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A novel conserved *evx1* enhancer links spinal interneuron morphology and cis-regulation from fish to mammals

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ABSTRACT

Spinal interneurons are key components of locomotor circuits, driving such diverse behaviors as swimming in fish and walking in mammals. Recent work has linked the expression of evolutionarily conserved transcription factors to key features of interneurons in diverse species, raising the possibility that these interneurons are functionally related. Consequently, the determinants of interneuron subtypes are predicted to share conserved cis-regulation in vertebrates with very different spinal cords. Here, we establish a link between cis-regulation and morphology of spinal interneurons that express the Evx1 homeodomain transcription factor from fish to mammals. Using comparative genomics, and complementary transgenic approaches, we have identified a novel enhancer of *evx1*, that includes two non-coding elements conserved in vertebrates. We show that pufferfish *evx1* transgenes containing this enhancer direct reporter expression to a subset of spinal commissural interneurons in zebrafish embryos. Pufferfish, zebrafish and mouse *evx1* downstream genomic enhancers label selectively Evx1$^+$ V0 commissural interneurons in chick and rat embryos. By dissecting the zebrafish *evx1* enhancer, we identify a role for a 25 bp conserved cis-element in V0-specific gene expression. Our findings support the notion that spinal interneurons shared between distantly related vertebrates, have been maintained in part via the preservation of highly conserved cis-regulatory modules.

Introduction

Despite considerable divergence in the cellular composition between the spinal cord of fish, birds and mammals, most vertebrates share a remarkably similar organization of spinal neurons, including the arrangement of their somata into discrete dorso-ventral layers (Rexed, 1952; Brown, 1981) and axon projections into descending, ascending or commissural pathways (Soffe et al., 1984; Landmesser and O'Donovan, 1984; Roberts and Alford, 1986; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Roberts, 2000; Hanson and Landmesser, 2003). These morphological similarities are consistent with the functions that spinal neurons share in coordinating rhythmic motor behaviors in all vertebrates (Grillner, 1975; Orlovsky et al., 1999). Thus when fish swim or mice walk, ventral descending interneurons provide rhythmic excitatory inputs to motor neurons that in turn activate peripheral muscles, while commissural interneurons prevent the bilateral activation of motor neurons to ensure alternating contractions (Roberts, 2000; Kiehn, 2006).

The similarities in morphology and function between interneurons of distantly related vertebrates reflect intrinsic developmental programs that have been conserved in evolution. Indeed, studies in chicks and mice have established that conserved homeodomain proteins label, and in many cases specify, discrete populations of interneurons or their progenitors along the dorso-ventral (DV) axis of the neural tube. In the ventral neural tube, transcription factors Evx1, En1, Chx10, and Sim1 label four primary interneuron subtypes, known as V0, V1, V2 and V3, respectively (Burrill et al., 1997; Ericson et al., 1997; Matise and Joyner, 1997; Briscoe et al., 1999; Pierani et al., 1999). As the molecular signals that pattern progenitors along the DV axis are evolutionarily conserved (Davis et al., 1991; Corbo et al., 1997; Jessell, 2000; Wilson and Maden, 2005), it is likely that interneurons derived from these progenitor domains will also express common transcription factors in most vertebrates. Despite this likely scenario, the precise level of correspondence between transcription factor expression and interneuron morphology across distant vertebrates remains

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unknown. Comparative analysis of transcription factor expression in the spinal cord of many vertebrate species may provide insights into the evolution of specific cell types in the vertebrate nervous system (Belting et al., 1998; Murakami et al., 2005).

Studies in zebrafish, frogs and mice have in fact demonstrated a link between transcription factor expression and spinal interneuron morphology and function in distantly related vertebrates (Higashijima et al., 2004a; Li et al., 2004; Sapir et al., 2004; Gosgnach et al., 2006). For instance, a bacterial artificial chromosome (BAC) driving green fluorescent protein (GFP) from the *eng1b* locus labeled a single class of ipsilateral inhibitory ascending interneurons in zebrafish (Higashijima et al., 2004a), consistent with the morphology and function of similar, but more heterogeneous, V1 interneurons in chicks and mice (Wenner et al., 2000; Sapir et al., 2004; Gosgnach et al., 2006). Kimura et al (2006) showed that *vsx2* (*alx*) marks a ventral subset of descending excitatory interneurons in zebrafish, consistent with the expected role of V2 interneurons in mice (Goulding et al., 2002). While interneuron morphology and function are apparently linked to specific transcription factors, it is less clear if common regulatory mechanisms underlie interneuron-specific expression in distant vertebrates. For example, *Gata2* expression in mouse V2 spinal interneurons is driven by a small intronic enhancer (Zhou et al., 2000), whereas expression in zebrafish spinal neurons is generated by unrelated cis-elements upstream of the *gata2* promoter (Meng et al., 1997). Similarly, expression of *Hb9* in spinal motor neurons can be generated by one enhancer in mice and a different one in zebrafish (Lee et al., 2004; Nakano et al., 2005). Hence, while gene expression may be directed by conserved cis-regulatory elements in spinal neurons, it is also possible that multiple cis-regulatory codes may have evolved independently in different vertebrate lineages. Clarification of the ancestral regulatory code underlying gene expression in interneurons requires the functional identification of enhancers from multiple vertebrates. Comparative analysis of such enhancers may unravel potential cis-elements and factors associated with the evolution of specific neurons and circuits.

