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A Homeodomain Protein Code Specifies Progenitor Cell Identity and Neuronal Fate in the Ventral Neural Tube

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Summary

Distinct classes of neurons are generated at defined positions in the ventral neural tube in response to a gradient of Sonic Hedgehog (Shh) activity. A set of homeodomain transcription factors expressed by neural progenitors act as intermediaries in Shh-dependent neural patterning. These homeodomain factors fall into two classes: class I proteins are repressed by Shh and class II proteins require Shh signaling for their expression. The profile of class I and class II protein expression defines five progenitor domains, each of which generates a distinct class of postmitotic neurons. Cross-repressive interactions between class I and class II proteins appear to refine and maintain these progenitor domains. The combinatorial expression of three of these proteins—Nkx6.1, Nkx2.2, and Irx3—specifies the identity of three classes of neurons generated in the ventral third of the neural tube.

Introduction

In many developing tissues, the generation of distinct cell types is initiated by the action of extracellular signals provided by local organizing centers. Certain signals have the additional feature of directing distinct cell fates at different threshold concentrations, and thus function as morphogens (Wolpert, 1969). In *Drosophila*, the patterning of embryonic segments and imaginal discs involves the graded signaling activities of the Hedgehog, Wingless, and TGFβ-related proteins (Lawrence and Struhl, 1996). In vertebrate embryos, the specification of mesodermal cell types has similarly been suggested to depend on the graded signaling activity of members of the TGFβ family (Smith, 1995; McDowell and Gurdon, 1999). The generation of cell pattern through morphogen signaling demands an effective means of converting graded extracellular activities into all-or-none distinctions in cell fate. But the mechanisms used to achieve such conversions have been poorly defined, particularly in vertebrate tissues.

In the developing vertebrate nervous system, Sonic hedgehog (Shh) appears to function as a gradient signal. The secretion of Shh by the notochord and floor plate controls the specification of ventral cell types (Marti et al., 1995; Roelink et al., 1995; Chiang et al., 1996; Ericson et al., 1996). Five distinct classes of ventral neurons can be generated in vitro in response to progressive 2- to 3-fold changes in extracellular Shh concentration (Ericson et al., 1997a, 1997b). Moreover, the position at which each of these neuronal classes is generated in vivo is predicted by the concentration of Shh required for their induction in vitro: neurons generated in progressively more ventral regions of the neural tube require correspondingly higher concentrations of Shh for their induction (Ericson et al., 1997a). These observations have led to the view that the position that ventral progenitor cells occupy within a ventral-to-dorsal gradient of extracellular Shh activity directs their differentiation into specific neuronal subtypes (Ericson et al., 1997b).

In turn, these findings have focused attention on the steps by which graded Shh signaling directs the diversification of neural progenitor cells. Several homeodomain proteins, Pax7, Pax3, Pax6, Dbx1, Dbx2, and Nkx2.2 are expressed by ventral progenitor cells and their expression is regulated by Shh signaling (Goulding et al., 1993; Ericson et al., 1996, 1997a; Briscoe et al., 1999; Pierani et al., 1999) Moreover, the pattern of generation of certain ventral neuronal subtypes is perturbed in mice carrying mutations in these *Pax* genes and in the *Nkx2.2* gene (Ericson et al., 1997a; Mansouri and Gruss, 1998; Briscoe et al., 1999), supporting the view that homeodomain proteins expressed by ventral progenitor cells regulate neuronal subtype identity. However, two important aspects of the link between Shh signaling and neuronal identity remain obscure. First, it is unclear how the presumed extracellular gradient of Shh activity results in stable and sharply delineated domains of homeodomain protein expression within ventral progenitor cells. Second, the spatial information provided by the homeodomain proteins characterized to date is insufficient to explain the diversity of neuronal subtypes generated at different dorsoventral positions.

In this paper we address these two issues. We show first that the homeodomain proteins Nkx6.1 and Irx3 are expressed by progenitor cells in discrete domains of the ventral neural tube and are regulated by graded Shh signaling. The differential expression of five class I (Shh-repressed) proteins, Pax7, Irx3, Dbx1, Dbx2, and Pax6, and two class II (Shh-induced) proteins, Nkx6.1 and Nkx2.2, subdivides the ventral neural tube into five cardinal progenitor domains. By misexpressing individual proteins in the neural tube in vivo, we provide evidence that cross-repressive interactions between class I and class II proteins establish individual progenitor domains and maintain their sharp boundaries, suggesting a mechanism by which graded Shh signals are converted into all-or-none distinctions in progenitor cell identity. In addition, we show that the spatial patterns of expression of Nkx6.1, Irx3, and Nkx2.2 are sufficient to direct both the position and fate of three neuronal subtypes generated in the ventral third of the neural tube. These findings suggest a model of ventral neuronal

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patterning that may provide insight into how extracellular signals are interpreted during the patterning of other vertebrate tissues.

Results

A Homeodomain Protein Code for Ventral Progenitor Cells

Shh signaling controls the generation of five distinct classes of neurons, each at a different dorsoventral position in the ventral neural tube (Ericson et al., 1997a; Briscoe et al., 1999; Pierani et al., 1999). The spatial information provided by the five homeodomain proteins examined previously—Pax7, Dbx1, Dbx2, Pax6, and Nkx2.2—is not sufficient to establish distinct progenitor domains for each postmitotic neuronal subtype (Ericson et al., 1996, 1997a; Briscoe et al., 1999; Pierani et al., 1999), prompting us to search for other relevant homeodomain proteins. We have found that two additional proteins, Nkx6.1 (Qiu et al., 1998) and Irx3 (Funayama et al., 1999), are expressed by distinct sets of ventral progenitor cells.

We compared the patterns of expression of Nkx6.1 and Irx3 with the homeodomain proteins characterized previously. The combinatorial expression of this set of seven homeodomain proteins is sufficient to define five ventral progenitor cell (p) domains, which we term the p0, p1, p2, pMN, and p3 domains, in dorsal-to-ventral progression (Figure 1A). The ventral limit of Pax7 expression defines the dorsal/p0 boundary (Figure 1Bi) (Ericson et al., 1996); the ventral limit of Dbx1 expression defines the p0/p1 boundary (Figure 1Bii) (Pierani et al., 1999); the ventral limit of Dbx2 expression defines the p1/p2 boundary (Figure 1Biii) (Pierani et al., 1999); the ventral limit of Irx3 expression defines the p2/pMN boundary (Figures 1Biv and 1Bvi); and the ventral limit of Pax6 expression defines the pMN/p3 boundary (Figures 1Bv and 1Bvii) (Ericson et al., 1997a). The dorsal limit of Nkx6.1 expression complements the ventral limit of Dbx2 expression at the p1/p2 boundary (Figure 1Biii); and the dorsal limit of Nkx2.2 expression complements the ventral limit of Pax6 expression at the pMN/p3 boundary (Figure 1Bvii) (Ericson et al., 1997a).

These seven homeodomain proteins can therefore be divided into two major subclasses. Five proteins—Pax7, Dbx1, Dbx2, Irx3, and Pax6—exhibit ventral limits of expression that delineate progenitor domain boundaries, and we term these class I proteins (Figure 1A). Two proteins—Nkx6.1 and Nkx2.2—exhibit dorsal limits of expression that define progenitor domain boundaries, and we term these class II proteins (Figure 1A).

