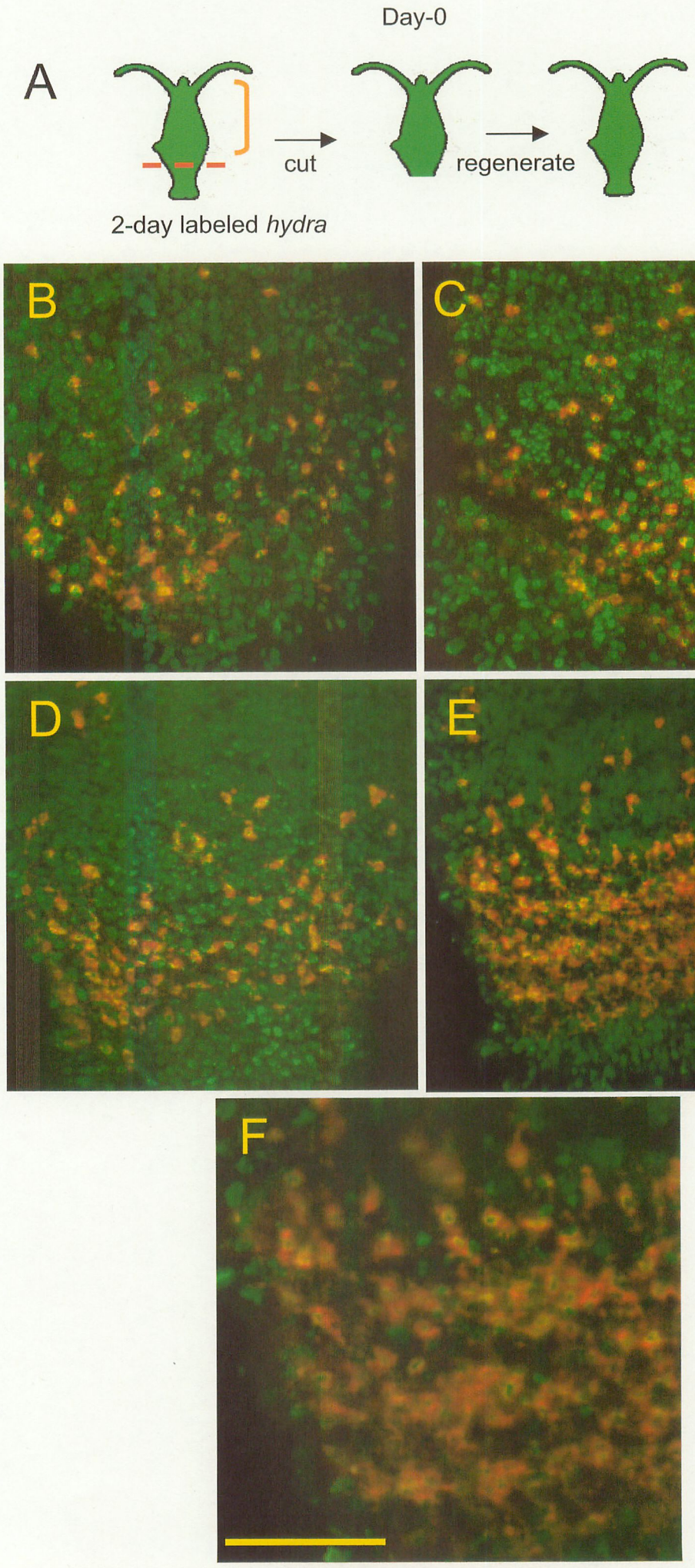
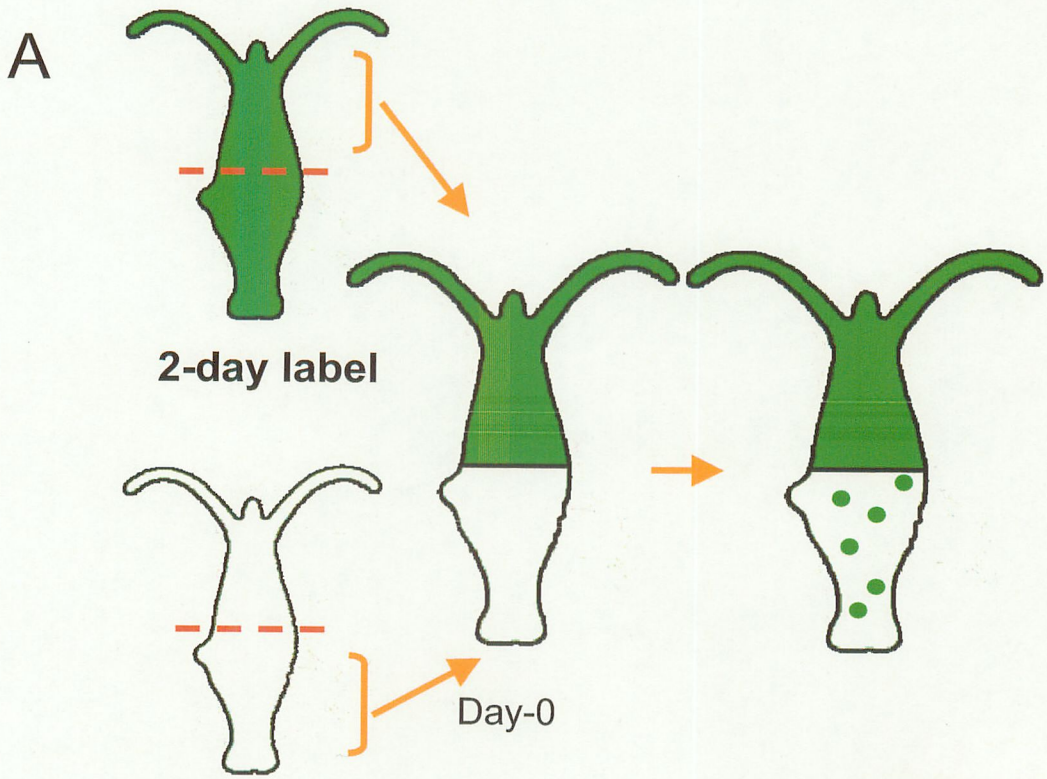

