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Generation and Analysis of Transgenic Zebrafish and Mice for the Study of Dix Function in the Forebrain

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Generation and Analysis of Transgenic zebrafish and Mice for the study of Dlx function in the forebrain

Purushothama Nanjappa

Thesis is submitted as a partial fulfillment of the Master of Science program in Cellular and Molecular Medicine

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Abstract

The vertebrate *Dlx* homeobox genes are organized as three convergently transcribed bigene clusters and show overlapping expression patterns in the forebrain, pharyngeal arches, sensory placodes and limb/fin buds. Little is known about how the *Dlx* genes are targeted to their sites of expression, or what particular roles individual genes play in development. *Cis*-acting regulatory elements have been identified in the intergenic and upstream regions of paired *Dlx* genes and are thought to play a major role in the *Dlx* regulation in the forebrain. My study was focused on two of the enhancer elements from the *Dlx1/Dlx2* locus, I12b and URE2. We used these two elements to target reporter transgene expression to the zebrafish forebrain and analyzed their activity. We also successfully demonstrated the use of Fluorescent Activated Cell Sorting to isolate *Dlx*-Green Florescent Protein-expressing cells from the zebrafish forebrain. These results contribute to our understanding of *Dlx* regulation, function and evolution.

Contents

List of figures

List of abbreviations

AER, apical ectodermal ridge

AIHHT, amelogenesis imperfecta hypoplastic-hypomaturation with taurodontism

AmphiDll, amphioxus *Distal-less*

bab , bric a brac

bHLH, basic helix-loop-helix

BMP, bone morphogenetic protein

Bp, base pair

BSA, bovine serum albumin

BSP, bone sialoprotein; extracellular matrix component involved with biomineralization

C-, carboxy

cDNA, complementary deoxyribonucleic acid

ChIP, chromatin immunoprecipitation

CS, calf serum

DAB, 3,3'-diaminobenzidine tetrahydrochloride

dac, dachshund

DEPC, diethylpyrocarbonate

DIP2, DLX interacting protein

Dll, *Distal-less* gene

Dlx, Distal-less related vertebrate homologue

DNA, deoxyribonucleic acid

dNTP, deoxyribonucleotides

DMSO, dimethyl sulfoxide

Dpf, days post-fertilization

Dpp, Decapentaplegic

EDTA, (disodium) Ethylenediamine tetraacetate (acid)

EGFP, enhanced green fluorescent protein

Exd, extradenticle

FACS, Fluorescence Activated Cell Sorting

FDG, fluorescein digalactoside

Fez, forebrain embryonic zinc finger

FGF, Fibroblast growth factor

GABA, γ-amino butyric acid

GAD, glutamic acid decarboxylase

GAL-4, yeast transcriptional activator

GFP, green fluorescent protein

GnRH , Gonadotropin-releasing hormone

HBS, homeodomain binding site

Hox, homeobox transcription factor

Hpf, hours post fertilization

Kb, kilobase

lacZ, β-galactosidase

LGE, lateral ganglionic eminence

mb, megabase

MGE, medial ganglionic eminence

mRNA, messenger ribonucleic acid

Msx, msh homologue

My, million years

N-, amino

NLS, nuclear localization signal

OB, olfactory bulb

O/N, overnight

°C, degree Celsius

PBS, phosphate buffered solution

PBST, phosphate buffered solution and Tween-20

PCR, polymerase chain reaction

PFA, paraformaldehyde

RA, retinoic acid

RNA, ribonucleic acid

RT, room temperature

RT-PCR, reverse transcriptase-polymerase chain reaction

sal, spalt

SHFM, split hand/split foot mutation

Shh, sonic hedgehog

ss , spineless

TDO, Tricho-Dento-Osseous syndrome

Wg, *Wingless*

WT, wild-type

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1. INTRODUCTION

1.1. The *Dlx* gene family

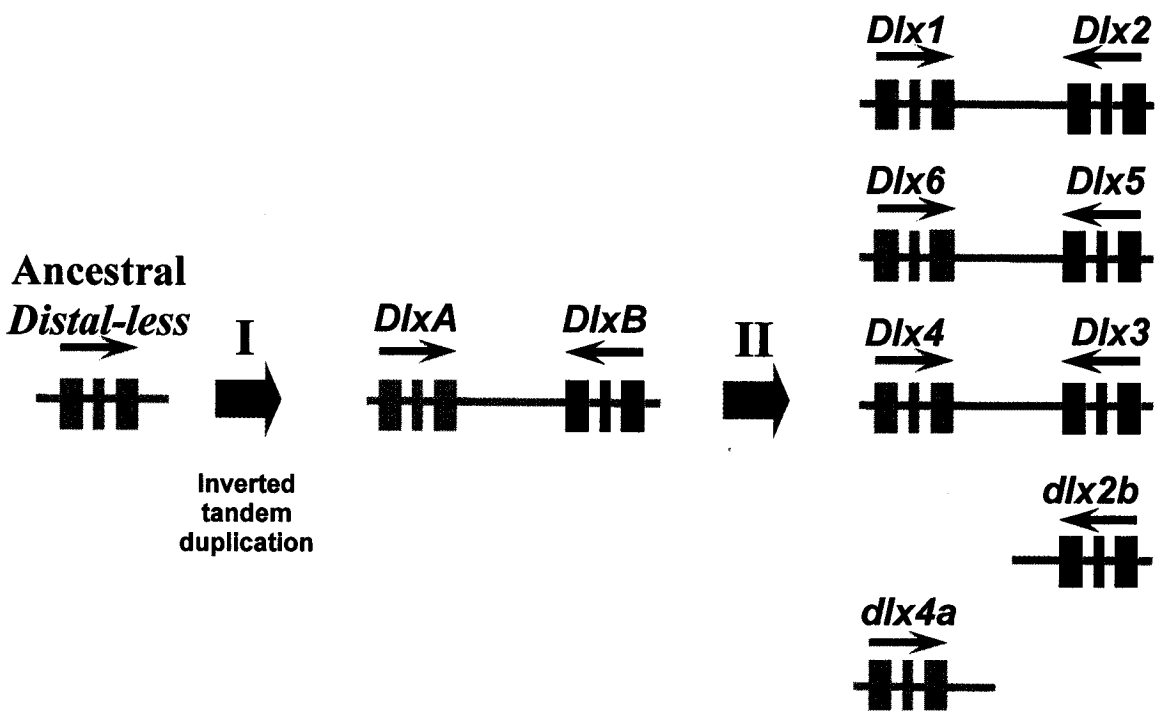
1.1.1. Vertebrate Dlx genes

Vertebrate *Dlx* genes encode a family of homeodomain-containing transcription factors related in sequence to the *Drosophila distal-less* gene product. *Distal-less* was initially characterized in *Drosophila melanogaster* and is specifically expressed in the developing appendages (Cohen *et al.*, 1989). In vertebrates, *Dlx* genes function in the proximodistal patterning for the pharyngeal arches, as well as several other morphological structures. The *Dlx* genes have been investigated in humans (Simeone *et al.*, 1994: Nakamura *et al.*, 1996) and in model organisms such as mouse (Porteus *et al.*, 1991, Simeone *et al.*, 1994; Nakamura *et al.*, 1996; Liu *et al.*, 1997) and zebrafish (Ekker *et al.*, 1992; Akimenko *et al.*, 1994, Stock *et al.*, 1996). Recently *Dlx* genes have also been investigated in two tetraodontid species, *Takifugu rubripe, Spheroids nephelus* (Ghanem *et al.*, 2003) and in a *leopard shark, Triakis semifasciata* (Stock, 2005). The vertebrate *Dlx* genes are primarily involved in the development of ventral forebrain, branchial arches, sensory organs and limbs/fin buds.

1.1.2. Genomic Organization of the Dlx genes

Vertebrate *Dlx* genes are organized as convergently transcribed bigene clusters separated by a relatively well conserved short intergenic space, ranging from 8-10kb in mammals and 4-7 kb in teleost species (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). In mouse and humans, there are sets of paired genes linked as shown in Fig. 1.1. The genes are linked

Figure 1.1 Model for the evolution of *Distal-less*-related genes. An initial inverted tandem duplication of an ancestral *Distal-less* gene resulted in an ancient bigene cluster (*DlxA* and *DlxB*). As a result of whole genome duplication, this ancient cluster gave rise to three linked pairs, *Dlx1/Dlx2, Dlx5/Dlx6* and *Dlx3/Dlx4*. *dlx2b* and *dlx4a* are the two additional genes found in zebrafish. They are unlinked. The three exons are indicated by boxes. The direction of transcription is indicated by arrows. Adapted from Zerucha and Ekker (2000).


Ancestral
Distal-less
I
Inverted
tandem
duplication
DlxA
DlxB
II
Dlx1
Dlx2
Dlx6
Dlx5
Dlx4
Dlx3
dlx2b
dlx4a

as *Dlx1/Dlx2, Dlx5/Dlx6* and *Dlx3/Dlx7* (*Dlx3/Dlx4),* according to the revised nomenclature by Panganiban and Rubenstein., (2002). Zebrafish, which diverged from a common vertebrate ancestor approximately 450 million years ago, has eight *dlx* genes organized in a similar manner as their mammalian orthologs, that is *dlx1a/dlx2a* (formerly *dlx1/dlx2*), *dlx5a/dlx6a* (formerly *dlx4/dlx6*), and *dlx3b/dlx4b* (formerly *dlx3/dlx7*).

The *Dlx* genes are located approximately 1 to 2 Mb from the *Hox* complexes (Stock *et al.*, 1996; Amores *et al.*, 1998; Ruddle *et al.*, 1999). In mouse and humans, *Dlx1* and *Dlx2* are linked to *Hoxd* (Ozcelik *et al.*, 1992; McGunines et al., 1996); *Dlx 3* and *Dlx4* are linked to *Hoxb* (Nakamura *et al.*, 1996); and *Dlx5* and *Dlx6* are linked to *Hoxa* (Simeone *et al.*, 1994). The *Hoxc* complex has no associated *Dlx* gene pair. This could be due to loss of *Dlx* genes or may be because the *Dlx* pair linked to the ancestral *Hoxc/Hoxd* cluster, which gave rise to the *Hoxc* and *Hoxd* clusters was not included in that duplication event (Amores et al., 1998).

Each Vertebrate *Dlx* gene has three exons and two introns, with a highly conserved intron splice site dividing the homeodomain between exons two and three (Ellies *et al.*, 1997b; Liu *et al.*,1997; Mcguinness *et al.*, 1996; Price *et al.*, 1991). Many of the *Dlx* genes have multiple transcripts of varying lengths, either due to several transcription initiation sites, as described for *Dlx1* (McGuinness *et al.*, 1996), or as a result of alternative splicing, as observed for *Dlx4* and *Dlx5* (Yang *et al.*, 1998; Liu *et al.*, 1997; Nakamura *et al.*, 1996). Antisense isoforms of unknown significance have also been

detected for *Dlx*1 and *Dlx6* (Liu et al., 1997). Furthermore, *Dlx5* was shown to produce cytoplasmic truncated proteins lacking the homeodomain and nuclear localization signal (Yang *et al.*, 1998; Liu *et al.*, 1997).

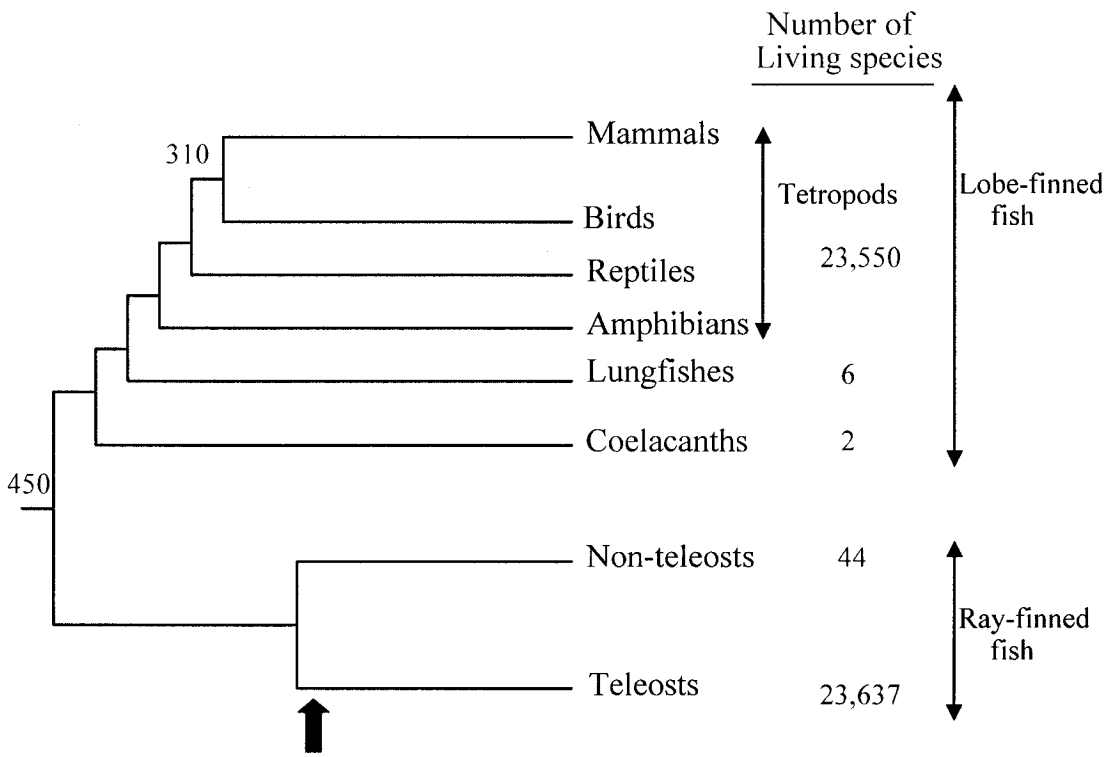
Outside the DNA-binding homeodomain, *Dlx* proteins share limited short regions of similarity. For example, all *Dlx* proteins possess at least two tryptophan residues that are C terminal to the homeodomain (Phelan *et al.*, 1995; Chang *et al.*, 1995). A tryptophan residue in a myogenic helix-loop-helix (bHLH) transcription factor has been found to mediate interaction with PBC-family co-factors such as pbx and Extradenticle (Exd) (Knoepfler *et al.*, 1999). *Dlx* proteins are proline-rich both upstream and downstream of their homeodomains. These proline-rich domains have been implicated in transcriptional activation and oligomerization (Xiao *et al.*, 2000). The proline-rich N and C termini of *Dlx3* cooperate in transcriptional activation (Feleday *et al.*, 1999) while N-terminal proline-rich sequences of *Dlx5* act as an activation domain when fused to the yeast Gal4 DNA-binding domain (Masuda *et al.*, 2001). In addition to the conservation of the coding region, both within and outside the homeodomain, non-coding intergenic regions separating linked pairs show approximately 400-500 bp sequence similarity. These conserved sites have important enhancer (*Cis*-acting) elements (Ghanem *et al.*, 2003; Zerucha *et al.*, 2000). These sequences will be discussed in more detail in section 1.3.2.

