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The relationship between *dlx* and *gad1* expression indicates highly conserved genetic pathways in the zebrafish forebrain

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Abstract

The *Dlx* genes encode a family of transcription factors important to the development of the vertebrate forebrain. These genes have very similar expression domains during the development of the telencephalon in mouse and play a role in GABAergic interneuron differentiation. We have used triple fluorescent *in situ* hybridization to study the relative expression domains of the *dlx* and *gad1* genes in the zebrafish telencephalon and diencephalon. We also generated transgenic zebrafish with regulatory elements from the zebrafish *dlx1a/2a* locus. The zebrafish *dlx* regulatory elements recapitulated *dlx* expression in the forebrain and mimicked the relationship between the expression of the *dlx* genes and *gad1*. Finally, we show that a putative enhancer located downstream of *dlx2b* can also activate reporter gene expression in a tissue specific manner similar to endogenous *dlx2b* expression. Our results indicate the *dlx* genes are regulated by an evolutionarily conserved genetic pathway and may play a role in GABAergic interneuron differentiation in the zebrafish forebrain.

Keywords

dlx; Forebrain; Transgenesis; Zebrafish; Regulatory elements; Fluorescent in situ hybridization

INTRODUCTION

The *Dlx* genes encode homeodomain containing transcription factors involved in the development of the mammalian forebrain (Anderson et al. 1997a; Anderson et al. 1997b; Marin et al. 2000; Pleasure et al. 2000). They have partially overlapping expression in the developing telencephalon of the mouse and follow a distinct spatial and temporal expression pattern: *Dlx1* and *Dlx2* are expressed in the ventricular zone and subventricular zone; *Dlx5* is expressed in the subventricular zone and *Dlx6* is expressed laterally in the mantle zone (Liu et al. 1997; Anderson et al. 1999; Eisenstat et al. 1999; Yun et al. 2003). *Dlx* gene expression highly overlaps with that of *glutamic acid decarboxylases (Gads)*, the enzymes responsible for the synthesis of γ -amino butyric acid (GABA). Furthermore, DLX proteins can induce *Gad* gene expression *in vitro* and *in vivo* (Anderson et al. 1999; Stühmer et al. 2002; Yun et al. 2003). *Dlx1*^{-/-}/*Dlx2*^{-/-} mutant mice show a loss of GABAergic interneuron differentiation in the ventral telencephalon, further implicating the *Dlx* genes in the differentiation of GABAergic interneurons (Anderson et al. 1997a).

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Vertebrate *Dlx* genes are typically organized in convergently transcribed bigene pairs, separated by a short intergenic region usually less than 10kb (Zerucha et al. 2000; Sumiyama et al. 2002; Ghanem et al. 2003). Zebrafish have eight *dlx* genes of which six show similar bigene genomic arrangements to the mouse: *dlx1a/dlx2a*; *dlx3b/4b*; and *dlx5a/6a* clusters orthologous to *Dlx1/2*; *Dlx3/4*; *Dlx5/6*, respectively (Quint et al. 2000). Two additional genes, *dlx2b* and *dlx4a*, are thought to be duplicates of ancestral *Dlx2* and *Dlx4*, respectively, after the teleost specific genome duplication event (Amores et al. 1998). The expression domains of two *Dlx* genes comprising a given bigene pair are very similar, both in mice and zebrafish, most likely due to shared regulatory regions (Liu et al. 1997; Eisenstat et al. 1999; Ellies et al. 1997). Regulatory regions have been identified both upstream of the transcription start sites and within the intergenic domains of a given bigene pair (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007). The zebrafish *dlx* genes share similar genomic arrangements to the mouse, including the presence of the regulatory elements I12a and I12b in the *dlx1a/2a* intergenic region, Upstream regulatory element 2 (URE2) upstream of *dlx1a*, I56i and I56ii between *dlx5a* and *dlx6a* (Zerucha et al. 2000; Ghanem et al. 2003). Five *dlx* genes (all but *dlx3b*, *dlx4b* and *dlx4a*) are expressed in the zebrafish forebrain with very similar expression domains in the telencephalon and diencephalon (Akimenko et al. 1994; Ellies et al. 1997). In particular zebrafish *dlx1a* and *dlx2a* have been proposed to mark cells closer to the ventricular wall than the *dlx5a* and *dlx6a* genes, reminiscent of the spatial-temporal succession of *Dlx* gene expression pattern in mouse (Zerucha et al. 2000). However, the precise spatial relationship between different *dlx* expression domains and *gad1* gene expression has not been determined.

In this study, we describe in detail the extent to which zebrafish *dlx* and *gad1* expression patterns overlap in the developing forebrain. The *gad1* gene, orthologous to *Gad67* in mammals, is used as a marker for GABAergic interneuron differentiation. The spatial-temporal expression pattern described in the mouse telencephalon appears to be similar in the zebrafish, with *dlx1a* and *dlx2a* being expressed in the ventricular zone and *dlx5a*, *dlx6a* and *gad1* expressed in more lateral differentiated cells. We tested whether the conserved regions linked to the *dlx1a/2a* cluster of the zebrafish have regulatory functions consistent to previously reported data in the mouse forebrain, by designing reporter constructs containing conserved sequences from the *dlx1a/2a* locus. We also utilized a previously described transgenic line, *Tg(dlx5a/6aIG:GFP)*, showing these regulatory regions are sufficient to mimic the endogenous nested expression patterns of the *dlx* genes in the zebrafish forebrain (Zerucha et al. 2000). We further explored the regulation of the *dlx2b* gene, paralogous to the *dlx2a* gene in the zebrafish. A small domain containing two putative *dlx* binding sites had been identified downstream of *dlx2b* (Ghanem et al. 2003), denoted *dlx2b* downstream regulatory element (*dlx2b*DRE). The forebrain expression of *dlx2b* is at least partially recapitulated by reporter gene expression when under the regulation of *dlx2b*DRE.

