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ScienceDirect
Gene 406 (2007) 36–41
GENE
www.elsevier.com/locate/gene

Developmental expression and transcriptional regulation of *Ci-Pans*, a novel neural marker gene of the ascidian, *Ciona intestinalis*

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Received 30 January 2007; received in revised form 11 May 2007; accepted 18 May 2007
Available online 16 June 2007

Abstract

A novel gene, named *Ci-Pans*, was isolated and characterized from the ascidian *Ciona intestinalis*. It is an 885-bp cDNA, is thought to encode a protein with no sequence similarities to known proteins and shows a spatial and temporal specific expression pattern. In fact, besides a transient early localization in the muscle precursors, it is expressed in a dynamic fashion in the nervous system, during *C. intestinalis* embryogenesis, reaching very high level of expression as the development proceeds. To study *Ci-Pans* transcriptional control, we isolated the predicted promoter region of *C. intestinalis Ci-Pans* using databases for this species. Analysis of transgenic embryos, with a green fluorescence protein (GFP) reporter, showed that approximately 1 kb of the 5′-flanking sequence of the *Ci-Pans* gene was implicated in its specific expression in the CNS.

The data on the expression pattern of *Ci-Pans* together with the strong activity exhibited by the 1 kb promoter region we have identified, indicate that a more deeply investigation on *Ci-Pans* could provide clues for exploring the complex network of nervous system-specific genes.

Keywords: *Ciona*; *Ci-Pans*; Nervous system; *In situ* hybridization; Promoter; Transgenesis; Electroporation

1. Introduction

Ascidians are popular organisms for developmental and evolutionary studies because of their small genomes, invariant cell lineages, simple tadpole larvae and phylogenetic affinity to vertebrates (Satoh and Jeffery, 1995; Jeffery, 2000). The tadpole has a primitive central nervous system (CNS) that provides us a chordate nervous system in miniature. The ascidian larval CNS consists, indeed, of a sensory vesicle, neck, visceral ganglion and tail nerve cord distributed along the anterior posterior axis (for reviews, see Meinertzhagen and Okamura, 2001; Lemaire et al., 2002; Meinertzhagen et al., 2004). Despite the complex organization, however, it is composed of only around 330 cells including 215 in the brain, 50 in the tail ganglion, and 65 ependymal cells of the caudal neural tube (Nicol and Meinertzhagen, 1991). This relative simplicity of organization compared with that of vertebrates is very fascinating, considering also that the expression of some genes along the dorsal–ventral and anterior–posterior axes of the CNS is conserved between ascidian and vertebrate embryos. Orthologues of *Otx*, *Pax2/5/8* and *Hox* genes are differentially expressed along the anterior–posterior axis, genes encoding *Hedgehog* and *HNF3* are expressed in the ventral neural tube while *Snail*, *Pax3/7*, *BMP2/4* and *Msx* are expressed in the lateral or dorsal part of the neural tube (Corbo et al., 1997; Miya et al., 1997; Wada et al., 1997, 1998; Aniello et al., 1999; Wada and Saiga, 1999a,b; Hudson and Lemaire, 2001; Imai et al., 2002; Takatori et al., 2002). Therefore, the two peculiar characteristics of ascidian, that is a small number of cells and far fewer genes than vertebrates, coupled with the nervous system architecture very close to that of vertebrates, make them an ideal model for exploring molecular mechanisms underlying the development of a complex nervous system, applicable to those of higher animals.

Abbreviations: CNS, Central Nervous System; cDNA, DNA complementary to RNA; PCR, polymerase chain reaction; GFP, green fluorescent protein; bp, base pair(s); kb, kilobases; nt, nucleotide(s); ORF, open reading frame.

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doi:10.1016/j.gene.2007.05.026

In recent years different neuronal markers have been characterized (Miya et al., 1997; Hudson and Lemaire, 2001; Kusakabe et al., 2001; Yagi and Makabe, 2002) and some steps of a so fundamental process are starting to be defined. Needless to say that we are only at the beginning and that most of the mechanisms that occur in the ascidian nervous system, at the molecular level, remain to be elucidated.

To this end one approach is to try to isolate as much molecular markers as possible to cover both small population of the neural cells and all of the neural cells in ascidians.

Here we report the isolation of a new early neural marker gene that demarcates most of the developing neural cells. One interesting aspect of this gene is that it represents an "unknown" gene, with a very strong expression in the CNS. Furthermore we have identified a region of about 1000 bases able to reproduce the endogenous gene expression. Therefore both the gene and its regulatory region can represent a useful tool to obtain novel findings on CNS differentiation during *Ciona* embryogenesis.

