The role of Phox2b in synchronizing pan-neuronal and type-specific aspects of neurogenesis

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SUMMARY

Within the developing vertebrate nervous system, specific subclasses of neurons are produced in vastly different numbers at defined times and locations. This implies the concomitant activation of a program that controls pan-neuronal differentiation and of a program that specifies neuronal subtype identity, but how these programs are coordinated in time and space is not well understood. Our previous loss- and gain-of-function studies have defined Phox2b as a homeodomain transcription factor that coordinately regulates generic and type-specific neuronal properties. It is necessary and sufficient to impose differentiation towards a branchio- and viscero-motoneuronal phenotype and at the same time promotes generic neuronal differentiation. We have examined the underlying genetic interactions. We show that *Phox2b* has a dual action on pan-neuronal differentiation. It upregulates the expression of proneural genes (*Ngn2*) when expressed alone and upregulates the expression of *Mash1* when expressed in combination with *Nkx2.2*. By a separate pathway, *Phox2b* represses expression of the inhibitors of neurogenesis *Hes5* and *Id2*. The role of *Phox2b* in the specification of neuronal subtype identity appears to depend in part on its capacity to act as a patterning gene in the progenitor domain. *Phox2b* misexpression represses the *Pax6* and *Olig2* genes, which should inhibit a branchiomotor fate, and induces *Nkx6.1* and *Nkx6.2*, which are expressed in branchiomotor progenitors. We further show that Phox2b behaves like a transcriptional activator in the promotion of both, generic neuronal differentiation and expression of the motoneuronal marker Islet1. These results provide insights into the mechanisms by which a homeodomain transcription factor through interaction with other factors controls both generic and type-specific features of neuronal differentiation.

Key words: Phox2b, Neurogenesis, Neural tube, Neuronal specification, Chick

INTRODUCTION

The vertebrate CNS is derived from the neuroepithelium, a single layer of proliferating cells with stem cell characteristics that line the lumen of the neural tube. During neurogenesis, the dividing neural stem cells generate neuronal precursors that migrate away from the ventricular zone (VZ), generally after completion of their last mitosis, and begin to express generic and type-specific neuronal genes. A key question in early CNS development is how specific subclasses of neurons are produced in proper numbers at the correct times and locations. This process involves the concomitant activation of programs that commit neural progenitors to differentiation and of programs directing neuronal subtype identity. Considerable advances have been made in defining the mechanisms that govern each of these programs in several model systems (reviewed by Cepko, 1999; Guillemot, 1999; Jessell, 2000; Briscoe and Ericson, 2001; Ohnuma et al., 2001). However, the question of how they are integrated to yield the highly

reproducible pattern of neurogenesis has been addressed only very recently (for a review, see Bertrand et al., 2002).

Generic neuronal differentiation in vertebrates, as in *Drosophila*, is regulated by basic helix-loop-helix (bHLH) transcription factors, which promote it, and the Notch signaling pathway, which inhibits it. Several vertebrate genes encoding bHLH proteins (such as *Ngn1*, *Ngn2* and *Mash1*) are thought to be equivalent to *Drosophila* proneural genes and to confer competence to become a neuron (for a review, see Kageyama and Nakanishi, 1997; Brunet and Ghysen, 1999; Bertrand et al., 2002). Pioneering studies in *Xenopus* and zebrafish have demonstrated that many of the neurally expressed bHLH factors promote neuronal differentiation during primary neurogenesis (Ma et al., 1996; Blader et al., 1997). Recent work in mouse and chick embryos shows that they function similarly in the neural tube of higher vertebrates (Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001). A key property of the early expressed bHLH proteins is that they restrict their own proneural action by activating the expression

of genes that inhibit neurogenesis. This 'lateral inhibition' has been well documented during primary neurogenesis in *Xenopus* and in the chick retina, where neural bHLH proteins up-regulate the expression of Delta that activates the Notch receptor on neighboring cells (Chitnis et al., 1995; Henrique et al., 1997; Koyano-Nakagawa et al., 1999). Activated Notch in turn inhibits expression and activity of the neural bHLH genes via its effector genes, the bHLH transcriptional repressors of the Hes family, which have been shown to inhibit neurogenesis in a variety of settings (Ishibashi et al., 1995; Wettstein et al., 1997; Kageyama and Nakanishi, 1997; Kageyama and Ohtsuka, 1999; Ohtsuka et al., 1999; Castella et al., 2000; Nakamura et al., 2000; Kondo and Raff, 2000; Cau et al., 2000; Kabos et al., 2002).

Another class of molecules that have been implicated in the control of neurogenesis are the Id HLH factors. Id proteins block differentiation and promote proliferation in diverse cell types, including neural cells, mainly by acting as dominant-negative inhibitors of positive regulatory bHLH proteins (for a review, see Norton, 2000). The four vertebrate Id family members are expressed in the embryonic neural tube in partially overlapping patterns (Jen et al., 1997). In mice double mutant for *Id1* and *Id3*, the neural tube shows signs of premature neuronal differentiation (Lyden et al., 1999). Conversely, overexpression of *Id2* leads to overgrowth of the neural tube (Martinsen and Bronner-Fraser, 1998), and forced expression of *Id1* or *Id2* blocks neuronal differentiation (Cai et al., 2000; Toma et al., 2000). However, how the expression of the Id family members is regulated in the neural tube and their precise mode of action in neural cells have not been elucidated.

In general, newly born CNS neurons acquire phenotypes that reflect their site of origin in the VZ. How this comes about has been best studied in the ventral spinal cord. In response to a gradient of sonic hedgehog secreted from ventral axial structures, the VZ is partitioned along the dorsoventral axis into discrete domains that express particular combinations of homeodomain (HD) transcription factors. These HD proteins appear to specify the subtype identity of their neuronal progeny through the action of a different set of HD proteins, which are switched on around the time of the last mitosis (for a review, see Jessell, 2000; Briscoe and Ericson, 2001; Lee and Pfaff, 2001). Most of the early expressed HD proteins appear to function as transcriptional repressors and are thought to specify neuronal identity by repressing alternative fates (Muhr et al., 2001; Vallstedt et al., 2001).

How the different molecular machineries that direct generic and type-specific aspects of neuronal differentiation are coordinated in any class of neurons remains poorly understood. One way this seems to be achieved is by the fate-specifying action of proneural genes themselves. Rather than merely drive a 'generic' pathway of neuronal differentiation, they also participate in the specification of neuronal types (Fode et al., 2000; Gowan et al., 2001; Scardigli et al., 2001; Parras et al., 2002). A striking recent example of the neural-fate determining properties of a bHLH factor is provided by *Olig2* function in spinal motoneuron progenitors (Mizuguchi et al., 2001; Novitch et al., 2001; Lu et al., 2002).

We have previously documented such a dual action on pan-neuronal and type-specific differentiation for the fate-determining HD protein Phox2b. In the ventral hindbrain, *Phox2b* is expressed by the progenitors of the two main classes of cranial motor neurons, the branchiomotor (bm) and visceromotor (vm) neurons (collectively termed bm/vm neurons), and by their postmitotic descendants, but not in somatic motor (sm) neurons (Pattyn et al., 1997). In the progenitors, *Phox2b* is necessary for cell cycle exit in proper numbers. In the postmitotic precursors, *Phox2b* function is required for all aspects of type-specific and generic differentiation. Conversely, forced expression of *Phox2b* in the spinal cord promotes pan-neuronal differentiation and emigration from the VZ and imparts a phenotype, which resembles that of bm/vm neurons (Pattyn et al., 2000; Dubreuil et al., 2000). In the *Phox2b*-expressing cells, the early postmitotic markers *Delta1* and *Math3/NeuroM* are induced prematurely, and the neurons thus generated ectopically express *Phox2a*, choline acetyltransferase (*ChAT*) and *Islet1*, but not *Islet2*, as do bm and vm neurons. However, the molecular interactions by which *Phox2b* accomplishes this have not been elucidated.