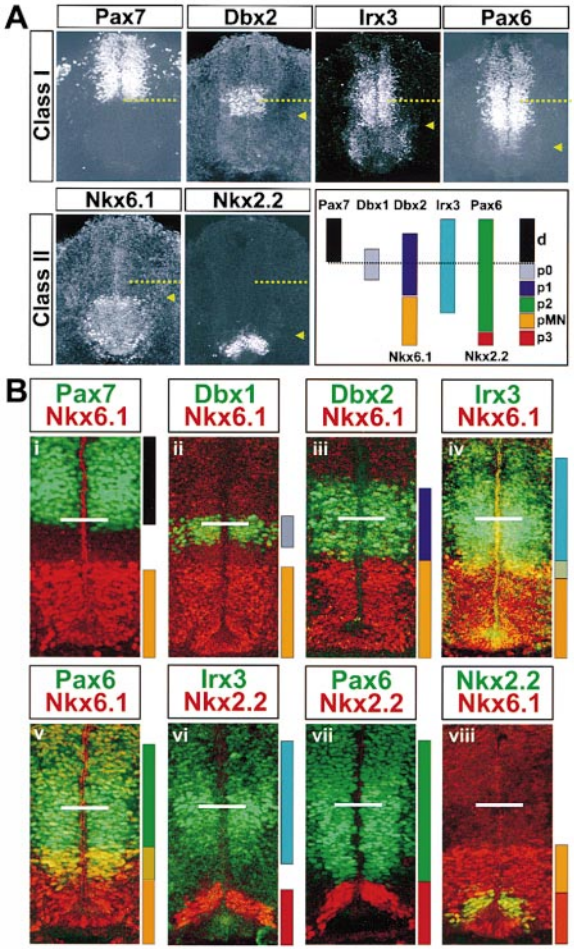

Figure 1. Homedomain Proteins Define Five Ventral Progenitor Domains

(A) Localization of homeodomain proteins in the neural tube of HH stage 20 chick embryos. Class I proteins (Pax7, Dbx2, Irx3, and Pax6) have different ventral boundaries (yellow arrowheads). Class II proteins (Nkx6.1 and Nkx2.2) have different dorsal boundaries (yellow arrowheads). The dorsoventral (DV) boundaries of the neural tubes are indicated by dotted lines. Composite of expression domains shown in (B), p = progenitor domain.

(B) The combinatorial expression of class I and class II proteins defines five ventral progenitor domains. Images show protein expression in the neural tube of HH stage 22 chick embryos.

Progenitor Homeodomain Protein Expression Is Initiated by an Early Period of Graded Sonic Hedgehog Signaling

The expression of certain class I (Pax7, Dbx1, Dbx2, and Pax6) and class II (Nkx2.2) proteins is controlled by Shh signaling in vitro (Ericson et al., 1996, 1997a; Briscoe et al., 1999; Pierani et al., 1999). The expression of class I proteins is repressed by Shh signaling, and the more ventral the boundary of class I protein expression in vivo, the higher is the concentration of Shh required for repression of protein expression in vitro (Ericson et al., 1997a). Conversely, Shh signaling is required to induce expression of the class II protein Nkx2.2 in vitro (Ericson et al., 1997a; Briscoe et al., 1999).

We examined whether this relationship extends to Irx3 and Nkx6.1 by assaying the expression of these two proteins in intermediate neural plate [i] explants exposed to different Shh-N concentrations. Repression of Irx3 required ~3 nM Shh-N (Figure 2A), a concentration greater than that required for repression of Pax7, Dbx1, and Dbx2 expression (Figure 2A) (Ericson et al., 1996; Pierani et al., 1999), but less than that required for complete repression of Pax6 (Ericson et al., 1997a). Conversely, induction of Nkx6.1 required ~0.25 nM Shh-N—a concentration lower than that required for induction of Nkx2.2 (3–4 nM) (Ericson et al., 1997a) (Figure 2B). Thus, the link between the domains of expression

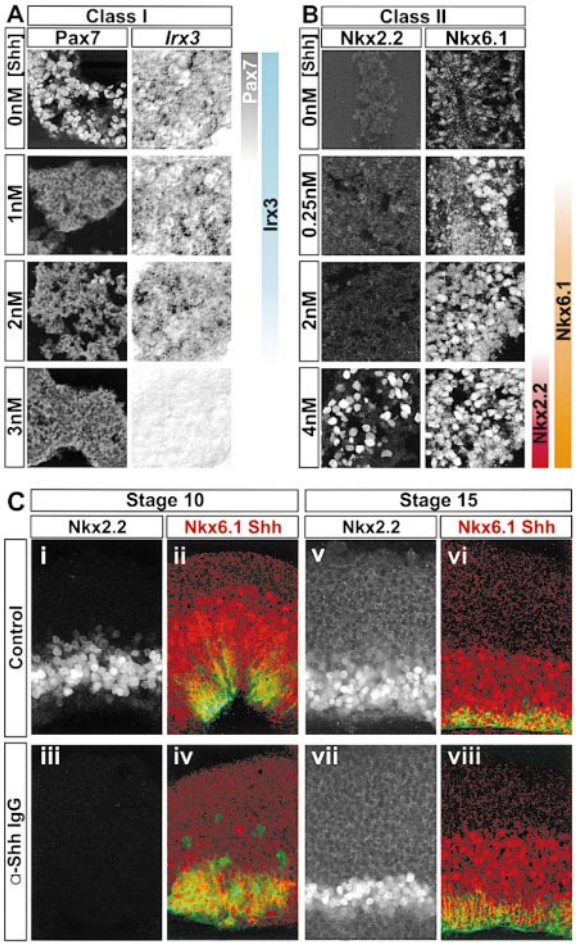

Figure 2. Shh Signaling Is Required to Establish but Not to Maintain the Expression of Progenitor Homeodomain Proteins

(A) Repression of class I gene expression by Shh. Expression of Pax7 and *Irx3* in [i] explants grown for 24 hr alone or in the presence of Shh-N. Repression of Pax7 requires ~1 nM Shh-N (Ericson et al., 1996) whereas repression of *Irx3* requires ~3 nM Shh-N. Images representative of 12 explants.

(B) Shh induces class II proteins. Expression of Nkx2.2 and Nkx6.1 in [i] explants exposed to Shh-N for 24 hr. Nkx2.2 expression requires ~4 nM Shh-N whereas Nkx6.1 expression requires ~0.25 nM Shh-N. Images representative of 12 explants.

(C) Expression of class II proteins requires Shh signaling at stage 10 but not at stage 15. [vf] explants taken from HH stages 10 or 15 embryos grown in the presence of anti-Shh IgG and analyzed for the expression of Nkx2.2, Nkx6.1 and Shh at 24 hr. Stage 10 [vf] explants grown alone express Nkx2.2 and Nkx6.1. Exposure of stage 10 [vf] explants to anti-Shh IgG blocks the expression of Nkx2.2 and Nkx6.1. Nkx6.1 expression continues in the floorplate of [vf] explants grown in the presence of anti-Shh IgG. Stage 15 [vf] explants grown alone or with anti-Shh IgG express Nkx2.2 and Nkx6.1 in similar domains. The slight narrowing of the domain of Nkx2.2 expression could reflect an influence of Shh on cell proliferation. Anti-Shh IgG blocks Shh signaling effectively in stage 15 [vf] explants (data not shown) (see Briscoe et al., 1999). Images representative of 12 explants.

of class I and class II proteins in vivo and the Shh concentration that regulates their expression in vitro extends to Irx3 and Nkx6.1 (Figures 2A and 2B). These findings support the idea that the differential patterns of expression of all class I and class II proteins depend initially on graded Shh signaling.

