Int. J. Dev. Biol. 50: 71-75 (2006)
doi: 10.1387/ijdb.052087hz

Developmental Expression Pattern

Dlx3 is expressed in the ventral forebrain of chicken embryos: implications for the evolution of the *Dlx* gene family

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ABSTRACT The archetypal genomic arrangement of vertebrate *Dlx* genes is as three bigene clusters (*Dlx1/2, Dlx3/4, Dlx5/6*). Phylogenetic sequence analysis of mouse and zebrafish *Dlx* clusters supports the notion that the *Dlx3/4* cluster is more derived and the absence of expression of either *Dlx3* or *Dlx4* in the central nervous system, as reported to date, is consistent with this. Together, these observations have prompted a model in which cis-regulatory elements, responsible for directing *Dlx* gene transcription in the forebrain, were lost from the *Dlx3/4* bigene cluster prior to the divergence of tetrapods from fish. Here, we describe *Dlx3* expression in the forebrain of chicken embryos; this constitutes the first documented evidence of expression of either *Dlx3* or *Dlx4* in the central nervous system of a vertebrate. Our observations have implications for models of the evolutionary history of the *Dlx* gene family, for the genomic organization of *Dlx* genes in birds and for functional redundancy of *Dlx* gene function during avian forebrain development.

KEY WORDS: *Dlx3, transcription factor, chicken embryo, ventral forebrain, neurogenesis*

The homeobox gene family that encodes Dlx transcription factors represents an example of a common paradigm in genome evolution wherein an archetypal gene (e.g. *Distal-less*) in a euchordate ancestor was serially duplicated over evolutionary time to give rise to a family of homologous genes in living vertebrates (e.g. *Dlx1-Dlx6* in mammals). In extant mammals, the six *Dlx* genes are arranged as three linked pairs (or bigene clusters) on different chromosomes within syntenic regions that include three of the four *Hox* clusters (McGuinness *et al.*, 1996, Nakamura *et al.*, 1996, Liu *et al.*, 1997, Zerucha *et al.*, 2000, Sumiyama *et al.*, 2003). Zebrafish also have this genomic organization of six *Dlx* orthologues in three bigene clusters, although further large-scale duplications have left *Danio rerio* with an additional orphan paralogue for each of *dlx2* and *dlx4* (Stock *et al.*, 1996, Ellies *et al.*, 1997). Of the mammalian genomic loci cloned thus far, the *Dlx* intergenic region ranges from a minimum of 8.3 kb in the mouse *Dlx1/2* cluster (McGuinness *et al.*, 1996) to a maximum of 17.6 kb in the human *DLX3/4* cluster (Sumiyama *et al.*, 2003). Intergenic regions for the genomically compact pufferfish *Takifugu rubripes* and *Spheroides nephalus* are smaller at 3-5 kb (Ghanem *et al.*, 2003), with *Danio rerio* having intermediate sizes of 3.5 - 7.3 kb (Ellies *et al.*, 1997). Despite the significant variation in their size, several highly conserved non-coding sequence elements have been identified in the intergenic regions of *Dlx* bigene clusters and have been shown to behave as tissue-specific enhancers (Zerucha *et al.*, 2000, Sumiyama *et al.*, 2002, Ghanem *et al.*, 2003, Ruest *et al.*, 2003, Sumiyama and Ruddle, 2003, Park *et al.*, 2004). Intergenic enhancer sharing therefore accounts for much of the overlap in expression of members of a *Dlx* bigene cluster.