In this study, we have focused on identifying genomic regulatory elements that direct cell-type specific expression of *evx1* in the embryonic spinal cord, in order to establish a link between cis-regulation and morphology of spinal interneurons from fish to mammals. Evx1 is a determinant of spinal V0 commissural interneurons in chicks and mice, and these are derived from Dbx1$^+$ p0 progenitors (Burrill et al., 1997; Moran-Rivard et al., 2001; Pierani et al., 2001). In zebrafish embryos, *evx1* and its paralogue *evx2*, are both dynamically expressed in discrete groups of spinal neurons (Thäeron et al., 2000; Avaron et al., 2003; Sordino et al., 1996). Using a combination of comparative genomics, transient and stable transgenesis and *in ovo* and *in utero* electroporation, we identified a spinal neuron enhancer of *evx1* that consists of both conserved and non-conserved DNA elements within the proximal downstream region of the gene. GFP expression driven by this enhancer from pufferfish, labeled a subset of *evx1*$^+$ commissural interneurons in zebrafish, and V0 interneurons in chick and rat embryos. By analyzing the effects of deletions in the zebrafish *evx1* enhancer, we identified a short conserved cis-element that restricts V0 gene expression when tested in the chick neural tube. Our results support the view that distinct classes of spinal interneurons in diverse vertebrates have been maintained, at least in part, through conservation of cis-regulation in key cell fate determinants.

Materials and methods

Comparative genomic sequence analysis

Ensembl (http://www.ensembl.org) v6 genomic sequences were aligned with Mulan (http://www.dcode.org) using a default window size of 100 bp and minimum 65% sequence identity. Repetitive sequences were masked in Mulan prior to generating alignments with ClustalW. Putative transcription factor binding sites were identified in Mulan and *MatInspector* (Genomatix, Germany).

Construction of reporter plasmids for transient expression in zebrafish

A 7 kb *ApaI–BamHI evx1* fragment from pufferfish (*Takifugu rubripes*) BAC 240-G7 and a 2.1 kb *SalI–NotI* fragment from pufferfish cosmid 1-F5 (Geneservice Ltd, UK) were cloned into a modified version of the *Tol2* transposon vector pT2XIGΔIN (Kawakami et al., 2004) to create pT2S*evx1*:GFP. In pT2S*evx1*:GFP, farnesylated GFP is fused in frame with the second exon of *evx1* at a unique SmaI site (1217 bp from the ATG translation start) and downstream from a 7 kb promoter, exon 1 and part of exon 2. Next, a 19 kb *evx1*:GFP minigene (10 k in Fig. 2A) was constructed by inserting a 10 kb *NotI–XhoI* fragment into pT2S*evx1*:GFP at the unique *NotI* site. To test smaller 3′ *evx1* fragments we used PCR to amplify them and subcloned them into the *SacII* site of pT2S*evx1*:GFP. PCR primers are listed in Table S1. Further details of plasmid construction are available upon request.

Construction of stable transgenic fish carrying Gal4 and UAS:GFP transgenes

Stable transgenic fish expressing Gal4 under the control of pufferfish *evx1* regulatory elements were generated by *Tol2*-mediated transgenesis (Kawakami et al., 2004). UAS:GFP transgenic fish have been recently described (Asakawa et al., 2008). For the Gal4 driver, we first replaced farnesylated GFP in pT2S*evx1*:GFP with Gal4FF, a modified version of the yeast transcriptional activator Gal4 (Asakawa et al., 2008) to create pT2S*evx1*:Gal4. Then a 4338 bp *PmeI–StuI* 3′*evx1* fragment was cloned into *EcoRV*-digested pT2S*evx1*:Gal4 to create E:Gal4 (Fig. 2A). E:Gal4 contains in the following order 4.3 kb 3′ and 1.6 kb 5′ *evx1* fragments, followed by exon 1 and exon 2 fused in frame to Gal4FF. After microinjection of plasmid DNA and *Tol2* transposase mRNA, injected fish were raised and crossed to UAS:GFP homozygous fish. Progeny from 15 independent F1 transgene carrriers all shared identical GFP expression patterns in the central nervous system. One of these lines carried a single insertion and was used in this study. To create a ~65 kb pufferfish *evx1*:Gal4 BAC transgene (BAC:Gal4 in Fig. 2A), Gal4FF-SV40 pA was introduced in frame with exon 2 of *evx1* into pufferfish BAC 240-G7Δ45 k (with 45 kb upstream and 11 kb downstream) by homologous recombination with *galK* selection (Warming et al., 2005; Table S1). BAC:Gal4 transgenic fish were generated by *Tol2*-mediated transgenesis (M.L.S. and K.K., manuscript in preparation). Two lines carrying single insertions of BAC:Gal4 with essentially identical expression patterns were identified by crossing founder fish to homozygous UAS:GFP fish. In our experiments, we used double heterozygous embryos carrying Gal4 and UAS:GFP. Further details of plasmid construction are available upon request.

Plasmid DNA injection into zebrafish blastomeres

Zebrafish (*Danio rerio*) embryos were obtained according to established procedures (Westerfield, 1995) and raised at 28.5 °C and staged according to hours (hpf) or days (dpf) post-fertilization (Kimmel et al., 1995). Plasmid DNA (25–100 ng/μl) was dissolved in 0.1 M KCl and 0.1% Fast Green and pressure microinjected into the cytoplasm of one-cell stage embryos (0.2–0.5 hpf) according to standard procedures. All animal experiments were performed in compliance with the guidelines stipulated by the Canadian Council for Animal Care, McGill University and the IRCM.

Construction of reporter plasmids for expression in chick and rat embryos

For electroporation in rat (*Rattus norvegicus*) or chick (*Gallus gallus*) embryos, we used ptkGFP (Uchikawa et al., 2003) or pTATAGFP (Lee et al., 2004). Pufferfish and zebrafish 3′ *evx1* genomic fragments