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A modular *cis*-regulatory system controls isoform-specific *pitx* expression in ascidian stomodæum☆

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Abstract

Pituitary homeobox (*pitx*) genes have been identified in vertebrates as critical molecular determinants of various craniofacial ontogenetic processes including pituitary organogenesis. Accordingly, a prominent conserved feature of *pitx* genes in chordates is their early expression in the anterior neural boundary (ANB) and oral ectoderm, also known as the stomodæum. Here we used the ascidian model species *Ciona intestinalis* to investigate *pitx* gene organization and *cis*-regulatory logic during early stages of oral development. Two distinct *Ci-pitx* mRNA variants were found to be expressed in mutually exclusive embryonic domains. *Ci-pitx* and vertebrate *pitx2* genes display remarkably similar exon usage and organization, suggesting ancestry of the *pitx* transcriptional unit and regulation in chordates. We next combined phylogenetic footprinting, transient transgenesis, and confocal imaging methods to study the *Ci-pitx cis*-regulatory system, with special emphasis on the regulation of isoform-specific ANB/stomodæal expression. Among 10 conserved noncoding sequences (CNSs) interspersed in *C. intestinalis* and *Ciona savignyi pitx* loci, we identified two separate *cis*-regulatory modules (CRMs) that drive ANB/stomodæal expression in complementary spatiotemporal patterns. We discuss the developmental relevance of these data that provide an entry point to investigate the gene regulatory networks (GRNs) that position and shape oral structures in chordates.

Keywords: Ascidian; Gene regulation; Pituitary homeobox; Oral development; Differential isoform expression; Evolution

Introduction

In vertebrates, craniofacial development involves cells of diverse ontogenetic origins including anterior mesendoderm, cranial neural crest, neural tube, placodes, and epidermis (Le Douarin et al., 1997). In particular, olfactory and oral epithelia, as well as the adenohypophysis, derive from a morphogenetic unit known as the stomodæum, which eventually invaginates, fuses with endodermal pharyngeal epithelium, and forms the oral opening. Lineage studies showed that the stomodæum arises from an anterior ectodermal territory that encompasses the anterior neural boundary (ANB) and contacts anterior endoderm (Couly and Le Douarin, 1985; Kouki et al., 2001). Thus, stomodæal cell specification must integrate a dual epidermal/neural origin, a pivotal position in the body plan, and maintain diverse developmental potentialities (Wardle and Sive, 2003).

During organogenesis, field-specific selector genes are key components of the gene regulatory networks (GRNs) that specify homogenous morphogenetic fields through the integration of various developmental cues. These integrative properties are mostly hardwired in selector gene *cis*-regulatory systems, which exhibit transcriptional modularity, and a complex logic for each autonomous *cis*-regulatory module (CRM; Carroll et al., 2001; Davidson, 2001).

Pituitary homeobox (*pitx*) genes are conserved early markers of the stomodæal ectomere in chordates (Boorman and Shimeld, 2002b; Essner et al., 2000; Lanctôt et al.,

Abbreviations: ANB, anterior neural boundary; CRM, *cis*-regulatory module; GRN, gene regulatory network; CNS, conserved noncoding sequence.

☆ *Ci-pitxa/b* mRNA variant, complete cds: GenBank accession no. AY677185. *Ci-pitx*: gene model ci0100147848 at http://genome.jgi-psf.org/ciona/.

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1997; Schweickert et al., 2001b; Uchida et al., 2003). In humans, mutations of *PITX2* in Axenfeld–Rieger patients cause severe facial defects (Semina et al., 1996). In addition, numerous functional studies have demonstrated that various aspects of craniofacial development require *pitx* genes (Kioussi et al., 2002; Schweickert et al., 2001a; Suh et al., 2002; Szeto et al., 1999), suggesting these latter are critical components of the selector gene battery specifying the ANB/stomodæum morphogenetic field. The above-mentioned considerations raise further questions about upstream mechanisms that trigger and precisely define *pitx* stomodæal expression and to which extent these mechanisms are conserved in chordates.

In osteichthyans, *pitx* gene regulation occurs at both transcriptional and posttranscriptional levels. For instance, the conserved *pitx2* transcriptional unit uses distinct distal and proximal promoters to produce different isoforms with identical homeodomains and carboxy termini, but different amino termini (Arakawa et al., 1998; Essner et al., 2000; Schweickert et al., 2000).

In addition, *pitx* paralogues and *pitx2* isoforms are expressed in both specific and common domains (Essner et al., 2000; Lanctôt et al., 1997; Schweickert et al., 2001b). Interestingly, functional and comparative gene expression data suggest that a conserved genetic hierarchy controls isoform-specific *pitx2* expression during the establishment of left–right asymmetry (Boorman and Shimeld, 2002a). Thus, key aspects of *pitx* pre- and posttranscriptional gene regulation rely on an ancestral genomic organization and probably emerged before the early radiation of vertebrates. Investigations in a broad range of chordate species are therefore required to address key issues regarding *pitx cis*-regulatory systems (Goodyer et al., 2003; Shiratori et al., 2001).

