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Regulation of motor neuron subtype identity by repressor activity of Mnx class homeodomain proteins

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SUMMARY

In the developing spinal cord, motor neurons acquire columnar subtype identities that can be recognized by distinct profiles of homeodomain transcription factor expression. The mechanisms that direct the differentiation of motor neuron columnar subtype from an apparently uniform group of motor neuron progenitors remain poorly defined. In the chick embryo, the Mnx class homeodomain protein MNR2 is expressed selectively by motor neuron progenitors, and has been implicated in the specification of motor neuron fate. We show here that MNR2 expression persists in postmitotic motor neurons that populate the median motor column (MMC), whereas its expression is rapidly extinguished from lateral motor column (LMC) neurons and from preganglionic autonomic neurons of the Column of Terni (CT). The extinction of expression of MNR2, and the related Mnx protein HB9, from postmitotic motor neurons appears to be required for the generation of CT neurons but not for LMC generation. In addition, MNR2 and HB9 are likely to mediate the suppression of CT neuron generation that is induced by the LIM HD protein Lim3. Finally, MNR2 appears to regulate motor neuron identity by acting as a transcriptional repressor, providing further evidence for the key role of transcriptional repression in motor neuron specification.

Supplemental figures available online

Key words: Motor neuron, Motor neuron specification, Mnx class homeodomain proteins, Transcriptional repression

INTRODUCTION

A crucial early step in the assembly of neural circuits is the generation of neurons with distinct identities and patterns of connectivity. In the vertebrate central nervous system (CNS), the specification of neuronal identity is initiated by inductive factors that are secreted from local cell groups (Jessell and Melton, 1992). One role of such factors is to impose a specific profile of transcription factor expression on neural progenitor cells, thus restricting their developmental potential and directing the production of specific classes of neuron (Goridis and Brunet, 1999). The later differentiation of postmitotic neurons is also marked by distinct profiles of transcription factor expression and there is emerging evidence that the postmitotic expression of these proteins contributes to the assignment of neuronal subtype identity (Moran-Rivard et al., 2001; Gross et al., 2002; Muller et al., 2002). Despite many advances in the identification of neuronal determinants, for most classes of neuron, the logic by which hierarchies of transcription factors specify distinct neuronal subtype identity has not been adequately resolved.

One region of the CNS in which some progress has been made in linking inductive signaling and transcription factor expression to neuronal fate is the developing spinal cord (Jessell, 2000; Shirasaki and Pfaff, 2002), where the pathway of motor neuron specification has been defined in greater detail than for other neuronal classes (Briscoe and Ericson, 2001). The generation of motor neurons depends initially on the graded signaling activity of Sonic hedgehog (Shh), an inductive factor secreted by the notochord and floorplate (Chiang et al., 1996; Ericson et al., 1996; Patten and Placzek, 2000; Litingtung and Chiang, 2000). Shh signaling specifies the identity of motor neuron progenitors by regulating the pattern of expression of a set of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors that fall into two major groups: a set of class I proteins that are repressed by Shh signaling; and a set of class II proteins that are activated by Shh (Briscoe et al., 2000; Briscoe and Ericson, 2001). These proteins function primarily as transcriptional repressors (Muhr et al., 2001), and their selective cross-regulatory interactions help to establish specific neural progenitor domains and to sharpen the boundaries between these domains (Briscoe et al., 1999; Briscoe et al., 2000; Jessell, 2000; Vallstedt et al., 2001).

Motor neuron progenitors are restricted to a narrow region of the ventral neural tube that has been termed the pMN domain (Briscoe et al., 2000; Jessell, 2000; Pierani et al.,

2001). The cross-regulatory interactions between class I and class II proteins that establish the pMN domain lead, in turn, to the expression of a distinct set of downstream transcription factors that include the HD proteins MNR2 and Lim3 (Tsuchida et al., 1994; Tanabe et al., 1998; Sharma et al., 1998). MNR2 is a member of an evolutionarily conserved subgroup of Mnx class HD proteins (Ferrier et al., 2001), which includes the vertebrate HB9 (Hlxb9) (Pfaff et al., 1996; Saha et al., 1997; Ross et al., 1998; Tanabe et al., 1998) and *Drosophila* HB9 (Broihier and Skeath 2002) proteins, and homologs in sea urchin and amphioxus (Bellomonte et al., 1998; Ferrier et al., 2001). The vertebrate HB9 protein is expressed by postmitotic motor neurons, and genetic studies in mouse have revealed its role in the consolidation of motor neuron identity (Arber et al., 1999; Thaler et al., 1999). More recently, a similar function for *Drosophila* HB9 has been demonstrated during *Drosophila* motor neuron development (Broihier and and Skeath, 2002). In the chick, the expression of MNR2 differs from Lim3 and all other progenitor HD proteins in that its expression is restricted to cells in the pMN domain (Tanabe et al., 1998). Moreover, gain-of-function studies have provided evidence that the ectopic expression of MNR2 in dorsal progenitor cells specifies many aspects of motor neuron identity, while concomitantly suppressing spinal interneuron fates (Tanabe et al., 1998).

After motor neurons have left the cell cycle, they acquire columnar subtype identities that have classically been revealed by the position of motor neuron cell bodies in the spinal cord and by the pattern of motor axon projections in the periphery (Landmesser, 1978a; Landmesser, 1978b; Tosney et al., 1995). Five major columnar groups of motor neuron can be recognized on the basis of these criteria. Two of these groups are found within the median motor column (MMC): a set of medial MMC neurons that is generated at all rostrocaudal levels of the spinal cord and that extends axons to axial muscles. At thoracic levels, a set of lateral MMC neurons is generated that project their axons to body wall muscles (Tosney et al., 1995). A third set, pre-ganglionic autonomic motor neurons [termed Column of Terni (CT) neurons in chick], is also generated selectively at thoracic levels and these neurons project axons to sympathetic neuronal targets (Prasad and Hollyday, 1991). The final two columnar groups are found within the lateral motor column (LMC) at limb levels of the spinal cord: medial LMC neurons project axons to ventrally derived limb muscles and lateral LMC neurons project their axons to dorsally derived limb muscles (Landmesser, 1978b; Tosney et al., 1995).

Molecular insights into the specification of motor neuron columnar identity have derived, in part, from the observation that each columnar subclass of motor neurons is distinguishable by a distinctive profile of LIM HD transcription factor expression (Tsuchida et al., 1994; Ensini et al., 1998; Liu et al., 2001). Moreover, genetic studies in mice have begun to provide evidence that the combinatorial expression of LIM HD proteins regulates the subtype identity and connectivity of spinal motor neurons (Shirasaki and Pfaff, 2002). For example, Isl1 function is required for the generation of all spinal motor neurons (Pfaff et al., 1996), Lim3 (Lhx3) and Gsh4 (Lhx4) impose aspects of medial MMC identity (Sharma et al., 1998; Sharma et al., 2000), and the expression of Lim1 by lateral LMC neurons establishes dorsal motor axonal trajectories in the limb (Kania et al., 2000). The initial specification of lateral LMC neuronal identity appears to be achieved by local retinoid signals provided by motor neurons themselves, through the induction of Lim1 and the repression of Isl1 expression (Sockanathan and Jessell, 1998). However, many of the extrinsic and intrinsic signaling pathways that specify motor neuron columnar identity remain to be defined.

Some of the HD proteins expressed by motor neuron progenitors, notably Lim3 and Nkx6.1, continue to be expressed by subsets of postmitotic motor neurons (Tsuchida et al., 1994; Sharma et al., 1998; Cai et al., 2000). One potential strategy for assigning motor neuron columnar identity may, therefore, involve the persistent expression of progenitor cell transcription factors in subsets of postmitotic motor neurons. We show that expression of the progenitor HD protein MNR2 persists in a subset of postmitotic motor neurons, primarily those destined to populate the medial MMC, whereas its expression is rapidly extinguished from CT neurons and most LMC neurons. By contrast, HB9 is more widely expressed in postmitotic somatic motor neurons. The differential expression of MNR2 and HB9 in postmitotic neurons appears to contribute to the assignment of spinal motor neuron subtype identity. In particular, the extinction of expression of MNR2 and HB9 from postmitotic motor neurons is required for the generation of CT neurons. Moreover, the action of Lim3 in suppressing CT generation (Sharma et al., 2000) appears to be mediated through its ability to activate expression of MNR2 and HB9. Thus, in neural progenitor cells, MNR2 appears to function in the initial specification of motor neuron identity, whereas its later expression appears to regulate motor neuron columnar subtype identity. In addition, our results indicate that the ability of MNR2 to regulate motor neuron identity reflects its role as a transcriptional repressor, providing further evidence for the key role of transcriptional repression in motor neuron specification.

MATERIALS AND METHODS

In ovo electroporation

DNA solutions [5 mg/ml in TE buffer (pH 7.5), with 0.2% Fast Green to permit visualization of injected solution in the embryo] were injected into the lumen of the neural tube of Hamburger Hamilton (HH) (Hamburger and Hamilton, 1951) stage 10-15 embryos. Electroporation was performed using 5× 50 msecond pulses at 30 V applied across the embryo using a horizontal platinum/iridium wire (90% platinum/10% iridium; FHC) and a T820 BTX Electrosquare Porator (Genetronics). A solution of 1000 U/ml penicillin and streptomycin (Gibco BRL) was added to the egg chamber. Embryos were incubated for 2-4 days and analyzed at HH stages 23-29.