We have begun to examine the genetic interactions by which *Phox2b* promotes both, generic and type-specific aspects of neurogenesis. Ectopic expression studies provide evidence that *Phox2b* drives pan-neuronal differentiation by upregulating *Ngn2* in the absence and of *Mash1* in the presence of *Nkx2.2* and by repressing the negative regulators of neurogenesis chick *Hes5b* and *Id2*. Initiation of a bm/vm fate represents a third activity that implies downregulation of *Pax6* and *Olig2* and upregulation of *Nkx6.1* and *Nkx6.2*. Our data reveal how an HD transcription factor, through interaction with other factors expressed in the progenitor domain, coordinately regulates pan-neuronal and type-specific differentiation.

MATERIALS AND METHODS

Expression vectors

The coding regions of mouse *Hes5* (Takebayashi et al., 1995), chicken *Id2* (Martinsen and Bronner-Fraser, 1998), *Mash1* (Cau et al., 1997), mouse *Ngn2* (Cau et al., 1997), mouse *Nkx2.2* (Hartigan et al., 1996) and *mPhox2b* (Pattyn et al., 1997) were cloned into the *pCAGGS* vector that drives expression by a CMV/actin hybrid promoter (Koshiba-Takeuchi et al., 2000). *GFP* was expressed from the *pCAGGS-AFP* vector (Momose et al., 1999). Activator and repressor forms of Phox2a/b were constructed by fusing the PCR-amplified *Phox2a* homeobox (which is identical at the amino acid level to that of *Phox2b*) in frame 3′ to either the *Drosophila Engrailed* repressor domain [the construct is identical to the one described by Lo et al. (Lo et al., 1999) under the name pERPH] or the herpes simplex *VP16* transactivation domain (Triezenberg et al., 1988) and subcloning into the *pCAGGS* vector, yielding PHDEnR and PHDVP16, respectively. To express the isolated homeodomain, a Kozak sequence containing an initiator methionine was fused 5′ to the *Phox2a* homeobox before subcloning into *pCAGGS*. *Siamois*-based control constructs were as described previously (Lemaire et al., 1998). Correct expression of all constructs was verified by in situ hybridization with the appropriate probes. In all cases, expression of the transfected gene was co-extensive with that of GFP.

Electroporation

Chick embryos 44- to 52-hour-old (HH 12-14) were electroporated in ovo essentially as described (Dubreuil et al., 2000). The expression vectors were used at 1 mg/ml except for Nkx2.2 at low doses and the homeobox fusion constructs, which were used at 0.5 mg/ml, and *pCAGGS-AFP* (0.8 mg/ml). We always co-injected *pCAGGS-AFP* to visualize the transfected area. Embryos were allowed to develop at

38°C for different time periods. After harvesting, the embryos were fixed in 4% paraformaldehyde, embedded in gelatin and analyzed on transverse neural tube sections at the transfected level.

Histological methods

Antisense RNA probes for *Cash1* (Jasoni et al., 1994), *Delta1* (Henrique et al., 1997), *EGFP* (Clontech), *cHes5b*, *Id2* (Martinsen and Bronner-Fraser, 1998), *Islet2* (Tsuchida et al., 1994), *NeuroM* (Roztocil et al., 1997), *Ngn1* and *Ngn2* (Perez et al., 1999), *Nkx2.2* (Briscoe et al., 1999), *Nkx6.1* (Qiu et al., 1998), *Nkx6.2* (Cai et al., 1999), *Olig2* (Zhou et al., 2001), *Pax6* (kindly provided by T. Ogura), and *Phox2b* and choline acetyltransferase (ChAT) (kindly provided by T. Jessell) were labeled using a DIG-RNA labeling kit (Roche). In situ hybridization and combined in situ hybridization and immunohistochemistry on cryosections were carried out as described (Hirsch et al., 1998; Dubreuil et al., 2000). For immunohistochemistry, the following antibodies were used: monoclonal anti-BrdU (Sigma), monoclonal anti-Islet1/2 (Tsuchida et al., 1994) and rabbit anti-mouse Phox2b (Pattyn et al., 1997). BrdU incorporation and detection in chick embryos were carried out as described (Sechrist and Marcelle, 1996). Pictures were taken with Kappa DX30, Nikon DXM 1200 or Leica DC300F CCD cameras using Kappa, ACT-1 or Leica software and assembled using Adobe Photoshop.

Quantitative analyses

In situ hybridization signals were quantified by measuring the signal intensity captured with a CCD camera on transverse spinal cord sections. On each section, the mean signal intensity was recorded for the optimally transfected area, as determined by GFP expression on an adjacent section, and in an equivalent area from the non-transfected side. The results were expressed as the difference in mean signal intensities between the transfected and the non-transfected sides and statistical significance determined by two-tailed *t*-test.

RESULTS

To explore the genetic interactions by which Phox2b triggers generic and type-specific neuronal differentiation, we used in ovo electroporation in chick embryos. By this method, high-level expression of exogenous genes is achieved in neuroepithelial progenitors and persists in the differentiating mantle layer (ML) neurons. Misexpression of Phox2b should thus mimic the situation in the ventral hindbrain, where Phox2b is expressed in the VZ progenitors and persists during terminal differentiation of the bm/vm neurons born in this region.

Phox2b upregulates expression of proneural genes

One way by which *Phox2b* could drive neuronal progenitors to become postmitotic is by inducing or boosting the expression
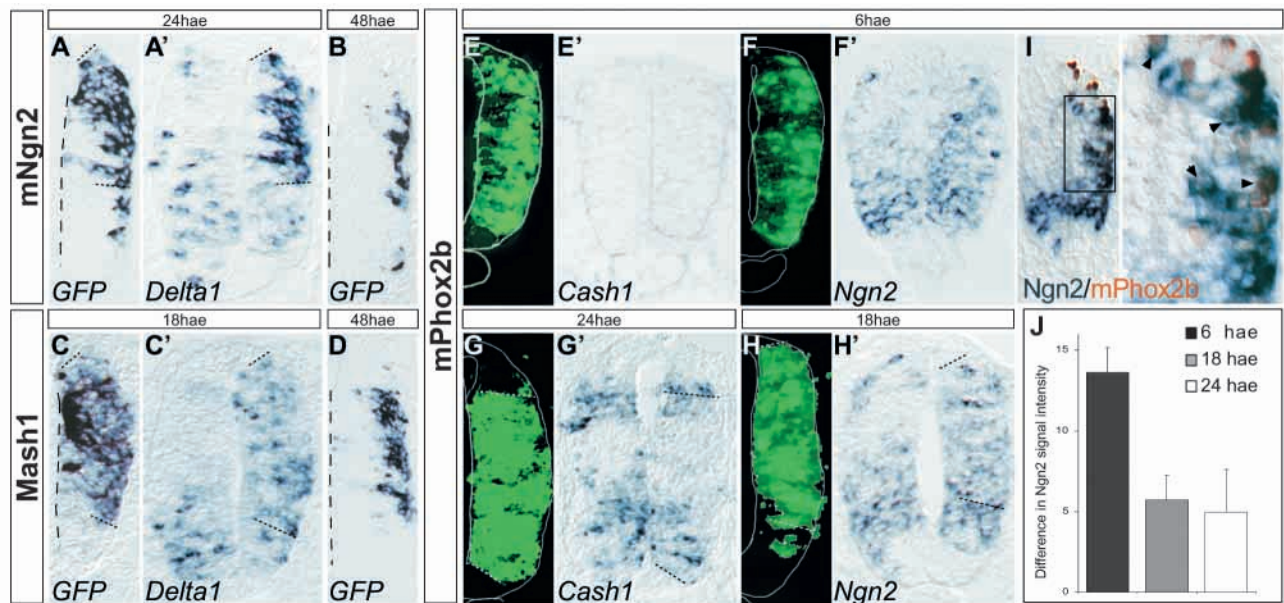