RESULTS

Partially overlapping expression domains of *dlx* and *gad1* genes in the zebrafish forebrain

The zebrafish *dlx* genes from a bigene pair have been proposed to have highly overlapping expression domains within the forebrain (Zerucha et al. 2000). Currently there are no antibodies against specific zebrafish *Dlx* proteins except *Dlx3b*, therefore we relied on fluorescent RNA *in situ* hybridization to determine the extent to which the *dlx* genes expression domains overlap. We examined the *dlx* expression in the zebrafish forebrain from 24 hours post-fertilization (hpf) until 48 hpf. At 48 hpf, the forebrain is beginning to undergo secondary neurogenesis, and *dlx* and *gad1* are expressed in the forebrain (Akimenko et al. 1994; Ellies et al. 1997; Martin et al. 1998). As observed by confocal imaging, the *dlx1a*, *dlx2a*, and the paralogous *dlx2b* genes are expressed in very similar domains in the subpallium of the zebrafish telencephalon at 48hpf (Fig. 1A-A'' and

delimited by solid lines in Fig. 1A'''). Within this domain, the three genes are expressed close to the ventricle, an area shown to be proliferative (Mueller and Wullimann, 2003). There may be quantitative differences within these domains as *dlx2b* appears to be more strongly expressed in the most dorsal domain of the subpallium (Fig. 1A'' and colored zone in A'''), while the *dlx2a* domain of expression is extended ventrally compared to the other two genes (see colored zone in Fig. 1A'''). The three *dlx* genes also show virtually identical expression domains in the diencephalon, more specifically in the prethalamus and preoptic area (Fig. 1B-B'''), with an apparently higher level of expression for *dlx1a* and *dlx2b* in the dorsal part of the prethalamus compared to the rest of their expression domains (Fig. 1B''').

The *dlx5a* and *dlx6a* genes display very similar expression domains in the subpallial telencephalon at 48hpf (Fig. 1C-C'') although *dlx6a* is highly expressed only in the dorsal-most part of the telencephalon. Both genes are expressed outside of the ventricular zone (Fig. 1F). In the diencephalon, transcripts of *dlx5a/6a* are localized throughout the prethalamus and preoptic area but *dlx6a* transcripts are strongly detected only in the dorsal-most part of the prethalamus (Fig. 1D). The expression limits of the *dlx1a/2a/2b* and *dlx5a/6a* genes appear to be, overall, very similar in the diencephalon (Fig. 1G). The highly overlapping domains of *dlx* forebrain expression are also observed at 24 and 36hpf (Suppl. 1 and Suppl. 2).

Consistent with previous reports, we find that *dlx2a* is transcribed in the ventricular zone of the zebrafish forebrain, where *zash1a* (*ascl1a*), *gad1*, and GABA expression can also be detected (Wullimann and Mueller, 2002; Mueller et al. 2006; Mueller et al. 2008). Based on the results presented in Fig. 1, it appears that expression of a gene in a *dlx* bigene cluster is representative of the second gene in the cluster. To further compare the expression domains of zebrafish *dlx* genes in the forebrain with areas of GABAergic interneuron differentiation, we studied the relative expression domains of one *dlx* gene from each bigene pair, *dlx1a* and *dlx5a*, alongside the specification marker *gad1*. The *dlx1a*, *dlx5a*, and *gad1* genes are expressed in very comparable domains within the telencephalon and diencephalon at 24hpf (Fig. 2A). Beginning at 36hpf and continuing at least until 48hpf, overt differences are observed between *dlx1a*, *dlx5a*, and *gad1* expression patterns within the telencephalon (Fig. 2B and C and see schematic on Fig. 2E). The medial limit of *dlx1a* expression was found at the level of the ventricle, while the medial limit of expression for both *dlx5a* and *gad1* was found more lateral. As for the lateral limits of *dlx5a* and *gad1* expression domains, these are found outside of the *dlx1a* expression domains. In addition, relatively stronger expression of *dlx1a* is detected close to the ventricle while stronger expression of *gad1* is located in the lateral part of the telencephalon (see Fig. 2C''', and Fig. 2E). In the diencephalon, the *dlx1a*, *dlx5a*, and *gad1* expression domains are very comparable in the preoptic area and prethalamus (Fig. 2D), although the limit of *gad1* expression is found more dorsal than that of the *dlx* genes. The levels of transcripts are not homogenous within the *dlx1a* and *gad1* domains, with *dlx1a* weakly expressed and *gad1* highly expressed in the dorsal-most domain of the prethalamus and vice versa in the preoptic area (Fig. 2D''').