Phylogenetic sequence analysis of mouse and zebrafish *Dlx* clusters supports the notion that the *Dlx3/4* cluster is more derived (Sumiyama *et al.*, 2003). Consistent with this, *Dlx1*, *-2*, *-5* and *-6* show more commonality in their sites of expression than *Dlx3* and *Dlx4* (reviewed in Bendall and Abate-Shen, 2000, Merlo *et al.*, 2000, Zerucha and Ekker, 2000, Panganiban and Rubenstein, 2002). The principle cited differences have been novel expression of *Dlx3* in epidermis (Beauchemin and Savard, 1992, Morasso *et al.*, 1993) and of *Dlx3* and *Dlx4* in the placenta (Quinn *et al.*, 1998, Morasso *et al.*, 1999) as well as the demonstrated or reported lack of expression of *Dlx3* in the central nervous system of all model vertebrate species examined (Papalopulu and Kintner, 1993, Akimenko *et al.*, 1994, Robinson and Mahon, 1994, Pera and Kessel, 1999). Indeed, forebrain-specific enhancers that are conserved in mammalian and zebrafish *Dlx1/2* and *Dlx5/6* clusters are absent in the murine *Dlx3/4* cluster (Sumiyama *et al.*, 2002, Ghanem *et al.*, 2003). This has resulted in the prevailing model of *Dlx* gene evolution in which a forebrain-specific enhancer was lost from the intergenic region of the *Dlx3/4* cluster

Abbreviations used in this paper: BA, branchial arch; ORF, open reading frame; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

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0214-6282/2006/$25.00

Printed in Spain
www.intjdevbiol.com

prior to the divergence of teleost fish from tetrapods (Quint *et al.*, 2000). Here, we report that *Dlx3* is expressed in the forebrain of chicken embryos in a pattern that very closely resembles that of *Dlx5*. Our results have implications for the sequence conservation and genomic organization of *Dlx* genes in the chicken, functional redundancy among Dlx transcription factors during neurogenesis in the avian basal forebrain, as well as current views of *Dlx* gene evolution, particularly with respect to sequence elements that control forebrain expression.

Dlx3 expression in the developing chicken brain

In ongoing experiments aimed at characterizing *Dlx* gene expression during chicken embryogenesis, we unexpectedly detected a *Dlx3*-specific fragment following reverse transcription-polymerase chain reaction (RT-PCR) from embryonic forebrain. Specifically, a fragment corresponding to the *Dlx3* open reading frame (ORF) was amplified from embryonic forebrain as well as the mandibular process where *Dlx3* is known to be expressed (Pera and Kessel, 1999) (Fig. 1). *Prx2* is expressed in the first branchial arch but is not expressed in the brain (Leussink *et al.*, 1995). Thus, to rule out the possibility that the forebrain sample was contaminated with *Dlx3*-expressing tissue or cDNA from the developing jaw, we performed PCR using gene-specific primers for *Prx2* from the same pools of first-strand cDNA. *Prx2* cDNA was detected in the mandibular process, but not in the forebrain (Fig. 1). These results are therefore consistent with a novel domain of expression for *Dlx3* during chick development.

Since expression of either member of the *Dlx3/4* pair in the vertebrate central nervous system is unprecedented, we characterized this expression further using *in situ* hybridization. A previous account of *Dlx3* expression during chick embryogenesis reported an absence of expression in the forebrain following whole mount *in situ* hybridization (Pera and Kessel, 1999). Anticipating that expression levels of *Dlx3* may be low and difficult to detect in the context of whole embryos, we dissected intact the brain and rostral spinal cord from stage 21-32 embryos and hybridized with a *Dlx3* antisense riboprobe. We also hybridized with an antisense *Dlx5* riboprobe as a marker for known *Dlx* expression territories in the chicken brain. Forebrain expression of *Dlx3* in whole brains was difficult to detect at the youngest stages examined, was obvious between stages 27 and 29 and continued through to the oldest stage examined (stage 32). We therefore chose to focus our subsequent analysis between stages 27 and 29. *Dlx3* was detected in a pattern largely indistinguishable from that of *Dlx5* and included a large domain of expression in the subpallial telencephalon (striatum, globus pallidum and septum) and diencephalon (ventral thalamus and hypothalamus, Fig. 2A,B). Hybridization of sense *Dlx3* riboprobe to dissected brains did not result in any consistent staining pattern (data not shown).

