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SHORT COMMUNICATION

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Isolation of an early neural maker gene abundantly expressed in the nervous system of the ascidian, *Halocynthia roretzi*

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Abstract Ascidian tadpole larvae possess a primitive nervous system, which is a prospective prototype of the chordate nervous system. It is composed of relatively few cells but sufficient for complex larval behavior. Here we report on *HrETR-1*, a gene zygotically expressed in a large proportion of the developing neural cells of the ascidian, *Halocynthia roretzi*. *HrETR-1* is an early neural marker which can be used for analyzing neural differentiation. *HrETR-1* expression intensified in most neural cells of genes isolated to date, in both central and peripheral nervous systems including palps as early as the 110-cell stage. Using this gene as a probe, we characterized neural cells in the nervous system as well as confirming their origins. Also, we recognized three types of peripheral epidermal neurons which presumably correlate to the larval neurons previously reported for another ascidian. Among these, five bilateral neurons located in the anterior region of the trunk appeared to be derived from a8.26 blastomeres.

Keywords Ascidian · Neural marker gene · ETR · Central nervous system · Peripheral nervous system

Introduction

Ascidians are one of the lower chordates with the basic chordate body plan. The ascidian larva has a primitive central nervous system (CNS) which develops in a typical manner with dorsal neural tube formation. However, the CNS is, composed of only about 330 cells (Nicol and Meinertzhagen 1991), including 215 in the brain, 50 in the tail ganglion, and 65 ependymal cells of the caudal neural tube. This relative simplicity of organization compared with that of vertebrates has fascinated biologists for more than a century. Nicol and Meinertzhagen (1988a, b) reported cell lineage and development of the CNS by elaborate scanning electron microscopy (SEM) observation. It is promising that analysis of the architecture of the ascidian nervous system using molecular markers will provide the fundamental information necessary to understand development of the nervous systems of higher animals. Along the anteroposterior axis of the embryo, there are several regional differences identified in the ascidian nervous system (Wada et al. 1998). From a cross-sectional view, clonal analysis by Nishida (1987) revealed that the neural tube in the tail region consists of four rows of cells: a dorsal row derived from the b8.19 pair, a ventral row from the A7.4 pair, and two lateral rows mainly from the A8.15 pair and partly from the A8.16 pair. In addition, the single neural tube cell at the tip of the tail is derived from either of the b8.17 pair. As in vertebrates, in which there are known regional differences of gene expression along the dorsoventral axis in a neural tube, there are several papers which describe genes showing regional expression in the ascidian neural tube (Shimauchi et al. 1997). On the other hand, Takamura (1998) investigated a peripheral nervous system (PNS) of ascidian larvae using the monoclonal antibody UA301, showing that three types of peripheral neurons were present, two located in the larval trunk and one in the larval tail.

To study how ascidian nervous systems develop at the cellular and molecular level, it is essential to identify the localities of neural cells at the single-cell level using molecular markers. However,there is no, or only a low number of, neural gene marker(s) which cover all of the neural cells in ascidians, or the markers studied so far are only expressed in a small population of the neural cells. Here we report an early neural marker gene which is expressed in most of the developing neural cells of the genes isolated so far. Using this as a probe, we obtained novel findings on both central and peripheral nervous systems.

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Materials and methods

Animals and embryos

Adult *Halocynthia roretzi* ascidians were obtained during the spawning season from fishermen near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan and Asamushi Marine Biological Station, Tohoku University, Aomori, Japan. Naturally spawned eggs were artificially fertilized with a suspension of non-self sperm. Fertilized eggs were cultured at 12°C. Eggs and embryos were collected at appropriate stages by low-speed centrifugation and fixed for whole-mount in situ hybridization.

cDNAs and sequence analysis

The cDNA was sequenced using an automated DNA sequencer (ABI PRISM 377, Perkin Elmer Japan, Chiba). Homology search for the predicted amino acid sequence was performed using the BLAST network service (NCBI). The amino acid sequence of the gene product was aligned and gaps introduced for maximal similarity.