The activity of zebrafish *dlx* enhancers is similar to their mouse counterparts

The I12a and I12b enhancers, located in the mouse *Dlx1/2* intergenic region, have regulatory functions when tested in transgenic mice, with only I12b displaying forebrain activity (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007). The mouse URE2 enhancer is located upstream of *Dlx1* and drives expression in a number of tissues, one of which is the developing forebrain (Ghanem et al. 2007). To test if orthologous sequences from the zebrafish genome have regulatory function in the zebrafish, we designed two reporter constructs containing these genomic sequences (Fig. 3A, B). We selected 6kb of the *dlx1a/2a* intergenic region, including I12a and I12b, and 900bp encompassing URE2. These sequences were cloned upstream of a β -globin minimal promoter associated with the coding

sequences of GFP to produce the *Tg(dlx1a/2aIG:GFP)* and the *Tg(URE2dlx1a/2a:GFP)* reporter transgenes, respectively.

Embryos harboring the *Tg(URE2dlx1a/2a:GFP)* transgene show GFP expression in the telencephalon and diencephalon starting at approximately 24hpf (Fig. 3C). GFP expression can be detected in the telencephalon and diencephalon from 24hpf until at least 72hpf (Fig. 3D and E). Expression of GFP in the *Tg(URE2dlx1a/2a:GFP)* line can also be detected in the mesenchyme of the visceral arches at 96hpf (data not shown). The *Tg(dlx1a/2aIG:GFP)* embryos show GFP expression in domains highly similar to those observed in *Tg(URE2dlx1a/2a:GFP)* embryos with fluorescence in the telencephalon and diencephalon starting at 24 hpf (Fig. 3F). Transgene expression can also be detected in the jaw region starting at approximately 48hpf (Fig. 3G). The overall forebrain GFP expression of *Tg(dlx1a/2aIG:GFP)* and *Tg(URE2dlx1a/2a:GFP)* lines closely mimics the endogenous forebrain expression of the *dlx1a* and *dlx2a* genes (Fig. 4). However, the transgenes are only active in small domains within the branchial arches and not at all in the olfactory or otic placodes, other areas of known *dlx1a/2a* expression (Akimenko et al. 1994; Ellies et al. 1997). The *Tg(dlx1a/2aIG:GFP)* transgene also targets GFP expression in the retina at 48hpf, an area where expression of *dlx1a* or *dlx2a* has not been previously reported (Fig. 4F).

***dlx* enhancer activity closely mimics endogenous *dlx* expression in the telencephalon**

To determine if the *dlx* regulatory elements are active in cells that express Gad proteins, we co-labeled transverse sections of the zebrafish forebrain with antibodies against GFP and Gad proteins (Fig. 5). At 48hpf, *Tg(dlx1a/2aIG:GFP)* reporter expression is located close to the ventricle and dorsally in the telencephalon but Gad proteins are localized more laterally (Fig. 5A). Very few cells are co-expressing Gad and GFP and those rare cells are located in the dorsolateral domain of the telencephalon (Fig. 5A). Expression of the *Tg(URE2dlx1a/2a:GFP)* reporter transgene is detected close to the ventricle and ventrally in the subpallium of the telencephalon, with only very few cells co-expressing GFP and Gad, again located in a dorsolateral domain (Fig. 5B). To assess the activity of *dlx5a/6a* regulatory elements in the developing zebrafish forebrain we utilized the *Tg(dlx5a/6aIG:GFP)* transgenic line containing the two enhancers, I56i and I56ii, that recapitulate endogenous *dlx5a/6a* expression (Zerucha et al. 2000; Mione et al. 2008). The *Tg(dlx5a/6aIG:GFP)* embryos show reporter expression in the telencephalon and diencephalon of zebrafish embryos and most Gad positive cells co-express GFP (Fig. 5C, data not shown) (Mione et al. 2008). These data suggest that the enhancers associated with the *dlx1a/2a* bigene cluster drive expression in the ventricular zone of the subpallium, while the enhancers associated with the *dlx5a/6a* bigene cluster drive expression in more lateral cells expressing Gad proteins. The expression domains described for GFP in transgenic lines are therefore consistent with the spatially restricted expression of the *dlx* genes (Fig. 6).

Co-expression of paralogous genes and insights into the regulation of zebrafish *dlx2b* expression

The I12b and URE2 enhancers appear to be sufficient to recapitulate expression of *dlx1a/dlx2a* in the forebrain. The paralogous gene to *dlx2a*, *dlx2b*, is also expressed in similar domains of the forebrain (Fig. 1). These highly overlapping domains of expression for *dlx1a*, *dlx2a*, and *dlx2b* suggest the existence of a regulatory sequence at the *dlx2b* locus that would be similar, in sequence and/or in function, to those described for the *dlx1a/2a* locus. Phylogenetic footprinting of the *dlx2b* locus identified a 60bp sequence located in the 3' region of the gene and showing similarity to other *dlx* enhancers, including two putative *dlx* transcription factor-binding sites (Ghanem et al. 2003). We amplified a 200 bp region downstream of *dlx2b*, including these 60bp, that we named *dlx2b* Downstream Regulatory

Element (*dlx2b*DRE), and placed it in a β -globin:GFP transgene which was tested in primary transgenic zebrafish embryos (Fig. 3B). This transgene targets GFP expression in the telencephalon and diencephalon of 84% (77/92) of primary injected zebrafish at 24hpf (Fig. 3I). This expression persists until at least 48hpf in the telencephalon and diencephalon and closely resembles that observed in the *Tg(URE2dlx1a/2a:GFP)* and *Tg(dlx1a/2aIG:GFP)* transgenic lines (Fig. 3J and K).