Fig. 1. *Phox2b* misexpression promotes *Ngn2* but not *Cash1* expression. In this and the following figures, light stippled lines demarcate the transfected area. A,A′,A″ means that these are either consecutive sections from the same embryo (for in situ hybridization) or the same sections photographed through different filters (for GFP fluorescence and fluorescent antibody labeling). A broken line indicates the lumen of the neural tube. The time of incubation after electroporation (h.a.e.) is indicated above each panel. (A-D) *Ngn2* and *Mash1* have proneural activity in the chick neural tube. Overexpression of mouse *Ngn2* or *Mash1* in spinal regions of the chick neural tube upregulates Delta1 (A,A′,C,C′). At 24 h.a.e. of mouse *Ngn2*, the transfected cells start to relocate to the mantle layer (ML) (A), and migration is complete at 48 h.a.e. (B). At 18 h.a.e. of *Mash1*, the transfected cells are still in the neuroepithelium (C), but they have relocated to the ML at 48 h.a.e. (D). In control transfections, most cells were still in the neuroepithelium at 48 h.a.e. (see Dubreuil et al., 2000). (E-H′) In situ hybridization with *Ngn2* and *Cash1* probes on spinal cord sections at different times after electroporation of *mPhox2b*. GFP fluorescence shows the extent of electroporation. (F′,I) Forced expression of *mPhox2b* expands the *Ngn2* expression domain at 6 h.a.e.; the effect becomes less pronounced at 18 h.a.e. (H′). Double-labeling with a *Ngn2* probe and anti-Phox2b antibodies (I) shows that most cells expressing *Ngn2* ectopically also express *mPhox2b* (arrowheads in right panel). The dorsalmost region of the neural tube appears refractory to *Ngn2* induction. *Cash1* is not induced at 6 h.a.e. (E′) or at 24 h.a.e. (G′). Note that at the earlier time point (HH 15/16), *Cash1* is not yet expressed at spinal levels of the neural tube (E′). (J) Quantitative analysis of *Ngn2* induction by *mPhox2b* at different times after electroporation. Results are expressed as the difference in *Ngn2* signal intensity between the transfected and the non-transfected sides (arbitrary units). Data points represent mean values±s.e.m. from more than 35 sections from at least five transfected embryos.

of bHLH transcription factors with proneural activity, which should promote pan-neuronal differentiation. *Ngn2* overexpression in neuroepithelial progenitors, for example, has recently been shown to promote their exit from the cell cycle, migration to the ML and expression of pan-neuronal markers (Mizuguchi et al., 2001; Novitch et al., 2001). We show in addition that, as does misexpression of *Phox2b* (Dubreuil et al., 2000), misexpression of *Ngn2* also upregulates *Delta1*, the earliest known marker of postmitotic cells in the VZ (Myat et al., 1996) (Fig. 1A). When overexpressed in the chick neural tube, *Mash1* behaved basically like *Ngn2*. It promoted cell cycle exit as assessed by BrdU incorporation, *Delta1* expression and relocation to the ML (Fig. 1C,D, and not shown). After transfecting GFP alone, most electroporated cells were still in the VZ (not shown) (Dubreuil et al., 2000). Most cells transfected with *Ngn2* were positioned laterally already at 24 hours after electroporation (h.a.e.) (Fig. 1A), while after transfection of *Mash1* and *Phox2b*, an equivalent effect was observed only at 48 h.a.e. (Fig. 1C,D) (Dubreuil et al., 2000). This suggests that at the doses used, *Ngn2* is more potent than *Mash1* or *Phox2b*.

Two observations made Cash1, the chicken ortholog of Mash1, an obvious candidate for mediating the effect of *Phox2b* on pan-neuronal differentiation. First, *Mash1* is co-expressed with *Phox2b* in the cranial bm/vm progenitors (Pattyn et al., 2000) and is in fact the only bHLH gene we found expressed in this progenitor population (M. R. H., unpublished). Second, in the absence of *Phox2b* function, *Mash1* expression in the bm/vm progenitors is downregulated (Pattyn et al., 2000). However, misexpression of mouse *Phox2b* (*mPhox2b*) did not induce *Cash1* in the presumptive spinal cord either at 6 or at 24 h.a.e. (Fig. 1E,G).

We thus reasoned that another bHLH factor with proneural activity might mediate the effect of *Phox2b* in spinal regions of the neural tube. Among the three genes studied (*Cash1*, *Ngn1* and *Ngn2*), *Ngn2* was prominently expressed in the HH18-20 spinal cord, in a broad ventrally located area, where the sm neurons are being born at this stage, and in some scattered cells further dorsally. Misexpression of *Phox2b* resulted in a marked dorsal expansion of the *Ngn2* expression domain. The increase in *Ngn2* expression was greatest at 6 h.a.e. and declined thereafter (Fig. 1F,F′,I,H,H′,J). Double-labeling with a *Ngn2* probe and anti-Phox2b antibodies showed that most cells expressing *Ngn2* ectopically also expressed Phox2b indicating that *Phox2b* functions cell-autonomously (Fig. 1I). Cells in the dorsalmost region of the neural tube consistently failed to respond to *Phox2b* transfection with *Ngn2* induction, in line with our observation that *Phox2b* was also unable to induce early postmitotic markers at this location (Dubreuil et al., 2000). *Ngn1* expression was upregulated by *Phox2b* at 12 h.a.e. but not at 6 h.a.e. (not shown).

The failure to induce *Cash1* could be explained by the requirement for a co-factor present in bm/vm progenitors, but absent in the dorsal spinal cord. Reasoning that *Nkx2.2*, the expression of which in the hindbrain bm/vm progenitors coincides precisely with that of *Phox2b* (Pattyn et al., 2000), may be the missing factor, we co-transfected *Nkx2.2* together with *Phox2b*.

This resulted in a marked induction of *Cash1* throughout the transfected area at 20 but not at 6 h.a.e. (Fig. 2A,A′,B,B′). *Nkx2.2* expressed alone at the same dose did not induce *Cash1* (Fig. 2C,C′). At higher concentrations, *Nkx2.2* transfected alone also promoted *Cash1* expression, but much more weakly than in combination with *Phox2b* (Fig. 2D,D′). Hence, at physiological expression levels, the combinatorial action of *Nkx2.2* and *Phox2b* may be required to induce or maintain expression of *Mash1*. These results raised the possibility that co-expression of *Phox2b* and *Nkx2.2* may also promote *Ngn2* expression. However, *Ngn2* expression was repressed rather than activated by transfecting *Phox2b* together with *Nkx2.2* (Fig. 2B″), which can be attributed to the negative regulation of *Ngn2* in the spinal cord (Zhou et al., 2001).

