An Early Phase of Embryonic Dlx5 Expression Defines the Rostral Boundary of the Neural Plate

Lu Yang, Hailan Zhang, Gezhi Hu, Hongyu Wang, Cory Abate-Shen, and Michael M. Shen

1Center for Advanced Biotechnology and Medicine, and Departments of 2Pediatrics and 3Neuroscience and Cell Biology, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Relative little is known about the molecular events that specify the rostrocaudal axis of the neural plate. Here we show that a member of the Distal-less (Dlx) homeobox gene family, Dlx5, is one of the earliest known markers for the most rostral ectoderm, before the formation of an overt neural plate. During late gastrulation Dlx5 expression becomes localized to the anterior neural ridge, which defines the rostral boundary of the neural plate, and also extends caudolaterally, marking the region of the presumptive neural crest. Subsequently, Dlx5 is expressed in tissues (olfactory epithelium, ventral cephalic epithelium) that are believed to derive from the anterior neural ridge, based on the avian fate map. The early phase of Dlx5 expression in the anterior neural ridge and its derivatives is distinct from a later phase of expression in the ventral telencephalon and diencephalon and also appears to be unique for Dlx5 among members of the Dlx family. Another distinctive feature of Dlx5 expression is the occurrence of an alternative transcript (\(\delta Dlx5\)), which encodes a truncated protein lacking the homeodomain, and represents a significant fraction of total Dlx5 transcripts at all embryonic stages that were examined. In contrast with full-length DLX5, the \(\delta Dlx5\) truncated protein is deficient in DNA-binding activity and does not interact with the homeoprotein partner MSX1. Taken together, our findings suggest that Dlx5 activity may be regulated via the expression of an alternative transcript and demonstrate that Dlx5 marks the anterior boundary of the neural plate.

Key words: homeobox gene; transcription factor; alternative transcripts; anterior neural ridge; neural crest; forebrain

During gastrulation stages of vertebrate embryogenesis the rostrocaudal axis of the neuroectoderm is specified via planar and vertical signaling events (for review, see Wilson and Hemmati-Brivanlou, 1997). Several studies indicate that the subsequent patterning of the rostral neuroectoderm is mediated by the activities of local organizing centers that confer regional identity within the forebrain and midbrain. In the mouse embryo it has been demonstrated that the anterior neural ridge, which is located at the rostral boundary of the neural plate, produces organizing activities that pattern the adjacent rostral prosencephalon at early postgastrulation stages (Shimamura and Rubenstein, 1997). Furthermore, it recently has been shown in the zebrafish embryo that forebrain organizing activities exist in the anterior neural boundary during gastrulation stages (Houart et al., 1998). These early patterning events presumably are dependent on the restricted expression of regulatory genes during gastrulation and neurulation (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Shimamura et al., 1995). Thus, the identification of regulatory genes that have specific boundaries of expression in the rostral ectoderm at the onset of neural plate formation should facilitate our understanding of neural patterning.

Members of the Distal-less (Dlx) gene family previously have been implicated in processes of neural patterning, particularly during later stages of forebrain development (Qiu et al., 1995; Anderson et al., 1997a,b). The Dlx genes comprise a highly conserved family of vertebrate homeobox genes that, like other members of the homeobox superfamily, are thought to act as regulatory molecules via the actions of their protein products as transcription factors. In the mouse, seven Dlx genes have been identified, with numerous homologs found in Amphioxus, zebrafish, newt, Xenopus, chick, rat, and human [compiled by Stock et al. (1996)]. Members of the Dlx family share a conserved homeodomain that mediates sequence-specific DNA binding as well as interactions with other homeoproteins, such as MSX1 (Zhang et al., 1997). Other conserved regions in DLX proteins are important for mediating their actions as transcriptional activators ((Zhang et al., 1997; H. Zhang, G. Hu, and C. Abate-Shen, unpublished data). Furthermore, the similarity among the murine Dlx genes extends to their expression patterns during development, because several members of this family (Dlx1, Dlx2, Dlx3, Dlx5, and Dlx6) are expressed in overlapping spatial domains during forebrain development (Porteus et al., 1991, 1994; Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993a,b; Simeone et al., 1994).