We previously reported *pitx* gene expression in the anterior neural boundary and stomodæum during ascidian embryonic development (Christiaen et al., 2002). *Ciona intestinalis* and *Ciona savignyi* species are particularly well suitable for the analysis of gene regulation (for recent review, see Satoh et al., 2003). Indeed, genomic information is readily available through interfaced data mining tools (Dehal et al., 2002), transient transformation of numerous embryos can be achieved by simple egg electroporation (Corbo et al., 1997), and reduced cell number throughout embryogenesis allows developmental gene activities to be studied at high resolution (Munro and Odell, 2002; Sardet et al., 2003). We therefore combined interspecific sequence comparison, transient transgenesis, and fluorescent imaging to study *pitx* locus organization and *cis*-regulation during development of the ascidian stomodæum (also referred to as "oral siphon primordium" or "primordial pharynx" in the literature). Our results suggest that the organization and usage of *pitx* exons are conserved in chordates. Moreover, we identified distinct *cis*-regulatory modules that govern isoform-specific ANB/stomodæal expression in complementary spatiotemporal patterns during embryogenesis.

Materials and methods

Animals and embryos

C. intestinalis adults were purchased at the Station Zoologique de Roscoff (Brittany, France). Embryos were obtained by in vitro cross-fertilization in artificial seawater as described (Hudson and Lemaire, 2001). Single cell zygotes were dechorionated according to Mita-Miyazawa et al. (1985), except that the protease was inactivated with glycine (1 mg/ml final in dechorionation solution), which substantially improves survival of the eggs. Electroporation and X-gal staining were performed as previously described (Bertrand et al., 2003; Corbo et al., 1997).

Sequence comparison and analysis

C. intestinalis and *C. savignyi* genomic sequences were obtained from JGI (Dehal et al., 2002, http://genome.jgi-psf.org/ciona/) and from the Broad Institute (http://www.broad.mit.edu/annotation/ciona/), respectively, and handled with the Vector NTI Suite (Informax). The VISTA algorithm (Mayor et al., 2000) was used for sequence alignment and comparison with the following parameters: 80 bp windows, 65% identity threshold. Conserved non-coding sequence (CNS) boundaries were refined by local alignment.

Molecular cloning and dissection of Ci-pitx genomic fragments

A mixture of 5′ and 3′ *Ci-pitx* RACE fragments was used as ^{32}P-labeled probes to screen a *C. intestinalis* genomic cosmid library (Burgtorf et al., 1998). The distal fragment was PCR amplified from cosmid clone MPMGc119J0337Q3 using vector-anchored forward primer and a reverse primer annealing in *Ci-pitx* exon1 (primer details in Supplementary Materials and methods) with the proofreading ThermalAce DNA polymerase (Invitrogen). The PCR fragment was inserted in pCRII-TOPO (Invitrogen), mapped by restriction analysis, and subcloned in pSP1.72-pPD27 expression vector (Corbo et al., 1997). Deleted constructs were obtained using native restriction sites and PCR-based constructs. The 8.5-kb proximal region was excised as an *Xho*I fragment from the cosmid DNA, subcloned into pSP1.72-pPD27, and recut with *Not*I. A first series of deleted constructs was made using native restriction sites. P1 to P3 were amplified using *Pfu* DNA polymerase (Promega) and corresponding primer pairs (Supplementary Materials and Methods), digested and cloned into the pCES vector, which contains the *Ci-fkh* core promoter and first codons fused in frame with the *lacZ* coding sequence (Harafuji et al., 2002). The *lacZ* reporter gene was replaced in *pSP1.72-pPD27* by the *venus* enhanced yellow fluorescent protein (EYFP; Nagai et al., 2002) coding sequence to create *pSD1:venus*. Distal and

proximal fragments were cloned into *pSD1:venus* as described above.

Whole mount in situ hybridization

The common 3′ probe was generated from a 3′RACE clone as previously described (Christiaen et al., 2002). Isoform-specific fragments were amplified by PCR from the cosmid DNA, cloned into pCRII-TOPO, linearized, purified, and used for cRNA probe synthesis and digoxigenin labeling (Roche) by in vitro transcription with the Sp6 RNA-polymerase (Promega). In situ hybridization was carried out in an InSituPro automate (Intavis), following an established protocol (Moret et al., in press).

Fluorescent imaging

Electroporated embryos were fixed with 3.7% formaldehyde in calcium and magnesium-free artificial seawater (CMF-ASWH: 463 mM NaCl, 11 mM KCl, 25.5 mM Na_2SO_4, 2.15 mM $NaHCO_3$, 10 mM HEPES, pH 8.1, recipe courtesy of Dr. P. Lemaire), permeabilized with PBTT1 (0.4% Triton X-100, 0.2% Tween 20 in phosphate buffer saline (PBS)), aldehyde autofluorescence was quenched with 50 mM NH_4Cl in PBTT2 (0.2% Triton X-100, 0.1% Tween 20 in PBS) and counterstained with Alexafluor®568-phalloidin (1/500) and DAPI (3 nM), diluted in PBTT2. Labeled embryos were equilibrated and mounted in glycerol/PBS (1:1, v/v), supplemented with 2% DABCO (w/v, Sigma D-2522). Images were captured within 2 days on a Leica DM RXA2 microscope equipped with a TCS SP2 confocal scanning system.