Recombinant retroviral vectors and expression constructs

MNR2 cDNA was isolated as described previously (Tanabe et al., 1998), and a *MNR2ΔC*-terminal construct was prepared by PCR-based cloning, fusing amino acids 1-218 of MNR2 in frame with a series of five Myc-epitope tags (Turner and Weintraub, 1994). The MNR2 N-terminal deletion series was generated using a PCR-based approach, adding a methionine to truncated MNR2 proteins. N-terminal deletions of 5, 14, 27, 45 and 70 amino acid residues were generated, and truncated sequences were cloned into *RCASBP(B)* constructs using a SLAX shuttle vector (Hughes et al., 1987; Morgan and Fekete, 1996). MNR2 HD constructs containing amino acids 146-218 were fused to a series of Myc tags (MNR2 HD), to the Engrailed

repressor domain [MNR2-EnR (Smith and Jaynes, 1996)], to the VP16 activation domain [MNR2-VP16 (Triezenberg et al., 1988)] or to amino acids 185-243 of E1a [MNR2-E1a (Boyd et al., 1993)]. A mutant C-terminal domain of E1a (MNR2-E1a^{mut}) was prepared by PCR mutagenesis of nucleotides encoding amino acids 235-237 of E1a to alanines (PLDLS→PLAAA). A CMV enhancer, β-actin promoter-based CAGGS plasmid was used to express MNR2-EnR.

Immunocytochemistry and in situ hybridization histochemistry

RALDH2 was detected using a rabbit polyclonal antibody (Sockanathan and Jessell, 1998). Monoclonal (4D5), rabbit polyclonal (K5) and guinea pig polyclonal sera were used to detect Isl1/2 proteins (Tsuchida et al., 1994; Tanabe et al., 1998). Isl2 was detected with monoclonal antibody (mAb) 4H9 and Isl1 was detected with rabbit polyclonal sera A8 (Tsuchida et al., 1994); Lim1/Lim2 was detected with mAb 4F2 and rabbit antibody T2 (Tsuchida et al., 1994); Lim3 was detected with mAb 4E12 (Ericson et al., 1997); and Chx10 was detected with a rabbit polyclonal serum (Ericson et al., 1997). Guinea pig polyclonal serum was used to detect Olig2 (Novitch et al., 2001). Rabbit polyclonal serum was used to detect Irx3 (Novitch et al., 2001) and a monoclonal antibody was used to detect Nkx2.2 (Ericson et al., 1997).

Chick embryos were fixed and prepared for immunocytochemistry as described (Novitch et al., 2001). Double- and triple-label analyses were perfomed with a BioRad 1024 confocal microscope using Cy3-, Cy5- and FITC-conjugated secondary antibodies (Jackson). β-galactosidase staining was performed as described (Kania et al., 2000), in situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993; Tsuchida et al., 1994) using *MNR2*, *HB9*, *ChAT*, *BMP5*, *BMP4*, and *ephrinA5* probes. *BMP5* and *BMP4* cDNAs were obtained from Dr A. Kottman.

Gal4 transcription assay

Protein sequence N-terminal to the MNR2 HD was cloned into the Gal4 DNA-binding domain vector pSG424 (Sadowski and Ptashne, 1989). COS-1 cells were co-transfected with pSG424 constructs, Gal4x5-E1b Luciferase and pRL-TK (Promega) plasmids using Fugene-6 lipofection reagent (Roche). Cells were harvested 48 hours later, and luciferase activity measured using a Dual-Luciferase Assay Kit (Promega). Gal4-luciferase activity was normalized to TK-Renilla luciferase activity. Additional Gal4-constructs included pSG424-MyoD (Weintraub et al., 1991) and pSG424-Engrailed repressor domain. Luciferase activity was compared with values obtained with transfection of the Gal4 reporter plasmid alone.

RESULTS

MNR2 expression persists in a subset of postmitotic motor neurons

MNR2 is expressed by all motor neuron progenitors but its expression is rapidly extinguished from many postmitotic motor neurons (Tanabe et al., 1998). To examine whether MNR2 expression persists in postmitotic motor neurons, we analyzed the spinal cord of chick embryos at stage 29, by which time motor neurons have segregated into molecularly distinct columnar subtypes (Sockanathan and Jessell, 1998). We compared the profile of expression of MNR2 with that of the closely-related HD protein HB9, and with the LIM HD proteins Isl1 and Isl2.

At stage 29, MNR2 expression was detected in medial MMC neurons at all axial levels of the spinal cord (Fig. 1A,C,E). At thoracic levels of the spinal cord, MNR2 was expressed in only a few lateral MMC neurons (Fig. 1C) and was absent from preganglionic autonomic motor neurons of the Column of Terni (CT) (Fig. 1C; supplementary Fig. S1 at dev.biologists.org/supplemental). MNR2 was not expressed by hindlimb level lateral motor column (LMC) neurons (Fig. 1E),

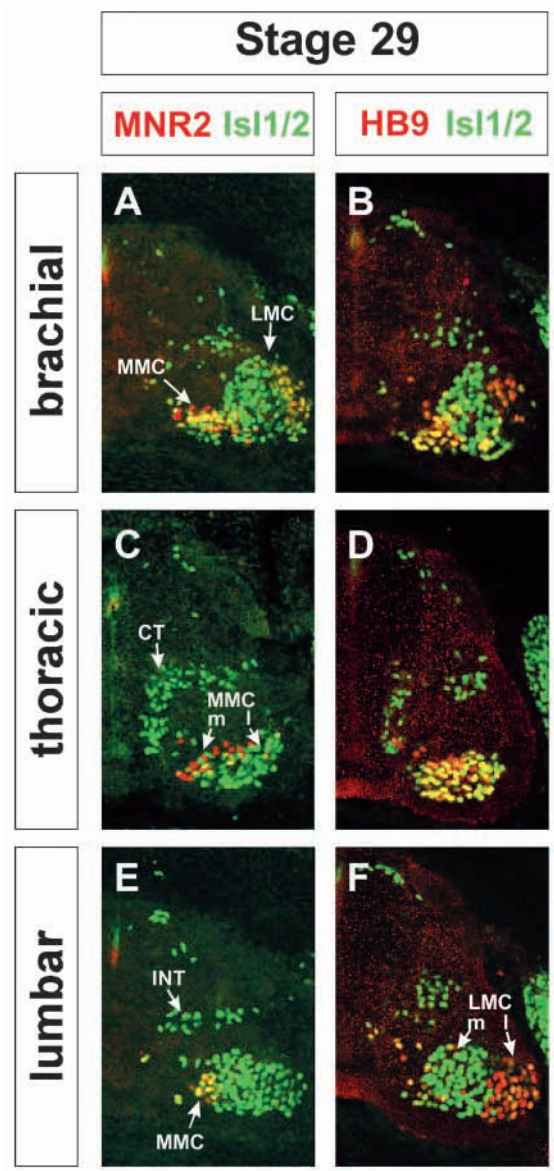

Fig. 1. Restricted expression of MNR2 and HB9 by postmitotic spinal motor neurons. Transverse sections through stage 29 chick spinal cord reveal the columnar restriction of MNR2 (A,C,and E) and HB9 (B,D,F) expression within motor neurons. (A) At brachial levels of the spinal cord, MNR2 is expressed in the medial MMC and in a lateral population of motor neurons, located within the confines of the LMC. (B) At brachial levels, HB9 is expressed by medial MMC and a subset of lateral LMC neurons. Isl1/2 is expressed in all medial MMC and LMC neurons. A dorsal population of interneurons also expresses Isl1. (C) At thoracic levels, MNR2 is expressed in all medial (m) MMC neurons but by only a few lateral (l) MMC neurons and not by CT neurons. (D) At thoracic levels, HB9 is expressed in all MMC neurons but not in CT neurons. (E) At lumbar levels, MNR2 is expressed in medial MMC neurons but not in LMC neurons. (F) At lumbar levels, HB9 is expressed in medial MMC and lateral LMC neurons. INT, interneuron.

and at forelimb levels was expressed by only a few laterally positioned neurons within the LMC (Fig. 1A). Many of these laterally placed MNR2$^+$ neurons co-expressed Lim3 (data not shown) and appeared to correspond to rhomboideus motor neurons: a set of laterally displaced medial MMC neurons (Tsuchida et al., 1994) (data not shown).

The expression of MNR2 is extinguished from LMC and CT motor neurons by stages 17-20, soon after motor neurons exit the cell cycle (see supplementary Fig. S1 at dev.biologists.org/supplemental). In these respects, the developmental profile of MNR2 expression in spinal motor neurons closely parallels that of Lim3, which is also rapidly extinguished from LMC and CT neurons but is maintained in medial MMC neurons (Tsuchida et al., 1994). In contrast to MNR2, the expression of HB9 persists in essentially all MMC neurons and in many lateral LMC neurons at both forelimb and hindlimb levels. However, as with MNR2, expression is not detected in CT neurons (Fig. 1B,D,F).

Restrictions in the columnar identity of ectopic motor neurons induced by MNR2

The persistence of MNR2 expression in medial MMC neurons raised the issue of whether the ectopic motor neurons that are induced in more dorsal locations in the spinal cord by MNR2 possess specific columnar identities. To assess this, we induced ectopic dorsal motor neurons by electroporation of *MNR2* into the dorsal neural tube of stage 10-12 chick embryos, permitting embryos to develop until stages 20-29 for analysis of generic and columnar markers of motor neuron identity. Electroporation of MNR2 into one side of the spinal cord (Fig. 2A) induced unilateral ectopic expression of four HD transcription factors normally associated with motor neuron differentiation (Lim3, Isl1, Isl2 and HB9) in neurons distributed along the dorsoventral axis of the spinal cord (Fig. 2B-E) (see also Tanabe et al., 1998). MNR2 also induced ectopic expression of the motor neuron Ig-family protein SC1 (Fig. 2F) and of the gene encoding the acetylcholine synthetic enzyme choline acetyltransferase (ChAT) (Tanabe et al., 1998). Moreover, the ectopic dorsal motor neurons induced by MNR2 projected axons out of the spinal cord (supplementary Fig. 2 at dev.biologists.org/supplemental). Together, these findings show that MNR2 is an effective inducer of ectopic motor neuron differentiation.