We next asked whether Shh signaling is required continuously to maintain the early pattern of progenitor homeodomain protein expression. To address this, we examined whether the expression of class II proteins, once initiated, can be maintained under conditions in which ongoing Shh signaling is eliminated. Explants of ventral neural tube, including the floor plate ([vf] explants), were isolated from stage 10 or stage 15 embryos and grown in vitro, alone or in the presence of a function blocking anti-Shh antibody (Ericson et al., 1996). Both stage 10 and stage 15 [vf] explants grown alone generated a narrow domain of Nkx2.2$^+$ cells and a broad domain of Nkx6.1$^+$ cells (Figures 2Ci, 2Cii, 2Cv, and 2Cvi). Addition of anti-Shh IgG to stage 10 [vf] explants blocked the expression of both Nkx2.2 and Nkx6.1 in neural progenitors (Figures 2Ciii and 2Civ). In contrast, in stage 15 [vf] explants, the domains of Nkx2.2 and Nkx6.1 expression persisted in the presence of anti-Shh IgG (Figures 2Cvii and 2Cviii). These results provide evidence that the pattern of class II protein expression becomes independent of Shh signaling over a period of ~12–15 hr, between stages 10 and 15.

Cross-Repressive Interactions between Class I and Class II Proteins Refine Progenitor Domain Boundaries

The boundaries of progenitor domains are sharply delineated in vivo (Figure 1), raising questions about the steps that operate downstream of Shh signaling to establish the nongraded domains of expression of class I and class II proteins. We examined whether the domain of expression of class I proteins might be constrained by the action of the class II protein that abuts the same domain boundary, and vice versa. To test this, we misexpressed individual homeodomain proteins in the chick neural tube in mosaic fashion, and assayed the resulting pattern of class I and class II protein expression. Ectopic protein expression was achieved using either retroviral transduction or electroporation.

Interactions at the pMN/p3 Boundary

We first analyzed the interaction between the class I protein Pax6 and the class II protein Nkx2.2—proteins that exhibit complementary domains of expression at the pMN/p3 boundary. To assess the influence of Pax6 on Nkx2.2, we misexpressed Pax6 ventral to its normal limit and examined the resulting pattern of expression of Nkx2.2 (Figures 3A–3C). After electroporation of *Pax6*, small clusters of ectopic Pax6$^+$ cells were detected within the p3 domain (Figures 3A and 3B). These cells lacked Nkx2.2 expression (Figures 3A and 3B), whereas expression of Nkx2.2 was maintained by neighboring p3 domain cells that lacked ectopic Pax6 expression (Figures 3A and 3B), arguing for a cell-autonomous action of Pax6. The expression of other class I and class II proteins was not affected by the deregulated expression of Pax6 (data not shown). Thus, Pax6 acts selectively to repress Nkx2.2 expression in p3 domain cells. These results complement studies showing a requirement for Pax6 activity in defining the dorsal limit of the p3 domain in vivo (Ericson et al., 1997a).

To examine whether Nkx2.2 normally limits the ventral boundary of Pax6 expression, we misexpressed Nkx2.2 in regions dorsal to the p3 domain. The vast majority (>95%) of progenitor cells that ectopically expressed

Nkx2.2 lacked Pax6 expression (Figure 3D). Since these experiments used a replication-competent retroviral expression system, the coexpression of both homeodomain proteins in a small minority of cells is likely to reflect the secondary infection of cells at later stages, with the consequence that Nkx2.2 may be expressed for too brief a period to repress Pax6 completely. Neighboring cells that lacked ectopic Nkx2.2 retained Pax6 expression (Figure 3D), indicating a cell-autonomous action of Nkx2.2. The expression of Nkx6.1 and Pax7 was unaffected by the ectopic expression of Nkx2.2 (Figures 3E and 3F). Thus, the repressive action of Nkx2.2 on Pax6 expression is selective and cell autonomous. These results provide evidence for mutually repressive interactions between Pax6 and Nkx2.2 at the pMN/p3 boundary.

Nkx2.9, a gene closely related to *Nkx2.2* (Pabst et al., 1998), is expressed in a pattern that overlaps transiently with *Nkx2.2* in the p3 domain (Briscoe et al., 1999). To test whether these two genes have similar activities, we expressed *Nkx2.9* ectopically and examined the pattern of Pax6 expression. Most (>95%) cells that expressed Nkx2.9 ectopically lacked Pax6 expression (Figure 3G). Moreover, the repression of Pax6 occurred in the absence of Nkx2.2 induction (Figure 3H), showing that Nkx2.9 acts independently of Nkx2.2. Thus, Nkx2.2 and Nkx2.9 have similar abilities to repress Pax6 expression and are likely to act in parallel in defining the ventral boundary of the pMN domain in vivo (Briscoe et al., 1999).

Interactions at the p1/p2 Boundary

We next examined whether cross-regulatory interactions occur between the class I protein Dbx2 and the class II protein Nkx6.1—proteins with complementary domains of expression at the p1/p2 boundary. We first misexpressed Dbx2 in regions ventral to the p1 domain and monitored the pattern of homeodomain protein expression. Most (>95%) ventral cells that ectopically expressed Dbx2 lacked expression of Nkx6.1 (Figure 3J), whereas neighboring cells that lacked Dbx2 maintained Nkx6.1 expression (Figure 3J). Misexpression of Dbx2 did not alter the expression of Pax6 or Pax7 (Figures 3K and 3L). Thus, the repressive action of Dbx2 is selective and cell autonomous. We also examined the consequences of misexpression of Nkx6.1 on the expression of Dbx2. Most (>95%) progenitor cells that ectopically expressed Nkx6.1 lacked Dbx2 expression (Figure 3M), whereas neighboring cells that lacked ectopic Nkx6.1 maintained Dbx2 expression (Figure 3M). Ectopic expression of Nkx6.1 did not repress Pax6 or Pax7 (Figures 3N and 3O). Thus, Nkx6.1 acts selectively and in a cell-autonomous manner to repress Dbx2 expression.

These results reveal that the two pairs of class I and class II proteins that share a common progenitor domain boundary exhibit mutual repressive interactions. Such interactions are likely to contribute to the establishment and sharp delineation of progenitor domain boundaries evident in vivo.

