

The proneural determinant MASH1 regulates forebrain *Dlx1/2* expression through the I12b intergenic enhancer

Luc Poitras, Noël Ghanem, Gary Hatch and Marc Ekker*

Establishment of neuronal networks is an extremely complex process involving the interaction of a diversity of neuronal cells. During mammalian development, these highly organized networks are formed through the differentiation of multipotent neuronal progenitors into multiple neuronal cell lineages. In the developing forebrain of mammals, the combined function of the *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* homeobox genes is necessary for the differentiation of the GABAergic interneurons born in the ventricular and subventricular zones of the ventral telencephalon, as well as for the migration of these neurons to the hippocampus, cerebral cortex and olfactory bulbs. The 437 bp I12b enhancer sequence in the intergenic region of the *Dlx1/2* bigene cluster is involved in the forebrain regulation of *Dlx1/2*. Using DNase I footprinting, we identified six regions of I12b potentially bound by transcription factors. Mutagenesis of each binding site affected the expression of reporter constructs in transgenic mice. However, the effects of impairing protein-DNA interactions were not uniform across the forebrain *Dlx1/2* expression domains, suggesting that distinct regulatory interactions are taking place in the different populations of neuronal precursors. Analyses of protein-DNA interactions provide evidence of a direct role for MASH1 in *Dlx1/2* regulation in the forebrain. DLX proteins play a crucial role in the maintenance of their own expression, as shown by transgenic and co-transfection experiments. These studies suggest that the seemingly continuous domains of *Dlx* gene expression in the telencephalon and diencephalon are in fact the combination of distinct cell populations within which different genetic regulatory interactions take place.

KEY WORDS: Telencephalon, Diencephalon, Interneuron, Homeobox, Enhancer, Distal-less, bHLH, Mouse

INTRODUCTION

The establishment of a diversity of neuronal cell types in the central nervous system requires complex genetic regulatory mechanisms. Through combinatorial codes of transcription factors, neural progenitor cells commit themselves to a specific neuronal cell fate (Briscoe and Ericson, 2001). Recent studies have revealed that the developing vertebrate brain can be subdivided into multiple regions based on genetic interactions between genes involved in neural differentiation (Manuel and Price, 2005; Pasini and Wilkinson, 2002; Trujillo et al., 2005). The fate of neuronal progenitors present in these defined regions is controlled by distinct regional molecular microenvironments that give them their own identity. Thus, characterization of the different genetic interactions controlling the differentiation of the neuronal progenitors will provide new insights in the understanding of brain development and various neurological disorders.

The homeobox genes of the *Dlx* family, which encode homeodomain-containing transcription factors, play an important role during the development of the forebrain (Bendall and Abate-Shen, 2000; Panganiban and Rubenstein, 2002; Zerucha and Ekker, 2000). This family comprises six members in mammals (Stock et al., 1996), organized as three bigene clusters – *Dlx1/2*, *Dlx3/4* and *Dlx5/6* – in an inverted and convergent configuration. Of the six mammalian *Dlx* genes, only *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* are expressed in the telencephalon and in the diencephalon (Bulfone et al., 1993; Robinson et al., 1991; Liu et al., 1997; Eisenstat et al., 1999).

Expression of the *Dlx* genes in the telencephalon is restricted to the differentiating GABAergic (γ -aminobutyric acid releasing) projection neurons and interneurons (Anderson et al., 1997b; Stuehmer et al., 2002a; Stuehmer et al., 2002b). Most of these neurons are born in the ventricular and subventricular zones (VZ and SVZ, respectively) of the lateral and medial ganglionic eminences (LGE and MGE, respectively). Soon after, they undergo a radial or a tangential migration through the SVZ to their final destinations in the piriform, cerebral cortex, hippocampus and olfactory bulb (Anderson et al., 1997a; Chapouton et al., 1999; de Carlos et al., 1996; Lavdas et al., 1999; Sussel et al., 1999; Tamamaki et al., 1997; Tamamaki et al., 1999; Wichterle et al., 1999). *Dlx* genes have highly overlapping but distinct patterns of forebrain expression. As reviewed by Panganiban and Rubenstein, *Dlx2* is mainly expressed in the VZ and SVZ of the MGE and LGE of the telencephalon of E12.5 mouse embryos, where early differentiation occurs (Panganiban and Rubenstein, 2002). *Dlx1* expression is found in all three zones (ventricular, subventricular and mantle). Expression of *Dlx5* and *Dlx6* is confined to the more-differentiated migrating neurons found in the subventricular and mantle zones.

The inactivation of individual *Dlx* genes implicated in forebrain development results in a subtle phenotype (Acampora et al., 1999; Anderson et al., 1997b; Qiu et al., 1997; Qiu et al., 1995; Robledo et al., 2002). For example, mice lacking *Dlx1* display a reduction in calretinin⁺ (also known as calbindin 2 – Mouse Genome Informatics) and somatostatin⁺ interneuron subtypes (Cobos et al., 2005). Mutant mice harboring this subtype-specific loss of interneurons show behavioral and histological signs of epilepsy. Inactivating the function of both *Dlx1* and *Dlx2* results in a massive reduction of GABAergic interneurons of the cerebral cortex, which is mainly due to a lack of tangential migration of the immature interneurons born in the VZ and SVZ of the ventral

Center for Advanced Research in Environmental Genomics (CAREG), Department of Biology, University of Ottawa, 20 Marie Curie, K1N 6N5, Ottawa, Ontario, Canada.

*Author for correspondence (e-mail: Mekker@uottawa.ca)

telencephalon (Anderson et al., 1997a). These mice also show a reduction in the number of interneurons in the striatal and in the olfactory bulb.

Interestingly, in *Dlx1 Dlx2* double mutants, the expression of the *Dlx5* and *Dlx6* genes is reduced. The DLX2 protein has been shown to bind to the I56i sequence, an enhancer found in the intergenic region of the *Dlx5/6* bigene cluster (Zerucha et al., 2000). This finding was supported by chromatin immunoprecipitation studies (Zhou et al., 2004). Phylogenetic footprinting and transgenic analyses have also revealed the existence of at least two cis-acting regulatory elements, called I12a and I12b, in the intergenic region separating *Dlx1* and *Dlx2* (Ghanem et al., 2003; Park et al., 2004). We also found one conserved cis-acting regulatory element, URE2, in the 5' flanking region of *Dlx1* (Hamilton et al., 2005).

Of the cis-acting regulatory elements identified thus far in the *Dlx1/2* and *Dlx5/6* bigene clusters, four – URE2, I12b, I56i and I56ii – contribute to *Dlx* gene expression in the forebrain. Analysis of reporter gene constructs in transgenic mice indicated that the activity of the I56i, I12b and URE2 enhancers was very similar when examined on whole-mount preparations (Ghanem et al., 2003) (N.G. and M.E., unpublished). However, in-depth analysis of sections from these transgenic embryos at different stages of development revealed subtle differences in the expression of the transgenes driven by each of the cis-acting elements (N.G., M. Yu, J. Long, G.H., J. L. R. Rubenstein and M.E., unpublished).

Despite their overlapping activities, the various forebrain enhancers are highly divergent in their sequence (Ghanem et al., 2003; Zerucha et al., 2000), suggesting that they could respond to distinct trans-acting factors. In an attempt to characterize the genetic pathways responsible for the expression of the *Dlx* gene family in the forebrain, we performed DNase I footprinting analysis of I12b. Mutations were introduced into the I12b enhancer at each putative forebrain transcription factor-binding site identified by DNase I footprinting and these mutant enhancers were used in a transgenic assay. Our results suggest that *Dlx1/2* expression is auto- or cross-regulated by DLX proteins in the telencephalon and diencephalon. We also provide evidence for the regulation of *Dlx1/2* expression by transcription factors such as MASH1 (also known as ASCL1 – Mouse Genome Informatics), MEIS1 and MEIS2 (also known as MRG1 – Mouse Genome Informatics).

MATERIALS AND METHODS

Nuclear extract preparation

Nuclear extracts were prepared from the telencephalon of E13.5 mouse embryos (1 ml of tissue) as described by Sambrook and Russell (Sambrook and Russell, 2001). In addition, the protein extract was dialyzed against the resuspension buffer using a Spectra/Por dialysis cuvette (VWR) overnight at 4°C. Aliquots of 50 µL were frozen in liquid nitrogen and stored at –80°C.

