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Evolution of Developmental Control Mechanisms

A cluster of non-redundant Ngn1 binding sites is required for regulation of *deltaA* expression in zebrafish

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ABSTRACT

Proneural genes encode bHLH transcription factors that are key regulator of neurogenesis in both vertebrates and invertebrates. How these transcription factors regulate targets required for neural determination and/or specification is beginning to be understood. In this study, we show that zebrafish *deltaA* is a transcriptional target of proneural factors. Using a combination of transient and stable transgenic reporters, we show that regulation of *deltaA* by one such proneural factor, Ngn1, requires three clustered E-box binding sites that act in a non-redundant manner. Furthermore, we show that as for other proneural targets, members of the different proneural families regulate *deltaA* expression via distinct cis-regulatory modules (CRMs). Interestingly, however, while the *deltaA* CRM regulated by a second proneural factor, Ascl1, has been conserved between *delta* genes of different species, we show that the Ngn1 CRM has not. These results suggest that evolutionary constraints on the mechanism by which Ngn1 regulates gene expression appear less strict than for Ascl1.

Introduction

The central nervous system of both vertebrates and invertebrates is capable of highly complex functions. This is achieved thanks to the staggering variety of neurons that compose it and the circuitry into which these neurons are integrated. The development of the CNS requires that the appropriate neurons be born at the correct times and places and in the correct numbers. A key molecular step in this process is the expression of so-called proneural genes, which are both necessary and sufficient for the formation of neurons, and are highly conserved from invertebrates to humans (Powell and Jarman, 2008).

Proneural genes encode basic-Helix–Loop–Helix (bHLH) transcription factors. In the developing nervous system, two large families of proneural genes have been identified on the basis of sequence homology in the bHLH domain with either *Drosophila* Atonal (Ato) or members of the Achaete–Scute family (Asc); the so-called Ato family can be further subdivided based on more subtle differences in the bHLH sequences (Bertrand et al., 2002). Interestingly, despite comparable molecular structures and roles during neurogenesis, Asc and Ato-like proteins display functional specificity with respect to the type of neurons they are involved in generating. For instance, while misexpression of Atonal in *Drosophila* predominantly induces the generation of ectopic chordotonal organs, Scute misexpression induces the formation of ectopic external sense organs (Chien et al., 1996). Similarly, reciprocal knock-in experiments in mice have shown that Mammalian achaete–schute Homolog 1 (Mash1 or Ascl1) and Neurogenin 2 (Ngn2), respectively homologues of the *Drosophila achaete–scute* and *atonal* genes, also have divergent functions (Parras et al., 2002). Moreover, in zebrafish overexpression of Ngn1 but not Ascl1a leads to formation of ectopic Rohon–Beard sensory neurons in the non-neural ectoderm (RM and PB, unpublished observations; (Blader et al., 1997). These results argue that while members of the Ascl and Ngn families share common transcriptional targets implicated in the generation of generic or immature neurons, in parallel they also direct the expression of distinct, non-overlapping sets of targets that impose aspects of specific neural identity on these neurons (Bertrand et al., 2002; Guillemot, 2007). Understanding how different bHLH proneural proteins regulate their targets is beginning to shed light on the molecular mechanisms that underlie the different activities of these factors (Powell and Jarman, 2008).

For the most part, proneural factors regulate transcription as heterodimers with ubiquitously expressed partners, or E proteins. Such heterodimers bind to the general consensus CANNTG, known as E-boxes, in the genomic sequence flanking target genes. Accumulated evidence suggests that different proneural/E-protein dimers display preferences for the two variable nucleotides in the E-box sequence (Bertrand et al., 2002; Powell et al., 2004). Nevertheless, this alone is unlikely to explain the divergent functions of the Ato and Asc families as, given their small size, even specific E-boxes are overrepresented in the genome relative to the number of proneural targets. Insights into the mechanisms underlying the distinct regulatory activities of different proneural factors have been provided by studies of the