Results and discussion

Ci-pitx encodes distinct isoforms expressed in complementary domains

Previous studies independently reported cDNA cloning of a single *C. intestinalis pitx* ortholog (Boorman and Shimeld, 2002b; Christiaen et al., 2002). Detailed comparison with current genomic and EST data led us to hypothesize the existence of an alternative Ci-pitx isoform (Supplementary Fig. S1) and to refine the current gene model (Fig. 1A): the *Ci-pitx* transcriptional unit, which encompasses a 14-kb region, produces two different mRNA isoforms composed of exons3–5 and either exon1 or exon2. We called these isoforms *Ci-pitxa/b* and *Ci-pitxc* by analogy with vertebrates *pitx2a/b* and *pitx2c*, which use distal and proximal isoform-specific upstream exons, respectively (Fig. 1A; note that in addition to comparable linear organization, *Ci-pitx* falls within the size range 5–20 kb of vertebrate *pitx* genes).

Differential isoform expression was assessed in parallel in situ hybridization experiments using isoform-specific and common probes (see Fig. 1A for probe mapping).

At mid-tailbud stage, *Ci-pitxa/b* is only expressed in three cells of the anterior neural boundary (ANB, Figs. 1B and E, and Christiaen et al., 2002). In hatching larvae, the *Ci-pitxa/b*-specific probe labels tail muscles and a crown-shaped domain restricted to the deepest (ventral) part of the stomodæum (Figs. 1C, D, F, and G).

In mid-tailbud embryos, the *Ci-pitxc*-specific probe revealed an epidermal expression in an anterior cap and dorsolateral caudal stripe (Figs. 1B and H), which corresponds to the asymmetric epidermal expression reported in *C. intestinalis* (Boorman and Shimeld, 2002b) and *Halocynthia roretzi* (Morokuma et al., 2002). In addition, transient *Ci-pitxc* expression was observed in two cells of the posterior cerebral vesicle (Figs. 1B and H). At hatching, *Ci-pitxc* is expressed in photoreceptor cells, left visceral ganglion, and left posterior trunk endoderm (Figs. 1C, D, I, and J, and Supplementary Fig. S2). Interestingly, only *Ci-pitxc* is expressed asymmetrically, which further supports its homology with vertebrate *pitx2c* isoforms (e.g., Essner et al., 2000; Schweickert et al., 2000).

Strikingly, isoform-specific expression patterns are mutually exclusive and the pitx3′ probe labels every above-mentioned domain (Figs. 1B–D), showing that isoform-specific *Ci-pitx* expression profiles are complementary (Figs. 1K–M). In particular, ANB/stomodæal cells uniquely express *Ci-pitxa/b* from mid-tailbud stage till hatching.

Ci-pitx regulation involves separate basal promoters and cis-regulatory regions

To investigate *Ci-pitx cis*-regulation, the spatiotemporal transcriptional activity of two non-overlapping genomic fragments was assessed by transient transgenesis. These fragments map upstream from exons1 and 2 and will henceforth be referred to as distal and proximal, respectively (Fig. 2A).

The distal fragment is active in tail muscles and posterior stomodæum at hatching, extending ectopically in dorso-posterior stomodæal cells (Fig. 2C). At mid-tailbud stage, its activity is restricted to the ANB (Fig. 2B). We obtained identical data with the Venus reporter (Figs. 2H and I) and by in situ hybridization (Supplementary Fig. S2), showing that the distal construct retains stomodæal activity during the whole embryonic period, while expression in tail muscles starts by the end of late tailbud stage (Figs. 2F and G). According to key temporal aspects of *Ci-pitx* ANB/stomodæal expression, the distal construct is not active before early tailbud stage (data not shown).

The proximal construct exhibits a more pleiotropic expression pattern. At mid-tailbud stage, endogenous *Ci-pitx* expression is recapitulated in the posterior cerebral vesicle, but neither in the ANB nor in the epidermis (Fig.