Day-0
A
cut
regenerate
2-day labeled *hydra*
B
C
D
E
F

Fig. 12. Repopulation of BrdU labeled Hym-176A+ neurons in the epithelial peduncle. A. Experimental procedure (see Materials and Methods for detail). B. Two days after grafting. C. Three days after grafting. D. Five days after grafting. E. Higher magnification of D. Hym-176A+ neurons in red and BrdU labeled nuclei in green. Bar, 50 um



epithelial *Hydra*: non label

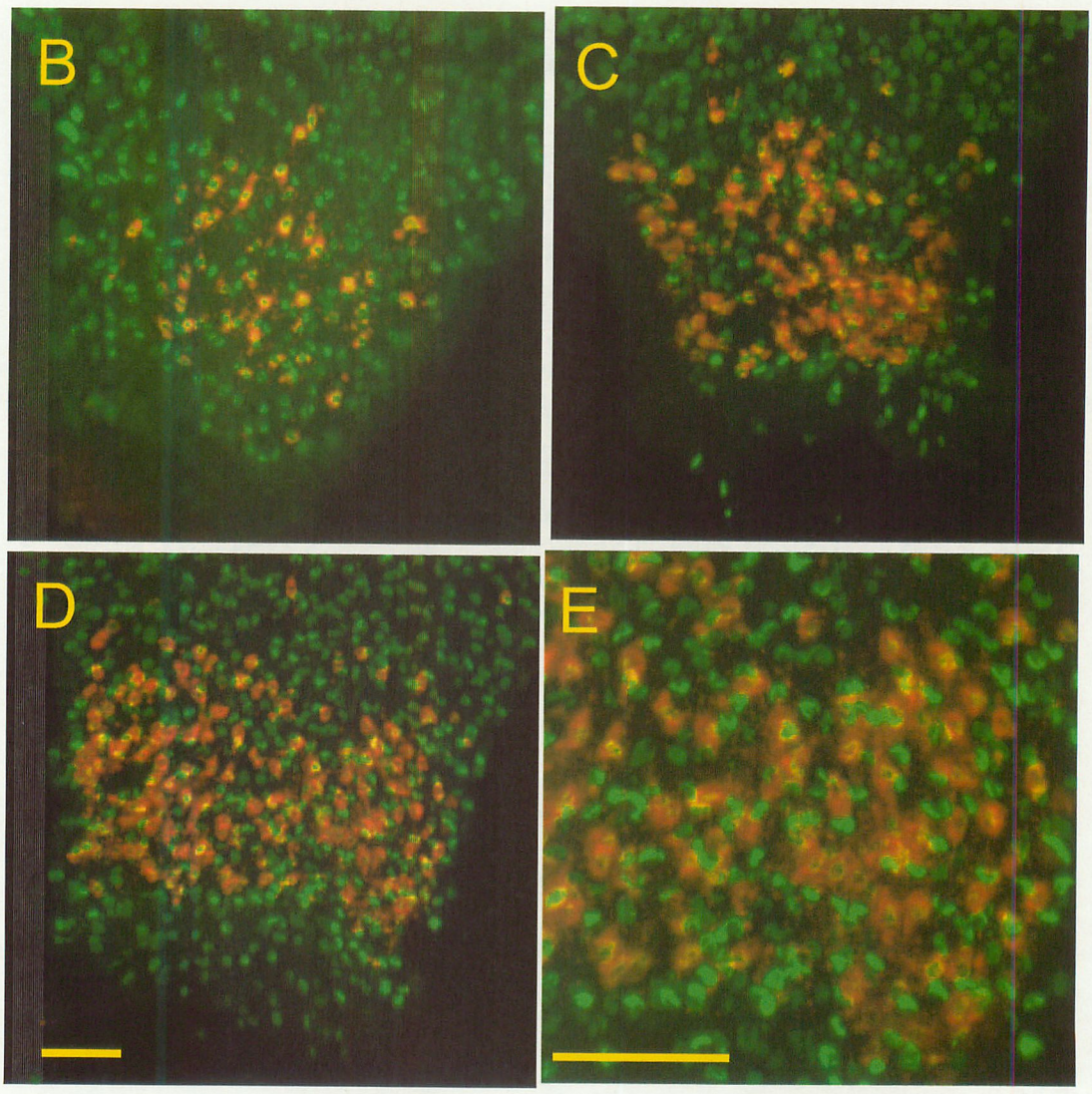


Fig. 13. Whole mount in situ hybridization on newly detached polyps treated with 2 mM LiCl. A-C. Expression of *Hym-301* (blue) and *Hym-176A* (red). A. Untreated 5-day control. B. 3-day treatment. C. 5-day treatment. D-E. Expression of *Hym-176B*. D. Untreated 5-day control. E. 3-day treatment. F. 5-day treatment. G. 7-day treatment. H. Expression of *HyBMP* in the ectoderm of a lower half of the body of untreated animal. I. Expression of *HyBMP* in the ectoderm of a lower half of the body after 7 day-treatment The arrows show the boundary of activated head and foot regions by LiCl.