Previously, we have shown that *Phox2b* misexpression in spinal regions of the neural tube, where it is normally never expressed, promotes the generation of neurons that migrate to the ML and induces aspects of a bm/vm phenotype (Dubreuil et al., 2000). This holds true also at hindbrain levels of the neuraxis, where *Phox2b*-expressing vm neurons are born at this stage. At 48 h.a.e in the caudal hindbrain, most ectopically *Phox2b*-expressing cells had relocated to the ML, an effect not seen after transfecting *GFP* alone (Fig. 3A-B′). Most of them expressed Islet1/2 (Fig. 3C-C″) and the bm/vm marker *Phox2a*, as do bm/vm neurons, but neither *Islet2* nor *Hb9*, which are specific for sm neurons (not shown). Forced expression of *Phox2b* also very efficiently induced the endogenous *Phox2b* gene (Fig. 3B′), as it does at spinal levels (see Fig. 5B″). We then examined whether ectopic *Phox2b* would also upregulate *Ngn2* when expressed alone and *Cash1*
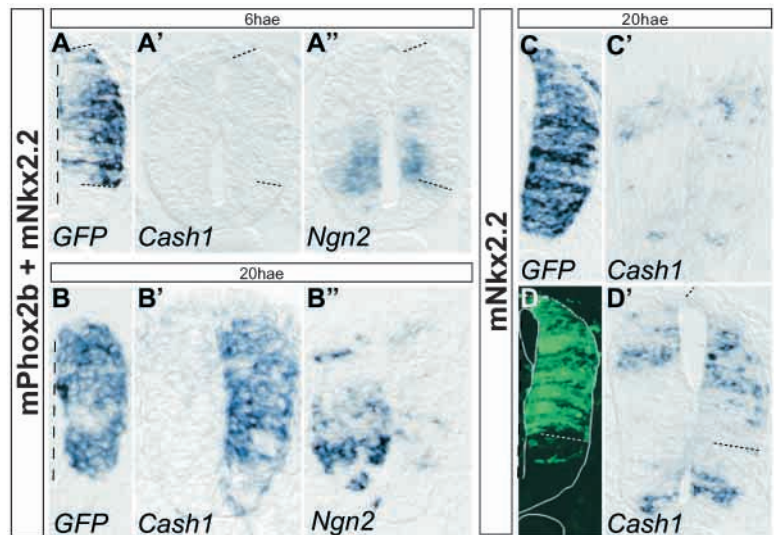

Fig. 2. *Phox2b* induces *Cash1* in combination with *Nkx2.2*. In situ hybridization on transverse sections of the spinal cord with *Cash1* (A′,B′, C′,D′) and *Ngn2* (A″,B″) probes at 6 (A-A″) and 20 (B-B″) h.a.e. of *mPhox2b* plus mouse *Nkx2.2* (0.5 mg/ml) or at 20 h.a.e. of 0.5 mg/ml (C,C′) or 1 mg/ml (D,D′) mouse *Nkx2.2* alone. The extent of transfection is shown by in situ hybridization with a *GFP* probe (A-C) or by GFP fluorescence (D). Mouse *Nkx2.2* (0.5 mg/ml) does not induce *Cash1* (C′), but does so in combination with *mPhox2b* (B′). *Ngn2* is repressed in the area where *Cash1* has been induced (B″). At 1 mg/ml, *mNkx2.2* transfection alone elicits a modest increase in *Cash1* expression (D′). No effects on *Cash1* or *Ngn2* expression were observed at 6 h.a.e. (A′ and A″).

when expressed together with *Nkx2.2*. Misexpression of *Phox2b* in the caudal hindbrain resulted in dorsal expansion of *Ngn2* expression at 16 h.a.e., while *Cash1* was not induced (Fig. 3D-E'). By contrast, *Cash1* was induced by co-transfecting *Phox2b* together with *Nkx2.2* (Fig. 3F-F'), but not after transfecting *Nkx2.2* alone at the same concentration (Fig. 3G-G'). Clearly, then, the effects of *Phox2b* misexpression on

Fig. 3. Effects of Phox2b overexpression in the hindbrain. (A-C″) *Phox2b* promotes migration to the ML and induces endogenous Islet1/2 and *Phox2b* expression. In situ hybridization on transverse sections of the caudal hindbrain with *GFP* (A), *mPhox2b* (B) and chicken *Phox2b* (B′) probes and double-labeling with anti-Islet1/2 (C) and anti-mPhox2b (C′) antibodies. At 48 h.a.e. of *mPhox2b* into the hindbrain, the transfected cells have relocated to the ML (B), an effect not seen in control transfections with GFP alone (A). The *mPhox2b*-expressing cells also express the endogenous *Phox2b* gene (B′). The ventral expression domain of endogenous *Phox2b* corresponds to the vm precursors. Islet1/2 is expressed in ventral motoneurons and by the cells that ectopically express *mPhox2b* (C,C′). (D-G′) *Phox2b* upregulates *Ngn2* and *Cash1* in combination with *Nkx2.2*. In situ hybridization on transverse sections of the caudal hindbrain using *mPhox2b* (D-F), mouse *Nkx2.2* (G), *Ngn2* (D′) and *Cash1* (E′,F′,G′) probes. *mPhox2b* alone upregulates *Ngn2* (D′), but not *Cash1* (E′). *Nkx2.2* (0.5 mg/ml) alone does not induce *Cash1* (G′), but does so when co-electroporated with *Phox2b* (F′).

pan-neuronal and type-specific differentiation can also be observed at rostrocaudal levels of the neural tube, where *Phox2b* is expressed and known to be required for the specification of vm progenitors.

These results suggest that in its normal expression territory in the ventral hindbrain, *Phox2b* promotes neurogenesis by upregulating *Mash1* in combination with *Nkx2.2*. When misexpressed in more dorsal regions of the neural tube, it appears to do so by upregulating *Ngn2* in cooperation with as yet unknown factors.

Phox2b inhibits expression of negative regulators of neurogenesis

We next examined if *Phox2b* affects the expression of genes known to play a role as negative regulators of neuronal cell cycle exit and differentiation. Among such negative regulators, we focused on the Hes and Id genes, which function as effectors of Notch signaling and as natural inhibitors of bHLH factor activity, respectively (Kageyama and Nakanishi, 1997; Kageyama and Ohtsuka, 1999; Norton, 2000).

Two members of the Hes family have been reported to be expressed in the chick neural tube: *Hairy1* and *Hairy2* (Jouve et al., 2000). In the early chick spinal cord, *Hairy1* is expressed in a narrow dorsal stripe and the floor plate (Jouve et al., 2000) and thus unlikely to function as negative regulator of neurogenesis in the lateral neural tube. Strong expression of *Hairy2* is confined to cells adjacent to the floor plate. Weaker expression is found throughout the alar plate (Jouve et al., 2000), which was not affected by misexpression of *Phox2b* (not shown). More recently, additional chick homologs of Hes genes have been identified, which are most similar by sequence to mouse *Hes5* (D. Henrique, personal communication). Among them, chick *Hes5b* is expressed in a broad lateral region of the neural tube (C. J., O. Pourquié and D. Henrique, unpublished; see Fig. 4L′,N′). Like mouse *Hes5* (Kageyama and Nakanishi, 1997), chick *Hes5b* responds to activated Notch and is upregulated in the chicken neural tube by expression of a constitutively active *Notch* construct (D. Henrique, personal communication). *Hes5* also behaved as a negative regulator of neurogenesis in the chick neural tube. Overexpression of mouse *Hes5*, which we used to distinguish enforced from endogenous expression, downregulated expression of the early postmitotic markers *Delta1* and *NeuroM* (Fig. 4C-D′). We then investigated whether Phox2b affected its expression. Six hours after misexpression of *Phox2b*, we already detected a decrease in chick *Hes5b* expression in the transfected area in a sizeable fraction of the embryos (Fig. 4K,K′). At 24 h.a.e., chick *Hes5b* expression was virtually extinguished on the transfected side of all embryos (Fig. 4L,L′).