In this report we present evidence indicating that one member of the murine Dlx family, Dlx5, is regulated via alternative mRNA processing and that it is expressed at the boundaries of the rostral neural plate. First, we show that Dlx5 is represented by at least two alternative transcripts, a full-length transcript that encodes an intact and biochemically active protein and a second transcript that is lacking a homeodomain (\(\delta Dlx5\)). Second, we demonstrate that an early phase of Dlx5 expression specifically...
defines the anterior boundary of the neural plate and, subsequently, the anterior neural ridge.

**MATERIALS AND METHODS**

Isolation of Dlx5 cDNA clones and biochemical analysis of DLX5 protein products. We isolated a region of the Dlx5 cDNA by reverse-transcriptase PCR (RT-PCR), using oligonucleotide primers that correspond to the 5′ and 3′ ends of the coding sequence of the rat ortholog (Shirasawa et al., 1994). The sequences of these primers are 5′-GAG TCT GGA TCC ATG ACA GGA GTG TTT GAC AGA AGA-3′ and 5′-CAT GTG GAA TTC AAG CTT CTA ATA AAG CTT CCC GGA GGC-3′. The resulting PCR product was cloned and used to screen a λExlox (Stratagene, La Jolla, CA) cDNA library that we prepared from day 12.5 embryonic head mRNA. Five independent cDNA clones were isolated and sequenced. Four of these cDNA isolates are overlapping and ~1 kb in size, with the longest isolate being identical in sequence to the published murine Dlx5 CDNA (Stock et al., 1996), which we confirmed was full-length by Northern blot analysis (data not shown). The fifth cDNA isolate, which we term Dlx5, has an 82 bp internal deletion of nucleotides 514–595 [numbering is based on the cDNA sequence of Stock et al. (1996)] but is otherwise identical to the other Dlx5 cDNAs. The GenBank accession number for the full-length Dlx5 cDNA clone is AF072452 and for the Dlx5 variant is AF072453.

To produce the DLX5 and Dlx5 proteins, we cloned the inserts from the full-length and the Dlx5 cDNA isolates into pgEM11zf (Promega, Madison, WI). RNA was prepared in vitro by using T7 RNA polymerase, followed by translation with rabbit reticulocyte lysates (Promega, Madison, WI). To examine DNA binding activity, we performed gel mobility shift analysis by using in vitro translated proteins as described (Zhang et al., 1997). The sequences of the DNA sites that were used are shown in Figure 3A and have been described (Catron et al., 1993). To examine protein–protein binding, we performed glutathione S-transferase (GST) interaction assays by using 35S-labeled DLX5 or Dlx5 protein and GST–agarose or a GST–MSX1 fusion protein as described (Zhang et al., 1997).

**Analysis of Dlx5 and Dlx5 expression.** To examine Dlx5 expression, we dissected tissues or intact mouse embryos at the indicated developmental stages (see Fig. 1A). We used an antisense probe for the Dlx5 transcript (sequence numbering of Stock et al. (1996)). Oligonucleotides 5′-AAA GCT GTG GCG TGA TGG-3′ and 5′-AAA GCT GTG GCG TGA TGG-3′ were used in RT-PCR analysis. The sequences of these primers are 5′-GACC GCC GCC GTC GCAG AGA AGA ACA-3′ and 5′-GTG GGT GGT GGT GTG GGT GGC-3′. The resulting PCR product comprised sequences directly flanking the homeodomain (indicated by shaded boxes). Two of these conserved regions are serine-rich (indicated by S) and tyrosine-rich (indicated by Y), whereas the third comprises sequences directly flanking the homeodomain [the extended homeodomain (Zhang et al., 1997)]. Because of a frameshift, Dlx5 contains the N-terminal conserved domains, lacks the homeodomain and other C-terminal regions, and has a novel stretch of 28 amino acids (indicated by the cross-hatched box).}

By screening a cDNA library derived from 12.5 dpc embryonic head mRNA, four of five cDNA clones isolated in this screen are full-length or nearly full-length (~1400 base pairs), in agreement with the published sequence (Stock et al., 1996). Conceptual translation of the coding region reveals three notable features of the Dlx5 protein product: (1) a homeodomain that is flanked by conserved sequences [known as the extended homeodomain (Zhang et al., 1997)]; (2) a serine-rich region that contains putative phosphorylation sites, because Dlx5 is phosphorylated in vitro (H. Zhang and C. Abate-Shen, unpublished data); and (3) an unusual tyrosine-rich motif, for which the function presently is unknown (Fig. 1A).