The Relationship between Progenitor Domain and Neuronal Fate

We next examined the relationship between the five progenitor domains defined by class I and class II protein

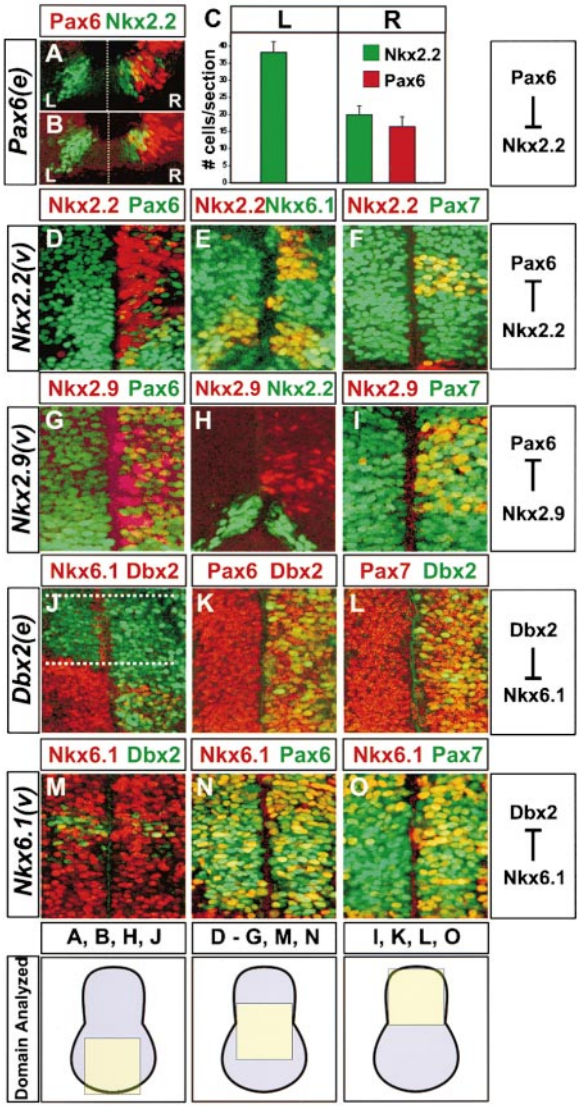

Figure 3. Repressive Interactions at the pMN/p3 and p1/p2 Boundaries

Pax6, Nkx2.2, Nkx2.9, Dbx2, and Nkx6.1 were ectopically expressed using in ovo electroporation (*e*) or retroviral transduction (*v*) and the pattern of expression of other progenitor homeodomain proteins was analyzed at HH stages 22–24.

(A and B) Ectopic expression of Pax6 in the p3 domain results in the cell-autonomous repression of Nkx2.2. A similar level of expression of Pax6 does not repress Dbx2 (data not shown).

(C) Number of $Pax6^+$ and $Nkx2.2^+$ cells within the p3 domain of untransfected (left, L) and transfected (right, R) halves of the neural tube (mean ± SEM, n = 5).

(D–F) Misexpression of Nkx2.2 dorsal to the p3 domain results in the cell-autonomous downregulation of Pax6 (D). Neither Nkx6.1 (E) or Pax7 (F) are repressed by ectopic Nkx2.2 expression. Images representative of 10 embryos. Similar results were obtained after misexpression of *Nkx2.2* by electroporation (not shown).

(G–J) Ectopic expression of Nkx2.9 represses Pax6 expression in a cell-autonomous manner (G). Nkx2.9 does not induce Nkx2.2 expression (H). Nkx2.9 does not repress Pax7 expression (I). Images representative of 10 embryos. (J) Ectopic ventral expression of Dbx2 results in the cell-autonomous repression of Nkx6.1. Ectopic expression of Dbx2 does not repress Pax6 (K) or Pax7 (L).

(M–O) Misexpression of Nkx6.1 dorsal to the p2 domain represses Dbx2 (M) but not Pax6 (N) or Pax7 (O) expression. Images representative of 10 embryos.

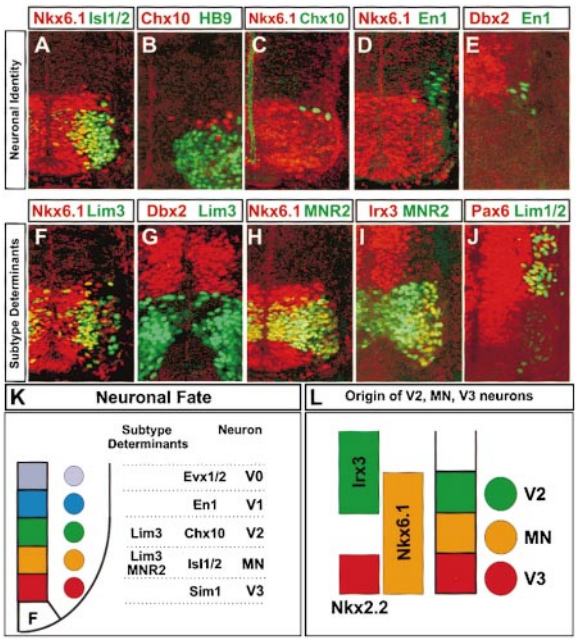

Figure 4. Each Progenitor Domain Generates a Distinct Neuronal Subtype

(A–E) Relationship between class I and class II proteins and neuronal markers. The domain of Nkx6.1 expression encompasses Isl1/2 MNs (A) and Chx10 V2 neurons (C) but is positioned ventral to En1 V1 neurons (D). Chx10 V2 neurons are generated dorsal to HB9 MNs (B). En1 V1 neurons are generated at the ventral extent of the Dbx2 domain (E). Images from HH stage 22–24 embryos.

(F–J) Relationship between class I and class II proteins and neuronal subtype determinants. The domain of Nkx6.1 expression encompasses the domain of generation of Lim3 (F) and MNR2 cells (H). Lim3 cells are positioned ventral to the domain of Dbx2 expression (G). MNR2 cells are positioned ventral to the domain of Irx3 expression (I). Lim1/2 cells derive from Pax6 progenitors (J).

(K) The relationship between progenitor domain identity and neuronal fate.

(L) The progenitor homeodomain code within the three ventral-most domains of neurogenesis.

expression and the pattern of neurogenesis in the ventral neural tube. We have found previously that Evx1/2$^+$ V0 neurons derive from cells within the p0 domain (see Ericson et al., 1997a; Pierani et al., 1999), that En1$^+$ V1 neurons derive from cells within the p1 domain (Ericson et al., 1997a; Pierani et al., 1999) (Figures 4D and 4E) and that *Sim1*$^+$ V3 neurons derive from cells within the p3 domain (Briscoe et al., 1999). We show here that Chx10$^+$ V2 neurons derive exclusively from cells within the p2 domain (Figures 4B and 4C) (Ericson et al., 1997a) and that HB9$^+$ motor neurons (MNs) derive only from cells within the pMN domain (Figures 4A and 4B) (Tanabe et al., 1998; Arber et al., 1999). Thus, a precise register exists throughout the neural tube between the dorsoventral extent of individual ventral progenitor domains and the position at which specific neuronal subtypes are generated.

Progenitor cells express a separate set of homeodomain proteins at late stages in the pathway of ventral neurogenesis. The final division of V2 neuron and MN progenitors is accompanied by the onset of expression of Lim3 (Ericson et al., 1997a; Sharma et al., 1998; Tanabe et al., 1998). Late stage MN progenitors express MNR2 (Tanabe et al., 1998). Lim3 and MNR2 appear to function respectively as determinants of V2 neuron and MN identity (Sharma et al., 1998; Tanabe et al., 1998). We therefore examined whether the expression of Lim3 and MNR2 also conforms to the domains defined by class I and class II protein expression. Lim3 expression was excluded from the p0 and p1 domains but was detected within both the p2 and pMN domains (Figures 4F and 4G and data not shown), whereas MNR2 expression was confined to the pMN domain (Figures 4H–4J). Thus, the expression of these two ventral neuronal subtype determinants also respects progenitor domain subdivisions defined by class I and class II protein expression. The concordance in expression of progenitor homeodomain proteins, late stage progenitor determinants, and neuronal fate supports the idea that the subdivision of the neural epithelium into five progenitor domains is a fundamental step in the allocation of cell fate in the ventral neural tube.

