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Expression of the *Distalless-B* gene in *Ciona* is regulated by a pan-ectodermal enhancer module☆

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ABSTRACT

The *Ci-Dll-B* gene is an early regulator of ectodermal development in the ascidian *Ciona intestinalis* (Imai et al., 2006). *Ci-Dll-B* is located in a convergently transcribed bigene cluster with a tandem duplicate, *Ci-Dll-A*. This clustered genomic arrangement is the same as those of the homologous vertebrate Dlx genes, which are also arranged in convergently transcribed bigene clusters. Sequence analysis of the *C. intestinalis Dll-A-B* cluster reveals a 378 bp region upstream of *Ci-Dll-B*, termed B1, which is highly conserved with the corresponding region from the congener *Ciona savignyi*. The B1 element is necessary and sufficient to drive expression of a *lacZ* reporter gene in a pattern mimicking the endogenous expression of *Ci-Dll-B* at gastrula stages. This expression pattern which is specific to the entire animal hemisphere is activated preferentially in posterior, or b-lineage, cells by a central portion of B1. Expression in anterior, or a-lineage cells, can be activated by this central portion in combination with the distal part of B1. Anterior expression can also be activated by the central part of B1 plus both the proximal part of B1 and non-conserved sequence upstream of B1. Thus, *cis*-regulation of early *Ci-Dll-B* expression is activated by a required submodule in the center of B1, driving posterior expression, which works in combination with redundant submodules that respond to differentially localized anterior factors to produce the total animal hemisphere expression pattern. Interestingly, the intergenic region of the cluster, which is important for expression of the Dlx genes in vertebrates, does not have a specific activating function in the reporter genes tested, but acts as an attenuator in combination with upstream sequences.

Introduction

The Distalless or Dlx family of homeodomain transcription factors have been identified as important developmental regulatory proteins in a wide range of animal groups. First identified in *Drosophila*, *distalless* is required for proper limb and central nervous system (CNS) development. In vertebrates, the *distalless* orthologs, termed Dlx genes, are found in two-gene clusters. Mammals have 3 two-gene clusters, for a total of 6 Dlx genes, while teleost fishes have as many as 5 two-gene clusters and 8 Dlx genes (Sumiyama et al., 2003). Major functional roles for the vertebrate genes have been found in the brain, neural crest and branchial arch derivatives, bone and cartilage formation, and limb development (Panganiban and Rubenstein, 2002).

In protochordates, one Dlx gene has been found in the cephalochordate amphioxus, and three in ascidian tunicates, such as *Ciona intestinalis*. Two of the *Ciona* genes, *Dlx-A* and *Dlx-B*, are tightly linked in a cluster, with the same convergent transcriptional orientation seen in all vertebrate Dlx clusters, suggesting that this cluster may be homologous to those in vertebrates (Caracciolo et al., 2000; Irvine et al., 2007). The *C. intestinalis* Dlx genes are expressed in several tissues: *Ci-Dll-A* is expressed in the adhesive and sensory palps and precursors of the atrial siphon; *Ci-Dll-C* transcripts are found in the adhesive papillae; and *Ci-Dlx-B* is expressed in the early ectoderm and is soon restricted to the epidermal lineage (Caracciolo et al., 2000; Imai et al., 2004; Irvine et al., 2007). Consistent with this expression pattern, *Ci-Dll-B* has been identified as a pivotal gene in the regulatory network for the epidermis (Imai et al., 2006).

The mRNA expression patterns for the Dlx genes in vertebrates have been shown to be largely overlapping between the two genes from one cluster (Ellies et al., 1997; Sumiyama et al., 2002). This finding has led to the notion that shared enhancer elements may control the coordinate expression of both genes of a cluster (Ellies et al., 1997). Subsequent analysis of genomic regulatory elements in zebrafish and mouse has focused on intergenic enhancer elements that are highly conserved between orthologous clusters in all vertebrate groups (Ghanem et al., 2003). These elements have been shown to separately regulate expression of Dlx genes in the brain, limbs, branchial arches and branchial arch derivatives (Ghanem et al., 2003; Park et al., 2004; Sumiyama et al., 2002; Sumiyama and Ruddle, 2003).

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In order to better understand the genomic regulation of a simple 2-gene cluster, we have examined the genomic regulation of *Ci-Dll-B*, using reporter transgene analysis. We have located a 400 bp enhancer upstream of the transcription start site that drives the total early endogenous pattern of expression in prospective ectoderm. We have further dissected this element to find that the anterior and posterior portions of the expression are controlled by separate portions of the enhancer. We also have evidence that the organization of the *cis*-regulatory elements in *C. intestinalis* differs considerably from the regulatory organization of the vertebrate Dlx clusters despite the fact that the convergently transcribed genomic arrangement of the genes is the same.

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. Gametes were collected by dissection and spawned *in vitro*. Embryos for electroporation were chemically dechorionated at spawning.

Reporter transgenes

The CiDB-A construct was made from a lambda clone (D5) 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, U.S.A.) and a 15 kilobase clone containing the entire *Dll-B–Dll-A* cluster and flanking sequence (Fig. 1) was subcloned in pBlueSTAR-1 by *Cre*-mediated excision. The reporter gene cassette from TV13 (Irvine et al., 2008), was inserted into a BglII site in the fifth exon of *Ci-Dll-B* using the InFusion® Dry-Down PCR Cloning Kit (Clontech) to complete the CiDB-A construct.

Other reporter constructs depicted in Fig. 1B were made by amplifying the respective putative regulatory region from lambda clone D5 using primers with restriction sites designed on the 5′ ends. Upstream fragments were cloned into the AscI and NotI sites of TV13. Downstream fragments were cloned into the RsrII and PacI sites.