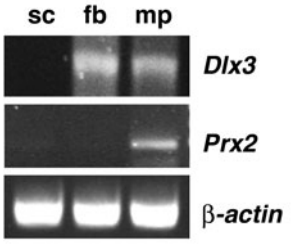

Fig. 1. *Dlx3* is expressed in the developing chicken forebrain. *Ethidium bromide stained agarose gels following RT-PCR of total RNA from tissues dissected from stage 28 chicken embryos.* Dlx3 *transcripts are present in the forebrain (fb) and mandibular process (mp) but not in the spinal cord (sp) whereas* Prx2 *transcription was only detected in the developing mandible. Gene-specific* β-actin *primers were used to demonstrate that an equivalent amount of first strand cDNA was added to each PCR.*

Given the high degree of similarity between the *Dlx3* and *Dlx5* expression patterns, we sought to rule out the possibility that our antisense *Dlx3* riboprobe had cross-hybridized with *Dlx5* mRNA. We next hybridized *Dlx3* or *Dlx5* riboprobes with coronal sections of whole embryonic heads such that both telencephalon and branchial arch tissue were included in the same section. Low-level *Dlx3* expression was detected in the ventral telencephalon in a region corresponding to *Dlx5* transcription (Fig. 2C, E). In contrast, more robust *Dlx3* expression was restricted to the lateral distal ectomesenchyme of the mandibular process of the first branchial arch (BA1) and hyoid arch (BA2), whereas *Dlx5* expression extended more medially and proximally, including a small area of expression in the maxillary process of BA1 (Fig. 2D, F). Thus, the *Dlx3* riboprobe was not cross-hybridizing with *Dlx5* transcripts.

To further characterize this novel domain of *Dlx3* expression, we examined when during neurogenesis *Dlx3* was expressed. Hybridization with a *Dlx3* riboprobe to transverse and coronal sections through the striatum and ventral thalamus revealed scattered *Dlx3*-positive neurons in the ventricular zone while most neurons in the subventricular zone were expressing *Dlx3*. *Dlx3* expression was low to undetectable in the mantle. Again, this paralleled *Dlx5* expression in these zones with scattered *Dlx5*-positive cells in the ventricular zone, high-level expression in the sub-ventricular zone and lower expression in the mantle (Fig. 2 G-J). Thus, while *Dlx3* expression levels are consistently lower than those of *Dlx5*, both genes are transcribed in very similar domains during forebrain neurogenesis in the chick embryonic telencephalon and diencephalon.

Finally, we asked whether this previously undetected feature of *Dlx3* expression was specific for avian embryos. Since a 79 kb reporter construct in which *lacZ* had been knocked into the murine *Dlx3* locus did not express beta-galactosidase in the brain of transgenic embryos (Sumiyama *et al.*, 2002) we did not anticipate seeing *Dlx3* expression in the brains of mouse embryos. However, to rule out the possibility that our particular combination of riboprobe and hybridization conditions would permit detection of *Dlx3* expression in a mammalian species, we re-examined *Dlx3* expression in the forebrain of mouse embryos by hybridizing coronal, sagittal and transverse sections of 14.5, 16.5 and 18.5 days post coitum mouse embryos with orthologous antisense riboprobes corresponding to the full-length ORF of murine *Dlx3*, using *Dlx5* as a marker for telencephalic and thalamic tissue. No *Dlx3* expression was detected in the brains of mid- to late gestation mouse embryos (data not shown). In summary then, we have detected expression of *Dlx3* in differentiating neurons of the developing ventral telencephalon and diencephalon of chicken embryos. This may represent a unique feature of *Dlx* gene expression in birds.

Explanations for the expression of *Dlx3* in the avian brain are currently frustrated by a lack of genomic sequence information; the genomic organization and, indeed, functional complement of *Dlx* genes in *Gallus* remains unknown. While full-length ORFs encoding highly conserved chicken orthologues have been isolated for *Dlx5* (Ferrari *et al.*, 1995), *Dlx3* (Pera and Kessel, 1999),