Nkx6.1 Activity Directs Motor Neuron and V2 Neuron Generation

If the combinatorial expression of class I and class II proteins within progenitor cells directs the fate of ventral neurons, then changing the expression profile of these proteins would be expected to alter patterns of neurogenesis. We have focused our analysis of this issue on the three ventral-most progenitor domains, from which V2 neurons, MNs, and V3 neurons are generated (Figure 4K). The combinatorial expression of Nkx6.1, Irx3, and Nkx2.2 distinguishes these three domains of neurogenesis (Figure 4L), and poses three questions about their role in the assignment of neuronal subtype identity. First, is the expression of Nkx6.1 in the absence of expression of Irx3 or Nkx2.2/Nkx2.9 sufficient to result in the generation of MNs? Second, does the coincidence in expression of Nkx6.1 and Irx3 result in the generation of V2 neurons, at the expense of MNs? Third, does the expression of Nkx2.2/Nkx2.9 and Nkx6.1 result in the generation of V3 neurons rather than MNs?

To test whether Nkx6.1 activity is able to generate MNs, we searched for a way of misexpressing Nkx6.1 in neural progenitor cells in the absence of high-level *Irx3* expression. All progenitor cells dorsal to the p2/pMN boundary express *Irx3* (data not shown). The onset of *Irx3* expression occurs only after neural tube closure, later than that of Nkx6.1, and is excluded from the ventral-most region of the neural tube (Supplemental Figure S1; available at http://www.cell.com/cgi/content/full/101/4/435/DC1). We reasoned therefore that misexpression of *Nkx6.1* by dorsal neural cells, prior to the onset of *Irx3* expression, might establish an initial homeodomain protein code (Nkx6.1$^+$, Irx3$^-$) that mimics that found normally in the pMN domain, and thus leads to ectopic MN generation.

Two approaches were taken to achieve early ectopic expression of Nkx6.1. First, *Nkx6.1* was misexpressed in stage 5–6 embryos by retroviral transduction (Figure S1A). With this method, the onset of ectopic protein expression occurs about 12–16 hr later, at approximately stages 12–14 (Figure S1B). At this stage, only at

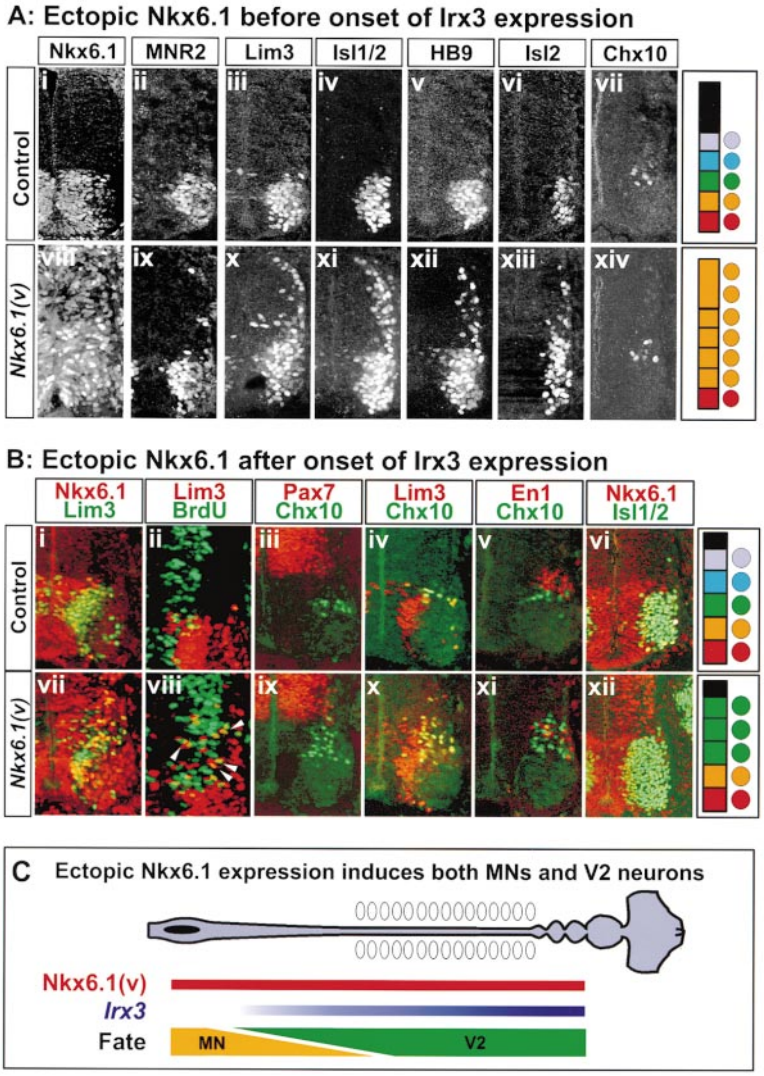

Figure 5. Nkx6.1 Induces both Motor Neurons and V2 Neurons

Patterns of protein expression obtained after misexpression of *Nkx6.1* at caudal (A) and rostral (B) levels of retrovirally infected embryos.

(A) At caudal (lumbar) regions, misexpression of *Nkx6.1* results in ectopic dorsal expression of MNR2 (ii and ix), Lim3 (iii and x), Isl1 (iv and xi), HB9 (v and xii), and Isl2 (vi and xiii). Misexpression of Nkx6.1 induces ectopic Chx10 expression at low incidence and only within the p0 and p1 domain (vii and xiv and data not shown). Electroporation of stage 10 embryos with *Nkx6.1* results in ectopic MNs, at both rostral and caudal levels of the spinal cord (data not shown).

(B) In rostral (cervical/thoracic) regions of infected embryos, misexpression of *Nkx6.1* results in the ectopic induction of V2 neurons. Ectopic expression of Chx10 (ix, x, and xi) and Lim3 (vii, viii, and x) is detected ventral to the boundary of Pax7 expression (ix) in the p1 and p0 domains. The misexpression of *Nkx6.1* decreases the number of En1 V1 neurons (xi) and Evx1 V0 neurons (data not shown), but does not induce MNs (xii). Many ectopic Lim3 cells are labeled by a 30 min BrdU pulse, indicating that Nkx6.1 induces Lim3 expression in progenitor cells. Images representative of 10 experiments.

(C) The relationship between the domains of ectopic dorsal Nkx6.1 expression (red), the pattern of expression of *Irx3* (blue) at the time of onset of ectopic Nkx6.1 expression, and the fate of neurons that emerge from the domain of ectopic Nkx6.1 expression.

the most caudal levels of infected embryos was ectopic neural expression of Nkx6.1 detected before the onset of expression of *Irx3* (Figure S1C). At more rostral levels, the onset of ectopic protein expression occurs at a stage when neural cells already express *Irx3* (Figure S1D). We also misexpressed *Nkx6.1* by electroporation in stage 10 embryos (Figure S1E). In this case, expression of transgenes was detected within ~2–4 hr (Figure S1F) (Muramatsu et al., 1997). Under these conditions, *Nkx6.1* was expressed ectopically prior to the onset of *Irx3* expression over a broader rostrocaudal region of the neural tube (Figures S1F–S1H). Based on these observations, we permitted embryos that had been retrovirally infected or electroporated in ovo with *Nkx6.1* constructs to develop until stages 22–24, and examined the resulting pattern of neurogenesis.

