Abstract To identify cholinergic neurons, we isolated a choline acetyltransferase (Ci-ChAT) gene from Ciona intestinalis by PCR methods. In the cloning process, we also obtained the gene encoding the vesicular acetylcholine transporter (Ci-vAChTP). These two genes shared the same 5′-UTR sequence as well as similar expression patterns. In both cases, the gene expression was first detected by whole-mount in situ hybridization in the anterior-dorsal region of the caudal nerve cord at the early tailbud stage. In the larva, the expression was seen in several cells of the visceral ganglion. These results suggest that ascidian larval motor neurons exist in the visceral ganglion.

Keywords Ascidian · Acetylcholine · Choline acetyltransferase · Vesicular acetylcholine transporter · Cholinergic neuron

The central nervous system (CNS) of the ascidian tadpole larva is thought to be the most simplified one of all vertebrate-type CNSs (Nicol and Meinertzhagen 1991; Satoh 1994; Meinertzhagen and Okamura 2001; Wada and Satoh 2001). The number of cells composing the CNS and of neurons is estimated at about 300 and less than 100, respectively (Nicol and Meinertzhagen 1991). Additionally, several types of peripheral sensory neurons exist around the trunk and caudal epidermis, and connect with the CNS (Takamura 1998). The identification and functional classification of these neurons, however, remains to be carried out. We have tried to analyze the genes specifically expressed in the adult brain, the so-called neural complex (Takamura et al. 2001), and isolate those genes that were related to the synthesis of the neurotransmitters.

Choline acetyltransferase (ChAT) catalyzes the synthesis of acetylcholine (ACh) from acetyl CoA and choline in cholinergic neurons and is a marker enzyme for these neurons. We amplified a homologous fragment with a primer set for the ChAT-conserved region (LCMK/AQYY and YGKTFIK, underlined in Fig. 2) by degenerate PCR. Based on the sequence of this fragment, we prepared a new specific reverse primer and performed 5′-RACE. Next, we performed 3′-RACE with a specific forward primer corresponding to the 5′-end of the 5′-RACE product. By this process, we cloned two kinds of genes related to acetylcholine synthesis. These two genes shared the same 5′-UTR sequence (data not shown, see the DDBJ database). However, each predicted ORF encoded different amino acid sequences. From the results of a homology search against a protein database (Swissprot), we identified one of the gene products as vesicular acetylcholine transporter (Ci-vAChTP, Fig. 1A) and the other as choline acetyltransferase (Ci-ChAT, Fig. 2). Ci-vAChTP (DDBJ accession number AB071998) was 2,547 bp long and had a predicted amino acid sequence of 657 residues. Ci-ChAT (DDBJ accession number AB071999) was 2,839 bp long and had a predicted amino acid sequence of 759 residues. The predicted proteins were longer than those of other organisms and differed especially at the C′-end (Figs. 1A and 2).

To investigate the genomic organization of these genes, we performed LA-PCR with Ciona genomic DNA and sequenced the amplified product (about 5.5 kbp). The location of the primers used is indicated by arrows in Fig. 1B. The result showed that the Ci-vAChTP gene contained no intron in its ORF and was located between the shared 5′-UTR and the Ci-ChAT gene (Fig. 1B). This genomic sequence can be seen in the
DDBJ database (accession number AB072000). Similar types of genomic organizations have been evolutionarily conserved at cholinergic loci in invertebrates and vertebrates (Kitamoto et al. 1998).

To analyze the spatial expression pattern of these genes in embryo and larva, whole-mount in situ hybridization was performed with DIG-labeled antisense RNA probes specific to Ci-vAChTP and Ci-ChAT (choline acetyltransferase) loci of Ciona intestinalis. The location of the PCR primers used is indicated by arrows. Two genes share the same 5′-UTR. A closed box shows the exon and an open box the UTR.

Neither gene was expressed until the neurula stage (Fig. 3A, F). At the early tailbud stage, both genes were first detected in part of the caudal neural tube (Fig. 3B, G). Although this positive region appeared first in the tail rather than the trunk, it moved anteriorly during tail extension and finally entered the visceral ganglion of the larval trunk (Fig. 3). Additionally, the positive region divided in a double line along the neural tube of the tailbud (Fig. 3C) and in the visceral ganglion of the larva (Fig. 3I). The number of positive cells in the