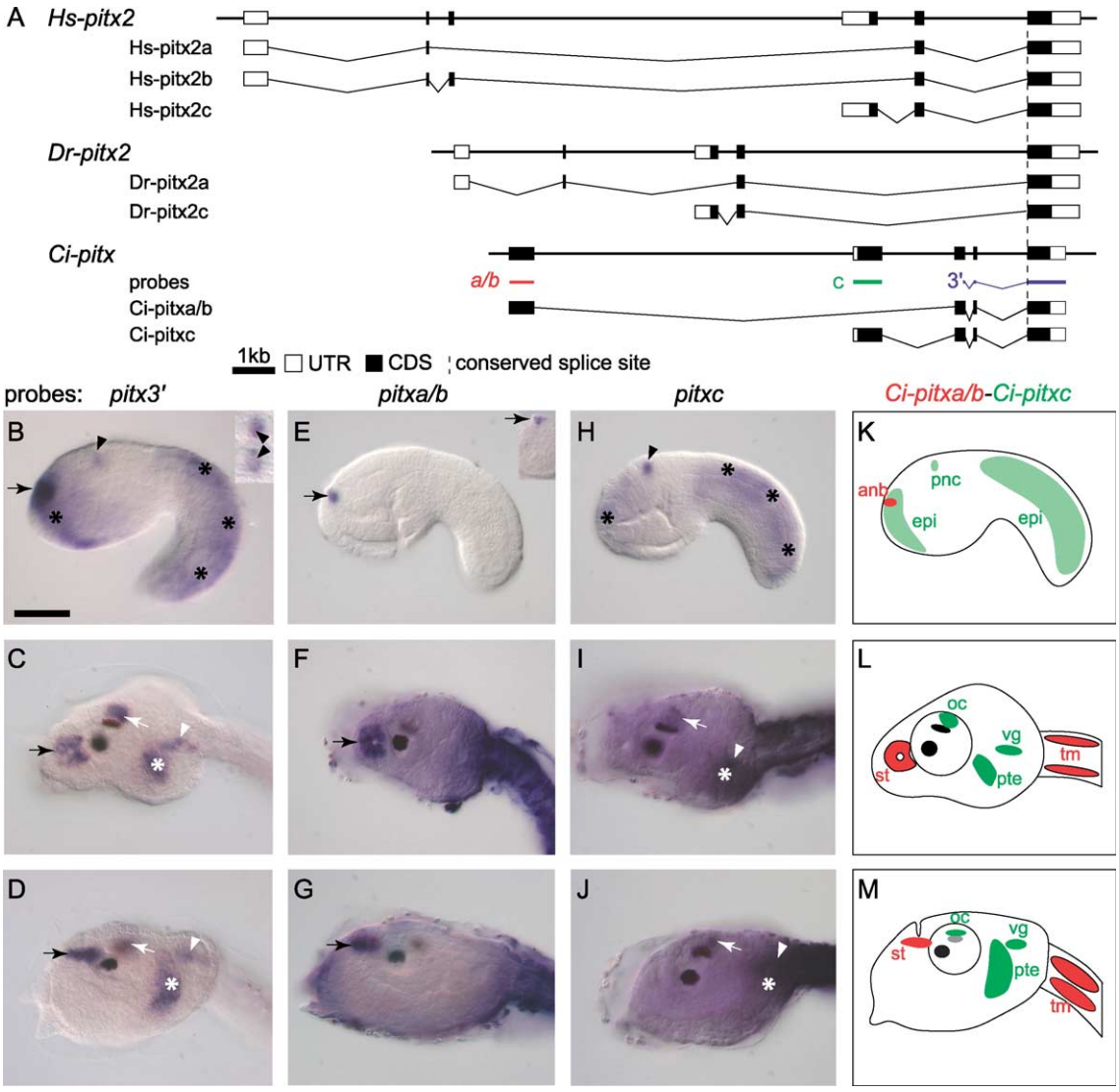

Fig. 1. *Ci-pitx* isoforms are expressed in mutually exclusive domains. (A) Comparative maps of Ciona *pitx*, human and zebrafish *pitx2* loci, and isoforms. Scale bar (1 kb) applies to all gene maps. (B–M) *Ci-pitx* isoforms expression patterns. (B–G) *Ci-pitxa/b*-specific expression in anterior neural boundary (anb, black arrows), stomodæum (st, black arrows), and tail muscles (tm, out of focus in C and D, obscured by background in F and G). (B–D and H–J) *Ci-pitxc*-specific expression in posterior neural cells (pnc, black arrowheads in B and H), left epidermis (epi, black asterisks), ocellus (oc, white arrows), left visceral ganglion (vg, white arrowheads), and left posterior trunk endoderm (pte, white asterisks). (K–M) Summary of *Ci-pitx* isoforms expression. Scale bar: 25 μm.

2D). In addition to widespread ectopic staining (compare Figs. 1D and 2E), the proximal construct labels endogenous *Ci-pitx* expression domains at hatching, including the anterior stomodæum, ocellus, left visceral ganglion, and dorso-posterior cerebral vesicle (Figs. 2E and G; the latter is stained by β-galactosidase activity remaining from the tailbud stage, see Fig. 2D). Expression dynamics was assessed by in situ hybridization, showing that the proximal construct activity is restricted to the anterior stomodæum, ocellus, and visceral ganglion at hatching (Fig. 2G and Supplementary Fig. S2).

The distal and proximal regulatory regions are non-overlapping (Fig. 2A). Nonetheless, both can drive reporter gene expression, indicating that the *Ci-pitx* transcriptional unit contains distinct distal and proximal basal promoters.

The overlap between endogenous and reporter patterns fits the topological relationship between regulatory regions and isoform-specific exons (Figs. 1K–M and 2F and G), raising the simple straightforward conclusion that transcription of each isoform is driven by its own promoter and tissue-specific *cis*-elements. In particular, the distal regulatory region is sufficient for early *Ci-pitxa/b* expression in ANB (Figs. 2B and F).

However, each construct drives reporter gene expression in larval stomodæum (Fig. 2G), which in contrast expresses only *Ci-pitxa/b* (Figs. 1F and G). Confocal imaging revealed that expression of the distal construct extends up to a flexure in the antero-ventral stomodæal layer that faces an actin-rich area in endoderm (Figs. 2J and N). Interestingly, the proximal fragment drives reporter gene expression in an anterior crescent-shaped stomodæal domain (Figs. 2M