Dlx1 and *Dlx6* (Brown *et al.*, 2005), it is not yet clear whether the chicken expresses a functional Dlx4 protein (Brown *et al.*, 2005; T. Coleman and A.J.B., unpublished). Additionally, no chicken *Dlx* bigene cluster has been cloned to date and the public chicken genome database does not contain any *Dlx*-bearing contigs large enough to reveal bigene cluster organization (Ensembl version 32.1h). Indeed, current *Dlx3* and *Dlx4* exon-bearing contigs have not been anchored to a specific chromosome. Based on human, mouse and zebrafish synteny, one would anticipate that a chicken *Dlx3/4* bigene cluster would fall between *Wnt3* and the *Hoxb* cluster (Sumiyama *et al.*, 2003). Indeed, *Wnt3* lies within 0.47 Mb of *Hoxb-8* on chicken chromosome 27, in a region that shares synteny with human chromosome 17 and murine chromosome 11. This region of chicken chromosome 27 is not well characterized though, with *Hoxb-8* being the only chicken *Hoxb* gene so far annotated. Interestingly, recent cloning of the *Dlx* loci from the Leopard shark *Triakis semifasciata* by genomic PCR yielded a *Dlx1/2* and *Dlx5/6* cluster, but failed to amplify *Dlx3* and *Dlx4* genes on a single fragment (Stock, 2004). Until a *Dlx3/4* cluster is cloned from these species, it remains possible that the *Dlx3* and *Dlx4* genes have become separated in some lineages.

Previous descriptions of *Dlx* gene expression patterns in various vertebrate model organisms (Papalopulu and Kintner, 1993, Akimenko *et al.*, 1994, Robinson and Mahon, 1994), including an earlier report of *Dlx3* expression in the chicken embryo (Pera and Kessel, 1999), have been consistent with an evolutionary scenario in which *cis*-regulatory elements responsible for directing *Dlx* gene transcription in the forebrain were lost from the *Dlx3/4* bigene cluster prior to the divergence of tetrapods from fish (Quint *et al.*, 2000). Our data, presented here, are inconsistent with such a straightforward model. Minimally, we need to consider that forebrain expression was secondarily acquired as a derived state in birds. Other scenarios would require independent loss of a forebrain enhancer in multiple diverged lineages. Focussed attempts to clone the chicken *Dlx3* genomic locus should provide the necessary data to discriminate between these possibilities.