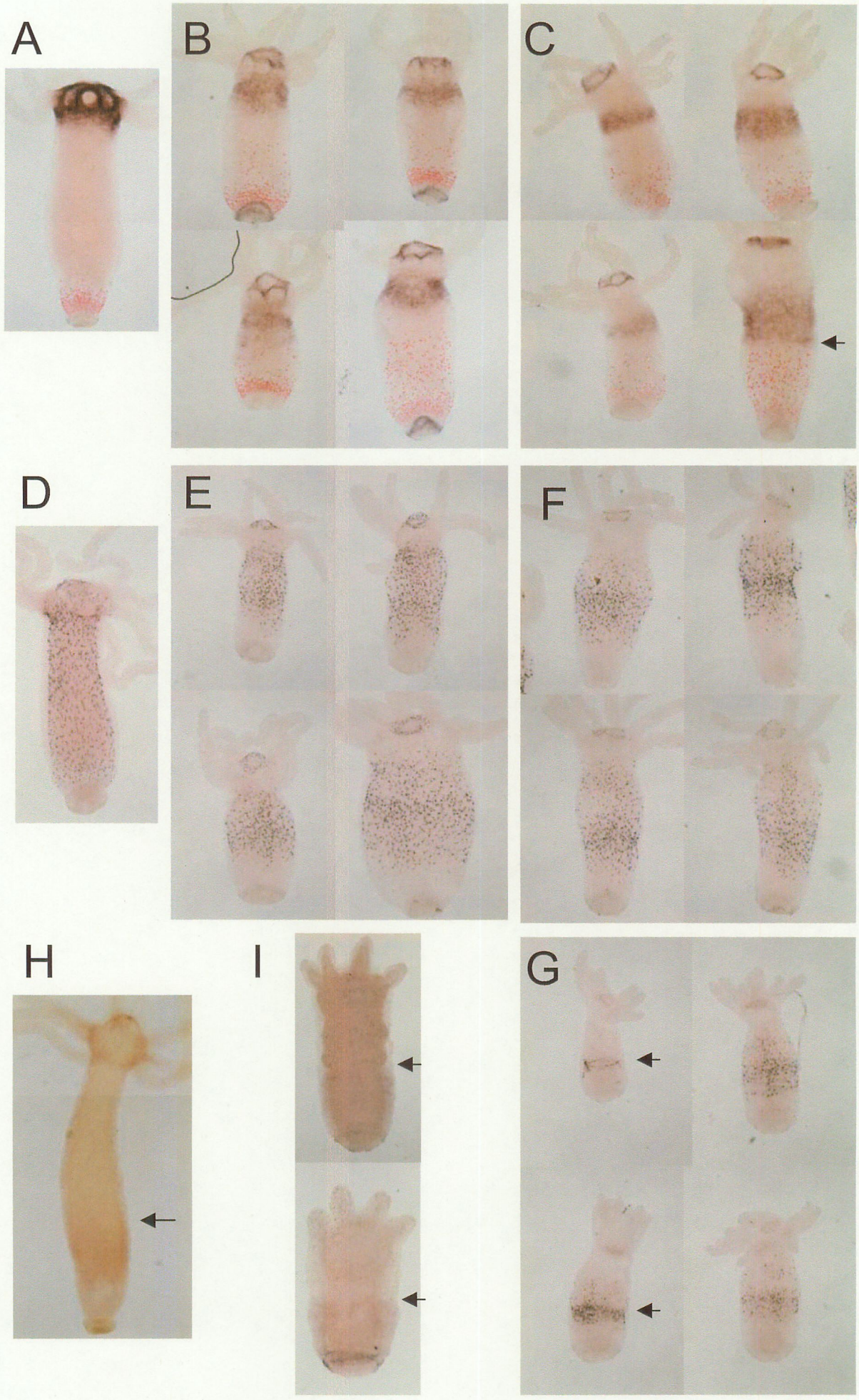

A
B
C
D
E
F
H
I
G

Fig. 14. Whole mount in situ hybridization on adult polyps treated with 2 mM LiCl. A-D. Expression of *Hym-301* (red) and *Hym-176A* (blue). A. Untreated control. B. 3-day treatment. C. 5-day treatment. D. 7-day treatment. E-H. Expression of *Hym-176B*. E. Untreated control. F. 3-day treatment. G. 5-day treatment. H. 7-day treatment. I. Expression of *CnNK-2* in the endoderm of a foot of untreated animal. J. Expression of *CnNK-2* in an animal treated with LiCl for 5 days. The arrows show the upper boundary of the expressed region.

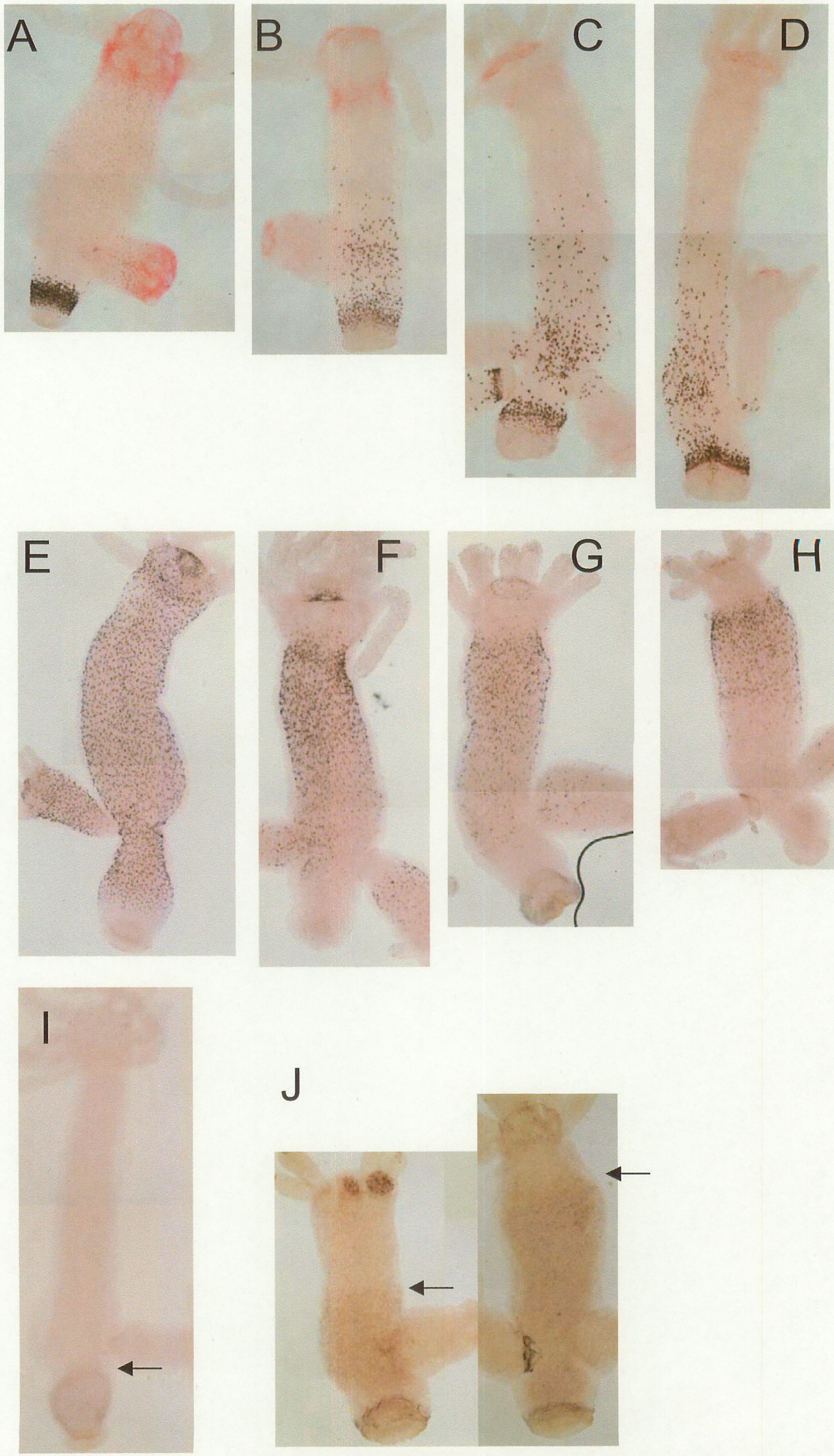
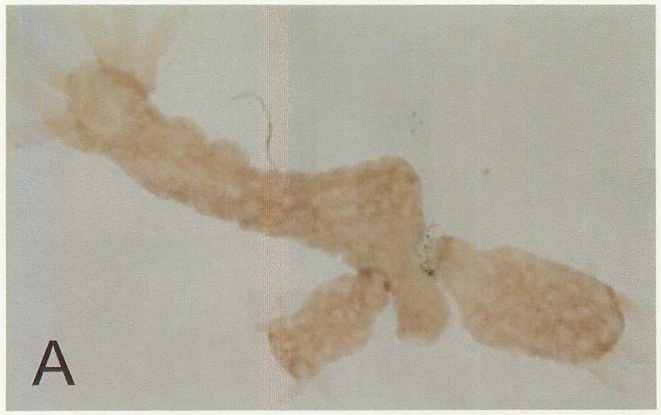
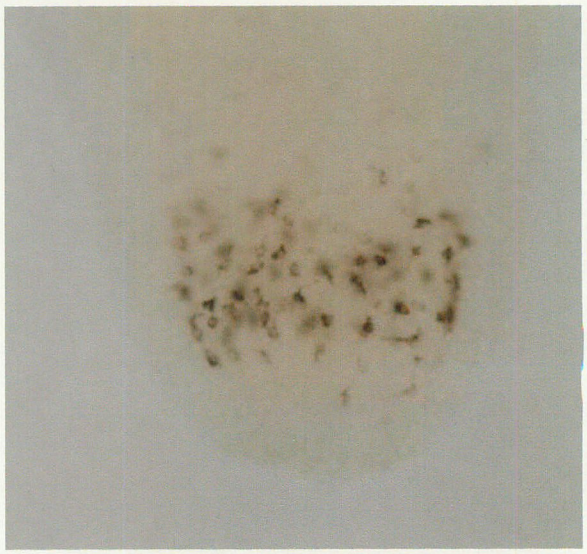