We first examined levels of the neural tube where ectopic dorsal neural expression of *Nkx6.1* occurred prior to that of endogenous *Irx3*. At these levels, the MN subtype determinants MNR2 and Lim3 were detected in ectopic dorsal positions, in both progenitor cells and postmitotic neurons (Figures 5Aix and 5Ax and data not shown). In addition, ectopic dorsal expression of the postmitotic MN markers Isl1, Isl2, and HB9 was detected (Figures 5Axi–5Axiii and data not shown). The ectopic expression of Isl1, Isl2, and HB9 was, however, limited to postmitotic MNs located in the lateral margin of the neural tube (Figures 5Axi–5Axiii). This finding is consistent with previous studies documenting that MNR2 can induce these MN markers only after cells have acquired postmitotic status (Tanabe et al., 1998). Strikingly, the expression of MN markers was detected both dorsal to the p2 domain boundary in the ventral neural tube, and throughout the dorsal extent of the neural tube (Figure 5A and data not shown). Under these conditions, additional ectopic $Chx10^{+}$ V2 neurons were occasionally detected within the p0 and p1 domains, but were not detected in the dorsal spinal cord (Figure 5Axiv and see below). These results show that misexpression of Nkx6.1 in neural cells at stages before the onset of *Irx3* expression can induce ectopic MN generation (Figure 5C).

We next examined the fate of cells at levels of the neural axis where ectopic expression of Nkx6.1 occurred together with *Irx3*. Misexpression of Nkx6.1 at this level

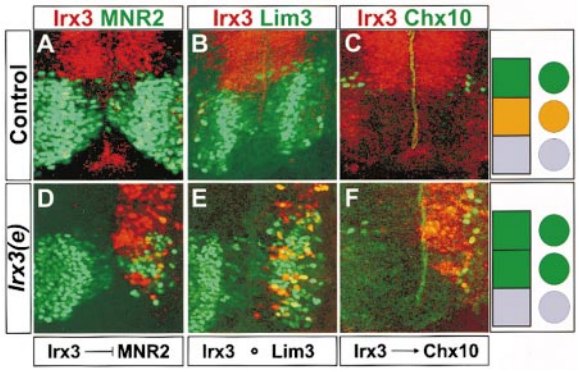

Figure 6. Irx3 Represses Motor Neuron Generation and Induces V2 Neurons

(A) The ventral limit of Irx3 expression corresponds to the dorsal extent of MNR2$^+$ cells in control embryos. Progenitor cells in the ventral-most domain of Irx3 expression give rise to V2 neurons that express Lim3 (B) and Chx10 (C). After ventral misexpression of *Irx3* by electroporation there is no change in the pattern of Lim3 expression (E) but MNR2$^+$ cells are repressed (D) and Chx10$^+$ V2 neurons are generated within the pMN domain (F). Images representative of 10 experiments.

resulted in the ectopic generation of many Chx10$^+$ V2 neurons within the p0 and p1 domains (Figures 5Bix–5Bxi). Many ectopic Lim3$^+$ cells were also detected within these domains, some of which were mitotic progenitors (Figure 5Bviii). In addition, the ectopic expression of Nkx6.1 within the p0 and p1 domains resulted in a marked decrease in the number of En1$^+$ V1 (Figure 5Bxi) and Evx1/2$^+$ V0 neurons (data not shown). Ectopic MN markers were not detected, suggesting that the coincident expression of *Irx3* attenuates the ability of Nkx6.1 to induce MNs (Figure 5Bxii). Together, these results support the idea that Nkx6.1, in the context of *Irx3* activity, promotes the generation of V2 neurons (Figure 5C).

Misexpression of Irx3 Directs V2 Neuron Generation at the Expense of Motor Neurons

To test more directly whether the expression of Irx3 in progenitor cells that express Nkx6.1 results in a switch from MN to V2 neuron fate, we misexpressed Irx3 in regions ventral to the p2 domain and examined the resulting pattern of neurogenesis. Cells that ectopically expressed Irx3 failed to express the MN markers MNR2, Isl1/Isl2, or HB9 (Figures 6A and 6D and data not shown). Neighboring pMN cells that lacked ectopic Irx3 expression maintained expression of these MN markers (Figure 6D), indicating the cell autonomy of Irx3 action. In addition, V2 neurons, defined by Chx10 expression, were generated at markedly more ventral positions, within the normal domain of MN generation (Figures 6C and 6F). The pattern of Lim3 expression was not altered by ventral misexpression of Irx3 (Figures 6B and 6E), consistent with the normal overlap of Lim3 and Irx3 expression within the p2 domain.

These findings, taken together with the results of late Nkx6.1 misexpression described above, indicate that coexpression of Irx3 and Nkx6.1 by ventral progenitor cells specifies V2 neuron identity. The domain of the ventral neural tube in which Nkx6.1 is able to generate MNs thus appears to be limited by the expression of Irx3 in cells dorsal to the p2/pMN domain boundary.

Nkx2.2 Constrains the Ability of Nkx6.1 to Induce Motor Neurons

We next examined whether the expression of Nkx2.2 within the pMN domain is sufficient to repress MN generation. To test this, we misexpressed Nkx2.2 in regions dorsal to the p3 domain and examined the resulting pattern of neurogenesis. We detected a marked repression in the expression of MNR2, Lim3, Isl1, Isl2, and HB9 in cells that expressed Nkx2.2 (Figure 7A and data not shown). A few ectopic Nkx2.2-labeled cells that coexpressed HB9 were detected in a lateral position, characteristic of postmitotic neurons (Figure 7A). The coexpression of Nkx2.2 and MN markers in these cells is likely to reflect the late onset of expression of Nkx2.2, after cells have committed to a MN fate. These results show that Nkx2.2 activity is sufficient to repress MN differentiation, extending findings that Nkx2.2 activity within the p3 domain is required to suppress MN fate (Briscoe et al., 1999).

Nkx2.2 Expression Directs V3 Interneuron Generation

The role of Nkx2.2 in repressing MN generation raised the additional issue of whether Nkx2.2 activity is sufficient to generate V3 neurons. To test this, we analyzed the pattern of expression of the V3 neuron marker *Sim1* in *Nkx2.2*-infected embryos. Misexpression of Nkx2.2 directed the ectopic expression of *Sim1* both within the domain of Nkx6.1 expression and throughout the dorsal neural tube (Figures 7Bi–7Biv). Nkx2.2 did not induce ectopic Nkx6.1 expression (data not shown), and Nkx6.1 was not sufficient to induce V3 neurons (Figures 7Bv and 7Bvi). Thus, Nkx2.2 is able to induce V3 neurons independently of Nkx6.1 activity. Nkx2.9 mimicked the ability of Nkx2.2 to induce V3 neurons (Figures 7Bvii and 7Bviii), supporting the idea that these two proteins have equivalent patterning activities. These findings, taken together with studies of *Nkx2.2* mutant mice (Briscoe et al., 1999), establish the critical role of Nkx2.2 in suppressing MN and promoting V3 neuron fates.

Discussion

The results described in this study fit most easily into a three-step model that links graded Shh signaling, the expression of class I and class II proteins by neural progenitor cells, and the pattern of neuronal subtype generation in the ventral neural tube (Figure 8). In a first step, the expression of progenitor cell homeodomain proteins is differentially repressed or activated by graded Shh signaling (Figure 8A). In a second step, cross-repressive interactions between class I and class II proteins establish, refine, and stabilize progenitor domains (Figure 8B). In a third step, the profile of homeodomain proteins expressed within each progenitor domain directs the generation of specific sets of postmitotic neurons (Figure 8C). We discuss each step of this model in the context of the interpretation of graded extracellular signals during the patterning of embryonic tissues.