Notably, we found that one of our Dlx5 cDNA isolates, which we call Dlx5, contains an internal nucleotide deletion within the coding region but is otherwise identical in sequence to the other four cDNAs. This internal deletion creates a frameshift such that the predicted protein product (Dlx5) lacks the homeodomain and C-terminal sequences but includes 28 novel amino acids (Fig. 1B). These findings are in agreement with a recent study by Liu and colleagues (1997), in which they reported alternative transcripts for both Dlx5 and Dlx6, including one (Dlx-5γ) that is similar to Dlx5. Inspection of the nucleotide sequences adjacent to the internal deletion of Dlx5 reveals the presence of imperfect splice sites in the full-length cDNA (specifically a 5′-donor splice site at nucleotide 513 and a 3′-acceptor splice site at nucleotide 596; Fig. 1A). Therefore, this variant transcript may result from alternative splicing.

To verify that this Dlx5 transcript is expressed in vivo and is not an artifact of cDNA synthesis, we performed ribonuclease protection analysis, using total RNA obtained from midgestation mouse embryos (Fig. 2A). For this analysis the riboprobe corre-

**RESULTS**

**Dlx5 encodes two transcripts, one of which lacks the homeodomain.**

To study the expression of murine Dlx5 as well as the biochemical properties of its protein product, we isolated Dlx5 cDNA clones by screening a cDNA library derived from 12.5 dpc embryonic head mRNA. Four of five cDNA clones isolated in this screen are full-length or nearly full-length (~1400 base pairs), in agreement with the published sequence (Stock et al., 1996). Conceptual translation of the coding region reveals three notable features of the Dlx5 protein product: (1) a homeodomain that is flanked by conserved sequences [known as the extended homeodomain (Zhang et al., 1997)]; (2) a serine-rich region that contains putative phosphorylation sites, because Dlx5 is phosphorylated in vitro (H. Zhang and C. Abate-Shen, unpublished data); and (3) an unusual tyrosine-rich motif, for which the function presently is unknown (Fig. 1A).

Notably, we found that one of our Dlx5 cDNA isolates, which we call Dlx5, contains an internal nucleotide deletion within the coding region but is otherwise identical in sequence to the other four cDNAs. This internal deletion creates a frameshift such that the predicted protein product (Dlx5) lacks the homeodomain and C-terminal sequences but includes 28 novel amino acids (Fig. 1B). These findings are in agreement with a recent study by Liu and colleagues (1997), in which they reported alternative transcripts for both Dlx5 and Dlx6, including one (Dlx-5γ) that is similar to Dlx5. Inspection of the nucleotide sequences adjacent to the internal deletion of Dlx5 reveals the presence of imperfect splice sites in the full-length cDNA (specifically a 5′-donor splice site at nucleotide 513 and a 3′-acceptor splice site at nucleotide 596; Fig. 1A). Therefore, this variant transcript may result from alternative splicing.

To verify that this Dlx5 transcript is expressed in vivo and is not an artifact of cDNA synthesis, we performed ribonuclease protection analysis, using total RNA obtained from midgestation mouse embryos (Fig. 2A). For this analysis the riboprobe corre-
responsive to the homeobox region of *Dlx5* such that the intact (*Dlx5*) and alternative (*δDlx5*) transcripts can be distinguished on the basis of their size (see Fig. 1A). Although the overall abundance of *Dlx5* transcripts varies among the developmental stages that were examined, both *Dlx5* and *δDlx5* are detected at each stage (Fig 2A). Moreover, quantitation of these results shows that the abundance of *δDlx5* represents ~10% of the total *Dlx5* transcripts in each of these RNA populations.

We further verified that *δDlx5* is expressed during development by performing RT-PCR analysis. For this purpose we designed oligonucleotide primers that distinguish the *Dlx5* and *δDlx5* transcripts on the basis of size (see Fig. 1A). As shown in Figure 2B, two distinct PCR products were detected in 9.5 and 11.5 dpc total RNA isolated from embryonic heads. The upper band is of greater abundance and comigrates with the product obtained from the control *Dlx5* cDNA, whereas the lower band is less abundant and comigrates with the product obtained from the control *Dlx5* cDNA.