1.1.3. Evolution of the *Dlx* gene family

A model of evolution is revealed in the increase in genes arising from multiple large or whole genome duplications correlated with the increase in body complexity. Increasing

complexity is observed in comparing the simplest urochordates such as the ascidians and larvaceans, followed by the cephalochordates exemplified by amphioxus and at the apex of the phylum the vertebrates. All members of the chordate phylum share primitive characteristics that were inherited from a common ancestor (plesiomorphic traits). Such traits include a notochord, paired and segmented muscles, post-anal tail, and pharyngeal gill slits. Conversely, differences between species within a class, or comparisons between classes reveal apopmorphic or derived characteristics specific to those animals. Examples of derived traits include such morphological innovations as paired limbs, sensory placodes, true teeth and a jaw (Shimeld *et al.*, 2000). Many of the morphological evolutionary innovations are thought to be as a result of increase in the number of regulatory genes (Chipman, 2001), evolution of the developmental mechanisms they control (Rudel *et al.*, 2003) and the mutations in *cis*-regulatory sequences (Fondon and Garner, 2005). It has been proposed that the multiple vertebrate *Dlx* genes arose during the same duplication events that gave rise to the multiple *Hox* clusters of vertebrates (Stock *et al.*, 1996). Analysis of genomes from three different chordate classes indicate that urochordate species and amphioxus have only one *Hox* cluster (Dehal *et al.*, 2002; Schilling *et al.*, 2001; Holland *et al.*, 1994). Vertebrates have multiple *hox* clusters, with gnathostomes, jawed vertebrates, such as tetrapods having at least four (Amores *et al.*, 1998). Teleost species, such as zebrafish have seven *hox* cluster correlating with their large increase in phenotypic diversity and species radiation (Fig. 1.2) (Venkatesh, 2003; Amores *et al.*, 1998). The genomic organization and the linkage to the *Hox* genes of *Dlx* gene family suggest a model for their evolution (Zerucha and Ekker, 2000). Only one *Dll*-like/*Dlx* gene has been characterized in *Drosophila* and other invertebrates, such as

Figure 1.2 Evolution of bony vertebrates. The numbers at the nodes are the divergence time in million years. The estimated divergence time of teleosts is ~235 My old. Arrows indicates whole-genome duplication event that has been proposed to have occurred in an ancient teleost. Adapted from Byrappa Venkatesh, 2003.


Number of
Living species
Mammals
310
Birds
Reptiles
Amphibians
Tetropods
23,550
Lungfishes
6
Coelacanths
2
Lobe-finned
fish
450
Non-teleosts
44
Ray-finned
fish
Teleosts
23,637

the nematode, *Caenorhabdidits elegans* (Aspock *et al.*, 2001) suggesting that a single *Dlx* gene existed in the common metazoan ancestor (Fig. 1.1). A tandem duplication event might have occurred during the primitive chordate radiation, resulting in *DlxA* and *DlxB* cluster. Examples for this include the single *Dlx*-related gene in the hemichordate species *Ptychodera flava* (Harada *et al.*, 2001) and three *Dlx* homologs in the urochordate *Ciona intestinalis*, organized as one convergently linked pair and a third unlinked gene. (Caracciolo *et al.*, 2000; Di Gregorio *et al.*, 1995). Two subsequent polyploidization events associated with the linked *hox* gene cluster duplications occurred in the common vertebrate ancestor. Four *hox* clusters were generated during these genomic duplications; only three *Dlx* bigene clusters exist in mammals, suggesting that one pair was lost (Stock *et al.*, 1996).

The agnathan lamprey, *Petromyson marinus,*, which is a basal level vertebrate, has four *Dlx* genes organized as one bigene cluster (*DlxA/DlxD*) and two unlinked genes (*DlxB, DlxC*) suggesting loss of paralogs (Neidert *et al.*, 2001; Sumiyama *et al.*, 2003a). Recent work from our own lab showed that *DlxD* is linked with *DlxC* and not DlxA (Maurya and Ekker, unpublished observations.

In more advanced ray-finned fishes, an additional round of polyploidization or partial genomic duplication event occurred resulting in two additional *dlx* genes in zebrafish, *dlx2b* (formerly *dlx5*), and *dlx4a* (formerly *dlx8*) (Stock *et al.*, 1996; Zerucha *et al.*, 2000). This genome duplication event is thought to have occurred after the divergence of the lineage that gave rise to mammals (Stock *et al.*, 1996; Robinson-Rechavi *et al.*,

2001). This is evident from the presence of seven *Hox* clusters in zebrafish compared to only four in mammals and it has been proposed that the multiple vertebrate *Dlx* genes arose during the same duplication events that gave rise to multiple *Hox* clusters of vertebrates (Stock *et al.*, 1996). Consistent with this, recent genome sequencing data has revealed more number of genes in zebrafish compared to mammals. It is yet to be determined if *dlx2b* and *dlx4a* are physically linked with any unidentified *dlx* genes.

1. 2 Function of *Dlx* genes

1.2.1 Function of *Distal-less* (*Dll*) genes in Invertebrates

Distal-less is specifically expressed in developing *Droshophila* limbs and is expressed dynamically in the limbs throughout the development. *Dll* is also required for the elaboration of distal pattern elements in the antenna, legs, the limb-derived gnathal structures and the anal plate (Boekhoff-falk, 2005; Cohen *et al.*, 1989; Dong *et al.*, 2000).

The *Distal-less* mutations affect the segmented appendages of the adult as well as several of the larval sense organs (Cohen *et al.*, 1989). The adult structures include: the head appendages associated with the mouth such as the maxillary pulps, the labium, the labrum, the proboscis, a subset of the maxillary cirri; the antennae; and the legs. The larval structures affected include the labral, antennal, maxillary and labial sense organs of the head and the Keilin's sense organs of the thorax. The Keilin's larval sense organs are homologous to the distal sensory apparatus of the legs of lower insects and are associated with developing appendages. These larval sense organs are rudimentary structures compared to the morphologically well developed limbs of simpler insects in that they

consist solely of the distal sensory apparatus. Sense organs not associated with developing limbs are unaffected in mutant embryos. In *Dll* mutants, the larval sense organs are deleted and the distal structures of the adult appendages are reduced in size and fused together. A number of different *Dll* mutations have been compared (Cohen and Jurgens, 1989; Cohen *et al.*, 1989) and while there is some variability in the severity of the heterozygous mutations (homozygous mutations are embryonic lethal), discrete adult structures are never deleted. This indicated that *Dll* is involved in patterning the formation of the distal limb as a whole.

The appendages of arthropods are thought to have evolved from simple, non-segmented appendages present in annelid-like ancestors. Modern segmented appendages are believed to have evolved as a result of specializations added to the ancestral unsegmented appendage. Thus the proximal domain of modern segmented appendages is representative of an evolutionary ground state upon which modern segmented specializations have been built. Based on its apparent role in distal but promixal appendage structures, *Dll* has been proposed to be the gene which promotes the development of appendage structures above the developmental and evolutionary ground state (Cohen and Jurgens, 1989).

Caenorhabditis elegans Dll ortholog, *Ceh-43* function and expression pattern has been investigated by Aspock and colleagues (Aspock *et al.*, 2001). The expression is mainly detected in the head and tail hypodermal cells, neuronal support cells and neurons during developmental stages (Aspock *et al.*, 2001).

The Regulation of *distal-less* in *Droshophila* has been thoroughly investigated by several workers. Embryonic expression of *Dll* in the thorax is activated by *Wingless* (Wg) and repressed by a bone morphogenetic protein (BMP) homolog, *Decapentaplegic* (Dpp) and by the epidermal growth factor (EGF) signaling pathway (Raz and Shilo 1993). However, both *Wg* and *Dpp* are required for the maintenance of *Dll* expression (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). There are no known direct targets of *Dll* regulation, but a variety of genes have been shown to lie genetically downstream, and represent candidate targets for direct regulation by *Dll.* These include *aristaless* (Cobos *et al.*, 2005b; Campbell and Tomlinson, 1998), *Spineless* (*ss*) (Duncan *et al.*, 1998), *bric a brac* (*bab*) (Campbell and Tomlinson, 1998; Gorfinkiel *et al.*, 1997), BarH1/BarH2 (Kojima *et al.*, 2000). *Ser*, a gene encoding the notch ligand serrate is repressed by *dll* in the tarsus (Rauskolb, 2001). The *Drosophila* antenna encompasses both ear and nose. Numbers of genes have been identified as potential targets of *Dll* activation in the developing retina and all of them have vertebrate homologs that are expressed in limb or ear suggesting a possibility that all these genes are evolutionarily conserved *Dll/Dlx* targets. For example, *dachshund (dac)* and *spalt (sal)* (Dong *et al.*, 2002) identified as potential target of *Dll* activation have vertebrate homologs namely *sal/SallI and dac/Dac* (Davis *et al.,* 2001).
It is interesting to note that vertebrate *Dlx* genes have been implicated in the development of the ear (Solomon and Fritz, 2002; Acampora *et al.*, 1999), nose (Quint *et al.*, 2000; Acampora *et al.*, 1999), mandible and maxilla (Depew *et al.*, 2002) and nervous system (Qiu *et al.*, 1995), suggesting evolutionary conservation of *Dll/Dlx* function.

1.2.2 Function of *Dlx* genes in vertebrates

Dlx genes are mainly expressed in hard tissues of the head, the axial and appendicular skeleton, forebrain, pharyngeal arches, sensory placodes, and developing limb/fin buds (Merlo *et al.*, 2000; Panganiban and Rubenstein, 2002). These expression patterns are well conserved between highly divergent vertebrate species such as mouse and zebrafish. Generally, *Dlx1/Dlx2* and *Dlx5/Dlx6* are expressed in similar patterns with subtle differences. *Dlx3/Dlx7*, however, is expressed in more neomorphic vertebrate organs such as placenta and erythropoietic lineages (Quinn *et al.*, 1998; Morasso *et al.*, 1999).

The roles of *Dlx* genes in vertebrate development has primarily been defined by generation of genetic null mutations for most of the mouse *Dlx* genes both individually as well as combination of two genes. Double mutants have more severe abnormal phenotype with novel malformations, indicating genetic compensation between linked genes. Role of *Dlx* genes in the specific organs will be discussed in the following sections.

1.2.2.1 *Dlx* function in limbs/fin buds

In tetrapods, *Dlx* genes are expressed in the apical ectodermal ridge (AER) of the developing limbs (Ferrari *et al.*, 1999; Morasso *et al.*, 1995; Ferrari *et al.*, 1995; Zhao *et al.*, 1994). In zebrafish, the *dlx* genes are expressed in the apical ectodermal cells of the pectoral fin buds and in the developing median fin fold (Akimenko *et al.*, 1994). Single as well as double *Dlx1/Dlx2* mutants revealed no changes in the limb morphology (Depew et al., 2002; Acampora et al., 1999; Qiu et al., 1997). However, *Dlx5/Dlx6* double mutants showed a split hand/foot malformation (SHFM) like phenotype (Robledo *et al.*, 2002). Spatiotemporal-specific transgenic overexpression of *Dlx5* in the apical

ectodermal ridge of *Dlx5/Dlx6* null mice resulted in rescue of *Dlx* function in limb outgrowth (Robledo *et al.*, 2002). *Dlx5* and *Dlx6* are also involved in the development of genital bud and external ear lobes of the mice (Merlo *et al.*, 2000). Therefore, *Dlx* gene function is necessary in several outgrowths of a body.

1.2.2.2 *Dlx* function in sensory organs, sensory placodes

The role of *Dlx* gene expression in sensory organs is conserved throughout evolution (discussed in the section 1.2.1). *Dlx5* plays a major role in chick and murine ear as well as olfactory bulb development (Bhattacharyya et al., 2004; Long *et al.*, 2003; Merlo *et al.*, 2002; Levi *et al.*, 2003). In zebrafish, *dlx3b and dlx4b* perform a prominent role in the development of Rohon-Beard (RB) sensory neurons and trigeminal (TG) placodes (Kaji *et al.*, 2004), as well as olfactory and otic placodes (Depew *et al.*, 1999; Quint *et al.*, 2000). *Dlx1/Dlx2* have been found in sensory neuronal precursors (Eisenstat *et al.*, 1999) and *Dlx3* in the optic cup and neural retina (Dhawan *et al.*, 1997). Recent evidence from chick suggests that all the members of the *Dlx* gene family except *Dlx*4 are necessary for vestibular and neural development of the inner ear (Brown *et al.*, 2005).

Dlx genes have been also shown to play a role in patterning of neural and non-neural surface ectoderm. Neural border-derived features include neural crest, sensory placodes and dorsal midline of the neural tube (Copp et al., 2003). *Dlx* genes participate in the specification of the border, which is necessary for subsequent fate determination of neural crest and sensory placodes (Woda et al., 2003). In mouse and chick, *Dlx5* specifies

the neural crest or fully formed sensory placodes at the border of the neural plate and is subsequently expressed in the olfactory epithelium (McLarren *et al.*, 2003). In early zebrafish embryos, *dlx3b/dlx4b* are expressed in the bilateral ectodermal stripes adjacent to the olfactory and otic placodes, while *dlx5a/dlx6a* are expressed in the sensory primordia (Ellies *et al.*, 1997b; Akimenko *et al.*, 1994). In mouse, *Dlx3* expression in the anterior neural ridge ectoderm is weak; however it is apparently compensated by the *Dlx5/Dlx6* expression (Quint *et al.*, 2000; Yang *et al.*, 1998). The amphioxus *Dlx* homologue, *AmphiDll*, is expressed in the ectodermal cells surrounding the neural plate, suggesting an ancestral role of the chordate *Dlx* gene in specifying the neural crest cells (Holland et al., 1996). *Dlx2a* knockdown experiments in zebrafish have revealed that *dlx2a* in necessary for the differentiation of the sensory ganglia and maintenance of hindbrain neural crest (Steven Sperber, PhD thesis, 2004). Thus, *Dlx* genes have a multifunctional role in sensory organ development.