2. Materials and methods

2.1. Animals and embryos

Adult *Ciona* were collected in the bay of Naples by the fishing service of the Stazione Zoologica. Naturally spawned eggs were fertilized *in vitro* with a suspension of sperm and fertilized eggs were raised in filtered sea water at 18–20 °C. Tadpole larvae hatched about 18–20 h after fertilization. Samples at appropriate stages were collected by low speed centrifugation and were fixed for whole-mount *in situ* hybridization or observed directly under the microscope after electroporation.

2.2. cDNA and sequence analysis

The cDNA was sequenced using an automated DNA sequencer (ABI 3730 DNA analyzer, Applied Biosystem) and blasted against *Ciona intestinalis* Genome Collection (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html). The gene analysis was performed by Genscan engine (http://bioweb.pasteur.fr/seqanal/interfaces/genscan.html). Homology search for the predicted amino acid sequence was performed using the BLAST network service (NCBI), DomPred (http://bioinf.cs.ucl.ac.uk/dompred/) and PredictProtein (http://www.predictprotein.org/).

2.3. In situ hybridization

The cDNA, corresponding to the clone AK112198, was used as template for *in vitro* transcription by using Boehringer Mannheim DIG RNA labeling Kit, according to the supplier's instruction, and stored in H_2O at −80 °C. *In situ* hybridization analysis was done according to Ristoratore et al. (1999). Embryos were analysed and images were captured with a Zeiss Axio Imager M1 microscope.

2.4. Ci-Pans constructs and electroporation

A 1130 kb DNA fragment, upstream the *Ci-Pans* cDNA start site, was amplified by PCR on genomic DNA using two oligonucleotides (*pCi-Pans4*: 5′-TAAAGTTAAACAAACAA-CAAACACAACAAT-3′ and *pCi-Pans6r*: 5′-GCGTGATTT-CTCTTTGTTTCTTTCATTCTC-3′) designed on the basis of the genome sequence available at the JGI *Ciona* genome project database. The fragment was fused to GFP into pBluescript vector and the construct was electroporated into fertilized eggs according to the protocol by Corbo et al. (1997).

Electroporated larvae were observed with epifluorescence microscopy (Zeiss Axio Imager M1 microscope).

3. Results and discussion

3.1. Ci-Pans cDNA and sequence analysis

In situ hybridization screen was used to find genes potentially involved in the CNS differentiation. The factors were selected both by choosing at random clones from a *C. intestinalis* cDNA collection (kindly provided by N. Satoh) and by searching, through available expression pattern databases and published literature, for genes expressed in CNS.

The screen led us to the identification of a new gene, strongly expressed in the CNS at the larval stage (Fig. 3H,H′). The clone, whose sequence was already submitted to the Genbank Database with accession number AK112198 (Satou et al., 2002) (Fig. 2), did not show any homology with known genes and was named by us as *Ci-Pans*.

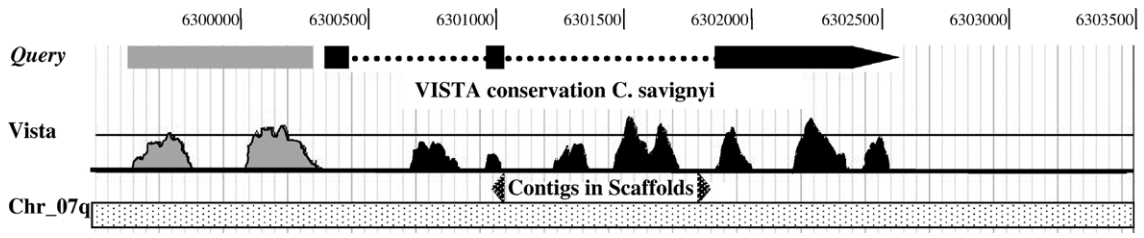

Fig. 1. Results of the BLAST analysis against the JGI genome project. The major homology was found on chromosome 7, but no annotation was identified. The black bars represent the putative exons interposed with introns. The grey box indicates the 1 kb promoter region isolated in this study and able to recapitulate the profile of the endogenous gene. On the bottom is the visual representation of the alignment with *Ciona savignyi*. The conserved regions, found with the Vista program, are indicated as grey areas, for the promoter region, and as black areas for the gene.