Commissural neuron identity is specified by a homeodomain protein, Mbh1, that is directly downstream of Math1

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Summary

Proneural basic helix-loop-helix (bHLH) proteins are key regulators of neurogenesis. However, downstream target genes of the bHLH proteins remain poorly defined. *Mbh1* confers commissural neuron identity in the spinal cord. Enhancer analysis using transgenic mice revealed that *Mbh1* expression required an E-box 3′ of the *Mbh1* gene. *Mbh1* expression was lost in *Math1* knockout mice, whereas misexpression of *Math1* induced ectopic expression of *Mbh1*. Moreover, Math1 bound the *Mbh1* enhancer containing the E-box in vivo and activated gene expression. Generation of commissural neurons by Math1 was inhibited by a dominant negative form of Mbh1. These findings indicate that Mbh1 is necessary and sufficient for the specification of commissural neurons, as a direct downstream target of Math1.

Key words: Math1 (Atoh1), Proneural, Mbh1 (Barhl2), Homeobox, Spinal cord, Mouse

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Introduction

An enormous variety of neuronal types are generated during vertebrate neurogenesis. Two critical steps in neurogenesis, generation of neural progenitor cells and their commitment to the neuronal fate, are controlled by proneural genes, which encode basic helix-loop-helix (bHLH) transcription factors, such as Mash1 (Ascl1 – Mouse Genome Informatics) and Math1 (Atoh1 – Mouse Genome Informatics) (reviewed by Bertrand et al., 2002). Proneural genes are expressed in distinct domains or populations of cells and are thought to integrate positional information into the program of neuronal differentiation, resulting in the specification of neuronal identity. In sympathetic ganglia, Mash1 controls noradrenergic phenotypes through activation of a homeobox gene, *Phox2a* (reviewed by Goridis and Rohrer, 2002). Math1 controls the differentiation of cerebellar granule cells (Ben-Arie et al., 1997), hair cells in the inner ear (Bermingham et al., 1999) and commissural neurons in the spinal cord (Bermingham et al., 2001; Gowan et al., 2001). Many bHLH proteins activate transcription through binding to an E-box nucleotide sequence motif. A neuronal differentiation bHLH protein, NeuroM, has been shown to regulate *Hb9*, which is necessary for differentiation of motoneurons (Lee and Pfaff, 2003). Less is known, however, about downstream targets of proneural bHLH proteins and their molecular mechanisms for specifying neuronal identity.

In the developing dorsal spinal cord, domains of progenitor cells are distinguished by the expression of bHLH genes, which are initially established by TGFβ-like signals (reviewed by Lee and Jessell, 1999; Caspary and Anderson, 2003; Helms and Johnson, 2003). Each domain produces a distinct set of neurons, which are marked by combinatorial expression of homeobox genes. *Math1* is expressed by dorsalmost cells adjacent to the roof plate, which give rise to dI1 cells positive for LIM-class homeodomain proteins, Lhx2 (LH2A) and Lhx9 (LH2B). dI1 cells are lost in *Math1* knockout mice (Bermingham et al., 2001; Gowan et al., 2001), whereas misexpression of *Math1* increases the number of dI1 cells and commissural neurons (Gowan et al., 2001). A Bar-class homeobox gene, *Mbh1* (*Barhl2* – Mouse Genome Informatics), is also expressed by dI1 cells, and *Mbh1*-positive cells give rise to commissural neurons (Saba et al., 2003). Dorsal cells that express *Mbh1* ectopically are transfated to commissural neurons in the spinal cord, suggesting that *Mbh1* is sufficient for the specification of commissural neuron identity (Saba et al., 2003).

In this study, we identified an enhancer directing *Mbh1* expression in the spinal cord by analyzing transgenic mice that carried *lacZ* with *Mbh1*-flanking genome sequences. An E-box, which was conserved among mouse, rat and human sequences, was critical to drive *lacZ* expression in the dorsal spinal cord, suggesting that *Mbh1* expression is regulated by a bHLH protein. Furthermore, chromatin immunoprecipitation (ChIP) experiments revealed that Math1 bound the *Mbh1* enhancer containing the E-box in the spinal cord. Transfection assays in the mouse spinal cord indicated that expression of a reporter gene carrying the E-box was activated specifically by Math1. These results, taken together with *Mbh1* expression in the gain- and loss-of-function experiments of *Math1*, indicate that *Mbh1* is a downstream target gene of Math1. The function of Mbh1 was analyzed using chimeric proteins containing the homeodomain of Mbh1 and functional domains that can

modulate transcription. A chimeric protein containing the Engrailed repressor domain generated commissural neurons, as Mbh1 and Math1 did. By contrast, a chimeric protein containing the VP16 activator domain inhibited generation of commissural neurons. These findings suggest that transcriptional repressor activity of Mbh1 is necessary and sufficient for the specification of commissural neurons. Thus, these studies revealed a cascade of events for specifying commissural neuron identity in the spinal cord.

Materials and methods

Analysis of the *Mbh1* gene

Eleven overlapping lambda genomic DNA clones were obtained by screening a 129SvJ mouse genomic library (Stratagene) using *Mbh1* cDNA (GenBank, AB004056) as a probe. These clones covered a 22.5 kb genome sequence including 4.5 kb 5′, 12.5 kb 3′ and introns. The transcription initiation site was determined using RNA from embryonic day (E) 12.5 mouse embryos with a 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Gibco BRL).

Generation and analysis of transgenic mice

The *lacZ*-coding region with SV40 polyadenylation site derived from BGZA (Yee and Rigby, 1993; Helms and Johnson, 1998) was inserted downstream of the translation start site of Mbh1, which matched the translation start site of β-gal. In Tg5 and Tg6, the basal *β-globin* promoter was used in place of the 1.0 kb 5′ sequence. Tg18 was constructed by linking three tandem copies of the 123 bp fragment containing the E-box. The mutation was introduced into the E-box using an In Vitro Mutagenesis Kit (Takara).

Transgenic mice were generated and analyzed as described previously (Saba et al., 2003). Briefly, injected eggs were implanted into ICR female mice, and founders were collected and stained for β-gal activity. Stable transgenic lines were also generated for Tg2 and Tg4. Transgenes were detected by PCR for the *lacZ* gene using placenta DNA for embryos and tail DNA for pups.

