Int. J. Devl Neuroscience 29 (2011) 681–691

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Activity of *dlx5a/dlx6a* regulatory elements during zebrafish GABAergic neuron development

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ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 10 June 2011
Accepted 16 June 2011

Keywords:
cis-Regulatory elements
dlx genes
Forebrain
GABAergic neuron
Gene regulation
Transgenesis
Zebrafish (*Danio rerio*)

ABSTRACT

During vertebrate forebrain formation, *Dlx* homeobox genes play essential roles in the differentiation, migration and survival of subpallial precursor cells that will later give rise to diverse subtypes of γ-aminobutyric acid (GABA)-expressing neurons, including inhibitory cortical interneurons in mammals. They also participate in the regulation of the *Gad* genes encoding the enzymes necessary for GABA synthesis. In mice, at least four *cis*-regulatory elements (CREs) control *Dlx* expression in the telencephalon and diencephalon: URE2 and I12b in the *Dlx1/Dlx2* bigene cluster, and I56i and I56ii in the *Dlx5/Dlx6* bigene cluster. However, little is known so far with respect to the function of orthologous *dlx* genes and their regulatory elements during zebrafish GABAergic neuron development. To investigate whether similar *dlx*-mediated pathways exist in the early developing zebrafish forebrain, we generated independent lines of transgenic zebrafish carrying two distinct GFP reporter constructs driven by a β-globin minimal promoter: one containing a ~1.4 kb *dlx5a/dlx6a* intergenic sequence (encompassing I56i and I56ii) and one with a ~1.1 kb fragment containing only the I56i CRE, respectively. The expression patterns of these two transgenes were compared with that obtained with another construct containing the ~1.4 kb *dlx5a/dlx6a* intergenic sequence and driven by a ~3.5 kb *dlx6a* 5′-flanking fragment. Our comparative analysis showed that GFP expression of the three transgene is largely overlapping throughout the ventral forebrain. Intriguingly, the *dlx6a* 5′-flanking fragment has a major impact on transgene expression in the mesencephalic tectum. Furthermore, comparison of transgene expression between the ~1.4 kb and ~1.1 kb intergenic fragments did not show any specific spatial expression conferred by I56ii. Almost all GFP-expressing cells in the transgenic zebrafish are GABA-positive and also express various GABAergic interneuron markers. Together, our data suggest that zebrafish *dlx5a/dlx6a* intergenic CREs may be involved in a conserved genetic pathway necessary for proper *dlx* expression during zebrafish GABAergic neuron development.

1. Introduction

The *Dlx* gene family encodes homeodomain-containing transcription factors that play essential roles in the development of the mammalian forebrain (Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). In particular, mouse *Dlx* genes are required for correct differentiation, migration and survival of subpallial-derived progenitors that will later give rise to diverse subtypes of GABAergic interneurons (Panganiban and Rubenstein, 2002). In the developing mouse forebrain, four *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) are sequentially expressed starting around embryonic day 9.5 (E9.5) and exhibit highly overlapping but distinct patterns in the ganglionic eminences of the subpallial telencephalon, prethalamus and hypothalamus (Liu et al., 1997; Yang et al., 1998; Eisenstat et al., 1999). Specifically, *Dlx1* and *Dlx2* are expressed in the immature and proliferating cells located in the ventricular zone (VZ) close to the ventricle, while *Dlx5* and *Dlx6* are predominantly restricted to the postmitotic, migrating and differentiating neurons in the subventricular and mantle zones (SVZ and MZ) (Eisenstat et al., 1999; Panganiban and Rubenstein, 2002). *Dlx* forebrain expression largely overlaps with that of the two *glutamic acid decarboxylase* (*Gad*) genes, *Gad1* and *Gad2*, which encode the enzymes responsible for the synthesis of γ-amino butyric acid (GABA) (Yun et al., 2002; Long et al., 2009).

Vertebrates possess multiple *Dlx* genes, which are generally organized in convergently transcribed bigene clusters on distinct chromosomes, separated by a relatively short intergenic (3.5–16 kb) region (Zerucha and Ekker, 2000). Mammals have six

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doi:10.1016/j.ijdevneu.2011.06.005