We used this RT-PCR assay to examine further the expression of *Dlx5* and *δDlx5* transcripts in earlier developmental stages and tissues (Fig. 2C). We found that *Dlx5* was barely detected in 6.75 dpc egg cylinders and was abundant in 7.5 dpc embryonic tissues, but not extraembryonic tissues, and also was detected readily in 9.5 dpc ventral head, dorsal head, branchial arches, and tail (Fig. 2C). *δDlx5* was expressed in parallel with *Dlx5* at each of these stages but at lower abundances (Fig. 2C). Together with the results of the ribonuclease protection analysis, these findings demonstrate that *δDlx5* is, indeed, expressed *in vivo*, representing ~10% of the total *Dlx5* transcripts throughout development.

The *δDLX5* protein does not interact with DNA or its protein partner MSX1

Because the predicted *δDLX5* protein lacks a homeodomain, we anticipated that its biochemical properties might differ significantly from those of the full-length DLX5 protein. To test this prediction, we performed gel mobility shift assays, using DLX5 and *δDLX5* proteins that were obtained by *in vitro* transcription/translation of the corresponding cDNAs. The DNA sites used for this analysis were variants of a homeodomain consensus DNA site, which contains an essential TAAT core and flanking nucleotides that contribute to DNA-binding specificity (Catron et al., 1993). As shown in Figure 3A, full-length DLX5 interacts with these DNA sites to varying degrees, although it does not bind to a DNA site containing a mutated TAAT core. In contrast, *δDLX5* does not interact with any of the DNA sites that were tested, consistent with its lack of a homeodomain.

In previous studies we have shown that the biochemical properties of DLX proteins, including DLX5, are modulated via their interactions with the MSX1 homeoprotein and that the MSX-DLX interaction is mediated by their respective homeodomains (Zhang et al., 1997). Therefore, we further compared the biochemical properties of DLX5 and *δDLX5* by examining their ability to form protein complexes with MSX1. As shown in Figure 3B, the full-length DLX5 protein interacts well with a GST-
Dlx5 expression marks the rostral-lateral boundaries of the neural plate

In the course of our analyses of expression patterns of Dlx family genes (Zhang et al., 1997), we observed that Dlx5 is expressed at much earlier stages of development than have been reported previously for any member of the murine Dlx family (Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993a; Simeone et al., 1994; Sheng et al., 1997). Therefore, we have examined Dlx5 expression by in situ hybridization during gastrulation and neurulation stages of mouse embryogenesis.

Notably, our findings demonstrate an early phase of Dlx5 expression that demarcates the rostral-lateral boundaries of the prospective neuroectoderm. In particular, we have found that Dlx5 expression first can be detected at the late-streak stage of gastrulation (6.75 dpc), which precedes the formation of an overt neural plate, but is not expressed at earlier stages (Figs. 4, 5; data not shown). (It is important to note that, because Dlx5 and Dl5x differ only in a small region, the resolution of the in situ approach does not distinguish expression of these transcripts; thus we refer to the total signal detected as Dlx5 expression.) During early gastrulation, at 6.75 dpc, Dlx5 expression is detected in a graded rostrocaudal distribution as a lateral band on the embryonic side of the embryonic/extraembryonic junction (Fig. 4A,B). Sections from whole-mount embryos at this stage show that this stripe of Dlx5 expression is localized to the ectoderm layer and is not found in the overlying mesoderm or visceral extraembryonic endoderm (Fig. 5A). At early neural plate stages (7.25 dpc) Dlx5 is expressed in a circumferential stripe around the embryonic half of the egg cylinder (see Fig. 4C,D). The rostral extent of this stripe overlaps the position at which the head folds will emerge, whereas the caudal end curls dorsally at the base of the allantois. Interestingly, the anterior limit of Dlx5 expression at these stages is localized to the most rostral region of the ectoderm and abruptly terminates where it adjoins the amnion (Fig. 5B,C).