DNase I footprinting analysis

To obtain an optimal footprinting resolution, the I12b enhancer was divided into two overlapping fragments (nucleotides 1–292 and 259–437; see Fig. 2B). The primers used to amplify the two fragments were: mI12b-1 (5'-GGAATTCGCGTACAGCTGCAAACC-3') and mI12b-292 (5'-CGG-TACCTACCGGAGAAATTGCAGAG-3'); mI12b-259 (5'-GGAATTCATAGGTGCGAGCTGCCA-3') and mI12b-437 (5'-CGGTACCAAGTGGGAAAGGTTGGG-3'); restriction sites (underlined) were incorporated at the 5' (*EcoRI*) and 3' (*KpnI*) ends of each fragment. PCR fragments were cloned into the pDrive vector (Qiagen). Each fragment was recovered by digestion with *EcoRI* and *KpnI* followed by gel purification (QIAquick Gel Extraction Kit, Qiagen). Directional labeling of each fragment was performed by 5' end-fill using the large fragment of DNA polymerase I (Invitrogen). Labeled fragments were then purified from low melting point agarose gels using β-agarase (New England Biolabs).

Binding reactions (50 µL) comprised 25 µL of binding buffer [20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 20 mM HEPES pH 7.9, 4% poly(vinyl alcohol)], 0.5 µL of 1 mg/ml poly(dAdT) (Sigma), 2–3 ng of end-labeled DNA fragment, nuclear extract and 60 mM KCl. The reaction mixtures were incubated on ice for 20 minutes. One volume of 10 mM MgCl₂ 5 mM CaCl₂ was added. Each reaction mixture was then treated with increasing amounts of DNase I, from 0.01 to 0.1 Kunitz units (Worthington) for 120 seconds and stopped by adding 100 µL STOP solution (1% sodium dodecyl sulphate, 200 mM NaCl, 20 mM EDTA pH 8.0, 40 mg/mL tRNA). Reactions were extracted twice with one volume of phenol-chloroform, once with one volume of chloroform and precipitated with two volumes of ethanol at –80°C for 20 minutes. Reactions were centrifuged for 15 minutes at 10,000 g. DNA pellets were washed with 80% ethanol and dried in a vacuum desiccator. Pellets were resuspended in 10 µL of loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). Reactions were loaded on a 6% (19:1 acrylamide:bis-acrylamide) polyacrylamide sequencing gel containing 7 M urea and 1×TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA). The gel was run at 80 volts in 1×TBE and dried. Radioactive bands were visualized by phosphorimaging (Molecular Dynamics). Guanine+adenine chemical sequencing was performed as previously described (Maxam and Gilbert, 1980).

The sequences of protected regions were compared with a library of matrix descriptions for transcription factor-binding sites using MatInspector software (Genomatix).

Co-transfection experiments

A zebrafish *dlx2a* cDNA (845 bp) encompassing the full-length coding sequence was PCR-amplified and cloned into the *EcoRI* site of the pTL2 or pcDNA3/HisB expression vector (Zerucha et al., 2000). A *Dlx2a* homeodomain mutant was obtained by changing essential amino acids of the third loop of the homeodomain: Trp170, Phe171, Gln172, Asn173, Arg174 and Arg175 were changed to glycine using overlapping PCR.

The mouse I12b enhancer was PCR-amplified and inserted upstream of the *thymidine kinase* (*tk*) minimal promoter driving expression of the *chloramphenicol acetyltransferase* (CAT) gene (pBLCAT2 vector) (Luckow and Schutz, 1987). zI56i-pBLCAT2 (Zerucha et al., 2000) was used as positive control. MASH1 and E47-pCDNA3 constructs were kindly provided by François Guillemot (National Institute for Medical Research, London, UK). Cell culture, transfection and the CAT assay were carried out as previously described (Zerucha et al., 2000). Experiments were performed in duplicate and repeated a minimum of three times.

Mutagenesis

Mutant enhancers were generated by PCR using overlapping fragments. A mix (1:3) of Taq and Pfx DNA polymerases (Invitrogen) was used to avoid unwanted mutations. A first fragment was amplified from the I12b enhancer (I12b-pBluescript®KS) using oligonucleotides I12b-437 (5'-CGGT-ACCAGTGAGGGAAAGGTTGGG-3') and T7 primer (annealing site in pBluescript vector). A second overlapping fragment was generated with an oligonucleotide containing the second mutation and an adaptor sequence corresponding to the SP6 promoter (mutations are underlined): Mut FP1, 5'-CTGAAATTACTGTCTAGATGTTGTCTTTGA-3'; Mut FP2, 5'-AAC-TGCATTAGAGGAGCTCAACCTGAAATTA-3'; Mut FP3, 5'-ATGGG-GAAACTGCTCTAGAAATAAATAAACCTG-3'; Mut FP4, 5'-GCGAAA-AAATTGCTCATCTAGACCAGAGAGATGGG-3'; Mut FP5, 5'-ATAC-TCTAGAAATGGAGCTCGCGGGCTCCGGTA-3'; and Mut FP6, 5'-GC-TAAGTCTGTCTTCTAGACTTCGCTGCTGTGC-3'.

After purification from agarose gels, the two fragments were used as template for a final PCR with SP6 and T7 primers. Finally, the mutant enhancer fragments were purified from agarose gels, TA-cloned and sequenced.

Transgenic experiments

The I12b mutant enhancers were subcloned into p1230 vector (Yee and Rigby, 1993) that contains a human β-globin minimal promoter and *lacZ* reporter cassette. Transgenic animals were produced and analyzed as previously described (Zerucha et al., 2000). For each construct, at least three

primary transgenic embryos were obtained. Embryos shown in Figs 4, 5 and 6 are representative transgenic animals of two or more independent integration events.

Gel mobility shift and supershift assays

Gel mobility shift assays were performed using double-stranded oligonucleotides corresponding to the identified putative DLX/MSX/NKX (FP1) and MASH1 (FP5) binding sites and also to a mutant version of FP5: mI12b-FP1.for, 5'-ACACAACATAATTACAGTAA-3' and mI12b-FP1.rev, 5'-TTACTGTAATTATGTTGTGT-3'; mI12b-FP5.for, 5'-GCCCGCAA-ATTGCTTGCAGGTGTATGG-3' and mI12b-FP5.rev, 5'-CCATACA-CCTGCAAGCAATTTGCGGGC-3'; mI12b-FP5mut.for, 5'-GCCCCG-CGAGCTCCATTCTAGAGTATGG-3' and mI12b-FP5mut.rev, 5'-CC-ATACTAGAATGGAGCTCGCGGGC-3'.

The complementary oligonucleotides were annealed and end-labeled using [³²P]γ-ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs). Electromobility shift assay (EMSA) reactions were performed by incubating 1 ng of labeled DNA with recombinant MASH1 and E47 proteins (TNT Quick Coupled Transcription/Translation Kit, Promega) or GST-Dlx2a in reaction buffer (7 mM Tris pH 7.5, 81 mM NaCl, 2.75 mM dithiothreitol, 5 mM MgCl₂, 0.05% NP-40, 1 mg/mL bovine serum albumin, 25 μg/mL poly(dIdC), 10% glycerol) at 0°C for 30 minutes. Reactions were loaded on a polyacrylamide gel (6% acrylamide, 0.16% bis-acrylamide) in 0.5×TBE. The gel was run at 80 volts in 0.5×TBE and dried. Radioactive bands were visualized by phosphorimaging (Molecular Dynamics).

For supershift assays, nuclear extracts were preincubated overnight at 4°C with increasing amounts (100 ng, 1 μg, 3 μg) of anti-MASH1 and anti-MEIS1 antibodies (sc-13222X and sc-25412X, Santa Cruz Biotechnology).

Chromatin immunoprecipitation

The LGE and MGE were dissected from E11.5 mouse telencephalon. Chromatin extraction and immunoprecipitation were performed essentially as previously described (Mac et al., 2000) with a few modifications. First, tissues were disaggregated using a dounce homogenizer and were resuspended in cell lysis buffer (50 mM Hepes pH 8, 75 mM KCl, 0.5% NP-40, plus protease inhibitors) prior to nuclei release. Second, protein-A sepharose (Sigma, P3391) was used for the immunoprecipitation. Third, chromatin was precipitated using 1 μg of anti-MASH1 antibody (Santa Cruz). Washing and elution were carried out as previously described (Boyd and Farnham, 1997). After elution, reactions were incubated at 65°C for 4 hours to reverse the DNA-protein cross-link, then treated with proteinase K (Invitrogen), extracted with phenol-chloroform and precipitated with ethanol. DNA was collected by centrifugation and resuspended in 100 μL of H₂O and analyzed by PCR. The I12b and Hes6 enhancers were amplified using the following primers: mI12b-ChIP.For (5'-GAGGGTCAGCAT-CATTTAC-3') and mI12b-ChIP.Rev (5'-GCAAGCTGTGGACCATAG-3'); mHes6-ChIP.For (5'-GCCGAGGCTGCTGTCTG-3') and mHes6-ChIP.Rev (5'-TGGGCGTCTGGCCGACA-3'). After 28-30 cycles, PCR products were analyzed on ethidium bromide-stained agarose gels.