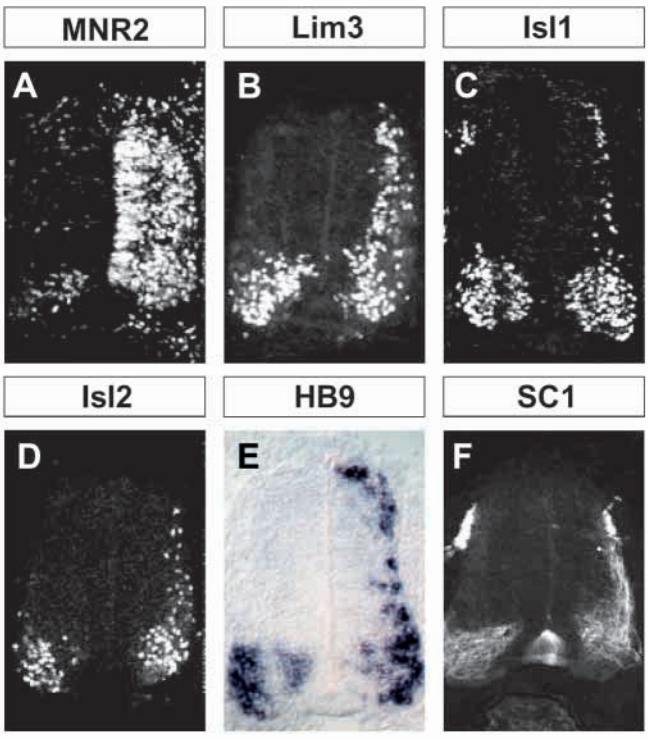

Fig. 2. Motor neuron inducing activity of MNR2 in chick spinal cord. (A) Widespread unilateral expression of MNR2 in stage 24 chick spinal cord, obtained by unilateral (right side) in ovo electroporation at stages 10-12. (B-F) Ectopic dorsal expression, after *MNR2* electroporation, of Lim3 (B), Isl1 (C), Isl2 (D), HB9 (E) and SC1 (F). Images are representative of more than 40 electroporated embryos.

Motor neurons induced by MNR2 were assayed for defining markers of columnar subtype identity. Medial MMC neurons were defined by expression of Lim3 (Fig. 3A) (Tsuchida et al., 1994), LMC neurons were defined by expression of retinaldehyde dehydrogenase-2 (RALDH2; Fig. 3B) (Sockanathan and Jessell, 1998), and the co-expression of Isl2 and Lim1 was used to define lateral LMC identity (Fig. 3C,D) (Tsuchida et al., 1994). Previous studies have not defined molecular markers that selectively identify CT neurons (Tanabe and Jessell, 1996). In a search for CT markers, we found that three genes, *BMP4*, *BMP5* and *ephrinA5*, are preferentially expressed by CT neurons. At stage 29, at thoracic levels, expression of *BMP5* is restricted to CT neurons, and it is not expressed by somatic motor neurons at any segmental level of the spinal cord (Fig. 3E,F,I,J). *BMP5* expression was evident at stage 23 in presumptive CT motor neurons prior to their dorsal migration (Fig. 3I,J). *BMP4* was similarly restricted to CT motor neurons at stage 29 but was not expressed at stage 23 (data not shown). *EphrinA5* was expressed selectively by CT neurons at thoracic levels, although the gene was also expressed by some LMC neurons (Fig. 3G,K,L). Thus, *BMP5* serves as the most reliable molecular marker of CT neuron identity.

We used these molecular markers to analyze, at stages 24-29, the identity of ectopic motor neurons induced by MNR2. Approximately 50% of ectopic motor neurons found at brachial, thoracic and lumbar levels co-expressed Isl1/2 and Lim3 (Fig. 3M,N), indicating that they possess a medial MMC-like identity. By contrast, ectopic dorsal motor neurons induced at limb levels of the spinal cord did not express RALDH2 (see Fig. 3P), nor did they co-express Isl2 and Lim1 (see Fig. 7F), indicating that they have not acquired LMC identity. In addition, the ectopic dorsal motor neurons induced at thoracic levels did not express *BMP5* (Fig. 3O), *BMP4* or *ephrinA5* (data not shown), indicating that they have not acquired CT neuronal identity.

Together, these findings provide evidence that ectopic dorsal motor neurons induced by MNR2 do not acquire a transcription factor profile characteristic of LMC or CT neurons. The coexpression of Isl1/2 and Lim3 in the absence of definitive LMC or CT markers suggests that some of the MNR2-induced ectopic motor neurons possess a medial MMC-like identity. The columnar identity of the remaining MNR2-induced motor neurons is uncertain, but their LIM HD profile is indicative of motor neurons with a lateral MMC-like character (Tsuchida et al., 1994).

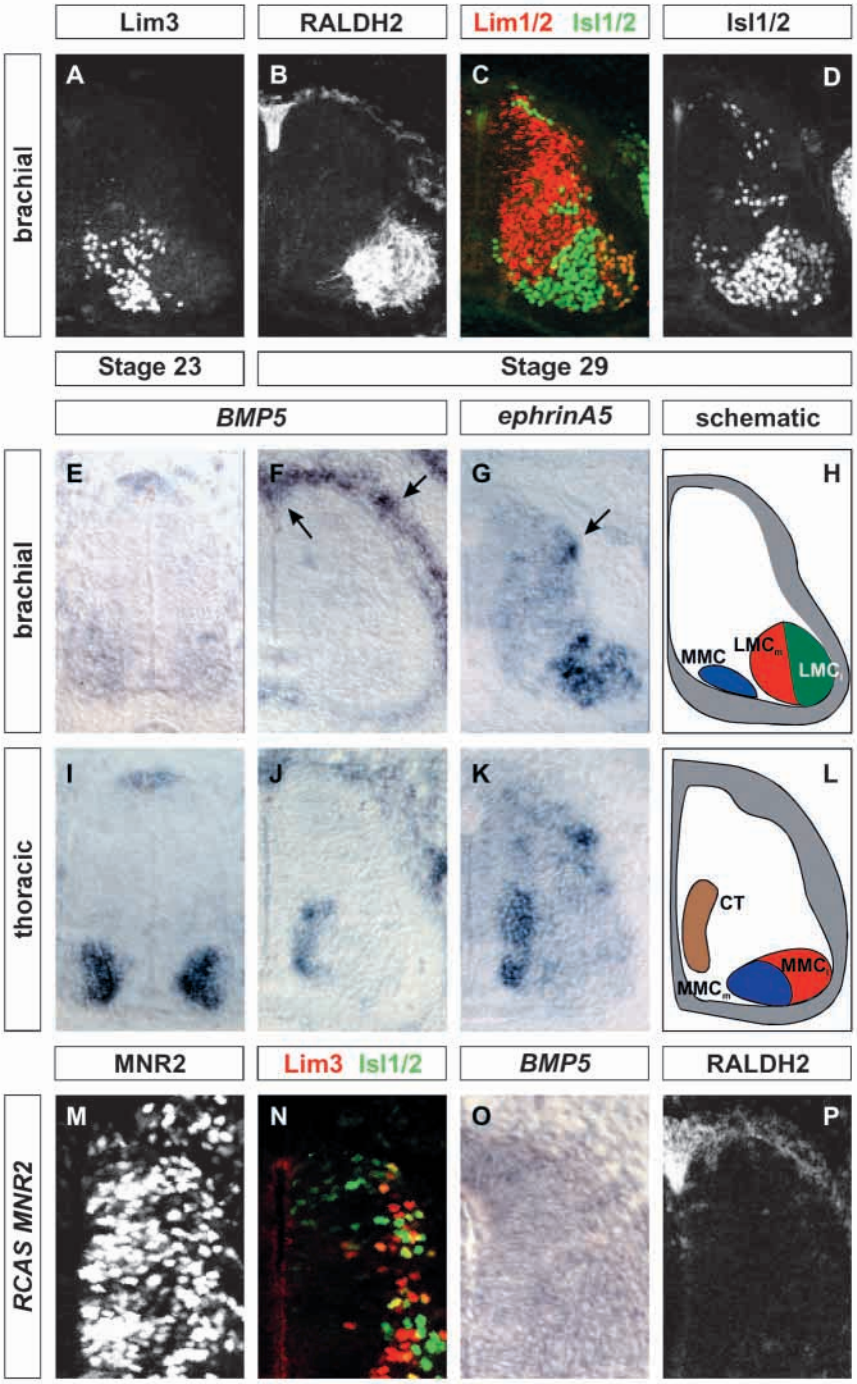

Fig. 3. Molecular markers of CT and LMC motor neuron subtype identity. (A) Selective expression of Lim3 by medial MMC neurons at brachial levels of stage 29 spinal cord. (B) Selective expression of RALDH2 by LMC neurons at brachial levels of stage 29 spinal cord. RALDH2 is also expressed by roof-plate cells. (C) Co-expression of Isl2 and Lim1 by lateral LMC neurons at brachial levels of stage 29 spinal cord. (D) Pattern of Isl1/2 expression in motor neurons and dorsal interneurons at brachial levels of stage 29 spinal cord. (E,F) Absence of expression of *BMP5* in motor neurons at brachial levels of the spinal cord. Arrows (F) indicate sites of *BMP5* expression in roof-plate (left arrow) and mesenchymal cells surrounding the spinal cord (right arrow). (G) Expression of ephrinA5 in a subset of LMC motor neurons at brachial levels of the spinal cord. Arrow indicates ephrinA5 expression in a small group of cells just ventral to the dorsal root entry zone. (H) Motor neuron columnar organization at brachial levels of the spinal cord. (I,J) Selective expression of *BMP5* in CT motor neurons at thoracic levels of the spinal cord. *BMP5* is also expressed by roof-plate cells. (K) Expression of ephrinA5 in CT motor neurons at thoracic levels of the spinal cord. (L) Motor neuron columnar organization at thoracic levels of the spinal cord. (M) Widespread expression of MNR2 in dorsal spinal cord after in ovo electroporation of *MNR2*. Analysis performed at stage 29. (N) Co-expression of Lim3 by Isl1/2+ ectopic motor neurons after *MNR2* electroporation. (O) Lack of expression of *BMP5* in ectopic motor neurons at thoracic levels after *MNR2* electroporation. (P) Lack of expression of RALDH2 in ectopic motor neurons at limb levels after *MNR2* electroporation. Images are representative of over 30 electroporated embryos