Among the *Id* family members, we focused on *Id2*, the overexpression of which has been found to cause overgrowth of the chick neural tube (Martinsen and Bronner-Fraser, 1998). We found *Id2* expression to be very dynamic over the time period studied. Strong expression at early stages and in caudal regions, where development is less advanced, was confined to the dorsal-most neural tube with weak expression more ventrally. Upon further development, expression became strong in a broad lateral domain (Fig. 4K″,L″,N″). *Id2* overexpression had little effect on expression of *Delta1* (Fig. 4G,G′), but inhibited neuronal differentiation as judged from downregulation of *NeuroM* (Fig. 4H,H′). As *Delta1* precedes
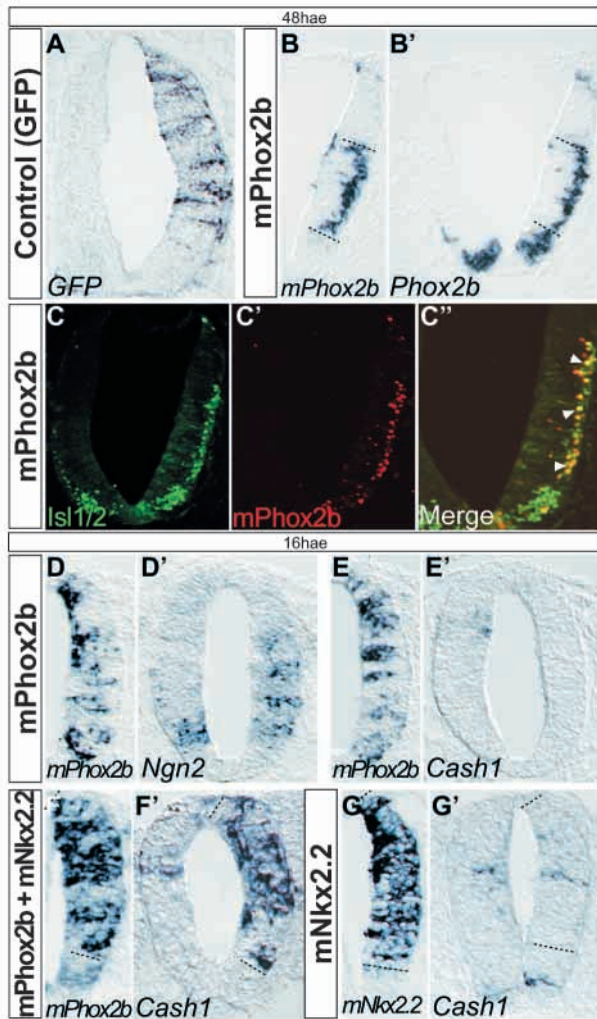
Fig. 4. *Phox2b* represses, while *Ngn2* upregulates negative regulators of neurogenesis. (A-F) *Hes5* overexpression inhibits neuronal differentiation and counteracts the increase in *Delta1* expression in response to *Phox2b*. In situ hybridization with *Delta1* (A′,C′,E′) and *NeuroM* (B′,D′) probes on transverse spinal cord sections. The extent of transfection is shown by *GFP* in situ hybridization as indicated. *Hes5* overexpression downregulates *Delta1* (C′) and *NeuroM* (D′) expression. The promotion of *Delta1* expression by *mPhox2b* (A′) is reduced by co-transfecting *Hes5* (E′). (F) Quantitative analysis of *Delta1* expression in response to *mPhox2b*, *Hes5* or *mPhox2b* plus *Hes5*. Results are expressed as the difference in *Delta1* signal intensity between the transfected and the non-transfected sides. Data points represent mean values±s.e.m. (arbitrary units) from more than 35 sections from at least four transfected embryos. *$P<0.05$, **$P<0.001$ with respect to transfection of *mPhox2b* alone. (G-J′) *Id2* overexpression inhibits neuronal differentiation and counteracts the increase in *Delta1* and *NeuroM* expression in response to *Phox2b*. In situ hybridization with *Delta1* (G′,I′) and *NeuroM* (H′,J′) probes on transverse spinal cord sections. The extent of transfection is shown by *GFP* in situ hybridization as indicated. *Id2* overexpression

downregulates *NeuroM* expression (H′); expression of *Delta1* is only marginally affected (G′). The promotion of *Delta1* and *NeuroM* expression by *mPhox2b* (A′,B′) is reduced by co-transfecting *Id2* (compare A′ and B′ with I′ and J′). (K-N″) *Phox2b* represses chick *Hes5b* and *Id2*, while *Ngn2* upregulates their expression. In situ hybridization with chick *Hes5b* (K′,L′,N′) and *Id2* (K″,L″,N″) probes alone or combined with anti-BrdU immunohistochemistry (M) (brown nuclei) on spinal cord sections of embryos electroporated with either *mPhox2b* (K-M) or mouse *Ngn2* (N-N″). In situ hybridization with a *GFP* probe (L,N) or GFP fluorescence (K) show the transfected area. Repression of chick *Hes5b* and *Id2* is already observed at 6 h.a.e. of *mPhox2b* (K′,K″) and has become pronounced at 24 h.a.e (L′,L″). (M) After an 1 hour BrdU pulse, virtually all BrdU-positive cells on the untransfected (left) side are chick *Hes5b*- or *Id2*-positive, while in the transfected area (right) chick *Hes5b* or *Id2* expression is absent in both BrdU-positive and -negative cells. The broken lines in M outline the lumen of the neural tube. In contrast to *mPhox2b*, mouse *Ngn2* overexpression promotes chick *Hes5b* (N′) and *Id2* (N″) expression.

NeuroM in postmitotic cells, this result may be taken to mean that Id2 affects neurogenesis mainly at a step downstream of *Delta1* expression. As in the case of chick *Hes5b*, a slight decrease in *Id2* expression at 6 h.a.e. of *Phox2b* foreshadowed a massive repression at 24 h.a.e. (Fig. 4K,K″,L,L″).

