Neuron, Vol. 31, 773–789, September 13, 2001, Copyright ©2001 by Cell Press

Coordinate Regulation of Motor Neuron Subtype Identity and Pan-Neuronal Properties by the bHLH Repressor Olig2

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Summary

Within the developing vertebrate nervous system, the mechanisms that coordinate neuronal subtype identity with generic features of neuronal differentiation are poorly defined. We show here that a bHLH protein, Olig2, is expressed selectively by motor neuron progenitors and has a key role in specifying the subtype identity and pan-neuronal properties of developing motor neurons. The role of Olig2 in the specification of motor neuron subtype identity depends on regulatory interactions with progenitor homeodomain proteins, whereas its role in promoting pan-neuronal properties is associated with expression of another bHLH protein, Ngn2. Both aspects of Olig2 function appear to depend on its activity as a transcriptional repressor. Together, these studies show that Olig2 has a critical role in integrating diverse features of motor neuron differentiation in the developing spinal cord.

Introduction

A critical early step in the development of the vertebrate central nervous system (CNS) is the generation of specific subclasses of neurons in constant numbers, in distinct positions, and at defined times. The precision with which these diverse aspects of neuronal differentiation are coordinated implies that neural progenitor cells possess mechanisms for integrating independent developmental decisions into a coherent program of neurogenesis. Despite considerable progress in defining individual steps in the specification of neuronal subtype identity (Cepko, 1999; Jessell, 2000), and in the control of generic neuronal character (Anderson and Jan, 1997; Guillemot, 1999), the mechanisms through which neuronal identity, number, and position are regulated in coordinate fashion within any specific class of neuron remains poorly defined.

The spinal cord represents one region of the CNS in which some progress has been made in defining the steps involved in the conversion of progenitor cells into specific subclasses of neurons. Here, distinct neuronal classes emerge in a precise spatial and temporal order from progenitor cells arrayed along the dorsoventral axis of the neural tube (Lee and Jessell, 1999; Briscoe and Ericson, 2001). This fine-grained pattern of neurogenesis is initiated by secreted signals that partition neural progenitor cells into spatially discrete domains (Briscoe et al., 2000). Within the ventral spinal cord, the secretion of Sonic hedgehog (Shh) from the notochord and floor plate has a key role in establishing ventral neuronal fates (Briscoe and Ericson, 2001). Graded Shh signaling controls the patterned expression of a set of homeodomain (HD) transcription factors that have been subdivided into two major groups, termed class I and II proteins, on the basis of their mode of regulation by Shh signaling (Briscoe and Ericson, 2001). The class I proteins are constitutively expressed by neural progenitors and are repressed by Shh signaling, whereas the class II proteins depend on Shh signaling for their expression (Briscoe et al., 2000). Most of these HD proteins have been shown to function directly as transcriptional repressors (Muhr et al., 2001), and their repressive activities underlie the crossregulatory interactions between class I and II proteins that refine boundaries between adjacent ventral progenitor domains (Briscoe and Ericson, 2001; Vallstedt et al., 2001 [this issue of *Neuron*]). Moreover, once established, the profile of progenitor HD proteins expressed within an individual domain appears to direct the subtype identity of its neuronal progeny (Briscoe and Ericson, 2001).

Many of these basic features of ventral neural patterning have emerged through an analysis of the generation of motor neurons. All motor neurons in the spinal cord, whether destined to supply somatic or visceral targets, arise from a common and apparently uniform set of progenitor cells within the pMN domain (Jessell, 2000). The pMN domain is flanked dorsally by the p2 domain which generates V2 interneurons, and ventrally by the p3 domain which generates V3 interneurons (Briscoe et al., 2000). In the chick neural tube, pMN domain progenitors express three HD proteins, Pax6, Nkx6.1, and Nkx6.2 (Briscoe et al., 2000; Cai et al., 2000; Vallstedt et al., 2001). The distinction in identity of the pMN and p3 domains depends on crossrepressive interactions between Pax6 and the p3 progenitor HD proteins Nkx2.2 and Nkx2.9 (Ericson et al., 1997; Briscoe et al., 1999). How the distinction in pMN and p2 progenitor identity is achieved is less clear. The class I HD protein Irx3 is expressed by p2 progenitors, and its repressive action constrains the dorsal limit of the pMN domain and prevents motor neuron generation (Briscoe et al., 2000). The existence of a complementary class II protein expressed selectively within pMN progenitors is predicted by derepression models of neuronal patterning (Briscoe et al., 2000; Muhr et al., 2001), but such a class II protein has not been identified.

Within the pMN domain itself, where progenitors express Nkx6.1 and Nkx6.2 but not Nkx2.2 or Irx3, the unconstrained activities of these two Nkx6 repressor proteins promotes the expression of downstream determinants of motor neuron identity, including the HD proteins MNR2 and Lim3 (Tanabe et al., 1998; Sharma et al., 1998; Briscoe et al., 2000; Vallstedt et al., 2001). MNR2 is noteworthy in that its expression begins during the final division cycle of motor neuron progenitors, and

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its activity directs many features of post-mitotic motor neuron differentiation (Tanabe et al., 1998). Nevertheless, the motor neuron promoting activity of MNR2 is only functional in post-mitotic neurons, and MNR2 expression itself does not drive progenitors out of the cell cycle (Tanabe et al., 1998). Indeed, none of the HD proteins implicated in the control of ventral neuronal fate in the spinal cord have been shown to control neuronal cell cycle exit or to promote the expression of pan-neuronal markers.

Generic programs of neuronal differentiation in vertebrates, as in *Drosophila* (Anderson and Jan, 1997), are regulated by members of the basic-helix-loop-helix (bHLH) class of transcription factors. Many neurally expressed bHLH proteins have the ability to promote the expression of neuronal differentiation markers (Ma et al., 1996; Guillemot, 1999), and in some instances these proteins can also direct the exit of neural progenitors from the cell cycle (Morrow et al., 1999; Farah et al., 2000). Moreover, studies in both insects and vertebrates have begun to provide evidence that bHLH proteins can impose certain subtype-specific features of post-mitotic neuronal identity (Chien et al., 1996; Jarman and Ahmed, 1998; Morrow et al., 1999; Fode et al., 2000). In the developing spinal cord, however, most of the well-characterized bHLH proteins are broadly expressed over several progenitor domains (Gradwohl et al., 1996; Sommer et al., 1996; Ma et al., 1997; Roztocil et al., 1997), and their specific functions in neuronal differentiation have not been resolved.

Recently, a pair of closely related bHLH genes, *Olig1* and *Olig2*, have been found to exhibit a restricted pattern of expression within the ventral spinal cord (Lu et al., 2000; Zhou et al., 2000), and at later stages of spinal cord development, these two genes are expressed by oligodendrocyte precursors as they disperse within the spinal cord. Misexpression of the *Olig* genes has further been reported to induce the expression of oligodendrocyte markers (Zhou et al., 2000; Lu et al., 2000). Based on these studies, the *Olig* genes have been proposed to play a role in oligodendrocyte differentiation (Lu et al., 2000; Zhou et al., 2000). Nevertheless, *Olig1* and *Olig2* are expressed by cells in the ventral neural tube before the onset of oligodendrocyte differentiation (Lu et al., 2000; Takebayashi et al., 2000), raising the possibility that these bHLH proteins participate in other aspects of ventral cell differentiation.

In this study, we provide evidence that one of these two proteins, Olig2, has a pivotal role in coordinating diverse aspects of motor neuron differentiation: integrating motor neuron subtype specification, generic neuronal differentiation, and cell cycle exit. During the period of motor neuron generation, Olig2 is expressed selectively by progenitor cells within the pMN domain, and appears to function as a class II transcriptional repressor that complements the activity of the class I HD protein Irx3 in establishing a distinction between the pMN and p2 domains. We provide evidence that the repressor activity of Olig2 within pMN progenitors directs the expression of motor neuron subtype determinants. In addition, Olig2 appears to direct expression of the neural bHLH protein Neurogenin2 (Ngn2) within pMN domain progenitors and, directly or through Ngn2, drives pMN progenitor cells to leave the cell cycle and express differentiation markers characteristic of post-mitotic neurons.

Results

Selective Expression of Olig2 by Motor Neuron Progenitors

To examine the role of Olig2 in early neural differentiation, we first defined the pattern of *Olig2* expression in prospective brachial and thoracic levels of the chick neural tube from Hamburger-Hamilton stages 10 to 27. Expression of *Olig2* was first detected at stage 10 in a restricted group of cells in the ventral neural tube at rostral levels, adjacent to the floor plate (Figures 1B and 1D). Between stages 10 and 15, the domain of *Olig2* expression expanded, and at stage 15, the onset of motor neuron generation (Ericson et al., 1992; Hollyday and Hamburger, 1977), *Olig2* expression encompassed much of the ventral third of the neural tube (Figures 1E and 1J; data not shown). From stages 16 to 25, the peak period of motor neuron generation, *Olig2* expression persisted in progenitor cells within the ventral spinal cord, but was rapidly extinguished in post-mitotic neurons (Figures 1F, 1G, and 1J). From stage 26 onward, after motor neuron generation, *Olig2* expression persisted in a medial progenitor domain within the ventral spinal cord, and was also evident in cells dispersed throughout the spinal cord (Figure 1H). This late, dispersed pattern of *Olig2* appears to be associated with oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000, 2001 [this issue of *Neuron*]).