In situ hybridization and immunohistochemistry

Section and whole-mount in situ hybridization were performed as described in Saito et al. (Saito et al., 1996) and Wilkinson (Wilkinson, 1992), respectively. Antisense RNA probes were synthesized from plasmids carrying mouse cDNA clones: pMH4-1 and pNH10 for *Mbh1*; *Lhx9* (gift of T. M. Jessell, Columbia University); *Math1* (gift of R. Kageyama, Kyoto University). Immunofluorescent studies were performed as described (Saba et al., 2003). The following primary and secondary antibodies were used for visualizing the signals: rabbit anti-Math1 (Helms and Johnson, 1998); goat anti-β-gal (Biogenesis); mouse monoclonal anti-bromodeoxyuridine (BrdU) (Sigma); donkey anti-mouse and anti-rabbit IgGs conjugated with Cy3 (Jackson ImmunoResearch); donkey anti-goat IgG conjugated with Alexa Fluor 488 (Molecular Probes).

In vivo electroporation

In vivo electroporation was performed as described before (Saba et al., 2003). Solution (1 μl) containing 140 nmol/l each plasmid in PBS was injected into the central canal of the spinal cord of E11.5 mouse embryos. Half-ring type electrodes were used to transfect DNA through the spinal cord. pEYFP, which carried *EYFP* downstream of a CAG promoter (Saito and Nakatsuji, 2001), was used as a control. pEYFP-Math1 and pEYFP-Mash1 were constructed by inserting the coding region of *Math1* and *Mash1* downstream of the second CAG promoter of pCAG-EYFP-CAG (Saito and Nakatsuji, 2001), respectively. The *Math1-HA* gene was constructed by inserting oligonucleotides encoding the HA tag immediately upstream of the translation termination codon of Math1. The *En-Mbh1* and *VP16-Mbh1* chimeric genes were constructed by fusing the sequences encoding the *Drosophila* Engrailed repressor domain (Jaynes and O'Farrell, 1991) and the VP16 activator domain (Clontech) to the sequence encoding the C-terminal portion of the Mbh1 protein, respectively. These three genes were inserted downstream of the second CAG promoter of pCAG-EYFP-CAG to construct pEYFP-Math1-HA, pEYFP-En-Mbh1 and pEYFP-VP16-Mbh1. Each result of electroporation was confirmed by using at least two independently isolated clones with the same structure.

ChIP assay

Chromatin was prepared as described in Forsberg et al. (Forsberg et al., 2000) with minor modifications. To examine binding of endogenous Math1, the spinal cord from ~28 mouse embryos at E10.5 was used for one assay. To analyze binding of misexpressed Math1, EYFP$^+$ sides of the spinal cord were dissected out 24 hours after electroporation at E11.5, and ~10 electroporated embryos were used for one assay. The dissected spinal cord was fixed with 1% formaldehyde in PBS for 3 hours on ice. After cell lysis and sonication, ChIP was performed following the manufacture's protocol in the ChIP Assay Kit (Upstate). The following antibodies and IgG (3 μg) were used: rabbit anti-Math1; rabbit anti-neurofilament 200 (Sigma); rat anti-HA (3F10, Roche); rat IgG (Immunotech). Immunocomplexes were pulled down using protein A and protein G-agarose beads for rabbit and rat IgGs, respectively. A 502 bp fragment spanning nucleotides +5507 to +6009 containing the E-box of the *Mbh1* enhancer was amplified by semi-quantitative PCR using the following primers: sense, TTCCAGGTGCCCGCCTCTTCTGA; antisense, TTCGCGGATCCAAGCACAACTCATT. As a negative control, a 546 bp DNA fragment spanning nucleotides –6 to +540 of the *Mbh1* gene was amplified using the following primers: sense, GTAGAAATGACAGCAATGGAAGG; antisense, CCTGAAGCT-CTCGTGTGC. The intensity of PCR bands was analyzed using a Typhoon 9410 fluorescence imager (Amersham Bioscience). For all experiments, immunoprecipitated DNA templates were well under the saturation level.

Reporter assay

Forty-eight hours after electroporation, EYFP$^+$ regions in one side of the spinal cord were dissected out in cold PBS under a fluorescent stereomicroscope and suspended with lysis buffer from the High Sensitivity β-galactosidase Assay Kit (Stratagene). Approximately 150 μg of protein was obtained from the EYFP$^+$ region of one embryonic spinal cord. β-gal activity was measured using the Assay Kit according to the manufacturer's protocol. The efficiency of transfection was normalized with the intensity of EYFP fluorescence, which was measured using the Typhoon 9410 fluorescence imager.

Results

Expression pattern of *Mbh1*

Mbh1 was expressed in several areas of the developing nervous system, including the ventral telencephalon, diencephalon, mesencephalon, hindbrain and spinal cord (Fig. 1). In the E10.5 spinal cord, the expression pattern of *Mbh1* was similar to that of *Math1*, as previously analyzed by sectioning (Saba et al., 2003). At E11.5, *Mbh1* expression extended ventrally to the deep dorsal horn in the spinal cord, reflecting ventral migration of *Mbh1*$^+$ cells (Saba et al., 2003).

A genomic fragment downstream of the *Mbh1* gene is sufficient to recapitulate endogenous *Mbh1* expression in the dorsal spinal cord

The mouse *Mbh1* gene spanned 5.5 kb and was composed of three exons. The 5′ end of the *Mbh1* transcript was mapped to

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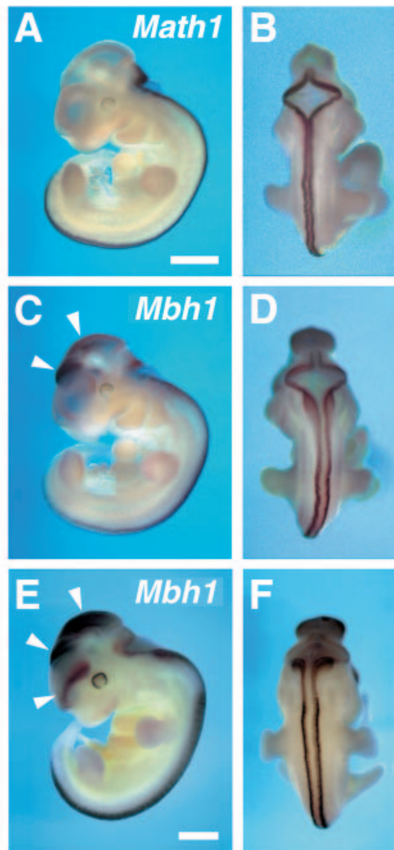

Fig. 1. *Mbh1* expression in mouse embryos. Lateral (A,C,E) and dorsal (B,D,F) views of whole-mount in situ hybridization of E10.5 (A-D) and E11.5 (E,F) embryos with antisense cRNA probes for *Math1* (A,B) and *Mbh1* (C-F). Arrowheads indicate the ventral telencephalon (only in E), dorsal diencephalon and mesencephalon, which expressed *Mbh1* but not *Math1*. Scale bars: in A, 1 mm for A-D; in E, 1 mm for E,F.

an A residue 641 bases upstream of the translation start site (Fig. 2A). The transcription initiation site was located in a GC-rich region, which contained a CAAT box, and binding sites for Sp1, ETS and CREB/ATF, but not a discernible TATA box (the nucleotide sequence was deposited as AB063281 in GenBank).