Our previous results have shown that ectopic *Phox2b* expression promotes cell cycle exit (Dubreuil et al., 2000). It was thus possible that repression of chick *Hes5b* and *Id2*, which are expressed mainly in VZ progenitors, was a mere consequence of cell cycle exit. Two types of evidence argue
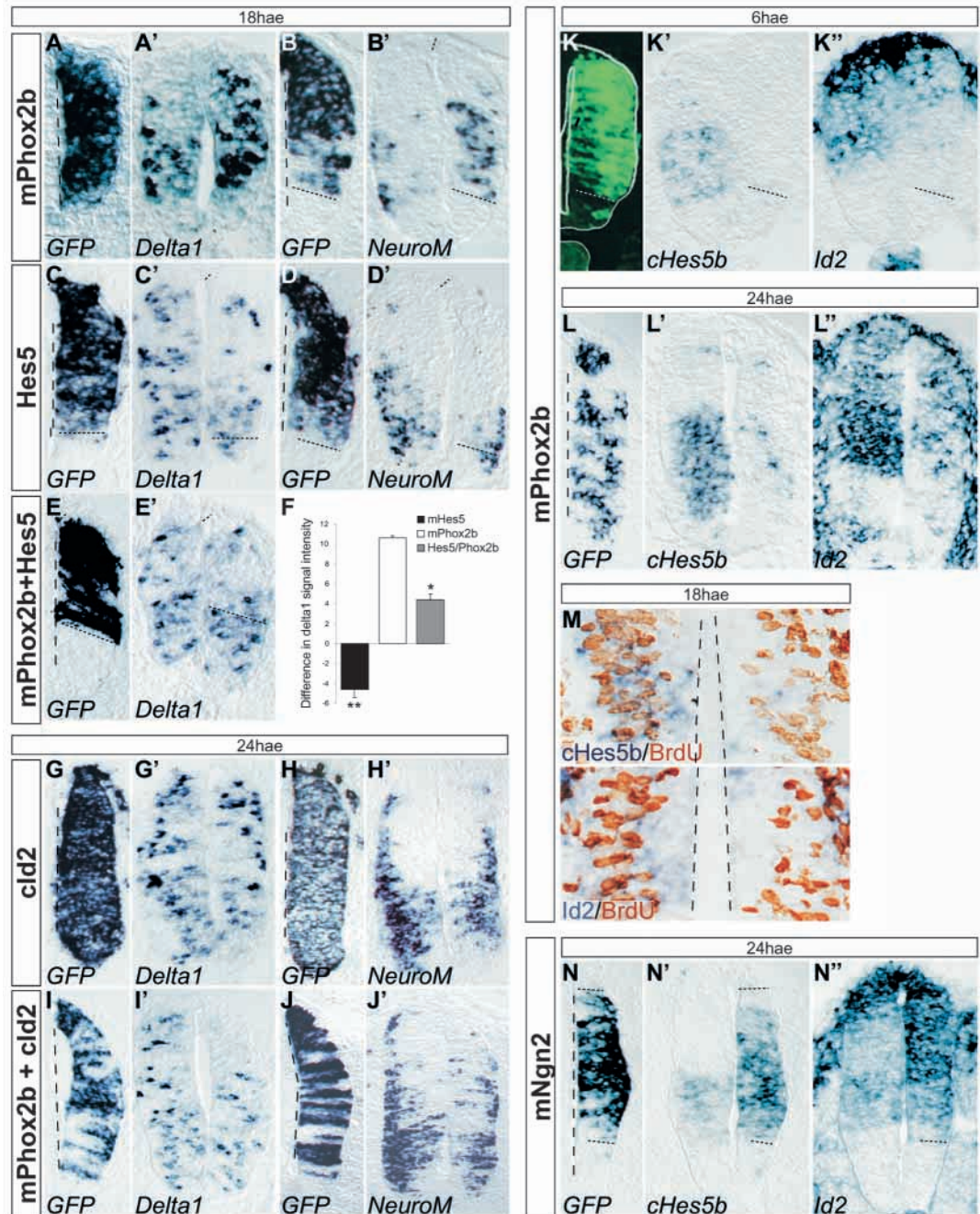
against this explanation: (1) at 6 h.a.e., when we detected the first signs of repression, equivalent numbers of BrdU-incorporating cells were present on the transfected and the non-transfected side (not shown); and (2) double-labeling for BrdU incorporation and chick *Hes5b* or *Id2* expression at 18 h.a.e. showed that either gene was downregulated both in cells that had incorporated BrdU and were thus in the S-phase of the cell cycle and in BrdU-negative cells, which are a mixture of postmitotic precursors and of progenitors in other phases of the cycle (Fig. 4M). Hence, downregulation of chick *Hes5b* and *Id2* appears to precede withdrawal from the cell cycle.

To provide evidence that inhibition of Hes and Id genes plays a role in mediating the neurogenesis-promoting activity of Phox2b, we co-expressed *Hes5* or *Id2* together with *Phox2b*. Co-transfection of *Hes5* antagonized the increase in the number of *Delta1*-expressing cells observed after misexpression of *Phox2b* (Fig. 4E,E'). Quantification of the results showed that in the presence of mouse *Hes5*, the increase in *Delta1+* cells seen after transfecting *Phox2b* alone was reduced by around 60% (Fig. 4F). Similarly, *Id2*, when co-transfected with *Phox2b,* consistently prevented the increase in *NeuroM+* cells caused by *Phox2b*; the effect on *Delta1* expression was more variable, but on most sections, *Delta1* expression was not increased in response to Phox2b (Fig. 4I-J').

Finally, we tested the idea that downregulation of chick *Hes5b* and *Id2* in response to Phox2b may be a consequence of increased *Ngn2* expression. Forced expression of *Ngn2*, however, resulted in a marked upregulation of the chick *Hes5b* and *Id2* expression levels (Fig. 4N-N''). Although induction of chick *Hes5b* probably reflects activation of the Notch pathway, the mechanism by which *Ngn2* promotes *Id* gene expression remains undefined.

Together, these results show that, in addition to activating proneural genes, Phox2b inhibits expression of negative regulators of neuronal differentiation by a separate pathway. They also raise the possibility that downregulation of *Hes5* and *Id2* may be essential components of the response to Phox2b. As *Hes5* expression can be taken as a read-out for Notch activity (de la Pompa et al., 1997; Ohtsuka et al., 1999), Phox2b may affect expression of any of the components involved in this signaling pathway.

Regulatory interactions between Phox2b and transcription factors expressed in the progenitor domain

We then explored the transcriptional regulations by which Phox2b specifies a bm/vm phenotype. There is now a large body of evidence to suggest that, in addition to driving generic neuronal differentiation, the bHLH factors with proneural activity play key roles in the specification of neuronal subtype identity (Perez et al., 1999; Fode et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Lo et al., 2002; Parras et al., 2002). We therefore tested whether the activation of a bm/vm phenotype in response to Phox2b could be mediated by Ngn2 or Mash1. Overexpression of *Ngn2*, although promoting premature neurogenesis, did not induce bm/vm markers (not shown). Misexpression of *Mash1* resulted in the appearance of ectopic Islet1+, Islet2− cells in the dorsolateral spinal cord (Fig. 5A-A''). However, neither ectopic expression of the motoneuronal marker *ChAT* (Fig. 5A''') nor
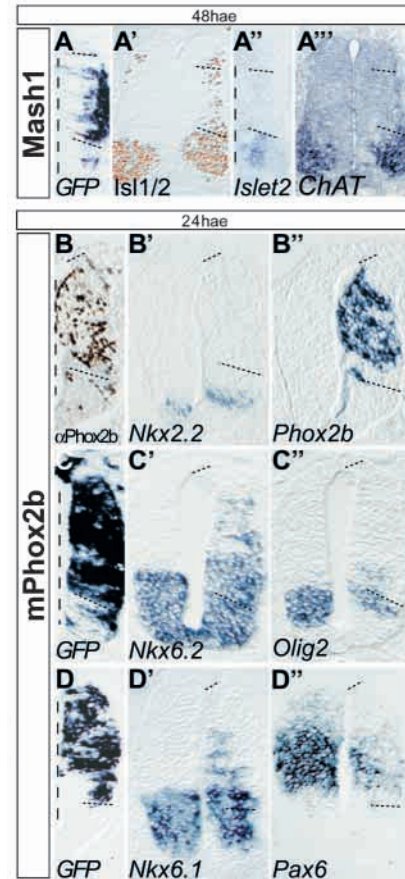

Fig. 5. Regulatory interactions between *Phox2b* and transcription factors expressed in the progenitor domain. (A-A''') *Mash1* induces Islet1, but not other motoneuronal markers. Anti-Islet1/2 immunostaining (A') and in situ hybridization with *Islet2* (A'') and *ChAT* (A''') probes at 48 h.a.e. of *Mash1* on spinal cord sections. At this time point, the transfected cells have relocated to the mantle layer, as shown by in situ hybridization with a *GFP* probe. (A) Forced expression of *Mash1* in the spinal cord induces ectopic Islet 1/2+ (A') cells, but neither *Islet2* (A'') nor *ChAT* (A''). The effect of *Mash1* overexpression on Islet1/2 expression has been quantitated by counting the Islet1/2+ cells in the transfected area and in an equivalent area of the non-transfected side, excluding the region of the Islet1/2+ spinal motoneurons. A total of 692 Islet1/2+ cells were counted on the transfected versus 296 cells on the control side, which amounts to an average difference of 9.7±0.27 cells/section (mean±s.e.m., *P*<0.0001, 40 sections from six embryos). (B-D'') *Phox2b* upregulates *Nkx6.1* and *Nkx6.2* and represses *Pax6* and *Olig2*. In situ hybridization on spinal cord sections with *Nkx2.2* (B'), chick *Phox2b* (B''), *Nkx6.2* (C'), *Olig2* (C''), *Nkx6.1* (D') and *Pax6* (D'') probes at 24 h.a.e. of *mPhox2b*. The extent of electroporation is shown on adjacent sections by anti-mPhox2b immunohistochemistry (B) or in situ hybridization with a *GFP* probe as indicated. *mPhox2b* misexpression induces the endogenous *Phox2b* gene (B'') and expands the expression domains of *Nkx6.1* (D') and *Nkx6.2* (C'). By contrast, *Olig2* (C'') and *Pax6* (D'') expression is repressed. *Nkx2.2* expression is not induced by *mPhox2b* (B').