Sequence analysis

The mouse and human *Hes6* sequences were obtained from public databases (<http://www.ensembl.org>): mouse *Hes6*, Ensembl gene ID ENSMUSG00000067071; human *HES6*, Ensembl gene ID ENSG00000144485. Pairwise sequence alignment was performed with PipMaker (available at <http://bio.cse.psu.edu/pipmaker/>).

RESULTS

Forebrain transcription factors bind to the intergenic I12b enhancer

The I12b intergenic enhancer (Fig. 1A) was previously identified in a phylogenetic footprinting analysis of the *Dlx1/2* intergenic region from five different vertebrates (Ghanem et al., 2003; Zerucha et al., 2000). Initial characterization of the I12b enhancer activity in transgenic mice indicated that this enhancer targets reporter gene expression exclusively to the forebrain (Ghanem et al., 2003) (Fig. 1B). Enhancer activity of I12b is first detectable at

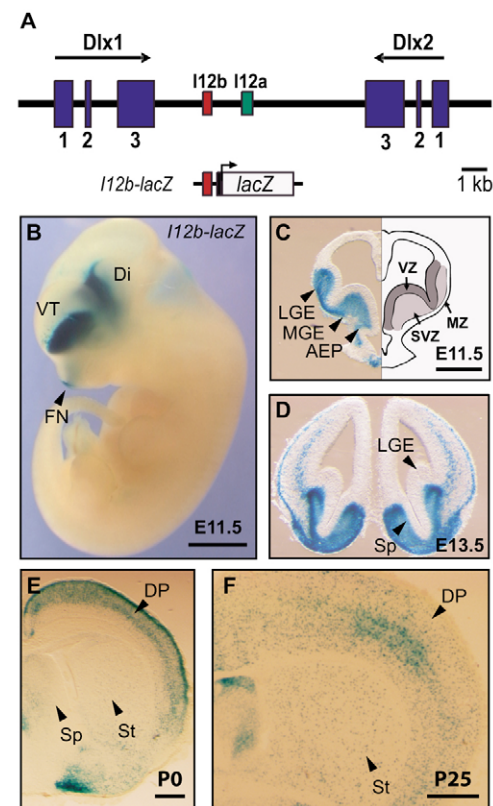


Fig. 1. The I12b intergenic enhancer targets the expression of a *lacZ* reporter construct to the developing mouse forebrain.

(A) The convergently transcribed (arrows) mouse *Dlx1* and *Dlx2* genes. Exons are shown as blue boxes. Two enhancers, I12a and I12b, are located in the intergenic region. The reporter construct used for the production of mouse transgenic lines is shown beneath. (B) The I12b enhancer targets expression of the reporter gene to the ventral telencephalon (VT), the diencephalon (Di) and the frontonasal prominence (FN), in an E11.5 mouse embryo (whole-mount preparation). (C-F) Transverse sections (half a section is shown in E,F) through the telencephalon of E11.5 (C) and E13.5 (D) transgenic embryos and rostral brain of P0 (E) and P25 (F) transgenic mice. Scale bars: 1 mm in B; 500 μm in C-F.

E10 in the diencephalon, followed by expression in the basal telencephalon at E10.5 (data not shown). Transverse sections of telencephalon at E11.5 revealed *lacZ* expression in the subventricular and mantle zones of the LGE and MGE, but also in the anterior entopeduncular area (AEP), the preoptic area and the suprachiasmatic band (SCB). (Fig. 1C) (N.G. and M.E., unpublished). The *I12b-lacZ* reporter transgene persisted in the SVZ of the LGE and MGE from E12.5 to E15.5 and reporter gene expression was found in cells migrating to the dorsal pallium (Fig. 1D). Expression of *I12b-lacZ* was still detected in the neocortex after birth (P0) and at P25 (in the somatosensory cortex), the latest stage tested (Fig. 1E-G). However, expression of the transgene in the septum and the striatum was much reduced at this stage (Fig. 1E-G, Sp and St). At P25, expression of the transgene is also observed in all layers of the olfactory bulb containing GABAergic neurons (data not shown).

To better define the important sequence elements within I12b and as a first step in the identification of trans-acting factors binding to this enhancer, we performed DNase I footprinting experiments using

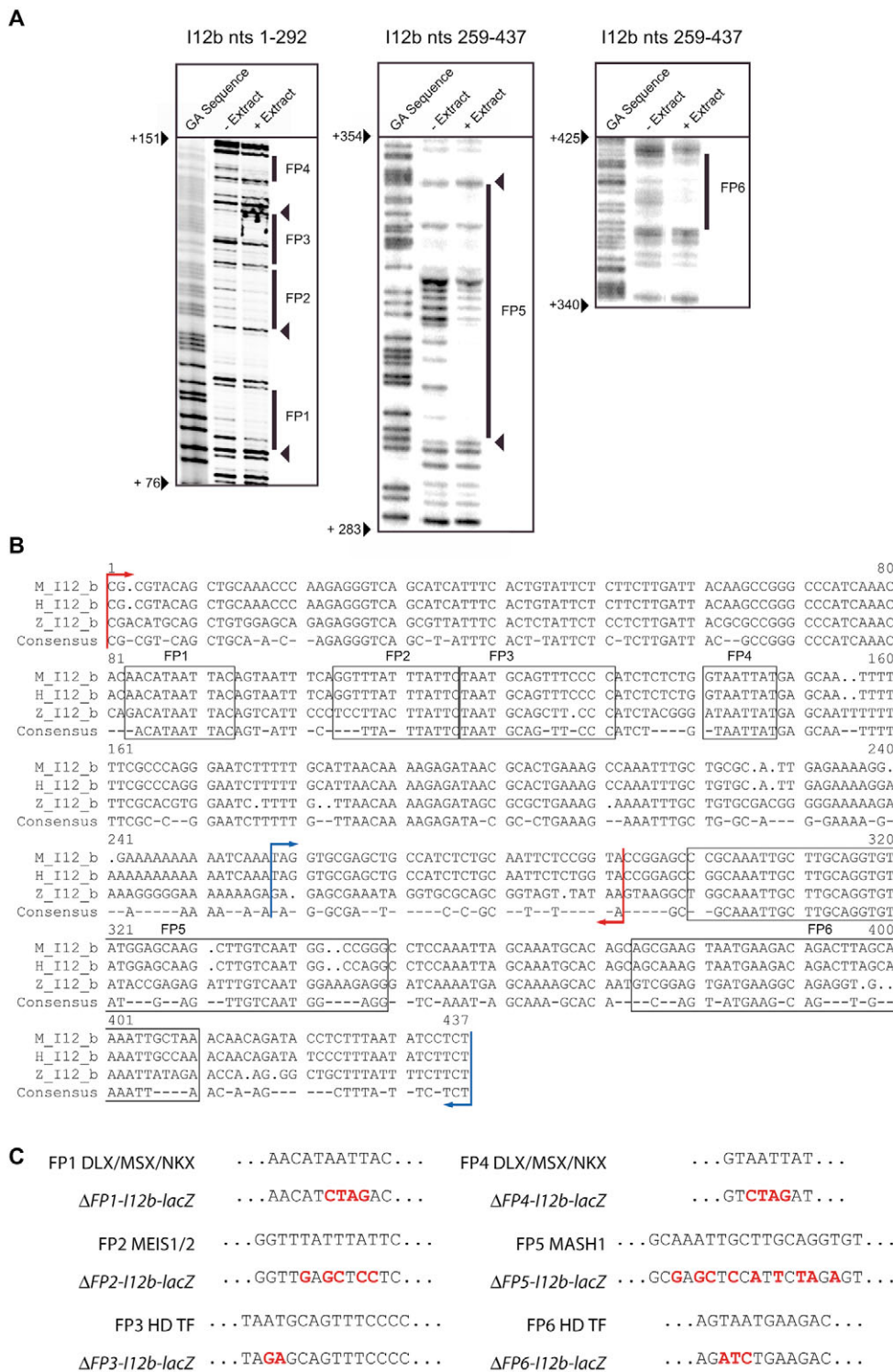


Fig. 2. Trans-acting factors from E13.5 mouse embryonic brain bind to the I12b enhancer.

