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during Ascidian Embryogenesis

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The ascidian embryo, a model for the primitive mode of chordate development, rapidly forms a dorsal nervous system which consists of a small number of neurons. Here, we have characterized the transcriptional regulation of an ascidian *synaptotagmin* (*syt*) gene to explore the molecular mechanisms underlying development of synaptic transmission. *In situ* hybridization showed that *syt* is expressed in all neurons described in previous studies and transiently in the embryonic epidermis. Neuronal expression of *syt* requires induction from the vegetal side of the embryo, whereas epidermal expression occurs autonomously in isolated ectodermal blastomeres. Introduction of green fluorescent protein reporter gene constructs into the ascidian embryos indicates that a genomic fragment of the 3.4-kb 5′ upstream region contains promoter elements of *syt* gene. Deletion analysis of the promoter suggests that *syt* expression in neurons and in the embryonic epidermis depends on distinct *cis*-regulatory regions. © 2002 Elsevier Science (USA)

Key Words: **neuronal gene expression; synaptotagmin promoter; ascidian; neural induction; motor neuron; GFP; epidermis.**

INTRODUCTION

Recently, there has been a rapid surge in studies of the molecular mechanisms of neural development. However, most studies have focused on early neuronal development, while the mechanisms that establish physiological properties of neurons, such as electrical excitability and synaptic transmission are largely unknown. Previous studies suggest that the transcriptional regulation of genes responsible for these neuronal functions is complex in vertebrates (Chong *et al.,* 1995; Weber and Skene, 1997; Myers *et al.,* 1998; Goodman and Mandel, 1998; Heicklen-Klein and Ginzburg, 2000).

Ascidians are marine invertebrates that form a sister phylogenetic group to the vertebrates. Their larval central nervous system consists of a small number of cells (about 100 neurons in *Ciona intestinalis*) (Nicol and Meinertzhagen, 1991; Meinertzhagen and Okamura, 2001), and it is generated by folding of the dorsal ectoderm of the gastrula embryo. Expression of neural phenotypes in the animal blastomeres of the ascidian embryo requires interaction from vegetal blastomeres, suggesting conservation of the neural induction that has been documented in vertebrate embryos (Nishida, 1991; Okado and Takahashi, 1988, 1990b). Many molecular mechanisms of neural patterning appear to be conserved between ascidians and vertebrates, such as the expression patterns of homeobox genes along the anterior–posterior and dorsal–ventral axes (Corbo *et al.,* 1997a; Katsuyama *et al.,* 1996). The small number of cells

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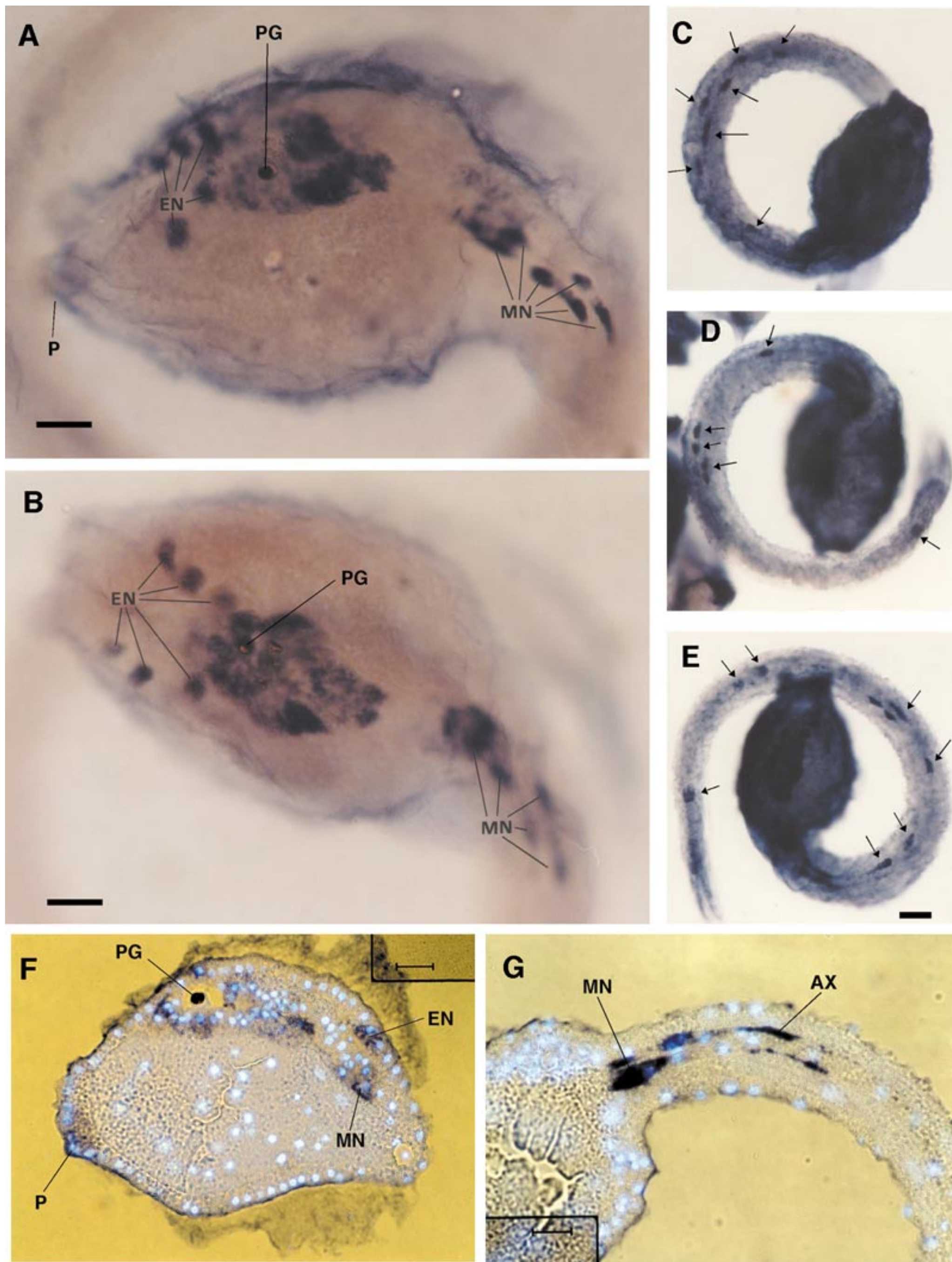