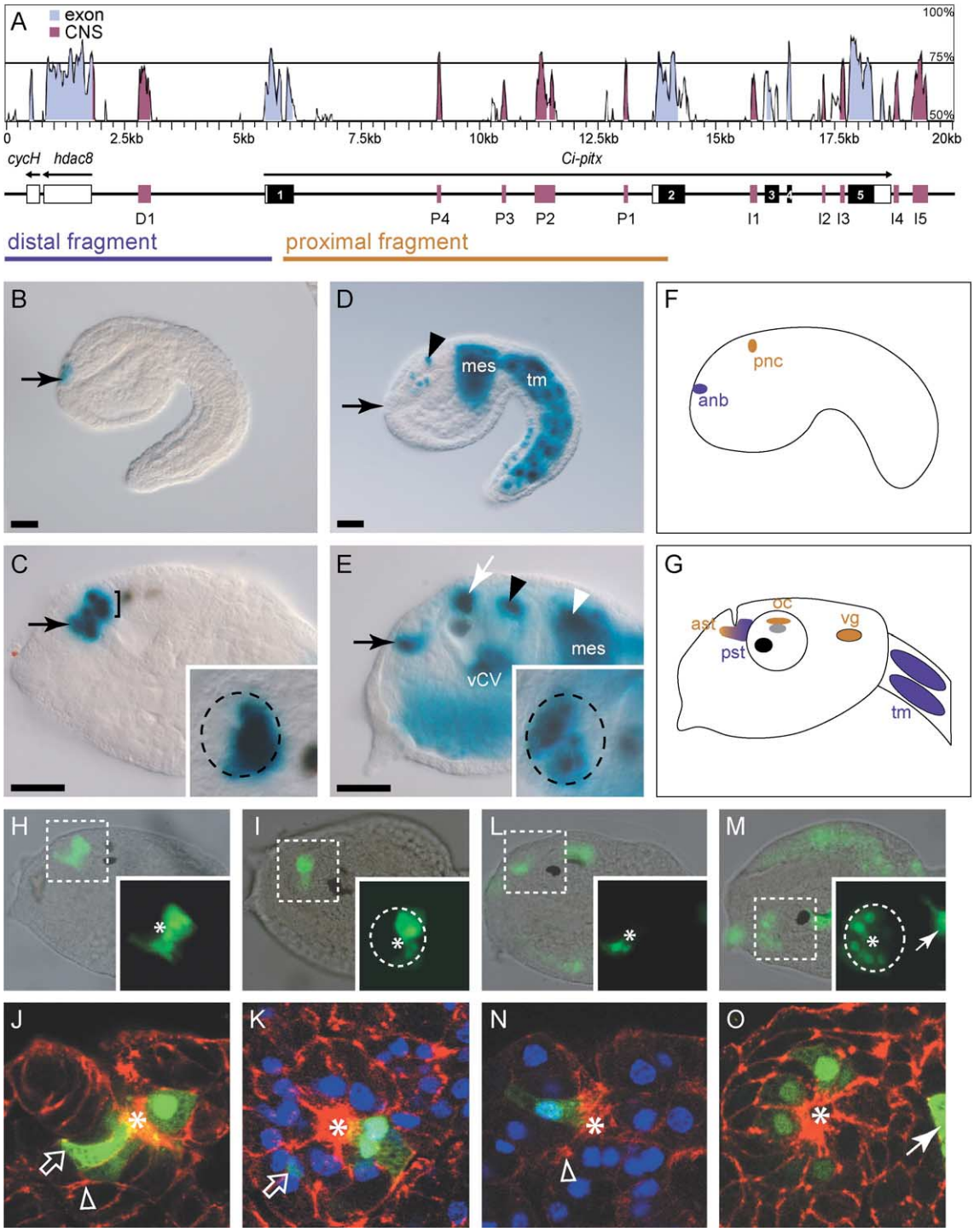

Fig. 2. Distinct *cis*-regulatory regions control *Ci-pitx* expression in complementary stomodæal patterns. (A) VISTA plot (percent identity with *Cs-pitx*) and map of *Ci-pitx* locus showing distal and proximal constructs, exons (open and black boxes), and conserved noncoding sequences (CNSs, purple boxes). (B and C) Distal construct activity in ANB and posterior stomodæum (black arrows, lateral views, inset: close-up, dorsal view). Note the ectopic expression in dorso-posterior stomodæal cells (squared bracket). (D and E) Proximal construct activity in posterior neural cells and derivatives (pnc, black arrowheads), anterior stomodæum (ast, black arrow; inset: close-up, dorsal view), ocellus (oc, white arrows), and visceral ganglion (white arrowhead). Ectopic expression in mesenchyme (mes), tail tissues (tt), and ventral cerebral vesicle (vCV). (F and G) Summary of distal and proximal construct expression in endogenous *Ci-pitx* expression domains, lateral views. Note that asymmetric expression is recapitulated by none of the constructs, suggesting that it requires additional elements, possibly located in the downstream CNS (I1-5) as it is the case in mice (Shiratori et al., 2001). (H–O) Detailed stomodæal activities of distal (H–K) and proximal (L–O) constructs using EYFP (green), lateral (H, J, L, and N) and dorsal (I, K, M, and O) views. Confocal sections (J, K, N, and O) show cell cortices (F-actin, red), nuclei (DAPI, blue); note the antero-ventral stomodæal flexure and endodermal actin-rich area (open arrowheads). Open arrows point to the tip of anterior-most expressing cells. Scale bar: 10 μm.

and O), which lays two cells above this stomodæal flexure (Fig. 2N) and is therefore adjacent to the distal expression domain. Taken together, these data show that distinct *cis*-regulatory elements control *Ci-pitxa/b* expression in complementary stomodæal territories at hatching.

Phylogenetic footprinting of putative cis-regulatory modules in Ci-pitx locus

Next, we sought to identify *cis*-elements regulating *Ci-pitx* with special emphasis on stomodæal expression. Conserved noncoding sequences (CNSs) have recently been emphasized as valuable predictive footprints of functional elements (e.g., Frazer et al., 2003), and actual transcription factor binding sites within *Ci-otx* early neural enhancer showed sequence conservation between *C. intestinalis* and *C. savignyi* (Bertrand et al., 2003). Aiming at uncovering regulatory modules, we extended interspecific sequence comparisons to the 20-kb *pitx* loci.