Whole-mount in situ hybridization

Whole-mount specimens were hybridized in situ at 42°C using digoxigenin-labeled antisense probes, as described previously (Miya et al. 1994). Some specimens were manually sectioned with a fine razor under a light microscope to confirm the localization of hybridization signals in the embryos. To render specimens transparent, they were dehydrated in ethanol and cleared in a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate.

Results and discussion

Early ascidian embryogenesis is characterized by mosaic development in which maternally localized cytoplasmic factors determine the developmental fates of blastomeres. To investigate maternally localized mRNAs, we performed a screen of randomly selected clones from a *Halocynthia roretzi* fertilized egg cDNA library for localized messages by whole-mount in situ hybridization to the 8-cell embryos. Simultaneously, we investigated the spatial expression patterns of zygotic transcription in the 110-cell and early tailbud embryos using the maternal messages as probes, since approximately 90% of zygotically active genes are reportedly also functional in oogenesis in the fruit fly, nematode and sea urchin (Davidson 1986). In this study, we isolated numerous genes which are expressed not only maternally in the egg but also zygotically in the developing nervous systems of the tailbud embryo. Among them, some genes were found to be expressed in subsets of the nervous systems, which can serve as useful molecular markers to categorize and characterize the neural cells in ascidian embryos.

In addition, in the ascidian tadpole larvae, several neural structures, including palps (adhesive organs) and the dorsal and ventral epidermal neurons along fins, are formed from the surface of the body which do not express epidermis-specific genes (Ishida et al. 1996). Here we report a gene also expressed in the areas generating those neural organs.

In this paper, we have adopted the following terminology concerning the CNS of the ascidian tadpole: brain, which is derived from a-line blastomeres; brain stem, which is derived from A- and b-line blastomeres and composed of posterior sensory vesicle and visceral ganglion; and finally caudal nerve cord of the tail, which is derived from A- and b-line blastomeres.

HrETR-1 (accession no. AB047035) cDNA is 3,933 bp long and encodes a 594 amino acid peptide which has three RNA-recognition motifs (RRMs). These regions of the deduced protein showed a remarkably high similarity especially to vertebrate ETR-1 proteins (Fig. 1). ETR-1 is an ELAV-class RNA-binding protein which was first isolated as a neural specific gene from *Xenopus* and is known to play roles in neural development (Knecht et al. 1995). In the early tailbud embryo, *HrETR-1* was found to be widely expressed in both central and peripheral nervous systems (Fig. 2). These sites included presumptive palps and the surrounding neurons which give rise to rostral trunk epidermal neurons (RTEN) reported by Takamura (1998), brain, brain stem, caudal nerve cord including a few pairs of motor neurons which are derived from A5.2 cells (namely, A8.15) and constitute the several lateral cells of the anteriormost nerve cord (Okada et al. 1997), and peripheral epidermal neurons. The peripheral neurons in the tail which express this gene were detected as a cluster of cells in the most proximal region of the tail, some bilateral pairs on the dorsal midline in the caudal region of the tail, and two sites on the ventral midline. The peripheral neurons located in the most proximal region of the tail on the dorsal midline were also *HrCA1*-positive (Araki et al. 1996). Observation of the staining pattern from the dorsal view suggested that the expression was only present in the lateral row of ependymal cells in the caudal nerve cord and was completely absent from the dorsal and ventral cells. This was also confirmed by cross-sectioning of the tail (Fig. 2G). The expression pattern is complimentary to that of *HrWnt-7* which is the first report showing a dorsal and ventral ependymal cell-specific expression pattern (Sasakura and Makabe 2000).

Since *HrETR-1* is expressed in most developing neural cells of genes isolated so far, we examined the detailed expression pattern during embryogenesis. Maternally derived mRNA appeared not to be abundant but was ubiquitously present in the egg and early embryos (Fig. 3A–C). At the early 24-cell stage when only A5.1, A5.2 and B5.1 had started to divide, the zygotic signals were initially detected in all animal blastomores, i.e. in a5.3, a5.4, b5.3 and b5.4 pairs (Fig. 3D). This expression was transient and disappeared from b-line blastomeres first followed by a-line blastomeres. At the late 32-cell stage, the signals were again detected in all animal blastomeres suggesting that this expression is perhaps regulated in a cell-cycle related manner. Although the signals were observed in the animal hemisphere of the 44-cell embryo (data not shown), we did not detect any nuclear