FIG. 1. *syt* gene expression in the *Halocynthia* larva examined by whole-mount *in situ* hybridization. (A) Lateral view. (B) Dorsal view. Nonspecific staining is observed weakly in the tunic. (C–E) Variable locations of the signals associated with epidermal sensory neurons in the tail. Prolonged staining caused nonspecific staining of the tunic. (F) Longitudinal section of the trunk region of a larva processed by *in situ* hybridization with a syt probe. (G) Horizontal section of the region at the junction of trunk and tail in a larva. Nuclei were counterstained by DAPI in (F) and (G). AX, axon of motor neuron; BR, brain; EN, epidermal sensory neurons; MN, motor neurons; P, papilla; PG, pigmented cells. Bar, 25 μm.

constituting the larval ascidian nervous system and rapid time course of its larval development provide advantages for studying these mechanisms in a simple context. Furthermore, there are now established techniques for microinjection (Hikosaka *et al.,* 1992) and blastomere manipulations (Nishida, 1991; Okado and Takahashi, 1988) that

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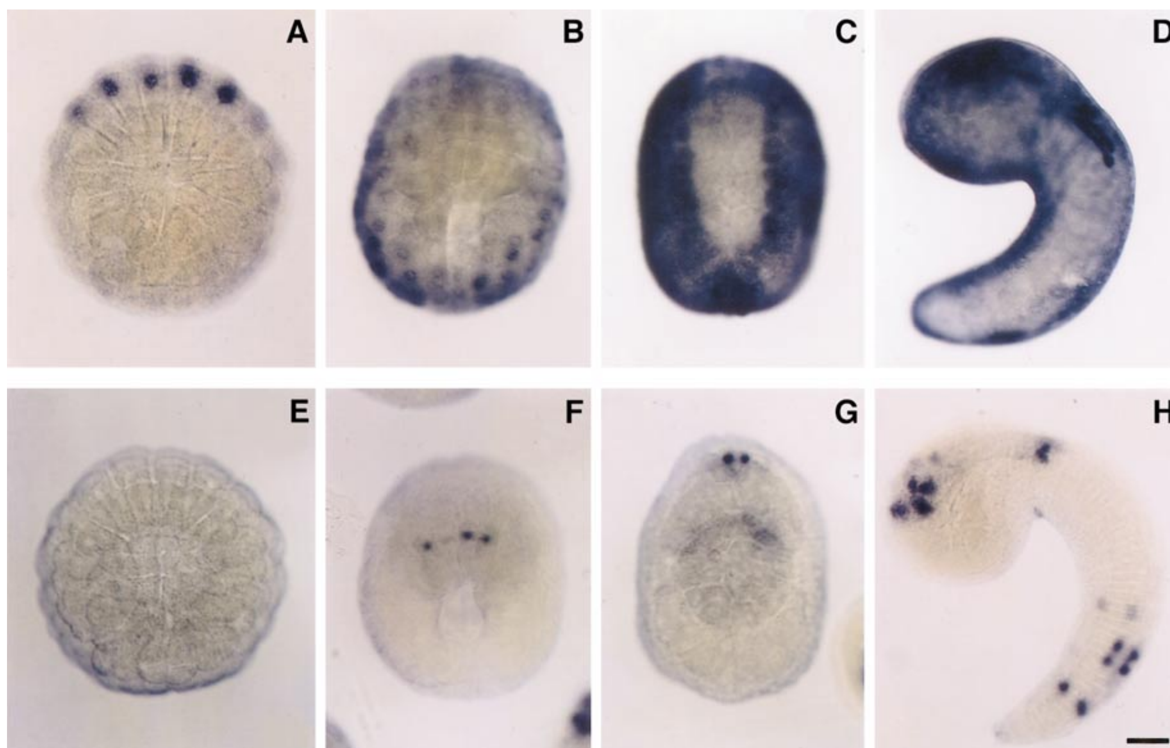

FIG. 2. Expression pattern of *syt* (A–D) and *TuNa1* (E–H) genes during ascidian embryogenesis. (A, E) 110-cell stage. (B, F) Neural plate stage. (C, G) Neurula stage. (D, H) Tailbud stage. (D) and (H) show lateral views, and the others show dorsal views. Anterior is to the top in all pictures. Bar, 25 μm.

facilitate studying gene regulation of chordate-type neural development using ascidian embryos as a model system.

We have focused on voltage-gated channels, which are essential molecular components of neuronal electrical activities, and characterized their expression patterns in ascidians (Nagahora *et al.,* 2000; Okada *et al.,* 1997; Okagaki *et al.,* 2001; Okamura *et al.,* 1994; Ono *et al.,* 1999). *TuNa1*, an ascidian sodium channel gene, is expressed in several types of neurons (Okada *et al.,* 1997), but at low levels, making it difficult to study detailed mechanisms underlying its neuronal gene expression. Other expressed ion channel genes do not necessarily serve as reliable neuronal markers, since they are also expressed in nonneuronal cells such as mesenchyme (Ono *et al.,* 1999) or muscle cells (Okagaki *et al.,* 2001). On the other hand, synaptic transmission is a functional property unique to neurons. Synaptotagmin (syt) is a key protein for Ca^{2+}-regulated fusion of synaptic vesicles at synapses. It is tethered to surfaces of synaptic vesicles and regulates vesicle cycling by sensing calcium ions at presynaptic membranes (reviewed in Fernández-Chacón *et al.,* 2001; Südhof and Rizo, 1996). Analyses of fly, nematode, and mouse mutants indicate that syt function is essential for synaptic transmission (Geppert *et al.,* 1994; Littleton *et al.,* 1993a; Nonet *et al.,* 1993). *syt* genes are expressed specifically in neurons in invertebrate embryos (Littleton *et al.,* 1993b; Nonet *et al.,* 1993). Thus, *syt* may serve as a unique neuronal marker to understand morphology and development of the ascidian nervous system.

Here, we report cloning, expression pattern, and transcriptional regulation of the *syt* gene of the ascidian *Halocynthia roretzi*. *Syt* is expressed in all types of neurons that have been previously described in ascidian swimming larvae. In addition, it exhibits transient expression in the embryonic epidermis. Although early expression patterns of *syt* are different from that of another neuronal marker, a sodium channel gene, both require signals from the vegetal blastomere for their expression in neurons. However, *syt* expression does not need induction for epidermal expression. The 3.4-kb upstream genomic sequence of *syt* activates transcription of a reporter gene, recapitulating its endogenous expression pattern. Analysis of deleted constructs of this promoter sequence suggests that distinct molecular mechanisms are responsible for *syt* expression in larval neurons and embryonic epidermal expression.