(A) DNase I footprinting analysis of two overlapping DNA fragments of the I12b enhancer (nt 1-292 and 259-437) using a nuclear protein extract from E13.5 mouse forebrain. Six regions of the enhancer (FP1 to FP6) are bound by nuclear proteins. Protected areas (footprints) are represented by thick lines and hypersensitive sites by arrowheads. Numbers to the left of each panel indicate relative positions on the enhancer. (B) Protein-DNA interactions mapped (using GA Sequence in A) on the mouse I12b enhancer sequence. The protected areas identified by DNase I footprinting (FP1-FP6) are boxed. The two fragments used for the footprinting analysis are indicated by colored arrows (red arrows, nt 1 to 292; blue arrows, nt 259-437). Sequences of the human (H) and zebrafish (Z) I12b sequences are compared with mouse (M). Protein-DNA interactions are mostly located in regions with a high degree of sequence similarity. (C) Reporter constructs containing mutations (red) of the FP sites in the I12b enhancer.

nuclear extracts from E13.5 mouse embryonic forebrain. Two overlapping DNA fragments from the I12b enhancer (Fig. 2B) were used. DNase I footprinting revealed six protein-DNA interactions (Fig. 2A, FP1 to FP6). The sequence of these protected regions (Fig. 2B) were compared with a library of matrix descriptions for transcription factor-binding sites using MatInspector software. The position of the protected area on the enhancer sequence and relevance to

telencephalic development were used as the main criteria to discriminate between the 87 candidate transcription factor-binding sites found by MatInspector.

Using this selection process, FP1 and FP4, which both contain the core sequence 5'-ATAATTA-3', were identified as possible binding sites for DLX or MSX homeodomain proteins, or as low affinity binding sites for the NKX2.5 (also known as NKX2-5 – Mouse

Genome Informatics) homeodomain protein. Previous studies showed that DLX2 is able to bind in vitro to two such binding sites present in the *Dlx5/6* intergenic enhancer I56i (Zerucha et al., 2000; Zhou et al., 2004). To test the hypothesis that DLX2 can bind and activate transcription through the FP1 and FP4 binding sites present in I12b, a transfection reporter assay was performed. P19 murine embryonic carcinoma cells were co-transfected with a reporter construct in which the CAT reporter gene was placed under the control of the mouse I12b enhancer (mI12b-pBLCAT2). A plasmid expressing the zebrafish Dlx2a protein (Dlx2-pTL2) was co-transfected. In the presence of Dlx2a, an 11-fold increase in relative CAT activity was observed (Fig. 3A). Similar results were also obtained when vectors expressing other zebrafish Dlx proteins (Dlx1a, Dlx3b, Dlx5a, Dlx6a) were used in this assay (data not shown). This activation is dependent on the DLX homeodomain because transfection of a Dlx2a homeodomain mutant (W170G, F171G, Q172G, N173G, R174G and R175G) did not show any transcriptional activation in the reporter assay (Fig. 3B). Furthermore, recombinant Dlx2a synthesized in vitro was shown to bind to a double-stranded oligonucleotide corresponding to FP4 in an EMSA (see Fig. S1 in the supplementary material). Taken together, our data show that DLX proteins are able to bind to and activate transcription from the I12b enhancer.

In addition to the two DLX/MSX/NKX2.5-binding sites (FP1 and FP4), a potential binding site for another homeodomain-containing transcription factor (referred to here as HD TF) was found in FP3, located between the two DLX/MSX/NKX2.5 sites. FP2 was identified as a possible binding site for MEIS1 (myeloid ecotropic viral integration site 1). MEIS transcription factors encode homeodomain proteins of the TALE (Three Amino acids Loop Extension) class. *Meis1* and *Meis2* are expressed in the developing telencephalon from E10.5 until birth, with patterns that partially overlap those of the *Dlx1/2* genes (Toresson et al., 1999).

Analysis of the sequences surrounding FP5 suggests the presence of an E-box element – a sequence recognized by a subset of basic helix-loop-helix (bHLH) proteins including the products of the neurogenin (Neurog), NeuroD, Mash (Ascl), Olig and E (Tcf) gene families (reviewed by Ross et al., 2003). Three of these bHLH factors have been shown to be expressed in the telencephalon: neurogenin 1 (*Neurog1*), neurogenin 2 (*Neurog2*) and the mammalian achaete-scute homolog 1, *Mash1* (*Ascl1*) (Guillemot and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996). *Mash1* controls the transition of the multipotent cortical progenitors from proliferation to neurogenesis (Cau et al., 1997; Guillemot et al., 1993; Hirsch et al., 1998; Sommer et al., 1995). *Mash1* is expressed in the proliferative zone of the LGE and MGE of the telencephalon. Many studies provide evidence suggesting that this proneural factor is a potential upstream regulator of *Dlx2*, but so far only genetic evidence supports this hypothesis (Casarosa et al., 1999; Fode et al., 2000; Horton et al., 1999; Letinic et al., 2002; Porteus et al., 1994; Yun et al., 2002). First, *Mash1* and *Dlx1/2* show overlapping patterns of expression in the VZ and SVZ of the ventral telencephalon (Porteus et al., 1994). Second, Horton and collaborators have shown that mice lacking *Mash1* have a reduced number of cells expressing Dlx genes in the SVZ of the ganglionic eminence at E12.5 (Horton et al., 1999). Third, ectopic expression of *Mash1* leads to an upregulation of *Dlx1/2* in the neocortical neurons (Fode et al., 2000). However, evidence of a direct regulation of *Dlx1/2* by the MASH1 protein has yet to be demonstrated at the molecular level.

The MatInspector software identified FP6 as possible binding site for yet another homeodomain-containing transcription factor. Sequence comparisons between different species show that the FP6 homeobox (TAAT) is present in human and mouse, but not in the zebrafish I12b sequence (Fig. 2B).

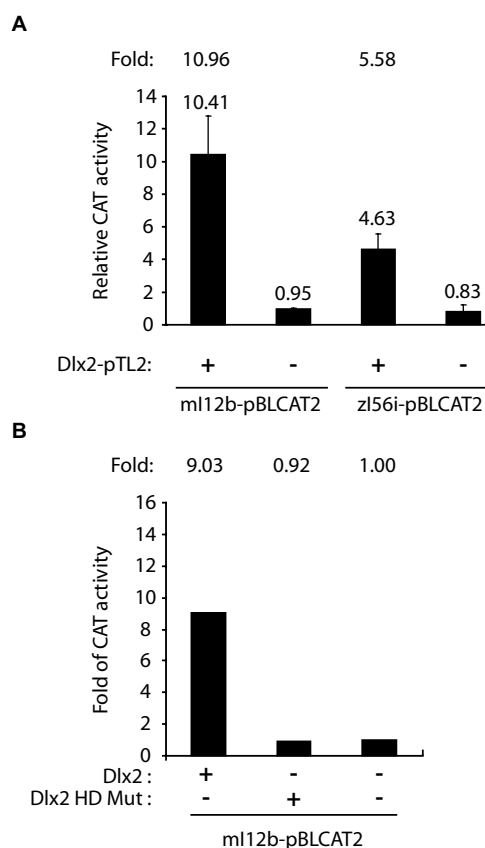


Fig. 3. Dlx2a can bind and activate transcription through the intergenic I12b enhancer. (A) In a transient transfection assay, zebrafish Dlx2a upregulates transcription of a CAT reporter construct containing the I12b enhancer (mI12b-pBLCAT2) by a factor of 11. By comparison, expression of a CAT reporter gene under control of the I56i enhancer (Zerucha et al., 2000) was activated 5.6-fold in the presence of Dlx2a. (B) The disruption of the Dlx2a homeodomain completely abolishes this activation. In the presence of zebrafish Dlx2a, a 9.03-fold increase in CAT activity was observed, whereas the Dlx2a homeodomain mutant showed only a basal level of CAT activity. Values shown represent the mean of relative CAT activity obtained from two to three independent experiments \pm s.e.m.