A
B
C
D
E
F
G
H
I
J

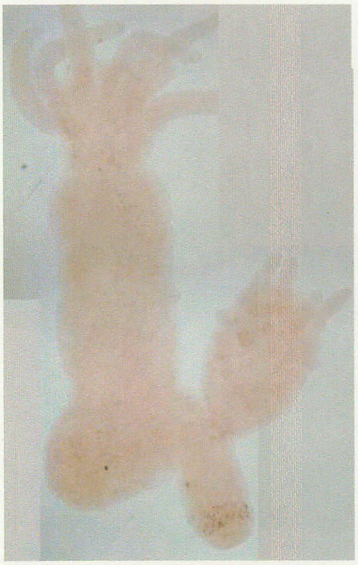
Fig. 15. Effect of ALP on the expression of Hym-176 A,B,E and Hym-301 genes in adult polyps. A. Expression of Hym-301 in a polyp treated with 4 uM ALP for 3 days. White spots throughout the body are tentacle anlage. B. Expression of Hym-176A in polyps treated with 4 uM ALP for 3 days. Right panel is high magnification of left panel. C. Expression of Hym-176B in polyps treated with 4 uM ALP for 3 days. Right panel is high magnification of left panel. D. Expression of Hym-176E in the tentacles of polyps treated with 4 uM ALP for 3 days. E. Higher magnification of tentacles shown in D. F. Expression of Hym-176E in the tentacles of an untreated control polyp.

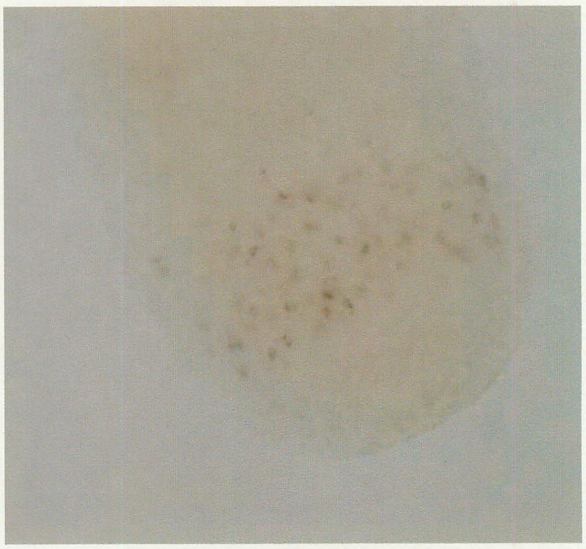


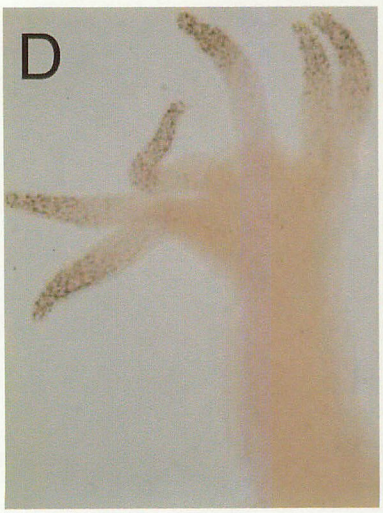
B

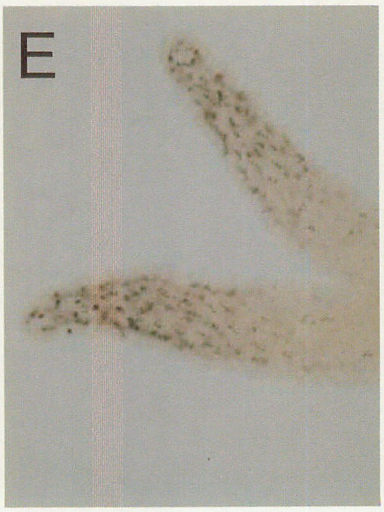


C









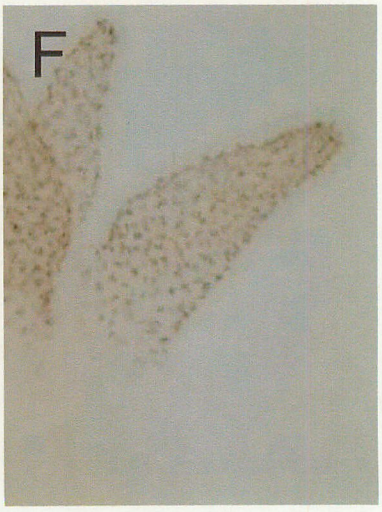
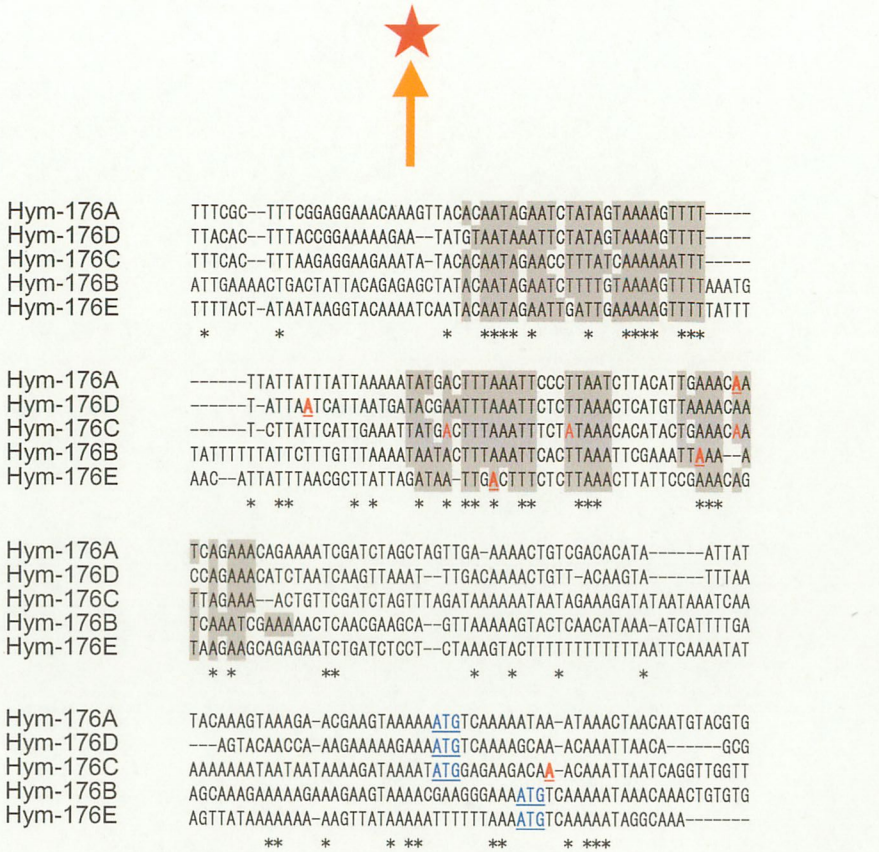


Fig. 16. Alignment of 5'-flanking sequences of Hym-176 paralogues. Sequence motifs shared between genes which are expressed in the same region are shaded in colored box. TATA-like consensus motifs are shaded in gray box. Motifs shaded in red are shared between genes which are expressed in the different region. Transcription start sites determined with the oligo-capping RACE are shown in red. The first ATGs of coding regions are shown in blue..