Extinction of MNR2 expression is necessary for the specification of CT identity

The rapid extinction of MNR2 and HB9 from CT neurons during normal development raised the issue of whether downregulation of Mnx-class HD proteins is required for the acquisition of CT columnar identity. To test this, we electroporated *MNR2* into the ventral neural tube at prospective thoracic levels of stage 12-14 embryos and analyzed the pattern of motor neuron generation at stages 27-29. Expression of MNR2 resulted in the appearance of Isl1/2$^+$ ectopic motor neurons in dorsal regions of the spinal cord (Fig. 4A,B). In addition, there was a striking loss of Isl1$^+$ CT motor neurons that are normally located in a dorsomedial position (Fig. 4A,B). To test whether the loss of these medially positioned Isl1$^+$ motor neurons reflects a failure in differentiation of CT neurons or simply a mispositioning, we assayed the expression of *BMP5*, *BMP4* and *ephrinA5* after *MNR2* electroporation. Expression of *BMP5*, *BMP4* and *ephrinA5* was lost on the electroporated side of the spinal cord (Fig. 4C,D; data not shown). Thus, maintaining expression of MNR2 in postmitotic motor neurons at thoracic levels of the spinal cord prevents motor neurons from assuming a CT identity (as assessed by

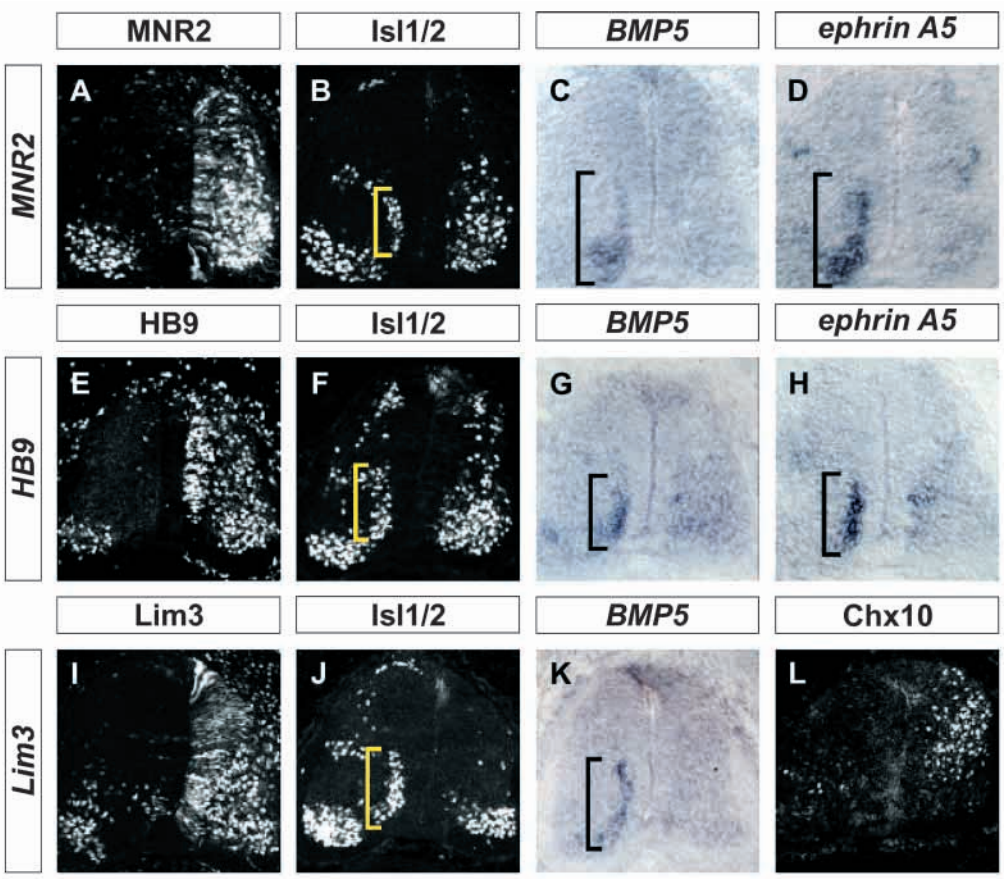

Fig. 4. Suppression of CT neuron generation by MNR2, HB9 and Lim3. (A) Widespread ectopic expression of MNR2 protein on the right side of spinal cord after *MNR2* electroporation. (B-D) Loss of dorsomedially located Isl1$^+$ motor neurons (B), and absence of expression of *BMP5* (C) and ephrinA5 (D) after unilateral *MNR2* electroporation. (E) Widespread ectopic expression of HB9 protein on the right side of spinal cord after *HB9* electroporation. (F-H) Loss of dorsomedially located Isl1$^+$ motor neurons (F) and absence of expression of *BMP5* (G) and ephrinA5 (H) after unilateral *Hb9* electroporation. (I) Widespread ectopic expression of Lim3 protein on the right side of spinal cord after *Lim3* electroporation. (J-K) Loss of dorsomedially located Isl1$^+$ motor neurons (J) and absence of *BMP5* expression (K), after unilateral *Lim3* electroporation. (L) Ectopic dorsal Chx10$^+$ (V2) interneurons induced by *Lim3* electroporation. Brackets indicate area of expression on the untreated side. Images are representative of 10-30 electoporated embryos.

their migratory route and profile of gene expression). Expression of HB9 also resulted in the loss of dorsomedially positioned Isl1$^+$ motor neurons, and similarly inhibited expression of *BMP5*, BMP4 and ephrinA5 (Fig. 4E-H; data not shown). These findings suggest that the extinction of expression of Mnx class HD proteins is a prerequisite for postmitotic motor neurons to progress to a CT identity.

The expression of Lim3, like MNR2, is extinguished from CT neurons, prompting us to examine whether Lim3 also suppresses the generation of CT neurons. We found that maintained expression of Lim3 in thoracic level motor neurons resulted in the loss of dorsomedially positioned Isl1$^+$ neurons, and in the inhibition of expression of *BMP5*, BMP4 and ephrinA5 (Fig. 4I-K; data not shown). These findings are consistent with studies in mice, where a suppression of pre-ganglionic autonomic neuron generation is observed after Lim3 (Lhx3 in mice) expression (Sharma et al., 2000). However, unlike MNR2, misexpression of Lim3 outside the context of motor neurons did not induce ectopic motor neurons but instead induced ectopic V2 interneurons, as revealed by the ectopic expression of Chx10 (Fig. 4L) (Tanabe et al., 1998; Thaler et al., 2002). Together, these findings show that MNR2, HB9 and Lim3 are each able to repress the differentiation of CT neurons.

We next addressed the issue of whether MNR2, HB9 and Lim3 act in parallel or sequential pathways to suppress the generation of CT neurons. We first examined whether misexpression of MNR2 within the normal domain of motor neuron generation maintains expression of Lim3 in postmitotic motor neurons. MNR2 was misexpressed at stages 12 to 14 and embryos were analyzed at stage 23, a time when *BMP5* expression has normally been initiated, and MNR2 and HB9 expression extinguished from prospective CT neurons. Thoracic level motor neurons in which MNR2 expression was maintained lacked *BMP5* expression (Fig. 5A,B), but did not exhibit prolonged expression of Lim3 or HB9 in prospective CT neurons (Fig. 5C-E). Similarly, prolonged expression of HB9 in thoracic level motor neurons failed to maintain expression of Lim3 or MNR2 (data not shown). Thus, the suppression of CT neuron specification by MNR2 and HB9 does not involve the maintenance of Lim3 expression in postmitotic motor neurons.

Conversely, we considered whether the ability of Lim3 to suppress the generation of CT neurons might result from the maintained expression of MNR2 or HB9 in prospective CT neurons. This possibility was suggested by two prior findings. First, the expression of Lim3 in dorsal spinal interneurons and dorsal root ganglion neurons (neurons that express Isl1) activates expression of HB9 (Tanabe et al., 1998). Second, the co-expression of Isl1 and Lim3 in neural progenitors is sufficient to induce HB9 expression in postmitotic neurons (Tanabe et al., 1998; Thaler et al., 2002). Prolonged expression of Lim3 in motor neurons at thoracic levels of the spinal cord resulted in the maintained expression of MNR2 or HB9 in postmitotic motor neurons (Fig. 5H-J) under conditions in which *BMP5* expression was extinguished (Fig. 5F,G).