The expression of *Olig2* in the ventral neural tube over the period of motor neuron generation raised the possibility that Olig2 participates in the specification and/or differentiation of motor neurons. To begin to test this possibility, we mapped *Olig2* mRNA and protein expression in relation to distinct ventral neural progenitor domains that have been defined on the basis of the profile of HD transcription factor expression (Briscoe et al., 2000). The three ventral-most progenitor (p) domains, termed the p2, pMN, and p3 domains, can be distinguished by the expression of the HD transcription factors Nkx6.1, Nkx6.2, Irx3, and Nkx2.2 (Briscoe et al., 2000; Cai et al., 2000; Vallstedt et al., 2001). Expression of Nkx6.1 and Nkx6.2 alone defines pMN progenitors; coexpression of Nkx6.1, Nkx6.2, and Irx3 defines p2 progenitor cells; and coexpression of Nkx6.1, Nkx6.2, and Nkx2.2 defines p3 progenitors (Figure 1T; Briscoe et al., 2000; Cai et al., 2000; Vallstedt et al., 2001)

The onset of expression of *Nkx6.1* occurred prior to that of *Olig2* and *Nkx6.2*, and before neural tube closure (Figures 1A–1D; Cai et al., 2000; data not shown). By stages 16 to 19, the domain of Olig2 expression was contained within a broader dorsoventral domain of expression of Nkx6.1 and Nkx6.2 (Figure 1I; data not shown). From its initiation at stage 10, the dorsal limit of *Olig2* expression coincided with the ventral limit of *Irx3* expression (Figures 1J and 1K; data not shown), indicating that the dorsal limit of Olig2 expression is positioned at the p2/pMN domain boundary (Briscoe et al., 2000). Consistent with this, the dorsal limit of Olig2 was always separated from the p1/p2 domain boundary, marked by the ventral limit of Dbx2 expression (data not

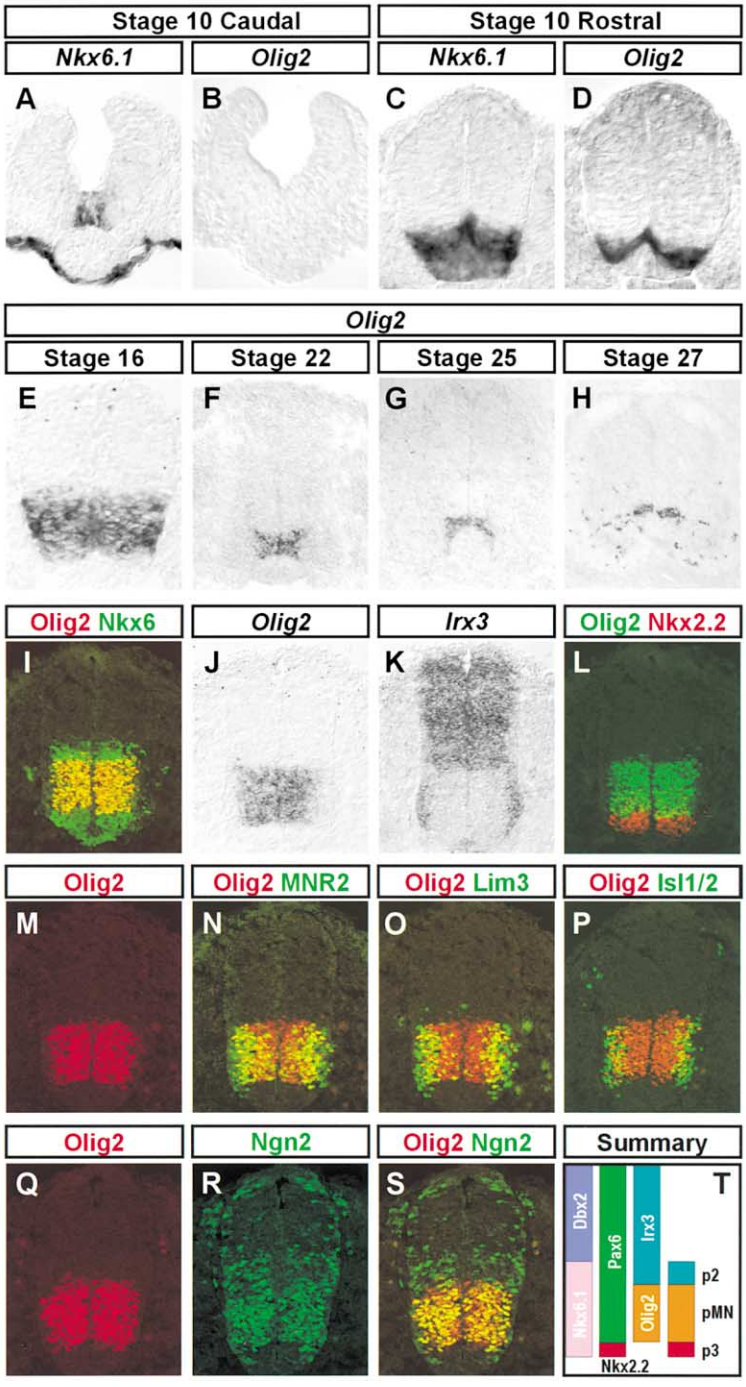

Figure 1. Expression of Olig2 in the Chick Spinal Cord

(A–D) Analysis of *Olig2* and *Nkx6.1* expression at rostral and caudal levels of the chick neural tube at stage 10.

(E–H) Analysis of *Olig2* expression at brachial levels at stages 16–27.

(I–S) Pattern of expression of Olig2 and other transcription factors at brachial levels at stage 18.

(T) Summary of the patterns of Olig2 and HD protein expression in ventral progenitor domains. For details see Briscoe et al. (2000).

shown; Briscoe et al., 2000). *Irx3* expression was also detected in post-mitotic motor neurons at the lateral margin of the ventral spinal cord (Figure 1K), whereas *Olig2* expression was confined to progenitor cells (Figure 1J). Thus, *Olig2* and *Irx3* are expressed in complementary patterns in the ventral spinal cord. We also defined the ventral limit of Olig2 expression. From stages 10 to 25, the ventral limit of Olig2 expression coincided with the dorsal limit of Nkx2.2 expression (Figure 1L; data not shown). Thus, over the entire period of motor neuron generation, Olig2 is expressed by progenitor cells in the pMN domain and is excluded from the adjacent p2 and p3 domains (Figure 1T).

We next examined the temporal pattern of Olig2 expression in the pMN domain in relation to several of the HD factors implicated in the specification of motor neuron identity. The onset of expression of Olig2 preceded that of the motor neuron subtype determinants MNR2 and Lim3, which are not expressed until stages 12 to 13 (data not shown; Tanabe et al., 1998). Moreover, at stage 18, Olig2 was expressed by progenitor cells along the entire mediolateral axis of the pMN domain,

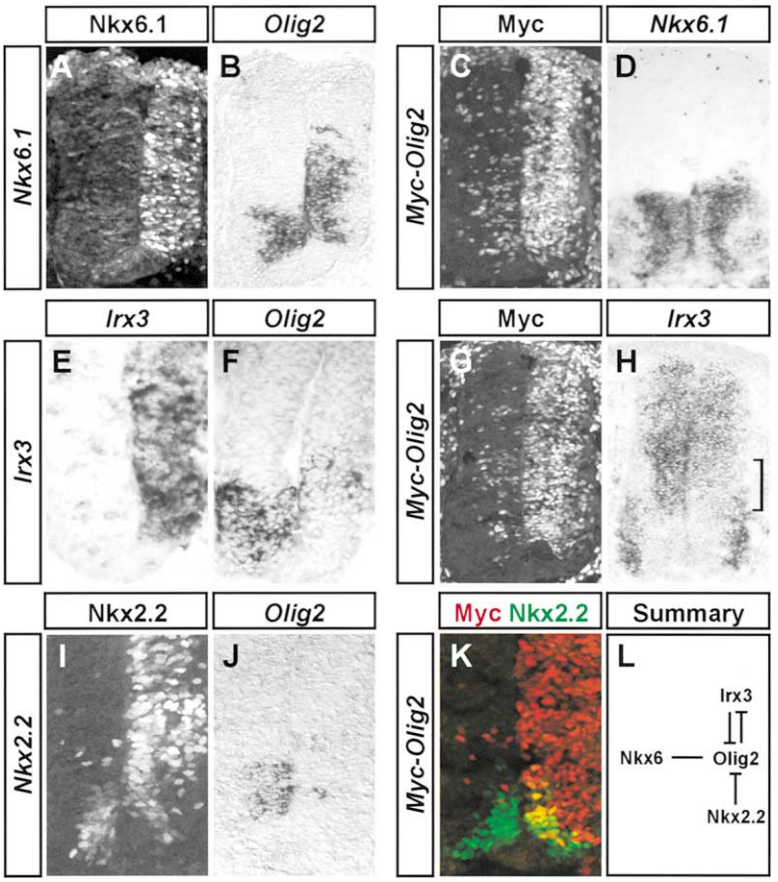

Figure 2. Regulatory Interactions between Olig2 and Progenitor Homeodomain Proteins in the Chick Neural Tube

(A and B) Misexpression of *Nkx6.1* leads to a dorsal expansion in *Olig2* expression.

(C and D) Misexpression of myc-tagged *Olig2* leads to a small dorsal expansion in *Nkx6.1* expression.

(E and F) Misexpression of *Irx3* represses *Olig2* expression.

(G and H) *Olig2* misexpression represses *Irx3* expression, in a position-dependent manner (see Figure 4A and Results). The bracket in (H) denotes the region of effective *Irx3* repression. Ventrolateral expression of *Irx3* corresponds to motor neurons.

(I and J) Misexpression of Nkx2.2 represses *Olig2* expression.

(K) Misexpression of *Olig2* does not repress Nkx2.2 expression.

Images representative of 5–10 embryos for each experiment.

(L) Regulatory interactions between Olig2 and progenitor HD proteins (see Supplemental Figure S1 on *Neuron* website).

whereas the levels of MNR2 and Lim3 expression were higher in more laterally positioned progenitors (Figures 1M–1O). Over this period, the pattern of expression of Olig2 showed little overlap with Isl1, Isl2, and HB9, markers of post-mitotic motor neurons (Figure 1P; data not shown; Ericson et al., 1992; Tanabe et al., 1998). Together, these findings indicate that the expression of Olig2 by motor neuron progenitors precedes that of MNR2 and Lim3, and is extinguished as progenitors leave the cell cycle and express markers of post-mitotic motor neurons.

Crossregulatory Interactions between Olig2 and Progenitor Homeodomain Proteins

To determine whether Olig2 has a role in establishing the pMN domain, we assayed regulatory interactions between Olig2 and selected progenitor HD proteins through misexpression experiments in chick embryos using a replication-competent retroviral expression vector, and through analysis of mouse mutant embryos.

We first examined whether the Nkx6 class proteins are involved in promoting the expression of *Olig2* in pMN domain progenitors. Ectopic expression of *Nkx6.1* in the neural tube resulted in a dorsal expansion in the domain of *Olig2* expression (Figures 2A and 2B). Conversely, *Olig2* expression was virtually absent from pMN domain progenitors in mice lacking both *Nkx6.1* and *Nkx6.2* function (Figures 3A and 3F; Vallstedt et al., 2001). Together, these results provide evidence that the Nkx6 class proteins regulate *Olig2* expression in the ventral spinal cord.

We then tested whether regulatory interactions between Olig2 and Irx3 establish the pMN/p2 domain boundary. Ectopic ventral expression of *Irx3* repressed *Olig2* expression within the pMN domain (Figures 2E and 2F), supporting the idea that Irx3 expression normally limits the dorsal extent of *Olig2* expression. Conversely, ectopic expression of *Olig2* repressed *Irx3* expression within the p2 domain (Figures 2G and 2H). This result, combined with prior data showing that *Olig2* requires Shh signaling for its expression (Lu et al., 2000), provides evidence that Olig2 functions as a class II protein, and exerts crossrepressive interactions with Irx3 to establish the pMN/p2 domain boundary. However, the efficiency with which Olig2 repressed *Irx3* varied along the dorsoventral axis of the neural tube (see Figure 4Ai). Within an intermediate region of the neural tube corresponding to the position of the p0 to p2 domains (a domain designated as the **r** domain—for *Irx3* **r**epressed) (bracketed region in Figure 2H), the expression of *Irx3* was repressed to low levels (Figures 4Aii and 4Aiii), whereas in more dorsal regions of the neural tube (designated the **m** domain—for *Irx3* **m**aintained), *Irx3* was repressed much less efficiently, and high levels of *Irx3* were maintained (Figures 2H, 4Aii, and 4Aiii). The impact of the differential repression of *Irx3* on neuronal specification is discussed below.

