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Cis-regulatory organization of the *Pax6* gene in the ascidian *Ciona intestinalis*

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Abstract

The *Pax6* gene has attracted intense research interest due to its apparently important role in the development of eyes and the central nervous system (CNS) in many animal groups. *Pax6* is also of interest for comparative genomics since it has not been duplicated in tetrapods, making for a direct orthology between the *Ciona intestinalis* gene *CiPax6* and *Pax6* in mammals. *CiPax6* has been shown to be expressed in the anterior brain, caudal nerve cord, and in parts of the brain associated with the photoreceptive ocellus. This information was extended here using *in-situ* hybridization, and shows that *CiPax6* transcripts mark the lateral regions of the nerve cord, remarkably similar to *Pax6* expression in the mouse. As a means of dissecting the *cis*-regulation of *CiPax6* we tested 8 kb of sequence using transient reporter transgene assays. Three separate regions were found that work together to drive the overall *CiPax6* expression pattern. A 211 bp sequence 2 kb upstream of the first exon was found to be a major enhancer driving expression in the sensory vesicle (the anterior portion of the ascidian brain). Other upstream sequences were shown to work with the sensory vesicle enhancer to drive expression in the remainder of the CNS. An "eye enhancer" was localized to the first intron, which controls specific expression in the central portion of the sensory vesicle, including photoreceptor cells. The fourth intron was found to repress ectopic expression of the reporter gene in middle portions of the embryonic brain. Aspects of this overall regulatory organization are similar to the organization of the *Pax6* homologs in mice and *Drosophila*, particularly the presence of intronic elements driving expression in the eye, brain and nerve cord.

Keywords: Transcriptional regulator; Urochordate; Tunicate; Enhancers

Introduction

The *Pax6* gene encodes a transcription factor that has been implicated in the development of eyes throughout the animal kingdom (Gehring, 1998, 2002). *Pax6* also has important roles in other parts of the central nervous system (CNS) in both flies and vertebrates (Gruss and Walther, 1992; Simpson and Price, 2002). *Pax6* is a member of the paired-box family of transcription factors, and contains both a paired domain and a homeodomain. It is of special interest in an evolutionary context since it has not been duplicated in the ascidian and tetrapod lineages, unlike many other developmental genes, and therefore the *Pax6* genes across chordate phylogeny can be related as orthologs without the complication of duplicated paralogs.

Pax6 is expressed in the CNS and eyes in many, if not all, animal groups. In ascidians *Pax6* is expressed strongly in the anterior and posterior portions of the brain, and in the dorsal nerve cord (Glardon et al., 1997; Mazet et al., 2003; this paper). Interestingly, its expression is absent from middle portions of the brain, reminiscent of its absence from the vertebrate midbrain, and thus it appears to be similarly deployed along the anterior–posterior axis of both ascidians and vertebrates.

The *cis*-regulation of *Pax6* has been studied extensively in mouse (Griffin et al., 2002; Kammandel et al., 1999; Kleinjan et al., 2004, 2006; Xu et al., 1999) and fly (Adachi et al., 2003; Hauck et al., 1999) and to a lesser extent in pufferfish (Griffin et al., 2002; Kammandel et al., 1999) and quail (Plaza et al., 1999; reviewed in Morgan, 2004). These studies provide much material for comparison of the *cis*-regulation of gene homologs in disparate metazoan species.

The ascidians *Ciona intestinalis* and *C. savignyi* are emerging as important model systems for the study of chordate

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gene regulation and networks (Cone and Zeller, 2005; Di Gregorio and Levine, 2002; Satoh and Levine, 2005). Ascidians have a very simple version of the chordate body plan. However, several studies have shown that some major developmental gene expression patterns are conserved with those of higher chordates, such as vertebrates (e.g. Corbo et al., 1997a; Wada et al., 1998). *C. intestinalis* has a very compact genome, which along with its congener *C. savignyi* has been completely sequenced, allowing for genomic comparisons. Often *cis*-regulatory elements are found quite close to the gene they regulate, which makes dissection of *cis*-regulation less strenuous than in many other taxa. Also, transient reporter transgenes for studying *cis*-regulation are easily delivered to many embryos simultaneously — a major technical advantage for this species (Zeller, 2004).

We present here an analysis of 8 kb of non-coding sequence upstream and in two large introns of the *Pax6* gene in *C. intestinalis (CiPax6)* using transient reporter transgenesis. This region encompasses all the conserved non-coding sequences found in comparisons of the *C. intestinalis* and *C. savignyi Pax6* regions. We identified major enhancers for expression in the central nervous system (CNS) upstream of the transcription start site. We also identified eye (ocellus) specific enhancers in the first intron. Finally, we found evidence that highly conserved sequences in the fourth intron act as repressors of ectopic expression in the CNS. We also examined in more detail than previously reported the transcript expression pattern of *CiPax6,* and discuss its similarities and differences with patterns seen in other animals.