Together, these findings suggest that Lim3 suppresses CT

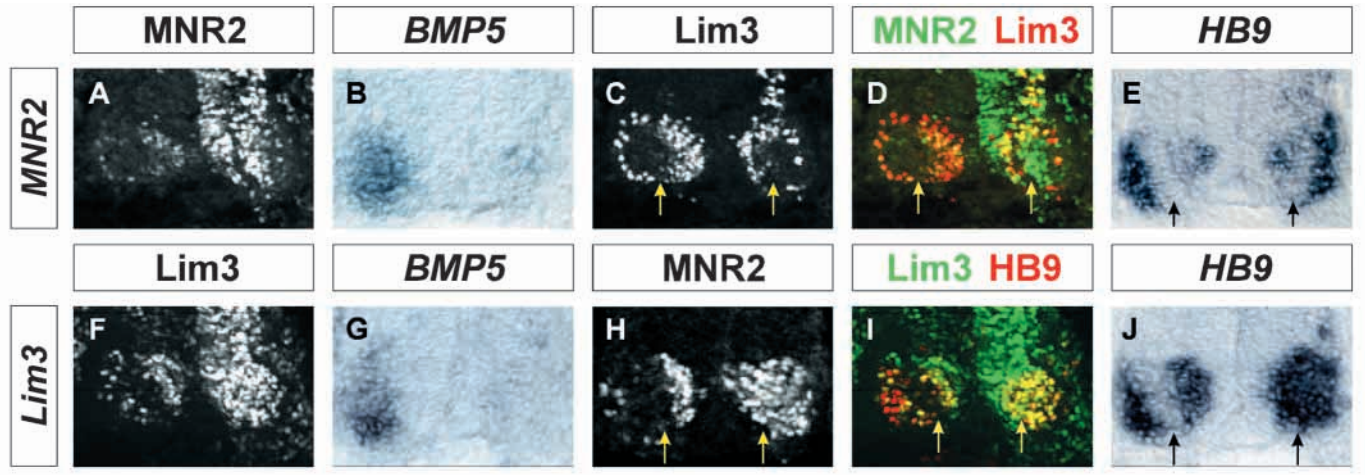

Fig. 5. Hierarchical interactions between MNR2, HB9 and Lim3 in the suppression of CT neuron generation. (A-E) Electroporation of MNR2 within the normal domain of motor neuron generation at thoracic levels (A) represses *BMP5* expression (B), but does not result in maintained Lim3 (C,D) or HB9 (E) expression in motor neurons. (F-J) Electroporation of *Lim3* within the normal domain of motor neuron generation at thoracic levels (F) represses *BMP5* expression (G), and results in maintained MNR2 (H) and HB9 (I,J) expression in postmitotic motor neurons. Images are representative of 20 electroporated embryos. Arrows in C-E,H-J indicate the approximate position of newly generated CT neurons, prior to their dorsal migration.

identity indirectly, through the maintained expression of Mnx-class HD proteins, and also indicate that the downregulation of MNR2 and HB9 expression is a prerequisite for thoracic level motor neurons to progress to a CT identity.

Differential requirements for extinction of MNR2 and Lim3 expression in the specification of LMC identity

At limb levels of the spinal cord, the expression of both MNR2 and Lim3 is rapidly extinguished from LMC neurons, raising the question of whether the downregulation of expression of both transcription factors is also required for the specification of LMC identity. To test this, we misexpressed MNR2 or Lim3 in motor neurons at forelimb and hindlimb levels of the spinal cord. Maintained expression of MNR2 did not result in the upregulation of Lim3 expression in LMC neurons (Fig. 6A-C), nor did it eliminate two definitive markers of LMC neuronal identity: RALDH2, and the co-expression of Isl2 and Lim1 (Fig. 6E-H). Similarly, we found that maintained expression of HB9 did not induce Lim3 (Fig. 6D) or extinguish LMC markers (data not shown). These findings are not unexpected given the expression of HB9 by many LMC neurons during normal development and the similar activities of MNR2 and HB9.

We next tested the influence of Lim3 on MNR2 expression and LMC specification. Maintaining Lim3 in limb level motor neurons resulted in persistent expression of MNR2 (data not shown), suppressed expression of RALDH2 and prevented the generation of $Isl2^{+}/Lim1^{+}$ motor neurons (Fig. 6I-L). These findings suggest that the extinction of Lim3 expression from postmitotic motor neurons is a key step in their progression to an LMC identity, a finding consistent with the role of Lhx3 (Lim3) in repressing LMC neuron specification in mouse (Sharma et al., 2000). In addition, these data indicate that the ability of Lim3 to repress LMC differentiation is independent of MNR2 expression, which is in contrast with the likely involvement of MNR2/HB9 in the Lim3-mediated repression of CT neuron specification.

Evidence that MNR2 functions as a transcriptional repressor

Our findings, taken together with those of Tanabe et al. (Tanabe et al., 1998), indicate that MNR2 has three main activities in ventral neuronal specification: (1) promoting the generation of motor neurons; (2) suppressing ventral interneuron generation; and (3) suppressing the generation of CT subtype identity. The ability of MNR2 to promote certain neuronal fates and inhibit others raises the issue of whether its regulatory activities depend on transcriptional activation or repression. To address this issue, we attempted to identify functional domains of MNR2, monitoring activity on the basis of its ability to induce ectopic Lim3 expression and to repress the differentiation of CT neurons.

Expression of a form of MNR2 that lacks the entire C-terminal domain resulted in ectopic expression of Lim3 along the dorsoventral axis of the spinal cord, with a level of inductive activity similar to that of the wild-type protein (Fig. 7A,B). Thus, sequences C-terminal to the HD appear to be dispensable for the motor neuron inductive activity of MNR2. By contrast, deletion of the N-terminal 70 amino acids of MNR2 rendered the protein inactive (Fig. 7A). However, deletion of the N-terminal 44 or 28 amino acids of MNR2 resulted in only a partial reduction in the ability to activate expression of Lim3 (Fig. 7A). Deletion of the 14 N-terminal residues of MNR2 did not significantly reduce Lim3 inductive activity (Fig. 7A). These results suggest that motifs present in the domain between residues 15 and 70 of the N terminus of MNR2, in the context of the MNR2 HD, are necessary for motor neuron inductive activity.

To examine whether the N-terminal domain of MNR2 functions as a transcriptional repressor or activator, we fused the entire N-terminal region of MNR2 to the DNA-binding domain of the yeast transcription factor Gal4 (Gal4-$MNR2^{N}$) and expressed this fusion protein in COS-1 cells, together with a Gal4-UAS reporter construct (Perlmann and Jansson, 1995). Fusions of MyoD or the *Drosophila* Engrailed repressor (EnR)

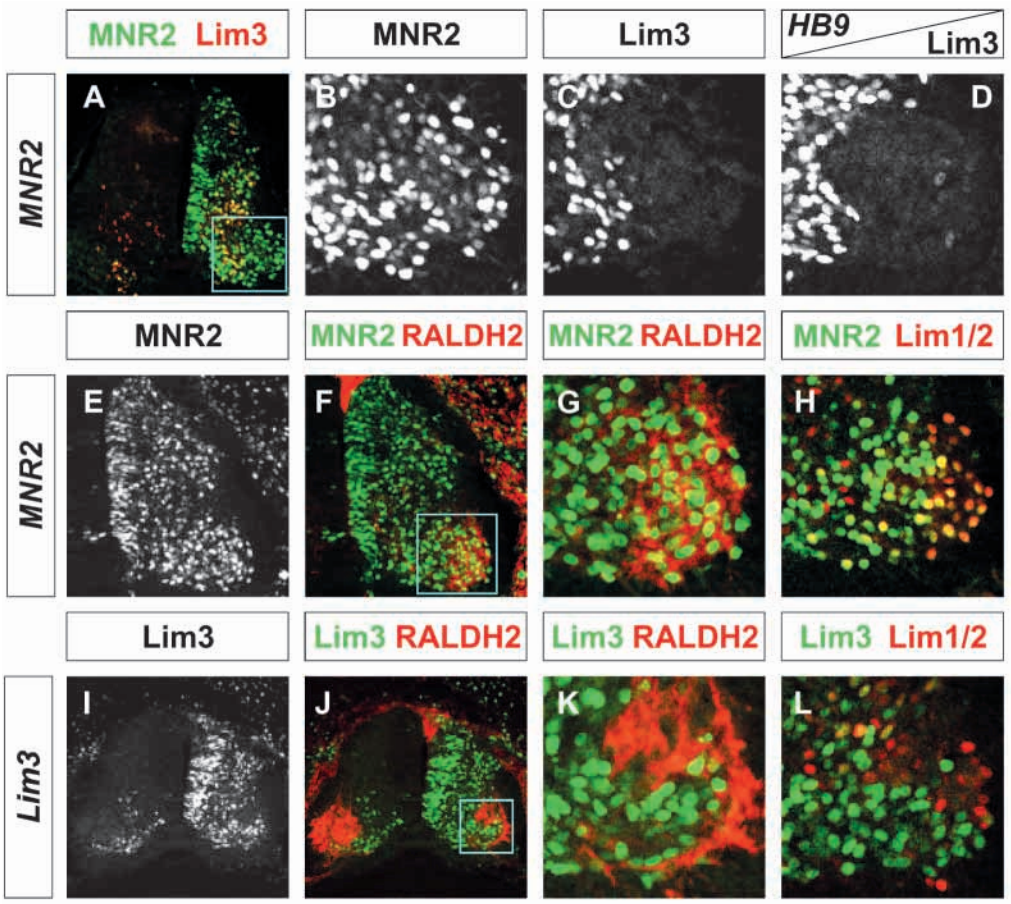

Fig. 6. Repression of LMC neuron specification by Lim3 but not by MNR2 or HB9.
(A-C) Electroporation of MNR2 within the normal domain of LMC neuron generation at brachial levels of the spinal cord does not maintain Lim3 expression in postmitotic LMC neurons. B and C, enlarged view of boxed area in A.
(D) Electroporation of HB9 within the normal domain of LMC neuron generation at brachial levels of the spinal cord does not maintain Lim3 expression in postmitotic LMC neurons. (E-H) Electroporation of MNR2 within the normal domain of LMC neuron generation at brachial levels of the spinal cord does not repress expression of RALDH2 or the generation of motor neurons that co-express Isl2 and Lim1. G and H, enlarged view of boxed area in E.
(I-L) Electroporation of Lim3 within the normal domain of LMC neuron generation at brachial levels of the spinal cord represses expression of RALDH2 and the generation of motor neurons that co-express Isl2 and Lim1. K and L, enlarged view of boxed area in J. Images are representative of 10-30 electroporated embryos.

domain to Gal4 provided controls for transcriptional activation and repression, respectively (Fig. 7C). We found that expression of the Gal4-MNR2^{N} fusion protein repressed transcription as efficiently as the Gal4-EnR protein (Fig. 7C). Thus, the N-terminal domain of MNR2 possesses transcriptional repressor activity in vitro, which suggests that MNR2 functions as a repressor in its neural patterning activities in vivo.