By the head-fold stages of gastrulation (7.5 dpc), Dlx5 expression appears more intense in the rostral ectoderm of the emerging head folds, whereas its expression laterally and caudally is less intense (see Fig. 4E–G). At these stages the expression of Dlx5 clearly defines the emerging anterior neural ridge (Fig. 5D–F). After gastrulation, at early somite stages of development (8.0 dpc), Dlx5 expression continues to be restricted to the anterior head folds, whereas its expression laterally and caudally fades (Figs. 4H,I,J,L, 5H). In addition, it is noteworthy that Dlx5 expression disappears from the rostral midline starting at this stage (see Fig. 4I,M, arrowheads). Thus, these findings demonstrate that Dlx5 is an early marker of the rostral ectoderm before the formation of the anterior neural ridge and that its subsequent expression specifically demarcates the anterior neural ridge.

In addition to marking the anterior neural ridge, Dlx5 also is
Figure 4. Expression of Dlx5 in whole-mount mouse embryos during gastrulation and neurulation stages. In all panels except G, I, and M, anterior (rostral) faces toward the left. A, Lateral bright-field view of a late-streak stage egg cylinder (6.75 dpc), showing expression in a lateral band just below the embryonic/extraembryonic junction. The gray lines mark the boundary between the embryonic and extraembryonic halves of the egg cylinder. B, High-power view of an embryo similar to that in A, showing the rostrocaudal gradient of Dlx5 expression, with the highest level at the rostral end (arrowhead). C, Early neural plate stage embryo (7.25 dpc), with expression in a broad circumferential band that is widest rostrally and curls dorsally at the base of the emerging allantois. D, Late neural plate stage (7.5 dpc), showing more intense rostral expression, with the band of expression fainter and narrower caudally. E, Early head-fold stage (7.75 dpc), showing expression at the lateral edges of the neural plate (arrowhead). F, Lateral dark-field view at the late head-fold stage (8.0 dpc), showing expression in a thin lateral stripe rostrally that is barely detectable caudally (arrowhead). G, Frontal bright-field view of an embryo similar to that shown in F. Expression is strongest at the anterior margin of the neural plate and diminishes caudolaterally (arrowhead). H, Lateral view of a two-somite embryo (8.25 dpc), with strong expression detectable only in the ventral ectoderm beneath the head folds and with weak expression in the otic placode (arrowhead). I, Frontal view of the embryo shown in H. Note that rostral expression is weaker in the midline (arrowhead). J, Lateral view of a four-somite embryo, showing expression at the lateral edges of the neural groove before neural tube closure (arrowheads). K, Higher-power view of the posterior neuropore of the embryo shown in J. Note that weak expression can be detected after neural tube closure (arrowhead). L, Lateral view of a six-somite embryo, showing expression in the ventral cephalic epithelium underneath the head folds and in the developing otic vesicles. M, Frontal view of an eight-somite embryo, showing bilateral expression in the ventral cephalic epithelium before anterior neuropore closure, but not at the midline (arrowhead). N, High-power lateral view of the embryo shown in M, showing staining in the ventral cephalic epithelium that continues faintly at the boundary of the anterior neuropore (arrowhead). Also shown is staining in the otic vesicle and in the newly forming mesenchyme of the first branchial arch. O, Lateral view of a 10-somite embryo (8.5 dpc), showing prominent expression in the ventral cephalic epithelium, the first branchial arch, the otic vesicles, and the tail bud. P, Higher-power view of an embryo similar in stage to that in O, showing staining in the tail bud and in the lateral edges of the posterior neuropore (arrowheads). Al, Allantois; AN, anterior neuropore; AVE, anterior visceral endoderm; BA, first branchial arch; C, caudal; HF, head folds; Ht, heart; Nd, node; NT, neural tube; OV, otic vesicle; PN, posterior neuropore; PS, primitive streak; R, rostral; RE, rostral ectoderm; Tb, tail bud; VCE, ventral cephalic epithelium; VYS, visceral yolk sac. Scale bars, 200 μm.

expressed at the lateral margins of the neural plate at late stages of gastrulation (7.75 dpc) (see Fig. 4E,G). During early somite stages (8.25 dpc) this lateral zone of Dlx5 expression is found in a narrow strip of cells adjacent to the neuroepithelium along the length of the neural plate, marking the position of the presumptive premigratory neural crest (Figs. 4J,K,P,5M,N). Unlike the rostral zone of expression, the lateral zone of Dlx5 expression disappears shortly after neural tube closure (see Fig. 4O). Thus, the lateral domain of Dlx5 expression may correspond to the premigratory neural crest.