To search for an enhancer responsible for *Mbh1* expression, we constructed several transgenes carrying parts of the *Mbh1* genome encompassing from –4.5 to +11 kb (Fig. 2A). The *lacZ* reporter gene was inserted immediately downstream of the translation start site of the *Mbh1* open reading frame. Each transgene was injected into fertilized eggs, and *lacZ* expression was analyzed in transgenic embryos developed from the eggs at E11.5, at which stage *Mbh1* expression was pronounced in the spinal cord. We had previously reported the result of Transgene (Tg)4 (Saba et al., 2003) and extended our analysis to other Tgs. Tg1 did not demonstrate *lacZ* expression, whereas transgenic embryos harboring Tg2 and Tg4, which contained 1.0 kb 5′ and 2.5 kb 3′ sequences, expressed *lacZ* in the dorsal spinal cord (Fig. 2B-D). The expression of *lacZ* closely resembled that of endogenous *Mbh1* (see Fig. 1 for whole embryos and Saba et al., 2003 for sections). Moreover, β-galactosidase (β-gal)$^+$ cells were labeled with anti-Mbh1 antibody (Saba et al., 2003), indicating that the expression of *lacZ* recapitulated endogenous *Mbh1* expression. Sections of the transgenic embryos showed β-gal$^+$ axons projecting to the floor plate and ventral funiculi, confirming that β-gal$^+$ cells gave rise to commissural neurons (Fig. 2F,G). The temporal expression patterns of *lacZ* were examined using transgenic mice bearing Tg2 and Tg4. The onset of *lacZ* expression matched that of endogenous *Mbh1* expression, which was first detected at E10.5 (Saba et al., 2003). Preceding *Mbh1* expression, *Math1* expression was detected at E9.5 (Helms and Johnson, 1998) (data not shown). At E10.5, when Math1$^+$ cells started to migrate ventrally, they expressed *lacZ* at the lateral border of the Math1$^+$ domain (Fig. 2H). Whereas *Math1* is expressed in both proliferating precursors and postmitotic cells (Helms and Johnson, 1998), only postmitotic cells expressed *lacZ* (see Fig. S1 in the supplementary material).

Embryos harboring Tg5 and Tg6, in which the 1.0 kb 5′ fragment of Tg4 was replaced with the *β-globin* basal promoter, expressed *lacZ* in the dorsal spinal cord (Fig. 2E), suggesting that the 2.5 kb 3′ fragment is sufficient to drive *lacZ* expression in an *Mbh1*-specific manner. The 3′ fragment functioned irrespective of its orientation as well as shorter fragments (see below).

An E-box is required for *lacZ* expression in the dorsal spinal cord

To identify a *cis*-regulatory element directing *Mbh1* expression in the dorsal spinal cord, we made a series of deletions of the 2.5 kb 3′ fragment (Fig. 3A). As demonstrated by Tg12, a 517 bp fragment, which was 5.7 kb downstream of the translation start site, was sufficient to drive *lacZ* expression in the dorsal spinal cord of some transgenic embryos. Further deletions suggested that the 5′ portion of the 517 bp fragment contained a critical site for *Mbh1* expression (see also Fig. S2C-F in the supplementary material). It should be noted that β-gal activity became weaker, as the genome sequences were progressively deleted. This finding suggests that several sites of the 3′ fragment are involved in upregulating *Mbh1* expression.

The 5′ portion of this 517 bp fragment, a 123-base sequence (Fig. 3E), was relatively well conserved among mouse, rat and human (95.6% identity between mouse and rat, and 76.7% identity between mouse and human). Conversely, the sequence encompassing from +4.5 to +11.0 kb downstream of the translation start site showed 79.0% identity between mouse and rat, and 60.0% identity between mouse and human. We searched the 123-base sequence for binding sites of transcription factors using the MatInspector *professional* (http://www.genomatix.de/index.html). Although there were some potential transcription-factor-binding sites, only one E-box (CAGCTG) was conserved among the three species.

To examine if the E-box was essential for *Mbh1* expression, we introduced a mutation into the E-box (ATTCTG) of the 517 bp and 308 bp fragments, thereby constructing Tg16 and Tg17. This mutation is known to disrupt the binding activity of Math1 to DNA (Helms et al., 2000). None of the transgenic embryos harboring these two mutant Tgs expressed *lacZ* in the dorsal spinal cord (Fig. 3A and see Fig. S2I,J in the supplementary material), suggesting that this E-box is required for *Mbh1* expression in the dorsal spinal cord.

The involvement of the E-box in *Mbh1* expression was further supported by Tg18. Transgenic embryos harboring