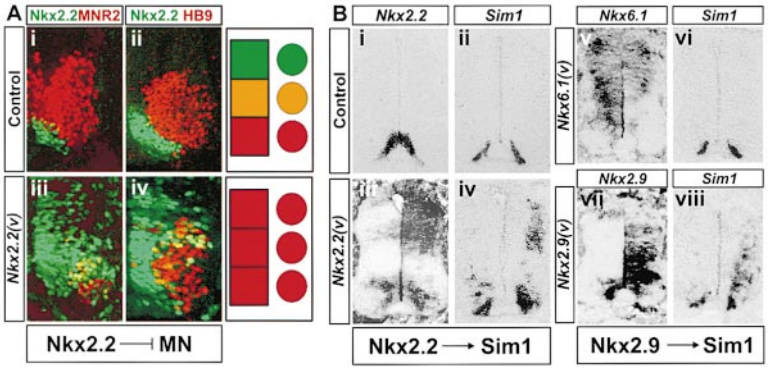

Figure 7. Nkx2.2 Activity Represses Motor Neuron Generation and Induces V3 Neurons

(A) MNR2$^+$ MN progenitors (i) and HB9$^+$ MNs (ii) are not generated from Nkx2.2 progenitors in control embryos (i and ii). Ectopic expression of Nkx2.2 (iii and iv) in pMN progenitors represses MNR2 (iii) and HB9 (iv) expression. Some more lateral cells coexpress Nkx2.2 and MN markers, probably because cells were infected with *Nkx2.2* virus after they had committed to a MN fate.

(B) *Sim1*$^+$ V3 neurons (ii) are generated from Nkx2.2 progenitors (i) in the p3 domain of control embryos. Misexpression of Nkx2.2 (iii) results in the ectopic dorsal expression of *Sim1* (iv). Nkx6.1 (v) has no effect on *Sim1* expression (vi). Nkx2.9 (vii) is sufficient to induce V3 neurons (viii). Images representative of 10 experiments.

Formation and Maintenance of Neural Progenitor Domains

Our findings address first the issue of how discrete progenitor domains are established in the ventral neural tube in response to Shh signaling. A ventral to dorsal gradient of Shh signaling activity appears to have an initial role in defining the dorsoventral domains over which individual class I and class II proteins are expressed. Yet, the existence of an extracellular gradient of Shh activity does not offer an easy explanation for the sharp boundaries that exist between progenitor domains. Our findings suggest that cross-repressive interactions that occur between class I and class II proteins may serve two early roles: first to establish the initial dorsoventral domains of class I and class II protein expression, and second to refine the initially imprecise pattern of homeodomain protein expression initiated by graded Shh signals. Support for this idea comes from the analysis of ventral patterning in mouse mutants lacking homeodomain protein function. The loss of Pax6 function leads to an expansion in the dorsoventral extent of the p3 domain, despite a constant level of Shh activity (Ericson et al., 1997a). Conversely, the loss of Nkx6.1 function results in a ventral expansion in the extent of the p1 domain, without any change in Shh signaling (M. Sander et al., submitted). It is noteworthy that the boundaries of each of the five progenitor domains are sharply defined, yet class II proteins have been identified only at the pMN/p3 and p1/p2 boundaries. Thus, additional class II proteins may exist, with patterns of expression that complement the three orphan class I proteins.

A second issue is how individual progenitor domains are maintained in relatively constant proportions over time. As neuronal fates are established, ventral progenitor cells undergo multiple rounds of proliferation (Langman et al., 1966) and the dorsoventral extent of the ventral neural tube increases markedly in size. Thus, the level of Shh activity at a given position in the ventral neural tube is likely to change significantly over time. Our findings show that by stage 15, ventral progenitor domains can be maintained despite the loss of Shh signaling. The cross-repressive interaction between class I and class II proteins may help to maintain progenitor domains over time, in the face of a changing level of Shh activity. Our findings suggest that these cross-repressive interactions relieve progenitor cells of a requirement for ongoing Shh signaling but do not exclude that Shh has a later role in regulating the proliferation of cells within individual progenitor domains (Rowitch et al., 1999).

How do neural progenitor cells initially perceive the extracellular gradient of Shh activity? Several components of the vertebrate hedgehog signaling pathway have been identified (Ingham, 1998). In particular, two zinc finger transcription factors, Gli1 and Gli2, have been proposed as intermediaries in Shh signaling (Ruiz and Altaba, 1999). One view of the initial steps in Shh signal transduction argues that the level of Gli activity varies in proportion to the concentration of extracellular Shh (Ingham, 1998), and thus, different levels of Gli activity may repress or activate different class I and class II homeobox genes. However, ventral neuronal pattern is surprisingly normal in mice containing mutations in both the *Gli1* and *Gli2* genes (Ding et al., 1998; Matise et al.,

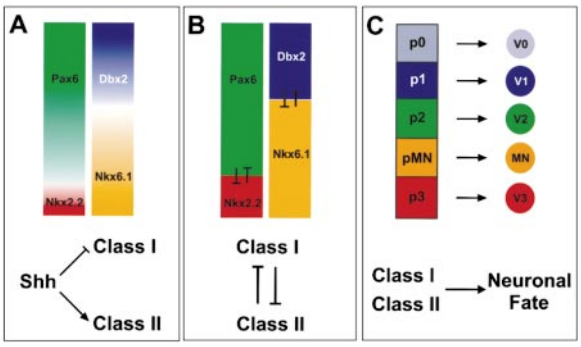

Figure 8. Three Phases of Ventral Neural Patterning

(A) Graded Shh signaling initiates dorsoventral restrictions in the domains of class I and class II protein expression within the ventral neural tube. Class I proteins are repressed by Shh signals and class II proteins require Shh signaling. Individual class I and class II proteins have different Shh concentration requirements for repression or activation.

(B) Cross-repressive interactions between class I and class II proteins that abut a common progenitor domain boundary refine and maintain progenitor domains.

(C) The profile of expression of class I and class II proteins within an individual progenitor domain controls neuronal fate.

1998). These findings raise the possibility (see Krishnan et al., 1997; Lewis et al., 1999) that additional transcriptional mediators participate in the initial interpretation of graded Shh signals within ventral progenitor cells.

The uncertainty that persists about the initial stages of Shh signal transduction in neural cells also leaves unresolved the issue of whether Shh acts independently to repress class I and to activate class II genes. The pairs of class I and class II proteins that form complementary domain boundaries are potent repressors of each other's expression. Thus, the repression of class I genes by Shh could depend on the activation of class II gene expression. Alternatively, the requirement for class II protein expression on Shh signaling may depend on the Shh repression of class I protein expression. A similar derepression mechanism has been suggested to operate during *Drosophila* development, in the dpp-mediated patterning of imaginal disc cells (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999).

The cross-regulatory interactions revealed for class I and class II proteins also have implications for the lineage relationship of neurons generated in the ventral neural tube. Lineage tracing studies have reported a temporal change in the extent to which clonally related cells disperse along the dorsoventral axis of the ventral neural tube (Leber and Sanes, 1995). After early stage marking of ventral progenitors, clonally related progeny spread widely along the dorsoventral axis of the ventral neural tube and acquire different neuronal identities (Leber and Sanes, 1995; Erskine et al., 1998). But, the progeny of clones marked at later developmental stages are restricted to narrower dorsoventral domains, and within these domains, cells acquire more uniform neuronal fates (Leber and Sanes, 1995). The timing of the cross-regulatory interactions between class I and class II proteins that seem to confer progenitor domain identity matches well with the time of restriction in clonal cell dispersal, suggesting a causal relationship between these two processes. The homeodomain proteins that define an individual ventral progenitor domain could control the surface properties of progenitor cells and restrict their intermixing along the dorsoventral axis, in a manner analogous with mechanisms that establish segmental domains along the rostrocaudal axis of the hindbrain (Lumsden and Krumlauf, 1996; Xu et al., 1999).