1.2.2.3 *Dlx* function in pharyngeal arches

Pharyngeal arches are paired structures that grow on either side of the future head and neck of the developing embryo and fuse at the centerline. Pharyngeal arches gives rise to cartilage, bone, nerves, muscles, glands, and connective tissue of the face and neck. The first two pairs give rise to the bones, muscles, and nerves of the ear, jaw and upper neck. The last three pairs of arches give rise to the bones, muscles, and thymus and thyroid glands. *Dlx* genes are known to participate in the proximodistal specification of the pharyngeal arches and their derived structures, including masticatory apparatus and teeth (Zhao *et al.*, 2000; Weiss *et al.*, 1995). In gnathostomes, *Dlx2* is the first family member

expressed in the cranial neural crest migrating from the rhombencephalon to the arches (Akimenko *et al.*, 1994; Bulfone *et al.*, 1993a). Subsequently, other *Dlx* genes are expressed in a dynamic manner in the arch primordia during pharyngogenesis (Akimenko *et al.*, 1994). *Dlx* genes are expressed in a nested pattern in the cranial neural crest derived ectomesenchymal cells (Schilling, 2003; Graham, 2002; Yelick and Schilling, 2002). Over expression of *Dlx2* in the branchial arch mesenchyme induced N-cadherin and NCAM and resulted in a dramatic increase in cell-cell adhesion and an increase in mesenchymal condensation, suggesting its role in regulating ectomesenchymal cell adhesion (McKeown *et al.*, 2005). In mouse, during the first molar development *Dlx2* is expressed in the epithelial and mesenchymal cells (Ma *et al.*, 2003). Expression of *Dlx1* and *Dlx2* has been detected in thymus (Woodside *et al.*, 2004). In the arch primordial, *Dlx1/Dlx2* expression is in a broad continuous rostral to caudal domain from the proximodorsal aspect of the maxilla portion of the first arch to the distoventral tip of the mandible. *Dlx5/Dlx6* expression overlaps with *Dlx1/Dlx2* in the mandible and *Dlx3/Dlx7* in the most distal part of the first arch, creating a nested pattern (Qiu *et al.*, 1997). The functional significance of the nested pattern is evident in mouse double mutants. Targeted loss of *Dlx1/Dlx2* results in dysmorphology of proximal soft and hard tissue features including an absence of maxillary molars (Qiu *et al.*, 1997). *Dlx5/Dlx6* double mutants exhibit craniofacial defects including enantiomorphism of both hard and soft tissues of the proximal maxillary portion of the first arch, replacing the distal mandibular features (Depew *et al.*, 2002; Beverdam *et al.*, 2002). Furthermore, *Dlx5/Dlx6* double mutants exhibit defects such as abnormal middle ear and jaw bone dysmorphology (Depew *et al.*, 2002). Knockdown of *dlx1a* in zebrafish resulted in craniofacial abnormalities (Steven

Sperber, Ph.D thesis, 2004). The nested expression pattern has also been attributed to the presence of *Cis*-acting regulatory elements in the intergenic region of *Dlx* bigene clusters. In fact, I12a and I56i, two distinct enhancer elements, identified in the intergenic regions of the *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters target transgene expression to ectomesenchymal and mesenchymal cells of the first arch (Park *et al.*, 2004; Ghanem *et al.*, 2003)

1.2.2.4 *Dlx* role in cartilage and skeleton development

Lately, the role of *Dlx* genes in cartilage formation is being investigated in precursors of skeletal tissue. *Dlx2* is involved in BMP-mediated expression of collagen type II (*colal*), a fibril-forming extracellular matrix protein prevalent in cartilage (Xu *et al.*, 2001). *Dlx5* and *Dlx6* are expressed in the endochondral and membranous-derived skeletal tissues of ectomesenchyme, and skeletal tissues of mesodermal origin (Zhao *et al.*, 1994; Acampora *et al.*, 1999; Depew *et al.*, 1999; Ghoul-Mazgar *et al.*, 2005). In chick and mouse, *Dlx5* participates in chondrocyte proliferation and maturation (Bendall *et al.*, 2003; Ferrari and Kosher, 2002). Additionally, BMP signals have been shown to regulate *Dlx5* expression in the neural crest derived membranous bone of the chick skull (Holleville *et al.*, 2003), and alveolar bone of the mouse jaw (Zhang *et al.*, 2003). Microarray investigation of BMP2 responsive gene in osteoblasts show that *Dlx2* and *Dlx5* are induced and maintained until mineralization (Harris *et al.*, 2003). Loss of *Dlx5* function causes reduction in the endosteal component of long bone diaphyses, and these enamels have a decrease in the periiosteal lamina causing delays in certain dermal bone maturation (Depew *et al.*, 1999; Acampora *et al.*, 1999).

1.2.2.5 *Dlx* role in tooth development

Mammalian teeth are derived from the oral ectoderm, and underlying ectomesenchyme exclusively in the maxillomandibular processes. *Dlx* genes are expressed in the ectoderm, and mesenchyme of the maxillomandibular process of the first arch (Bulfone *et al.*, 1993a). As jaw development progresses, *Dlx* genes participate in tooth development (Weiss *et al.*, 1995; Zhao *et al.*, 2000), and alveolar bone formation which supports the dentition (Zhang *et al.*, 2003).

Teeth are ectodermal appendages that undergo several stages of development beginning with oral epithelial thickening at the site of tooth formation. *Dlx1* and *Dlx2* genes are initially expressed in the mesenchyme. The dynamic expression of the *Dlx* genes in the oral epithelium and ectomesenchyme reveals the complex reciprocal epitheliomesenchymal interactions necessary for tooth development (Weiss *et al.*, 1995; Thomas *et al.*, 1995). Additionally, *Dlx* paralogues have differing dynamic expression patterns during tooth development, suggesting a differential regulation between paired genes (Zhao *et al.*, 2000).

Dlx function in tooth development is yet to be investigated thoroughly. However, a human disorder, Tricho-Dento-Osseous (TDO) syndrome, characterized by abnormal hair, tooth and bone development, is attributed to a *Dlx3* mutation (Haldeman *et al.*, 2004). A human enamel genetic disease named amelogenesis imperfecta hypoplastic-hypomaturation with taurodontism (AIHHT), is associated with a *Dlx3* mutation within the homeobox domain (Dong et al., 2005). The targeted *Dlx5* mouse mutant has poor

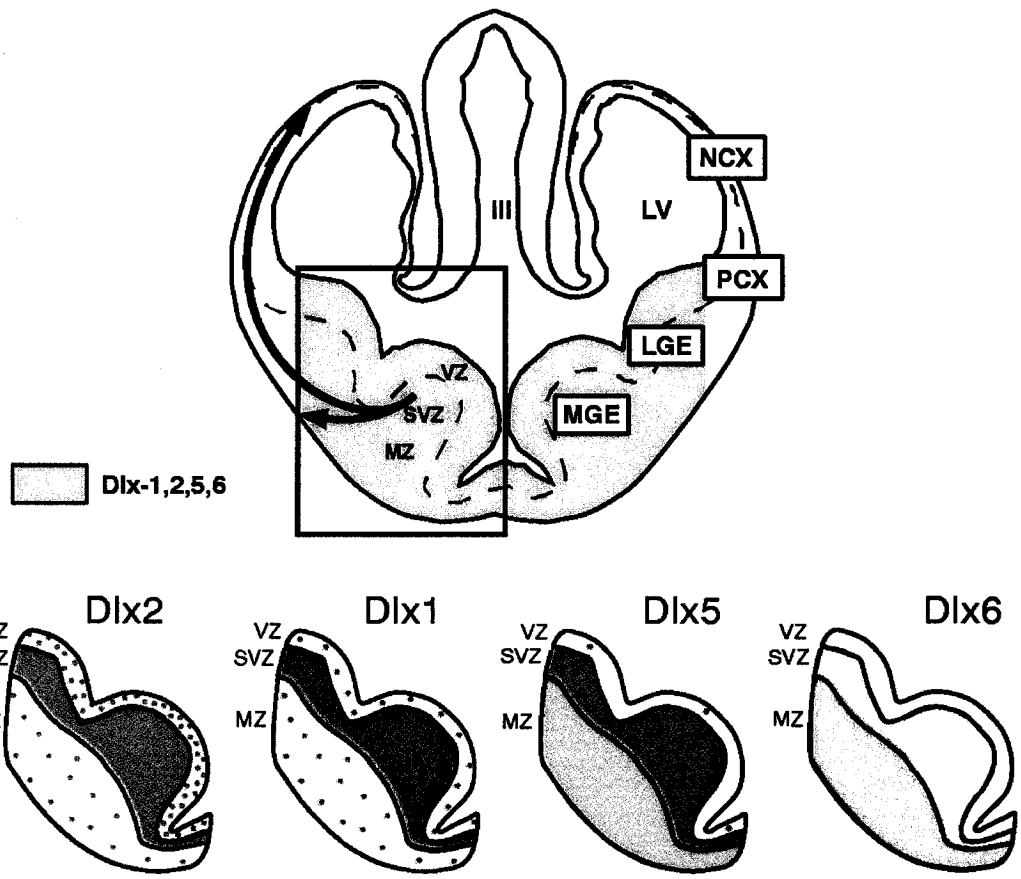
mineralization of the molar crowns suggesting a role in odontoblast differentiation (Depew *et al.*, 1999). *Dlx1/Dlx2* mutant mice replace the loss of maxillary molars with cartilaginous nodules, suggesting that the mesenchyme cannot respond to epithelial cues necessary for tooth development (Thomas *et al.*, 1997). Therefore, the role of the *Dlx* genes extends to patterning of all types of hard tissues in the body.

1.2.2.6 *Dlx* expression and function in the forebrain

Forebrain, also recognized as prosencephalon, is the most anterior of the three primary regions of the embryonic brain from which the telencephalon and diencephalon develop. The telencephalon consists of the cerebral cortex, basal ganglia, corpus striatum and olfactory bulb. The diencephalon includes the thalamus, hypothalamus, epithalamus and third ventricle. The *Dlx* genes are involved in regional specification of the vertebrate forebrain. Among the 6 known *Dlx* genes in vertebrates, four genes, *Dlx1, Dlx2, Dlx5 and Dlx6* are expressed in diencephalic and telencephalic domains. These two domains are also present in chickens, frogs, turtles, zebrafish and lampreys (Panganiban and Rubenstein, 2002). In the forebrain, the expression of *Dlx* follows a temporal sequence:

Dlx2, Dlx1 and Dlx5, then Dlx6 (Figure 1.3). *Dlx2* is expressed in subsets of neuroepithelial cells in the ventricular zone. *Dlx1* is expressed in the ventricular and subventricular zones during neuroblast proliferation. *Dlx5* is expressed in the subventricular and mantle zone in the differentiating post-mitotic cells, whereas *Dlx6* is

Figure 1.3 Expression domains of *Dlx*1, *Dlx*2, *Dlx*5 and *Dlx*6 during mouse brain development. Schematization of a transverse section through the mouse telencephalon showing the combined expression of *Dlx* genes. *Dlx*1 and *Dlx*2 are mostly expressed in subventricular zone and in few cells in ventricular and mantle zone. *Dlx*5 and *Dlx*6 are expressed in differentiated cells in the mantle zone. Adapted from Panganiban, G. *et al.* Development 2002



Model:

Dlx2 → Dlx1 → Dlx5 → Dlx6

mostly expressed in the mantle, the site of terminally differentiated neurons and glia (Eisentat *et al.*, 1999; Liu *et al.*, 1997; Porteus *et al.*, 1994). The spatiotemporal patterns of *Dlx* expression suggest a regulatory cascade for organizing differentiation of the basal ganglia (Liu *et al.*, 1997).

Dlx1, Dlx2, Dlx5 and Dlx6 are expressed in an overlapping manner in the developing forebrain suggesting genetic redundancy (Eisenstat *et al.*, 1999; Bulfone *et al.*, 1993b). For example, *Dlx2* mutants have reduced numbers of dopaminergic neurons in the olfactory bulb while the *Dlx1/Dlx2* double mutants exhibit a major block in neurogenesis within the sub-cortical telencephalon and the differentiation of several types of GABAergic, dopaminergic, and cholinergic interneurons (Andrews *et al.*, 2003; Anderson *et al.*, 2001; Marin *et al.*, 2000; Pleasure *et al.*, 2000; Anderson *et al.*, 1997a; Anderson *et al.*, 1997b). Assessment of the retinal phenotype in the *Dlx1/Dlx2* double knockout mice indicated that *Dlx* function is necessary for terminal differentiation of late-born retinal ganglion cell (RGC) progenitors (de Melo *et al.*, 2005).

Dlx1 knockouts have reduced numbers of cortical and hippocampal interneurons, leading to symptoms of epilepsy in animals over 2 months age (Cobos *et al.*, 2005a). *Dlx1* is also implicated in the development of thalamus in the mouse and monkey forebrain (Jones *et al.*, 2004).

Dlx genes are also involved in the development of neurons that utilize the Gama-aminobutyric acid (GABA) neurotransmitter and that contribute to the cerebral cortex, hippocampus, and olfactory bulb (Anderson *et al.*, 1997a; Anderson *et al.*, 1997b;

Bulfone *et al.*, 1998; Anderson *et al.*,2001; Stuhmer *et al.*, 2002b). *Dlx1, Dlx2* and *Dlx5* have been shown to activate a *cis*-regulatory sequence of the glutamic acid decarboxylase (GAD) gene, the rate limiting enzyme in the synthesis of the GABA neurotransmitter (Panganiban *et al.*, 2002). Expression of *Dlx1* and *Dlx2* in the lateral (LGE) and medial (MGE) ganglionic eminences and subpallial embryonic structures, is required for generation of telencephalic interneurons, which migrate and populate the cortex, hippocampus, and olfactory bulb (OB) (Saino-Saito et al., 2003). Ectopic expression of *Dlx2 and Dlx5* in cortical neurons induces a GABAergic phenotype of large nuclei and expression of GAD65 and GAD67genes (Anderson *et al.*, 1999; Stuhmer *et al.*, 2002a). Therefore, *Dlx* genes, in addition to their role in development of limbs/fin buds, sensory organs and branchial arches, are required for distinct stages of neuronal development in the forebrain.

1.3 Regulation of *Dlx* genes

1.3.1 Regulation by signaling molecules

Efforts to identify the substances, signal transduction pathways, and *cis*-elements that regulate *Dlx* expression are just beginning. Thus far, signaling factors like Sonic hedgehog (*Shh)*, BMPs and FGFs have been implicated in influencing *Dlx* gene expression. BMPs regulate *Dlx* expression both positively and negatively depending on tissue type and developmental stage. Bone morphogenetic protein 2 (BMP2) induces *Dlx2/Dlx5* expression during chondrogenesis (Xu *et al.*, 2001). BMP4 induces *Dlx5* expression in osteoblasts (Miyama *et al.*, 1999) and *Dlx1/Dlx2* in late dental mesenchyme (Bei *et al.*, 1998). However, BMP4 signaling induces *Dlx2* expression in oral epithelium

and inhibits mesenchymal expression prior to odontogenesis, suggesting a stage-dependent regulatory mechanism (Thomas *et al.*, 2000). *Shh* can induce *Dlx* expression in the forebrain (Gaiano *et al.*, 1999). FGF signals have been shown to stimulate *Dlx* expression in tissues such as denervated axolotol limb ectoderm (Mullen *et al.*, 1996), and in chick limb primordia (Ferrari *et al.*, 1999). In an inverse manner to BMP4, FGF8 inhibits *Dlx2* expression in the oral epithelium (Thomas *et al.*, 2000). However, FGF8 induces *Dlx1 and Dlx2* expression in the dental mesenchyme (Shigetani *et al.*, 2002; Bei *et al*, 1998). FGF9 and FGF4 signaling in the oral epithelium also contributes to *Dlx1/Dlx2* and *Dlx5* maintenance (Trump *et al.*, 1999). In the mandibular arch, FGF8 and FGF9 have a positive influence on expression of reporter transgenes placed under the control of the I12a and I56i enhancers, found in the *Dlx1/Dlx2* and in the *Dlx5/Dlx6* intergenic regions, respectively. However, BMP4 exhibited a negative influence on both regulatory elements (Park *et al.*, 2004). Another signaling molecule shown to influence *Dlx* gene expression is retinoic acid. Zebrafish embryos treated with retinoic acid during cranial neural crest migration showed reduced *Dlx* expression in ectomesenchymal cells (Ellies *et al.*, 1997a).

Various transcriptional regulators have been shown to influence *Dlx* expression. Pitx2, a bicoid-like transcription factor, expressed during tooth development, induces a 3.2kb 5'-flanking sequence of the *Dlx2* promoter in Chinese hamster ovary cells (Espinoza et al., 2002). Misexpression of Fez, an early forebrain marker, ectopically induces *dlx2a* and *dlx6a* expression (Yang et al., 2001). Msx1 has been shown to maintain *Dlx2* expression in the arch mesenchyme (Bei and Mass, 1998). Studies in mouse and zebrafish indicate

familial cross-regulatory interactions between *Dlx* genes. Examples of cross-regulation include *Dlx1/Dlx2* expression in the subventricular zone of the LGE and MGE that is necessary for *Dlx5/Dlx6* subsequent expression (Zerucha *et al.*, 2000). *dlx3b* expression in the zebrafish otic placode is necessary for *dlx5a* subsequent expression (Zerucha *et al.*, 1997; Solomon and Fritz, 2002)

1.3.2 Regulation by *cis*-acting regulatory elements

Phylogenetic footprinting analysis of the non-coding regions surrounding the *Dlx* genes has revealed many sites of conservation between highly divergent species, such as teleosts and mammals (Fig 1.4 and Fig 1.5). When tested in transgenic animals, these conserved elements drive reporter gene expression to the regions of the endogenous expression patterns, indicating regulatory function (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b). The conserved elements drive reporter gene expression in the forebrain, the limb buds, and in the pharyngeal arches, recapitulating the endogenous *Dlx* expression patterns, and provide a mechanism for shared expression (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b). A high degree of overlap in expression pattern of each *Dlx* pair suggests its regulation by shared enhancer elements in the intergenic region.