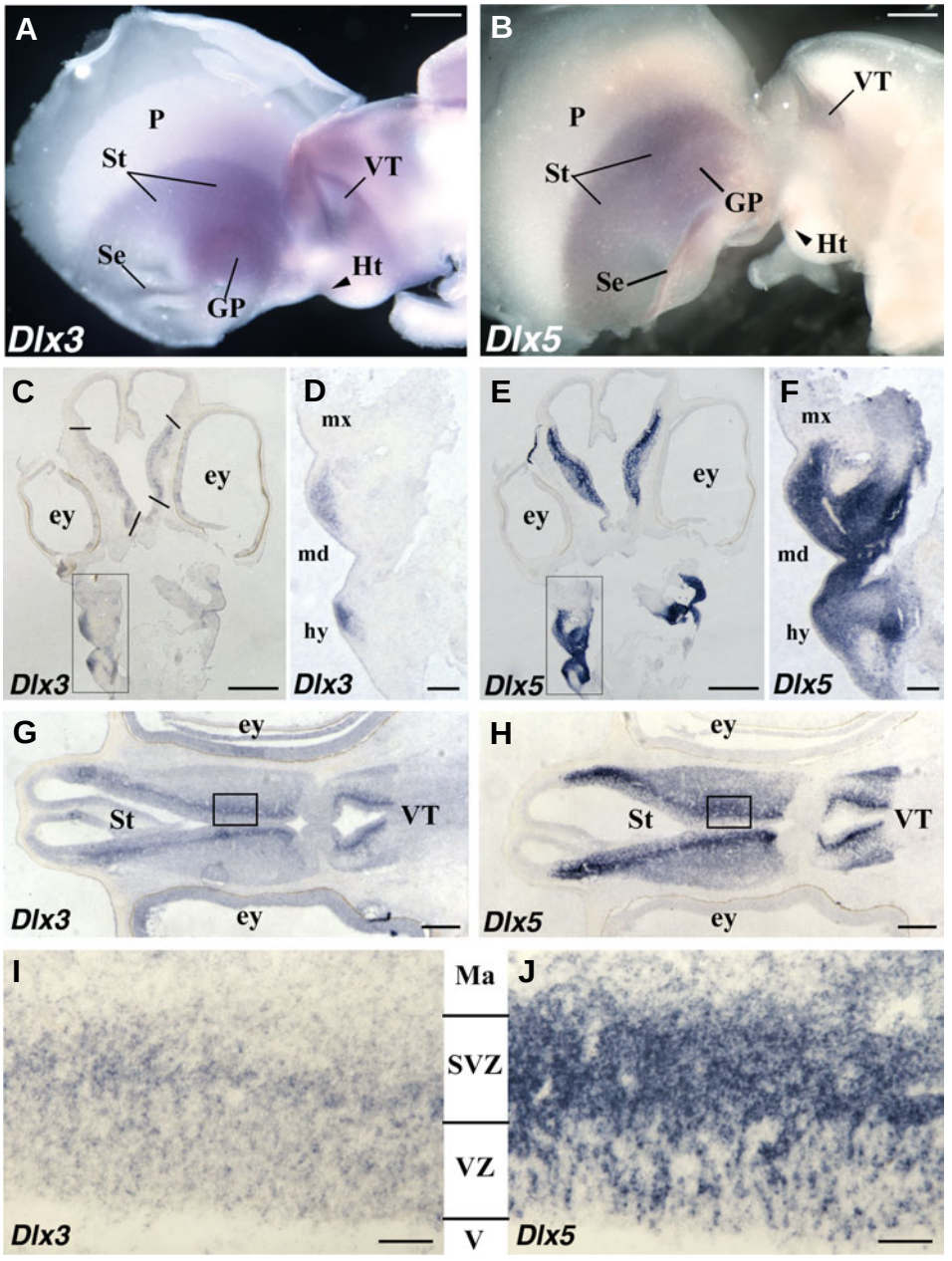

Fig. 2. Overlap of *Dlx3* and *Dlx5* expression in differentiating neurons in the basal forebrain and ventral thalamus. (A,B) *Whole mount* in situ *hybridization of dissected brains from stage 28 embryos with* Dlx3 *or* Dlx5 *riboprobe showing expression of both genes in the subpallial region of the telencephalon (St, GP and Se), in the ventral thalamus (VT) and in the hypothalamus (Ht). Brains have been bisected along the midline and are viewed from the ventricular side. Rostral is to the left, dorsal is up.* **(C-F)** *Coronal sections through a stage 27 embryo showing low level bilateral expression of* Dlx3 *(demarcated by the short black lines) that overlaps with* Dlx5 *in the basal telencephalon but a more restricted pattern of* Dlx3 *expression compared to* Dlx5 *in the first and second branchial arches. The boxed areas in C and E approximate the regions shown in D and F. Panels C and E show adjacent sections, as do panels D and F.* **(G-J)** *Transverse sections through the head of a stage 29 embryo at the level of the ventral thalamus showing highest levels of* Dlx3 *and* Dlx5 *in the sub-ventricular zone. The boxed areas in G and H approximate the regions shown in I and J. Panels G and H show adjacent sections, as do panels I and J. Rostral is to the left. Abbreviations: ey, eye; GP, globus pallidum; Ht, hypothalamus; hy, hyoid arch; Ma, mantle; md, mandibular branch of the first branchial arch; mx, maxillary branch of the first branchial arch; P, pallium; Se, septum; St, striatum; SVZ, sub-ventricular zone; V, ventricle; VT, ventral thalamus; VZ, ventricular zone. Scale bars, 500 μm (A-C,E,G,H), 100 μm (D,F,I,J).*

Materials and Methods

Embryos

Fertile eggs from Barred Plymouth Rock chickens were obtained from a flock maintained at the Arkell poultry barn (Guelph, ON) and incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951).