MATERIALS AND METHODS

Cloning of Ascidian Synaptotagmin

A partial fragment of *syt* cDNA was amplified by RT-PCR using larval RNA of the ascidian *H. roretzi* and the degenerate primers

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5′-AARAARATGGAYGTNGGNGG-3′ and 5′-GTRTGCCAYTG-NGCDATNGG-3′. A PCR product of the expected size was subcloned, sequenced, and used as a probe to screen an ascidian larval λZAP cDNA library. Five candidate positive clones were completely sequenced. Four of five candidate clones have basically identical sequences with subtle differences at the 5′ end (see Results).

RNA Detection

Northern hybridization, *in situ* hybridization, and RT-PCR were carried out as previously described (Nagahora *et al.,* 2000; Ono *et al.,* 1999; Wada *et al.,* 1995). Primers used for RT-PCR were as follows: for *syt*, 5′-ATTGGCTGAAGTGTGTGCC-3′ and 5′-CC-TTGACTATGATCGCATGG-3′; for *HrEpiB*, 5′-CCGTTACTG-GTATGTGCACG-3′ and 5′-ACAGTGGCAAGACGAAGTCC-3′; for *HrEpiD*, 5′-GCAGCAGATGGTTATGAGAGG-3′ and 5′-TT-ACTGCGAGCAGAATTCCG-3′; and for rRNA, 5′-TCAATCCT-ACCTGTGTCCGG-3′ and 5′-CGTTACCATGACGACCTTCC-3′, and *TuNa1* primers as previously described (Okamura *et al.,* 1994). For sectioning of *in situ* hybridization specimen, stained embryos were dehydrated in ethanol and embedded in polyester wax (BDH Laboratory Supplies). Serial sections were cut 5 μm thick and mounted on slides. After removal of the polyester wax with absolute ethanol, the specimens were mounted in 75% glycerol in PBS.

Plasmid Construction

Syt cDNA was used as a probe to screen a *Halocynthia* genomic library (EMBL3), and nine candidate clones containing *syt* sequences were obtained. The 5′ upstream region of the *syt* locus was amplified by PCR using primers Syt5′-forward (5′-GAACC-TTCGGTAGCAGATTGTGC-3′) and Syt5′-reverse (5′-ATGC-CATGGTGTCCGACTTATGC-3′), and candidate genomic clones as templates. The longest PCR product (approx. 3.7 kb, including vector arm sequence) was subcloned into a pCR2.1 TA cloning vector (Invitrogen), generating sytpr-pCR2.1. The green fluorescent protein (GFP) coding sequence was amplified by PCR from pEGFP-N1 (Clontech) using primers GFP-forward (5′-GCTA-GCGCTACCGGACTCAG-3′) and GFP-reverse (5′-GAGCTCA-CGCTTACAATTTACGCCT-3′). The GFP and the promoter sequence were subcloned between the *Xho*I and *Sac*I sites of pBluescript SK−, generating the sytpr-GFP mother construct. Δd and Δi mutant constructs were generated by digesting mother plasmid with restriction enzymes, as shown in Fig. 5, by blunting the ends of religation.

Introduction of DNA into the Ascidian Embryo and Microscopy

Egg microinjection was carried out as previously described (Hikosaka *et al.,* 1992). Briefly, fertilized eggs were immobilized on the bottom of a culture dish by actinase E treatment, which renders the surface of the egg chorion sticky. Eggs were injected in a chamber maintained at 5–7°C. Circular DNA (10 ng/μl) in H_2O was injected with 0.2% fast green.

For detecting GFP signals, live whole embryos were examined under a Leica dissecting microscope with fluorescent illumination (Leica, MZ FLIII) and a GFP filter set, or were fixed in 1% paraformaldehyde in sea water and observed under epifluorescence (Zeiss Axiophot) with a 10× objective lens (Fluar, N.A. 0.5, or Plan-neofluar, N.A. 0.3; Zeiss). Enhanced GFP (Clontech) was excited at 450 nm. A band pass filter (width 50 nm, centered at 510 nm) was used to record the emission. For imaging the picture seen in Fig. 4H, a low pass filter (over 485 nm) was used. Images were taken through a cooled charge-coupled device camera and stored in personal computers.

Embryo Manipulation

The chorion of each ascidian embryo was removed manually by using sharp tungsten needles. Embryos were dissected by using fine glass knives. Dechorionated embryos were cultured in sea water on agarose-coated petri dishes. To arrest cell cleavage, the embryos were treated with 2% cytochalasin B in sea water for 1 h, transferred to the sea water containing 0.2% cytochalasin B, and then cultured until the required stage (Okado and Takahashi, 1990a). Embryos and blastomeres were washed three times in fresh filtered sea water just before collection.

RESULTS

Cloning of Ascidian Synaptotagmin Gene

Ascidian syt is a 383-amino-acid protein, a size similar to that of mammalian and fly syt. Its overall structure shows high similarity to vertebrate syt I, II, and V, but less to syt III and IV. A well-conserved region is found in the middle of the C terminus of the protein. It includes the C2A and C2B domains, which, in other animals, are important for calcium sensing and interactions with other synaptic proteins (Fernández-Chacón *et al.,* 2001; Südhof and Rizo, 1996). The critical amino acid residues for calcium sensing are completely conserved in ascidian syt protein. Because the degenerate PCR primers used in this study were designated to target vertebrate *syt* I, but not *syt* III/V, it is possible that ascidians have more than one *syt* gene.

Northern hybridization to ascidian embryonic RNA detected a band at 2.6 kb, consistent with the length of the *syt* cDNA clones we isolated here (GenBank Accession No. AB044144). A strong expression was detected from the onset of gastrulation, while a faint band was detected at earlier stages as well as in unfertilized eggs (data not shown), indicating the presence of maternal *syt* transcripts.

Synaptotagmin Is Expressed in Neurons of the Ascidian Larva

syt expression in the ascidian larva was examined by whole-mount *in situ* hybridization. The *syt* transcript was abundant in the sensory vesicle, a region homologous to the vertebrate brain, which contains two types of pigment cells, the otolith and ocellus. In this region, several clusters of cells were densely stained, and other cells between them were weakly stained (Figs. 1A and 1B). These observations were confirmed in sections of *in situ* specimens (Fig. 1F). *Syt* expression was also detected as discrete pairs of cells in the region around the junction of the trunk and the tail of the larva (Figs. 1A and 1B). Observation of sections indi-

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cates that these spots are localized to large cells in the neural tube (Fig. 1G). These most likely correspond to the neurons previously described as those innervating muscle (Okada *et al.,* 1997). Detailed analysis of these *syt*-positive cells in the accompanying paper (Okada *et al.,* 2002) establishes that these cells are motor neurons.