DISCUSSION

Conserved activity of *dlx* regulatory elements between zebrafish and mouse

Several conserved regulatory elements have been identified that are sufficient to mimic many aspects of endogenous *Dlx* expression in the mouse (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007). Similarly, the distinct expression domains of the *dlx* genes in the forebrain seem to be completely recapitulated by orthologous zebrafish enhancers. Therefore, our results show that zebrafish and mouse regulatory elements not only show strong sequence conservation, but also are sufficient to drive similar expression in homologous tissues (forebrain and pharyngeal arches) indicating their activity has been conserved throughout vertebrate evolution. The expression domains of the *Tg(URE2dlx1a/2a:GFP)* and *Tg(dlx1a/2aIG:GFP)* transgenes are similar in the forebrain, although the former shows an extended expression domain in the ventral subpallium while the latter has an extended expression domain in the dorsal subpallium. Heterologous transgenic experiments have indicated that zebrafish *dlx5a/6a* regulatory sequences are functional in the mouse forebrain, mimicking the endogenous *Dlx* gene expression patterns (Zerucha et al. 2000; Stühmer et al. 2002). Consistent with these results, the URE2 element from distantly related vertebrates has conserved function in the forebrain of zebrafish and mice (R.B.M., M.D.T, and M.E., unpublished observations). Taken altogether, the high levels of conservation of the sequence and regulatory function strongly suggest that the genetic cascades upstream of the *Dlx* genes are conserved between distantly related vertebrates.

Spatial-temporal expression of *dlx* and *gad* genes is conserved between mouse and zebrafish

The *Dlx* genes have been shown to play an important role in the differentiation and migration of GABAergic interneurons in mice (Anderson et al. 1997a; Anderson et al. 1997b; Stühmer et al. 2002a; Stühmer et al. 2002b). The *Dlx1/2* genes are expressed in immature neurons and *Dlx5/6* in more mature neurons, eventually differentiating into *Gad* positive interneurons (Liu et al. 1997; Eisenstat et al. 1999; Stühmer et al. 2002b; Yun et al. 2003). Previous studies of gene expression and cell proliferation revealed similarities in the topological relationships of mouse and zebrafish telencephalon (For reviews see: Wullimann and Mueller, 2004; Mueller and Wullimann, 2009; Wullimann, 2009). Thus, the ventricular (proximal) zone is a site of proliferation and expresses *dlx2a* (Mueller et al. 2002; Mueller et al. 2008). The expression domain of *dlx5a* (and by extension, *dlx6a*) is shifted laterally compared to *dlx1a* (*dlx2a*), and overlaps partially with the more lateral of *gad1* expression domain. These relative expression patterns can be indicative of a temporal sequence of gene expression (*dlx1a/2a* \rightarrow *dlx5a/6a* \rightarrow *gad1*) in cells moving from the proliferative zone to more lateral regions of the telencephalon and may be related to the various stages of GABAergic neuron differentiation. This apparent movement of cells from the ventricular zone to more lateral domains may be due to tangential and/or radial migration, as both modes of migration have been previously identified in the zebrafish telencephalon (Mueller et al. 2006; Mueller et al. 2008; Mione et al. 2008). As the domains of enhancer activity are consistent with gene expression data (Fig. 6), transgenic zebrafish lines may be useful to isolate neural progenitors from the forebrain at different stages of GABAergic interneuron differentiation.

Conserved genetic mechanisms lead to the highly overlapping expression domains of *dlx1a/2a* and *dlx2b*

The two *Dlx* genes of a bigene pair have highly overlapping expression domains that have been suggested to be due to shared regulatory regions (Ellies et al. 1997). In this study, we report the very similar expression domains of *dlx1a*, *dlx2a* and *dlx2b* in the forebrain starting at 24hpf and continuing until at least 48hpf. We explored the possibility that *dlx2b* expression was controlled by sequences resembling *dlx1a/2a* enhancers, such as URE2 or I12b. Sequence comparisons of the forebrain enhancers (I12b, I56i, and I56ii) to the *dlx2b* locus had identified a 60bp sequence containing two putative *dlx* binding sites located downstream of *dlx2b* (Ghanem et al. 2003). Given the position of this element, it could be paralogous to the I12b sequence located between *dlx1a* and *dlx2a*, but would have accumulated mutations since the whole-genome duplication event specific to teleosts. Here we show that this *dlx2b*DRE region is sufficient to drive reporter gene expression in the forebrain, with patterns similar to those observed with the *dlx1a/2a* transgenes, suggesting that similar regulatory mechanisms may be involved in *dlx2b* and *dlx1a/2a* expression. The *dlx2b*DRE forebrain activity may also be attributable to the presence of the two *dlx* binding sites within a conserved 60bp region. A region from the I56i enhancer containing two *Dlx* binding sites was sufficient to drive forebrain expression of a reporter gene in transgenic mice (Zerucha et al. 2000; Ghanem et al. 2003). The conservation of the *dlx* binding sites is suggestive of potential cross-regulation of *dlx2b* expression by *dlx1a* and/or *dlx2a*, as the mouse Dlx2 protein has been shown to bind directly to DNA sequences organized in a similar context (Zerucha et al. 2000; Zhou et al. 2004; Potter et al. 2009). Cross-regulation between *dlx1a/2a* and *dlx2b* via the *dlx2b*DRE would explain the strong overlap in expression. Overall, the highly overlapping expression of the *dlx1a/2a* bigene pair with the unlinked *dlx2b* is suggestive of similar gene regulation mechanisms at the two loci.