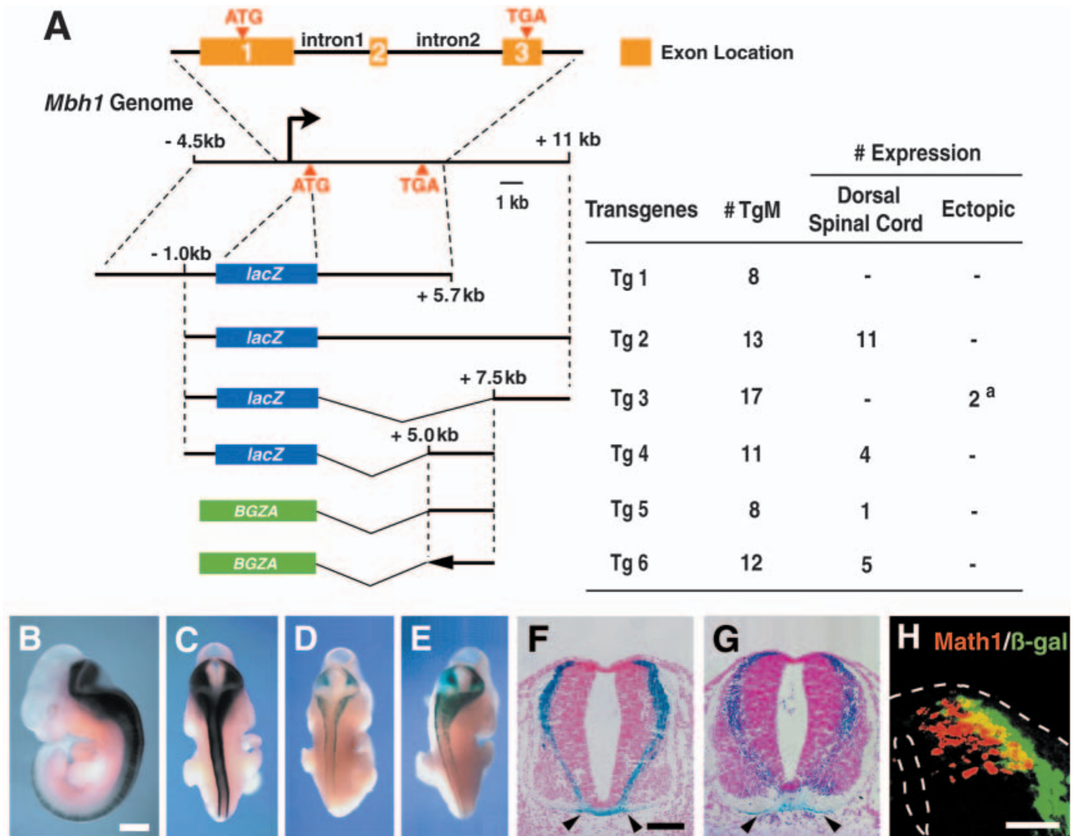

Fig. 2. Identification of an enhancer to drive *lacZ* expression in the dorsal spinal cord. (A) Schematic representation of the genomic structure of *Mbh1* and *Mbh1/lacZ* transgenes. The orange boxes indicate exons, and translation start site (ATG) is designated as nucleotide number 1. The blue and green boxes represent *lacZ* and the β-*globin promoter/lacZ* construct (*BGZA*), respectively. The 3′ fragment was reversed in Tg6. The number of transgenic (#TgM) and β-gal$^+$ (#expression) embryos are shown in the table; [a]the two embryos expressed *lacZ* at low levels in the midbrain. Lateral (B) and dorsal (C-E) views of representative β-gal$^+$ embryos harboring Tg2 (B,C), Tg4 (D) and Tg5 (E). Transverse sections of the embryos carrying Tg2 (F) and Tg4 (G). Arrowheads indicate β-gal$^+$ ventral funiculi. (H) Transverse section of the embryo carrying Tg4 was immunostained with antibodies against Math1 (red) and β-gal (green). Scale bars: in B, 1 mm for B-E; in F, 100 μm for F,G; in H, 50 μm.

Tg18, which carried a trimer of the 123 bp fragment, expressed *lacZ* in the dorsal spinal cord (Fig. 3D and see Fig. S2G,H in the supplementary material). However, *lacZ* expression from Tg18 was also detected at ectopic sites, the midbrain and somites, in most of the β-gal$^+$ embryos, suggesting that the sequence outside these 123 bp is involved in restricting *Mbh1* expression in the dorsal spinal cord.

Math1 is necessary and sufficient for *Mbh1* expression

The E-box necessary for *Mbh1* expression completely matched the site to which the Math1 protein could bind efficiently in vitro (Akazawa et al., 1995; Helms et al., 2000). *Mbh1* is expressed by a lineage of cells that have expressed Math1 (Saba et al., 2003). Moreover, *Mbh1* expression started in cells expressing Math1 (Fig. 2H). To clarify a genetic relationship between *Mbh1* and *Math1*, *Mbh1* expression was examined in *Math1* knockout embryos (Fig. 4 and see Figs S3, S4 in the supplementary material). *Mbh1* expression was lost in the spinal cord of *Math1*$^{-/-}$ embryos, indicating that Math1 was necessary for *Mbh1* expression. Expression of *Lhx9*, which resembled that of *Mbh1*, was also lost in the *Math1*$^{-/-}$ spinal cord (Fig. 4G). By contrast, in the developing dorsal diencephalon, where *Math1* was not expressed, *Mbh1* expression was not perturbed by the *Math1* null mutation (Fig. 4H).

We also performed gain-of-function analysis using mouse in vivo electroporation (Fig. 5). On *Math1*-transfected sides of the developing spinal cord, *Mbh1* expression was induced ectopically, indicating that Math1 was sufficient for *Mbh1* expression in the spinal cord (Fig. 5B). Math1 also induced ectopic expression of *Lhx9* (Fig. 5C). These results indicate that Math1 is both necessary and sufficient for *Mbh1* expression in the spinal cord.

Math1 binds the Mbh1 enhancer

To determine whether Math1 bound the E-box in vivo, we performed ChIP experiments. Chromatin was prepared from the spinal cord at E10.5, at which stage endogenous *Math1* expression peaked. An anti-Math1 antibody specifically immunoprecipitated *Mbh1* enhancer DNA fragments, which contained the E-box (Fig. 6A). *Mbh1* genomic fragments that were 5.8 kb far from the E-box were not immunoprecipitated. To examine binding of misexpressed Math1 to the E-box, *Math1-HA* was transfected to the spinal cord. Math1-HA exhibited the same activity as Math1 in the spinal cord (data not shown). A monoclonal anti-HA-antibody specifically immunoprecipitated the DNA fragments containing the E-box (Fig. 6B). The monoclonal antibody did not co-precipitate the DNA fragments after transfection of *Math1*, which did not contain the HA tag (data not shown). These findings indicate that Math1-DNA complexes were specifically immunoprecipitated by these antibodies, and that both endogenous and misexpressed Math1 bound the *Mbh1* enhancer in the spinal cord.