Fig. 1 A comparison of amino acid sequences. The deduced amino acid sequence of *Halocynthia roretzi ETR-1* was compared with those of vertebrate proteins such as *Xenopus* V16800 and zebrafish AB032725, which showed the highest degree of similarity to HrETR-1 in a BLAST search. Identical residues are enclosed by *black boxes* and three RNA-recognition motifs (RRMs) are *boxed by rectangles*. For maximal similarity, *gaps* were introduced

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ascidian      1  MVMAMHGATQVLQHTLTSGGGIGGCGAPSTSLGFQVINGVVPMAANAHIPMDASLLPFGT   60

ascidian     61  GFVQPNPPTSTHYITTTQAQPQLCSTPSNVSMKEFQDKDDDAVKLFIGQVPKNWTEHELR  120
Xenopus       1  ------------------------------MKE-----PDAIKLFIGQIPRNLDEKDLK   24
zebrafish     1  ------------------------------MKE-----ADAIKLFIGQIPRNLEEKDLK   24

ascidian    121  PIFEPYGEIYELSVLHDKYTGMHKGCAFLTYCKKTPAINAQNFLHEQKTLPGMNHPMQVK  180
Xenopus      25  PIFEQFGKIYELTVIKDKFTGMHKGCAFLTYCARESALKAQSALHEQKTLPGMNRPIQVK   84
zebrafish    25  PIFEQFGKIYELTVIKDKYTGMHKGCAFLTYCARESALKAQNALHEQKTLPGMNRPIQVK   84

ascidian    181  PADTVNKGEDRKLFVGMLGKRQTEEDIRQLFEKFGHIEECTILRTPDGQSKGCSFVKLST  240
Xenopus      85  PADSESRGEDRKLFVGMLGKQQTDEDVRRMFEPFGNIDECTVLRGPDGTSKGCAFVKFQT  144
zebrafish    85  PADSEGRG-DRKLFVGMLGKQLSDADVRKMFEPFGSIEECTVLRGPDGASKGCAFVKYQS  143

ascidian    241  STGARAAIDALHGSQTMPGASSSIVVKLADTDKERAIRKMQQMAQNYGLVSPVTLQLGPV  300
Xenopus     145  HTEAQAAINALHGSRTLPGASSSLVVKFADTEKERGLRRMQQVANQLGMFSPIALQFGAY  204
zebrafish   144  NAEAQAAISALHGSRTLPGASSSLVVKFADTEKERGIRRMQQVASQLGVISPMNLHLGAY  203

ascidian    301  GSPLPQMAQHPVM-ATTPGVMPAAAGWS-PLATAFGTATQLGQMTNLGPASAIMQATNGH  358
Xenopus     205  SAV-TQAVSDQLMQQQAALV--AAHSAYLNPMATMAAV--QMQQMATINPNGII--ATP--  256
zebrafish   204  NAY-TQA----LVQQQA-LV--AQSAYLSPVATVAAV--QMQQLAALNPSSII--ATP--  249

ascidian    359  GAQIAGI-PSTPQSPVASITTLNLVQPPMASQSGMTTPQEIYPLQAVPGTRLAAPVVTTT  417
Xenopus     257  ---ITQINPITSSS-GTS--TP-----PTLTATQVSAIPATL-GVNGVSAV----PTQSTV  301
zebrafish   250  ---IASITP---SS-GTS--TP----PTIAATPVPALPPPI-AVNSYPPV----PAAPNG  291

ascidian    418  HSMTPSLAVHNGSSSDPSPMTLCASQTPPTM-EMIQAPAYPQ-P-YTVVYVPPGQVAPVP  474
Xenopus     302  QPSSEAIYT-NGLH-----PYP--A-QSPVAQLDPLQQ-AYAGMQHYTAAY-P-AAYGLVS  350
zebrafish   292  QSASEALYT-NGVH-----PYQ--A-QSP-A-LDPLQQ-AYTGMQHYTATY-P-AAYGLVG  338