We next examined the interactions of Olig2 and

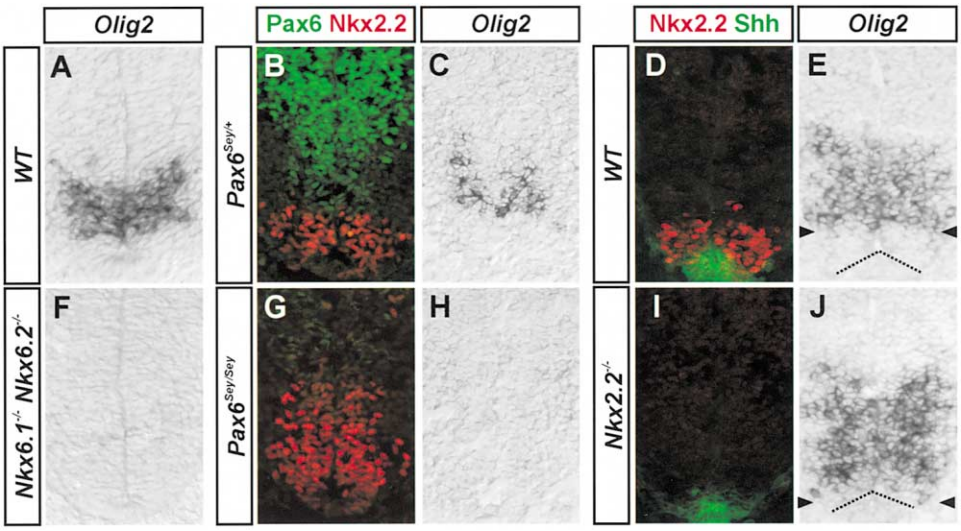

Figure 3. Genetic Evidence for Regulation of *Olig2* Expression by Progenitor Homeodomain Proteins

(A and F) *Olig2* expression at cervical levels of e10.5 *Nkx6.1*; *Nkx6.2* mouse mutant embryos (Vallstedt et al., 2001). A low level of *Olig2* expression was detected at lumbar levels (see Vallstedt et al., 2001).

(B, C, G, and H) *Olig2* and Nkx2.2 expression at rostral spinal cord levels in *Pax6* mutants.

(D, E, I, and J) *Olig2* expression in *Nkx2.2* mutants. Dotted lines indicate the lateral edge of floor plate marked by expression of Shh; arrowheads indicate the ventral limit of *Olig2* expression.

Nkx2.2 at the pMN/p3 domain boundary. Dorsal misexpression of *Nkx2.2* in the chick neural tube repressed *Olig2* expression within the pMN domain (Figures 2I and 2J). In addition, at rostral levels of the spinal cord of *Pax6* mutant mouse embryos, the dorsal expansion in the domain of Nkx2.2 expression and loss of somatic motor neurons (Ericson et al., 1997) were accompanied by the extinction of *Olig2* expression (Figures 3G and 3H). However, *Pax6* is not directly required for *Olig2* expression since in the caudal spinal cord, where the extent of Nkx2.2 expansion and loss of somatic motor neurons is more limited, *Olig2* was expressed (data not shown; Ericson et al., 1997). In *Nkx2.2* mutant embryos (Briscoe et al., 1999), *Olig2* expression expanded ventrally to occupy the p3 domain (Figure 3J). Thus, Nkx2.2 expression defines the ventral boundary of *Olig2* expression. In contrast, misexpression of *Olig2* within p3 domain progenitors did not extinguish expression of Nkx2.2 (Figure 2K). This finding indicates that the repressive relationship between Nkx2.2 and Olig2 is not reciprocal, consistent with prior studies showing that Nkx2.2 repression at the pMN/p3 boundary is controlled by Pax6 (Ericson et al., 1997; Briscoe et al., 2000).

Class II proteins can also repress the expression of class I proteins that do not exhibit a common progenitor domain boundary (Sander et al., 2000; Vallstedt et al., 2001). We therefore tested whether misexpression of *Olig2* represses class I genes other than *Irx3*. Misexpression of *Olig2* did not repress the expression of Pax7 (data not shown), but expression of Dbx2 within the p0 and p1 domains was repressed by Olig2 (see Supplemental Figures S1A and S1B at http://www.neuron.org/cgi/content/full/31/5/775/DC1). Since the dorsal limit of Nkx6.1 expression is normally constrained by expression of Dbx2 (Briscoe et al., 2000), we examined whether the repression of Dbx2 expression by Olig2 might result, indirectly, in the deregulation of Nkx6.1 expression. Misexpression of *Olig2* did occasionally result in an expansion in the domain of *Nkx6.1* expression into the p1 domain (Figures 2C, 2D, and 4E), but the pattern of *Nkx6.2* expression was not altered (data not shown). Thus, *Olig2* misexpression can indirectly influence Nkx6.1 expression, albeit subtly, and probably through regulation of Dbx2 expression. Ectopic expression of *Olig2* did not change the pattern of Shh expression (data not shown), indicating that the patterning changes observed after *Olig2* misexpression do not result from a change in extrinsic inductive signaling.

In summary, these results provide evidence that the restriction in expression of *Olig2* to motor neuron progenitors is regulated by the actions of selected class I and II HD repressor proteins, and suggest that Olig2 itself has a role in establishing the identity of pMN progenitors, primarily through its crossrepressive interaction with Irx3 at the pMN/p2 boundary (Figure 2L).

Olig2 Directs Expression of Motor Neuron Transcription Factors

The restricted expression of Olig2 in pMN progenitors raised the question of whether the protein has a role in the specification of motor neuron fate. To explore this possibility, we examined the pattern of expression of markers of motor neuron and ventral interneuron differentiation after misexpression of *Olig2* in the neural tube. Irx3 has previously been found to inhibit motor neuron generation (Briscoe et al., 2000), and we therefore compared changes in neuronal pattern in the **r** domain (in which *Irx3* expression is repressed to low levels by Olig2) with changes in the **m** domain (in which *Irx3* expression is maintained at high levels).

Misexpression of *Olig2* resulted in a marked dorsal expansion in the domain of MNR2 expression (Figures

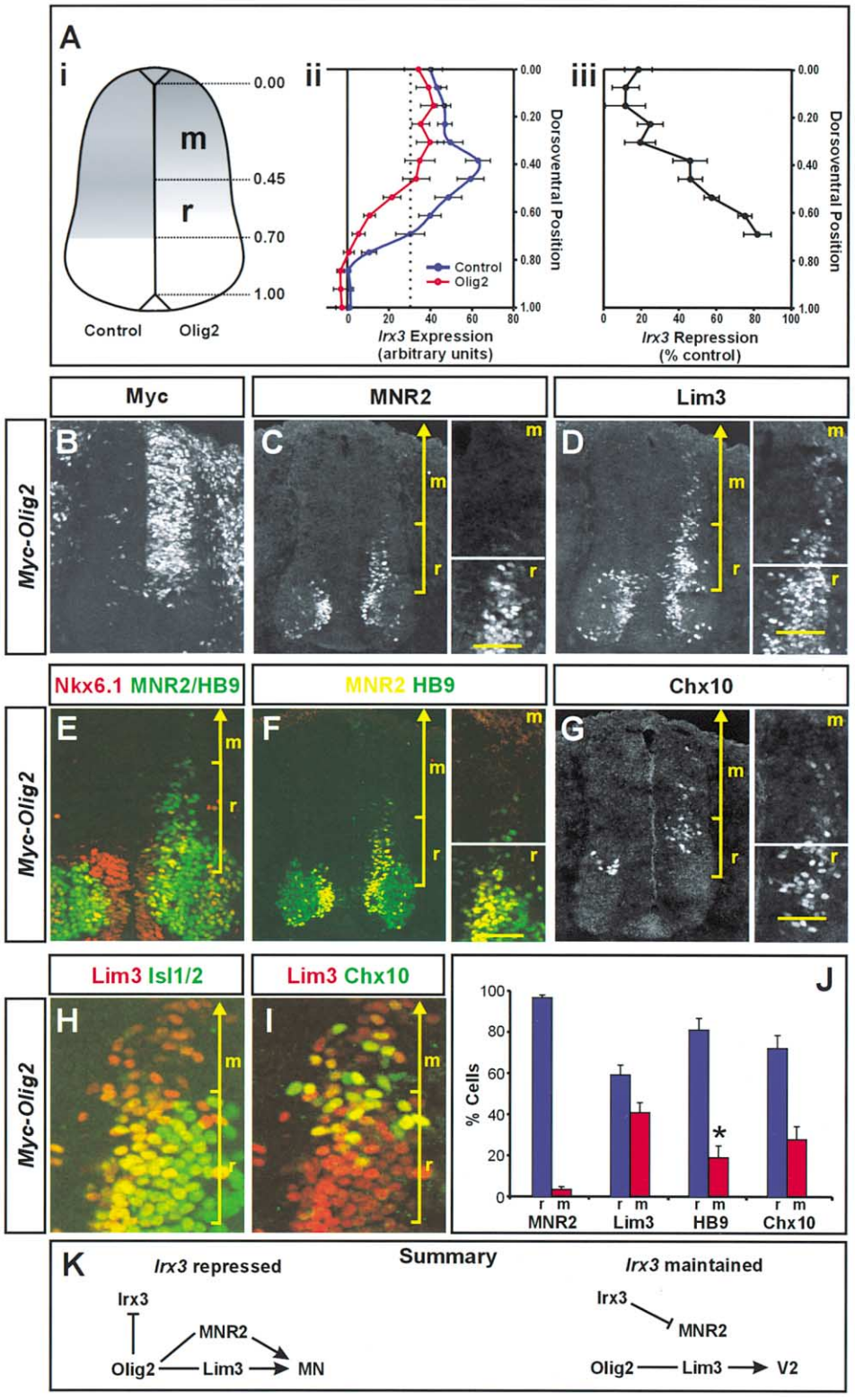

Figure 4. Olig2 Directs the Expression of Motor Neuron Transcription Factors

(A) Position-dependent repression of *Irx3* by Olig2.

(Ai) Diagram summarizing the graded repression of *Irx3* expression. The 0.00 value on the position scale corresponds to the nadir of the roof plate, and the 1.00 value corresponds to the apex of the floor plate. The **m** or **r** designation indicates regions of the spinal cord in which *Irx3* expression is maintained (**m**) or repressed (**r**). The **r** domain corresponds approximately to the p0, p1, and p2 domains.