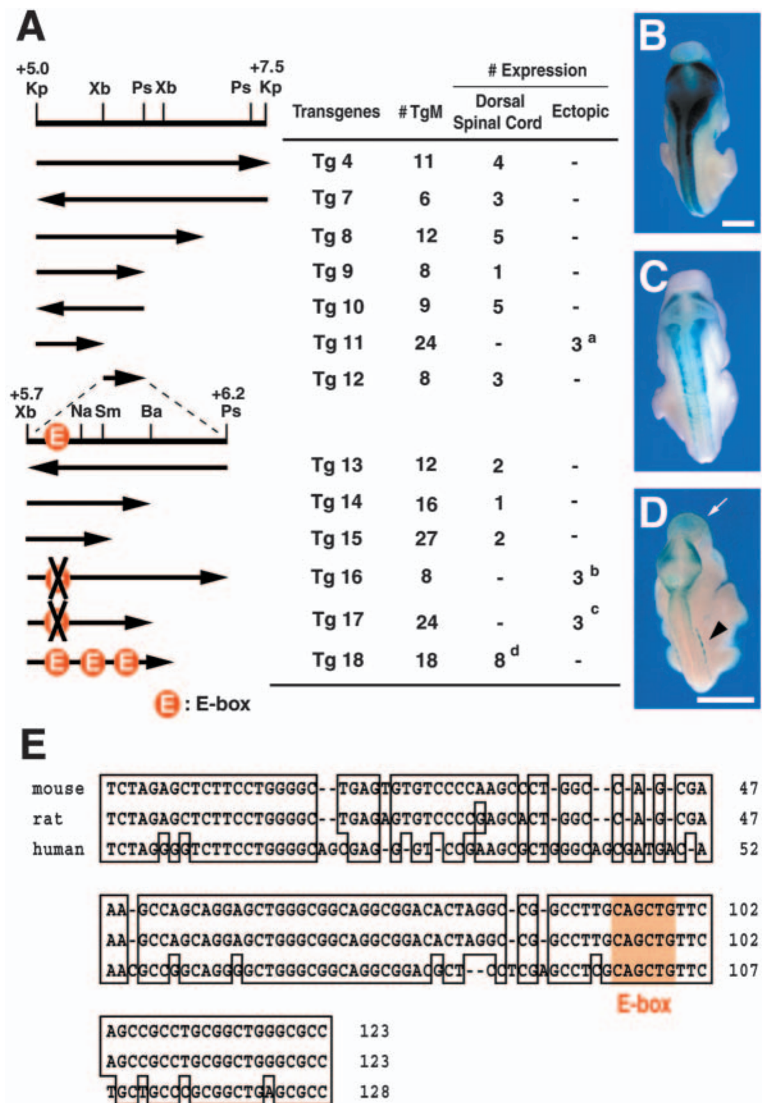

Fig. 3. An E-box 3′ of the *Mbh1* gene was required for *lacZ* expression in the spinal cord. (A) Structure of the transgenes, and the number of transgenic and β-gal⁺ embryos recorded. The 3′ fragments were reversed in Tg7, Tg10 and Tg13. Restriction enzyme sites used for cloning are indicated: Ba, *Bam*HI; Kp, *Kpn*I; Na, *Nar*I, Ps, *Pst*I; Sm, *Sma*I; Xb, *Xba*I. [a]The three embryos expressed *lacZ* in the midbrain, floor plate or whole body. [b]*lacZ* was expressed in the branchial arch or midbrain. [c]The three embryos expressed *lacZ* in the telencephalon, limb or skin. Expression levels of *lacZ* in these ectopic sites ([a-c]) were low. [d]Seven embryos also expressed *lacZ* in the midbrain and somite. (B-D) Dorsal views of representative β-gal⁺ embryos harboring Tg8 (B), Tg14 (C) and Tg18 (D). An arrow and an arrowhead indicate the β-gal⁺ midbrain and somite, respectively. Scale bars: in B, 1 mm for B,C; in D, 1 mm. (E) Nucleotide sequence comparison of the 123-base sequence among the three species. Residues identical in the three species are boxed.

Math1 activates expression of a transgene carrying the E-box

To examine if Math1 specifically regulated expression of a transgene, we performed transient transfection assays in the developing spinal cord. Tg12 was transfected into the E11.5 mouse spinal cord with an expression vector, pEYFP-Math1, using in vivo electroporation. E10.5 embryos could not survive after electroporation into the spinal cord. *EYFP* and *Math1* were co-expressed in the same cells by a double promoter vector, which carried the two genes under two separate promoters on the same plasmid (Saito and Nakatsuji, 2001; Saba et al., 2003). Two days after electroporation, fluorescence of EYFP was detected through the spinal cord (Fig. 7A,E). Strong *lacZ* expression was detected, when Tg12 was co-transfected with *Math1* (Fig. 7F). By contrast, transfection of Tg12 with *EYFP* alone generated only a few β-gal⁺ cells close to the roof plate (arrow in Fig. 7D), reflecting that endogenous *Math1* expression is limited to a smaller number of dorsal cells after E11.5. Since most dorsal commissural neurons had already migrated away from the ventricle at E11.5 (Saba et al., 2003), these genes were mostly transfected into cells that had not been fated to commissural neurons. Therefore, transfection of *EYFP* alone labeled less commissural neurons (Fig. 7C and also see Fig. 8A). By contrast, transfection of *Math1* generated more commissural neurons (Fig. 7G and also see Fig. 8C), as dorsal neurons were transfated into commissural neurons by *Math1* (Saba et al., 2003). Many of the transfated neurons were β-gal⁺ (Fig. 7H).

To measure the transcriptional activity of Math1, EYFP⁺ regions of the electroporated spinal cord were dissected out, and cell extracts were analyzed (Fig. 7I). *lacZ* expression from Tg12 was activated ~5-fold by Math1 but not by Mash1. This result was consistent with the data of transgenic embryos, where *lacZ* was not expressed from Tg12 in Mash1⁺ domains, such as the dorsal half of the spinal cord and autonomic nervous system. These results indicate that the expression of the transgene is specifically activated by Math1.