We found that *pitx* exons are conserved in both sequence and relative positions (Fig. 2A and data not shown). Moreover, conserved noncoding sequences allowed us to define putative *cis*-regulatory modules (pCRMs). We identified one (D1, 307 bp) and four (P1–4; 69, 470, 210, and 118 bp) pCRMs in the above-mentioned distal and proximal noncoding regions, respectively (Fig. 2A).

Noticeably, pCRMs could be readily identified in a global alignment, suggesting that the evolutionary distance between *Ciona* species is adequate to efficiently pinpoint functional elements (see below) by phylogenetic footprinting of large genomic data sets.

D1 is an autonomous ANB/stomodæal enhancer

Sequence conservation suggests that D1 may contribute to distal regulatory inputs in ANB/stomodæal cells. A first series of deleted constructs was generated and tested by transient transgenesis and X-gal staining at larval stage (Fig. 3A, #3–7). These data show that specific abolishment of stomodæal expression correlates with a deletion of the first 188 bp of D1.

We next generated internal deletions that remove D1 (Fig. 3A, #8, 9). These constructs were unable to drive reporter gene expression in the stomodæum, showing that the first 188 bp of D1 is absolutely required for stomodæal expression of the transgenes.

We then asked whether D1 could drive stomodæal expression in a minimal context (Fig. 3A, #10,11). Constructs #10 and #11 drive expression uniquely in the stomodæum, showing that partial D1 is sufficient to enhance transcription specifically in stomodæal cells. The complete and partial D1 fragments could also activate expression in tailbud ANB and larval stomodæum when

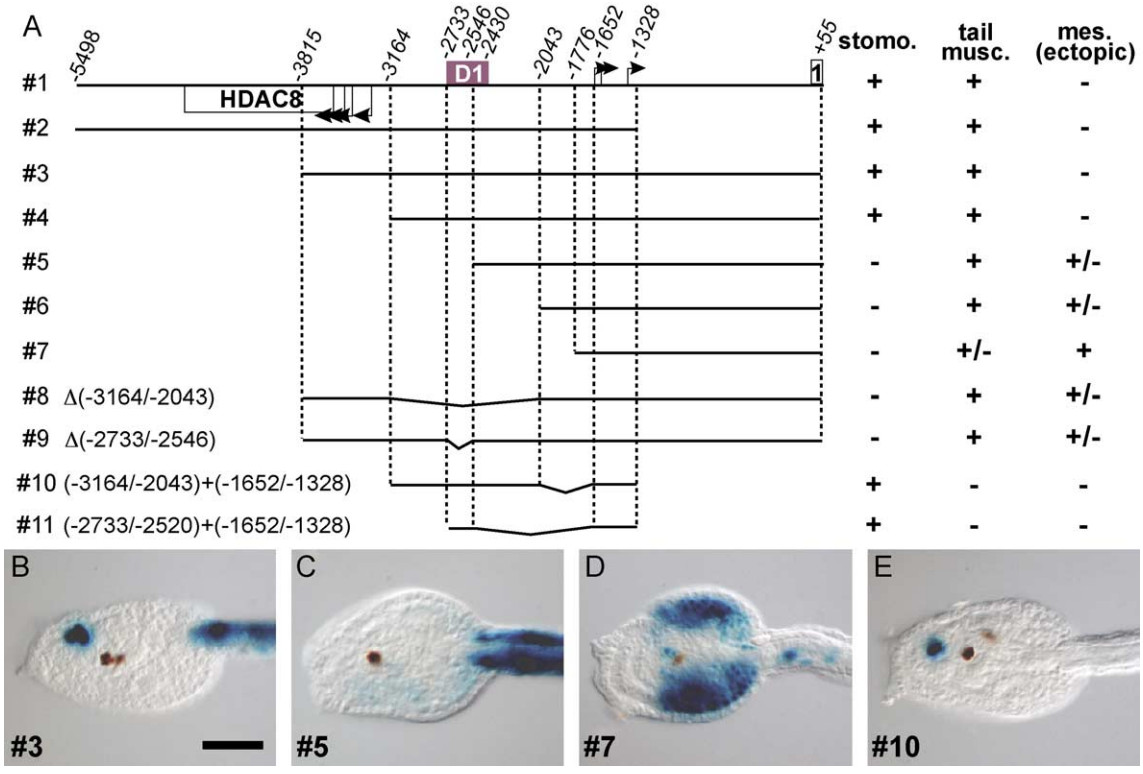

Fig. 3. D1 is necessary and sufficient for stomodæal expression. (A) Molecular dissection of the distal construct. SL *trans*-splicing occurs during the maturation of the *Ci-pitxa/b* primary transcript (Christiaen et al., 2002), distal promoter was roughly mapped between −1652 and −1328 bp (#2 and data not shown). Expression is represented as + or −, meaning that stomodæal expression is "all or nothing" (>85% or 0%), +/− means reduced expression relative to maximum (e.g., compare C and D for expression in tail muscles, and mesenchyme). Scale bar: 20 μm.

fused to the heterologous *Ci-fkh* basal promoter (pFkh in pCES construct, data not shown). Taken together, these data show that D1 is a bona fide stomodæal activation module that could be efficiently identified upon sequence conservation and accounts for ANB/stomodæal activity of the distal regulatory region during embryogenesis.