ATG: translation start

X, X: transcription start (major transcript start underlined)

- motifs shared between genes expressed in lower peduncle
- motifs shared between genes expressed in gastric region
- motifs shared between genes expressed in upper peduncle
- TATA-like consensus
- unattributed motifs


Hym-176A
Hym-176D
Hym-176C
Hym-176B
Hym-176E
TTATTTGCAATCTTTAGTTGATTCTAATGAGCAATATAAAGAGGTGGAATAACTAATTTT
AAGTTAGAAAAATGTAAAATAATTTAGAAAAAAAAAAGTTATATCTTTTCTTATTTAGCAA
ACGTTTAAAACGCATTTTTATGCTTTTCGTTTAAAACGCAAATTGTAGATAACAAAATGT
ATCTTAGTTTAAAAAAAAACAACGTTAAAAACATAGACCAGATAATTGGTTAAAAATTAA
TATTTTTACCGCTAATCTAAA----GTTATTTATGTTTAATAACTCAATTATATTTTAT
--------AATTCACTCAAAA----AGTCTTTAGATCTAA--AATAAAGTTATTTGTTCC
GTTTGTTATTACT--TCCAAA----ATAATACAAATCTATTTTGGTAAAATGTTTGATAA
TTTTTTTTTTTTTTTTTCAGATTTAAAAATATGCGCCTAAAATCTGCACCTATTTCATAA
TTTATCTCAAATCAGTTGAAAAT-AAAAATCTATAAAATAGGTCGAAAAACAAATCGGTC
TACAAAGTGAGC---GTCTTTGTTTGTCGCTACTTTGAATTATCATTTCGTATTCTATTT
CACT--TTTGAA---ATACTAAATTGCA--TGTTTTGAAATACGCAATTACATGTTTTGA
AATAATGTAAAAAAGGAATTAAATTTTT-TTAACTCTTGTTTTTCATTAACTAGAATTTC
AAACTAGCTGGGT--ATATTAAAATTAG--GATTCTGGGTAATTTAGTAACTAAAAATTA
GAAAACGAAAAC---GTCATAAATCGGG-----TGAAAATAAAAAAAGAATATTATCGTT
ATTCTAATGAGGAAAGCCGGTGAGAACATATATT-AATAATGAATTACATTCAGAT-AAA
AGGATTGCACTTAAAGTTAAAAAGTTTAGCTGGT-AGTACTG-------TTTTGAT-GAC
ATTGTGGTGCTTAGCCGCCCTACAACATATTTTCAATACTAAAAGGATATCAGGTGAAA
CCCAGAACCCTTATTTTTTATGTAACCAATTGTTCAAAACTTATGAAAAATTAACCAGAA
CGAAAATAATTTATGTCTTAGTGGGTTATTTGAAAAGTAGTT--CCACTTTTTAGTAGAT
TAACATGTGGGTTTTTGA--AAAAAAAAGTAAA--TT-CTAAACAAAATACAAAAATATT
TCGC-TCCAGAAATCCAA--AAATATAATGATA--CA-GTAATTGGGATAATATGTAATC
CTTTAAATGTGTTAACGA--ATGTAAATTTAGA--TTTGTAAAAAACTTATTGTTTTATT
TCCTTATCTTTAATATAA--TCAAAAGTTTAAAACTTTTGAATTTTTTTACTGGGTTGTT
GTTCATTTTTTAGAACAACTTAGTAGGGCAGGATTTTTGTAGGTCTAACTATCTGACACG
TA----TTT-TTT-ATTAAAGTAACATATTTAAAACA-AGTTTGTATTTATATTCTTGAG
AA----TTT-ACA-AATTTAAAAATATCTCAATAACA-AA-----ACGTG-ACCATCAAA
GA-----CCTCACA-GTTGGGATAGCGGGGTGATGGGACAATATTTCGGTTTTTCACTATC
GGAACTCATTAAA-AATAAAATCAAAAGTTTTAAACAAAAATAAATTGTTTATTTTT-TC
GCTCTACCCACTACGCCACAGCCGCTTGATAATTACATATGTTGTAATAAAATTAATCAG
GTTACGTTGACTCATTTTATTTTAAATCAAACATTTCAAACAATTACTCCCAAAGTTATA
ATTACAATAAG-CATACTTGTATATAAGAAAAA-----GAAAAATTATTTCAAAGATTT--
GTTATATTTAACTATTTCAATTTGTTTCTTTAGACAACACGAAATATT----GGGTTA-A
TTTATAACGAGAGGGACAGTTACATTATATTACTAATTAGACTTTACTT-AAAAATGCAG
TTAAAGTCTAATTAATCTCATCAAAATGAAGAAACCAACACTAATCTCATCAACATGAAG
AGTAAATTGTTTTTCTTTTAAACAATGACATTTTAAC-----CATTATTAACAAATATAA
ATTTTATTCCAAAATCTTTATTTAACTATAGGTAA--------ATGGGAAAAACTTAAAA
ATTGAATCCAATAAGGAAACAGCAACTAAAATTAAAC-----GATGTTCACTCACTTAAA
ACAAAAT--ATAAATTAATAAAAAAAAAAAGTTTA-------CAACACCAGGATACGGAG
GAACCAATACTAGCCTCATCAATAAGAAGGGACAAACGCTTATCTCATAAACATGAAGAA
TTTAA-----ACAAGAAAAGTAGACGGAAGATAAGCAAAAAACAAACAAA-CTG-GAAAC
GTTTA-----AACATAAAAGTAGACGGAAGATGTAGTATACGGAAACAAT-TAATGTTTC
CATTACT---ACTATGACATCTAATTGAAATTGTGCAGAAA--AAACAGT-CTG--AACA