Mutational analysis of putative binding sites in I12b

To investigate the functionality of the putative transcription factor-binding sites found in the I12b enhancer, and to understand their relative contributions to Dlx gene regulation in the forebrain, we tested the effect of mutating these sites in a transgene reporter assay. Mutations affecting 2-8 nucleotides were introduced for each putative binding site (Fig. 2C). Mutated enhancers were subcloned upstream of a β -globin minimal promoter-*lacZ* cassette (Yee and Rigby, 1993). These constructs were injected into fertilized mouse eggs to produce transgenic animals. Staining of E11.5 primary transgenic mouse embryos revealed that mutagenesis of each of the DLX/MSX/NKX2.5 (FP1 and FP4) or MASH1 (FP5) binding sites resulted in a reduction of the *lacZ* expression in the ventral telencephalon (Fig. 4, compare A,B,E,F). Transverse sections through the brain of the transgenic embryos indicated that the mutations impair enhancer activity in both the LGE and MGE of the ventral telencephalon (Fig. 5, compare C,D,I-L with A,B). The effects of the mutations appeared to be stronger in the more-rostral cells (Figs 4, 5).

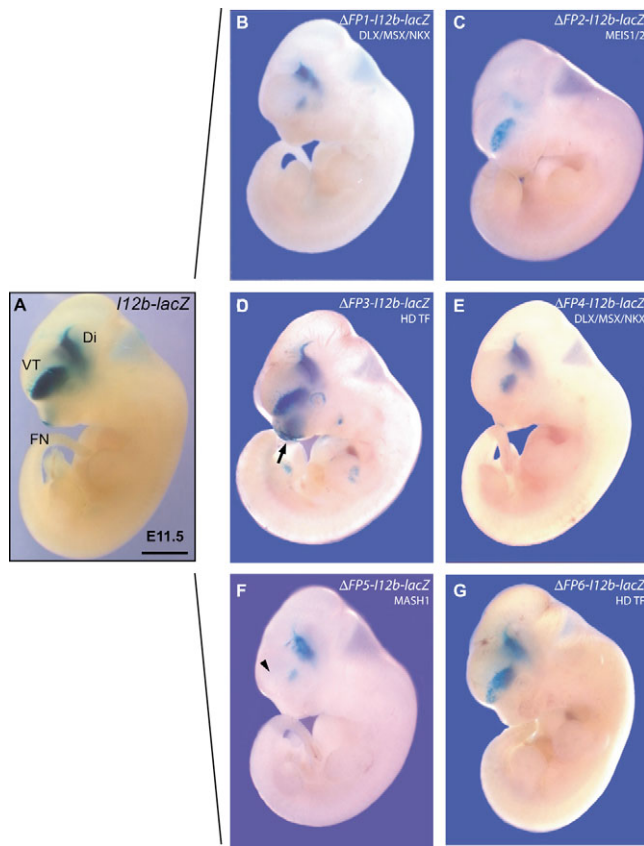


Fig. 4. Effect of mutations in FP sites on I12b enhancer activity.

Lateral views of E11.5 transgenic mouse embryos showing expression of the *lacZ* reporter in the forebrain. Wild-type (*I12b-lacZ*) enhancer (A) and enhancers with mutagenized (B,E) DLX/MSX/NKX2.5 sites, (C) MEIS1 site, (D) FP3 homeodomain-binding site, (F) MASH1 site, and (G) FP6 homeodomain site. VT, ventral telencephalon; Di, diencephalon. FN, frontonasal prominence. Scale bar: 1 mm.

In embryos carrying the $\Delta FP1-I12b-lacZ$ reporter transgene (affecting one of the two DLX/MSX/NKX2.5 sites), expression was observed in only a subset of cells located at the border between the ventral MGE and AEP (Fig. 5D). Expression was also observed in the SCB in the diencephalon (Fig. 5D, arrow). Reporter gene expression in primary transgenic embryos carrying the $\Delta FP4-I12b-lacZ$ transgene (affecting the second DLX-binding site, Fig. 5J) or the $\Delta FP5-I12b-lacZ$ transgene (affecting the MASH1-binding site, Fig. 5L) was scattered throughout the superficial mantle zone of the MGE and intermediate zone and in the AEP. All three mutations abolished β -galactosidase activity in the rostral LGE (Fig. 4A,B,E,F and Fig. 5A,C,I,K).

At E13.5, primary transgenic embryos carrying $\Delta FP1-I12b-lacZ$ or $\Delta FP4-I12b-lacZ$ transgenes showed an overall reduction in reporter gene expression in the LGE and MGE and reduced expression in interneurons migrating to the cortex (data not shown). Interestingly, E13.5 transgenic embryos carrying $\Delta FP2$ (MEIS1/2), $\Delta FP5$ (MASH1) or $\Delta FP6$ (HD TF) *-I12b-lacZ* transgenes showed full restoration of the expression obtained with the wild-type mI12b enhancer construct (*I12b-lacZ*), suggesting that these sites play a role in establishing *Dlx1/2* expression, whereas DLX-binding sites (FP1 and FP4) are responsible for maintaining this expression (data not shown).

In the diencephalon, *lacZ* expression driven by $\Delta FP1-I12b-lacZ$ (Fig. 6B) was reduced compared with that driven by the wild-type I12b sequence (Fig. 6A). This reduction occurred in both domains of expression, the prethalamus and the SCB. In the $\Delta FP4-I12b-lacZ$ and $\Delta FP5-I12b-lacZ$ transgenic embryos, *lacZ* expression was absent in the prethalamus and reduced in the SCB as compared with the wild-type enhancer (Fig. 6E,F, arrows).

Transgenic embryos produced with the $\Delta FP2-I12b-lacZ$ construct displayed a slight reduction in *lacZ* expression in the ventral telencephalon as compared with wild-type *I12b-lacZ*, and an almost complete abolition of reporter expression in the diencephalon (Fig. 4C). Transverse sections of the ventral telencephalon revealed that *lacZ* expression was weaker in the MGE and AEP and was absent in the LGE and preoptic area (Fig. 5E,F). Expression in the MGE was concentrated at the intermediate zone. An overall reduction in *lacZ* staining was found in the diencephalon (Fig. 6C). Reduction in expression mainly occurred in the prethalamus (Fig. 6C, arrow). However, the effect of FP2 mutation on the targeted activity of the I12b enhancer to the diencephalon was variable, as two independently generated $\Delta FP2-I12b-lacZ$ transgenic embryos showed different levels of reduction (Fig. 4C, Fig. 6C).

Mutagenesis of FP3 resulted in a relative increase in reporter transgene expression in all domains compared with the wild-type *I12b-lacZ* transgene (Fig. 4D, Fig. 5G,H, Fig. 6D). For example, the faint frontonasal prominence staining present in the control embryo (Fig. 4A) was greatly increased following mutagenesis of the FP3 site (Fig. 4D, arrow). This increase in staining was not the result of an integration effect, as three $\Delta FP3-I12b-lacZ$ transgenic embryos obtained from two separate experiments gave similar staining. This result suggests that the FP3 site is probably recognized by a homeodomain-containing repressor.

Primary transgenic embryos carrying the $\Delta FP6-I12b-lacZ$ construct showed a reduction in *lacZ* staining in particular cells (Fig. 4G, Fig. 5M,N, Fig. 6G). This reduction gave a granular aspect to the staining as compared with the wild-type *I12b-lacZ* embryos (Fig. 5B,N). Since *Dlx1* and *Dlx2* are expressed in at least four different subtypes of interneurons, the granular staining could be the result of a subtype-specific loss in which a mutation in FP6 affects one or more subtypes of interneurons. The same type of granular staining was observed in the diencephalon (Fig. 6G). Although this granular expression could correspond to specific progenitor populations, the paucity of specific markers at this early stage makes it difficult to confirm such a correlation.

A functional bHLH-binding site is present in the I12b enhancer

We found an E-box sequence in the I12b enhancer (FP5). To test the hypothesis that FP5 is a functional E-box and to determine if MASH1 is able to bind to that sequence and activate transcription through this site, we performed co-transfection and EMSAs. A plasmid expressing *Mash1* was transfected with a mouse I12b enhancer reporter construct (mI12b-pBLCAT2) into P19 murine embryonic carcinoma cells. In the presence of MASH1, a 4-fold increase in relative CAT activity was observed (Fig. 7A).