In the dorsal epidermis anterior to the brain, *syt* expression was detected in bilateral pairs of cells (Figs. 1A and 1B). With prolonged staining of the *in situ* specimens, syt expression was detected in a small number of cells in the dorsal epidermal layer of the tail (Figs. 1C–1E). These cells are observed bilaterally along the dorsal midline in a variable location, and they extend along the anterior–posterior axis. Based on these morphological features, *syt*-positive cells in the epidermal layer of the trunk and tail appear to be epidermal sensory neurons, which were previously characterized morphologically and immunocytochemically (Crowther and Whittaker, 1994; Ohtsuka *et al.,* 2001).

The papilla, an adhesive organ located at the anterior end of the larva, also expresses *syt* weakly (Figs. 1A and 1F). Prolonged staining confirms this expression (Figs. 1C–1E), although it also enhances nonspecific staining of tunic surrounding the trunk. *Syt*-expressing cells in the papillae may be the sensory neurons that were described in other species of ascidians (Torrence and Cloney, 1983).

In summary, *syt* is expressed in the larval neurons, which were described in previous studies, and the expression pattern in the larva is similar to those of a neuronal marker gene, *TuNa1* (Okamura *et al.,* 1994).

Distinct Expression Pattern of Two Neuronal Genes during Embryogenesis

The temporal pattern of *syt* expression was compared with another neuronal marker, *TuNa1* (Okamura *et al.,* 1994). *syt* expression is first detected at the 110-cell stage in blastomeres at the anterior edge (Fig. 2A), which has a neural fate (Nishida, 1987). This expression does not seem to be residual maternal transcript, because *syt* expression was not detected in the embryos before this stage. In contrast, *TuNa1* expression was not detected at this stage (Fig. 2E). *Syt* expression in anterior vegetal blastomeres subsides at gastrula stages and is initiated in cells of epidermal lineage, while *TuNa1* expression at this stage marks a different set of cells in a row in the neural plate. Based on their position, these cells appeared to be descendants of the A4.1 blastomere of the eight-cell-stage embryo (Fig. 2F). At neurula stages, expression of *syt* becomes stronger in the embryonic epidermis and is not evident in the neural tube-forming area (Fig. 2C). *TuNa1* expression at this stage is now detected in the anterior part of the neural tube-forming region (Fig. 2G), corresponding with derivatives of the a4.2 blastomere. At the tailbud stage, epidermal expression of *syt* is reduced and expression is detected in the presumptive brain and in the region around the junction of the tail and trunk in the neural tube (Fig. 2D). *TuNa1* expression at this stage is similar to that of *syt* in the neural tube, and it is also observed in the tail epidermis as some spots probably corresponding to signals in epidermal sensory neurons (Fig. 2H; Okamura *et al.,* 1994; Ohtsuka *et al.,* 2001). In summary, expression patterns of *syt* and *TuNa1* are different during ascidian embryogenesis, despite their final common patterns at the larval stage.

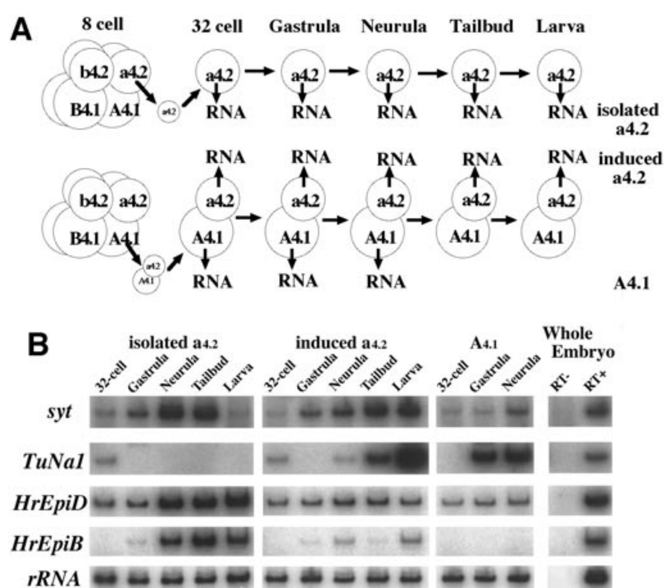

FIG. 3. The neural induction is involved in *syt* gene expression. The upper scheme depicts the procedure of this experiment. a4.2/A4.1 pairs were isolated from eight-cell embryos, and their cell cleavage was arrested by cytochalasin B. These blastomeres were cultured until the indicated developmental stages and harvested for RT-PCR. Neural differentiation of a4.2 in a4.2/A4.1 pairs was checked by *TuNa1* expression. Epidermal differentiation of a4.2 was confirmed by the expression of two epidermal genes, *HrEpiB* and *HrEpiD*. Ribosomal RNA (rRNA) was used as an internal control. Some basal level expression of *HrEpiD* persisted in both situations of a4.2, consistent with the expression pattern observed by *in situ* hybridization (Ueki and Satoh, 1994). Occasional expression of *HrEpiB* at low levels in the induced a4.2 cultures may be caused by incomplete neural induction due to loosened contact with the inducer blastomere in some cases, since incomplete contact allows a4.2 to have a weak epidermal phenotype (Okado and Takahashi, 1990b).

Neural Induction Regulates Synaptotagmin Gene Expression

Previous studies have suggested that neural differentiation of the animal blastomeres in the ascidian embryo requires induction from the vegetal hemisphere (Reverberi *et al.,* 1960; Okado and Takahashi, 1988; Nishida, 1991; Hudson and Lemaire, 2001; Ohtsuka *et al.,* 2001). a4.2/A4.1 pairs were isolated from eight-cell embryos, and their cell cleavage was arrested by cytochalasin B. Cell cleavage-arrested blastomeres were then cultured until their siblings