Two enhancer elements, I56i and I56ii, identified in the intergenic region of *Dlx5/Dlx6* clusters of mouse, human and zebrafish, are capable of recapitulating forebrain expression in zebrafish as well as in mouse (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). DNA-protein binding assays and Co-transfection experiments indicate that I56i is responsive to *Dlx*1 and *Dlx2* genes *in vivo* and *in vitro*, suggesting cross regulatory

interactions between bigene clusters (Zerucha *et al.*, 2000; Stuhmer *et al.*, 2002a). Mice homozygous for the *Dlx1/Dlx2* null mutation that also carry the I56i-lacZ transgene had no forebrain expression. This was comparable to the *Dlx5/Dlx6* loss, suggesting that *Dlx1/Dlx2* may function as positive regulators of *Dlx5/Dlx6* expression through the I56i enhancer (Anderson *et al.*, 1997b; Zerucha *et al.*, 2000).

One limiting factor in distinguishing the role of linked *Dlx* genes in a bigene cluster is their overlapping expression patterns, suggesting functional redundancy (Solomon and Fritz, 2002; Quint et al., 2000; Zerucha and Ekker, 2000) However, subtle differences in the expression patterns do exist as it can be seen in the forebrain (Fig. 1.3). The subtle differences do exist between endogenous expression pattern of *Dlx5* and *Dlx6* forebrain expression patterns with the I56i enhancer recapitulating *Dlx5* expression more closely, suggesting that the enhancer works differentially between the two linked genes (Zerucha *et al.*, 2000).

Recent phylogenetic footprinting comparing *Dlx1/Dlx2* clusters in mouse, human and zebrafish has revealed two enhancers, namely, I12a and I12b (Ghanem *et al.*, 2003) Mouse I12a drives reporter gene expression to a subset of mesenchymal cells in the mandibular component of the first branchial arch and in the second branchial arch, whereas, I12b targets reporter gene expression to the telencephalon and diencephalons domain of transgenic mice (Ghanem *et al.*, 2003). The activity patterns of both elements mimicked endogenous expression patterns (Ghanem *et al.*, 2003).

Phylogenetic footprinting analysis of human and mouse *Dlx3/Dlx4* clusters has revealed

Figure 1.4 Schematization of the *Dlx5/Dlx6* Genomic organization in Mouse, Human and Zebrafish. Arrows indicate direction of transcription. Conserved forebrain enhancers, I56i and I56ii are depicted. The third exons of the *Dlx* genes are indicated.

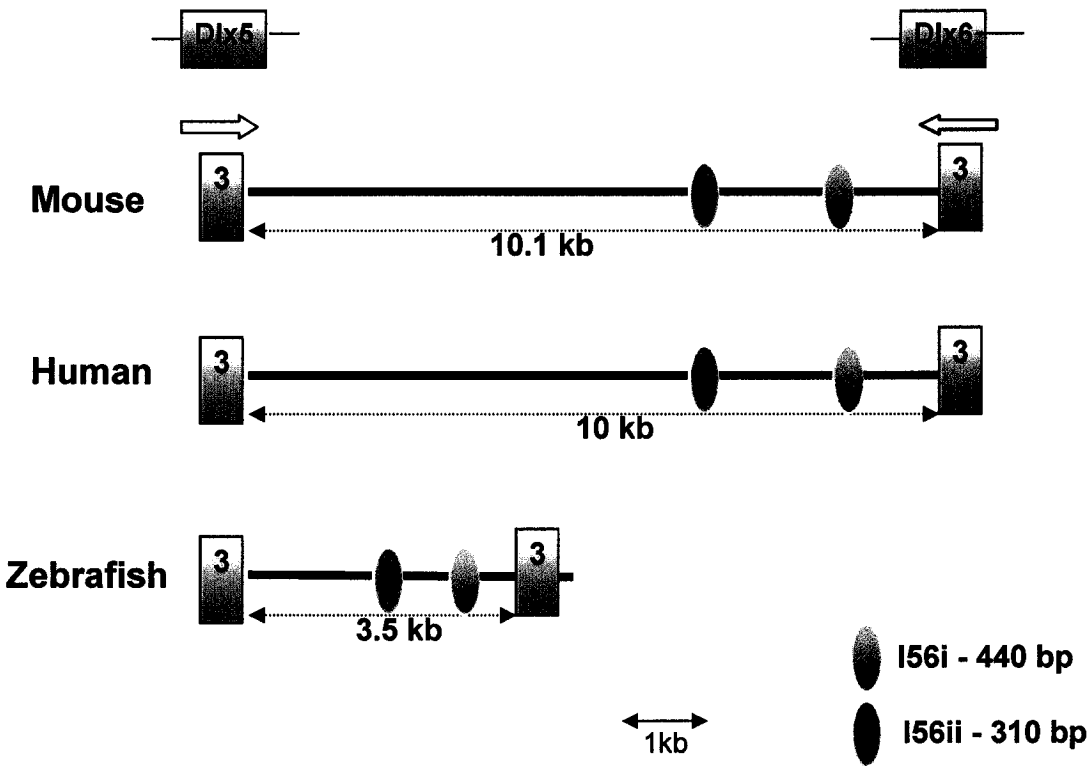
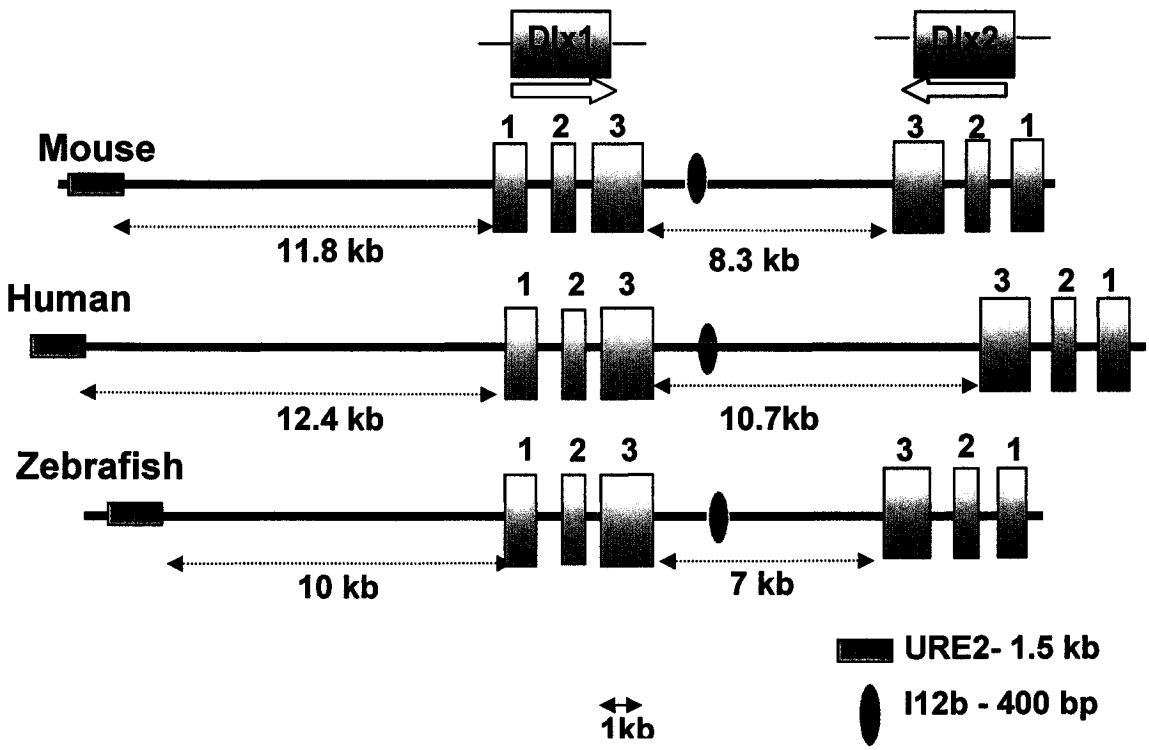

Dlx5
Dlx6
Mouse
3
3
10.1 kb
Human
3
3
10 kb
Zebrafish
3
3
3.5 kb
1kb
I56i - 440 bp
I56ii - 310 bp

Figure 1.5 Schematization of the *Dlx*1/*Dlx*2 Genomic organization in Mouse, Human and Zebrafish. Arrows indicate direction of transcription. Conserved forebrain enhancers, I12b and URE2 are depicted. The three exons of the *Dlx* genes are indicated. The sizes of the introns are not to scale.


Dlx1
Dlx2
Mouse
1 2 3
3 2 1
11.8 kb
8.3 kb
Human
1 2 3
3 2 1
12.4 kb
10.7kb
Zebrafish
1 2 3
3 2 1
10 kb
7 kb
URE2- 1.5 kb
I12b - 400 bp
1kb

conserved sequences capable of driving reporter gene expression to the arches and limbs of transgenic mice (Sumiyama *et al.*, 2002). The I37-2, 245 bp element, has high sequence similarity between human and mouse (88%). I37-2 contains a putative homeodomain binding site (HBS), and two conserved *DLX* binding sites similar in arrangement to I56i, and is necessary for *Dlx3* arch expression (Sumiyama and Ruddle, 2003b.) The element targets transgene expression to the mesenchyme of the first and second pharyngeal arches, recapitulating *Dlx3* expression (Sumiyama and Ruddle, 2003b). Upstream flanking sequences have also been investigated for regulatory activity. Analysis of *Dlx1* upstream region has revealed a 1.5 kb 5'-flanking sequence, named URE2, that confers lacZ expression in the mouse forebrain (Ghanem and Ekker, unpublished observations). Promoter analysis of *Dlx3* (*Xdll*-2) reveals a 1 kb 5'-flanking sequence that targets transgene expression to mouse keratinocytes and Xenopus epidermis, as well as distal portions of the limbs (Park and Morasso, 1999). The *Dlx2* promoter has also been examined. A 3.8kb 5'-flanking fragment targets *lacZ* transgene expression to a subset of the maxillomandibular oral epithelium identical to endogenous gene expression (Thomas *et al.*, 2000). The regulatory flanking region was further shown to be responsive to epithelial BMP4 and FGF8 signals similar to endogenous *Dlx*2 expression (Thomas *et al.*, 2000).

Patterns of *Dlx* expression are well conserved with few exceptions. Expression of the single *Distal-less* gene, *AmphiDll*, in amphioxus is similar to regions of *Dlx* gene expression in vertebrates (Holland *et al.*, 1996). For example, *AmphiDl*l expression in the presumptive ectoderm during gastrulation and the cerebral vesicles is considered to be

homologous to the vertebrate forebrain (Holland *et al.*, 1996). Therefore, aspects of *Dlx* regulation appear to be inherited from an ancestor that predates vertebrate radiation. Exceptions include early *dlx3b/dlx4b* and *Xenopus Dlx3* expression which appears to be performed by *Dlx5/Dlx6* in mammals and chicks (Quint *et al.*, 2000) suggesting that the genes are functionally equivalent (Zerucha and Ekker, 2000). In mice and chick *Dlx5/Dlx6* acts in an equivalent manner to *dlx3b/dlx4b* in zebrafish, specifying the neural plate border, suggesting that the enhancers responsible were originally in the initial bigene complex and were subsequently lost or degenerated during vertebrate species radiation (Quint *et al.*, 2000).

1.3.3 *Dlx* targets

Dlx proteins are DNA-binding dependent transcriptional activators, which are thought to control transcription of specific target genes but few of them have been identified so far. Apart from a few signaling molecules, little is currently known about the *Dlx* gene transcriptional properties. The homeodomain confers binding to DNA, as well as mediating protein:protein interactions (Zhang *et al.*, 1997). Overall, *Dlx* proteins are thought to have similar binding properties (Liu *et al.*, 1997; Zhang *et al.*, 1997; Zerucha *et al.*, 2000). *In vitro* binding studies indicate that the *Xenopus Dlx3* DNA-binding consensus sequence (Feledy *et al.*, 1999) works equally well for *dlx2a* (Zerucha *et al.*, 2000). *Dlx* proteins interact with both artificial DNA regulatory elements, as well as endogenous enhancers (Masuda *et al.*, 2001; Feledy *et al.*, 1999).

A few *Dlx* downstream transcriptional targets have been characterized at present.

Chromatin Immunoprecipitation (ChIP) experiments indicate that *Dlx1/Dlx2* proteins bind directly to enhancer I56i in the mouse forebrain and retina (Zhou *et al.*, 2004). *Dlx*2 is known to regulate Wnt1 directly in the developing telencephalon (Iler *et al.*, 1995). *Dlx3* regulates several genes though a TAAT core Homeodomain DNA-binding motif in trphoblasts, chriocarcinoma cellsand differentiating keratinocytes (Roberson *et al.*, 2001). *dlx3b* regulates *Pax8* and *Pax2a* during otic specification (Hans *et al.*, 2004). *Dlx*1, *Dlx*2 and *Dlx*5 all can activate transcription from the mouse and zebrafish *Dlx5/Dlx6* intergenic enhancer in the tissue culture cells (Zerucha *et al.*, 2000; Yu *et al.*, 2001). *Dlx* proteins also regulate the expression of the GAD gene, coding for glutamic acid decarboxylase, the enzyme responsible for the synthesis of the neurotransmitter GABA (Stuhmer *et al.*, 2002a). *Dlx5* is suggested to both positively and negatively regulate the osteocalcin gene during different stages of osteoblast differentiation (Miyama *et al.*, 1999). DLX proteins bind directly to the ATTA consensus sequences and regulate transcriptional activity of the Gonadotropin-releasing hormone (GnRH) promoter (Givens *et al.*, 2005). Ectopic expression of *Dlx* genes in mouse embryos and in the neural tube of chick embryos shows that *Dlx* genes are sufficient to induce *Aristaless* (*Arx*) ectopically in the developing forebrain (Cobos *et al.*, 2005b).

Regulation of gene expression by DLX proteins remains unclear. Outside the homeodomain, several short amino acid sequences are conserved among *Dlx* proteins that may have some significance. Within the *Dlx* clades, conservation of amino acids surrounding the homeodomain is apparent. The biological significance of these regions remains unclear. However, the amino- and carboxy-terminal domains of *Dlx3* potentiate

transcriptional activation, as well as function as a bipartite nuclear localization signal (NLS) necessary for DNA binding (Bryan and Morasso, 2000). Functional analysis of the *Dlx5* N-terminal sequence, in a GAL-4 DNA binding fusion protein, acts as an activation domain. Similarly, both *Dlx3* N-and C-terminal domains assist in transcriptional activation with in the homeodomain (Feledy *et al.*, 1999).

Dlx function is likely context-dependent, requiring additional factors to modify activator or repressor functions. *Dlx* genes have been shown to act in concert with other factors, such as *Dlxin1* to regulate transcriptional activity (Masuda *et al.*, 2001). Yeast two-hybrid screens indicate the GRIP1b PDZ-domain protein, previously designated *DLX* interacting protein-2 (DIP2), interacts with N-termini of *Dlx2* and *Dlx5* (Yu *et al.*, 2001; Eisenstat *et al.*, 1999). Chiba and colleagues have shown that *DLX1* interacts with Smad4 activin A signaling in hematopoetic cells (Chiba *et al.*, 2003). Therefore, the observed influence of *Dlx* genes in different cell-types and cellular processes may be the result of cell-specific cofactors.