ascidian    475  QQLTPTHLTPITTTQGAPALVNSPTAPQKEGPEGCNLFIYHLPQEFTDADLANVFQPFGN  534
Xenopus     351  PAFTQP---PAIIQQQ-PPQQQQ-----QREGPEGCNIFIYHLPQEFTDSEILQMFLPFGN  402
zebrafish   339  QPFPHQ---PTLVAQQ-PQQPQQLQ--QREGPEGCNIFIYHLPQEFTDSEMLQMFLPFGN  392

ascidian    535  VISAKVFIDRATNQSKCFGFVSYDNPVSAQTAIQLMNGFQIGMKRLKVQLKRPKEQSRPY  594
Xenopus     403  VISAKVFVDRATNQSKCFGFVSFDNPGSAQAAIQAMNGFQIGMKRLKVQLKRPKDANRPY  462
zebrafish   393  VISAKVFVDRATNQSKCFGFVSFDNPASAQAAIQAMNGFQIGMKRLKVQLKRPKDANRPY  452
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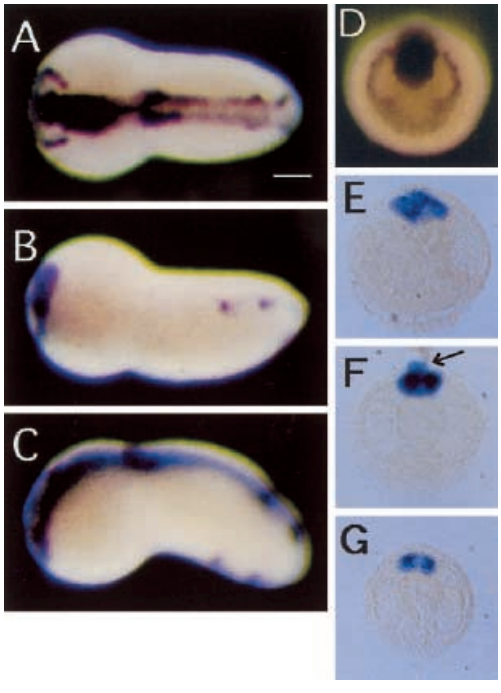

Fig. 2A–G Expression of *HrETR-1* genes at the early tailbud stage. Expression of each gene was detected by whole-mount in situ hybridization under the same conditions: **A** dorsal view, **B** ventral view, **C** lateral view, **D** frontal view. **E**–**G** A cross-section of an early tailbud embryo, though trunk (**E**), the most proximal area (**F**) and middle (**G**) of the tail. Hybridization signals of *HrETR-1* were detected in the whole of the brain (**E**) and lateral rows of the caudal neural tube (**F,G**). An *arrow* indicates a cluster of epidermal neurons. *Scale bar* 50 μm

signal in the 64-cell embryo (Fig. 3E). Since hybridization signals of zygotic transcripts always initially appear in the nuclei in *H. roretzi*, this suggests that the gene may temporarily be inactive during this period. In the 76-cell embryo, the signals were again detected in the nuclei of a7.9, a7.10, a7.13, b7.9 and b7.10 which contain neural fates of all the embryos examined (Fig. 3F), while some embryos showed the signals in blastomeres which give rise to epidermis, although the stained regions varied from embryo to embryo. Together with the finding that the stained cells were close to each other in each embryo, this transient expression in epidermal blastomeres may be regulated in a cell-cycle related manner. Until this stage, no signals were detected in any vegetal blastomeres, despite some containing neural fates.

From the 110-cell stage onward, zygotic transcription was restricted to precursor cells of the central and peripheral nervous system including palps and RTEN. In the 110-cell embryo, A8.7, A8.8 and A8.15 started to express the gene as well as a8.17, a8.19 and a8.25, fates of which are restricted to neural lineages (Fig. 3G,G′). It should be noted that blastomeres still containing fates other than neural lineages, such as A8.16 (muscle), b8.17 (endodermal strand and muscle) and B8.19 (muscle), did not express this gene.