Materials and methods

Animals

Adult *C. intestinalis*, sp. B (Nydam and Harrison, 2007) were collected from floating docks in the Point Judith Marina at Snug Harbor, Rhode Island. Some animals used for electroporation (*C. intestinalis*, sp. A) were obtained commercially from M-Rep, Carlsbad, California. Gametes were collected by dissection and spawned *in vitro*. Embryos for *in-situ* hybridization or electroporation were chemically dechorionated at spawning.

Reporter transgenes

Reporter constructs were based on a reporter vector, TV13, modified from 72-1.27 (Corbo et al., 1997b) (kindly provided by A. Di Gregorio and M. Levine) which in turn is a derivation of pPD1.27 (Fire et al., 1990). Rare-cutting restriction sites were added to 72-1.27 by cutting with *Pst*I and *Xba*I and inserting a cohesive-end oligonucleotide linker with *Asc*I and *Fse*I sites. Subsequently, this construct was cut at *Pfl*F1 and *Bgl*II sites downstream of the reporter gene and a linker with *Rsr*II and *Pac*I sites was inserted to make TV13. This vector has a multiple cloning site, nuclear localization signal, *lacZ* sequence, SV40 polyadenylation signal, and the *Rsr*II/*Pac*I downstream cloning site.

Individual constructs were made using the polymerase chain reaction (PCR) to amplify desired fragments of a lambda clone (P3A) obtained from a *C. intestinalis*, sp. B (Rhode Island, USA population) genomic library. This library was constructed in the BlueSTAR lambda vector (Novagen, Madison, WI, USA) and a 15-kb clone containing the *CiPax6* gene and flanking sequence was subcloned in pBlueSTAR-1 by *Cre*-mediated excision. PCR primers had restriction sites designed on the 5′ ends depending on the desired cloning sites in the TV13 vector.

Electroporation

Transgenes were delivered by electroporation. Fertilized eggs were dechorionated using 0.4 mg/ml Pronase E (P5147, Sigma, St. Louis, MO, USA) in 1% sodium thioglycolate in filtered sea water (FSW) pH 10.1 for 2 min. at 18°C. 150 µl of dechorionated single cell embryos in FSW (approx. 50 embryos) were transferred to the electroporation solution (50–100 ug super-coiled transgene DNA in mannitol for a final mannitol concentration of 0.5 M) in a 0.4 cm electroporation cuvette. A square wave pulse was delivered using a BTX ECM 830 electroporation device (Harvard Apparatus, Holliston, MA, USA). The contents of the cuvette were immediately decanted into a 150 mm × 15 mm gelatin-coated petri dish of FSW with antibiotics (approx. 15 U penicillin and 15 µg streptomycin per milliliter) and incubated at 14–18 °C to the desired stages.

β-Galactosidase detection

Expression of the *lacZ* transgene was detected using either standard X-gal histochemistry, or immunofluorescence. For immunofluorescence, an anti-β-galactosidase monoclonal antibody, 40-1a (Developmental Studies Hybridoma Bank) was used with a goat anti-mouse secondary antibody conjugated to AlexaFluor 488 (Invitrogen).

In-situ hybridization

A 1.4-kb *CiPax6* clone was obtained from a 9 h *C. intestinalis,* sp. B cDNA library in lambda-ZapII (kindly provided by T. Meedel). A riboprobe was made using the Maxi-Script kit (Ambion, Austin, TX, USA) with digoxygenin-UTP as label (Roche, Indianapolis, IN, USA). Whole mount *in-situ* hybridization was performed as previously described (Irvine, 2007; Irvine et al., 2007).

Results

Endogenous CiPax-6 transcript and expression pattern

We screened a *C. intestinalis*, sp. B cDNA library, and the longest clone obtained was 1.4 kb. This clone is highly similar to the Ghost cDNA Database (URL: http://ghost.zool.kyoto-u.ac.jp/indexr1.html) clone ci0100144072, being only 10 bp shorter at the 5′ end. We found no evidence for alternate splice variants in our screen, however, the Ghost Database lists another clone from an adult cDNA library, cima822k22, which is 216 bp longer at the 5′ end. This is a splice variant listed on the JGI v. 2 genome browser (URL: http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) as estExt_fgenesh3_pg.C_chr_09q0597. Since we were examining embryonic stages only, we confined our study to the shorter embryonic 5′ variant, which will be the cDNA referred to as *CiPax6*.

As a means of interpreting reporter transgene assays we examined the transcript expression pattern of *CiPax6* using WMISH. Portions of the *CiPax6* expression pattern have been previously described by others (Mazet et al., 2003; Satou et al., 2005), ANISEED: http://crfb.univ-mrs.fr/aniseed/index.php). We first examined the gastrula stage, which exhibits one bilateral pair of cells that hybridize with the *CiPax6* riboprobe in the neural plate (Fig. 1A). These are the A9.30 cells which are in the lineage contributing to brain and pigment cells (Nishida, 1987; Nicol and Meinertzhagen, 1988; Imai et al., 2004). By the neurula stage this expression has expanded to bilateral ranks of cells corresponding to the neural tube anlage, which further lengthen as the tailbud embryo develops (Figs. 1B–D). At mid-