of *Phox2a* (not shown) could be detected, two genes that are induced by *Phox2b* (Dubreuil et al., 2000). In some embryos, we observed ectopic expression of chicken *Phox2b* in the transfected area, but this effect was slight and inconsistent. We

thus conclude that neither Ngn2 nor Mash1 mediates the effect of Phox2b on bm/vm differentiation.

The current model of neuronal subtype specification in the spinal cord posits that the VZ is parcelled into different domains, each expressing a different set of transcription factors that cross-repress each other and ensure that only the appropriate type of neurons arises from each domain (Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001; Lee and Pfaff, 2001). Changing the transcriptional code of the progenitor domains may thus be a prerequisite for inducing ectopic bm/vm neurons. In the ventral hindbrain, the expression patterns of *Phox2b* and *Nkx2.2* are co-extensive (Pattyn et al., 2000), raising the possibility that Nkx2.2 may be involved in the fate-specifying activity of Phox2b. However, *Phox2b* misexpression did not yield ectopic *Nkx2.2*⁺ cells (Fig. 5B,B′).

We then tested whether *Phox2b* affects the expression of *Nkx6.1*, *Nkx6.2*, *Pax6* and *Olig2*. Among them, *Nkx6.1* and *Nkx6.2* are expressed by hindbrain bm/vm progenitors (Qiu et al., 1998; Briscoe et al., 1999; Cai et al., 1999) and are required for their proper development (J. Ericson and M. Sander, personal communication), albeit the Nkx6.1/6.2 expression domains also encompasses the progenitors of sm and V2 neurons. In both, mouse and chick, *Nkx6.2* is co-expressed with *Nkx6.1* in bm/vm progenitors, while in the spinal cord, the expression patterns of both genes are co-extensive in chick but not in mouse embryos (Cai et al., 1999; Vallstedt et al., 2001). The ventral limits of *Pax6* and *Olig2* expression define the dorsal boundary of the bm/vm progenitor domain (Ericson et al., 1997; Osumi et al., 1997; Mizuguchi et al., 2001). In the absence of *Pax6*, hindbrain sm neurons appear to acquire a vm identity (Ericson et al., 1997; Osumi et al., 1997). *Nkx6.1* and Nkx6.2 expression should thus favor a bm/vm fate, whereas

Pax6 and *Olig2* should suppress it. Forced expression of *Phox2b* resulted in dorsal expansion of the domains of *Nkx6.1* and *Nkx6.2* expression whereas *Pax6* and *Olig2* were repressed (Fig. 5C-D′′). These data show that *Phox2b* changes the pattern of transcription factor expression in the progenitor domains and suggest that to be able to initiate bm/vm differentiation, *Phox2b* needs to downregulate progenitor factors that are not permissive for this fate. They also suggest that Phox2b may cooperate with Nkx6.1 and Nkx6.2 in specifying a bm/vm identity.

An activator form of Phox2b mimics the auto-regulatory and neurogenesis-promoting activities of Phox2b and the induction of Islet1

Only activating functions have so far been ascribed to Phox2b (Yang et al., 1998; Lo et al., 1999; Yokoyama et al., 1999; Flora et al., 2001). To investigate whether Phox2b functions as an activator when promoting neurogenesis and bm/vm differentiation, we fused the Phox2a HD either to the trans-activating domain of the viral protein VP16 (*PHDVP16*) or to the repressor domain of *Drosophila* Engrailed (*PHDEnR*). The Phox2a HD could be used in place of the Phox2b HD, as the two HDs are identical at the amino acid level (Pattyn et al., 1997). In a previous study, the *PHDEnR* construct has been shown to behave as a dominant-negative form of Phox2a/b in neural crest cells (Lo et al., 1999). Similar fusions between the HD of S*iamois*, which is also a member of the *paired* homeogene family (Galliot et al., 1999), and the activator or repressor domains were used as controls (*SHDVP16* and *SHDEnR*).

Expression of *PHDVP16* mimicked the ability of *Phox2b* to elicit emigration to the ML and ectopic Islet1/2⁺ cells, albeit the effect on Islet1/2 expression was less pronounced. By contrast, expression of *PHDEnR* had no such effects (Fig. 6A-
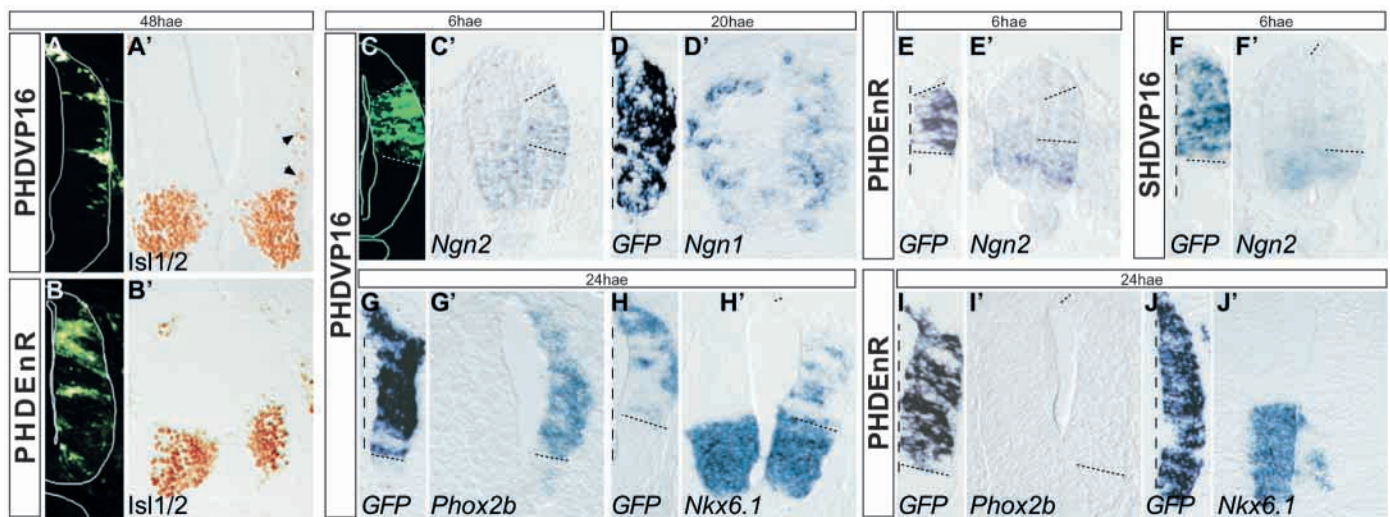