Before anterior neuropore closure (8.0 dpc) the rostral zone of Dlx5 expression becomes restricted to the ventral cephalic epithelium and olfactory placodes, whereas expression is not found in the prosencephalon (see Fig. 4L–N). Sections from this stage clearly demonstrate that Dlx5 expression is restricted to the non-neural cephalic epithelium and is excluded from the overlying neural tissue (Fig. 5H–J). After anterior neuropore closure (8.5 dpc) Dlx5 expression continues in the ventral cephalic epithelium and also is found in the cranial neural crest derivatives that form the mesenchyme of the first branchial arch (Figs. 4O,P,5K,L). At 10.5 dpc, expression of Dlx5 is evident in the olfactory epithelium of the nasal cavity (Fig. 5Q).
ues in the olfactory epithelium through 13.5 dpc and is also prominent in the vomeronasal (Jacobson’s) organs, which are derived from the olfactory epithelium (Fig. 5R–T).

It is noteworthy that the expression of Dlx5 in the anterior neural ridge and its derivatives contrasts with a distinct domain of its expression in the telencephalon and diencephalon, which has been described previously (Simeone et al., 1994; Chen et al., 1996; Sheng et al., 1997) and which resembles the expression pattern of several other Dlx genes (Porteus et al., 1991, 1994; Price et al., 1991; Robinson et al., 1991; Bullone et al., 1993a,b; Simeone et al., 1994). Notably, Dlx5 expression is not detected in the prosencephalon at 8.5 dpc (Fig. 5I,K), whereas beginning at 9.5 dpc Dlx5 expression is observed in two separate regions of the ventral telencephalon and diencephalon (Fig. 5O,P).

DISCUSSION

In this report we describe two aspects of Dlx5 expression that may offer insight into its function during neurogenesis. First, we show that a significant percentage of Dlx5 transcripts encodes a protein (δDLX5) that lacks a homeodomain and presumably would have altered biological functions in vivo. Second, our in situ hybridization studies define an early phase of Dlx5 expression at the rostral and lateral borders of the neural plate. Notably, the expression of Dlx5 at the rostral boundary of the neural plate and in the anterior neural ridge occurs at the developmental stages when these regions have organizing activities that pattern the adjacent rostral prosencephalon (Shimamura and Rubenstein, 1997; Houart et al., 1998). These observations underscore the complex regulation and potential activities of members of the Dlx family.

Potential for regulation of DLX5 activity by an alternative transcript

An unexpected finding of our study was the identification of a second Dlx5 transcript, δDlx5, which encodes a truncated polypeptide that lacks biochemical activities requiring the homeodomain. Interestingly, in their analysis of Dlx5 expression in later developmental stages, Rubenstein and colleagues also have reported recently the isolation of two alternative Dlx5 transcripts, one of which (Dlx-5γ) corresponds to the transcript we call δDlx5 (Liu et al., 1997). Here, we demonstrate that δDlx5 is deficient in several biochemical activities and is coexpressed with the full-length transcript in similar ratios at early developmental stages.

We can envisage several possible roles for truncated versions of the DLX5 protein. First, δDLX5 may be inactive in all functional respects, in addition to those that we have examined. In this regard, the production of an alternative transcript may provide a means of regulating the levels of active DLX5 protein in vivo. Second, δDLX5 may be active in some functional contexts, but not in others. If so, δDLX5 may provide a means of regulating DLX5 activity in a dominant-negative manner via competition with DLX5 for components of transcription complexes. Indeed, δDLX5 contains the N-terminal regions required for transcriptional activation, which are conserved among DLX proteins (G. Hu and C. Abate-Shen, unpublished data); thus δDLX5 could compete with full-length DLX5 for interactions with other transcription factors. Third, δDLX5 may have distinct biochemical activities that do not require the homeodomain. Notably, Ftz polypeptides lacking the homeodomain are nonetheless biologically active during Drosophila embryogenesis (Copeland et al., 1996). Furthermore, numerous homeobox genes, including HoxA1, HoxB6, CAPS, and CSX1/Nkx2.5, have alternative transcripts that encode truncated proteins lacking the homeodomain (Baron et al., 1987; LaRosa and Gudas, 1988; Shen et al., 1991; Shijoima et al., 1996; Lievens et al., 1997). Although the functions of these truncated proteins have not yet been elucidated, the prevalent occurrence of alternative transcripts among homeobox-containing genes suggests that such transcripts have biological significance.