In conclusion, we have described the spatial overlap between *dlx* bigene pairs and *gad1* expression in the embryonic zebrafish forebrain. The *dlx1a/2a* genes are expressed close to the ventricle, *dlx5a/6a* are expressed throughout the subpallium, and *gad1* is expressed in the lateral most regions consistent with the hypothesis that *dlx* genes play a role in GABAergic interneuron differentiation in the zebrafish forebrain, similarly to what is known in the mouse. The *dlx* regulatory elements have conserved function in the zebrafish and mouse forebrain, and mimic the spatial relationship between *dlx* and *gad1* within the zebrafish telencephalon. These results support the idea that genetic pathways controlling *dlx* gene expression and GABAergic interneuron development, in the forebrain of mammals and teleosts, may be conserved over approximately 430 million years of evolution.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Wild type zebrafish were raised according to standard procedures (Westerfield, 2000). Embryos were staged as hours post-fertilization (hpf) according to specific criteria outlined by Kimmel et al. 1995. All experiments were carried out in accordance with animal care guidelines provided by the Canadian Council on Animal Care and the University of Ottawa animal care committees approved all protocols.

dlx transgene constructs

The *dlx1a/2a*IG:GFP transgene construct contains a six kilobase (kb) *HindIII-HindIII* region from the *dlx1a/2a* intergenic (IG) region and comprises two potential enhancers I12a and I12b (Ellies et al. 1997; Ghanem et al. 2003). This region was cloned into a modified SP72 vector (Promega, Madison, WI) containing a human β -globin minimal promoter immediately upstream of the green fluorescent protein (GFP) coding sequence. This β -

globin-GFP cassette does not, on its own, produce any GFP expression in zebrafish. The URE2*dlx1a/2a*:GFP transgene construct contains 900 bp of a region 5' to the *dlx1a* transcriptional start site (Ghanem et al. 2008). This region was amplified by PCR using the following oligonucleotides: 5' GCAAAGCACAGAATTATTCT 3' and 5' CTTTTAGGGTTTTTGTTCGGA 3'. This 900 bp fragment was cloned into a second modified SP72 vector containing the β -globin:GFP cassette, and Tol2 transposase recognition sites.

Generating and visualization of transgenic zebrafish

Transgenic *dlx1a/2aIG*:GFP zebrafish lines were generated as described in Amsterdam et al. 1995. The *dlx1a/2aIG*:GFP construct was injected at 200ng/ μ l in standard DNA microinjection buffer (0.2 mM KCl, 0.1% phenol red). The URE2*dlx1a/2a*:GFP construct was co-injected at 50 ng/ μ l with 50 ng/ μ l of tol2 *transposase* mRNA following standard procedures as described by (Fisher et al. 2006). Approximately 200 zebrafish larvae expressing GFP were obtained for each construct and retained as founders. They were intercrossed and at least 100 embryos for each pair were screened for GFP fluorescence at 24-48 hpf. At least two independent transgenic lines were generated for *dlx1a/2aIG*:GFP and for URE2*dlx1a/2a*:GFP. In each case, the two transgenic lines obtained for a given construct yielded identical GFP expression patterns. GFP-expressing F1 embryos were raised to adulthood and intercrossed. Screening for GFP positive embryos was done using a Nikon NBZ 1500 dissecting microscope and imaging was done with a Nikon DXM 1200C digital camera.

In situ hybridization

Whole mount mRNA *in situ* hybridization was done as described in Thisse and Thisse (1998). The antisense mRNA probes were labeled with digoxigenin-11-UTP (Roche, 11277073910) and synthesized from cDNA clones: *dlx1a* (Ellies et al. 1997), *dlx2a* (Akimenko et al. 1994), *dlx2b* (Ellies et al. 1997), *dlx5a* (Akimenko et al. 1994), *dlx6a* (Ellies et al. 1997), *gad1* (Mueller et al. 2008). The GFP probe was synthesized as complementary to the cDNA fragment that was amplified with the oligonucleotides 5' AAGGGCGAGGAGCTGTTCAC 3' and 5' GAACTCCAGCAGGACCATGT 3' and cloned into the pDrive vector (Qiagen, Valencia, CA).

Fluorescent RNA *in situ* hybridization was carried out with a protocol modified from those described previously (Jowett and Yan, 1996; Welten et al., 2006). DNP labeled probes were revealed with tyr-Cy5, digoxigenin labeled probes were revealed using tyr-Cy3, fluorescein labeled probes were revealed with tyr-fluorescein (available from Perkin-Elmer). Our full tissue labeling protocols can be found online:

<http://wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ>

Imaging of fluorescent in situ hybridizations

Embryos were placed on glass slides and positioned under coverslips for confocal imaging. Confocal z-stacks were obtained by using a Zeiss LSM5 PASCAL (Carl Zeiss, Germany) and excitation lasers were at 488 (Fluorescein), 543 nm (Cy3), 633 nm (Cy5). Channels were acquired sequentially to avoid cross talk between the different filters. Images were processed using LSM Image Manager and Volocity LE software (Improvision).