As most HD proteins that function as transcriptional repressors require the recruitment of co-repressor proteins, we analyzed the N-terminal 70 residues of MNR2 for potential co-repressor recruitment motifs. Analysis of the N-terminal sequence of MNR2 and related Mnx family members revealed the presence of a highly conserved ~15 residue N-terminal domain (Fig. 7D) that exhibits sequence conservation with a motif initially characterized in the Engrailed protein, termed the eh1 domain, which functions to recruit Groucho co-repressor proteins (Smith and Jaynes, 1996). However, as described above, a truncated MNR2 protein lacking the N-terminal 14 amino acid eh1 motif is able to induce ectopic Lim3 expression with wild-type efficiency (Fig. 7A,E). Moreover, a fusion protein comprising the MNR2 HD fused to the Engrailed eh1 domain had little or no Lim3 inductive activity (Fig. 7A,F). These findings raise doubts as to whether the motor neuron inductive activity of MNR2 requires the N-terminal eh1 domain or acts exclusively through recruitment of Groucho class co-repressors.

In a search for additional structural motifs within the N-terminal domain of MNR2 that might be required for its activity, we noted the presence of motifs that resemble, albeit loosely, sequences required for recruitment of a distinct class of transcriptional co-repressor, the Ctbp proteins (Fig. 8A) (Deltour et al., 2002; Turner and Crossley, 2001; Chinnadurai, 2002). The N-terminal domains of Mnx class HD proteins in other organisms also possess similar motifs (Fig. 8A). We therefore considered whether fusion of the MNR2 HD to an efficient Ctbp recruitment domain might mimic the activity of wild-type MNR2. We fused the MNR2 HD to the C terminus of the oncoprotein E1a, which contains a potent Ctbp co-repressor recruitment domain (Fig. 8B) (Boyd et al., 1993; Molloy et al., 2001). Ectopic expression of the MNR2 HD-E1a fusion protein resulted in a potent induction of Lim3^{+} cells in dorsal regions of the neural tube (Fig. 8C,D). In addition, expression of the MNR2 HD-E1a fusion protein at thoracic levels effectively suppressed the formation of CT neurons (Fig. 8G,H). To test the potential involvement of Ctbp recruitment in the activity of this fusion protein we made use of the finding that the three C-terminal amino acids of E1a are critical for Ctbp recruitment (Turner and Crossley, 2001; Chinnadurai, 2002). We expressed a form of the MNR2 HD-E1a fusion that had been mutated at these three residues (Fig. 8B) and found that this protein did not induce Lim3 expression, nor did it repress the generation of CT neurons (Fig. 8E,F; data not shown). Together, these findings support the idea that MNR2 normally functions in motor neuron specification through its role as a transcriptional repressor and raise the possibility that

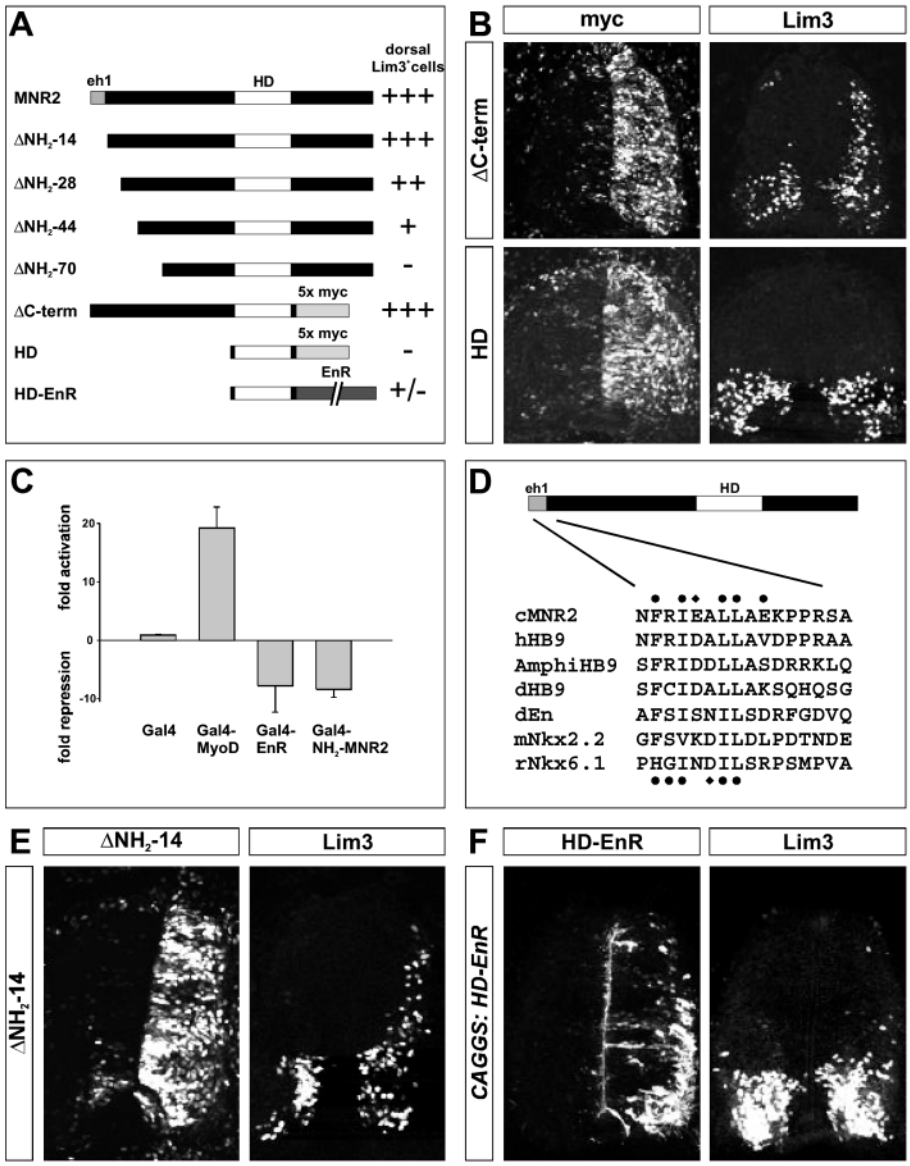

Fig. 7. Evidence that MNR2 can function as a transcriptional repressor. (A) Deletion isoforms of MNR2 tested for Lim3-inducing activity in vivo. Approximate activity of these proteins, assessed by induction of Lim3$^+$ cells, is indicated on the right, by comparison with wild-type MNR2. Electroporation of each of these constructs was performed at stage 10-12, and stage 25 embryos were analyzed for expression of Lim3. +++, more than 20 Lim3$^+$ cells per 12 μM section; ++, 10-12 Lim3$^+$ cells per section; +, 10 cells per section; –, no ectopic Lim3$^+$ cells per section. Six to ten embryos were analyzed for the activity of each construct. (B) Deletion of the C-terminal domain of MNR2 does not abolish Lim3 inductive activity. Expression of the MNR2 HD alone has no inductive activity. (C) The N-terminal domain of MNR2 functions as a potent transcriptional repressor when fused to a Gal4 DNA-binding domain in vitro. The repressive activity of the MNR2 domain is similar to that of a Gal4-Engrailed repressor domain fusion. A MyoD-Gal4 DNA-binding domain fusion acts as a potent transcriptional activator. COS-1 cells were co-transfected with Gal4-fusion constructs and a Gal4-E1b-luciferase reporter plasmid. In controls, a Gal4-MyoD construct activated E1b-luciferase activity 19.2±3.6-fold (s.e.m.) overexpression of Gal4 alone. A Gal4-EnR construct repressed E1b-luciferase activity 7.7±4.5-fold (s.e.m.). A fusion of Gal4 to protein sequence N-terminal to the MNR2 HD repressed E1b-luciferase activity 8.3±1.3-fold (s.e.m.). (D) Detection of an eh1 domain in all Mnx class HD proteins. MNR2 protein sequence is represented as a black bar. The HD is depicted as a white box and an N-terminal eh1 motif is depicted with a gray box. The sequence containing the eh1 motif in MNR2 has been aligned with other Mnx class HD proteins to illustrate the high degree of conservation of this motif. An alignment with the eh1 motif in Engrailed reveals several identical residues, highlighted by black circles. Eh1 motifs characterized in Nkx proteins are aligned below Engrailed. Black circles below the Nkx6.1 sequence highlight residues in either Nkx2.2 or Nkx6.1 that are identical to Engrailed. Black diamonds above MNR2 and below Nkx6.1 highlight acidic residues conserved between Mnx class and Nkx HD proteins. cMNR2, chick MNR2; hHB9, human HB9; AmphiHB9, *Amphioxus* homolog of HB9; dHB9, *Drosophila* homolog of HB9; dEn, *Drosophila* Engrailed; mNkx2.2, mouse Nkx2.2; rNkx6.1, rat Nkx6.1. (E) Expression of an MNR2 isoform lacking the 14 N-terminal residues retains wild-type Lim3 inducing activity. (F) Negligible Lim3 inductive activity of an MNR2 HD-Engrailed repressor domain fusion protein. Images are representative of over 10 electroporated embryos.

its repressor activity may involve recruitment of Ctbp-like co-repressors.