Control of Neuronal Identity by a Homeodomain Protein Code

This study has relied on ectopic expression methods to address the roles of Nkx6.1, Nkx2.2, and Irx3 in specifying the fate of V2 neurons, MNs, and V3 neurons. Our results show that Nkx2.2 activity is sufficient to induce V3 neurons, that Nkx6.1 activity in the absence of Irx3 induces MNs, whereas Nkx6.1 activity in the presence of Irx3 induces V2 neurons. The inferences derived from these gain-of-function studies are supported by the switches in neuronal fate that occur in mice in which individual class I and class II proteins have been inactivated by gene targeting. In mice lacking Pax6 activity, the dorsal expansion in the domain of Nkx2.2 expression is accompanied by an expansion in the domain of V3 neuron generation, and by the loss of MNs (Ericson et al., 1997a). Conversely, the loss of Nkx2.2 results in the loss of V3 neurons and in the ectopic generation of MNs within the p3 domain (Briscoe et al., 1999). In addition, the loss of Nkx6.1 activity depletes the ventral neural tube of many MNs and V2 neurons (M. Sander et al., submitted).

How do class I and class II proteins control neuronal subtype identity? The final cell division of certain ventral progenitors is accompanied by the onset of expression of a distinct set of homeodomain proteins, notably MNR2 and Lim3 (Tanabe et al., 1998; Ericson et al., 1997; Sharma et al., 1998). Ectopic expression of MNR2 is able to induce MN differentiation independent of dorsoventral position, and ectopic expression of Lim3 induces V2 neurons (Tanabe et al., 1998). Our studies indicate that class I and class II proteins function upstream of MNR2 and Lim3. Thus, within the pMN and p2 domains, the actions of progenitor homeodomain proteins in specifying neuronal subtype identity are likely to be mediated through MNR2 and Lim3. Subtype determinant factors with equivalent functions may therefore be expressed by cells in the other ventral progenitor domains.

Our findings provide further support for the idea that the activity of individual homeodomain proteins can direct specific neuronal fates in the developing spinal cord. We show here that Nkx2.2 can specify V3 neuronal identity. In previous studies MNR2 has been shown to specify MN identity and Lim3 to direct V2 neuronal identity (Tanabe et al., 1998). Thus, the fate of other classes of neurons in the ventral spinal cord and perhaps in other regions of the vertebrate central nervous system, may be controlled through the actions of similarly dedicated transcription factors. The activities of Nkx6.1 revealed in our studies also provide further insight into the hierarchical functions of homeodomain proteins in specifying spinal MN identity. Nkx6.1 can induce the expression of both MNR2 and Lim3 in MN progenitors, and like MNR2, is able to specify MN fate in dorsal neural tube cells. Thus, it seems possible that Nkx6.1 functions upstream of MNR2 in a linear pathway of MN generation in the chick embryo.

Linking Graded Extracellular Signals to Neuronal Subtype Diversity

A set of seven homeodomain proteins defines five neural progenitor domains with a fundamental role in the organization of ventral neural pattern. The analysis of these homeodomain proteins suggests that ventral patterning proceeds in three stages: (1) the regulation of class I and class II proteins by graded Shh signals, (2) the refinement and maintenance of progenitor domain identity by cross-repressive interactions between homeodomain proteins, and (3) the translation of a homeodomain protein code into neuronal subtype identity. The central features of this model may apply to other vertebrate tissues in which cell pattern is regulated by local sources of extrinsic signals. Consistent with this idea, cross-regulatory interactions between transcription factors have been suggested to refine cell pattern in the embryonic mesoderm and in the pituitary gland (Dasen and Rosenfeld, 1999; Papin and Smith, 2000).

Finally, we note that the principles of the model of ventral patterning outlined here resemble those involved

in subdividing the *Drosophila* embryo (Lawrence, 1992). Graded Shh signaling subdivides the ventral neural tube into five domains, just as graded levels of the dorsal protein establish five distinct regions of the early *Drosophila* embryo (Huang et al., 1997), suggesting an upper limit to the number of distinct cell fates that can be generated in response to a single gradient signaling system. In addition, the graded anterioposterior distribution of maternally supplied factors in the *Drosophila* embryo is known to initiate the expression of a set of proteins encoded by the *gap* genes (Struhl et al., 1992). Subsequent cross-regulatory interactions establish and maintain sharp boundaries in the expression of gap proteins, and their activities within individual domains control later aspects of cell pattern (Kraut and Levine, 1991; Wu et al., 1998). Thus, in the neural tube and the *Drosophila* embryo, the cross-repression of genes whose initial expression is controlled by graded upstream signals provides an effective mechanism for establishing and maintaining progenitor domains and for imposing cell type identity.

Experimental Procedures

Retroviral Transduction and In Ovo Electroporation

Mouse *Nkx2.2*, *Nkx2.9*, rat *Nkx6.1*, chick *Dbx2*, and *GFP* cDNAs were cloned into RCASBP(A) and (B) vectors (Hughes et al., 1987; Morgan and Fekete, 1996). Viral supernatants (Morgan and Fekete, 1996) were applied to Hamburger and Hamilton (1951) (HH) stage 5–6 chick embryos in ovo. Retroviral transduction resulted in expression of the target protein 12–14 hr postinfection (data not shown). For electroporation, cDNAs were cloned into RCASBP or pNES (gift of U. Lendhal) vectors. HH stage 10–12 chick embryos were electroporated unilaterally with cDNAs for mouse *Irx3*, *Pax6*, *RCASBP(Dbx2)*, and *RCASBP(GFP)* using a T820 electro-squareporator (BTX Inc.) and ectopic protein expression was detected after 2–4 hr. Embryos were analyzed at HH stages 20–24.

Immunocytochemistry and In Situ Hybridization Histochemistry

Guinea pig antisera were generated against peptides encoding the N-terminal 14 residues of mouse Irx3 and the N-terminal 12 residues of mouse Nkx2.9. Other antibody reagents and protocols have been described (Yamada et al., 1993; Ericson et al., 1997a; Tanabe et al., 1998; Briscoe et al., 1999; Pierani et al., 1999). In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using probes for *Irx3*, *Nkx2.2*, *Sim1*, *Nkx6.1*, and *Nkx2.9* (Briscoe et al., 1999).

BrdU Incorporation

To define mitotic cells, 100 μM BrdU was applied to HH stage 22 embryos, followed by incubation at 37°C for 30 min, at which time embryos were fixed and analyzed.

Neural Explant Culture

Neural explants were isolated from intermediate [i] regions of stage 10 chick neural plate or ventral + floor plate [vf] regions from stage 10 or stage 15 embryos, as described (Yamada et al., 1993; Ericson et al., 1996). Explants were cultured for 24 hr with or without Shh-N (Ericson et al., 1996), or in the presence of anti-Shh IgG (20 μg/ml) (Ericson et al., 1996). Explants were processed as described (Ericson et al., 1997a).

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