Shortly after the onset of gastrulation, a8.18, a8.20 and a8.26 at the 118-cell stage (Fig. 3H), and b8.17 descendants at the very early gastrula stage started to express the gene (Fig. 3I). We found a pair of positive cells oblique to the blastopore, which appeared to be b9.49 and b9.50. At about the gastrula stage, the signals were detected in all neural lineage cells except for b8.19 de-

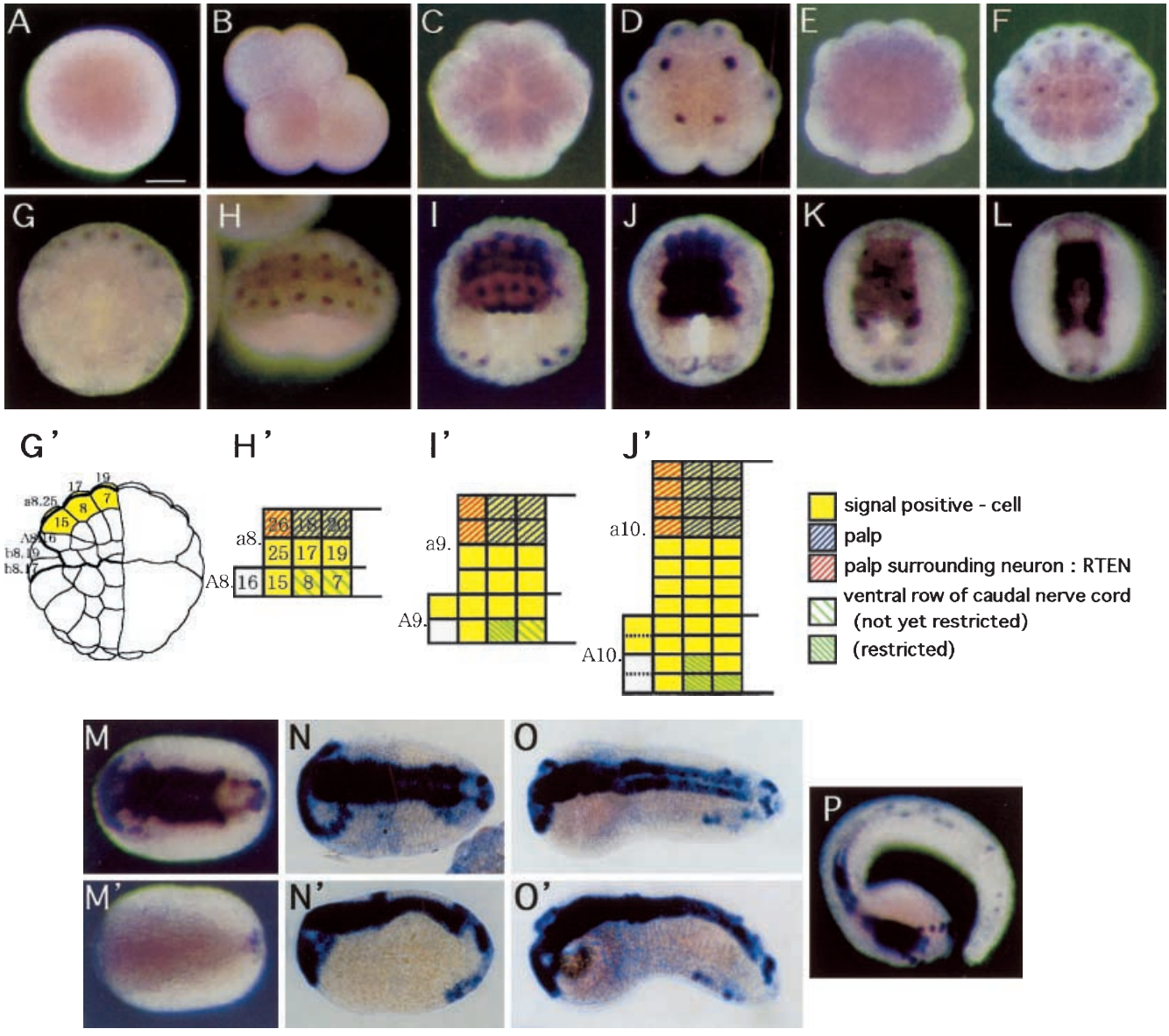

Fig. 3A–P Temporal and spatial expression of *HrETR-1*, as revealed by whole-mount in situ hybridization. **A** A fertilized egg after ooplasmic segregation. **B** An 8-cell embryo, lateral view. **C** A 16-, **D** an early 24-, **E** a 64- and **F** a 76-cell embryo, animal view. **G,G′** A 110-cell embryo and its diagram, vegetal view. Stained cells were colored in *yellow* in the left half embryo. The names of blastomeres that contain neural fates are indicated. **H** A 118-cell embryo, anterior view, the animal pole is to the *top*. **I** A middle gastrula embryo, dorsal view. **J** A neural plate stage embryo, dorsal view. **H′–J′** Left half of the presumptive neural plate cells in **H–J** schematically illustrated and colored as captioned in the figure. **K** An early neurula embryo, dorsal view. **L** A middle neurula embryo, dorsal view. Signals in cells around the blastopore that give rise to the ventral row of the ependymal cells became weak. **M,M′** A late neurula embryo, dorsal (**M**) and ventral (**M′**) view. **N,N′** An initial tailbud embryo, dorsal (**N**) and lateral (**N′**) view. **O,O′** An early taibud embryo, dorsal (**O**) and lateral (**O′**) view. **P** A mid tailbud embryo. *Scale bar* 50 μm

scendants. This lack of staining corresponds to our observation that the dorsal row of the tail nervous system including brain stem and the caudal nerve cord, which are derived from b8.19, did not express this gene. Interestingly, a8.26, from which only epidermis has been proposed to be differentiated, also showed the signal. Kobayashi et al. (1999) showed that *Hrsmad1/5*, which is expressed in an epidermis-restricted cell-specific manner, is not necessarily detected in a8.26 at the 110-cell stage in all embryos. Together with staining observation of *HrETR-1* in later stages, RTEN seemed to be derived from a8.26. At the neural plate stage, several cells showed weak signals in the posterior region of the embryo (Fig. 3J). These cells appeared to include a few descendants of b9.49 and b9.50, and some epidermal lineage cells. Nishida (1987) described two unidentified cells in the caudal tip region, which belong to neither a nerve cord nor epidermis that are derived from b7.13. It is likely that the stained cells in Fig. 3I are the precursors