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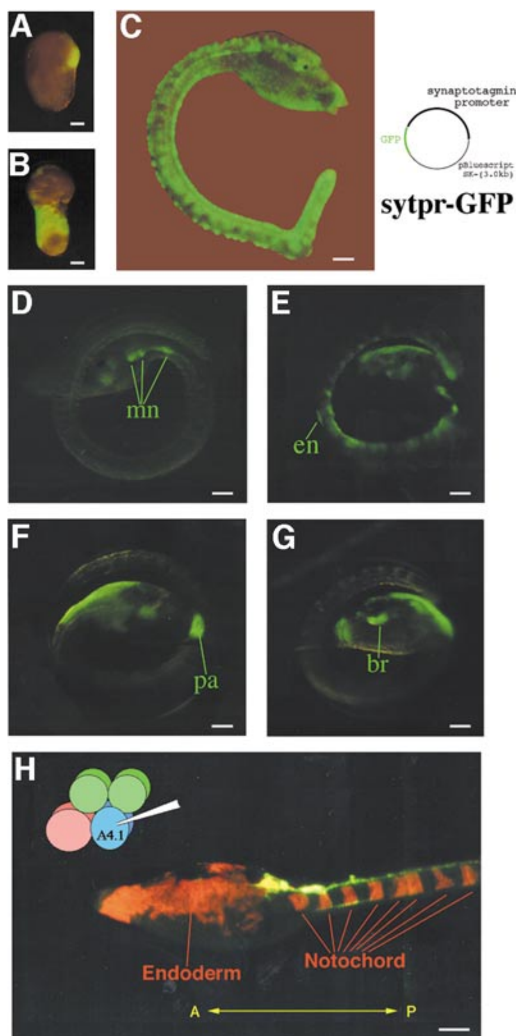

FIG. 4. Expression of a GFP reporter gene in the embryos injected with a *syt* promoter construct (a plasmid shown in inset). (A) Neurula embryo with weak GFP signal in the epidermis. (B) Tailbud-stage embryo with stronger GFP signal in the epidermis. (C–G) Examples of tadpole larva. (C) A larva in which GFP expression is observed in epidermal cells and neurons. Epidermal signals mask the neural expression where they overlap, making observation of neuronal GFP expression difficult. (D–G) Examples in which epidermal expression was weaker and internal neural cells could be observed. *Syt* expression was examined in distinct types of neural cells; putative motor neurons (mn in D), epidermal sensory neurons (en in E), papilla (pa in F), brain (br in **G**). Epidermal sensory neurons (en) show elongated cell body along the anterior–posterior axis of the larva, which is distinct from the square shape of epidermal cells. Papilla (or adhesive organ) is not a neural tissue, but contains sensory neurons (Torrence and Cloney, 1983). (H) A typical example of tadpole larva in which the promoter construct was injected into the A4.1 blastomere. This blastomere gives rise to neural, endodermal, and mesodermal fates, but not to epidermal fate. Coinjected lineage tracer (red) labels these cell types, but only neurons express GFP, confirming that this promoter does not drive gene expression in nonectodermal tissues. Bar, 50 μm.

reached various developmental stages (Fig. 3A). Because A4.1 adopts neural fate cell-autonomously (Okada *et al.,* 1997; Minokawa *et al.,* 2001), a4.2 was separated from the A4.1 just before harvesting cells to examine gene expression in the a4.2 blastomere. The expression of *syt* and *TuNa1* was examined semiquantitatively by RT-PCR. The expression of two genes, *HrEpiB* and *HrEpiD* (Ueki *et al.,* 1994; Ueki and Satoh, 1994), was also used to assay epidermal differentiation in these cultures. In isolated a4.2 blastomeres, the expression of *syt* peaked between neurula and tailbud stages and declined at the larval stage. In contrast, it gradually increased until the larval stage in a4.2 cultures left in contact with A4.1 (Fig. 3). *TuNa1* expression was detected in a4.2 cell cultures left in contact with A4.1, consistent with a previous report (Okamura *et al.,* 1994). *TuNa1* was not detected in isolated a4.2 blastomere cultures, except for weak expression at the 32-cell stage. The reason for *TuNa1* at this early stage is not clear, but it is possible that it is due to the residual maternal transcript or transient gene activation caused by cell dissociation. The two epidermal markers were more prominently expressed in isolated than in induced a4.2 cultures, showing that cell contact between a4.2 and A4.1 suppresses epidermal differentiation. This strengthens the idea that neuronal expression of *syt* is regulated by neural induction.

Cloning of the Synaptotagmin Promoter Region

To investigate transcriptional regulation of *syt* in the ascidian embryo, we isolated, from *Halocynthia* genomic DNA, 3.4 kb of 5′ upstream sequences of the *syt* gene (Accession No. AB044145). Because four independent cDNA clones basically have the same sequence at their 5′ ends, we tentatively defined the 5′ end of the cDNA to be the transcription initiation site (TIS). Several putative binding sites for TATA-binding proteins are observed just upstream of the TIS. One of these is located 30 bp upstream of TIS and may constitute a TATA box.

A plasmid carrying the GFP coding sequence downstream of the *syt* genomic fragment was microinjected into ascidian embryos (Fig. 4). Consistent with previous reports (Hikosaka *et al.,* 1992; Corbo *et al.,* 1997a,b), the reporter gene expression was observed mosaically in the embryos injected with the plasmid. The GFP signal was observed in epidermal precursors (Fig. 4A) in some neurula embryos, and became evident in most injected embryos by tailbud stages (Fig. 4B). At larval stages, the GFP signal is prominent in the epidermis, and identification of neurons inside the larvae is difficult in most injected larvae (Fig. 4C). However, epidermal signals do not overlap with the signals inside of some larvae, making observation of GFP expression in neurons clearer (Figs. 4D–4G). Spots of GFP signals are observed in the neural tube at around the junction of the tail and trunk regions. These cells have long neurites traversing dorsally, which resemble the motor neurons described in a previous study (Okada *et al.,* 1997). Some cells in the epidermal layer of the tail have an elongated

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shape along the anterior–posterior axis (Fig. 4E). They are clearly different from epidermal cells that are generally square. These elongated cells look similar to cells that were detected in the same region by *in situ* hybridization for *syt* mRNA (Fig. 1). Based on their morphology and location, these cells are judged to be epidermal sensory neurons that were characterized by expression of gelsolin (Ohtsuka *et al.,* 2001) and β-tubulin (Crowther and Whittaker, 1994). GFP expression was also observed in the adhesive organ (Fig. 4F), which is also known to contain sensory neurons (Torrence and Cloney, 1983). In many cases, GFP expression was observed in the brain of the larvae (Fig. 4G).

Because most larvae express GFP in the epidermis, observations described above do not rule out the possibility that this genomic sequence may also activate reporter gene expression in endoderm and mesoderm. To check this possibility, we injected the plasmid with lineage tracer (fast green) into the A4.1 blastomere of eight-cell-stage embryos. This blastomere is normally fated to form mesoderm, endoderm, and neural tissues, but not to form epidermis (Nishida and Satoh, 1983). Only cells with neurites in the neural tube expressed GFP, although other cell types, such as notochord and endoderm, from the A4.1 blastomere were labeled by the tracer ($n > 100$; Fig. 4H). Descending neurites were also detected in the dorsal part of the notochord, suggesting that GFP-positive cells in the neural tube are motor neurons. These findings suggest that the 3.4-kb genomic fragment is capable of driving reporter gene expression in neuronal and epidermal cells, but not elsewhere.