DISCUSSION

The differentiation of motor neurons with distinct subtype identities is a key step in the construction of functional spinal motor circuits. One early aspect of motor neuron diversification is the emergence of columnar identity, a process that is linked to the settling pattern of motor neuron cell bodies in the spinal cord and to the selectivity of motor axon projections into the periphery. The present studies of the developmental expression and function of MNR2 and HB9, two Mnx class HD transcription factors expressed by motor neuron progenitors and postmitotic motor neurons, provide evidence that the specification of motor neuron columnar identity involves three interrelated steps: (1) the persistence of expression of HD transcription factors that mark motor neuron progenitors within subsets of postmitotic motor neurons; (2) the differential extinction of HD transcription factors from different columnar subsets of motor neurons; and (3) the differential activities of HD transcription factors within different columnar subsets of motor neuron.

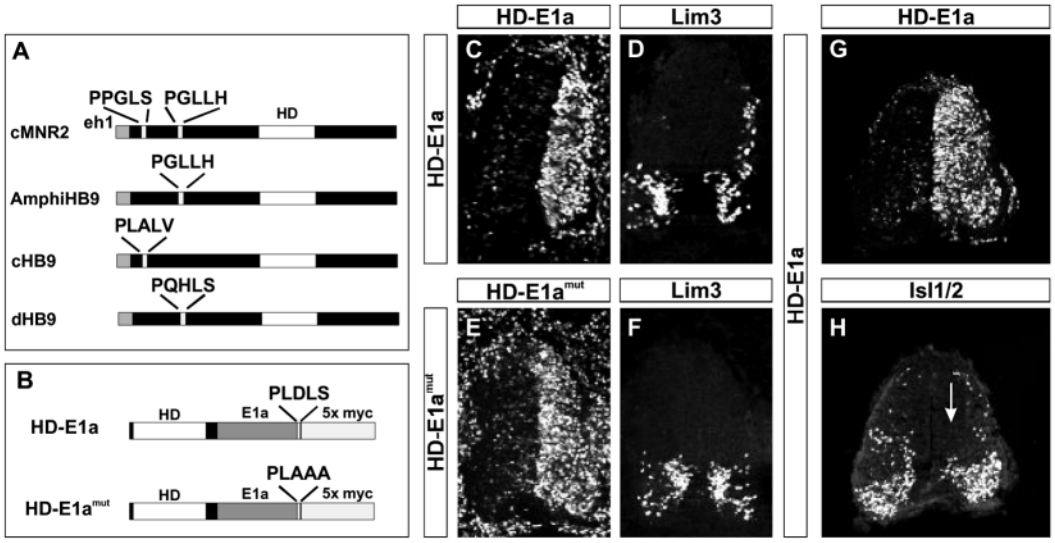

Fig. 8. Inductive activity of a MNR2-E1a C-terminal domain fusion protein. (A) Position of PxxL motifs in MNR2 and related Mnx class HD proteins. (B) Diagram of MNR2HD-E1a C-terminal fusion proteins. (C) Widespread ectopic expression of MNR2 HD-E1a C-terminal fusion protein. (D) Lim3 inductive activity of MNR2 HD-E1a C-terminal fusion protein in vivo. (E) Widespread expression of the mutated MNR2 HD-E1a C-terminal fusion protein. (F) Lack of Lim3 inductive activity of MNR2 HD-E1a C-terminal fusion protein mutated in its three C-terminal residues. (G) Widespread expression of MNR2 HD-E1a C-terminal fusion protein in spinal cord at stage 29 after in ovo electroporation. (H) Repression of CT neuron generation (arrow) by MNR2 HD-E1a C-terminal fusion protein. The MNR2 HD fused to a VP-16 activation domain was also inactive in inducing Lim3 expression (data not shown). Images are representative of over 20 electroporated embryos per construct.

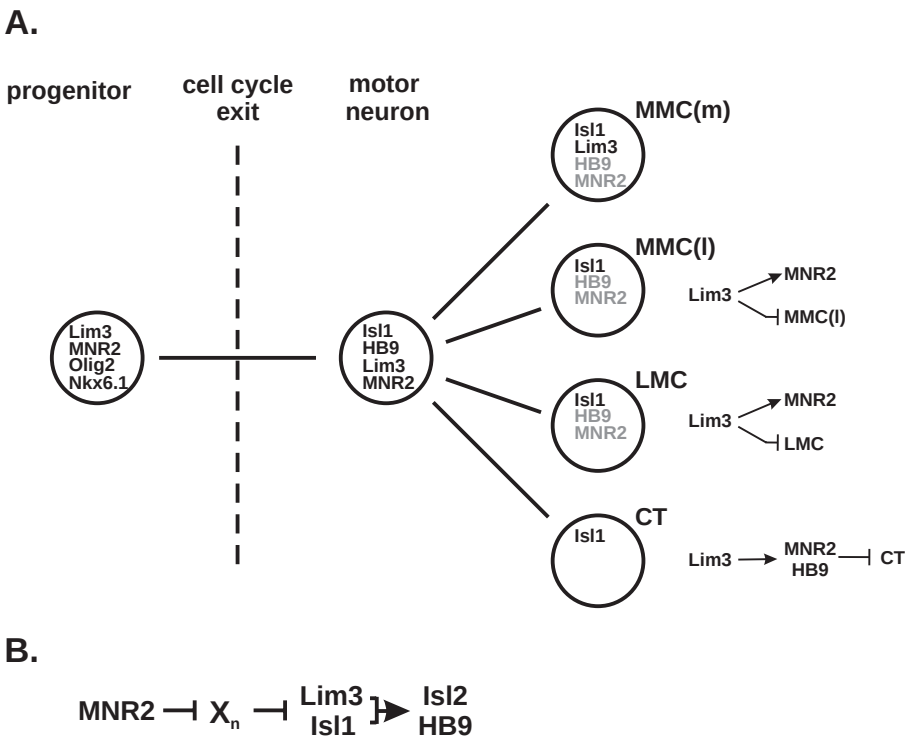

Fig. 9. Progressive restrictions in homeodomain protein expression and the assignment of motor neuron columnar identity. (A) Profiles of HD and Olig2 protein expression in motor neuron progenitors and postmitotic motor neurons. Proteins shown in black designate expression in columnar neuronal subsets where expression is required for progression to a specific motor neuron columnar fate. Proteins shown in gray indicate expression in a particular subset of motor neurons under conditions in which these proteins do not influence motor neuron subtype determination, as assessed by gain- and loss-of-function studies. Late-stage motor neuron progenitors express Nkx6.1, MNR2, Lim3 and Olig2. Cell-cycle exit is accompanied by the extinction of Olig2 expression, the loss of Nkx6.1 from most motor neurons, and by the onset of expression of Isl1, Isl2 (not shown) and HB9. The emergence of medial MMC [MMC(m)] neuronal fate is accompanied by the persistence of expression of Isl1, HB9, MNR2 and Lim3, whereas lateral MMC [MMC(l)] and LMC fates are associated with the extinction of expression of Lim3 and MNR2. CT neuronal fate is associated with the extinction of MNR2, Lim3 and HB9 expression. Within the lateral MMC and LMC lineages, Lim3 represses motor neuron columnar fates independent of its ability to induce MNR2 expression, whereas within the CT lineage, the Lim3-mediated repression of CT identity is likely to be mediated by induction of Mnx class HD protein expression. (B) In dorsal neural progenitors, the repressor activity of MNR2 appears to induce motor neuron differentiation by repression of intermediate repressors (Xn) that function to repress expression of Lim3 and Isl1, proteins that when co-expressed have the capacity to direct motor neuron generation.

Accordingly, the differential extinction of transcription factor expression has a key role in the assignment of motor neuron columnar subtype identity (Fig. 9A). At the time of cell-cycle exit, all spinal motor neurons in chick express several HD transcription factors: Isl1 and Isl2, HB9, Lim3 and MNR2. The persistent expression of all five HD proteins is associated with the acquisition of medial MMC identity. The loss of Lim3 and MNR2 is associated with the emergence of lateral MMC and LMC identity, and the loss of MNR2, Lim3 and HB9 is required for the establishment of CT identity. Below, we discuss the role that the differential extinction of HD transcription factor expression plays in the progressive specification of motor neuron columnar subtype identity.

Differential homeodomain protein expression and the establishment of motor neuron columnar identity

At thoracic levels of the spinal cord, two major classes of motor neurons are generated: somatic MMC neurons and visceral CT neurons. Studies of the mechanisms that impose the distinction in somatic and visceral motor neuron identity have been hindered by the lack of molecular markers that clearly distinguish visceral CT from somatic MMC neurons. Our findings show that prospective CT neurons can be defined by expression of genes encoding members of the BMP family, notably *BMP5*. The role of BMPs in the specification of CT identity remains to be defined, but the selectivity of *BMP5* expression has provided a means to examine the early specification of CT identity. Our analysis of *BMP5* expression reveals that the molecular distinction in CT neuronal identity begins soon after thoracic level motor neurons have left the cell cycle. Prospective CT neurons rapidly acquire expression of *BMP5* and concomitantly extinguish expression of three HD proteins MNR2, Lim3 and HB9. By contrast, medial MMC neurons lack *BMP5* expression and retain expression of all three HD proteins. Thus, there is a mutually exclusive relationship between the expression of *BMP5* and these three HD proteins during the early divergence of somatic and visceral motor neuron identities at thoracic levels of the spinal cord.