(Aii) Densitometric analysis of *Irx3* expression levels in *Olig2*-transfected and control sides of spinal cord as a function of dorsoventral position. Data points taken from six sections from three embryos (mean ± SEM). The vertical dotted line indicates the mean level of *Irx3* expression

4B, 4C, 4F, and 4J). However, the ectopic expression of MNR2 was limited almost exclusively to the **r** domain, with <5% of ectopic MNR2$^+$ cells evident in the **m** domain (Figures 4C, 4F, and 4J). Misexpression of *Olig2* also promoted ectopic expression of Lim3 within both the **r** and **m** domains (Figures 4B, 4D, and 4H–4J). Within the **r** domain, the incidence of ectopic Lim3 expression was greater than that of MNR2, suggesting that Olig2 is more efficient at inducing Lim3 than MNR2. Ectopic expression of MNR2 directs motor neuron generation, and in the absence of MNR2, Lim3 directs the generation of Chx10$^+$ V2 neurons (Tanabe et al., 1998). Consistent with these findings, the expression of Olig2 within the **r** domain was accompanied by the differentiation of Isl1/2$^+$, HB9$^+$ motor neurons and Chx10$^+$ V2 neurons. In contrast, very few Isl1/2$^+$, HB9$^+$ motor neurons were detected within the **m** domain (Figures 4F, 4H, and 4J). All ectopic motor neurons expressed Olig2, indicating that its patterning activity is cell autonomous. Misexpression of mouse *Olig2* led to similar changes in neuronal pattern, but misexpression of mouse *Olig1* or human *Olig3* had no activity (data not shown).

Nkx6.1 can direct expression of MNR2, Lim3, and post-mitotic motor neuron markers (Briscoe et al., 2000), raising the concern that the neuronal patterning actions of Olig2 might result solely from the expansion in the domain of Nkx6.1 expression (Figures 2C and 2D). We therefore compared the domain of ectopic expression of Nkx6.1 with that of motor neuron transcription factor markers. Most of the ectopic MNR2$^+$, Lim3$^+$, Isl1/2$^+$, and HB9$^+$ cells detected within the **r** domain after misexpression of *Olig2* lacked Nkx6.1 expression, and were found in positions dorsal to the position of ectopic Nkx6.1$^+$ cells (Figure 4E; data not shown). In addition, we found that *Olig2* misexpression occasionally induced MNR2 and Lim3 expression in sensory neurons of the dorsal root ganglion and in cells of the otic vesicle, tissues that lack Nkx6.1 expression (data not shown). Together, these findings provide evidence that Olig2 activity promotes the expression of transcription factors characteristic of motor neuron progenitors and post-mitotic motor neurons, in a manner downstream and independent of Nkx6 protein activity.

Misexpression of *Olig2* in the **m** domain, where Lim3$^+$ cells but few if any ectopic MNR2$^+$ cells were detected, resulted in the ectopic generation of many Chx10$^+$ V2 neurons (Figures 4B, 4G, 4I, and 4J). Consistent with this, the position of ectopic V2 neurons always extended more dorsally than that of ectopic motor neurons (Figures 4H and 4I; data not shown). At the border of the **r** and **m** domains, we occasionally detected ectopic neurons that coexpressed Isl1/2 and Chx10 (data not shown), suggesting that an intermediate level of *Irx3* repression results in a hybrid motor neuron-V2 neuron phenotype. The ectopic generation of motor neurons and V2 neurons within the p0 and p1 domains after expression of *Olig2* was associated with a corresponding loss of Evx1$^+$ V0 and En1$^+$ V1 neurons (data not shown).

We infer from these data that Olig2 activity is sufficient to direct the ectopic expression of both MNR2 and Lim3 in neural progenitor cells, but that the spatial pattern of expression of these two markers, and consequently the selection of motor neuron or V2 neuronal fate, may be gated by the efficiency with which Olig2 represses *Irx3* expression (Figure 4K). In the **r** domain, where more efficient *Irx3* repression is achieved, MNR2 is expressed and thus many cells give rise to motor neurons. But in the **m** domain, where a high level of *Irx3* expression is maintained, MNR2 expression is inhibited and thus ectopic Lim3$^+$ cells generate V2 neurons. In support of this, coexpression of *Olig2* with *Irx3* blocked the generation of ectopic MNR2$^+$ cells, but still permitted the ectopic appearance of Lim3$^+$ cells and V2 neurons (data not shown).

A Repressor Form of Olig2 Mimics the Patterning Activity of the Wild-Type Protein

Most of the class I and II HD proteins that control progenitor cell pattern act as transcriptional repressors (Muhr et al., 2001). Since Olig2 also appears to function as a class II protein, we examined whether its neural patterning activity reflects its function as an activator or repressor. To begin to analyze this issue, we measured the activity of Olig2 in a Gal4 transcriptional reporter assay (Sadowski and Ptashne, 1989; Muhr et al., 2001). COS-1 cells were cotransfected with a full-length Olig2 protein fused to the DNA binding domain of yeast Gal4, or with a similar Gal4 fusion with MyoD, a known transcriptional activator (Weintraub et al., 1991). Gal4-Olig2 reduced the level of transcription 8-fold, whereas Gal4-MyoD increased the level of transcription 6-fold, compared to basal levels (Figure 5B). These results suggest that Olig2 possesses transcriptional repressor activity, and prompted us to examine whether Olig2 also functions as a repressor in patterning the neural tube.

To test this possibility, expression constructs consisting of Olig2 subdomains, alone or fused to heterolo-

in the p2 domain. The blue and red plots indicate *Irx3* expression on the control and *Olig2*-electroporated sides, respectively.
(Aiii) Extent of *Irx3* expression on the *Olig2*-transfected side as a function of dorsoventral position.
(B–D) Misexpression of Myc-tagged *Olig2* leads to ectopic MNR2 and Lim3 expression. Yellow horizontal lines in inset panels indicate the dorsal limit of marker expression on the control side.
(E) Ectopic expression of MNR2/HB9 extends beyond the region of Nkx6.1 expansion.
(F) *Olig2* misexpression leads to ectopic HB9 expression.
(G) *Olig2* misexpression also leads to ectopic Chx10$^+$ V2 neuron generation.
(H and I) Ectopic Lim3$^+$, Chx10$^+$ V2 neurons are found dorsal to the position of ectopic Lim3$^+$, Isl1/2$^+$ motor neurons.
Images in (B)–(I) are representative of >10 embryos.
(J) Distribution of ectopic transcription factor expression in the **m** or **r** domains. Values obtained from >7 sections, from >3 embryos. Data are displayed as percentage of total ectopic cells, plotted as mean ± SEM. The asterisk above the **m** bar for HB9 indicates that some of the HB9$^+$ cells found in the **m** domain likely arise by virtue of the expression of Lim3 in dorsal D2 interneurons (Tanabe et al., 1998).
(K) The status of *Irx3* expression appears to determine whether *Olig2* expression generates ectopic motor neurons or V2 neurons.

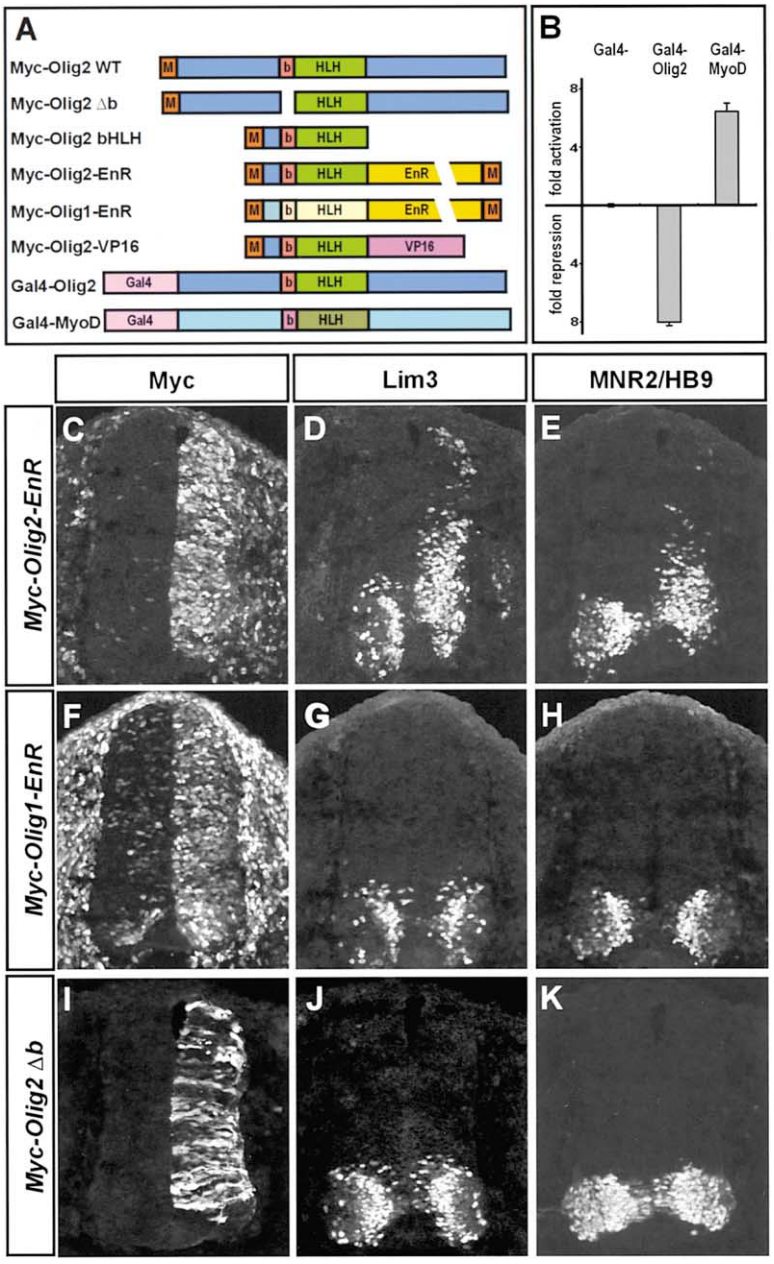

Figure 5. Olig2 Functions as a DNA Binding-Dependent Repressor

(A) Constructs used. M = Myc epitope tag.

(B) Gal4-repression/activation assay. COS-1 cells were cotransfected with Gal4-fusion constructs and a Gal4-E1b-luciferase reporter plasmid. Mean values from four experiments ± SEM are plotted. In controls, a Gal4-EnR construct repressed E1b-luciferase activity 66 ± 10-fold (data not shown).