We performed an EMSA using a 27 bp double-stranded oligonucleotide corresponding to the FP5 sequence, which was incubated with recombinant MASH1 protein and its co-factor E47 (also known as TCFE2A – Mouse Genome Informatics) produced by in vitro transcription/translation. Up to three protein-DNA complexes were observed on the EMSA (Fig. 7B, lanes 2-4, arrowheads). To further prove that the E-box present in I12b is a functional binding site, we used an oligonucleotide that lacked the

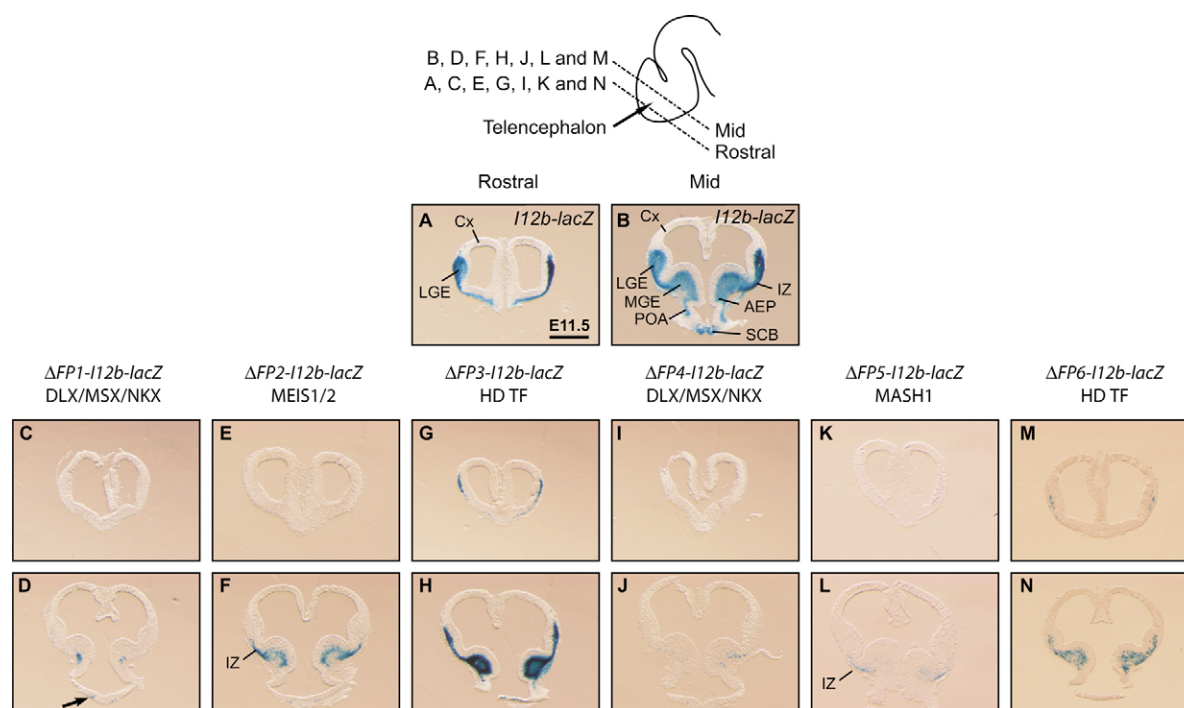


Fig. 5. Impaired I12b enhancer activity in the telencephalon following mutagenesis of protein-binding sites. Transverse sections through the telencephalon of E11.5 transgenic mouse embryos (such as those shown in Fig. 4). The plane of section is schematized. Two series of transverse sections corresponding to the rostral (A,C,E,G,I,K,M) and middle (B,D,F,H,J,L,N) regions of the telencephalon are shown. Expression obtained with the wild-type I12b enhancer (A,B) and following mutagenesis of (C,D) the first DLX/MSX/NKX2.5-binding site, (E,F) MEIS1 site, (G,H) FP3 site, (I,J) second DLX/MSX/NKX2.5 site, (K,L) MASH1 site, and (M,N) FP6 homeodomain site. Cx, cortex; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; AEP, anterior entopeduncular area; POA, preoptic area; VT, ventral telencephalon; SCB, suprachiasmatic band (a diencephalic structure). Scale bar: 500 μ m.

site as a result of introducing the same mutation that was used in the transgenic experiments shown in Figs 4-6. Lower motility complexes were not obtained with the mutant fragment in the presence of MASH1 and E47 in vitro translation extracts (Fig. 7B, compare lanes 6-8 with lanes 2-4). In a supershift assay, MASH1-E47-FP5 complex was completely eliminated by preincubation with an anti-MASH1 antibody, whereas in the control experiment using a non-specific antibody these complexes remained intact (Fig. 7C, compare lanes 7-9 with lanes 3-5, arrowheads).

MASH1 has been shown to form heterodimers with ubiquitously expressed E12, E47 and HES-like proteins, all of which have the ability to bind to DNA (Miyoshi et al., 2004; Murre et al., 1989). In our experiments, the recombinant MASH1 bound to FP5 with almost equal efficiency in the absence or presence of E47 (data not shown). Thus, MASH1 is likely to form a heterodimer preferentially with a co-factor present in the in vitro translation extract whose identity remains to be determined.

To determine if MASH1 is able to bind to the I12b enhancer in vivo, we developed a chromatin immunoprecipitation assay (ChIP) for this protein. To our knowledge, this procedure has not previously been used for MASH1 and little is known about MASH1-binding sites in vivo. Therefore, we also tested our ChIP assay on an independent site. We used a conserved sequence that has been suggested as the site of *Hes6* gene regulation by MASH1 in mouse prostate cells (Hu et al., 2004). Interestingly, *Hes6* is also expressed in the developing telencephalon and binding of MASH1 to *Hes6* regulatory element(s) should be detectable in the chromatin we isolated in our ChIP analysis of MASH1 with I12b. Phylogenetic footprinting analysis of the *Hes6*

promoter region allowed us to identify a 217 bp conserved element (*Hes6* enh) located 671 bp from the mouse *Hes6* exon1 (see Fig. S2A in the supplementary material). Three conserved E-box sequences were found in this element (see Fig. S2B in the supplementary material). In a ChIP assay performed on fixed chromatin isolated from the LGE and MGE of E11.5 mouse embryos, both the I12b enhancer and the conserved *Hes6* enh sequence were precipitated with the anti-MASH1 antibody (Fig. 8). Thus, MASH1 is able to bind, in vitro and in vivo, to the I12b enhancer.

DISCUSSION

The I12b enhancer, located in the *Dlx1/2* intergenic region, is at least in part responsible for the expression of these genes in the differentiating GABAergic projection neurons and interneurons. DNase I footprinting suggested that at least six forebrain transcription factors bind to I12b. Mutagenesis of the identified putative binding sites revealed that all were important for I12b enhancer activity, but that individual FP sites often had different impacts, both qualitatively and quantitatively. Using EMSA and ChIP, we demonstrated that the neuronal determinant MASH1 binds to an E-box present at the FP5 site of the I12b enhancer and is, therefore, likely to play a direct role in *Dlx1/2* gene regulation.

DLX proteins as factors necessary for optimal I12b enhancer activity

Two sequences in I12b, FP1 and FP4, are potential binding sites for DLX and MSX proteins. They could also correspond to a low affinity NKX2.5-binding site (5'-ATAATTA-3'). FP1 and FP4

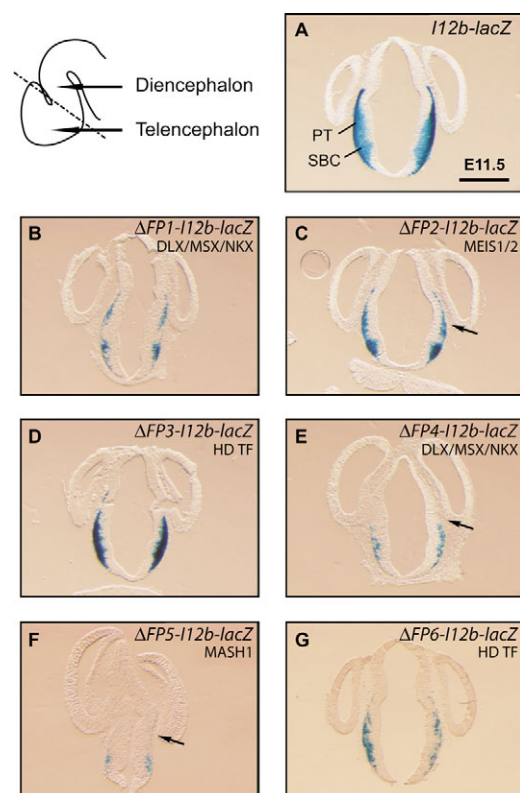


Fig. 6. Mutagenesis of protein-binding sites on the I12b enhancer affects its activity in the diencephalon. Transverse sections through the diencephalon, at the caudal limit of the telencephalon, in E11.5 transgenic mouse embryos (such as those shown in Fig. 4). The plane of section is schematized. Activity of the wild-type I12b enhancer (A) and following mutagenesis of (B) the first DLX/MSX/NKX2.5 site, (C) MEIS1 site, (D) FP3 site, (E) second DLX/MSX/NKX2.5 site, (F) MASH1 site, and (G) FP6 site. Scale bar: 500 μ m.