Expression of Dlx5 defines the anterior neural ridge

The molecular processes by which the rostral ectoderm is patterned are of particular interest because this region gives rise to both neural and non-neural derivatives. Thus, the fates of the rostral ectoderm have been elucidated primarily by chick–quail transplantation studies (Coulou and Le Douarin, 1985, 1987), which have been supported by DiI labeling experiments in cultured mouse embryos (Osumi-Yamashita et al., 1994). These fate-mapping studies suggest that the murine anterior neural ridge subsequently will generate ectodermal derivatives (adeno-hypophysis, ventral cephalic epithelium, and olfactory placodes), whereas regions that are caudal to the anterior neural ridge will generate structures of the forebrain (optic vesicles, hypothalamus, and ventral telencephalon) (Fig. 6). The anterior neural ridge extends caudolaterally and is contiguous with the lateral margins of the neural plate, which will generate the cranial neural crest (Coulou and Le Douarin, 1985, 1987; Baker and Bronner-Fraser, 1997).

Our description of Dlx5 expression in the mouse embryo is in good agreement with the avian fate map (Fig. 6), because murine Dlx5 is expressed first in the anterior neural ridge and subsequently in the ectoderm of the nasal cavity, olfactory placodes, and ventral cephalic epithelium. Indeed, murine Dlx5 expression persists in these derivatives of the anterior neural ridge, whereas the disappearance of Dlx5 expression in the rostral midline at early somite stages is consistent with its later absence in Rathke’s pouch (adeno-hypophysis). It is important to note that the later phase of Dlx5 expression in ventral telencephalon and diencephalon occurs in regions that, based on the avian fate map, are not derived from the anterior neural ridge (Fig. 6) (Coulou and Le Douarin, 1985, 1987), suggesting that these two phases of murine Dlx5 expression are distinct both in their temporal onset and embryological origin.

Among other molecular markers for regions of the anterior ectoderm in the mouse embryo are the homeobox genes Otx2 (Simeone et al., 1992, 1993; Ang et al., 1994), Pax6 (Li et al., 1994), and Six3 (Oliver et al., 1995) as well as the winged helix
Figure 5. Expression of Dlx5 in mouse embryo sections. A–N represent cryosections of embryos processed for whole-mount in situ hybridization, whereas O–T show results obtained from section in situ hybridization. Anterior (rostral) faces to the left, except for M, N, R, and S, where anterior faces down. A, Transverse section through a late-streak stage embryo (6.75 dpc), showing expression in the rostral and lateral ectoderm. Expression is not detected in the caudal ectoderm, near the primitive streak, nor in the newly formed mesoderm or in the extraembryonic visceral endoderm. B, C, Sagittal section through an early neural plate stage embryo (7.25 dpc), showing expression in the rostral and lateral ectoderm as well as in the posterior ectoderm overlying the primitive streak. (The tissue layers have separated slightly during processing for cryosectioning.) The gray lines mark the boundary between the embryonic and extraembryonic regions of the egg cylinder. In C, note that expression in the rostral ectoderm overlies the precardiac mesoderm (anterolateral plate mesoderm), with a sharp boundary rostrally at the junction with the extraembryonic amnion and a more diffuse boundary caudally (arrowheads). D, E, Sagittal view of an early head-fold stage embryo (7.75 dpc), showing restricted expression of Dlx5 in the anterior neural ridge. In E, note the relatively diffuse caudal boundary of expression (arrowhead). F, Sagittal section of a two-somite stage embryo, showing restricted expression of Dlx5 in the anterior neural ridge. G, Transverse section through the head folds at the two-somite stage. Expression is found in the ventral epithelium of the head folds, but expression does not extend into the neural groove and is nearly undetectable at the ventral midline (arrowhead). H, Higher-power sagittal view of a four-somite embryo, closer to the midline. Expression of Dlx5 extends through the anterior neural ridge but terminates at the border with the prosencephalon (arrowhead). I, J, Sagittal section of a six-somite embryo. Note that expression is not detected in any region of the brain at this stage. K, L, Sagittal section through an 8.5 dpc embryo, with expression in the ventral cephalic epithelium, menenchyse of the first branchial arch, and otic vesicle. In L, note that expression of Dlx5 in the ventral cephalic epithelium terminates caudally at the junction with the epithelium of the first branchial arch (arrowhead). M, N, Transverse sections through an 8.5 dpc embryo at different axial levels. In the open neural groove of the posterior neuropore (M), expression is found in cells adjacent to the columnar neuroepithelium (arrowheads). More rostrally, after neural tube closure (N), transient expression is found in the cells overlying the dorsal neural tube (arrowhead). O, P, Coronal sections through a 9.5 dpc embryo, showing the earliest detectable expression of Dlx5 in the forebrain. Expression primarily localizes to differentiating cells in the subventricular zone, although limited staining can be observed in the ventricular layer of the diencephalon. Q, Sagittal section through the head of a 10.5 dpc embryo, with expression in the olfactory...
transcription factor *BF1* (Tao and Lai, 1992). At early stages of neurulation these genes are expressed in overlapping patterns that include the anterior neural ridge and extend caudally to the forebrain (Oliver et al., 1995; Shimamura and Rubenstein, 1997). Subsequently, *Six3, Otx2, Pax6,* and *BF1* are expressed continuously in specific domains of the forebrain (e.g., optic vesicles, hypothalamus, and ventral telencephalon) (Tao and Lai, 1992; Simeone et al., 1993; Li et al., 1994; Oliver et al., 1995). In contrast, *Dlx5* is not expressed in any forebrain regions until 9.5 dpc. This comparison suggests that regional subdivisions of the rostral ectoderm already may be established at the time of the formation of an overt neural plate.