ATTAACC---AATCATAAAAATAATGTAAACCAGATTAATTATCAATAATACTACGACAC
ATTAACACTAATTGCATCAATACGAAGGAACCAGTATTAATCTCATCAATATGAAGAAAC
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AAGTAACATCTCTG-AAT--GGTAGTCAAAAGTA----TTTTTGGTCATAAATATACGAA
CTGTGATCATTTTATACTTTTATATTCATTGTAAAC--GAGTTTATTATATACAATCACA
CAACACTAATCTCA--TCAATATGAAGAAACCAACACTAATCTCATCAAAATGAAGGAAC
AAGTTATAAG----------TAA--GCTCTCATATTAAATTAGTTAGACGTCTAGATGCA
AACTTTTTAA----------TAATAATTTTAAAATAGACAAGCTAGACGTCTAGACGC-
AACTATTTATATCTTC---TTAAACGTTTTTTCCATATAAATAATTAACATGGAAGTTAT
AAGTTGCAATG--------ATAAAAGA----ATAATA-ACAACAACAACAACAAAACCAT
CAGCGCTAATCTCGTAAAAATGAAGGAGCCAACTCTA-ATCTCGTCAGTATGAAGTAAGC
AATTGTAATACTTTATAAAAACAAACCTTAATATCGTGTTCAGAGGCGAGACTGTTTTTT
--TTGTCGCGC---ACAACAACA----CTGATATTGTTGCCACGGATGGGTTTGTTTTTT
ACTATTAAAATTAGAAAGGAACT--TCCTAATCTTGTAGTCACGGTTTGTTTTTTTATTT
ACTGTTAAAGTTAGTAAAGAGTTTGCAATT-CATTAAATTGAGTAACGAGTTAAATATTT
AACATTAATCTATTTTTCAAGTTG--TAAAAGACTAAATCAAAAAATAGCACGATAAAAA
TA-----A--TTTCCTGAGAATCATTTTTTCATTGTGCGTATTTTTATAGGTTG-CCTTAT
TC-----AGTTTCCTTAAAAAAAAATTATATTTTCAAAGCCATTGTATGTGTT--CTTCAC
CCTTGTAAGTTCCTTTTTTA--ATTTTTTAATTTTGCGTATTTGTAGATGTTTACTTTAT
AAACGAGCAAGGCTGCCCACGTGAATACATAAATCACATCACTGGCGAGACTTGTTTTTT
AATAGTTGTTATGTAGCTAGAAAGCTAGATCACACCGGGCATAGGCTATTTTTAATTTAA
TTCCGRKAA>STATx-like
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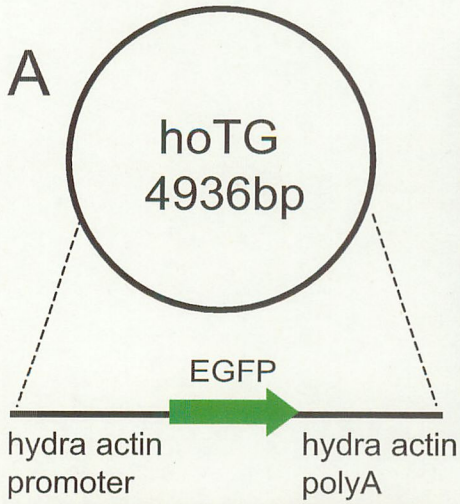
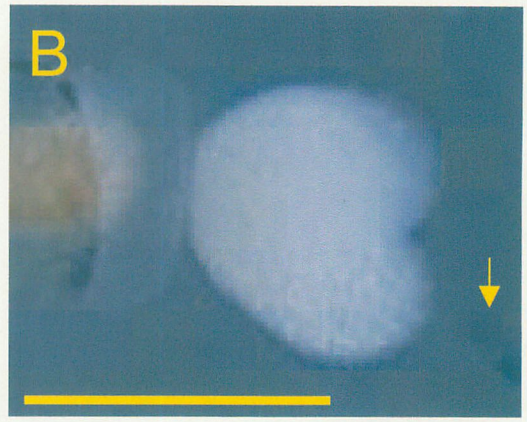
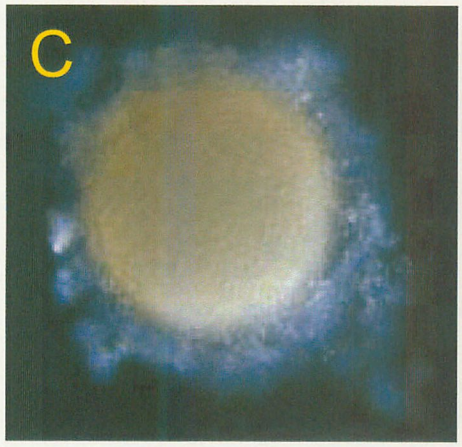
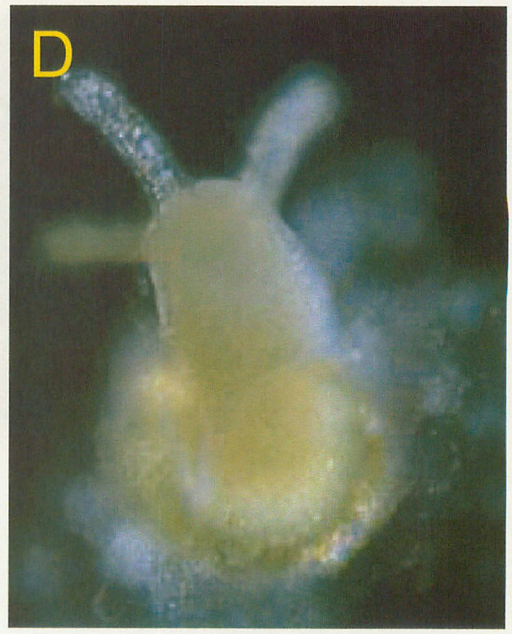
ATACAATGAGAAGAAATTGGTTTTAAAAAGA---GTTCCTTCCTGAAATAATAAATTCTA
AATTTTAAAAAAAAATTAGTTTTTTATTGTAAAGTTTTTCTGTGTTGCCTTTTGAGCACCG
GTAACAAATGGAAAATTGATTTTTATTAATAA-TGTTCTTTCTTGAAACAACAAGTTTTA

Fig. 17. GFP-transgenic Hydra. A. Schematic illustration of a DNA construct used for injection. GFP is under control of Hydra actin promoter. B. Embryos from 1 to 2-cell stage are injected. The embryo was sucked by a pipette and injected with a needle (shown by an arrow). Bar represents 0.5 mm. C. An embryo 2 days after injection. The embryo was surrounded by theca, a thick protective cuticle layer. D. Hatching. The earliest embryo came out three weeks after injection. E. Summarized table of injection. Fourteen percent of hatched embryos turned out to be stable transgenic lines. F. One of 4 founders shown in E. Expression of GFP was stable but in mosaic. G. Summarized table of backcrosses. Each of 4 founders was backcrossed to parental AEP polyps. Two lines were selected from F1 progenies. H. Most of the backcross F1 and F2 progenies expressed GFP stably but in mosaic fashion at the beginning and later all over the body. I,K. Hand made section of F1 progenies which showed GFP expression all over the body. I. GFP expression only in the ectodermal layer. J. GFP expression in one of the 4 founders to show the endodermal expression. K. GFP expression both in the ectodermal and endodermal layers.