1.4 Cell Sorting

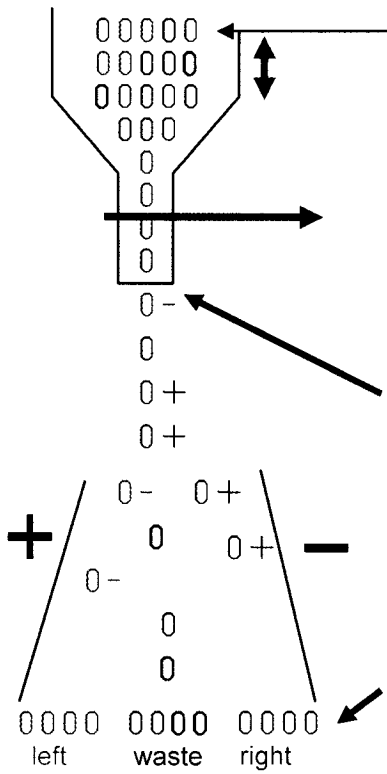
The main aim of generating transgenic animals expressing GFP under the control of enhancer elements shared between *Dlx1/Dllx2* and *Dlx5/Dlx6* clusters was to isolate GFP expressing cells from the transgenic animals and utilize them for further comparative studies by microarray analysis. Previous studies on transgenic animals using *LacZ* as a reporter gene have shown that the *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic enhancers drive the reporter gene to different subsets of cells of the forebrain. Similarly the GFP

transgenic animals generated under the control of different enhancer elements would represent different subsets of cells which could be selectively sorted by employing techniques such as cell sorting.

We decided to use GFP as a reporter gene, since for studies involving cell sorting, use of GFP as a reporter gene is more accurate as compared to other reporters. Cells expressing GFP constructs will fluoresce without addition of a substrate and can be sorted out live without any delay in the process. Reporter genes other than GFP need to be marked by fluorogenic substrates such as fluorescein digalactoside (FDG). The process of adding substrate is a time consuming and stressful process, which would result in change of original transcript of cells that are being studied. By using GFP as a reporter gene, enhancer driven GFP positive cells could be sorted from the brain of transgenic animals for each enhancer element and used for further study.

The cell sorter is an instrument with sophisticated optics, lasers and electronic processors, which has been programmed to identify and quantitatively analyze the individual cells, and separate and rapidly sort closely related cell populations. It has become an important tool for biomedical research and clinical medicine as it measures the physical and chemical properties of cells, such as fluorescence, and physically separate the cells while they are still alive. Although the Fluorescence Activated Cell Sorter (FACS) was invented in the late 1960s, it became more and more widely used through the 1990s and has been evolving slowly throughout this period (Herzenberg *et al.*, 2002).

Figure 1.6 Schematic representation of an Electrostatic sorter. Adapted from cytonetuk



Hydrodynamic focusing in nozzle vibrated by a transducer produces a stream breaking into droplets.

Laser interrogation and signal processing followed by sort decision: red sort right green sort left blue or black no sort

Electronic delay until cell reaches break off point. Then the stream is charged: +ve if red –ve if green.

Charged droplets deflect by electrostatic field from plates held at high voltage (+/- 3000 volts).

Various collection devices can be attached: tubes, slides, multi-well plates

The cell sorter has the ability to physically isolate sub-population of cells from a sample. The process of cell sorting by flow cytometry involves hydrodynamic focusing of mixture of cells or particles to be sorted to form a central core within a fluid sheath. The cells are then interrogated by a laser source with subsequent analysis of scatter and fluorescent signals. Finally gates and regions are applied to define subpopulations, which can be physically separated. There are two mechanisms to achieve this; electrostatic and mechanical sorting. Electrostatic sorters are most commonly used. Electrostatic sorting is achieved by ejecting the focused stream of cells through a nozzle into air (CytonetUK) (Fig. 1.6). The laser beam passes through the stream that leaves the nozzle. By vibrating the nozzle with a transducer, the stream coming out of the nozzle is forced to break up into droplets. By adjusting the relative flow of sample and sheath fluids it is possible to arrange for the majority of cells to be placed individually in droplets separated by a number of droplets not containing cells. As the sheath fluid is commonly PBS or a similar electrolyte the whole stream can be electrically charged (+,-, or 0) just before a droplet separates enabling the three populations to be selected after passing through an electric field. Decisions on which cells to sort are made rapidly after analyzing at the laser beam before the droplets separate. A MoFlo sorter can produce droplets between 20-50 thousand per second and can sort 5000 cells per second. The sorted populations have a constant cell density resulting from a discrete number of droplets being sorted per cell. Three droplets per cell are usually sorted to allow for the cell being in a drop adjacent to that expected and the sort is electronically aborted if this set of droplets is followed immediately by another cell. This avoids contamination with unwanted cells. For a well

resolved population of cells, total recoveries of up to 80% and purities better than 99% can be achieved.

1. 5 Statement of problem

The purpose of the project described in this thesis was to study the regulation of *Dlx* family during development, specifically forebrain. As stated previously, very little is known about the genetic hierarchies, both upstream and downstream, in which *Dlx* genes are involved. A number of explanations can be forwarded for the striking patterns of overlapping expression observed for the *Dlx* genes. One possibility is that DLX proteins, as part of their function may be involved in regulating *Dlx* gene expression. This type of cross-regulation has been discussed above. Another possibility, applicable to the paired *Dlx* genes which share virtually identical expression patterns, is that the paired *Dlx* genes share *cis*-acting regulatory elements. Previous work from our lab has revealed number of such elements, which are present in the intergenic and upstream regions of *Dlx* paralogs. Four of these identified enhancer elements are able to recapitulate endogenous *Dlx* expression patterns in the forebrain with subtle differences. These subtle differences have to be studied thoroughly in order to understand the role of each gene from the *Dlx* bi-gene clusters in the forebrain. Therefore, to understand the functional significance of the subtle differences observed in the expression of *Dlx* paralogs, we decided to generate transgenic zebrafish and mouse expressing the reporter gene, Green Fluorescent Protein (GFP) under the control of specific forebrain enhancer elements. The previous studies in our lab using the *lacZ* reporter gene have shown that forebrain enhancer elements are able to target reporter gene to the endogenous domains of *Dlx* expression. Since there is no

standardized method to sort live *lacZ* expressing cells from transgenic animals, we considered generating GFP transgenic animals. The enhancer driven GFP expressing forebrain cells could be sorted live using Fluorescence activated cell sorting technique.

The subtle differences seen in the expression pattern of *Dlx* genes can be studied by comparative analysis of different subpopulation of cells that are under the control of different enhancer elements in the generated GFP transgenic animals. The Fluorescent Activated Cell sorting (FACS) technique would facilitate the selective sorting out of the subpopulation of cells required for the study, which can be further analyzed by employing microarray technology. Microarray analysis of *Dlx*-GFP expressing cells from transgenic lines under the control of different enhancer elements would assist in identifying candidate genes involved in forebrain development and *Dlx* gene regulation. Furthermore, functional analysis of the identified genes would facilitate in uncovering the mechanisms and signaling pathways associated with *Dlx* gene expression and thus contributing significantly to our understanding of *Dlx* regulation, function and evolution.

2. Materials and Methods

2.1 Zebrafish studies

2.1.1 Construction of GFP reporter constructs

Green Florescent Protein reporter transgenes (GFP) constructs were generated under the control of enhancer elements from *dlx1a/dlx2a* loci for transgenic analysis in zebrafish. Constructs 1, 2, and 3 (Fig. 2.1) were made in the pEGFP-N1 vectors. pEGFP-N1 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in eukaryotic cells. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency (Kozak, M., 1987). A 2.5 kb zebrafish *dlx1* promoter fragment was inserted into the *Bam*H I site of pEGFP-N1, upstream of the EGFP. Reporter transgene construct containing the entire zebrafish *dlx1a/dlx2a* intergenic region (Fig.2.1, construct 1), was made first by subcloning an 8.3 kb intergenic fragment into the *Nhe* I restriction site of pEGFP-N1. A 1.5 kb URE2 fragment identified in the *Dlx1* upstream region (Ghanem and Ekker, unpublished observations) was subcloned into the *Eco*R I site of pEGFP-N1 (Fig. 2.1, construct 2). Construct 3 was made by inserting both fragments.

Constructs 4, 5 and 6 (Fig. 2.2) were made in the SP72 vector containing an EGFP reporter gene. A human β-globin promoter was subcloned into *Eco*RI and *Bam*HI sites of the vector. A 400bp I12b fragment, identified in the intergenic region of *dlx1a/dlx2a* (Ghanem *et al.*, 2003) (Fig. 2.2, Construct 4) was subcloned into *Kpn*I and *Cla*I restriction sites to make a reporter construct. Another construct was made by subcloning

Figure 2.1 Schematic representation of the zebrafish transgene constructs (1, 2 and 3) also containing the 2.5 kb zebrafish *dlx1* promoter fragment. Arrows indicate direction of transcription. Restriction sites are labeled as the following: B, *Bam*HI; E, *Eco*RI; N, *Nhe*I. The drawings are not to scale.

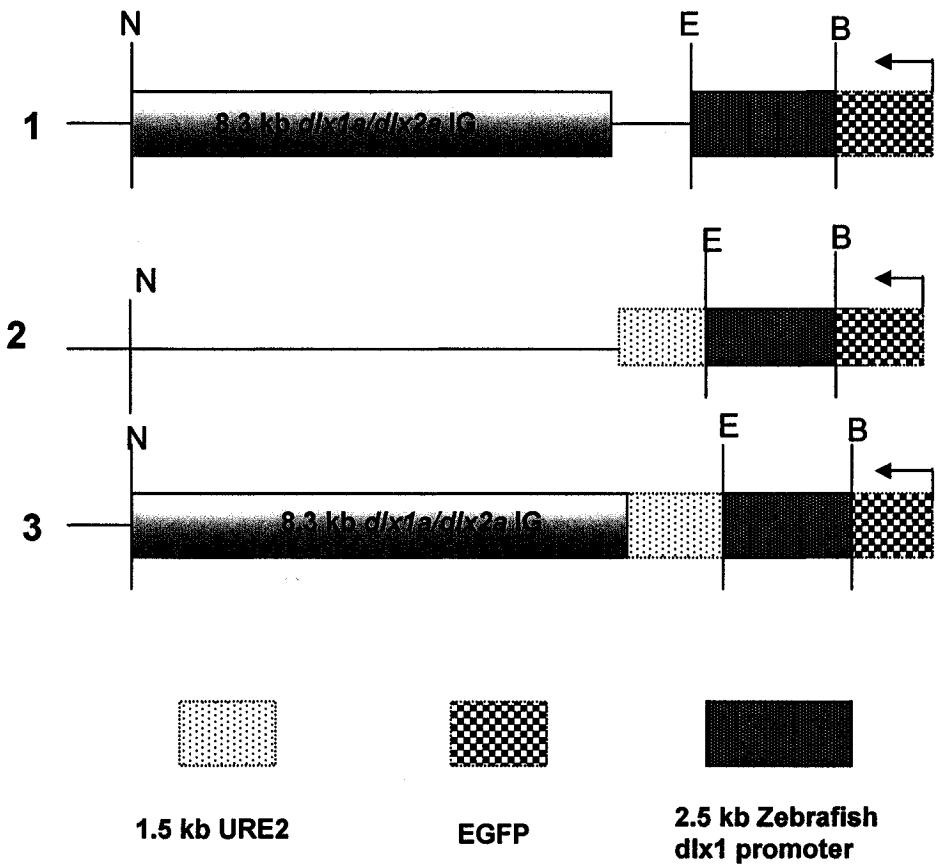
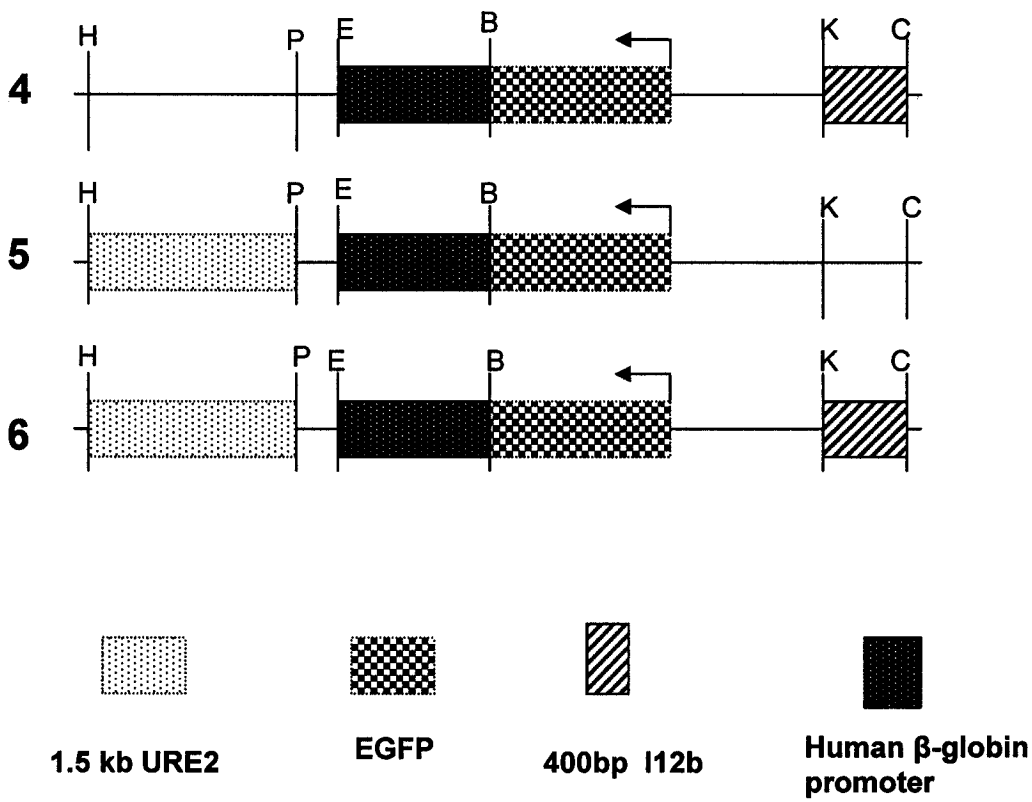

N
E
B
1
8.3 kb dlx1a/dlx2a IG
2
3
1.5 kb URE2
EGFP
2.5 kb Zebrafish
dlx1 promoter

Figure 2.2 Schematic representation of the zebrafish transgene constructs (4, 5 and 6) made with the human β-globin promoter. Arrows indicate direction of transcription. Restriction sites are labeled as the following: B, *bam*HI; E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I. The drawings are not to scale.


4
H
P
E
B
K
C
5
H
P
E
B
K
C
6
H
P
E
B
K
C
1.5 kb URE2
EGFP
400bp I12b
Human β-globin promoter

the 1.5kb URE2 fragment (Fig. 2.2, Construct 5) into *Pst*I and *HInd*III sites. Construct 6 contains both fragments.

2.1.2 Maintenance of Zebrafish

Fishes were maintained at standard conditions as described (Westerfield, 1995). For the immunochemistry experiments, founder transgenic embryos were raised. Embryonic chorions were removed with watchmaker forceps prior to fixation. Embryos and larvae were fixed in 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) and dehydrated in methanol before storage at -20°C.