(C–K) Analysis of ectopic Lim3 and MNR2/HB9 expression in embryos expressing the indicated constructs. Higher power images of embryos expressing Myc-tagged Olig2Δb confirmed that this protein is present in the nucleus of transfected cells (Supplemental Figure S2). Images representative of five embryos for each construct.

gous transcriptional activator or repressor domains (Figure 5A), were expressed in the neural tube, and the ectopic expression of MNR2, Lim3, and HB9 was assayed. Expression of the bHLH domain of Olig2 alone had no patterning activity, but the Olig2 bHLH domain fused to the repressor domain of the *Drosophila* Engrailed protein (Olig2-EnR; Smith and Jaynes, 1996) mimicked the patterning activities of full-length Olig2 (Figures 5C–5E; data not shown). The Olig2 bHLH-EnR fusion also effectively repressed *Irx3* expression from p2 domain progenitors (data not shown). In contrast, expression of an equivalent Olig1 bHLH-EnR fusion had no neural patterning activity (Figures 5F–5H). Similarly, expression of the Olig2 bHLH domain fused to the herpes simplex virus protein 16 transactivation domain (Olig2-VP16; Triezenberg et al., 1988) did not promote ectopic MNR2, Lim3, or HB9 expression (data not shown; see below). Lastly, expression of a full-length Olig2 protein lacking the basic region (Olig2Δb) did not result in ectopic MNR2, Lim3, or HB9 expression (Figures 5I–5K; Supplemental Figure S2 on *Neuron* website; data not shown), suggesting that DNA binding is required for Olig2 function. Together, these results support the idea that Olig2, like class II HD proteins, exerts its neural patterning activity through its function as a DNA binding-dependent transcriptional repressor.

Olig2 and Ngn2 Expression Coincides in Motor Neuron Progenitors

Olig2 shares functional features with class I and II HD proteins in ventral neural patterning, yet belongs to the bHLH family of transcription factors. Certain bHLH pro-

teins have been shown to regulate the expression of general neuronal markers and promote exit from the cell cycle (Morrow et al., 1999; Farah et al., 2000), raising the possibility that Olig2 might also promote the expression of pan-neuronal features of motor neuron differentiation.

To begin to test this idea, we compared the spatial and temporal patterns of expression of *Olig2* with that of other *bHLH* class genes in the ventral neural tube. *Ngn2* and *NeuroM* are expressed during the early phase of progenitor cell differentiation in the spinal cord and have been implicated in neurogenesis (Sommer et al., 1996; Roztocil et al., 1997; Perez et al., 1999; Scardigli et al., 2001; Simmons et al., 2001). We therefore compared the patterns of expression of Olig2, Ngn2, and *NeuroM*. In the ventral neural tube, the expression of Ngn2 begins at stage 12, after that of Olig2 (data not shown). By stages 15–18, as progenitors begin to generate post-mitotic motor neurons, Ngn2 is expressed at high levels within the pMN domain, whereas most other progenitor domains express little or no Ngn2 (Figure 1R). Virtually all cells within the pMN domain expressed Olig2 (Figure 1Q), but Ngn2 expression appeared more scattered (Figures 1R and 1S). *NeuroM* was also expressed at high levels by pMN domain progenitors at stages 15 to 20, but its expression was more prominent in cells in the lateral extent of the pMN domain, close to the position of newly differentiated motor neurons (data not shown). Together, these data indicate that, within pMN domain progenitors, Olig2 expression coincides with that of Ngn2 and other *bHLH* genes implicated in the control of neurogenesis.

Olig2 Regulates Ngn2 Expression

We next examined whether Olig2 expression influences the pattern of Ngn2 expression within the neural tube. Misexpression of *Olig2* resulted in a marked dorsal expansion in the domain of *Ngn2* mRNA and protein expression (Figures 6A–6D). Ectopic Ngn2$^+$ cells were detected in both the **r** and **m** domains, although ectopic Ngn2 expression was less extensive in the most dorsal region of the neural tube (Figures 6A–6D). Coexpression of *Olig2* with *Irx3* still led to the ectopic dorsal expression of Ngn2 (data not shown). A similar expansion in Ngn2 expression was detected after misexpression of Olig2-EnR, but not after expression of the Olig2-VP16 or Olig2Δb constructs (Figures 6E and 6F; data not shown), supporting the idea that Olig2 promotes the expression of Ngn2 through its function as a transcriptional repressor. Olig2 misexpression also led to a dorsal expansion in the domain of *NeuroM* expression (data not shown). The ability of *Olig2* to expand Ngn2 expression dorsally was not mimicked by MNR2 or Lim3 (Figures 6I–6L). Misexpression of *Ngn2* did not lead to ectopic expression of *Olig2*, or to the generation of Isl1/2$^+$ motor neurons, although the domain of *NeuroM* expression was expanded (Figures 6G and 6H; data not shown). These results therefore show that *Olig2* regulates the expression of *Ngn2* in neural progenitor cells.

The limited expansion of Ngn2 into the most dorsal region of the spinal cord after Olig2 misexpression, taken together with the absence of ectopic motor neurons in these dorsal regions, prompted us to examine whether the pattern of Ngn2 expression restricts the ability of Olig2 to generate motor neurons. To test this, we examined whether ectopic expression of *Ngn2* expands the spatial domain in which Olig2 can induce motor neurons. Coexpression of *Olig2* and *Ngn2* in the dorsal spinal cord, however, did not expand the domain of ectopic motor neuron generation (Supplemental Figure S3). This finding suggests that the ability of Olig2 to induce motor neurons is not limited by constraints on Ngn2 expression. Since both Ngn2 and MNR2 appear to function downstream of Olig2, we also examined whether Ngn2 alters the efficiency with which MNR2 generates motor neurons. Coexpression of *Ngn2* and *MNR2* resulted in a marked increase in the number of ectopic Isl1/2$^+$ neurons in dorsal regions of the spinal cord, compared with the actions of MNR2 alone (data not shown). Together, these findings suggest that HD and bHLH domain proteins induced in pMN domain progenitors by Olig2 act together to promote the efficient generation of motor neurons.

Olig2 and Ngn2 Direct Cell Cycle Exit and Neuronal Differentiation

We next turned to the issue of whether Olig2 or Ngn2 influence the acquisition of generic neuronal character, through assays of the expression of pan-neuronal markers and the exit of neural progenitor cells from the cell cycle. Forty-eight hr after misexpression of *Olig2* with a replication-competent retroviral vector, we detected an increase in the number of *Olig2*-transfected cells that expressed the cyclin-dependent kinase inhibitor p27 (Farah et al., 2000) and the neuronal cytoplasmic marker Cyn1 (Tanabe et al. 1998), proteins expressed by all post-mitotic neurons in the chick spinal cord and these embryonic stages (data not shown). However, the high incidence of secondary infection of cells with *Olig2* when expressed with replication-competent retroviral vectors precluded a detailed analysis of the ability of Olig2 to promote expression of pan-neuronal markers.

To overcome this problem, we performed an analysis of Olig2 and Ngn2 activity through misexpression of these two *bHLH* genes using either a replication-incompetent retroviral vector or a CMV-based expression system. Qualitatively similar results were observed with both expression systems, and we present data using the CMV-based vector. We first assayed the proliferative state of neural cells by measuring incorporation of bromodeoxyuridine (BrdU) after a 30 min pulse. 48 hr after misexpression of *Olig2*, ~80% of cells transfected with Olig2 failed to incorporate BrdU, whereas only ~30% of cells expressing a control marker lacked BrdU incorporation (Figures 7B, 7J, and 7N). In addition, most cells transfected with Olig2 were located in a lateral position, characteristic of post-mitotic neurons. Cells transfected with a neutral marker were evenly dispersed along the mediolateral axis of the neural tube (Figures 7A, 7I, and 7M). Similar results were seen with *Ngn2* misexpression, where >80% of transfected cells failed to incorporate BrdU and were positioned laterally (Figures 7E, 7F, 7M, and 7N). Analysis of electroporated embryos at 24 hr revealed that a substantial proportion of *Olig2*-transfected cells maintained a medial position in the neural tube and still incorporated BrdU (data not shown). In

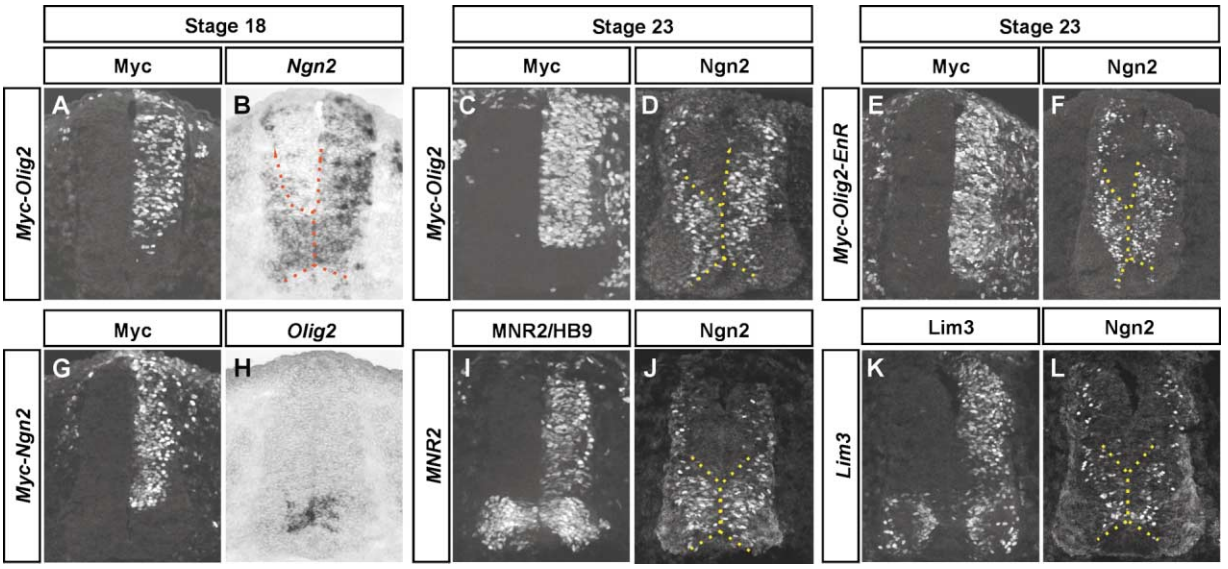

Figure 6. Olig2 Expands the Domain of Ngn2 Expression

(A and B) Myc-tagged *Olig2* misexpression expands *Ngn2* in the intermediate and dorsal spinal cord, assayed at stage 18 (red or yellow dotted lines indicate limit of Ngn2 expression in medial progenitor cells).

(C and D) Myc-tagged *Olig2* misexpression expands Ngn2 into the intermediate and dorsal spinal cord, analyzed at stage 23.

(E and F) Misexpression of Myc-tagged *Olig2-EnR* expands Ngn2.

(G and H) Equivalent misexpression of Myc-tagged *Ngn2* does not expand *Olig2*.

(I–L) Misexpression of *MNR2* or *Lim3* does not alter the pattern of Ngn2.

Images representative of at least five embryos for each construct.

contrast, at 24 hr, most cells transfected with Ngn2 had exited the cell cycle and were positioned laterally (data not shown). These data suggest that Ngn2 is more potent than Olig2 in promoting cell cycle exit.