of two bilateral pairs of neurons that locate at the tip of the tail and derive from b7.13. The other positive cells may give rise to the dorsal and ventral peripheral neurons in the caudal region.

From the gastrula to neurula stage, expression was detected exclusively in the presumptive CNS and PNS including palps (Fig. 3J–M). The signals were not restricted to the nuclei but were also noted in the cytoplasm. As neurulation progressed, intensive staining in the anterior part including the presumptive palps, RTEN and the brain was seen. At the same time, expression gradually disappeared from the ventral row of ependymal cells in the nerve cord. Finally, in the initial and early tailbud embryo (Fig. 3N,N′ and O,O′, respectively), there was intense staining of the CNS as well as the PNS, presumptive palps and RTEN as a ring-shaped region in the anteriormost part of the embryo (Fig. 2D), the brain, brain stem, and the caudal nerve cord - except for the dorsal and ventral rows of ependymal cells extending to the caudal area between the anterior and posterior peripheral neurons along the dorsal midline. After the mid tailbud stage, the signals became unclear in the caudal neural tube (Fig. 3P). This suggested that *HrETR-1* is activated transiently in most neural cells, and thereafter is down-regulated in the non-neuronal neural region.

This gene is expected to be useful as a good early neural marker, because its expression after the 110-cell stage is limited to the central and peripheral nervous systems including the palps, and the expression area is the largest and stongest of reported neural genes so far. This is the first report of a gene which can be used practically for monitoring neural tube differentiation in embryological experiments (Minokawa et al., in preparation).

Here we demonstrated that four rows of ependymal cells in the nerve cord differ in their gene expression pattern. *HrETR-1* was expressed in the lateral cells, while *HrWnt-7* is expressed in a dorsal and ventral ependymal cell-specific manner. Shimauchi et al (1997) described *Hrfkh* as being expressed in the ventral ependymal cells. These results clearly suggested that there are at least three kinds of cells in the nerve cord, which are different at the molecular level.

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