RT-PCR

Total RNA was prepared from the dissected tissues of stage 28 embryos with an RNeasy Protect midi kit (Qiagen). First strand cDNA was reverse transcribed with an oligo-dT primer and Superscript II (Invitrogen) and used for PCR with the following gene-specific primers in the presence of 10% dimethyl sulfoxide. Dlx3 forward: 5'-ATGAGCGGCTCCTTCGAC-3'; Dlx3 reverse: 5'-TTAGTAAACGGCGCCCGG-3'; Prx2 forward: 5'-GCCAAGAGGAAGAAGAAACAG-3'; Prx2 reverse: TTAGTTCACAG TCGGCACCTG-3'; β-actin forward: 5'-CATCACCATTGGCAA TGAGAGG-3'; β-actin reverse: 5'-GATTCATCGTACTCCTGCTTGC-3'. Amplification conditions were: 94°C for 5 min followed by 35 (Dlx3) or 30 (Prx2 and β-actin) cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec and a final extension step of 72°C for 10 min.

In situ hybridization

Probes: An 837 bp chicken *Dlx3* cDNA was amplified by RT-PCR from dissected embryonic tissue using restriction site tagged primers and cloned as a *Bsp*HI-*Bam*HI fragment into the *Nco*I and *Bam*HI sites of pSlax13 (Riddle *et al.*, 1993) whose polylinker had been modified to remove the redundant *Sal*I, *Xba*I and *Sac*I restriction sites between the *Nco*I and *Hin*dIII sites. Antisense riboprobe was synthesized with T7 RNA polymerase from a template linearized at the remaining *Xba*I site. Antisense chicken *Dlx5* riboprobe was synthesized with T7 RNA polymerase from a pBluescriptSK-Dlx5 template linearized with *Bam*HI (Bendall *et al.*, 2003). Full-length mouse *Dlx3* and *Dlx5* cDNAs were cloned as *Eco*RI-*Hin*dIII fragments into the modified pSlax13 plasmid. Antisense riboprobes were synthesized with T7 RNA polymerase from a template linearized with *Eco*RI (*Dlx3*) or *Bam*HI (*Dlx5*).

For whole-mount *in situ* hybridization, brains were dissected in phosphate buffered saline (PBS) and fixed overnight with rotation in 4% paraformaldehyde in PBS (pH 7.4) at 4°C. Tissue was rinsed twice in PBT (PBS with 0.1% Tween 20), dehydrated through a methanol:PBT series (25%, 50%, 75%, 2 x 100% methanol) and stored at –20°C in methanol before use. For section *in situ* hybridization, embryos were collected between stages 16 and 32, fixed overnight as above and prepared as follows. Embryos were transferred to 30% sucrose in PBS, pH 7.5 at 4°C with rotation until the embryos sank (usually overnight) then transferred to a 1:1 mixture of Cryomatrix (Thermo Electron Corp.) and 30% sucrose/PBS at 4°C with rotation overnight and, finally, transferred to Cryomatrix and rotated overnight at 4°C. Embryos were embedded in fresh Cryomatrix under a dissecting microscope to ensure desired orientation of specimens and snap-frozen on dry ice. Frozen blocks were stored at –80°C until use and equilibrated to –20°C prior to sectioning. Whole mount or 12 μm section *in situ* hybridization with digoxygenin-labelled antisense riboprobes was done as described in Shen (2001). Whole tissues or sections hybridized with *Dlx3* riboprobes were developed for the same time or longer than those hybridized with *Dlx5* riboprobes. All images were taken using a MicroPublisher color digital camera on a Leica MZ12.5 Stereomicroscope with Qcapture 2.68.6 software (QImaging) or on a Leica DMRA2 upright microscope with Openlab 4.0.1 software (Improvision) and processed using Adobe Photoshop 6.

Acknowledgments

We thank Babak Noamani for technical assistance. This work was supported by Operating grant MOP-62900 from the Canadian Institutes for Health Research and Discovery grant 249773-02 from the Natural Sciences and Engineering Research Council of Canada, to A.J.B.

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Received: July 2005
Reviewed by Referees: August 2005
Modified by Authors and Accepted for Publication: August 2005
Edited by Thomas D. Sargent