Immunohistochemistry on zebrafish forebrain sections

Zebrafish embryos were collected and fixed with 4% PFA overnight, washed in PBS, and placed in PBS containing 30% sucrose to equilibrate. Embryos were then embedded in Shandon cryomatrix (Thermo Scientific, 2860051) and frozen at -20 °C. Cryosections were

done on a Leica CM1850 (Leica Microsystems, Weltzar, Germany) at a thickness of 10 μm and stored at $-20\text{ }^{\circ}\text{C}$ until use. Sections were washed 3X in PBS 0.1% Triton X (PBST) for 10 minutes and blocked for 2 hours in 2% BSA in PBST. The sections were incubated with the primary antibody (see below) overnight in a humid chamber at $4\text{ }^{\circ}\text{C}$. After removal of the primary antibody with 3 X PBST washes, the appropriate secondary antibody was incubated on the slides for 2 hours at room temperature. After the final washes, the slides were mounted using Aquatex mounting media (VWR, 65036-62). Signals were visualized on a Nikon Eclipse E3600 stereomicroscope for both fluorescent stains. The following primary antibodies were used in this study: Rabbit anti-GFP (1:1000, Invitrogen, A-11122); Mouse anti-glutamic acid decarboxylase (BioMol International, GC3108). The following secondary antibodies were used in this study: Goat anti-rabbit Alexa Fluor488 (1:300, Invitrogen, A11008); Goat anti-mouse Alexa Fluor594 (1:300, Invitrogen, A11005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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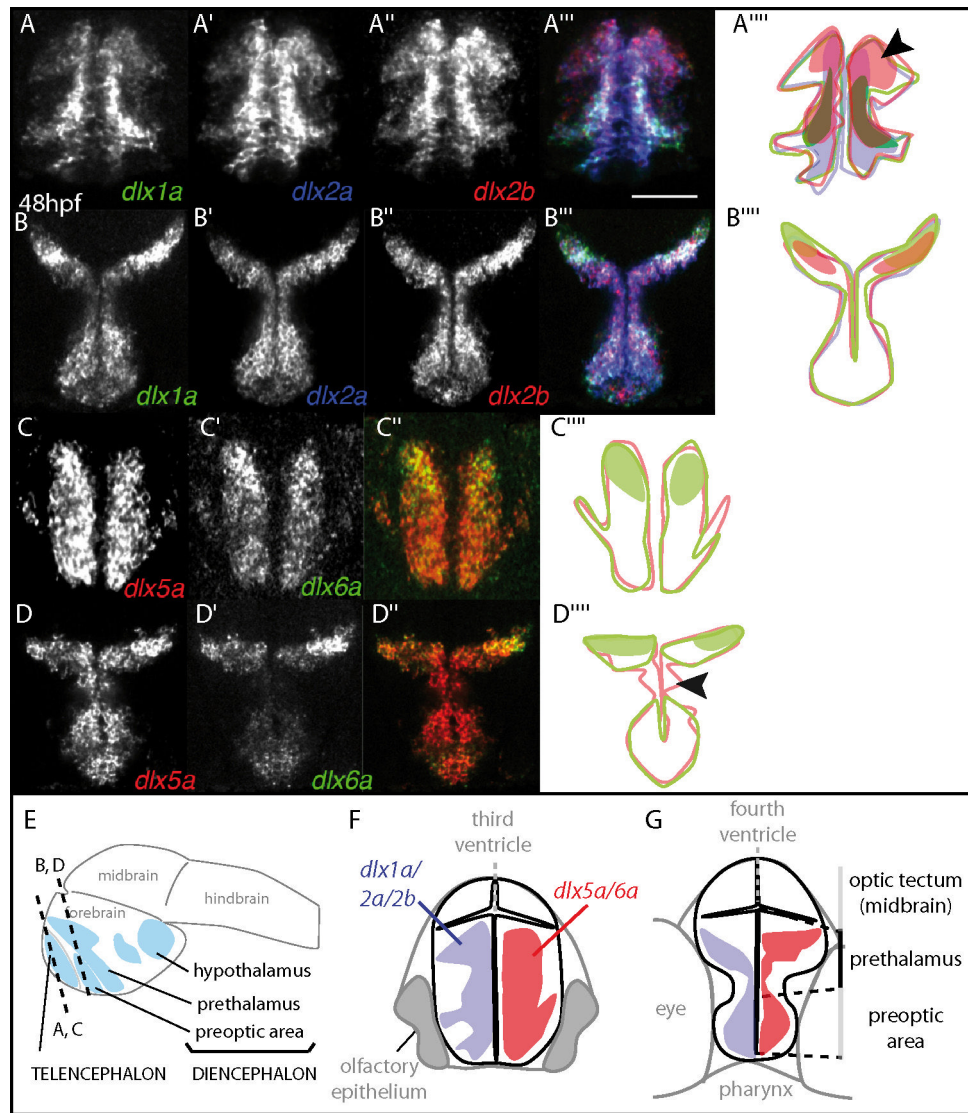