2.1.3 Microinjection

Fish were bred and embryos were collected as described (Westerfield, 1995). One or two cell stage embryos were microinjected using a Narashingi IM300 microinjector. The microinjection needles were pulled using a P90 micropipette puller (Browing and Flame). Plasmid constructs were diluted in 1M KCl and 0.05% phenol red. Embryos were injected with the diluted plasmid DNA at a concentration of 100ng/μl. The injected embryos were maintained in petri dishes at 28.5°C and primary embryos were examined for GFP expression at various time points thereafter. The embryos which showed GFP expression were further raised until maturity. The raised fishes were screened for the germline transmission of the transgene by mating them with wild-type fishes and observing the embryos under Fluorescent Microscope at various time points. Founder transgenic fishes were crossed with wild-type fishes and founder transgenic embryos were harvested at various time points and analyzed.

2.1.4 Immunohistochemistry

Fixed embryos were rehydrated using a series of decreasing concentrations of methanol mixed with a PBS solution and finally washed twice in PBST. The embryos were then embedded in a solution of 1.5% agar and 5% sucrose in PBS, melted and poured over the tissue. The tissue was oriented before the agar sets and the blocks were trimmed to size. The blocks were then transferred to 30% sucrose in PBS and stored at 4°C until the block sinks. For sectioning, the blocks were embedded in OCT and sections cut at 15μm onto TESPA-coated super frost slides. The sections were allowed to dry for 30mins and then transferred to a box with silica gel and stored at -70°C until antibody staining.

The slides were thawed at room temperature for 30 min. The sections were blocked overnight at 4°C using block solution containing 1%BSA, 5% calf serum, and 1% DMSO in PBST. The sections were incubated with 1/1000 diluted anti-GFP antibody overnight at 4°C. Incubation with the primary antibody was followed by four washes, 30 min each at RT with immunowash solution consisting of 1% BSA and 1% DMSO in PBST. The samples were then incubated with biotinylated secondary antibody for 4 hours at room temperature. Incubation with the secondary antibody was followed by four washes, 30 min each in immunowash solution. The samples were then treated with AB (Avidin DH: biotinylated enzyme, VECTASTAIN ABC Systems, Vector laboratories) complex for 45 min at RT. This was followed by three washes, 30 min each slide in immunowash and once in PBST. The samples were incubated in DAB peroxidase substate solution for 30 min. The reaction was stopped by several washes in PBST. The sections were mounted and stored until further use.

2.1.5 Cell sorting

Approximately 100 embryos were collected in a sterile tube containing 1X PBS. Prediluted trypsin (0.5 g/l trypsin, 0.14 M NaCl, 0.05 M KCl, 5 mM glucose, 7 mM NaHCO3, 0.7mM EDTA) was added, and embryos were triturated using a pipette until dissociated. Cells were centrifuged twice at 2000 r.p.m (Allegra® Benchtop Centrifuge) for 7 min at 4°C and resuspended in PBS before filtration through a 70mm nylon cell strainer. The filter was rinsed once with PBS. Fluorescence-activated cell sorting (FACS) was carried out by using an Elite FACS machine (Coulter).

2.2 Mouse Studies

2.2.1 Construction of GFP reporter constructs

Construct 7 (Fig. 2.3) was made in the SP72 vector containing an EGFP reporter gene. A 3.5kb 5' *Dlx6* promoter was subcloned into *Sst*I and *Xba*I sites. A 4.1 kb fragment of the mouse *Dlx5/Dlx6* intergenic region containing the I56i enhancer element was subcloned into *Kpn*I and *Cla*I sites of the SP72.

Construct 8 (Fig. 2.3) was made in another SP72 vector containing an EGFP reporter gene. A human β-globin promoter was subcloned into *Eco*RI and *Bam*HI sites of the SP72 vector. A 1.5 Kb URE2 fragment from 5'-flanking region of *Dlx1* was subcloned into the *Hin*dIII site.

Constructs 9 and 10 were obtained from John Rubenstein's lab, University of California, San Francisco. Both were made in a pBS KS bicistronic Vector containing the coding region of the Cre Recombinase (CreER) gene and EGFP under the control of β-globin

Figure 2.3 Schematic representation of the mouse transgene constructs (7 and 8). A 4.1kb Mouse *Dlx5/Dlx6* intergenic fragment is under the control of a 3.5kb 5' *Dlx6* promoter (construct 7). A mouse 1.5 kb URE2 fragment taken from 5'-flanking region of *Dlx1* is under the control of human β-globin promoter (construct 8). Both the constructs are made in Sp72 vector containing EGFP reporter gene. Arrows indicate direction of transcription. The drawings are not to scale.

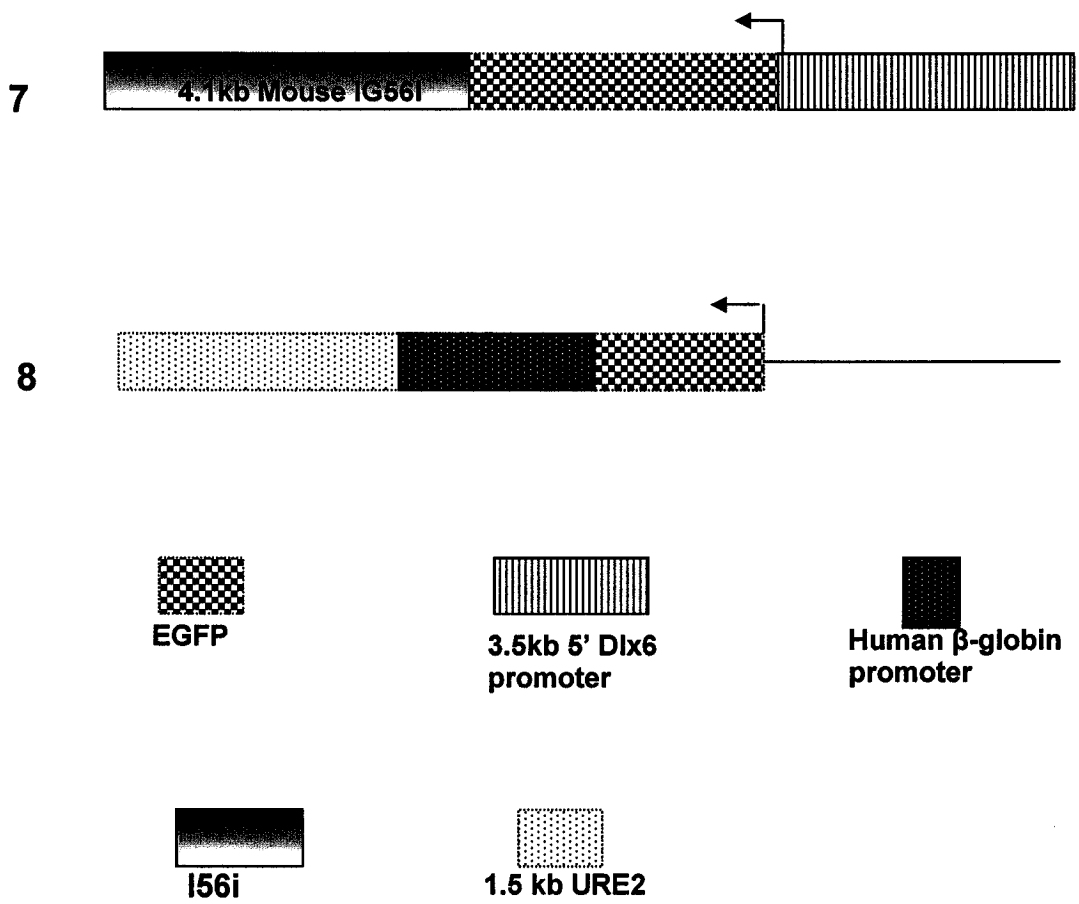
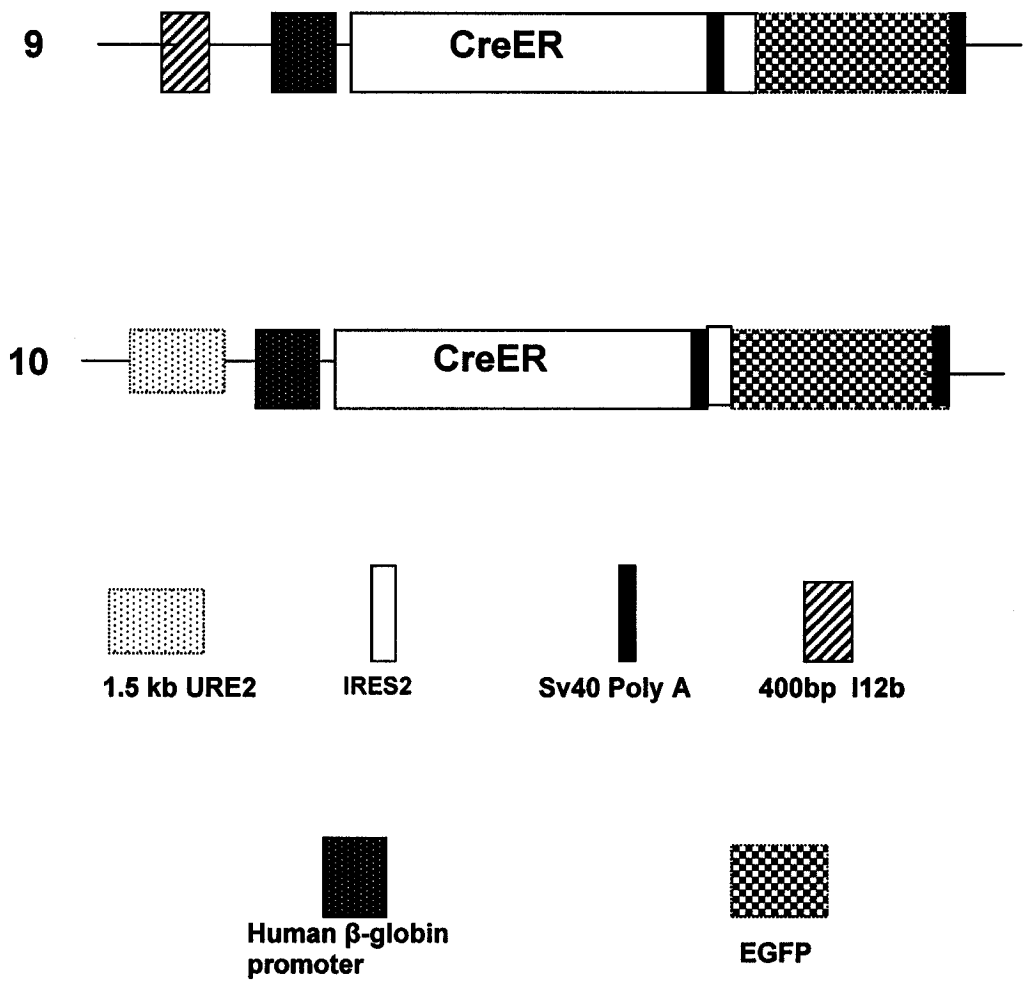

7
4.1kb Mouse I56i
8
EGFP
3.5kb 5' Dlx6 promoter
Human β-globin promoter
I56i
1.5 kb URE2

Figure 2.4 Schematic representation of the Mouse Cre transgene constructs (9 and 10). Both the constructs made in a bicistronic Vector containing the coding region of the Cre Recombinase (CreER) gene and EGFP under the control of β-globin minimal promoter. A 440bp fragment from mouse *Dlx1/Dlx2,* I12b (construct 9) and a 1.5kb fragment from *Dlx1* upstream, URE2 (construct 10) are subcloned upstream of β-globin minimal promoter in two separate pBS KS vectors.


9
CreER
10
CreER
1.5 kb URE2
IRES2
Sv40 Poly A
400bp I12b
Human β-globin promoter
EGFP

minimal promoter. The vector has an internal ribosome entry site (IRES2) of the encephalomyocarditis virus (ECMV) between the EGFP coding region and the CreER. The IRES sequence permits both the Cre and the EGFP gene to be translated from a single bicistronic mRNA. The vector also contains SV40polyadenylation signals downstream of the EGFP gene to direct proper processing of the 3' end of the bicistronic mRNA. A 440bp mouse I12b fragment (Construct 9, Fig. 2.4) has been inserted in the upstream of β-globin minimal promoter. Construct 10 contains 1.5kb URE2 fragment in the similar organization.

After submitting my thesis for reviewing it was discovered that the Cre constructs we used were designed faultily. A second SV40polyadenylation signal was placed downstream of the Cre (between Cre and EGFP) and this signal terminated the transcription before the EGFP reporter gene is transcribed. Therefore, no GFP expression could be obtained from the construct.

2.2.2 Production of transgenic mice and genotyping

The linearized plasmid constructs were prepared following GeneCAPSULE™ nucleic acids extraction method and were injected into fertilized oocytes at a concentration of 5ng/μl following standard procedures (Hogan et al., 1986). The injections were performed by Adrianna Gamarotta, animal care technician at Ottawa Health Research Institute, Ottawa. The presence of transgenes was tested in either founder embryos or from established lines by PCR on DNA prepared from extra-embryonic tissues. PCR was performed using the following set of oligonucleotide primers; GFP: Forward (5' to 3')

ATC CTG GTC GAG CTG CTG GAC; GFP: Reverse (5'to 3') CTT GAA GTT CAC CTT GAT GCC; Cre: Forward (5' to 3' AAC CTG AAG ATG TTC GCG; Cre: Reverse (5' to 3') CGG TAT TGA AAC TCC AGC. Transgenic expression was analyzed in founder transgenic embryos or embryos from the cross of a transgenic male with CD1 females at E10-E12 embryonic stages. PCR amplification was performed using the Mastercycler gradient Thermal Cycler (Eppendorf, Westbury, NY, USA). General PCR conditions used were as follows; 2μl of DNA template in 16μl autoclaved water, 2.5μl of 10X PCR reaction buffer, 2.5μl of dNTPs (2mM), 1μl of forward and reverse primers (10pM) and 1μl of Taq DNA Polymerase. The initial denaturation step was performed at 94°C for 4 min. This was followed by 30 cycles with a denaturation step at 93°C for 30 sec, an annealing step ranging between 50 and 60°C (depending on the primer set used) for 30 sec, and an extension step at 72 °C for 1 min. The final extension step was performed at 72°C for 10 min.

2.2.3 Immunohistochemistry

Embryos from hemizygous mice (transgenic FBV males × CD1 males) were harvested at E12 embryonic stage and immersion fixed in 4% paraformaldehyde in 0.1M PB overnight at 4°C and then placed in PBS with 30% sucrose for at least 24 hours at 4°C. The embryos were transferred to multiwell plates, the sucrose was aspirated and cryostat medium added. The plate was kept on a shaker at 4°C for one hour before transferring the embryos to plastic molds. The blocks were frozen on dry ice and stored at -70°C before

sectioning on a cryostat. The embryonic brains were sectioned at 20μm and mounted on superfrost slides and stored at -80°C before proceeding to immunostaining.