completely fulfill the DLX/MSX consensus binding site requirement, G-A/C-TAATT-A/G-G/C (Feledy et al., 1999). Co-transfection assays provided evidence that DLX proteins activate transcription from the I12b enhancer and that this is dependent on the presence of the homeodomain (Fig. 3). DLXIN-1 (MAGED1 – Mouse Genome Informatics), GRIP and MSX proteins (Masuda et al., 2001; Yu et al., 2001; Zhang et al., 1997) have been proposed as interacting partners for DLX proteins. For example, it has been shown that DLX and MSX proteins could form homo- and heterodimers (Zhang et al., 1997). Msx genes are expressed in the most dorsal parts of the forebrain where the Dlx genes are not expressed, suggesting that these transcription factors are unlikely to play a role in Dlx gene regulation. We cannot exclude co-expression of Msx and Dlx genes at the neural plate stage in the neural ridge.

Potential binding proteins for FP1 and FP4 also include NKX2.5 (Chen and Schwartz, 1995). Transcripts of the *Nkx2.5* gene are found in early cardiac cell progenitors, but not in the developing brain (Komuro and Izumo, 1993). However, another member of the Nkx2 family, *Nkx2.1*, was shown to play a role in the development of the ventral telencephalon. *Nkx2.1* (also known as thyroid transcription factor 1, *Ttf1*), is expressed in the developing thyroid, telencephalon, and lung (Guazzi et al., 1990; Lazzaro et al., 1991; Price et al., 1992). In the developing telencephalon, *Nkx2.1*

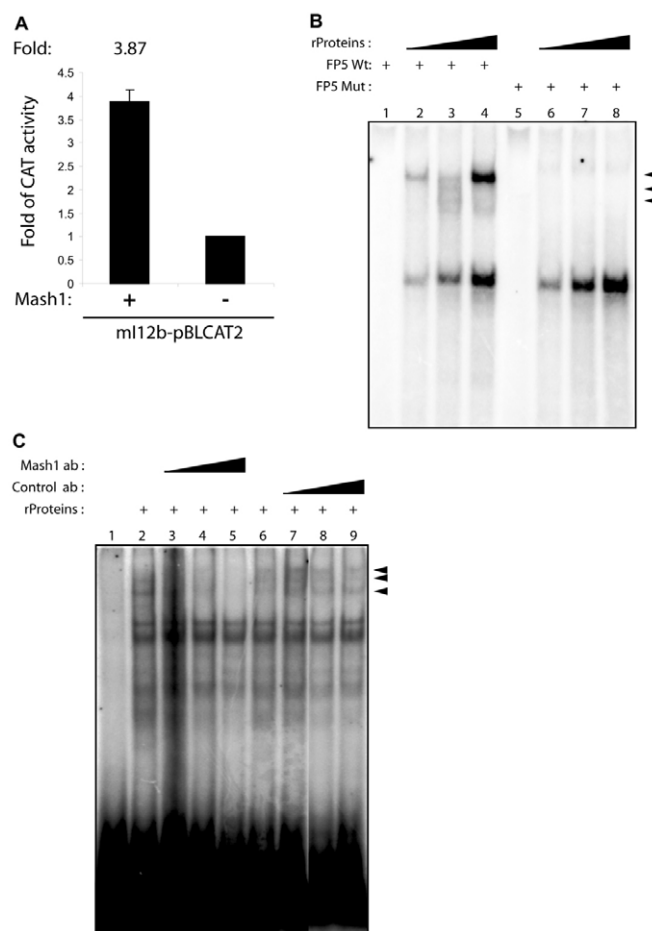


Fig. 7. The bHLH transcription factor MASH1 can bind and activate transcription through an E-box sequence in the I12b enhancer. (A) In a transient co-transfection assay, MASH1 activates transcription of a CAT reporter construct containing the I12b enhancer (ml12b-pBLCAT2) by a factor of about four. Values shown represent the mean relative CAT activity obtained from two independent experiments \pm s.e.m. (B) EMSA using the FP5 sequence (see Fig. 2) shows binding to a combination of recombinant (r) MASH1 and E47 (lanes 2-4, arrowheads). This binding is achieved through an E-box present in the FP5 sequence. rMASH1 and rE47 were unable to bind FP5 in which the E-box was mutated (lanes 6-8; same mutation as for the transgenic mouse experiments). (C) Preincubation of rMASH1 and rE47 with an anti-MASH1 antibody completely abolishes the formation of the rMASH-rE47-FP5 complexes (lanes 3-5, arrowheads), in contrast to a binding reaction that was preincubated with the control antibody (lanes 7-9).

expression is restricted to the MGE of the basal telencephalon as well as to parts of the septum, the entopeduncular area and preoptic area (Sussel et al., 1999). NKX2.1 has been shown to have high affinity for the 5'-CAAG-3' core motif and to inefficiently bind a general homeodomain core sequence (5'-TAAT-3') resembling FP1 and FP4 (Damante et al., 1994). Extrapolating from these data, we can propose that NKX2.1 could bind, with low affinity, to the FP1 and FP4 binding sites of the I12b enhancer, but this assertion remains to be confirmed.

The FP1 and FP4 DLX/MSX/NKX2.5-binding sites in the I12b enhancer seem to play a crucial role in Dlx gene regulation. Several areas of I12b reporter transgene expression disappear when individual

binding sites are mutated. Reductions in expression were also observed at E13.5. When mutations were introduced in both DLX/MSX/NKX2.5-binding sites, the reporter transgene expression targeted by I12b was totally abolished (data not shown). Interestingly, E13.5 transgenic embryos carrying I12b reporter transgenes with mutagenized MEIS1/2 or MASH1 sites showed transgene expression comparable to that obtained with the wild-type enhancer. Thus, we propose that *Dlx1/2* expression in the ventral telencephalon is first activated by neural determinants involving some of the sites we identified. Then, expression of *Dlx1/2* would be maintained by the newly synthesized DLX1 and/or DLX2 proteins through a positive-feedback loop. Products of the *Dlx5* and *Dlx6* paralogous genes, whose expression follows that of *Dlx1/2* chronologically (Liu et al., 1997) and depends on *Dlx1/2* function (Anderson et al., 1997b; Zerucha et al., 2000), could also act as positive regulators.

Previous studies have shown that DLX2 is able to bind to sequences on the I56i enhancer that show similarity to the I12b FP1 and FP4 sites (Zerucha et al., 2000; Zhou et al., 2004). The vertebrate *Dlx* bigene clusters are thought to have originated from duplications of an ancestral cluster (Stock et al., 1996; Zerucha and Ekker, 2000). Thus, cis-regulatory elements in the *Dlx1/2* and *Dlx5/6* loci might descend from a cis-acting element that existed in the ancestral *Dlx* bigene cluster. Nucleotide sequence comparisons between I12b and I56i enhancer sequences revealed that two DLX/MSX/NKX2.5 sites are present in both enhancers and the physical distance separating the two sites is similar for both enhancers (Ghanem et al., 2003). Footprinting analysis of the I56i enhancer revealed protein-DNA interactions at the two binding sites (L.P., N. Shipley and M.E., unpublished). However, the rest of the I12b and I56i enhancer sequences (more than 400 bp) are very different. Therefore, although it seems as if I12b and I56i may function through partially similar mechanisms involving auto- or cross-regulation by DLX proteins, it is not possible to say at this time whether the two enhancers result from the duplication of an ancestral regulatory element.