Figure 1. Similar forebrain expression of the two genes from a *dlx* cluster

Single z transversal sections of triple fluorescent *in situ* hybridization shows *dlx1a*, *dlx2a*, and *dlx2b* expression domains at 48hpf in the telencephalon (A-A''') and diencephalon (B-B'''), as well as *dlx5a* and *dlx6a* (C-C''') and (D-D'''). Schematic summaries representing the overlaps in *dlx* expression are shown in A''', B''', C''', and D''': the solid lines define the boundaries of detectable expression; the zones of relatively higher expression are colored when applicable. The boundaries of expression domains are comparable for *dlx1a*, *dlx2a* and *dlx2b* but *dlx2b* specifically displays a more intense zone of expression in a dorsal-lateral domain (arrow in A'''). Boundaries of expression domains for *dlx5a* and *dlx6a* are overall similar although *dlx6a* was hardly detected in the ventral domain of the prethalamus (arrowhead on D'''). Levels of section are shown on the schematic in (E). Shared expression domains of clustered genes (and their paralogs) are mapped on a schematic representation of a brain section at the level of the telencephalon (F) and of the diencephalon (G): *dlx1a/2a/2b* in blue and *dlx5a/6a* in red. Scale bar: 100 μ m.

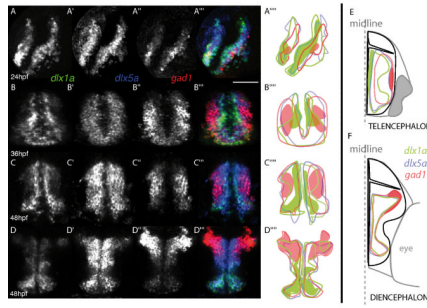


Figure 2. Expression domains of *dlx* genes with respect to *gad1* expression

Single z sections of triple fluorescent *in situ* hybridization for *dlx1a*, *dlx5a*, and *gad1* in the telencephalon at 24hpf (A-A'', para-sagittal section, anterior is on the left), 36hpf (B-B'', transverse section in the telencephalon, dorsal is to the top), and 48hpf (C-C'', transversal section in the telencephalon; dorsal is to the top) with a colored merge of all three channels on A'', B'', C'' and D''. For each section level, a schematic representation is given to localize the boundaries of each expression domain (solid line) and a domain of higher level of expression is represented as a colored surface. Panel E gives a schematic representation of the partially nested expression domains in the telencephalon (left half) and diencephalon (right half) at 48hpf, based on the data shown in panels C-C'' and D-D'': the proximal (ventricular) domain where only *dlx1a* is detected is shown in green and the lateral domain where *gad1* only is detected is colored red. Scale bar: 100 μ m.

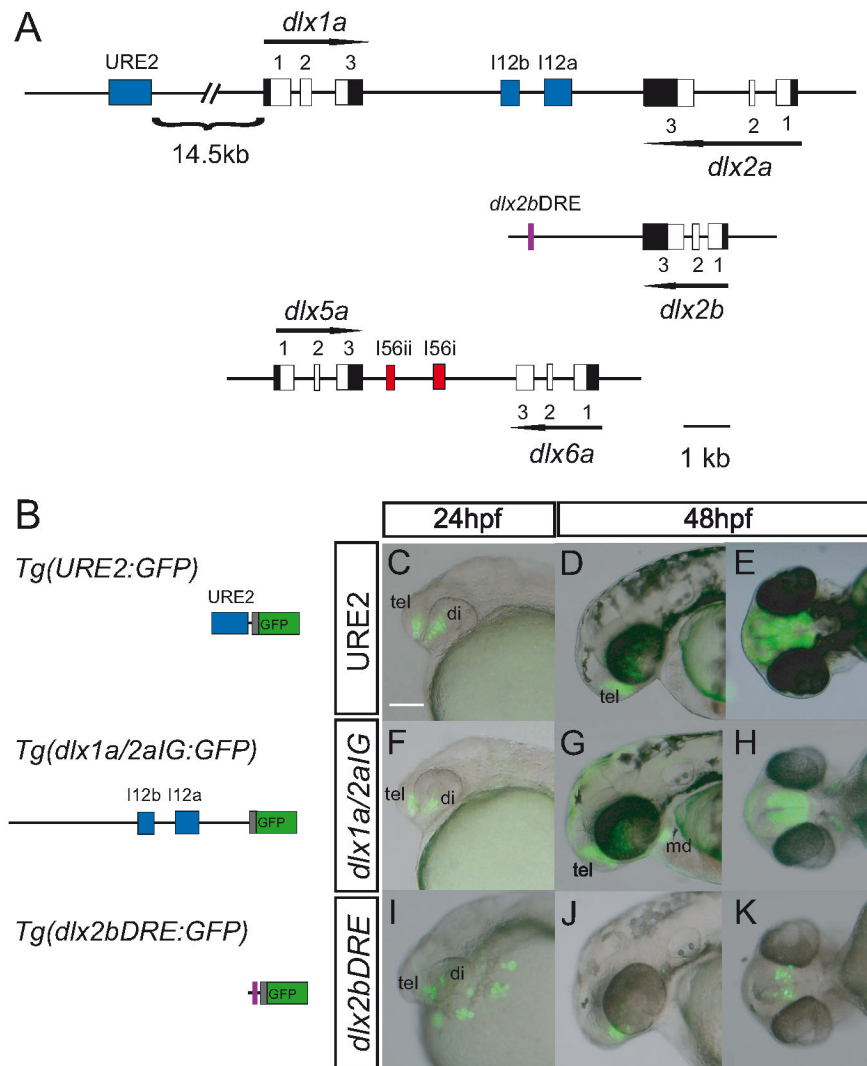


Figure 3. Regulatory elements of *dlx1a/2a* and *dlx2b* drive reporter gene expression in the zebrafish forebrain and branchial arches