P2 is a second major stomodæal activation module

We next sought to identify proximal *cis*-regulatory elements driving *Ci-pitx* expression in the anterior crescent-shaped stomodæal territory. We generated a preliminary set of 5′ deleted constructs and focused on stomodæal expression. These deletions were designed to remove successively larger fragments containing the above-mentioned pCRMs, P1 to P4 (Fig. 4A).

The first deletion does not affect reporter gene pattern (Fig. 4B), suggesting that P4 is not required for stomodæal expression during embryogenesis.

The next deletion removes P3 and leads to a substantial reduction of stomodæal staining at hatching (Figs. 4A and C), but not at later stages (data not shown). In addition, P3 is unable to drive stomodæal expression when fused to a heterologous basal promoter (Fig. 4A, pCES:P3). Thus, P3 is likely to be a co-activation module, not sufficient for stomodæal expression on its own.

The next deletion removes P2 and abolishes stomodæal expression, opening the possibility that this latter requires P2 (Fig. 4A). We next found that a P2:pFkh fusion drives reporter gene expression in anterior stomodæal cells (Figs. 4A and D). P2 thus appears as an additional autonomous stomodæal enhancer.

Given the topology of the *Ci-pitx* locus, *Ci-pitxa/b* expression implies that transcription is initiated from the distal core promoter in stomodæal cells (Figs. 1A and L). Therefore, we assessed the ability of P2 to drive stomodæal expression through the distal promoter (Figs. 4A and E, P2:pdist). This expression was enhanced by P3, which appears again as a co-activation module in stomodæal cells (Fig. 4A, P2P3:pdist).

The lack of detectable *Ci-pitxc* expression suggests that the proximal core promoter is isolated from the P2 input in stomodæal cells. The mechanism of P2 promoter selectivity remains elusive. However, close examination of core promoter sequences revealed the presence of a TATA-box in the distal core promoter, while the proximal promoter only possesses a putative downstream promoter element (Kadonaga, 2002; see supplementary Fig. S3). Such a difference in basal promoter sequence could account for P2 promoter preference, as reported for AE1 and IAB5 elements in *Drosophila* (Ohtsuki et al., 1998), thus leading to correct isoform expression in stomodæal cells.

These observations led us to propose that P2 is a second major stomodæal enhancer, its input on the distal core promoter being eventually reinforced by the P3 co-activation module (Fig. 5D). The transcriptional outcome

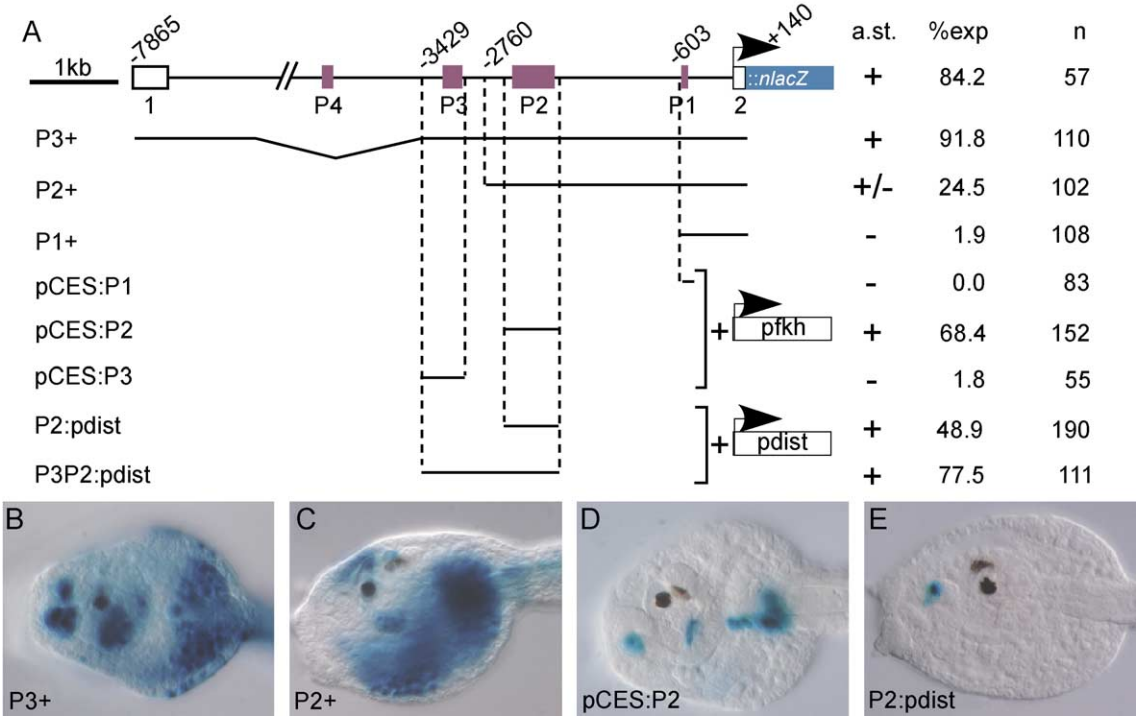

Fig. 4. P2 is a major stomodæal enhancer. (A) Molecular dissection of the proximal construct. Expression is scored both qualitatively (+, +/−, −) and semiquantitatively (%exp: percentage of expression, n: number of embryos). (B–E) Characteristic embryos and corresponding constructs. Anterior stomodæal expression in B, D, and E (black arrow). Note the ectopic epidermal staining in C. Mosaic embryos (such as D and E) were scored positive for stomodæal expression.