Promoter function was also examined in cleavage-arrested blastomeres. Epidermally differentiating animal blastomeres started GFP expression earlier than cells neurally induced by A4.1 (data not shown). This pattern is consistent with the native gene expression patterns revealed by the RT-PCR (Fig. 3), reinforcing the idea that the genomic fragment contains a promoter sequence that recapitulates neuronal and epidermal expression of *syt* gene in the ascidian embryo.

Organization of the syt Promoter

Distinct temporal patterns of *syt* expression in neuronal and epidermal cells suggest that distinct molecular mechanisms underlie gene expression. To investigate this, we generated a series of deletion constructs and tested them in ascidian embryos (Fig. 5). When the full-length construct was injected, all the larvae expressed GFP in both epidermis and neurons. Injection of Δd1 which lacks the distal most 1.6-kb sequence resulted in indistinguishable activity compared with the full-length promoter construct. However, the deletion of the distal region up to −1680 (Δd2) resulted in loss of epidermal expression of the reporter, whereas this construct exerts neuron-specific expression in most injected larva. Further deletions, Δd3 and Δd4, showed lower level but evident neuronal expression without any expression in epidermal cells. Deletion down to −246 (Δd5) shows neither neuronal nor epidermal expression of GFP. These results suggest that neuronal and epidermal expression depend on distinct *cis* element(s).

To test distinct regulation of *syt* transcription in neuronal and epidermal cells, the regions between −2223 and −1680 and between −2223 and −824 were deleted from the full-length fragment, respectively (Δi1, Δi2). Both Δi1 and Δi2 showed no epidermal expression but robust neuronal expression (Figs. 6A and 6B, for Δi1). On the other hand, Δi3 lacking the flanking downstream region (−1680 to −824) did not eliminate epidermal expression, consistent with results of the distal deletion series described above. To test whether the sequence between −2223 and −1680 is sufficient to drive epidermal gene expression, this region was added to a construct deleting all the regions down to −36 (Δi4). This plasmid produced clear GFP expression in epidermal cells both in the trunk and tail (Fig. 6C), indicating that this region contains the epidermal enhancer. Neuronal gene expression was not observed in any embryo injected with this construct.

Multiple Different Cis Elements Are Involved in Neuronal Expression of Synaptotagmin

The relatively low probability of neuronal gene expression with Δd4 as opposed to Δd2 (Fig. 5) indicates that the region between −1680 and −824 contains the ability to enhance neuronal expression. On the other hand, the Δi1 construct, lacking sequence between −2223 and −824, was still capable of inducing neuronal gene expression in all injected larvae (Figs. 6A and 6B), indicating that the region distal to −2223 has enhancing activity of neuronal expression which can substitute for the region between −2223 and −824.

Deletion analyses of the promoter above suggest that neuronal expression of *syt* depends on a wider region in the promoter in contrast with the short critical region for epidermal expression. This may be accounted for if distinct regions of the promoter drive gene expression in distinct subtypes of neurons in the nervous system. To test this possibility, the number of embryos was counted that showed GFP signals in epidermal sensory neurons, brain, motor neurons, and cells in the papilla, respectively. These numbers were compared between two constructs, Δi2 and Δd4 (Table 1). Δi2 contains the longest promoter sequence without epidermal expression ability, and Δd4 is the shortest one and is capable of driving neuronal expression. There was no significant difference in frequencies of GFP signals in individual types of neurons between the two constructs, although the probability of obtaining embryos with any neuronal signal per injected embryos was significantly lower in Δd4. These experiments indicate that the region between −824 and −246 is sufficient to drive gene expression in most types of neurons and that enhancement of neuronal gene expression by the other regions does not bias them toward any specific type of neuron.

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TABLE 1
Number of Larvae Expressing GFP in Subsets of Cells in the Nervous System

Name of construct	Δi2	Δd4
Total no. of injected embryos	17	38
No. of GFP-positive larva	16	13
Larva showing GFP expression in		
motor neuron	12	6
sensory vesicle	12	8
epidermal sensory neuron	7	7
papillae	4	2

DISCUSSION

Here, we reported cloning and expression pattern of the *syt* gene of the ascidian *H. roretzi*. Expression of *syt* is neuron-specific in the tadpole larva, but is also observed in epidermal cells at earlier developmental stages. Promoter analysis revealed that *syt* expression in these cell types is regulated by distinct mechanisms. The neuron-specific promoter construct obtained here is a useful tool for studying the ascidian nervous system.

Identity of syt-Expressing Cells in the Ascidian Larva

Whole-mount *in situ* hybridization shows three pairs of *syt*-expressing cells around the junction of the trunk and tail regions of the larval neural tube. This region is likely to correspond with the visceral ganglion of the larva of *C. intestinalis*, another species of ascidian. The visceral ganglion is known to contain motor neurons (Nicol and Meinertzhagen, 1991). In *Halocynthia* embryos, neural cells in the neural tube at the border between the trunk and the tail are derived from the anterior vegetal blastomeres, A4.1 of the eight-cell-stage embryo (Nishida, 1987). In our present study, GFP-labeled neurons have axons innervating muscle cells when *syt*-GFP plasmid was injected into A4.1 blastomere. This confirmed our previous observation that motor neurons are derived from A4.1 blastomere (Okada *et al.,* 1997). The identity of individual motor neurons is described in more detail in the accompanying paper (Okada *et al.,* 2002).

Neurons have not been described in the larval nerve cord posterior to the visceral ganglion by histological studies (Katz, 1983; Nicol and Meinertzhagen, 1991). Consistent with this observation, *syt*-positive cells were not detected in the posterior region of the larval central nervous system. On the other hand, the epidermal layer of the larval tail contains some *syt*-positive cells. Double staining using anti-gelsolin antibody, a molecular marker specific to epidermal sensory neurons (Ohtsuka *et al.,* 2001), has recently confirmed that *syt*-expressing cells are epidermal sensory neurons (Y. Ohtsuka, unpublished results).

Syt is expressed in the sensory vesicle region, which includes the two types of pigmented sensory organs. The sensory vesicle corresponds with the vertebrate forebrain and the midbrain based on the expression of ascidian Otx (Katsuyama *et al.,* 1996; Wada *et al.,* 1998). Several large neurons robustly express the *syt* gene in this region, and numerous small cells exhibit weak *syt* expression. The latter group of neurons may correspond to small neuron-like cells described by Nicol and Meinertzhagen (1991) in the postsensory vesicle region of the ascidian *C. intestinalis*, and possibly, to immature neurons which will form the adult ganglion after metamorphosis (Willey, 1893).