The slides were thawed at room temperature for 10mins and washed thrice in 1xPBS for 5 min and once in PBS-Tx 0.2%. The slides were incubated at RT in a blocking solution containing 10% Normal serum and 0.2% gelatin in PBS-Tx 0.2%. The slides were then incubated overnight at 4°C in 1/1000 diluted anti-GFP antibody prepared in a blocking solution. Incubation with the primary antibody was followed by three washes, 10 min each in PBS-Tx 0.2%. The samples were then incubated in 1/200 diluted biotinylated secondary antibody for 2 hours at room temperature. Incubation with the secondary antibody was followed by four washes, 30 min each in PBS-Tx 0.2%. The samples were then treated with AB (Avidin DH: biotinylated enzyme) (VECTASTAIN ABC Systems, Vector laboratories) complex for 45 min at RT. The slides were then washed thrice in PBS-Tx 0.2% for 5 min and once in 0.05M Tris buffer for 15 min. The samples were incubated in substrate containing DAB peroxidase and 0.01% hydrogen peroxide solution for 30 min. The slides were washed twice in Tris Buffer, dehydrated by several washes in Xylene and mounted.

2.2.4 RT-PCR

E12 embryos were harvested, the brain tissue was rapidly dissected out and flash frozen in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA was resuspended in 30μl RNAse-free water. DNase treatment was performed on 2 μg

RNA samples in a reaction tube containing 2µl 10X DNase I Reaction buffer, 2µl DNase I, and DEPC treated water up to 20µl. The reaction was mixed, centrifuged and incubated at RT for 15 min. DNase was inactivated by adding 2µl of 25mM EDTA and heating the mixture at 70°C for 15 min. First-strand cDNA synthesis was carried out using DNase treated total RNA and 1µl oligonucleotide dT. The reaction was heated to 70°C for 10 min to denature the RNA and primer, quickly chilled on ice, and centrifuged briefly. Four microliters of 5X reaction buffer (Invitrogen), 2µl of 0.1 M DTT, 1 µl of 10 mM dNTPs and 1 µl of RNAse inhibitor were added, the reaction mixture was gently mixed, and heated at 50°C for 2 min. 1µl of Superscript II– Reverse Transcriptase (Invitrogen) was added and the reaction was allowed to continue at 50°C. The reaction was inactivated at 70°C for 15 min and stored at -20°C until used. PCR amplification was performed using the Mastercycler gradient Thermal Cycler. General PCR conditions used were as follows; 2µl of cDNA template in 16µl PCR water, 5µl of 10X PCR reaction buffer, 1.5µl of $MgCl_2$ (50 mM), 1µl of dNTPs (10mM), 2µl of forward and reverse primers (10mM) and 1µl of Taq DNA Polymerase. The initial denaturation step was performed at 94°C for 4 min. This was followed by 35 cycles by a denaturation step at 93°C for 30 sec, an annealing step ranging between 50 and 60°C (depending on the primer set used) for 30 sec, and an extension step at 72 °C for 1 min. The final extension step was performed at 72°C for 10 min.

3. Results

3.1. Zebrafish Studies

3.1.1 I12b and URE2 targets reporter transgene expression to the forebrain

To study the activity of *cis*-acting regulatory sequences in the *dlx1a/dlx2a* intergenic region and in the upstream region of d*lx1, a* 8.3 kb intergenic fragment that includes the zebrafish *dlx1a/dlx2a* intergenic region and a 1.5 kb *dlx1* 5'-flanking sequence, URE2, were cloned into an EGFP reporter gene construct containing a 2.5 kb zebrafish *dlx1* promoter (Fig 2.1, Construct 1, 2 and 3) fragment. The constructs were injected and primary embryos were examined for transgene expression at a number of distinct developmental stages using fluorescence microscopy. All three constructs did not yield any GFP expression. This result could be probably due to the nature of the promoter, as it was a fragment containing the immediate 5'-flanking region and was never been tested for its promoter activity.

Another set of constructs were made by cloning a 400bp fragment (I12b) from *dlx1a/dlx2a* intergenic region and a 1.5 kb URE2 fragment into an EGFP vector containing a minimal human β-globin promoter (Yee and Rigby, 1993) (Fig 2.2, Construct 4, 5 and 6). Transgene expression was examined in primary embryos after injection at different time points. Construct containing I12b targeted expression of the green fluorescent protein reporter transgene to domains of *dlx* expression in the forebrain in about 45% of the injected embryos (Fig. 3.1A). URE2 containing construct targeted transgene expression to similar domains in 40% of the primary embryos (Fig. 3.1B). The construct containing both zebrafish I12b and URE2 showed forebrain expression in 50-60

Figure 3.1 Enhancer activity of conserved sequences I12b and URE2 in primary transgenic zebrafish (A-B). Head of 48 hpf primary primary transgenic zebrafish embryo, lateral view, injected with construct containing a 400bp fragment (I12b) from *dlx1a/dlx2a* intergenic region and a 1.5 kb *dlx1* 5'-flanking sequence, URE2 respectively. The transgene is expressed predominantly in the 2 domains of the forebrain, diencephalon and telencephalon. Abbreviations: Di, Diencephalon; Te, Telencephalon.

Fig. 3.1A

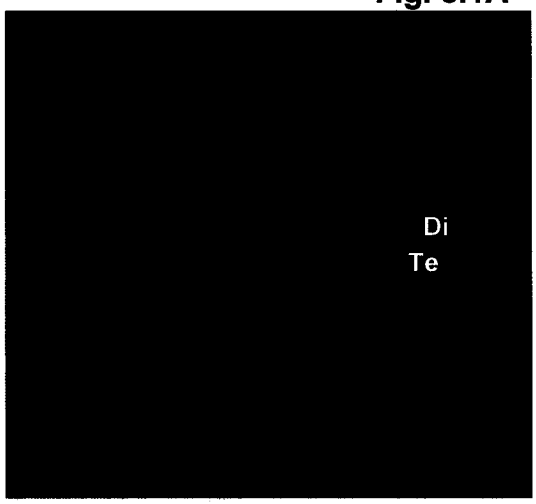


Fig. 3.1B

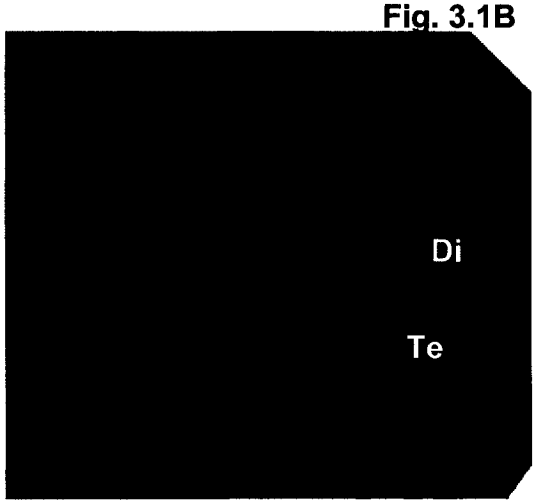


Table 1 Transient expression of GFP in embryos microinjected with transgene constructs containing EGFP reporter gene and enhancer elements, I12b and URE2.

Constructs	No. of Observed embryos	No. of embryos with GFP expression in the forebrain	No. of embryos with strong GFP expression in the forebrain *
I12b-EGFP	425	185 (43.5%)	54 (12.7%)
URE2-EGFP	390	162 (41.25%)	38 (9.7%)
I12b+URE2-EGFP	480	255 (53%)	55 (11.5%)

*Strong GFP expression means that each embryo has more than 15 green fluorescent cells in the forebrain

% of the injected embryos . Primary embryos from all three constructs had forebrain expression starting at 18 h postfertilization (hpf) and lasting until at least 72 hpf. These observations indicate that both the intergenic as well as upstream enhancer elements are capable of recapitulating the endogenous *dlx* expression pattern.

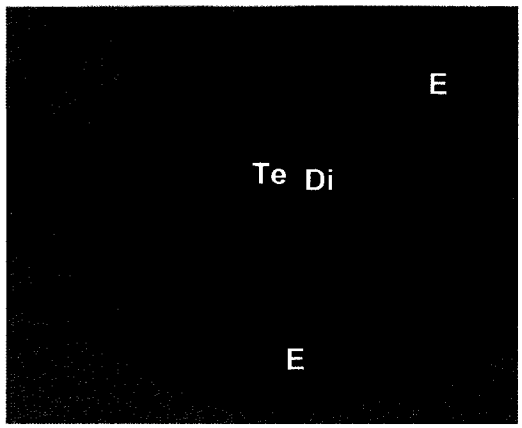
Similar conserved sequences are also found in the *dlx5a/dlx6a* intergenic region. As previously reported, when tested in transgenic zebrafish, constructs containing *dlx5a/dlx6a* intergenic elements, I56i and I56ii targeted expression of the GFP to the forebrain (Ghanem *et al.*, 2003; Zerucha *et al.*, 2000).

3.1.2 Analysis of I12b transgenic line

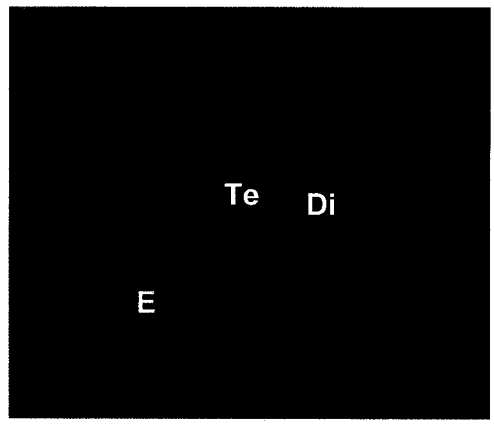
To establish stable transgenic lines, primary transgenic embryos with strong GFP expression were raised to adulthood and were crossed against wild-type or crossed with each other to generate F1 embryos. Of 147 founder fish tested, one female founder that was injected with the construct containing I12b fragment produced embryos expressing GFP in the forebrain. 50% of the progeny had GFP expression indicating that GFP was transmitted to progeny in a Mendelian fashion. The established I12b transgenic line showed strong and reproducible expression in two domains of endogenous *dlx1a/dlx2a* expression (Fig. 3.2). These included the telencephalic and diencephalic domains of expression in the forebrain. The forebrain expression was observed starting at 20 hpf and lasting until 72hpf with strongest GFP fluorescence signal at 38 hpf (data not shown). The number of GFP-positive cells in the forebrain ranged from a few to few hundred cells. To study the expression pattern of I12b driven GFP expressing cells in the forebrain, I12b transgenic female was crossed with a wild-type male and embryos

Figure 3.2 Lateral, dorsolateral, dorsal and ventral view of a 38 hpf zebrafish embryo from a transgenic line produced with a construct made with human β-globin promoter-GFP plasmid (Figure 2.2, construct 3, 4 and 5) that also contained a 400bp *dlx1a/dlx2a* intergenic fragment, I12b. The transgene is expressed predominantly in the 2 domains of forebrain, telencephalic and diencephalic. The GFP expressing cells could be seen in the both the domains. Abbreviations: E, eye; Di, Diencephalon; Te, Telencephalon.

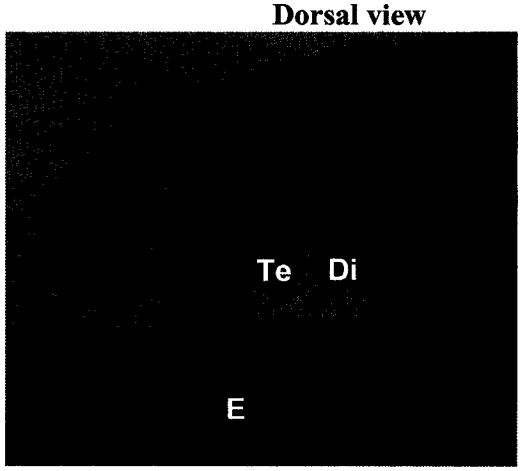
Lateral View



Dorsolateral view



Dorsal view



Ventral View

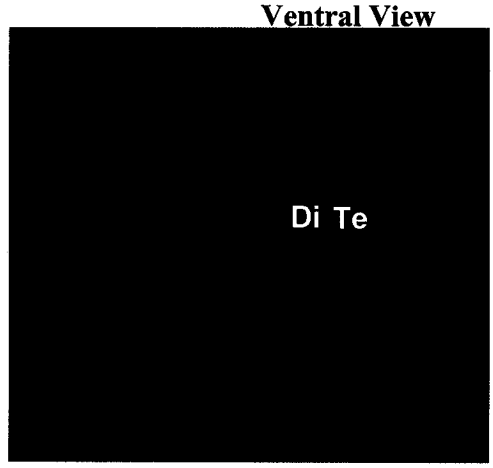
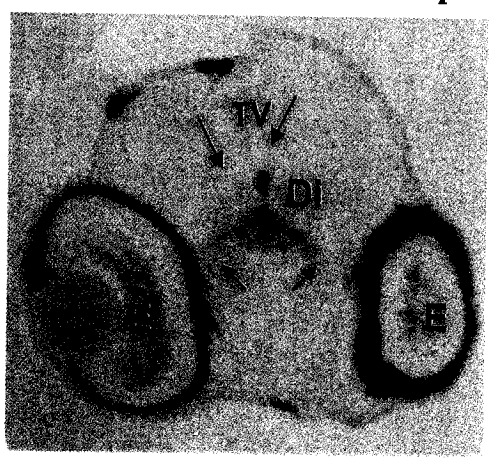
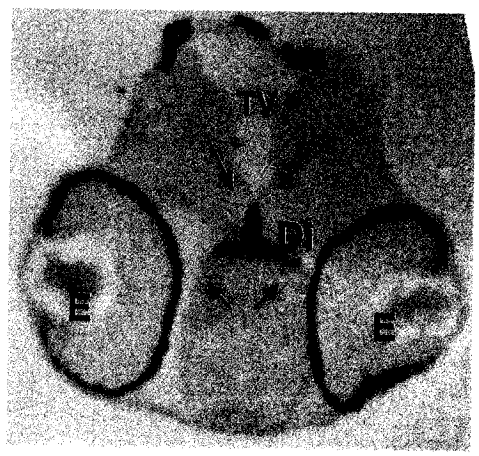


Figure 3.3. Immunohistochemistry: The top left and right panel and the bottom left panel represents transverse sections of zebrafish embryos at the level of diencephalon from a transgenic line produced with a construct containing a 400bp fragment, I12b. These sections are taken at different time points and are stained with an anti-GFP antibody. The I12b-GFP expressing cells are closer to the third ventricle compared to those that express *dlx5a/dlx6a* (figure not shown), confirming the fact that *dlx1a/dlx2a* are expressed in more immature cells (Akimenko *et al.*, 1994 and Zerucha *et al.*, 2000). The right bottom panel is a section from a wild-type embryo hybridized with *Dlx1* probe. Abbreviations: E, eye; Di, diencephalon; TV, third ventricle. The arrows indicate the more lateral cells which lack GFP expression.