Repressor activity of Mbh1 is required for the differentiation of commissural neurons downstream of Math1

We analyzed the function of Mbh1 using chimeric proteins, which were expected to exert opposite functions. As the C-terminal portion, which included the homeodomain, was well conserved among Bar-class homeodomain proteins, the N-terminal portion of the Mbh1 protein was replaced with a functional domain: the repressor domain of *Drosophila* Engrailed (Jaynes and O'Farrell, 1991) for En-Mbh1, or the activation domain of herpes simplex virus VP16 (Triezenberg et al., 1988) for VP16-Mbh1. Their genes were transfected into the E11.5 spinal cord by in vivo electroporation and co-expressed with *EYFP* in the same cells using the double promoter vector. More commissural neurons were generated by transfection of *Mbh1* as well as *Math1* (Fig. 8B,C), as described previously (Saba et al., 2003). Similarly, EYFP⁺ commissural neurons were generated by misexpression of *En-Mbh1* (Fig. 8D) but not *VP16-Mbh1* (Fig. 8E), suggesting that Mbh1 functions as a transcriptional repressor.

To clarify the role of Mbh1 in the differentiation of commissural neurons, these chimeric genes were co-

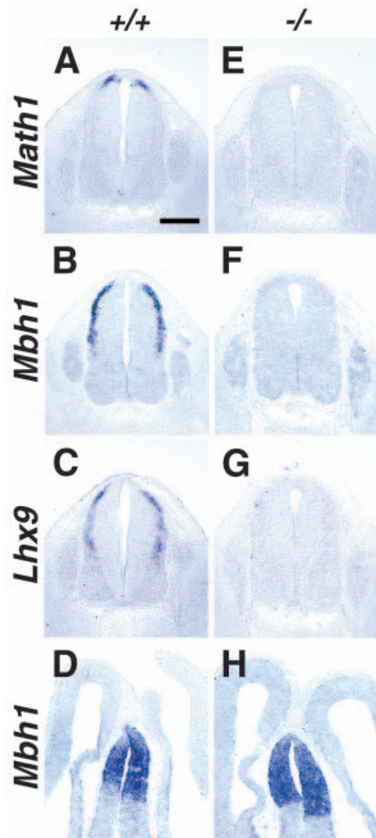

Fig. 4. *Math1* was necessary for *Mbh1* expression in the spinal cord. Transverse sections at brachial (A-C,E-G) and forebrain levels (D,H) of E11.5 *Math1*$^{+/+}$ (A-D) and *Math1*$^{-/-}$ (E-H) mouse embryos were hybridized with antisense cRNA probes for *Math1* (A,E), *Mbh1* (B,D,F,H) and *Lhx9* (C,G). Loss of *Mbh1* and *Lhx9* expression was observed through all axial levels of the spinal cord in all *Math1*$^{-/-}$embryos (*n*=3). Scale bar: 100 µm.

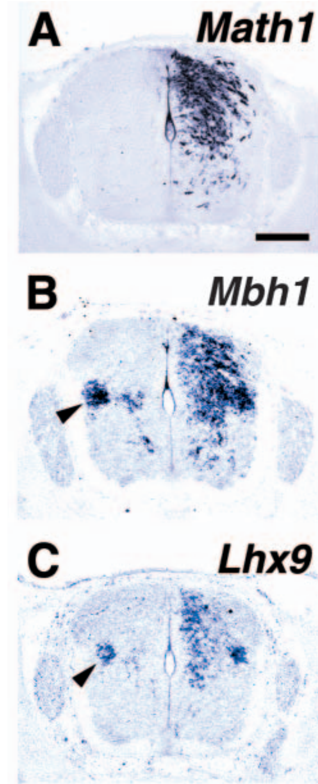

Fig. 5. Misexpression of *Math1* induced ectopic expression of *Mbh1* in the spinal cord. Two days after electroporation, mouse embryos were collected at E13.5. Transverse sections at brachial levels of the electroporated spinal cord were hybridized with antisense cRNA probes for *Math1* (A), *Mbh1* (B) and *Lhx9* (C). Right sides of sections were transfected with *Math1*. Arrowheads indicate endogenous *Mbh1*$^{+}$ and *Lhx9*$^{+}$ domains. Ectopic expression of *Mbh1* and *Lhx9* was also detected at E12.5, one day after electroporation (data not shown), and through all axial levels of the spinal cord at both E12.5 and 13.5 in all electroporated embryos (*n*=4). Misexpression of *EYFP* did not induce expression of either *Mbh1* or *Lhx9* (data not shown). Scale bar: 100 µm.

transfected with *Math1*. Whereas co-transfection of *En-Mbh1* with either *Math1* or *Mbh1* generated commissural neurons in a manner similar to the transfection of each gene (data not shown), the generation of commissural neurons by *Math1* and *Mbh1* was inhibited by *VP16-Mbh1* (Fig. 8F; data not shown). These results suggest that VP16-Mbh1 functions as a dominant negative form of Mbh1, and that transcriptional repressor activity of Mbh1 is required for the differentiation of commissural neurons downstream of Math1.

Discussion

Both gain- and loss-of-function experiments of *Math1*, taken together with *Mbh1* expression in a lineage of cells that have expressed *Math1*, indicate that *Mbh1* is expressed downstream of *Math1* in the spinal cord. Moreover, ChIP assays showed in vivo binding of Math1 to the enhancer containing the E-box, which was crucial for *Mbh1* expression in the spinal cord. These results indicate that *Mbh1* is a direct downstream target of a proneural protein, Math1. Previous analyses indicate that *Tag1* (*Cntn2* – Mouse Genome Informatics) and *Dcc* are induced by misexpression of *Mbh1* (Saba et al., 2003). As Mbh1 is a potential transcriptional repressor, Mbh1 may de-repress the expression of these two genes, by downregulating expression of a repressor. These findings delineate a cascade of genes in the differentiation of commissural neurons in the spinal cord (Fig. 9).

Downstream target of a proneural bHLH protein

Many homeobox genes are expressed downstream of proneural bHLH genes. Some homeodomain proteins are directly involved in the specification of neuronal identity, for example, Phox2a regulates the expression of the noradrenaline-synthesizing enzyme, dopamine-β-hydroxylase (reviewed by Goridis and Rohrer, 2002). However, it remained to be determined what genes are direct downstream targets of proneural bHLH proteins. In this study, we demonstrated that Math1 directly activated expression of a homeobox gene, *Mbh1*, which is necessary and sufficient for the specification of commissural neuron identity. This finding indicates that some aspects of neuronal identity are determined immediately downstream of proneural bHLH proteins.