To determine whether cells driven out of the cell cycle by Olig2 or Ngn2 expressed generic markers of post-mitotic neurons, we analyzed the expression of p27 and Cyn1 at 48 hr. About 85% of cells transfected with Olig2 expressed p27 and Cyn1, whereas $<30\%$ of cells transfected with a control marker were $p27^+$ or $Cyn1^+$ (Figures 7C, 7D, 7K, 7L, 7O, and 7P). Similarly, about 90% of cells transfected with Ngn2 expressed p27 and Cyn1 (Figures 7G, 7H, 7O, and 7P). The neurogenic activity of Olig2 was detected in cells located in both the **m** and **r** domains, implying that the ability of Olig2 to promote cell cycle exit and neuronal differentiation is not subject to the positional constraints imposed on motor neuron specification. These findings provide evidence that misexpression of Olig2 and Ngn2 in neural progenitor cells promotes their exit from the cell cycle and directs the expression of pan-neuronal markers.

An Activator Form of Olig2 Inhibits Motor Neuron Generation

Collectively, our findings suggest that Olig2 generates motor neurons through its ability to repress the expression of a set of target genes that themselves serve as repressors of motor neuron differentiation. We therefore considered whether an activator form of Olig2 might induce the expression of some of these target genes and thus interfere with endogenous Olig2 function. To examine this possibility, we tested whether misexpression of Olig2-VP16 within the pMN domain influenced the expression of transcription factors implicated in motor neuron fate specification.

We first examined the influence of Olig2-VP16 on the expression of Nkx6.1 and Nkx6.2, proteins that appear to function upstream of Olig2. Expression of Olig2-VP16 did not reduce the number of $Nkx6^+$ cells at the dorsoventral position of motor neuron generation (Figures 8A, 8G, and 8H; data not shown). In addition, no change in endogenous Olig2 was detected after expression of Olig2-VP16 (data not shown). Moreover, the expression of Nkx2.2 within the p3 domain was not altered (data not shown). Thus, Olig2-VP16 does not inhibit the expression of HD proteins that function upstream of Olig2, or the expression of Olig2 itself. We next examined the influence of Olig2-VP16 on expression of MNR2 and Lim3, motor neuron subtype determinants that appear to function downstream of Olig2. Expression of Olig2-VP16 within pMN domain progenitors reduced the number of $MNR2^+$ and $Lim3^+$ cells by $\sim$80% and $\sim$55%, respectively, compared to controls (Figures 8B, 8C, and 8H). In contrast, no reduction in $MNR2^+$ or $Lim3^+$ cells was detected after expression of an equivalent Olig1-VP16 construct (data not shown). We also examined whether the loss of $MNR2^+$ cells was accompanied by the derepression of *Irx3* within pMN domain progenitors. In some experiments, ectopic *Irx3* expression was detectable within the pMN domain, but only at very low levels (data not shown, see Discussion). We then examined the profile of expression of markers of post-mitotic motor neurons. Expression of Olig2-VP16 within the pMN domain resulted in a $\sim$50% reduction in the number of $Isl1/2^+$ motor neurons (Figures 8D and 8H). Together, these results confirm that Olig2 functions downstream of Nkx6

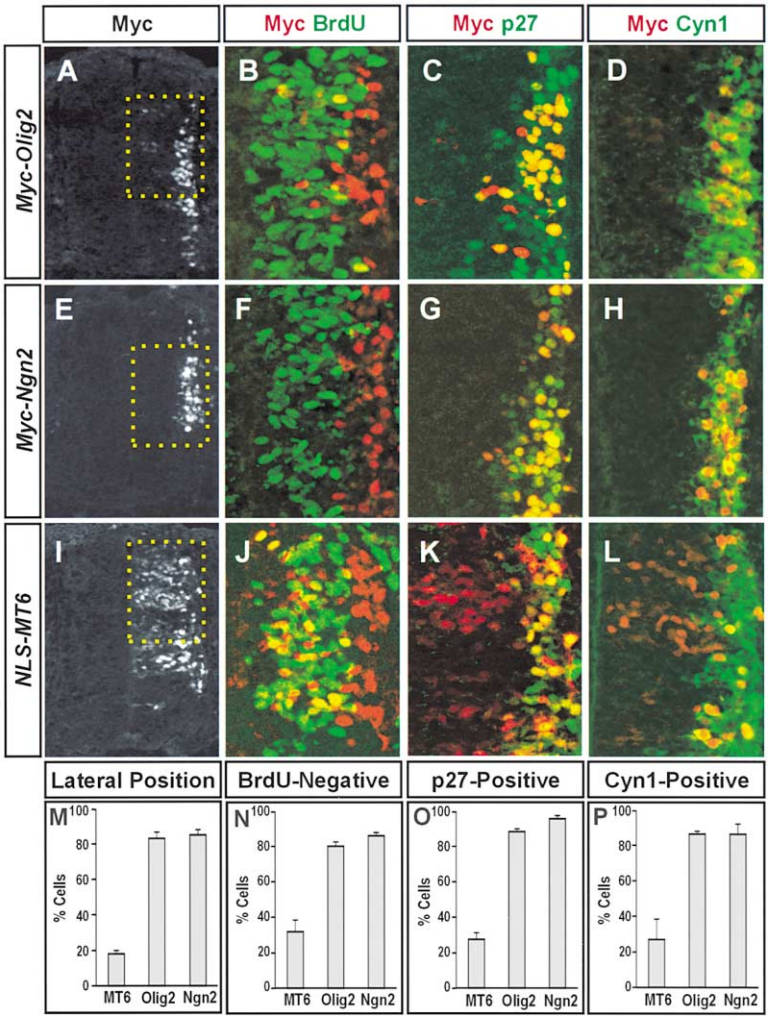

Figure 7. Olig2 and Ngn2 Promote Cell Cycle Exit and Neuronal Differentiation

(A, E, and I) Mediolateral location of cells transfected at stages 11–13 with CMV-based vectors driving the expression of either a nuclear localization signal fused to 6 Myc tags (*NLS-MT6*), or Myc-tagged forms of *Olig2* or *Ngn2*. The medial or lateral position of cells was defined by cell location within or lateral to the region of $BrdU^+$ cells.

(B, F, and J) BrdU incorporation, (C, G, and K) p27 expression, and (D, H, and L) Cyn1 expression in cells transfected with *Myc-Olig2*, *Myc-Ngn2, or NLS-MT6*.

Images representative of at least five embryos for each construct.

(M–P) Quantitative analysis of transfected cells. Data points represent mean values ± SEM from >600 cells, analyzed from at least three transfected embryos. Olig2 and Ngn2 promote cell cycle exit and neuronal marker expression, compared to the NLS-MT6 control ($p < 0.0001$).

proteins, but upstream of MNR2, Lim3, and Isl1/2 in a pathway of motor neuron specification. The loss of $Isl1/2^+$ motor neurons that resulted from Olig2-VP16 misexpression was not compensated for by the generation of V0, V1, or V2 interneurons within the motor neuron domain (data not shown).

This latter finding raised the issue of whether expression of pan-neuronal markers is also inhibited by Olig2-VP16. Olig2-VP16 expression reduced the number of $Ngn2^+$ cells within the pMN domain by ~70% (Figures 8E, 8G, and 8H). In addition, we detected an ~60% reduction in the expression of p27 and Cyn1 (Figures 8D, 8F, 8G, and 8H; data not shown). Together, these results show that expression of Olig2-VP16 results in a concomitant loss in subtype-specific and pan-neuronal features of motor neuron differentiation. We also examined whether the loss of neurons in the pMN domain is accompanied by an increase in the proportion of cells that retain progenitor characteristics. Since the Nkx6 proteins are expressed both by progenitor cells and post-mitotic motor neurons (Sander et al., 2000), we addressed this issue by determining the number of cells within the pMN domain that express Pax6, a protein extinguished as motor neuron progenitors exit the cell cycle and acquire neuronal markers (Ericson et al., 1997). We detected an increase (35 ± 4%; mean ± SEM, n = 12 sections from 3 embryos) in the number of $Pax6^+$ cells within the pMN domain after expression of Olig2-VP16, consistent with the idea that cells that lose neuronal markers retain progenitor cell characteristics.

Discussion

This study provides evidence that Olig2, a bHLH protein expressed selectively by motor neuron progenitors, coordinates subtype-specific and pan-neuronal features of spinal motor neuron differentiation (Figure 9A). Olig2 appears to specify the subtype identity of motor neurons through crossregulatory interactions with progenitor HD transcription factors, and to direct pan-neuronal properties by promoting Ngn2 expression, cell cycle exit, and neuronal marker expression. Olig2 directs both branches of this neuronal differentiation program through its actions as a transcriptional repressor, providing further evidence that repressive interactions lie at the heart of motor neuron specification (Muhr et al., 2001; Figure 9B). A companion paper, Zhou et al. (2001), provides evidence that Olig2 has a later function, in collaboration with Nkx2.2, in the control of oligodendrocyte differentiation in the ventral spinal cord. Together, these two

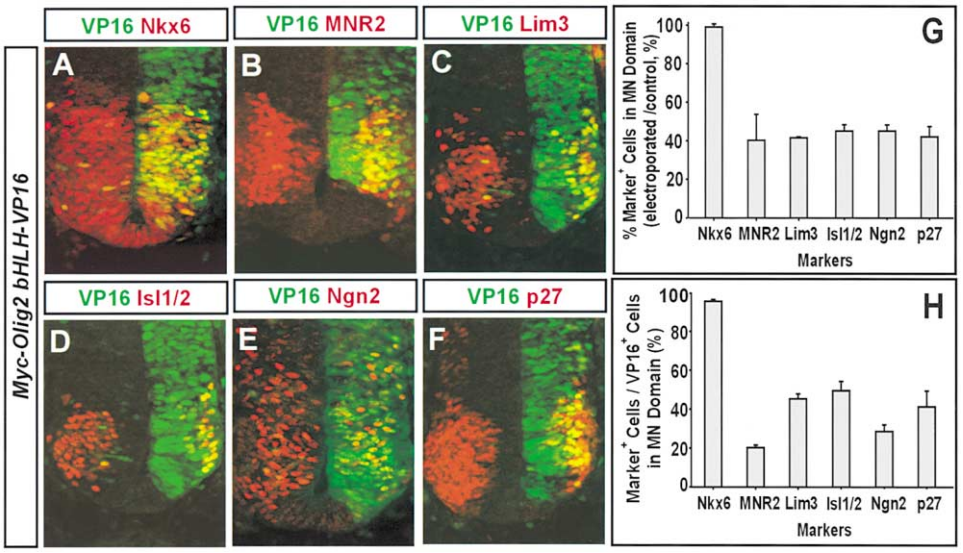

Figure 8. Olig2-VP16 Expression Inhibits Motor Neuron Differentiation

(A–F) Repressive effects of Myc-tagged *Olig2-VP16* misexpression on the expression of differentiation markers within the domain of motor neuron generation. Note that Nkx6 proteins are expressed by both progenitors and post-mitotic motor neurons.