Genomic organization of the *dlx1a/dlx2a* and of the *dlx2b* loci (A). Exons are represented in white and UTRs in black. Intergenic regulatory elements are shown as blue (URE2 and I12b) and red boxes (I56i and I56ii). The *dlx2bDRE* is shown as a purple box. Schematic representation of the transgene constructs (containing the β -globin minimal promoter (grey) linked to GFP (green)) used to generate transgenic zebrafish (B). The *Tg(dlx1a/2aIG:GFP)* construct contains a 6kb of the intergenic fragment from the *dlx1a/dlx2a* intergenic region, the *Tg(URE2dlx1a/2a:GFP)* contains a 900 bp fragment encompassing the URE2 enhancer and the *Tg(dlx2bDRE:GFP)* contains a 200 base pair fragment downstream of *dlx2b*. Activity of the transgene constructs in the forebrain at 24hpf and 48hpf (C-E) *Tg(URE2dlx1a/2a:GFP)* transgene (F-H) *Tg(dlx1a/2aIG:GFP)* transgene (I-K) *Tg(dlx2bDRE:GFP)*. tel, telencephalon; di, diencephalon; md, mandibular portion of branchial arches. Scale bar: 100 μ m.

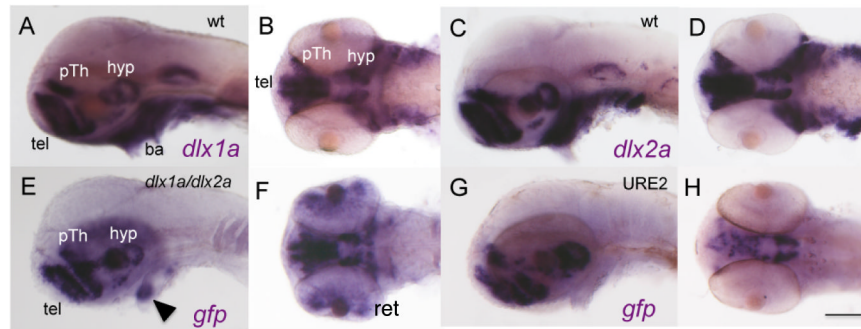


Figure 4. The *dlx* regulatory elements recapitulate endogenous *dlx* expression in the forebrain of the zebrafish at 48hpf

Whole mount *in situ* with probes against *dlx1a* (A,B), *dlx2a* (C,D) and *gfp* (E, F) *Tg(dlx1a/2aIG:GFP)* is expressed in the telencephalon, ventral thalamus, and hypothalamus. There is also expression in the pharyngeal arches (arrowhead), which recapitulates a small portion of the endogenous *dlx* arch expression domain. G,H) URE2 activity closely resembles the expression of *Tg(dlx1a/2aIG:GFP)* in the telencephalon, ventral thalamus, and hypothalamus and mimics *dlx* expression in these tissues. tel, telencephalon; pTh, prethalamus; hyp, hypothalamus; di, diencephalon; md, mandible; ba, branchial arches. Scale bar: 100 μ m.

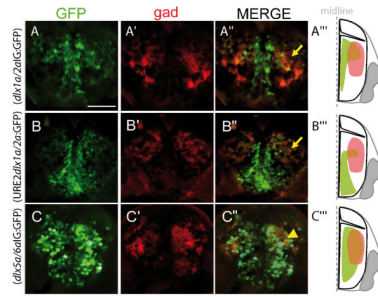


Figure 5. Immunolocalization of GFP and Gad proteins on transverse cryosections of the telencephalon at 48hpf

Expression of GFP (A, B, C) and Gad proteins (A', B', C') is detected in the three transgenic lines, a merged image is presented in panels A'', B'' and C'' to show overlap (yellow arrows). An interpretative schematic representation of a hemi-section is presented in each case in panels A''', B''' and C'''. Scale bars: 100 μ m.

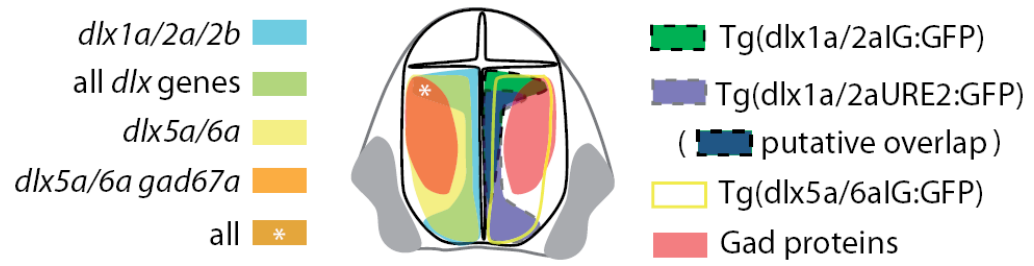


Figure 6. Schematic representation of the overlaps in *dlx* and *gad* expression (left) and of the activity of *dlx* regulatory elements (right) in the zebrafish forebrain.