Dlx genes arranged as three bigene pairs (*Dlx1*/*Dlx2*, *Dlx3*/*Dlx4* and *Dlx5*/*Dlx6*), whereas some teleost fish including zebrafish have eight *dlx* genes, among which six (*dlx1a*/*dlx2a*, *dlx3b*/*dlx4b* and *dlx5a*/*dlx6a*) are organized in a manner similar to their mammalian counterparts (Stock et al., 1996; Amores et al., 1998; Quint et al., 2000). Two additional unlinked genes, *dlx2b* and *dlx4a*, are thought to be duplicates of ancestral *Dlx2* and *Dlx4*, respectively, after a genome duplication event specific to the teleost lineage (Amores et al., 1998). Consistent with the expression of their mouse orthologs or co-orthologs, five *dlx* genes (*dlx1a*/*dlx2a*, *dlx2b* and *dlx5a*/*dlx6a*) are expressed with very similar domains in the zebrafish telencephalon and diencephalon (Akimenko et al., 1994; Ellies et al., 1997; MacDonald et al., 2010a). In particular, *dlx1a* and *dlx2a* have been proposed to mark cells closer to the ventricular wall than *dlx5a* and *dlx6a*, reminiscent of the spatio-temporal succession of *Dlx* expression in the mouse forebrain (Zerucha et al., 2000; MacDonald et al., 2010a). Proper *Dlx* expression in the developing mouse and zebrafish forebrain is transcriptionally modulated and concerted, at least in part, by a number of highly conserved *cis*-regulatory elements (CREs, enhancers) present in their surrounding genomic loci (Zerucha et al., 2000; Ghanem et al., 2003, 2007): URE2 located upstream of *Dlx1* (*dlx1a*), I12a and I12b within the *Dlx1*/*Dlx2* (*dlx1a*/*dlx2a*) intergenic region, I56i and I56ii within the *Dlx5*/*Dlx6* (*dlx5a*/*dlx6a*) intergenic region. Our studies using transgenic mice have previously shown that URE2, I12b and I56i CREs target GABA-expressing progenitors in the ganglionic eminences and GABAergic cortical interneurons in adult animals (Ghanem et al., 2007). In contrast, I56ii activity is mainly limited to a subpopulation of GABAergic striatal projection neurons at E11.5–E13.5 (Ghanem et al., 2008).

The roles of *dlx* genes and their corresponding individual CREs in the genetic cascades involved in GABAergic neuron formation have not yet been fully determined, particularly in non-mammalian vertebrates. Using triple fluorescence *in situ* hybridization techniques, we were able to show that *dlx* genes are expressed in highly overlapping domains with the *gad* genes within the subpallium of the developing zebrafish forebrain (MacDonald et al., 2010a). The spatio-temporal succession of *dlx* and *gad1* gene expression and the apparent migration of these cells from the proliferative zones (Mueller et al., 2008; MacDonald et al., 2010a), as in the mouse (Panganiban and Rubenstein, 2002; Marin and Rubenstein, 2003), indicates that these genes appear to follow a conserved genetic pathway mediating GABAergic neuron generation. To further understand the regulatory roles of the *dlx* CREs during zebrafish GABAergic neuron development, we generated transgenic zebrafish with two novel constructs: one in which GFP is under the control of a 1.4 kb *dlx5a*/*dlx6a* intergenic sequence (encompassing I56i and I56ii) and another containing a 1.1 kb fragment encompassing only I56i. Expression of both constructs was compared with that of the previously reported *dlx6a-1.4kbdlx5a*/*dlx6a-GFP* construct that contains a ~3.5 kb of *dlx6a* 5′-flanking region in addition to the 1.4 kb intergenic fragment (Ghanem et al., 2003). In addition to a comparative analysis conducted between the three transgenic lines, we also sought to determine whether any potential association exists between GFP expression and several GABAergic interneuron markers using double immunohistochemical staining in an effort to evaluate the functional importance of these two enhancers in the differentiation of zebrafish GABAergic neurons.

2. Materials and methods

2.1. Animal maintenance

Adult wild-type and transgenic zebrafish were housed in circulating aquaculture system water at 28.5 °C under a controlled photoperiod (a light/dark cycle of 14/10 h) according to standard methods (Westerfield, 2000). Fish were fed once or twice daily with No. 1 crumble (Zeigler™, Aquatic Habitats). Embryo-collection traps were placed inside each 9 L acrylic tank of 20–30 fish in the evening and embryos were collected at 9 a.m. in the next morning. Embryos were raised in an incubator at 28.5 °C, staged in days post-fertilization (dpf) according to specific criteria and euthanized with an overdose of tricaine mesylate (ethyl 3-amino-benzoate methanesulfonate, Sigma) when needed. All experimental procedures in this study were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the University of Ottawa animal care committee.