Embryos were electroporated at stages 11–13 and analyzed at stages 18–19. Images representative of at least five embryos.

(G and H) Quantitative analysis of marker expression. (G) Repression of marker expression is plotted as the relative number of cells that express each marker on electroporated and control sides. (H) Analysis of the expression of individual markers in $VP16^{+}$ cells. Data points represent the mean ± SEM from >300 cells counted from at least three embryos. The repression of MNR2, Lim3, Ngn2, p27, and Isl1/2 expression is significant at the $p < 0.0001$ level.

studies reveal that a single bHLH protein, Olig2, has key roles in specifying distinct ventral cell types, at different developmental stages and through divergent molecular programs.

Olig2 Specifies Motor Neuron Subtype Identity through Repressive Interactions with Homeodomain Proteins

The spatial precision of motor neuron and interneuron generation in the ventral spinal cord has been proposed to depend on the prior subdivision of the ventral neural tube into distinct progenitor domains that can be defined by the differential profiles of class I and II HD protein expression (Briscoe et al., 2000). Class II proteins are characterized by four main features: their dependence on Shh signaling, their involvement in establishing progenitor domains through crossrepressive interactions with class I proteins, their role in specifying the identity of the neuronal progeny that emerge from these domains, and their activity as transcriptional repressors (Briscoe and Ericson, 2001; Muhr et al., 2001).

Functionally relevant class II proteins have been defined for many ventral progenitor domains, but the two adjacent domains that generate motor neurons and V2

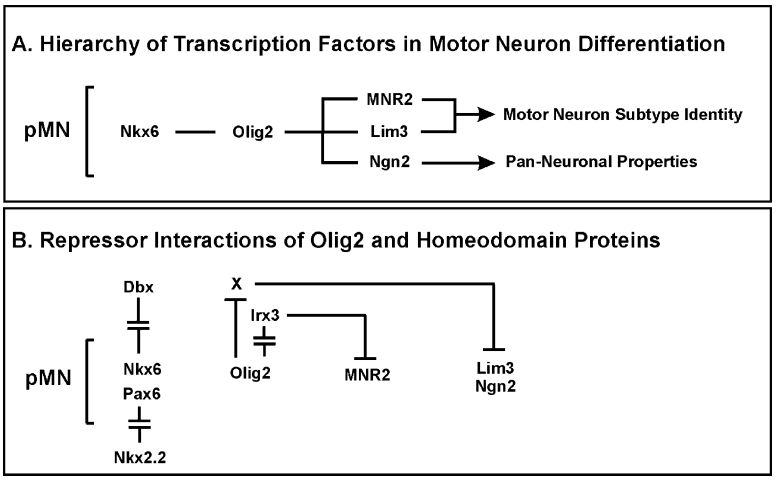

Figure 9. Proposed Role of Olig2 in the Regulation of Motor Neuron Subtype Identity and Pan-Neuronal Properties

(A) Within the pMN domain, Nkx6 proteins initiate motor neuron generation by promoting Olig2 expression. In turn, Olig2 promotes MNR2 and Lim3 expression to impose motor neuron subtype identity, and Ngn2 expression to direct general neuronal character.

(B) Selected repressive interactions between Olig2 and HD proteins during motor neuron generation. Nkx6 and Pax6 proteins promote Olig2 expression by repressing the Dbx and Nkx2.2 proteins, respectively. Once expressed, Olig2 appears to establish the dorsal limit of the pMN domain through a crossrepressive interaction with Irx3. Irx3 expression inhibits MNR2 expression (Briscoe et al., 2000). Olig2 is likely to repress additional targets (designated X) that repress Lim3 and Ngn2 expression within the pMN domain. For further details, see text.

neurons are not distinguishable by any of the known class II HD proteins. Our findings show that Olig2 is expressed selectively by progenitors in the pMN domain over the entire period of motor neuron generation, and possesses each of the four attributes of a typical class II protein. Nevertheless, this functional assignment of Olig2 is unusual since all of the previously identified class II proteins are HD transcription factors. Moreover, our data clearly place Olig2 downstream of the class II HD proteins Nkx6.1 and Nkx6.2 within pMN domain progenitors, and the dedicated role of Olig2 in motor neuron generation is more typical of neuronal subtype determinants such as MNR2. Thus, Olig2 appears to possess attributes of both a class II protein and a motor neuron subtype determinant. The finding that Olig2 functions as a motor neuron subtype determinant is noteworthy in view of the fact that a mouse homolog of MNR2 has not yet been identified. It is possible therefore that in the mouse, Olig2 acts downstream of Nkx6 proteins to promote the expression of motor neuron subtype markers without the intermediary function of MNR2.

Previous studies of neuronal specification in the vertebrate CNS have begun to suggest that certain widely expressed bHLH proteins, notably Mash1, Ngn1, and Ngn2, participate in the specification of regional neuronal identity in the forebrain (Guillemot, 1999; Fode et al., 2000), and in cell type specification in the developing retina (Cepko, 1999). Here, we extend these findings by showing that Olig2 is expressed in a single progenitor domain along the dorsoventral axis of the neural tube and has a dedicated role in the generation of one specific neuronal subtype. In the dorsal spinal cord, the bHLH protein Math1 is similarly expressed in a restricted domain, in the progenitors of D2 neurons (Helms and Johnson, 1998; Lee et al., 1998). Math1 activity is necessary for the differentiation of D2 neurons (Bermingham et al., 2001), and misexpression of Math1 in the spinal cord directs the ectopic generation of D2 neurons (K. Lee and T.M.J., unpublished data). Thus, Olig2 and Math1 may represent prototypic examples of domain-restricted bHLH proteins that possess dedicated roles in specifying the identity of individual neuronal subtypes in the developing spinal cord.

Although Olig2 is able to elicit the ectopic differentiation of motor neurons, its activity along the dorsoventral axis of the spinal cord appears to be constrained, in part, by its efficiency in repressing *Irx3*. Within the p2 domain, Olig2 is a potent repressor of *Irx3* expression, but its repressive activity is markedly less effective in more dorsal domains of the spinal cord. Since Irx3 inhibits MNR2 expression (Briscoe et al., 2000), ventrally where *Irx3* expression levels are reduced below threshold (the **r** domain), Olig2 activity directs both MNR2 and Lim3 expression and activates a pathway of motor neuron differentiation. Even within the **r** domain, however, Olig2 appears to promote Lim3 expression more efficiently than that of MNR2, and thus V2 neurons as well as motor neurons are generated. The basis of the dorsoventral difference in the efficiency of *Irx3* repression by Olig2 is not known, but may reflect the earlier onset of *Irx3* expression in more dorsal domains of the spinal cord (Briscoe et al., 2000), or a dorsoventral restriction in the expression of cofactors that mediate the repressive activity of Olig2. Olig2 also appears to function as a repressor in expanding Lim3 and Ngn2 expression well beyond the domain of ectopic MNR2 expression. Our findings indicate that Irx3 does not function as a relevant repressor of either Ngn2 or Lim3, implying that Olig2 represses additional and as yet undefined repressors of these two genes (Figure 9B).

A Role for Olig2 in Promoting Ngn2 Expression and Generic Neuronal Character

One function of Olig2 within pMN domain progenitors appears to be to promote the expression of MNR2 and other motor neuron subtype determinants. Once expressed, MNR2 can impose many subtype-specific features of motor neurons (Tanabe et al., 1998). However, the activity of MNR2 is evident only in conjunction with a parallel pathway of neurogenesis that directs progenitor cells out of the cell cycle and promotes the acquisition of generic neuronal character (Tanabe et al., 1998). Olig2 appears to regulate this other aspect of motor neuron differentiation since misexpression of Olig2 markedly increases the probability of cell cycle exit and promotes pan-neuronal marker expression.

How does Olig2 promote neurogenesis? The ectopic expression of Olig2 during the period of motor neuron generation markedly expands the dorsoventral domain of expression of Ngn2. Ngns have been shown to promote the expression of generic neuronal markers in a wide variety of settings (Ma et al., 1996; Perron et al., 1999; Farah et al., 2000), and we find that ectopic expression of Ngn2 effectively directs the expression of such pan-neuronal markers in the spinal cord. One line of evidence that favors the idea that Olig2 promotes generic neuronal character through the activity of Ngn2 comes from the analysis of the later function of Olig2 in oligodendrocyte differentiation (Zhou et al., 2001). Here, Olig2 expression in oligodendrocyte progenitors is preceded by the extinction of Ngn expression, and in the absence of Ngns, cells that express Olig2 retain their proliferative capacity. Moreover, in mice lacking *Ngn2*, the number of ventral $Lim3^+$ and $Isl1^+$ neurons is reduced, and in *Ngn1*; *Ngn2* double mutants, pan-neuronal marker expression is lost (Scardigli et al., 2001). Together, these results suggest that Ngns participate downstream of Olig2 in promoting the efficient expression of motor neuron subtype properties, and in imposing pan-neuronal character.

During neural tube development, the expression of Olig2 is likely to underlie the prominent early expression of Ngn2 within the pMN domain, in contrast to the sparse expression of Ngn2 evident in other progenitor domains at this stage. Thus, even though Ngn2 is widely expressed by progenitor cells in the spinal cord and has a general role in neurogenesis, its expression may be controlled independently in individual progenitor domains. In support of this idea, analysis of the *Ngn2* promoter in the developing spinal cord has revealed distinct domain-specific regulatory elements (Scardigli et al., 2001; Simmons et al., 2001). The early Olig2-dependent onset of Ngn2 expression within the pMN domain may therefore account for the predominance of motor neuron rather than interneuron generation during the initial stages of ventral spinal cord neurogenesis (Altman and Bayer, 1984). But if Olig2 and Ngn2 are expressed at

high levels within the pMN domain as early as stages 10 to 12, how are motor neuron progenitors amplified, and why do the first post-mitotic motor neurons not appear until stages 14 to 15? One possible reason could be that pMN domain progenitors transiently express inhibitory bHLH proteins (Jen et al., 1997; Kageyama and Nakanishi, 1997), and a temporal decay in the expression of these inhibitors may be needed to unleash the neurogenic activities of Olig2 and Ngn2.

Suppression of Motor Neuron Generation by an Activator Form of Olig2

The motor neuron subtype and pan-neuronal promoting activities of Olig2 appear to be mediated through the repression of a set of target genes that themselves repress motor neuron generation. Consistent with this, we find that the expression of genes whose domains are expanded by Olig2 in ectopic expression assays is inhibited within the pMN domain upon expression of Olig2-VP16. The simplest interpretation of these findings is that Olig2-VP16 selectively activates the expression of the set of target genes that are normally repressed by wild-type Olig2. This view is supported by the finding that Olig2-VP16 expression inhibits the expression of proteins that appear to function downstream of Olig2, such as MNR2, Lim3, and Ngn2, but not proteins that appear to function upstream of Olig2, such as Nkx6.1 and Nkx6.2.