Our results provide evidence that the extinction of MNR2, HB9 and Lim3 expression is an essential step in the specification of CT identity (Fig. 9A). Maintaining the expression of any of these HD proteins in thoracic level motor neurons prevents the molecular differentiation of CT neurons and eliminates their dorsomedial migration. Furthermore, analysis of the hierarchical relationship of these three HD proteins in postmitotic thoracic motor neurons indicates that the early downregulation of Lim3 expression from prospective CT neurons is required for the extinction of MNR2 and HB9. Thus, the suppression of CT neuron specification observed after maintained expression of Lim3 is likely to result from the persistence of expression of the Mnx class HD proteins MNR2 and HB9. Our findings on the specification of CT neuron identity also point to a crucial difference in the hierarchical relationship between MNR2 and Lim3 in motor neuron progenitor cells and postmitotic motor neurons. In progenitor cells, MNR2 activates Lim3 but not vice versa (Tanabe et al., 1998), whereas in postmitotic motor neurons Lim3 activates MNR2 but not vice versa. These findings suggest that the nature of interactions between HD transcription factors changes dramatically with the transition from motor neuron progenitors to postmitotic motor neurons.

However, the hierarchical relationship between MNR2, Lim3 and HB9 that emerges from the analysis of motor neuron columnar specification at thoracic levels of the spinal cord does not extend to limb levels. Although MNR2 is also extinguished from LMC neurons, expression of the related Mnx class protein HB9 is maintained in LMC neurons (Fig. 9A). Consistent with this observation, maintained expression of MNR2 does not inhibit the differentiation of LMC neurons. By contrast, maintained expression of Lim3 effectively represses the generation of LMC neurons (see also Sharma et al., 2000). Thus, despite their similar patterns of expression in motor neuron subsets, MNR2 and Lim3 appear to have distinct roles in the assignment of motor neuron columnar identity.

The restriction of MNR2 expression to medial MMC neurons observed during normal development appears to fit with the columnar subtype identity of motor neurons induced by ectopic expression of MNR2. Many of the MNR2-induced ectopic motor neurons express Lim3, which is consistent with the view that MNR2 expression is associated with motor neurons of a medial MMC-like identity. Moreover, at thoracic levels of the spinal cord, postmitotic motor neurons induced by MNR2 express HB9 and fail to express *BMP5*, consistent with the role of MNR2 in repressing CT neuronal differentiation. Moreover, at limb levels of the spinal cord, motor neurons induced by MNR2 fail to express RALDH2 and do not co-express Isl2 and Lim1. Thus, MNR2-induced motor neurons also fail to exhibit LMC character. These findings with MNR2 overexpression at limb levels contrast with results of notochord grafting at limb levels of the spinal cord, where ectopic dorsal LMC neurons are generated (Fukushima et al., 1996). Together, these findings point to a Shh-induced program of LMC neuronal differentiation that is not recruited by MNR2 expression alone.

Evolutionary conservation and divergence in Mnx class homeodomain protein function

One additional issue that emerges from our findings on the actions of MNR2 in motor neuron specification in chick concerns the apparent absence of a corresponding *MNR2* gene in mammals (Arber et al., 1999; Thaler et al., 1999). Of relevance here, is the finding that the bHLH protein Olig2 has a pattern of expression and role in motor neuron specification similar to that invoked for MNR2 (Novitch et al., 2001; Mizuguchi et al., 2001; Zhou et al., 2000; Zhou and Anderson, 2002; Lu et al., 2002). Olig2 is an efficient inducer of MNR2, Lim3 and Isl1 expression in chick spinal cord and is required for motor neuron generation in vivo (Novitch et al., 2001; Mizuguchi et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002; Takebayashi et al., 2002). As the combined expression of Lim3 and Isl1 is sufficient to induce HB9 expression (Tanabe et al., 1998; Thaler et al., 2002), it seems as if multiple transcriptional pathways converge at the point of regulation of Lim3 and Isl1 expression during the specification of motor neuron fate. The apparent absence of an MNR2 counterpart in mouse implies that the Olig2-dependent, MNR2-independent pathway is the primary route of motor neuron generation in mammals, whereas in chick both Olig2 and MNR2 have the ability to trigger Lim3 and Isl1 expression and motor neuron generation. Nevertheless, both Olig2 and MNR2 appear to

function as transcriptional repressors in the pathway of motor neuron specification (Novitch et al., 2001) (Fig. 9B). Thus, the activation of Lim3 and Isl1 expression by MNR2 and Olig2 is likely to result from the repression of a common repressor of genes involved in motor neuron generation (Fig. 9B).

HB9, a close relative of MNR2, is expressed in motor neurons in all vertebrate species examined (Pfaff et al., 1996; Saha and Grainger, 1997; Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999), raising the additional issue of whether HB9 might assume the functions of MNR2 in mouse. In chick, HB9 is confined to postmitotic motor neurons, and thus its actions are likely to be restricted to the control of later aspects of motor neuron differentiation. Moreover, in mouse, HB9 is also confined largely to postmitotic motor neurons (Arber et al., 1999; Thaler et al., 1999), and inactivation of *HB9* does not impair the initial generation of motor neurons. Thus, the mouse HB9 protein is unlikely to have acquired the progenitor cell functions performed by MNR2 in chick (Tanabe et al., 1998). However, the loss of HB9 expression in mouse results in the persistent expression of Lhx3 (Lim3) and the related LIM HD protein Lhx4 in many or all postmitotic motor neurons (Arber et al., 1999; Thaler et al., 1999). Thus, one possible function of HB9 in LMC and lateral MMC neurons may be to ensure the rapid extinction of Lim3 expression after cell-cycle exit. The deregulation of Lim3 expression observed in *HB9* mutants, could, however, also reflect the misassignment of motor neuron and V2 interneuron identity, as these interneurons also express Lim3 (Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2002). Nevertheless, these findings, taken together with the results of MNR2 overexpression in chick, support the idea that Mnx class HD proteins have significant roles in assigning motor neuron columnar subtype identity in vertebrates. These conclusions have also received support from recent observations that HB9 has a critical role in motor neuron specification in *Drosophila* (Broihier and Skeath, 2002).

Motor neuron specification by transcriptional repression

Several lines of evidence suggest that MNR2, and its relative HB9, function as transcriptional repressors during the process of motor neuron specification. First, the N-terminal domain of MNR2 essential for its activity in motor neuron specification can function as a potent transcriptional repressor in cell-based reporter assays. Second, the HD of MNR2, when fused to a known co-repressor recruitment domain, the E1a C-terminal domain (Chinnadurai, 2002; Turner and Crossley, 2002), can mimic the activity of the wild-type MNR2 protein, both in motor neuron specification and in repression of CT subtype identity. These findings are complemented by genetic studies of HB9 function in mouse, in which HB9 has been shown to repress its own expression (Arber et al., 1999; Thaler et al., 1999) and to repress expression of V2 interneuron determinants in motor neurons (Tanabe et al., 1998; Thaler et al., 2002).

The precise mechanism of MNR2- and HB9-mediated transcriptional repression remains unclear. MNR2, like many other HD proteins (Muhr et al., 2001), possesses a well conserved eh1 motif that, in other contexts, can recruit Groucho class co-repressors (Smith and Jaynes, 1996). However, elimination of the eh1 motif in MNR2 does not abolish its ability to induce motor neuron generation. Moreover, fusion of the HD of MNR2 to a potent Groucho recruitment domain results in poor motor neuron-inducing activity in vivo. Thus, the repressor functions of MNR2, and by inference of HB9, may not simply reflect the recruitment of Groucho class co-repressors. Our data show that the MNR2 HD-E1a C-terminal repressor domain fusion protein mimics the activity of the wild-type MNR2 protein, raising the possibility that MNR2 repressor activity involves the recruitment of Ctbp class co-repressors (Turner and Crossley, 2001; Chinnadurai, 2002). However, additional experiments are necessary to resolve whether the repressor functions of MNR2 normally involve the recruitment of Ctbp class co-repressors. In addition, studies on co-repressor function in *Drosophila* raise the possibility of cooperative interactions between eh1 Groucho recruitment and Ctbp recruitment domains present within the same transcription factor (Hasson et al., 2001; Barolo et al., 2002).

Regardless of the precise co-repressors recruited by MNR2, our evidence supports the view that MNR2 function in vivo is likely to reflect its role as a transcriptional repressor. These findings therefore add to the emerging view that the logic of motor neuron fate specification is grounded in transcriptional repression (Muhr et al., 2001). Many of the progenitor transcription factors involved in motor neuron specification at steps upstream of MNR2, e.g. Nkx6.1, Nkx6.2 and Olig2, also function as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001; Vallstedt et al., 2001). Unlike the Nkx6 and Mnx proteins, Olig2 does not possess a clear eh1 motif, further supporting the idea that the transcriptional repressors that function in motor neuron specification recruit distinct classes of co-repressor protein. Finally, the similarities in sequence and activities of Mnx class HD proteins, and genetic studies of HB9 in mouse and *Drosophila* indicate that all Mnx class proteins may function as transcriptional repressors (Arber et al., 1999; Thaler et al., 1999; Broihier and Skeath, 2002). As HB9 expression in spinal cord is restricted largely to postmitotic motor neurons, these observations imply that the key role of transcriptional repression in motor neuron fate specification extends from progenitor cells into postmitotic neurons.

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