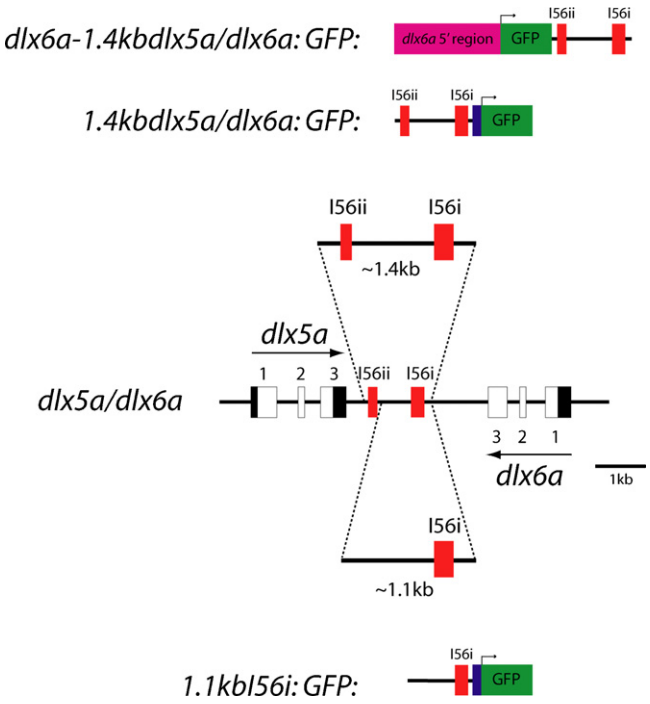

Fig. 1. A schematic drawing of GFP transgene constructs used to establish transgenic zebrafish. Similar to mammalian *Dlx* genes, zebrafish *dlx5a*/*dlx6a* genes are physically linked in a bigene arrangement and contain two highly conserved intergenic CREs named I56i and I56ii. The *dlx6a-1.4kbdlx5a*/*dlx6a:GFP* construct comprises a 1432 bp *dlx5a*/*dlx6a* intergenic sequence (nucleotide positions 745–2176, downstream of the stop codon of *dlx6a*) encompassing both I56i and I56ii CREs as well as a ~3.5 kb *dlx6a* 5′-flanking fragment (nucleotide positions −3435 to 100, relative to the start codon of *dlx6a*), while the *1.1kbI56i:GFP* construct comprises a 1137 bp fragment (nucleotide positions 750–1886, downstream of the stop codon of *dlx6a*) containing all elements of the 1.4 kb sequence, except for the I56ii CRE. It also contains a human β-globin minimal promoter. The *1.4kbdlx5a*/*dlx6a:GFP* transgene construct contains the same intergenic fragment as *dlx6a-1.4kbdlx5a*/*dlx6a:GFP* but contains the β-globin minimal promoter as in *1.1kbI56i:GFP*. Exons are numbered (black, untranslated region; white, coding sequences). I56i and I56ii regulatory elements are shown as red boxes. The pink and blue boxes represent a 5′ flanking sequence of *dlx6a* gene and a β-globin minimal promoter, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

2.2. Construction of dlx CRE transgene vectors

The *dlx6a-1.4kbdlx5a*/*dlx6a:GFP* transgene construct has been previously generated in the laboratory as described in (Zerucha et al., 2000; Ghanem et al., 2003) (Fig. 1). In brief, a 1432 bp *dlx5a*/*dlx6a* intergenic region containing both I56i and I56ii CREs (nucleotide positions 745–2176, downstream of the stop codon of *dlx6a*) was subcloned into a modified SP72-pEGFP-N1 vector using *Kpn*I/*Cla*I restriction sites and placed downstream of a 3536 bp fragment from the immediate 5′ flanking region of zebrafish *dlx6a* (nucleotide positions −3435 to 100, relative to the start codon of *dlx6a*) and the GFP coding sequence. This *dlx6a* 5′-flanking fragment by itself does not produce any tissue-specific expression in transgenic zebrafish (Ghanem et al., 2003).

The *1.1kbI56i:GFP* transgene construct was made up of a 1137 bp region (nucleotide positions 750–1886, downstream of the stop codon of *dlx6a*) from the *dlx5a*/*dlx6a* intergenic locus containing only I56i CRE and its flanking sequences. This fragment shares the same sequence with the ~1.4 kb *dlx5a*/*dlx6a* intergenic segment with the only difference being the lack of the ~290 bp I56ii CRE (Fig. 1). Specifically, a 1137 bp *Sal*I-*Sal*I I56i fragment was first PCR-amplified from the *dlx6a-1.4kbdlx5a*/*dlx6a:GFP* transgene plasmid using the following primer pair (forward: 5′-GTC GAC GCT CAA TTA TTA AGG TAT TGA CAA-3′; reverse: 5′-GTC GAC ACA AGC