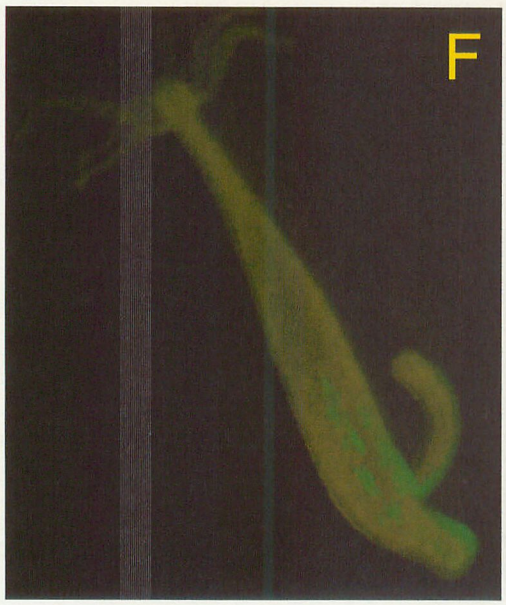


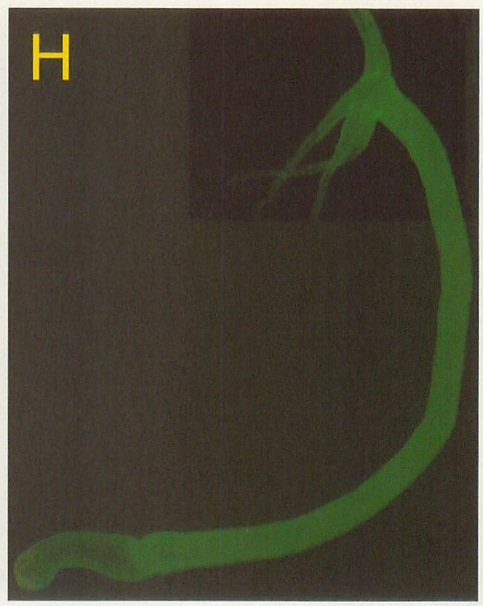
E

Injected	(%)	Hatched	(%)	Expression					
				Stable	(%)	Transient	(%)	None	(%)
81	(100)	28	(35)						
			(100)	4	(14)	7	(25)	17	(61)

G

	Hatched	(%)	Expression			
			+	(%)	−	(%)
Line 1	13	(100)	8	(62)	5	(38)
Line 2	13	(100)	9	(69)	4	(31)





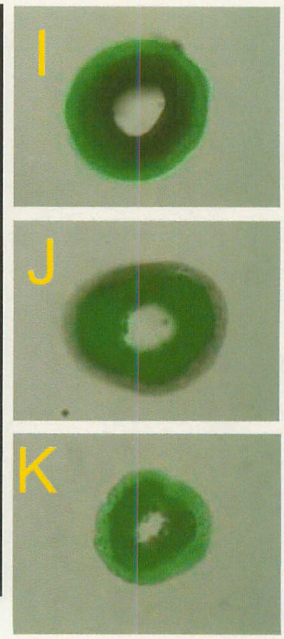
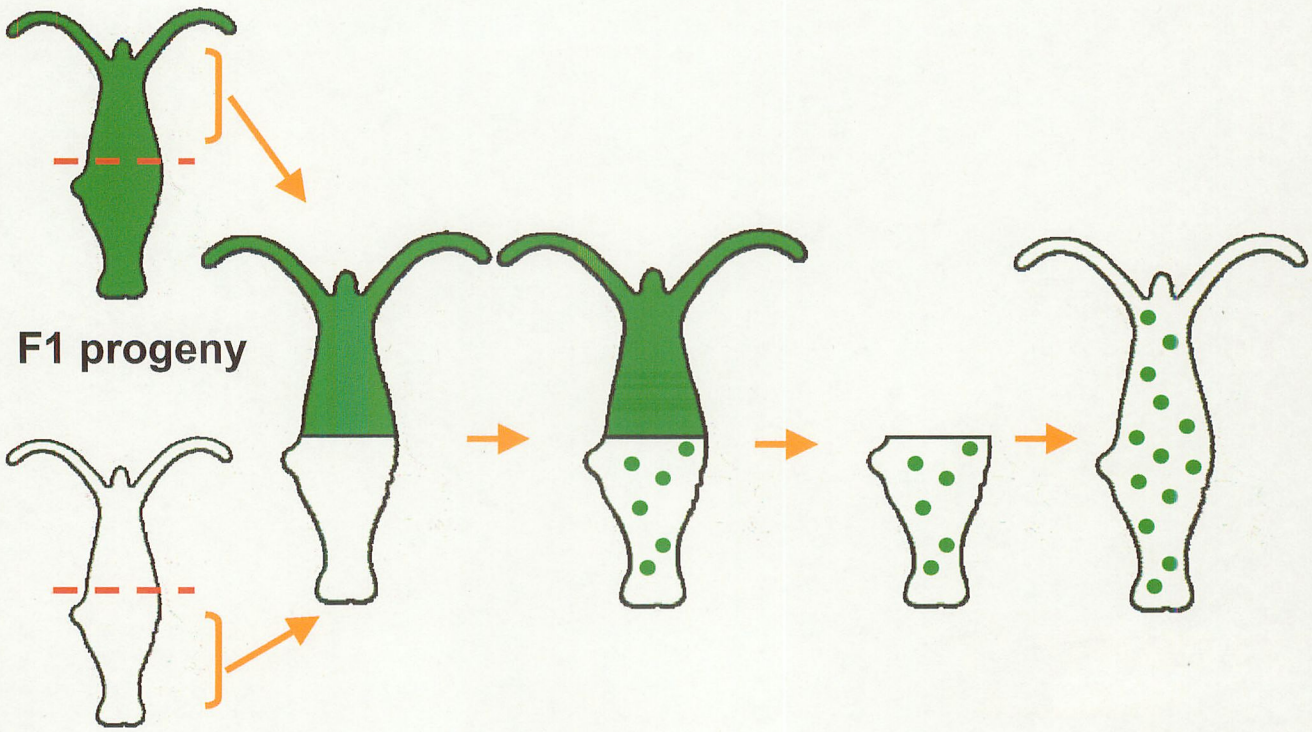


Fig. 18. Analysis of GFP expression in the I-cell lineage cells by grafting. A. Grafting scheme. An upper half of the GFP expressing polyp (F1 progeny) and a lower half of epithelial polyp was grafted. Three days later the lower half was isolated and allowed to regenerate for 5 days. During grafting only cells in the I-cells lineage migrated from the upper part to the epithelial tissue. B. GFP expressing cells in the regenerate. Most of them are neurons and presumably neuron precursors. No large I-cells and nematocytes were detected. Bar, 50 um