Enhancer analyses using transgenic mice showed the critical

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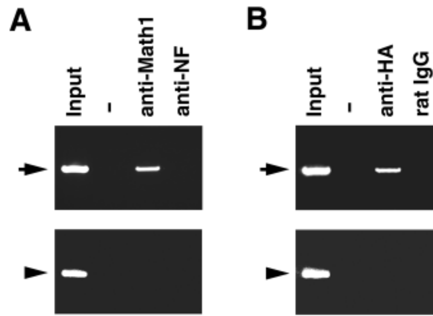

Fig. 6. Math1 bound the *Mbh1* enhancer containing the E-box in the spinal cord. (A) ChIP demonstrating binding of endogenous Math1 to the enhancer. Formaline-cross-linked chromatin from the E10.5 spinal cord was incubated without (–) and with rabbit polyclonal antibodies against Math1 and neurofilament. (B) Misexpressed Math1 also bound the enhancer. One day after electroporation of *Math1-HA*, cross-linked chromatin from the E12.5 spinal cord was incubated without (–) and with a rat monoclonal anti-HA antibody and IgG. Immunoprecipitates were analyzed by PCR using primers specific to the *Mbh1* enhancer (upper panels) and to the region 5.8 kb upstream of the E-box as a negative control (lower panels). Each input represents DNA purified from the chromatin before immunoprecipitation. These data are representative of two (A) and four (B) independent experiments. Arrows and arrowheads indicate the amplified 502 bp and 546 bp DNA fragments, respectively.

role of the E-box 3′ of the *Mbh1* gene for its expression. Math1 is suggested to autoregulate its expression through an E-box 3′ of the *Math1* gene (Helms et al., 2000). The nucleotide sequences of the two E-boxes were identical. There is another Bar-class homeobox gene, *Mbh2* (*Barhl1* – MGI), of which expression is lost in *Math1*$^{-/-}$ mice (Bermingham et al., 2001). We could not find a sequence similar to the 123 bp fragment containing the E-box 3′ of the *Mbh1* gene in the *Mbh2*-flanking genome sequences. Expression patterns of *Mbh1* and *Mbh2* are similar but not identical (T.S., T. Hama and R.S., unpublished), as is the case for their *Xenopus* orthologs, *Xbh1* and *Xbh2* (Patterson et al., 2000). Regulatory mechanisms of *Mbh1* and *Mbh2* may be different.

The sequence outside the 123 bp was required for efficient and restricted expression of *lacZ* in transgenic embryos, suggesting that Math1 regulates *Mbh1* by collaborating with another factor that may bind a site other than the E-box. Mash1 could not activate *lacZ* expression from Tg12 in reporter assays using the spinal cord. However, this finding may not simply imply that Mash1 cannot bind the E-box, because misexpression of *Mash1* efficiently activated *lacZ* expression from Tg18, which carried three copies of the E-box, in 10T1/2 cells (data not shown). These findings also suggest that there is a factor that specifically interacts with Math1 to activate gene expression in the spinal cord. Misexpression of *Math1* induced ectopic expression of *Mbh1* in more ventral regions, suggesting that the factor may not be restricted to the dorsalmost area of the spinal cord, where endogenous *Mbh1* expression starts. A bHLH protein, NeuroM, has been shown to interact with LIM-type homeodomain proteins and regulate *Hb9* (Lee and Pfaff, 2003). In the dorsal spinal cord, *Lhx9* is expressed downstream of Math1 (Figs 4, 5), but misexpression of *Lhx9* did not activate either endogenous *Mbh1* expression (Saba et al., 2003) or *lacZ* expression from Tg12 in reporter assays (data not shown).

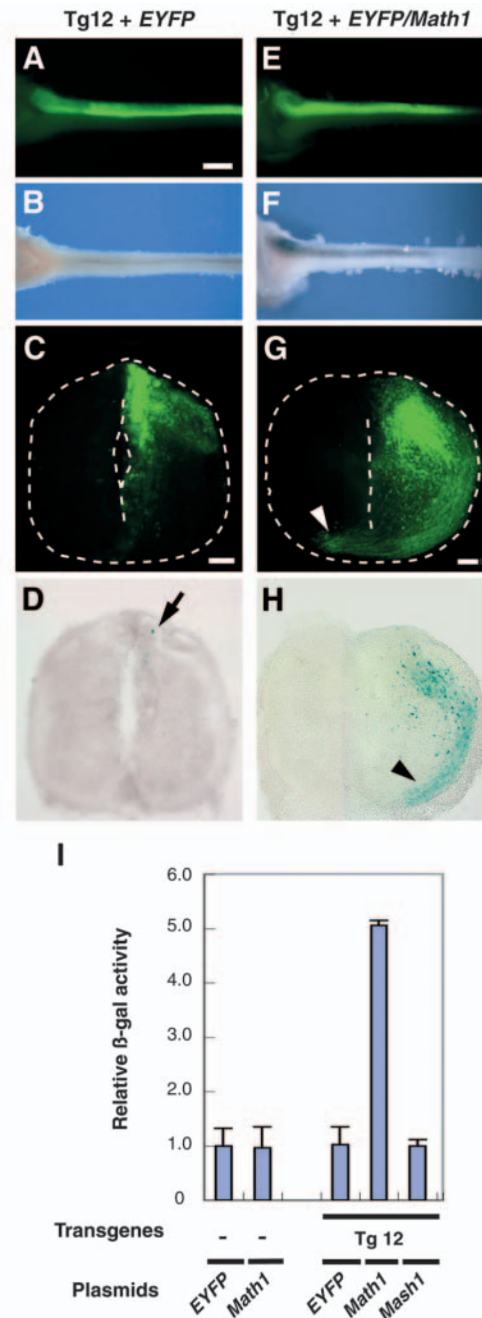

Fig. 7. Transcriptional activation by Math1 in the spinal cord. The E11.5 spinal cord was electroporated with Tg12 and either *EYFP* (A-D) or *EYFP/Math1* (E-H), and stained with X-gal, two days after electroporation. (A,B,E,F) Dorsal views of the spinal cord. (C,D,G,H) Transverse sections at brachial levels. Dark (A,C,E,G) and illuminated (B,D,F,H) views to show transfected (EYFP$^+$) and β-gal$^+$ cells, respectively. Upper (in A,B,E,F) and right (in C,D,G,H) sides were transfected with the genes. Arrow, β-gal$^+$ cell; arrowheads, commissural axons. Scale bars: in A, 1 mm for A,B,E,F; in C, 100 μm for C,D; in G, 100 μm for G,H. (I) Quantitative analysis of transcriptional activation by Math1. Two days after electroporation, cell extracts were prepared from EYFP$^+$ portions. After normalization to EYFP fluorescence, the data are expressed as β-gal$^+$ activity relative to the activity obtained by transfection of *EYFP* alone. Error bars indicate standard error of at least three independent experiments.