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cis-regulatory modules (CRMs) that these factors activate at target loci. Both invertebrate and vertebrate proneural factors regulate the expression of genes of the Delta family of transmembrane Notch ligands (Castro et al., 2006; Haenlin et al., 1994; Henke et al., 2009; Kunisch et al., 1994). Dissection of the regulatory DNA upstream of the mouse *Delta1* gene, a common target of both Ascl1 and Ngn2, identified two elements, HI and HII, that are capable of driving reporter gene expression in a subset of the pattern of expression of the endogenous *Delta1* gene, and that display high sequence identity to regions located upstream of the zebrafish *deltaD* gene (Beckers et al., 2000). Functional analysis in zebrafish has shown that, while HI is a CRM specifically responsive to Ngn1, HII is only regulated by Ascl1a/b (Hans and Campos-Ortega, 2002).

More recently, *Delta1* regulation in the mouse has been shown to involve synergistic interaction between Ascl/E12 heterodimers and a POU-domain transcriptional co-factor via a conserved octamer binding site located between the E-boxes in HII (Castro et al., 2006). The presence of an Ascl/POU consensus sequence in the regulatory regions of other Ascl targets suggests that there is a conserved logic for Ascl regulatory activity. For Ngn, however, things are less clear. It has been shown that Ngn2 and NeuroM synergise with LIM homeodomain containing transcription factors for the regulation of HB9 expression during specification of motorneurons (Lee and Pfaff, 2003). In addition, sequence analysis of the genomic DNA flanking genes identified by transcriptome analysis to be up-regulated by Ngn has highlighted potential Ngn-regulated CRMs containing E-boxes and potential transcriptional cofactor binding sites, including those for POU-domain transcription factors (Gohlke et al., 2008; Mattar et al., 2004; Seo et al., 2007). However, mutating these potential cofactor sites has no effect on the Ngn-responsiveness of these predicted CRMs (Seo et al., 2007).

In the hope of identifying an underlying code to Ngn regulation of its targets, as has been shown for at least 21 genes by Ascl1 in the mouse, we have dissected the upstream regulatory regions of the zebrafish *deltaA* gene; such a code can be thought of as a minimum combination of Ngn binding sites and associated co-factors that comprise a "generic" Ngn-responsive CRM. Here, we show that *deltaA* is a *bona fide* proneural target and provide evidence that, as for *deltaD*, members of the Ngn and Ascl families regulate *deltaA* via distinct CRMs. However, while the Ascl responsive CRM is conserved between *deltaA*, *deltaD* and *Delta1* conservation of a minimum CRM regulated by Ngn1 could not be found for the three genes. Finally, we have identified an Ngn-responsive CRM for *deltaA*, and show that it functions via the non-redundant activity of three clustered E-boxes.

Materials and methods

Fish lines and developmental conditions

Embryos were raised and staged according to standard protocols (Kimmel et al., 1995). Embryos homozygous for *ngn1*^{hi1059} mutations were obtained by intercrossing heterozygous carriers (Golling et al., 2002); adults heterozygous for the *ngn1*^{hi1059} allele were identified by PCR genotyping of tail-clip genomic DNA. Heat-shocks were performed in a water bath at 38.5 °C. Embryos were fixed overnight at 4 °C in 4% paraformaldehyde/1× PBS, after which they were dehydrated through an ethanol series and stored at −20 °C until use.

In situ hybridization and immunostaining

In situ hybridizations were performed as previously described (Oxtoby and Jowett, 1993). Antisense DIG labelled probes for *ngn1* (Blader et al., 1997), *gfp* (Blader et al., 2003), *deltaA* and *deltaD* (Haddon et al., 1998) were generated using standard procedures. *In situs* were revealed using either BCIP and NBT (Roche) or Fast Red (Roche) as substrate. Immunohistochemical stainings were performed as previously described (Masai et al., 1997), using either anti-GFP (1/1000 Torrey Pines Biolabs) or anti-Myc (1/10, "9E10") (Evan et al., 1985) as primary antibodies and Alexa 488-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1/1000) as secondary antibodies (Molecular Probes).