In summary, *syt* is expressed in multiple types of neurons, including motor neurons, epidermal sensory neurons, and cells of the brain. This expression pattern is basically the same as that of *TuNa1* (Okamura *et al.,* 1994), although the level of *TuNa1* expression is less abundant than that of *syt*. Pan-neuronal expression of *syt* gene is consistent with its function that is essential for evoked synaptic transmission both in vertebrates and invertebrates (Geppert *et al.,* 1994; Littleton *et al.,* 1993a).

Comparison of the Temporal Expression Patterns of Ascidian Neural Genes

Syt is expressed transiently in two phases of development. It turned on in the presumptive neural region of the 110-cell-stage embryos. This is one of the earliest cases of gene expression in the presumptive neural region among known neuron-specific genes in ascidian (Yagi and Makabe, 2000). This stage is around the period when early specification of neural cell fates occurs (Nishida, 1991; Okado and Takahashi, 1990b). Such early gene expression could be linked to early events of neural induction, which will be interesting to investigate further.

The second phase of *syt* expression occurs in the presumptive epidermal region at neurula and tailbud stages. In vertebrates, some *syt* isoforms are expressed in nonneuronal cells (Hudson and Birnbaum, 1995). Syt protein in nonneuronal cells is presumed to be involved in regulation of calcium ion-mediated vesicle transport. Thus, ascidian synaptotagmin could be involved in regulation of exocytosis in embryonic epidermis. Epidermal cells of the ascidian embryo express voltage-dependent calcium channel genes, which are known to trigger rapid calcium ion entry leading to exocytosis in synapses, endocrine cells, and egg cells (Hirano and Takahashi, 1987; Okagaki *et al.,* 2001). The embryonic epidermal cells also express other genes involved in exocytosis, such as synaptobrevin and syntaxin (Kawashima *et al.,* 2000). Because epidermal cells of the ascidian embryo secrete a large amount of extracellular matrix to form the tunic at tailbud and larval stages, they may have recruited the molecules associated with neuronal function to achieve efficient exocytosis.

Three neuronal genes, *TuNa1* (a sodium channel gene), neural *β-tubulin* (Miya and Satoh, 1997), and *HrETR* (a *ELAV*-related gene) (Yagi and Makabe, 2001) have been

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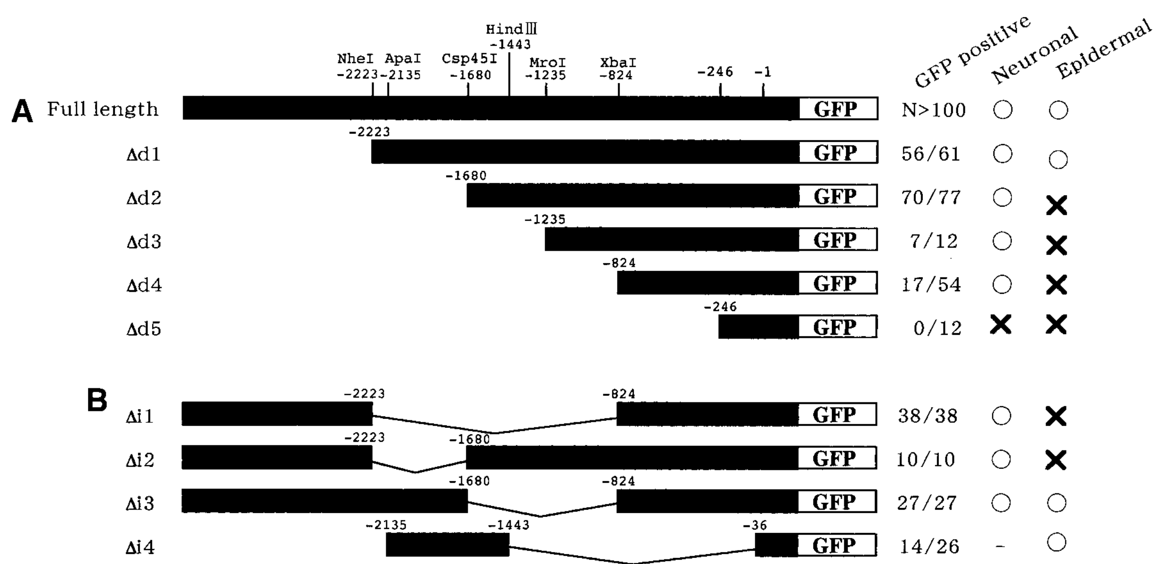

FIG. 5. Summary of the deletion analysis of the *syt* promoter. (A) Results of experiments analyzing the effect of a distal deletion series. (B) Results obtained with an internal deletion and a combination of distal and internal deletion experiments. In Δd1–5 and Δi-3, the number of embryos expressing GFP in at least one neural cell vs. the number of injected embryos is indicated in the right column. In Δi4, the number of embryos expressing GFP in epidermal cells vs. the total number of injected embryos is indicated. In the constructs of Δd1, Δi3, and the full-length sequence, epidermal expression was detected in all injected samples.

cloned from *H. roretzi*, and they exhibit extensive expression in the larval nervous system and papilla (Okamura *et al.*, 1994; Yagi and Makabe, 2001). The expression pattern of *syt* in the larva is similar to that of *TuNa1* and *β-tubulin*. However, expression patterns during embryogenesis are quite different among the four neuronal genes. *Syt* exhibits transient expression in the region that will form neuroectoderm in the 110-cell embryo and in the epidermal lineage in the neurula and tailbuds. Such expression is not observed in the other three genes. *TuNa1* expression appears in the presumptive neural region of the late gastrula embryos, where neither *β-tubulin* nor *syt* is expressed. When this transient expression is disregarded, neural expression of *tubulin* and *HrETR* starts earlier than that of *syt* and *TuNa1*. *HrETR* and *β-tubulin* are transiently expressed in the posterior region of the neural tube, the presumptive region of ependymal cells, whereas expressions of *TuNa1* and *syt* are lacking in this region. Moreover, based on whole-mount *in situ* hybridization, neural expression of *syt*, *β-tubulin*, and *HrETR* begins throughout the embryo within a short period, whereas timing of *TuNa1* expression varies among diverse neuron subtypes. These findings suggest that the molecular mechanisms that govern temporal gene expression could be diverse among those genes which exhibit neuron-specific gene expression at the larval stage.