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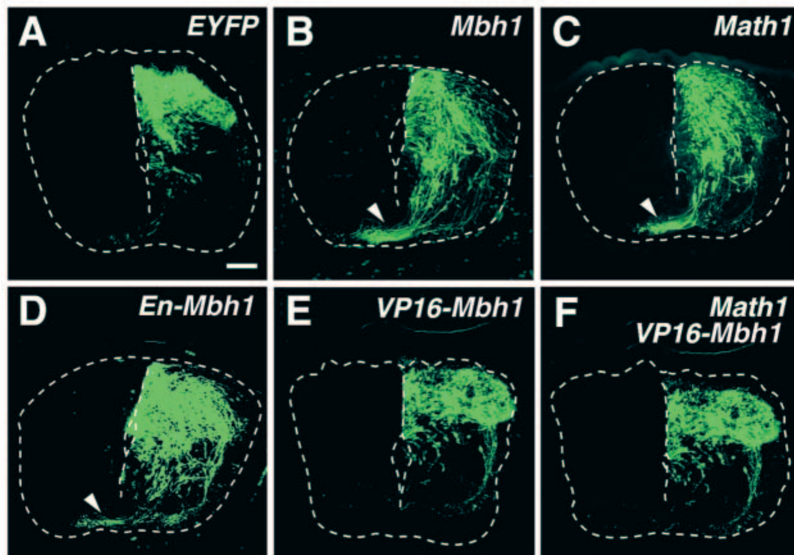

Fig. 8. Mbh1 function as a transcriptional repressor. Transverse sections at brachial levels of the spinal cord, two days after electroporation at E11.5 of *EYFP* alone (A), *EYFP/Mbh1* (B), *EYFP/Math1* (C), *EYFP/En-Mbh1* (D), *EYFP/VP16-Mbh1* (E) and *EYFP/Math1/VP16–Mbh1* (F). Similar patterns of EYFP+ axons were observed through all axial levels of the spinal cord in all electroporated EYFP+ embryos (*n*=20, 32, 20, 10, 10 and 10 for *EYFP*, *EYFP/Mbh1*, *EYFP/Math1*, *EYFP/En-Mbh1*, *EYFP/VP16-Mbh1* and *EYFP/Math1/VP16–Mbh1*, respectively). Arrowheads indicate EYFP+ commissural axons. Scale bar: 100 μm.

Spatiotemporal expression of *Mbh1*

Consistent with the cascade from *Math1* to *Mbh1*, *Mbh1* was expressed in many domains that expressed *Math1*, including the dorsal spinal cord and hindbrain. But *Mbh1* was not expressed in all places that expressed *Math1*, such as the inner ear. This finding suggests that Math1 is not sufficient and requires an additional factor for *Mbh1* expression. β-gal activity in the transgenic mice bearing Tg4 started to be detected at the same time as endogenous *Mbh1* expression. The β-gal activity faded after E13.5, whereas endogenous *Mbh1* expression persisted to at least E18.5. This finding indicates that the 2.5 kb 3′ fragment containing the E-box was sufficient for initiation, but not for maintenance, of *Mbh1* expression. In agreement with this, Math1 is abundant at the onset of *Mbh1* expression.

Mbh1 was also expressed in domains that did not express *Math1*, such as the dorsal diencephalon (Saito et al., 1998). *lacZ* expression was not detected in those areas even in transgenic mice carrying DNA fragments encompassing from –4.5 kb to +11 kb of the *Mbh1* genome, suggesting that *Mbh1* expression and its maintenance in various domains is controlled by many *cis*-regulatory elements dispersed throughout the *Mbh1* locus.

Function of Mbh1

Analysis using chimeric proteins, En-Mbh1 and VP16-Mbh1, indicated that Mbh1 is a potential transcriptional repressor. Bar-class homeodomain proteins contain a short sequence motif (FxIxxIL), called FIL, in their N-terminal regions (Saito et al., 1998). This motif closely resembles some examples of the eh1 motif (Smith and Jaynes, 1996), which mediates transcriptional repression by interacting with Groucho-family co-repressors. Many of the transcription factors that are expressed in progenitor domains of the ventral spinal cord function as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001; William et al., 2003). Transcriptional repression may be a general feature in regulating the neuronal fate. Interestingly, a number of genes expressed by postmitotic neurons are silenced by Nrsf/Rest in non-neuronal cells (reviewed by Schoenherr and Anderson, 1995). One of the genes, *Scg10* (*Stmn2* – Mouse Genome Informatics), which is a pan-neuronal marker, is de-repressed downstream of bHLH proteins. As Mbh1 is expressed by postmitotic neurons in the spinal cord, Mbh1 might also be implicated in pan-neuronal differentiation through the repression of *Nrsf* (*Rest* – Mouse Genome Informatics). However, misexpression of *Mbh1* in NIH3T3 cells could not activate *Scg10* expression (data not shown), suggesting that Mbh1 is involved only in the specification of commissural neuron identity. These findings indicate that Mbh1 functions in a cascade controlling specific differentiation to commissural neurons. In parallel with this cascade, another cascade controlling pan-neuronal differentiation may be activated by Math1.

In vivo analysis of transcriptional activation

Quantitative analysis of enhancers and promoters has been mostly performed using cell lines. Cell lines that are suitable for the analysis of a particular gene, however, are not always available. We have established a quick and efficient method to introduce DNA into the developing nervous system using in vivo electroporation, even if the size of DNA is larger than 12 kb (Saito and Nakatsuji, 2001; Saba et al., 2003). Our present work indicates that embryonic tissues can be a good source for transcriptional analysis using in vivo electroporation, because EYFP+ portions of the embryonic spinal cord provided enough protein for the analysis. This approach will be very powerful for examination of gene function, where suitable cell lines are not available.

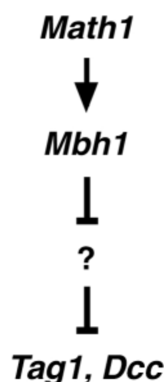

Fig. 9. Transcriptional cascade to generate commissural neurons downstream of Math1.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/9/2147/DC1

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