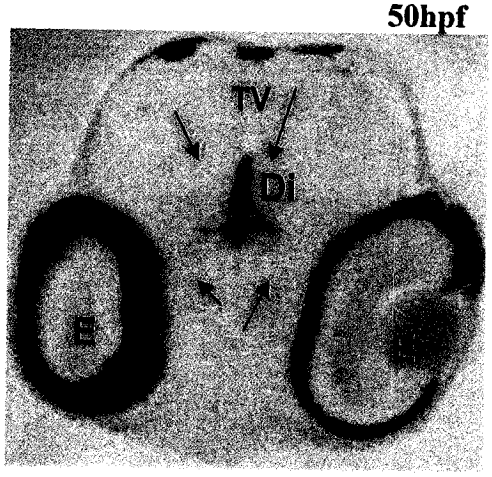
30 hpf



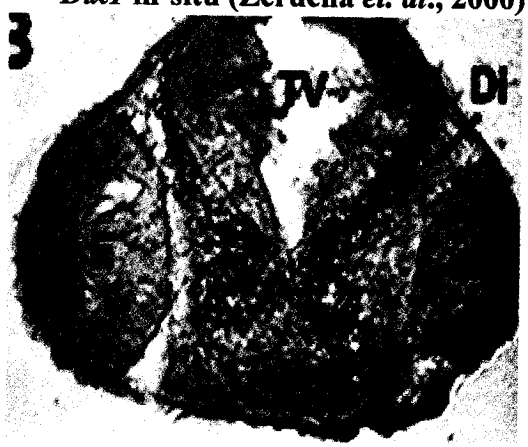
40hpf



50hpf



***Dlx1* in-situ (Zerucha *et. al.*, 2000)**



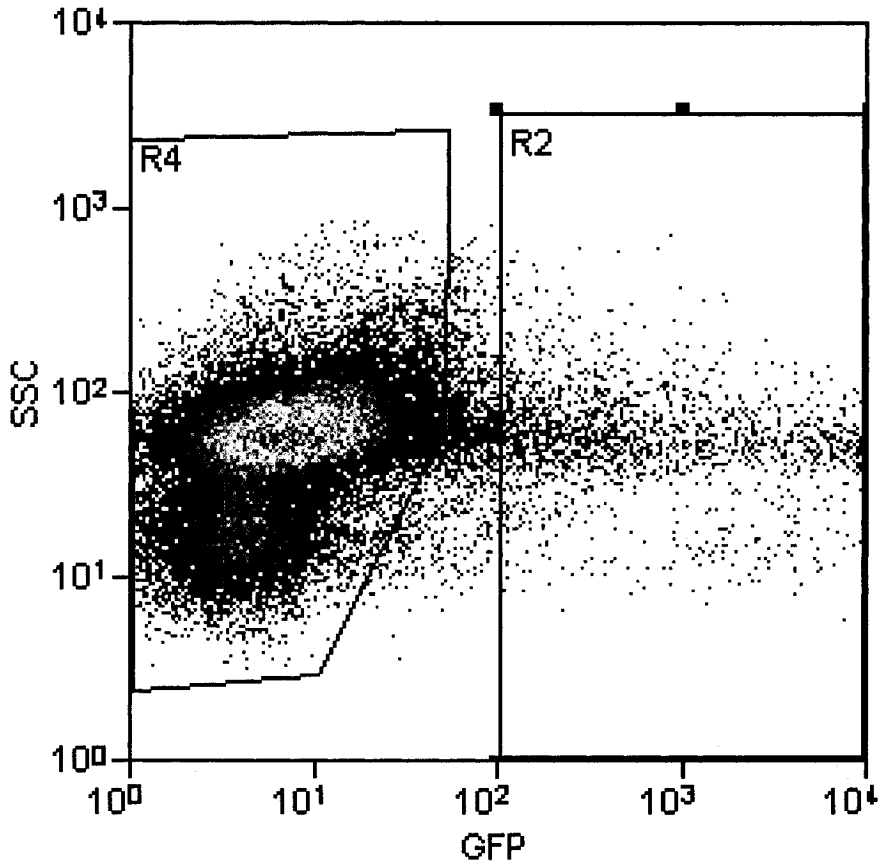
expressing GFP were collected at three time points and sectioned at the level of diencephalon. GFP protein expression was detected by immunohistolocalization with an anti-GFP antibody. GFP-expressing cells were found in the region that is closer to ventricle (Fig. 3.3) confirming the fact that endogenous *dlx1a/dlx2a* are expressed in more immature cells. As previously reported, patterns of expression of *dlx1a/dlx2a* in both the telencephalon and the diencephalon indicated that the two genes are expressed in more immature cells than the cells that express *dlx5a/dlx6a* (Zerucha *et al.*, 2000). However, when the results were compared with the endogenous expression pattern of *dlx1*, there is a paucity of cells expressing GFP in more lateral cells (Fig. 3.3). The possible explanation for this result could be due to the low insertion copies of GFP transgene in those sites. Overall, this transgene does not seem to recapitulate endogenous expression as faithfully as it is seen in the *dlx5a/dlx6a* transgenic line (Ghanem *et al.*, 2003).

3.1.3 FACS analysis of GFP expressing forebrain cells

A cell sorting experiment was conducted to get an estimate of yield and number of GFP-positive forebrain cells from an established *dlx5a/dlx6a* transgenic line (Ghanem *et al.*, 2003). A total of 100 two days-old embryos were dissociated using prediluted trypsin solution. The total number of cells in the processed sample was 9.77×10^5, out of which 4.87×10^4 (4.98%) were GFP-positive and 8.16×10^5 (83.57%) were GFP-negative (Fig. 3.4). After the cells were sorted, a small aliquot of the sorted fraction, comprising GFP-positive cells were taken and re-run on the flow cytometer. After analyzing this aliquot,

we looked at the percentage of GFP-positive cells that fall into the original region/gate that we used to sort the cells. We found that sorted fraction had now 91% of GFP-positive cells compared to only 4.98% GFP+ in the pre-sorted fraction. This is the enrichment: we went from 4.98% GFP cells to 90% GFP cells. The remaining fraction consisted of cell aggregates, dead cells and the contaminated sorted fraction. The basic idea of generating transgenic zebrafish and isolating *Dlx*-expressing cells from different lines was to use those cells for studying functional specificity of *Dlx* paralogs by employing a microarray technique. As all the enhancer elements are capable of recapitulating endogenous *Dlx* gene expression patterns, each enhancer element would represent the activity of respective *Dlx* genes. A standard affymetrix chip requires a total of 10ug of total RNA for each hybridization reaction. Based on the results obtained from the cell sorting experiments, it was estimated that approximately 1000 embryos would be required for each standard microarray hybridization reaction, considering an estimated RNA content of 20-35 picogram/live dissociated cell and an estimated total RNA requirement of 10ug. That means each microarray experiment with two duplicates will require at least 3000 embryos at a time. Practically this may not be possible, not only with respect to the the number of embryos but also the work involved in dechorinating such a large number of embryos before cell sorting. Chemical dechorination technique such as pronase treatment cannot be employed here for dechorination as it may influence the quality of sorted cells later. One probable solution for this problem could be isolating total RNA from as many embryos as possible and increasing the amount of RNA by employing an mRNA amplification technique.

Figure 3.4. Isolation and characterization of *dx5a/dlx6a-GFP*-positive cells from transgenic embryos. FACS profiles of cells isolated from two days old *dlx5a/dlx6a* transgenic embryos. Graph represents regions/gates selected for sorting, based on GFP fluorescence intensity. Colored spots represent the relative number of cells in the same region and go from red to green with increasing cell numbers. SSC (Side Scatter) - also called right-angle scatter provides information on the internal structure of cells such as granularity. R2 and R4 represent the regions containing GFP+ve (sorted fraction) and GFP-ve cells respectively.


R4
R2
SSC
GFP
10^0
10^1
10^2
10^3
10^4

3.2. Mouse Studies

3.2.1 Generation of GFP transgenic mice

To study the activity of *cis*-acting regulatory sequences in the *Dlx5/Dlx6* intergenic region of the mouse, a 4.1 kb intergenic fragment that includes the 440bp I56i enhancer element (Zerucha et al., 2000) was cloned into an EGFP reporter gene construct containing 3.5kb of zebrafish *dlx6* 5'-flanking region, including the promoter (Fig. 2.3, Construct 7). Another construct was made using the 1.5 Kb URE2 fragment from 5'-flanking region of *Dlx1*. It was cloned into an EGFP reporter gene vector containing a minimal human β-globin promoter (Fig.2.3, Construct 8).

The constructs were injected into the fertilized eggs of CD-1 donor mice. For the construct containing the I56i element, four founder males were obtained. For the URE2 construct, one founder female was obtained after screening primary pups. The URE2 female founder was raised until maturity, mated with a male and the pups born to them were screened. A F1 transgenic male was obtained after screening. The presence of the GFP fragment was confirmed in all the founders by isolating genomic DNA from placenta and performing PCR with EGFP primers.

Another set of constructs designed in Dr. John Rubenstein's laboratory, University of California, San Francisco, were also injected. These constructs contained the mouse *Dlx5/Dlx6* or *Dlx1/Dlx2* intergenic fragments, I12b and I56i, that were subcloned into a bicistronic construct also containing the coding sequences for the Cre recombinase (CreER) and EGFP. The CreER contains an estrogen receptor ligand-binding domain which allows induction of recombination at a given time in a specific cell type. After

screening primary pups, five founder males were obtained for the Cre-I12b construct and three founder females were obtained for the Cre-URE2 construct. One of the URE2 female founders was raised until maturity, mated with a male and the pups born to them were screened. A founder male was obtained after screening. The presence of the GFP and Cre fragments was confirmed in all the founders by isolating genomic DNA from placenta and running PCR with EGFP and Cre primers.

3.2.2 Analysis of GFP/Cre transgenic animals

The basic idea of generating transgenic lines under the control of different enhancer elements was to generate transgenic animals recapitulating endogenous *Dlx* gene expression pattern in a manner that *Dlx*-expressing cells and marking them with a reporter transgene so that they could be readily sorted out for further functional studies. GFP was used as a reporter gene, as GFP-expressing cells can be sorted out using Fluorescent Activated Cell Sorting technique.

In order to analyze the transgenic animals, founder males from the all the generated lines were crossed with wild-type female mice and E12 stage embryos were harvested. When the brains were dissected and observed under a fluorescence microscope, the GFP fluorescence could not be detected, However, 50% of the embryos were positive by PCR when genomic DNA from the placenta of the harvested embryos were tested using GFP or Cre primers. When transgenic animals were further analyzed by anti-Green Fluorescent Protein antibody staining, we could not detect GFP protein in any of the transgenic lines. Furthermore, RT-PCR experiments were conducted by isolating total

RNA from the E12 embryos brain to test the presence of GFP-RNA. Mouse β-actin was used as an internal control. All the transgenic lines were found to be negative for RT-PCR.

After submitting my thesis for reviewing it was discovered that the Cre constructs we used were designed faultily. A second SV40polyadenylation signal was placed downstream of the Cre (between Cre and EGFP) and this signal terminated the transcription before the EGFP reporter gene is transcribed. Therefore, no GFP expression could be obtained from the construct.

4. Discussion

4.1 Intergenic enhancers recapitulate forebrain *Dlx* expression

Conserved sequences from the intergenic region between the *dlx1a* and *dlx2a* genes is sufficient, once combined to a minimal promoter, to direct expression in cells of the telencephalon and diencephalon of zebrafish where *dlx* genes are normally expressed. Conserved sequences present in the upstream flanking sequence of *Dlx1a* are also capable of recapitulating endogenous *Dlx* gene expression. This strongly suggests that the regulatory mechanisms controlling *Dlx* expression in the forebrain have been conserved during vertebrate evolution and it provides support to the idea that *Dlx* function during forebrain development has also been conserved. Further evidence for conserved function of *Dlx* genes in forebrain development comes from the differential expression of *Dlx* genes in the telencephalon and diencephalon where more immature cells express *dlx1a* and *dlx2a* compared to *dlx5a* and *dlx6a* (Akimenko *et al.*, 1994).

Functional conservation of enhancer sequences between mammals and teleost fishes have been recently observed for elements present in the *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic regions (Ghanem *et al.*, 2003). Comparison of *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic regions from human, mouse, zebrafish and from two pufferfishes have revealed four intergenic conserved enhancer elements. Each intergenic sequence acted as an enhancer when tested in transgenic animals and three out of four of these conserved sequences were active in the forebrain (Ghanem *et al.*, 2003).

However, the constructs that contained the *dlx1* promoter did not target transgene expression in the forebrain. Detailed analysis of reporter transgene activity in the mouse

forebrain suggests that both I12b and URE2 enhancer sequences reproduce the endogenous *Dlx1* and *Dlx2* expression patterns (Ghanem *et al.*, 2003; Ghanem and Ekker, unpublished observations). Therefore, the observed result could only be attributed to the inability of the promoter to drive transgene expression. Further evidence for this comes from the fact that the promoter was taken from the immediate 5'-flanking region of *dlx1* and we did not know if it could act as a promoter.

When we compared the activity of I12b in the forebrain with the endogenous expression pattern of *dlx1a,* there was paucity of cells expressing GFP in more lateral cells (Fig. 3.3). One possible explanation for this result is that, although the intergenic enhancer can direct expression to the forebrain, its activity is modulated by specific interactions with other *cis*-acting regulatory elements, such as promoters of each of the two flanking genes, *dlx1a* and *dlx2a.* Another explanation is that, it is not just the enhancer that is responsible for targeting reporter transgene expression, the overall distinct set of transcriptional activators binding to upstream and intergenic regulatory sequences also play an important role.

4.2 Function of Intergenic enhancers and upstream regulatory elements in Evolution

The current three *Dlx* bigene clusters of vertebrates are probably the result of duplication of an ancestral bigene cluster, as two of the three characterized *Dll* genes of the ascidian *Ciona intestinalis*, *Dll*-A, and *Dll-B* are organized similarly with a short intergenic region (Di Gregorio *et al.*, 1995). It is possible that this paired organization arose after the

divergence of arthropods from the lineage that would give rise to vertebrates because insects are thought to have only one *distal-less* gene. However, enhancers have yet to be found in the intergenic region that separates the *Ciona Dll-A* and *Dll-B* genes. Of late, an enhancer located upstream of *Dll-A* was identified and shown to recapitulate most aspects of the endogenous expression pattern (Harafuji *et al.*, 2002). Recent studies in a basal level vertebrate, the agnathan lamprey *Petromyson marinus* has revealed a possibility of enhancer sequences in the *DlxD* and *DlxC* bigene cluster (Maurya and Ekker, unpublished observations).

The *Dlx* intergenic enhancers could have originated from a sequence found in the ancestral *Dlx* bigene cluster and diverged following the duplication events that took place early in vertebrate evolution, and that led to the three *Dlx* bigene clusters of modern vertebrates. This divergence happened before the separation of the lineages leading to modern-day teleosts and tetrapods. Since then, the regulatory mechanisms involving the intergenic enhancers from *Dlx* bigene clusters have been maintained.

The present study indicates an important role for the intergenic region in the *cis*-regulatory mechanisms that are responsible for many aspects of the expression of genes from *dlx1a/dlx2a* loci. Intergenic regulatory elements are not solely responsible for *Dlx* regulation, as a fragment from *Dlx1* upstream region was also shown to recapitulate expression in the forebrain. Thus, intergenic enhancers as well as enhancers located in the upstream region confer expression of a transgene to the forebrain suggesting a synergistic effect between multiple and distinct enhancers in forebrain regulation of *Dlx1* and *Dlx2*.

Recently, evidence has been presented for cross regulatory interactions between *Dlx1/Dlx2* and *Dlx5/Dlx6* through intergenic enhancers. Thus, *Dlx1* and *Dlx2* genes are expressed earlier in the forebrain and are involved in either the activation or maintenance of *Dlx5/Dlx6* intergenic region through the enhancers found in the *Dlx5/Dlx6* intergenic region (Zerucha *et al.*, 2000). Overall, these observations suggest the multiple roles of enhancer elements in regulating complex mechanism of *Dlx* expression in the forebrain.

4.3 FACS as a method to isolate *Dlx*-GFP-expressing forebrain cells

We have successfully shown that *Dlx*-GFP-expressing cells can be isolated from the transgenic zebrafish forebrain in real time by employing FACS technique. Furthermore, we demonstrate the utility of the *Dlx*-GFP reporter system in the isolation of forebrain cells from highly mixed populations, thus allowing the characterization of these cells under defined conditions. This technique could be employed in studies involving GFP reporter gene in other organs and other model organisms too. It could also be useful for studies involving comparative analysis of different subpopulation of cells within or between different species of model organisms.

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