**Expression of Dlx5 in the anterior neural ridge may be conserved evolutionarily**

Recent phylogenetic analyses of gene sequences have clarified the ortholog relationships among members of the vertebrate *Dlx* family, which has been complicated by a confusing nomenclature. In particular, the murine *Dlx5* gene is orthologous to rat *rDlx*, chicken *gDlx5*, *Xenopus X-Dll3*, and zebrafish *zDlx4* (Ferrari et al., 1995; Stock et al., 1996). Of these orthologous genes, the expression patterns of *X-Dll3* and *zDlx4* have been examined during gastrulation and neurulation. In *Xenopus, X-Dll3* is expressed during the neural plate stage along the anterior transverse ridge of the neuroectoderm, which is analogous to the murine anterior neural ridge, and in older embryos is expressed in the olfactory placodes, part of the cement gland, and in clusters of cells in the prosencephalon (Papalopulu and Kintner, 1993) in a pattern resembling that of murine *Dlx5*. In zebrafish, however, *zDlx4* is expressed in a pattern similar to that of murine *Dlx5* at late stages of neurulation, but it is not expressed during gastrulation (Akimenko et al., 1994), suggesting that another (as yet uncharacterized) member of the *zebrafish Dlx* family may mimic the early expression pattern of murine *Dlx5*.

Our findings demonstrate that *Dlx5* is expressed at much earlier stages than has been reported previously for other members of the murine *Dlx* family. Interestingly, the single *Distal-less* homolog in *Amphioxus, AmphiDil*, is expressed during gastrulation by ectodermal cells at the rostralateral margins of the neural plate and by presumptive neural crest cells (Holland et al., 1996). Holland and colleagues have proposed that *AmphiDil* represents an ancestral chordate *Dlx* gene, subserving all of the functions of vertebrate *Dlx* genes, and that these functions have been split among different members of the *Dlx* family during evolution (Holland et al., 1996). This proposal is consistent with the unique early expression of murine *Dlx5* in the anterior neural ridge and, in combination with its evolutionary conservation of expression, suggests a role in early neural patterning.

**REFERENCES**


Couly GF, Le Douarin NM (1987) Mapping of the early neural primordium in quail chick chimeras. II. The prosencephalic neural plate and...


Papalopulu N, Kintner C (1993) Xenopus Distal-less related homeobox genes are expressed in the developing forebrain and are induced by planar signals. Development 117:961–975.