A



epithelial *Hydra*

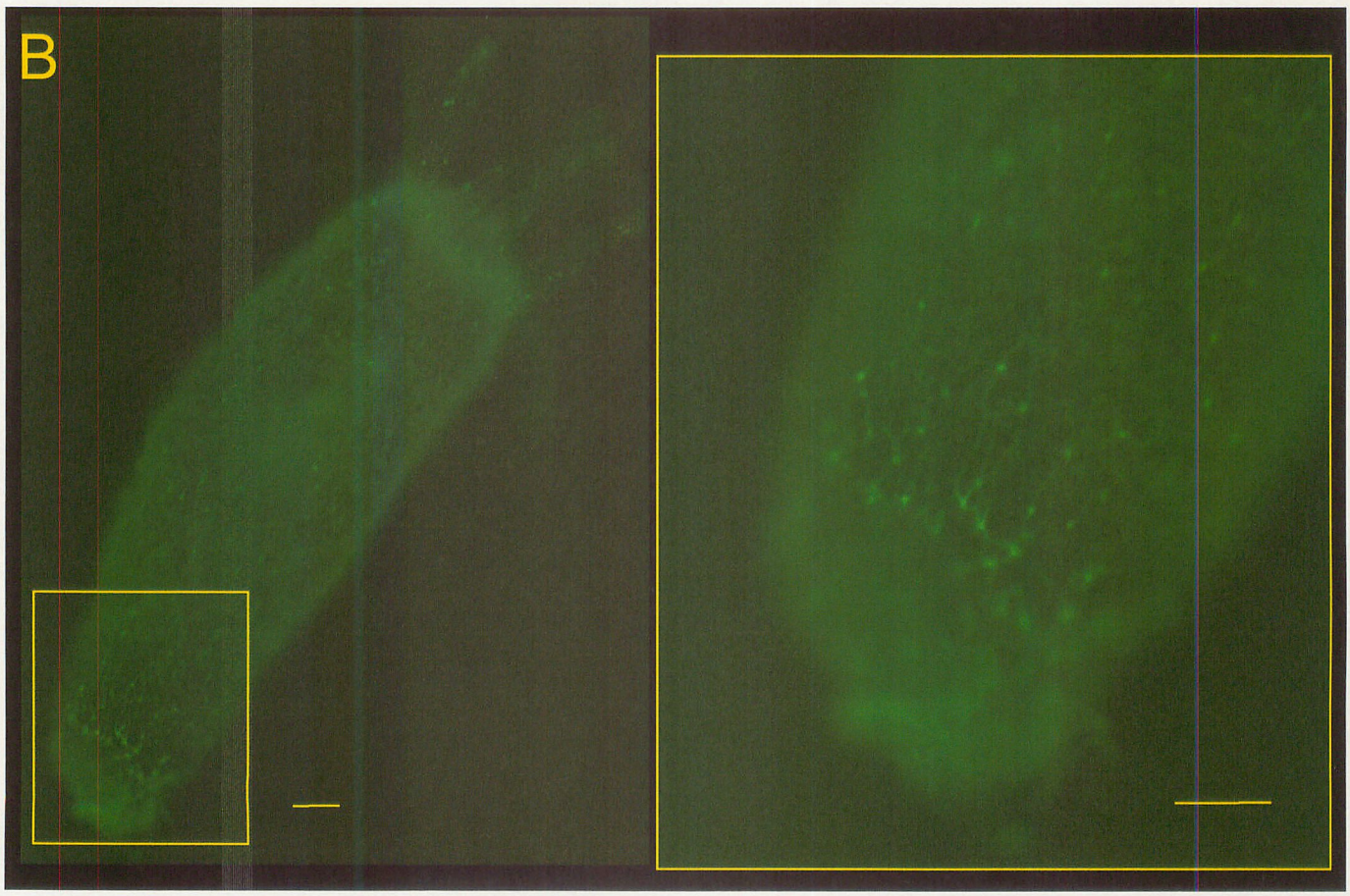
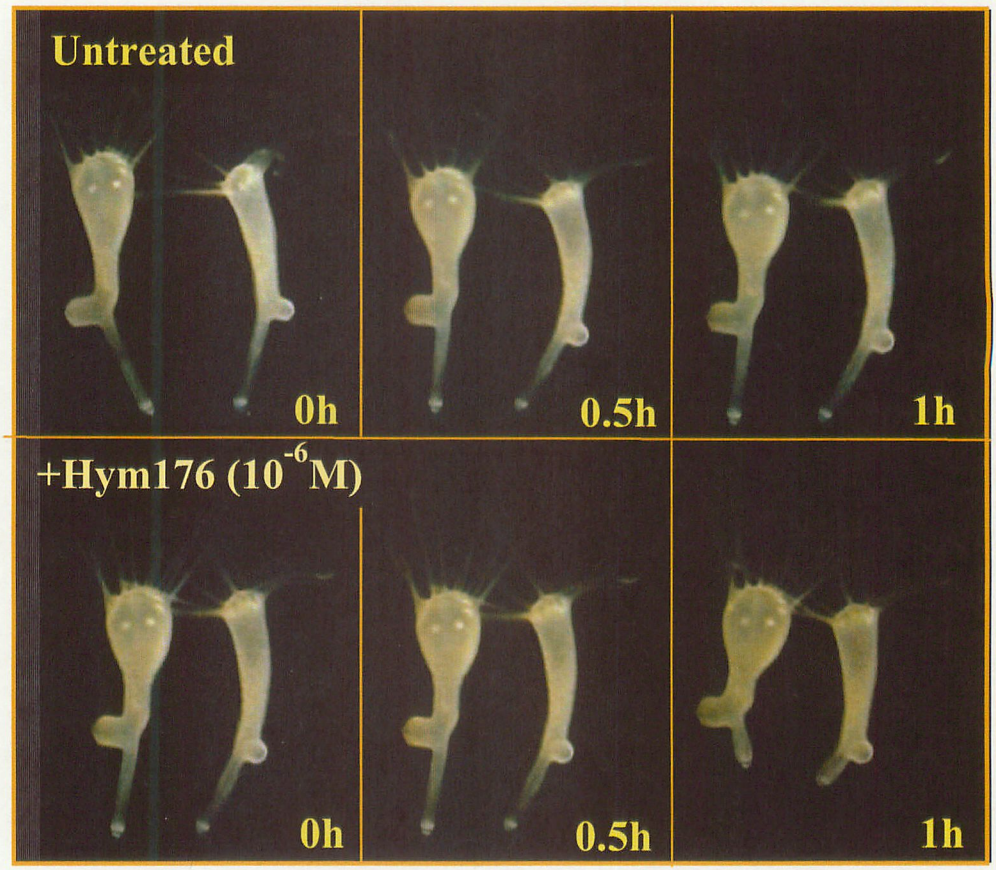


Fig. 19. Effect of a neuropeptide Hym-176 on epithelial polyps. Epithelial *Hydra* were treated with 10^-6 M of Hym-176 peptide for 0, 0.5 and 1hr.


Untreated
0h
0.5h
1h
+Hym176 (10^{-6}M)
0h
0.5h
1h

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