Plasmid construction

Tg(hs:ngn1) or *Tg(hs:myc-ngn1)* transgenes: In a first step, the previously reported zebrafish *hsp70* promoter (Halloran et al., 2000) was amplified by PCR and used to replace the CMV enhancer promoter of pCS2:CFP (a gift from Dr. U. Strähle). Subsequently, the coding region of CFP was replaced by a fragment containing the *ngn1* or *myc-ngn1* open reading frame (Blader et al., 1997). The resulting *hs:ngn1* or *myc-ngn1* cassettes were then transferred into *pI-SceI*, which has been described to enhance the frequency of stable transgenesis and minimise the mosaicism in transient transgenesis (Thermes et al., 2002).

deltaA promoter:reporter plasmids: A previously described genomic phage library was screened using a PCR fragment corresponding to the 5′UTR of *deltaA* (Appel and Eisen, 1998; Stachel et al., 1993). From a positive clone, a 10 kilobases BamHI genomic fragment extending 5′ from 75 basepairs upstream of the *deltaA* ATG was subcloned into pBluescript. Upstream deletions were made in this fragment by replacing restriction fragments with oligonucleotide linkers containing a NotI restriction site; the resulting plasmids are *pBL:7.1delA*, *pBL:4.5delA*, *pBL:2.9delA* and *pBL:1.6delA*. In parallel, a 75 basepair PCR fragment corresponding to the genomic DNA between the BamHI restriction site and the *deltaA* ATG was subcloned upstream of Chloramphenicol Acetyl Transferase (CAT), β-Galactosidase (βGal) and GFP:SV40pA cassettes; while CAT and βGal cassettes were in *pBluescript*, the corresponding GFP cassette was in *pI-SceI*. Finally, the 7.1, 4.5, 2.9 and 1.6 kilobase *delA* fragments were subcloned NotI/BamHI into the various *0.75delA:reporter* vectors. Further 5′ deletions of the *4.5delA:reporter* fragments and mutations in the various E-boxes were made by replacing a NotI/SphI fragment in the original *4.5delA:reporter* clone, corresponding to the genomic DNA from 4.5 to 2.9 kilobases upstream of the *deltaA* ATG, with an appropriately modified PCR fragment. The amplified DNA fragments were subsequently sequenced.

Plasmid Injection and establishment of transgenic lines

To establish stable transgenic lines, *pI-SceI-hs:ngn1* and the various *pI-SceI-deltaA:gfp* plasmids were injected into 1-cell stage embryos with I-SceI meganuclease to maximize the number of integration events (0.5× I-SceI Buffer, 10% I-SceI enzyme). Similarly, for transient expression of Myc-Ngn1, *pI-SceI-hs:myc-ngn1* was injected with I-SceI meganuclease. Stable transmission of the *Tg(hs:ngn1)* transgene was detected by PCR genotyping using genomic DNA extracting from at least 50 F1 embryos. In subsequent generations, PCR genotyping of adult carriers was performed on tail-clip genomic DNA; Transgenic lines for the various *delA* promoter:reporter constructs were identified by GFP fluorescence. At least two independent insertions were analysed for each transgenic construct.

CAT and βGal ELISA assay

The various *pBLdelA:CAT* plasmids were co-injected with *pBL7.1deltaA:lacZ* (20 ng/μl) and synthetic mRNA encoding either *ngn1* or *ascl1a* (500 ng/μl). The number of copies of the different CAT plasmids injected was normalised as a function of the length of the various constructs. mRNAs were produced using a mMessage Machine SP6 kit following the manufacturer's instructions (Ambion, Inc). Injected embryos were allowed to develop to 10 hpf at which stage total proteins were extracted and the relative levels of CAT and βGal expression were determined as previously described using a commercially available ELISA kit (Hans and Campos-Ortega, 2002;