The precise targets of Olig2-VP16 involved in the repression of motor neuron generation, however, remain unclear. The expression of Olig2-VP16 within the pMN domain does not lead to a consistent ventral expansion in the domain of expression of *Irx3*, as might be predicted from the mutual crossrepressive activity exhibited by these two genes. The lack of a marked ventral expansion in *Irx3* expression could result from the persistence of low levels of endogenous Olig2 activity within pMN domain progenitors after Olig2-VP16 expression, or could be a reflection of the relatively weak repressive influence of Olig2 on *Irx3* expression observed in ectopic expression assays. Recent derepression models of neuronal patterning in the spinal cord (Muhr et al., 2001; Vallstedt et al., 2001) imply the existence of many genes that serve as targets for repression by Olig2 and other motor neuron inducers. In principle, by activating rather than repressing one or more of these Olig2 targets, Olig2-VP16 could inhibit motor neuron generation independently of *Irx3* deregulation. One line of evidence that supports this view is that Olig2-VP16 expression within the pMN domain leads to the ectopic ventral expression of *Dbx2*, a class I gene that is normally repressed by Olig2 (Supplemental Figures S1C and S1D on *Neuron* website). Although the ectopic ventral expression of *Dbx2* is not sufficiently widespread to account for the marked suppression of motor neuron generation observed after Olig2-VP16 expression, its deregulation may be indicative of the ectopic ventral expression of other Olig2 targets that have the capacity to inhibit motor neuron generation.

An additional mechanism for the Olig2-VP16-mediated repression of motor neuron generation is suggested by the efficient suppression of Ngn2 expression within pMN domain progenitors. Since Ngn2 expression within pMN domain progenitors is required for the normal expression of Lim3 and Isl1 (Scardigli et al., 2001), the loss of Ngn2 expression elicited by Olig2-VP16 may account for the loss of motor neuron-specific transcription factors, as well as to the elimination of pan-neuronal markers. Together, the loss of motor neurons observed after expression of Olig2-VP16 supports the idea that Olig2 repressor function is required during the normal pathway of motor neuron differentiation, although loss of function studies will be needed to confirm this conclusion.

bHLH and HD Proteins as Integrators of Subtype-Specific and Pan-Neuronal Programs

The dual role of Olig2 in regulating motor neuron subtype-specific and pan-neuronal character revealed in these studies has parallels with the proposed roles of the bHLH protein Mash1 and the Phox2 HD proteins in the control of neuronal differentiation. Both Mash1 and Phox2b are required for the expression of pan-neuronal and subtype-specific aspects of the differentiation of peripheral sympathetic neurons (Brunet and Ghysen, 1999; Guillemot, 1999), suggesting that Phox2b and Mash1 have similar functions early in sympathetic neuronal development. In contrast, the expression of Phox2a is involved in the control of noradrenergic neurotransmitter phenotype, and its expression depends on Mash1 activity (Brunet and Ghysen, 1999; Guillemot, 1999). Thus, similar hierarchical relationships may exist between Olig2 and MNR2, and Mash1 and Phox2a in the differentiation of motor neurons and sympathetic neurons, respectively.

Within the hindbrain, Phox2b is expressed by visceral but not by somatic motor neurons (Pattyn et al., 1997), and in mice lacking Phox2b function, there are defects in the exit of neural precursors from the cell cycle and in neuronal marker expression (Dubreuil et al., 2000). Moreover, ectopic caudal expression of Phox2b at spinal levels of the neural tube promotes cell cycle exit and induces the expression of Isl1 and markers indicative of hindbrain visceral motor neuron identity (Dubreuil et al., 2000). It remains unclear, however, which of these actions of Phox2b are direct, and which depend on the activation of downstream bHLH proteins, perhaps Mash1. At caudal hindbrain levels, the expression of Olig2, like that of MNR2 (Tanabe et al., 1998), is restricted to somatic motor neuron progenitors (our unpublished data). Thus in the hindbrain, Phox2b and Olig2 may have comparable roles in coordinating pan-neuronal and motor neuron subtype properties, but acting in visceral and somatic motor neurons, respectively. One mechanistic difference in the actions of these two proteins is that Olig2 functions as a transcriptional repressor, whereas the Phox2 proteins are transcriptional activators (Lo et al., 1999).

Sequential Roles of Olig2 in Motor Neuron and Oligodendrocyte Specification

Olig2 is selectively expressed by pMN domain progenitors over the entire period of motor neuron generation, consistent with its proposed role in motor neuron specification. Strikingly, Olig2 expression persists in a similar ventral domain of the spinal cord long after motor neuron

generation is complete (Hollyday and Hamburger, 1977). This observation raises the possibility of a second, and later, role for Olig2 in cell differentiation in the ventral spinal cord. Previous studies have proposed a role for Olig2 in the control of oligodendrocyte differentiation (Lu et al., 2000; Zhou et al., 2000), but the evidence for this has been circumstantial. The accompanying paper, Zhou et al. (2001), provides direct evidence that at later stages of spinal cord development, Olig2 has a critical role in the differentiation of oligodendrocyte precursors, acting in conjunction with the repressor HD protein Nkx2.2. Together, these studies reveal a further level of diversity in Olig2 function. In effect, they indicate that temporal, as well as spatial, constraints on Olig2 activity dictate the divergent roles of a single bHLH protein in the generation of neurons and glial cells in the vertebrate CNS.

Finally, we note that the closest structural counterpart to Olig2 in the *Drosophila* genome, CG5545/Doli, is expressed at high levels in the ventral nerve cord (Moore et al., 2000). The HD repressor proteins implicated in ventral neuronal patterning in vertebrates have close structural and functional homologs in *Drosophila* (Cornell and Ohlen, 2000; Muhr et al., 2001). It may be informative to examine whether the functional inter-relationship between Olig2 and progenitor HD proteins revealed in vertebrates has an evolutionary parallel in neuronal specification in the insect nervous system.

Experimental Procedures

Mouse and Chick Embryo Preparation

Chick eggs (Spafas, Truslow Farms) were incubated and staged as in Hamburger and Hamilton (1951). *Pax6* (*Small Eye* allele) and *Nkx2.2* mutant mice were genotyped as in Ericson et al. (1997) and Briscoe et al. (1999). The generation of *Nkx6.1; Nkx6.2* double mutant mice was as in Vallstedt et al. (2001).

Cloning of Chick *Olig2*

The bHLH region of the chick *Olig2* was isolated through PCR-based cloning (details on request). A full-length chick *Olig2* clone was provided by Q. Zhou and D. Anderson (Zhou et al., 2001), and used in subsequent experiments.

In Situ Hybridization Histochemistry and Immunohistochemistry

In situ hybridization was performed as described (Tsuchida et al., 1994). Probes are as described: Roztocil et al., 1997; Perez et al., 1999; Pierani et al., 1999; Briscoe et al., 2000; Zhou et al., 2000, 2001; Vallstedt et al., 2001. Images were collected on a Zeiss Axioskop microscope. *Irx3* expression was quantified by digitizing in situ images and using Image Pro Plus software (Media Cybernetics).

Antibody staining was performed as described (Tsuchida et al., 1994). Guinea pig antisera were generated against a chick Olig2 peptide (APLPAHPGHPASHPAHHPILPPA). Additional antibodies: rabbit anti-Ngn2 (Zhou et al., 2001); rabbit anti-Nkx6.1/Nkx6.2 (provided by H. Edlund); guinea pig anti-Nkx6.1 (unpublished); mouse anti-VP16 (Santa Cruz Biotechnology, SC-7545); mouse anti-p27 (BD Transduction Laboratories); rat anti-BrdU (Accurate Chemical). Other antibodies are as described: Ericson et al., 1997; Tanabe et al., 1998; Pierani et al., 1999; Muhr et al., 2001.

Expression Constructs and In Ovo Electroporation

RCASBP(B)-myc-tagged chick *Olig2* and *Olig2* Δb replication-competent retroviral constructs were provided by Q. Zhou and D. Anderson (Zhou et al., 2001). RCASBP(B)-*Nkx6.1*, *Irx3*, *Nkx2.2, Ngn2* are described in Perez et al. (1999) and Briscoe et al. (2000). The mouse Olig1 (amino acids 63–153) and chick Olig2 (amino acids 92–180) bHLH domains were fused to the herpes simplex VP16 transactivation domain (Triezenberg et al., 1988) or the *Drosophila* Engrailed repressor domain (Smith and Jaynes, 1996), and subcloned into RCASBP(A) and (B) vectors. cDNAs were also cloned into the CMV-based vectors pCS2+ and pCS2+MT (Turner and Weintraub, 1994), and replication-incompetent retroviral vectors (provided by E. Laufer).

Misexpression of cDNAs was achieved by in ovo electroporation (Briscoe et al., 2000). Embryos were electroporated at stages 11–13 and analyzed at stages 21–23, unless indicated. RCASBP vectors were used in most experiments. For analysis of neuronal differentiation, pCS2-based vectors and replication-incompetent constructs were used to eliminate secondary infection. Results using the pCS2-based vectors are displayed.

Gal4-Reporter Assay

Full-length chick *Olig2* was cloned into the Gal4 DNA binding domain vector pSG424 (Sadowski and Ptashne, 1989). COS-1 cells were cotransfected with pSG424 constructs, Gal4x5-E1b Luciferase, and pRL-TK (Promega) plasmids using Fugene-6 lipofection reagent (Roche). Cells were harvested 48 hr 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 to the value of the Gal4-only control, defined as 1.

BrdU Incorporation

100 μM BrdU was applied to embryos in ovo, followed by incubation for 30 min at 38°C, at which time embryos were fixed and analyzed.

Acknowledgments

We thank D. Anderson, Q. Zhou, and G. Choi for reagents and helpful discussions; J. Ericson and M. Sander for the image of *Olig2* expression in *Nkx6.1*; *Nkx6.2* mutants; H. Edlund, A. Lassar, E. Laufer, C. Murtaugh, and D. Turner for reagents. We also thank F. Guillemot for a preprint of studies on Ngn2 function. We are grateful for the technical assistance of S. Tom, and thank R. Axel, J. Briscoe, and J. Ericson for discussions and comments on the paper. K. MacArthur provided expert help in preparing the manuscript. B.G.N. is a Marion Abbe Fellow of the Cancer Research Fund of the Damon Runyon-Walter Winchell Cancer Research Foundation, DRG-1569. A.I.C. was supported by an NIH training grant 5T32GM08224. T.M.J. was supported by grants from NINDS, Project ALS, and is an HHMI Investigator.

Received June 5, 2001; revised July 23, 2001.

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Accession Numbers

The *Olig2* sequence reported in this paper was deposited in GenBank with accession number AF405699.