The potential role of the MEIS2 in *Dlx1/2* gene regulation

Meis transcription factors are potent regulators of cell proliferation. Of the three mammalian Meis genes, only *Meis1* and *Meis2* have been shown to be expressed during telencephalic development (Cecconi et al., 1997; Oulad-Abdelghani et al., 1997; Toresson et al., 1999). *Meis1* transcripts are mainly present at the lateral edges of the LGE and MGE in E12.5 mouse embryos, whereas *Meis2* was found to be highly expressed in the SVZ of the LGE at the same stage. In the LGE, *Meis2* expression is restricted to the proliferating progenitors (Toresson et al., 1999). In the diencephalon of E11.5 embryos, *Meis1* is expressed in the optic stalk and the prosomere 1, whereas *Meis2* transcripts are present in the ventral thalamus. We have shown that a potential MEIS-binding sequence is present in I12b. The MatInspector software identified this site as a MEIS1-binding site. However, it was recently reported that MEIS2 is able to recognize the same sequence (Yang et al., 2000). Our transgenic experiments provided evidence that the putative MEIS site sequence contributes to I12b enhancer activity, more specifically in the LGE of the ventral telencephalon. Based on the expression pattern of the Meis genes, we can assume that MEIS2, but not MEIS1, is likely to be involved in this regulation. Toresson and collaborators showed that MEIS, PBX and DLX proteins colocalize in the SVZ of the LGE but not the MGE at E12.5 (Toresson et al., 2000). The FP3 site is a homeobox binding site, located not far from the MEIS FP2 site. We hypothesize that FP3 could be a binding site for a MEIS1 co-factor. However, sequence comparison of the PBX consensus binding site with the FP3 sequence did not reveal any similarities.

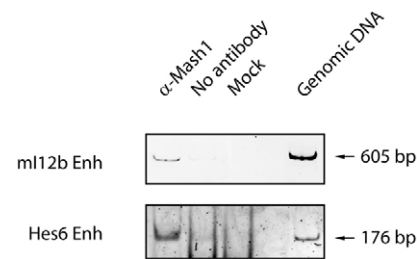


Fig. 8. MASH1 binds to the I12b enhancer in vivo. LGE and MGE were dissected from E11.5 mouse telencephalon, cross-linked and immunoprecipitated using an anti-MASH1 antibody (α -Mash1). PCR analysis was performed on the immunoprecipitated chromatin using primers corresponding to the I12b enhancer (I12b Enh) and the *Hes6* upstream enhancer (*Hes6* Enh). After 28–30 cycles, PCR products (605 bp for mI12b and 176 bp for *Hes6*) were electrophoresed on 1% agarose gel. As controls, amplifications were carried out on chromatin obtained from the elution of protein-A sepharose beads that was preincubated with the cross-linked chromatin or with the IP dilution buffer (No antibody and Mock, respectively). Different dilutions of total input chromatin (Genomic DNA) were used for each set of primers: 1/500 for I12b and 1/1000 for *Hes6* Enh.

A bHLH factor binds to an E-box in the intergenic I12b enhancer

Neurogenesis in the ventral telencephalon is a balancing act controlled in part by repressor-type and activator-type bHLH transcription factors. Neural stem cells are kept in an undifferentiated state by repressor-type bHLH proteins (for example, members of the HES family). In the absence of the repressor-type bHLHs, expression of the activator-type bHLHs is upregulated, leading to the commitment of multipotent stem cells to a neuronal cell lineage. Expression of the activator-type *Mash1* promotes neurogenesis of progenitors present in the VZ and SVZ of the ventral telencephalon. These committed neural progenitors will then be specified into multiple neuronal lineages, including various subtypes of GABA-expressing interneurons and projection neurons. *Mash1* has a dual function in cell fate specification and differentiation of ventral telencephalon progenitors (Yun et al., 2002). First, through a non-cell-autonomous function, *Mash1* delays the maturation of neighboring progenitors by using the Notch signaling pathway. Differentiation of these delayed progenitor cells gives rise to a population of cells called late-born neurons. *Mash1* also has a cell-autonomous function in the subcortical telencephalon. *Mash1* expression triggers the differentiation process of some progenitor cells. *Dlx1/2* are early markers of early-born neurons and their expression quickly follows that of *Mash1*. As proposed by previous genetic studies, *Mash1* could be a potential regulator of *Dlx1/2* expression in interneuron progenitors. However, whether the molecular mechanisms underlying this regulation are direct or indirect was not known. Our results provide the first evidence for a direct regulation of the *Dlx1/2* genes by MASH1. We have demonstrated the presence of a sequence (E-box) recognized by MASH1 in the FP5 site of the I12b enhancer. Transgenic embryos generated using a mutant I12b enhancer (Δ FP5-I12b-lacZ), in which the E-box was destroyed, displayed an almost complete loss of I12b enhancer activity in the telencephalon of E11.5 embryos. This result is surprising considering that *Dlx1/2* expression in the telencephalon is not completely abolished in *Mash1*^{−/−} embryos (Casarosa et al., 1999). In fact, *Dlx* gene expression in the LGE and MGE of these mutants is upregulated. This is only an apparent contradiction: it must be remembered that I12b activity recapitulates only part of the *Dlx1/2*

expression patterns. At least one other enhancer, URE2 (Hamilton et al., 2005) (N.G., M. Yu, J. Long, G.H., J. L. R. Rubenstein and M.E., unpublished) is also involved in *Dlx1/2* regulation in the forebrain in an overlapping but distinct population of cells. Thus, in *Mash1*-null mice, *Dlx1/2* expression can be increased in a cell population in which *Dlx* expression is I12b-independent but depends instead, for example, on URE2. This result can also be explained by the non-cell-autonomous function of *Mash1* in the developing telencephalon. Thus, in *Mash1*-null mice, neighboring progenitors could undergo premature differentiation and express *Dlx1/2* in an I12b-independent fashion.

Concluding remarks

The disruption of interneuron development could be linked to neurodevelopmental disorders (Berretta et al., 2004; Cobos et al., 2005; Cossart et al., 2005; Dykens et al., 2004; Horike et al., 2005; Levitt et al., 2004; Magloczky and Freund, 2005; Rubenstein and Merzenich, 2003; Samaco et al., 2005). Interestingly, *Dlx* genes have been shown to be linked to epilepsy and Rett syndrome (Cobos et al., 2005; Horike et al., 2005). Also, members of the *Dlx* homeobox gene family are found in two autism-susceptibility loci, chromosome 2q and 7q (International Molecular Genetic Study of Autism Consortium, 2001). Rubenstein and Merzenich recently proposed a model of autism in which an increased ratio of excitation/inhibition on a given neuronal network is suggested to cause some forms of autism (Rubenstein and Merzenich, 2003). Single nucleotide polymorphisms (SNPs) were found at the *Dlx1/2* and *Dlx5/6* loci, including in ultraconserved (Bejerano et al., 2004) regulatory elements controlling *Dlx* gene expression in the forebrain (URE2, I12b, I56i and I56ii) (Hamilton et al., 2005). The presence of a SNP in these ultraconserved regulatory elements is not only surprising, but also strongly suggests that the SNP could be causally related to the behavioral defects seen in the autistic individuals that carry it. Thus, elucidation of the genetic cascades controlling *Dlx* gene expression through these enhancers will enhance our knowledge of GABAergic interneuron development, as well as providing new insights in the understanding of important neurological disorders.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/9/1755/DC1>

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rDlx2 : +



4539 4549 4559 4569 4579
 : : : : :
 4530: AGCTCCTCCCCCTTCTATTCTTGGTTCCTGCAGGCCGAGGCTGCTGTCTG mHes6
 4251: -----.....A.....C..... hHes6

 : : : : :
 4580: GCCCCAGTAACCTTGATGCCCGTGA**CAGTTGGCAGCTG**CAGCCGCTTCCGC mHes6
 4268: .T..T-.....G.....G.. hHes6

 : : : : :
 4630: AGAGAAAAGCGGCA-----GGCCG-GCCAGGAGCGA**CAGGTG**TTGGC mHes6
 4317: G.....AG.AAAGCGGC....C....A..... hHes6

 : : : : :
 4680 4690 4700 4709 4719
 : : : : :
 4671: CGCGCCTTTGTCTAGCTGGCTTTGTCTGCAGGC-TGCAACAGGCACGCC mHes6
 4367:C..ACC.....G..G...AG..GG.G.--...T hHes6

 : : : : :
 4729 4739 4744 4754 4764
 : : : : :
 4720: TGTGTCGGCCAGACGCCCCAG----CCCCGCGTCGTCGGCGGTC**CATCT** mHes6
 4415: ...CC.....GC.ACTCC...AT..CG.CG...C.C..... hHes6

 : : : : :
 4773 4783 4793 4803 4813
 : : : : :
 4765: **G**CC-GGCCCTGCCTCGCCGCGGGAGGGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT mHes6
 4465: ..TG..TT.CT.T.G.....C.....C.....G.C..... hHes6