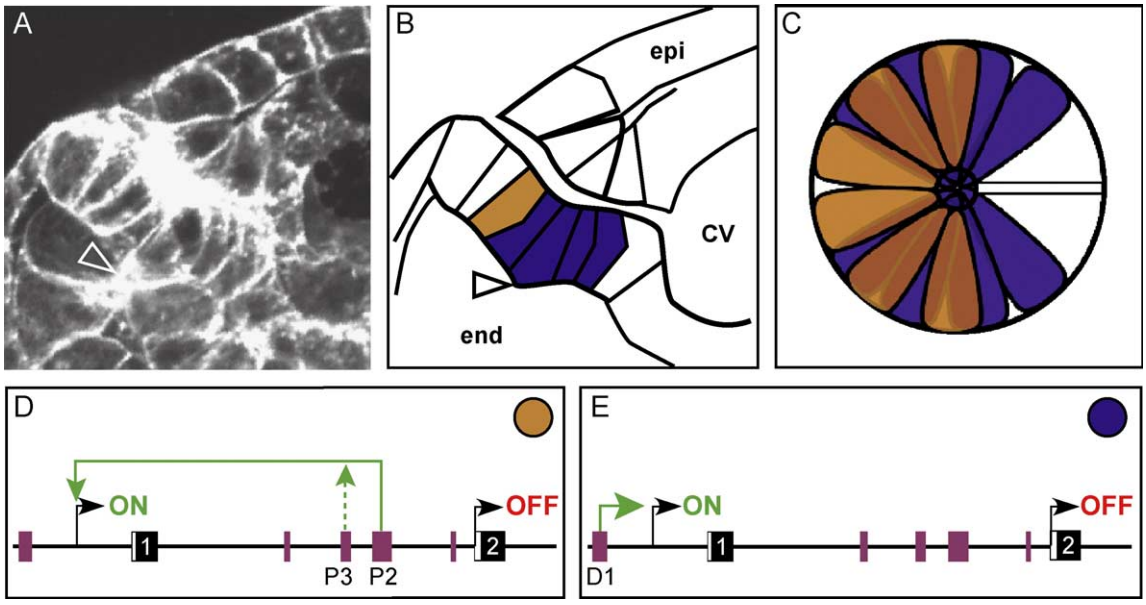

Fig. 5. Model of *Ci-pitx cis*-regulation in ascidian stomodæum. (A) Confocal section through the stomodæum of a hatching larva stained with Alexa488-conjugated phalloidin (labels F-actin), sagittal view, dorsal up, anterior left. Note the stomodæal flexure (open arrowhead), epidermal cells have a cuboidal shape, while stomodæal cells are rather prismatic. (B) Realistic drawing from A, the *cis*-regulatory logics (D and E) were mapped using the stomodæal flexure as morphological landmark (color codes apply to B–E). (C) Schematic mapping of *cis*-regulatory logic in larval stomodæum, dorsal view, anterior left. Importantly, the D1 *cis*-regulatory input (blue dot) also controls expression in the anterior neural boundary at mid-tailbud stage (epi, epidermis; cv, cerebral vesicle; end, endoderm).

of such combinatory interplay of individual *cis*-regulatory modules would result in accurate *Ci-pitxa/b* expression in the anterior crescent-shaped stomodæal territory (Figs. 5B and C), which is complementary to the D1 activation pattern in posterior stomodæal cells (Figs. 5B, C, and E) and ANB.

This current model, together with confocal imaging of reporter gene expression, lets us envision elements of stomodæum patterning in ascidians (Figs. 5A–C). In particular, distinct superficial and deep stomodæal compartments can be defined upon restricted *Ci-pitxa/b* expression in ventral/deep stomodæal cells. Lineage studies suggest that these superficial and deep stomodæal compartments originate from a/*a*6.7 and a/*a*6.5, sixth generation blastomeres, respectively (Nishida and Satoh, 1985). Moreover, neural induction overrides a-line epidermal default fate in a/*a*6.5 blastomeres (Hudson and Lemaire, 2001), suggesting that distinct stomodæal compartments in ascidians may reflect dual ontogenetic origin of the stomodæum in chordates.

Though clonal restriction of cell fate is of special importance in ascidians (reviewed in Satoh, 1994), it does not fully explain stomodæal cell specification. In fact, deep stomodæal cells originate from a/*a*8.19 and a/*a*8.17 blastomeres (descendants of a/*a*6.5), which share posterior position after the eighth cleavage, while their respective a/*a*8.20 and a/*a*8.18 anterior sister cells give birth to the palps (Nishida, 1987). In addition, the anterior cerebral vesicle also arises from a/*a*8.19 and a/*a*8.17 (Nishida, 1987). Taken together, these data suggest that stomodæal fate is established after the eighth cleavage, possibly by position-dependant induction events that overcome differences in clonal origins. Indeed, the stomodæal presumptive territory encompasses neural and nonneural ectoderm and contacts anterior endoderm. The complexity of *pitx cis*-regulation in ascidians might therefore witness combined developmental cues that position the ancestral chordate mouth and associated structures.

This study provides us with an entry point to investigate the genomic hardwiring of GRNs underlying oral development in chordates, with the hope that the *Ciona* system could lead the way toward a detailed comprehension of stomodæal GRN in the more complex vertebrate species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.10.008.

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