We also used isolated, cleavage-arrested blastomeres to test whether *syt* gene expression is regulated by neural induction as shown for *TuNa1* (Okamura *et al.*, 1994). *Syt* transcript in induced a4.2 blastomere at the larval stage depended on the cell contact to A4.1 blastomere, whereas only transient gene expression occurred in uninduced a4.2 blastomeres. Such transient expression in uninduced blastomeres probably occurs as the result of cell-autonomous regulation of epidermal differentiation. Expression of *syt* in neurally induced a4.2 blastomeres is presumed to be due to the upregulation of transcription factors as a result of neural induction. Expression of *syt* at epidermal differentiation and neuronal differentiation thus occurs via distinct molecular mechanisms. This view is supported by our finding that epidermal and neuronal gene expressions of *syt* depend on distinct *cis*-regulatory regions.

Functional Structure of Ascidian Synaptotagmin Promoter

Deletion constructs of the *syt* promoter differ in the probability of observing GFP-positive cells, when they are injected into the ascidian embryos. When the plasmid with intense promoter activity is injected, even cells incorporating a small number of plasmids show up as GFP-positive, resulting in a high probability of GFP-positive embryos. In the case of the construct with weaker promoter activity, only cells incorporating a larger number of plasmids could express GFP at a detectable level. Thus, the probability of a GFP-expressing embryo reflects the strength of the promoter activity. This view is consistent with a recent view about functions of enhancers (Walters *et al.*, 1995).

The genomic fragment isolated here was shown to recapitulate the expression pattern of the *syt* gene. Deletion experiments of the promoter suggest that the region be-

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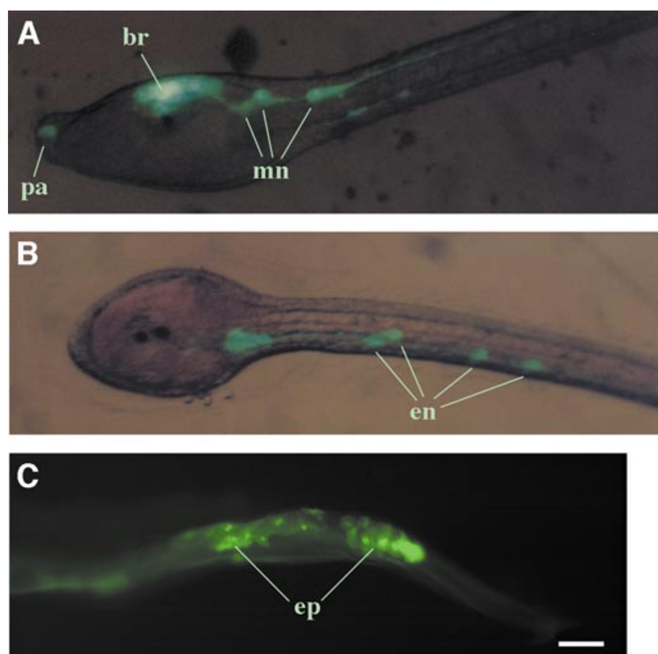

FIG. 6. Expression of GFP in tadpole larvae driven by two deleted *syt* promoter constructs, Δi1 (A, B) and Δi4 (C). Δi1 construct that lacks the sequence between −2223 and −824 drives GFP expression exclusively in neural cells, including cells in the brain (A) and motor neurons (B). (A) and (B) are different larvae. Δi4 drives only epidermal GFP expression (C). Bar, 50 μm.

tween −2223 and −1680 is required and sufficient for epidermal gene expression, and deletion of this region does not affect neuronal expression of the reporter gene. Thus, epidermal and neuronal expression of *syt* during ascidian embryogenesis are regulated by distinct transcriptional mechanisms. The promoter sequences of ascidian epidermal genes have been analyzed (Ueki and Satoh, 1995; Ishida and Satoh, 1999). However, the temporal pattern of *syt* expression in epidermal lineage differs from those genes. Consistent with this, we could not find any sequence similarity between regulatory elements of these epidermal genes and *syt*. Thus, it is likely that epidermal expression of *syt* is regulated by transcriptional factors distinct from other reported epidermal genes of the ascidian. Because the decrease in *syt* expression also occurs in blastomeres cultured in isolation, this temporal expression pattern is regulated in a cell-autonomous manner.

There are at least two distinct regions enhancing neuronal gene expression. One is the region between −1680 and −824, which was delineated by comparison between Δd2 and Δd4. The other region is upstream of −2223, as defined by comparison between Δd4 and Δi1. Residual neuronal gene expression by the construct Δd4 and absence of neuronal expression by the Δd5 construct suggest that a minimal region for neuronal gene expression exists between −824 and −246. In contrast with a relatively short critical region for epidermal expression, multiple regions over the larger promoter sequence are likely to regulate neuronal expression. Expression in distinct neuronal subpopulations could be controlled by distinct *cis*-regulatory elements. However, we could not find any region that regulates gene expression exclusively in certain subpopulations of larval neurons. The region up to −824 (Δd4) can drive GFP expression in a pattern indistinguishable from that by the full length and Δi2. To define detailed *cis*-regulatory elements that drive neuron-specific expression of *syt* gene, more quantitative or statistical analyses are required. So far, it is difficult to obtain sufficient numbers of injected embryos using *Halocynthia*. An electroporation method is available in another species, *C. intestinalis*, and we found that *Halocynthia syt* promoter sequence can drive neuron-specific expression in *Ciona* (Okada *et al.,* 2001). Thus, we are currently investigating this promoter by using both species.

Ascidian Synaptotagmin Promoter as a Molecular Tool

GFP expression under this promoter enables us to visualize the morphology of ascidian larval neurons. As shown above, the *syt* promoter can drive transgene expression exclusively in neurons when a critical region involved in epidermal expression is deleted (Δd2 and Δi1; Figs. 6A and 6B). This, combined with the nature of mosaic integration of transgenes in the ascidian embryos, provides us with a unique opportunity to reveal the morphologies of individual neurons which has been otherwise difficult. As an example, characterization of the development of individual motor neurons is described in the accompanying paper (Okada *et al.,* 2002). Another important consequence of this work will be the ability to measure activities of individual neurons *in vivo* by marking cells with GFP-based sensors. Moreover, such forced expression under the control of neuron-specific promoters may help us to understand the function of genes expressed in the neurons. We have successfully addressed the roles of one class of potassium channels in generating action potentials in the ascidian nervous system (Ono *et al.,* 1999). The *syt* promoter we have characterized here will thus serve as the powerful molecular tool for understanding anatomy, development, and function of the ascidian nervous system.

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