Reporter constructs CiDB-0.2–0.4; CiDB-0.35–0.62; CiDB-0.42–0.62; CiDB-0.38–0.62; and CiDB-0.35–0.51 were made by amplifying the non-coding sequences from lambda clone D5 by PCR with forward primers with a 5′ SalI site, and reverse primers with a 5′ BamHI site. These fragments were then cloned into the vector CiFk5′A cut with SalI and BamHI. CiFk5′A is a modification of the CiFk-1.77 reporter vector (kindly provided by A. DiGregorio and M. Levine) with a polylinker inserted between the XhoI and EcoNI sites. This produces a transcriptionally silent basal promoter *lacZ* reporter vector (similar to Harafuji et al., 2002). CiFk5′A contains 349 bp upstream of the *Ci-FoxA-a* mRNA sequence (Genbank NM_001078564), the 5′ UTR (39 bp), and the first 86 codons of the ORF (Di Gregorio et al., 2001) fused to a nuclear localization signal, *lacZ*, and the SV40 polyadenylation signal.

Constructs CiDB-1.0d4, d5, d7, and d10, were made by PCR deletion using the Phusion Site Directed Mutagenesis kit (New England Biolabs). Primer sequences are available upon request.

The mutated GATA site in CiDB-0.35–0.62mG was created by PCR using the primers CiDBgmut (5′-GACGCGCTGCTCTAAGAAGTCATT) and CiDBf45 (5′-GAAAAACAATGCATTTCTCGGTAGGCT). This converts the sequence on the reverse strand from GATAA to TCTAA. All ligations, deletions, and site-directed mutagenesis were confirmed by sequencing.

Electroporation

Transgenes were delivered to single-celled embryos by electroporation, and *lacZ* reporter signal detected, as previously described (Irvine et al., 2008). Embryos were reared to mid to late gastrula stage and the number of animal hemisphere quadrants with detectable ß-galactosidase staining counted for each normally developing embryo. Each construct was tested in 3 or more electroporation experiments (except for CiDB-1.0d5 tested twice), and the results were pooled to derive percentages of anterior and posterior quadrants with reporter gene expression (refer to Supplementary Table 1).

For statistical testing of differences between reporter experiments, percentages of anterior or posterior embryonic animal quadrants with reporter expression for individual electroporation experiments were taken as data points. The mean anterior or posterior percentages for sets of electroporation experiments for different reporter constructs were compared using a nonparametric test, the Kruskal–Wallis test, implemented in SPSS, since it is appropriate for the small sample sizes available, which may not be normally distributed. p-values less than or equal to 0.05 were considered significant.

Sequence analysis

Similarity between putatively homologous sequences were assessed using mVista (http://genome.lbl.gov/vista/index.shtml) (Mayor et al., 2000). For alignment the LAGAN option was used (Brudno et al., 2003). For LAGAN, all default parameters were used. Prediction of protein binding sequences in the conserved B1 element was done using CONSITE (Sandelin et al., 2004b) which uses transcription factor binding profiles from the JASPAR database (Sandelin et al., 2004a).

Results

A 15 kilobase DNA fragment encompassing the whole Ci-Dll-A–Dll-B cluster with a reporter gene inserted into Dll-B recapitulates the endogenous early Dll-B expression pattern

A 15 kilobase lambda clone (CiDB-A) was isolated from a *C. intestinalis* genomic DNA library which encompassed the *Ci-Dll-B* and *Ci-Dll-A* genes. This clone contains a number of conserved non-coding sequences revealed by a sequence alignment comparing the corresponding regions of the *C. intestinalis* Dll cluster with that of the congeneric ascidian *C. savignyi* (Fig. 1A). A *lacZ* reporter gene inserted into the fifth exon of *Ci-Dll-B* was able to recapitulate the early expression pattern of *Ci-Dll-B* from the 64-cell to late gastrula stages, which includes, and is specific to, all animal hemisphere cells (Figs. 2A–E). Endogenous transcript expression of *Ci-Dll-B* is downregulated at the neurula stage to a few cells in the anterior dorsal part of the embryo (Irvine et al., 2007). The CiDB-A construct does not show this downregulation, either in β-galactosidase protein expression or in *Ci-Dll-B* transcript expression, assayed by whole-mount *in situ* hybridization (data not shown). This paper will therefore concentrate on the control of the early expression pattern at the early and late gastrula stages.

To locate the regulatory elements driving the expression pattern we tested a series of reporter gene constructs with fragments both upstream of *Ci-Dll-B* and from the intergenic region between *Ci-Dll-B* and *Ci-Dll-A*. Fragments that included the conserved upstream sequence denoted B1 were able to drive the animal hemisphere expression in much the same way as the whole cluster CiDB-A construct (Figs. 1B, 2F–H). However, the reporter with 5 kb of upstream sequence (CiDB-5.0; Fig. 3A) was able to drive expression in a higher proportion of animal hemisphere cells, like the whole-cluster construct, than a shorter construct lacking 5 short segments of conserved sequence (CiDB-2.4; Fig. 3B). Interestingly, eliminating the next conserved segment, B2 in Fig. 1A (CiDB-1.5; Fig. 3C), increases the extent of expression, suggesting that B2 is an attenuator. Cutting another 500 bp of sequence reduces the extent of expression to the level of the 2.4 kb fragment, indicating that there may be enhancer binding sites in the non-conserved region from 1.0 to 1.5 kb upstream (Fig. 1B). This reporter, CiDB-1.0, which includes only the B1 conserved element was still able to drive expression in the endogenous gastrula stage pattern (Figs. 2H–J). A reporter gene with only 200 bp of