Fig. 6. Phox2b acts as an activator in promoting neurogenesis and inducing Islet1, *Nkx6.1* and endogenous *Phox2b*. (A-B′) at 48 h.a.e. of *PHDVP16* (A) but not of *PHDEnR* (B), the transfected cells have emigrated to the mantle layer as shown by GFP fluorescence. Anti-Islet1/2⁺ cells are found in ectopic location after expressing the *PHDVP16* (arrowheads, A′) but not the *PHDEnR* construct (B′). (C-J′) in situ hybridization with *Ngn2* (C′,E′,F′), *Ngn1* (D′), *Phox2b* (G′,I′) and *Nkx6.1* (H′,J′) probes at 6 h.a.e. of *PHDVP16* (C′), *PHDEnR* (E′) and *SHDVP16* (F′), at 20 h.a.e. of PHDVP16 (D′), or at 24 h.a.e. of *PHDVP16* (G′,H′) or *PHDEnR* (I′,J′). GFP fluorescence (A-C) or in situ hybridization with a *GFP* probe (D-J) shows the extent of electroporation. At 6 h.a.e. of *PHDVP16*, *Ngn2* is upregulated in the transfected area (C′) while *PHDEnR* or *SHDVP16* constructs have no effect (E′,F′). Forced expression of *PHDVP16* also promotes expression of *Ngn1* (D′), induces the endogenous *Phox2b* gene (G′) and expands *Nkx6.1* expression (H′), while *PHDEnR* does not induce *Phox2b* (I′) and represses *Nkx6.1* (J′).

B′). Similarly, *PHDVP16* expression, but not that of *PHDEnR* or *SHDVP16*, increased the number of *Ngn2*^{+} cells within the transfected area at 6 h.a.e., when the effect of *Phox2b* on *Ngn2* expression was greatest (Fig. 6C,C′,E-F′). *Ngn1* expression was not yet upregulated at 6 h.a.e. (not shown), but was so at 20 h.a.e. (Fig. 6D,D′). None of these effects was observed after expression of the HD alone (not shown). Positive autoregulation of *Phox2b* and upregulation of *Nkx6.1* also appeared to reflect an activator function, as *PHDVP16* transfection resulted in induction of the endogenous *Phox2b* gene and in dorsal expansion of *Nkx6.1* expression (Fig. 6G-H′), whereas *PHDEnR* had no effect on *Phox2b* and repressed *Nkx6.1* (Fig. 6I-J′). Together, these results suggest that Phox2b acts as a transcriptional activator in promoting neurogenesis and a bm/vm fate.

Activation of the endogenous *Phox2b* gene potentially complicates the interpretation of the *PHDVP16* phenotype, which could be ascribed to wild-type *Phox2b*. However,

Fig. 7. Chick *Hes5b* and *Id2* are repressed by activator and repressor forms of Phox2b and by expressing the native homeodomain. (A-D″) In situ hybridization with chick *Hes5b* (A′-D′) and *Id2* (A″-D″) probes at 24 h.a.e. of *PHDVP16* (A-A″), *PHDEnR* (B-B″), *PHD* (C-C″) and *SHDVP16* (D-D″) constructs. The extent of electroporation is shown by GFP fluorescence (C) or in situ hybridization with a *GFP* probe (all others). Both, Phox2b transcriptional activator (*PHDVP16*) or repressor (*PHDEnR*) forms repress chick *Hes5b* (A′,B′) and *Id2* (A″,B″). The repressive activity is shared by the Phox2 HD (C′,C′), but not by control constructs with the Siamois HD (*SHDVP16*) (D′,D″).

conversion of a transcriptional repressor to an activator, or the converse, generates a dominant-negative or antimorphic form that antagonizes the endogenous protein (Onichtchouk et al., 1998). In principle, then, if wild-type Phox2b acted as a repressor, PHDVP16 should block and not phenocopy its activity.

In contrast to the foregoing results, transfection of both *PHDVP16* and *PHDEnR* (but not of *SHDVP16*) reduced chick *Hes*5b and *Id2* expression, an effect also observed after expression of the HD alone (Fig. 7). These results suggest that inhibition of Hes and Id genes by full-length Phox2b may be mediated by the HD, and that the function of the Phox2 HD is dominant over heterologous repressor and activator regions. Equivalent results have been obtained for the HD transcription factor Xdbx (Gershon et al., 2000). Also in this case, the isolated HD and activator and repressor fusion constructs mimicked the repressive activity of the full-length protein. One possibility is that the Phox2 HD binds to and sequesters essential co-activators required for chick *Hes5b* and *Id2* expression. A similar mechanism has been shown to operate in the case of the inhibition of astrocyte differentiation by Ngn1 (Sun et al., 2001). Likewise, the Pou domain transcription factor Pit1 has been found to function as a DNA-binding dependent activator and a DNA binding-independent repressor (Scully and Rosenfeld, 2002). Clearly, further work is required to elucidate the mechanism by which Phox2b represses the expression of these negative regulators of neurogenesis.

DISCUSSION

Our previous loss-of-function (LOF) and gain-of-function (GOF) studies have provided evidence that the HD transcription factor Phox2b coordinates generic and subtype-specific aspects of cranial motoneuronal differentiation (Dubreuil et al., 2000). Here, we have used a GOF approach to explore the underlying molecular interactions. Phox2b appears to drive cell cycle exit and pan-neuronal differentiation by promoting expression of bHLH genes with proneural activity and relieving them from the negative influences exerted by Notch signaling and the Id2 repressor. In addition, Phox2b elicits changes in the expression of transcription factors in the progenitor domain that may underlie its capacity to initiate a bm/vm neuronal differentiation program. These results provide insights into the mechanisms by which a single transcription factor collaborates with other factors to coordinately regulate generic and type-specific neuronal differentiation. Fig. 8A,B present a schematic view of the postulated genetic interactions that underlie these activities, in bm/vm progenitors and upon ectopic expression of Phox2b in the dorsolateral spinal cord or caudal hindbrain, respectively. Our GOF approach also reveals a substantial degree of redundancy built into the system, which could not have been detected by LOF studies alone.

Phox2b upregulates proneural genes and represses negative regulators of neurogenesis

One function of Phox2b, whether misexpressed in the spinal cord or in its normal expression domain in the ventral hindbrain, is to increase the probability of cell cycle exit and to activate pan-neuronal markers (Dubreuil et al., 2000). Our present data strongly suggest that this activity is mediated in
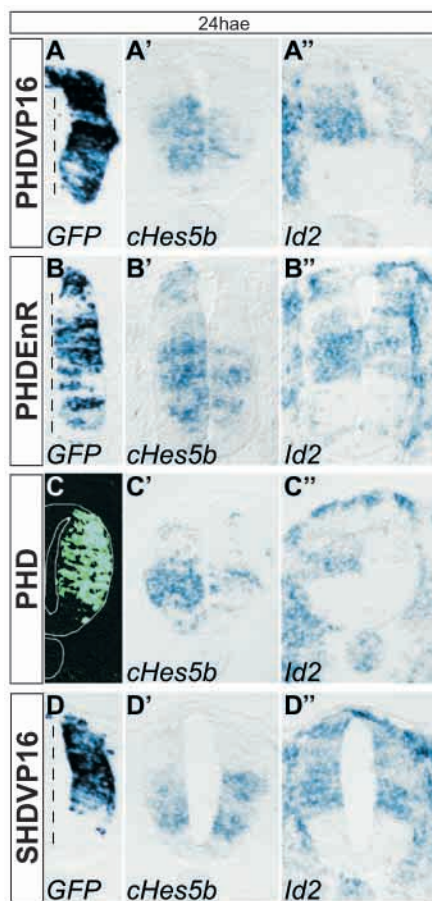
Fig. 8. Postulated genetic interactions during cranial motoneuron differentiation in the ventral hindbrain and upon misexpression of *Phox2b* in the dorsolateral neural tube. (A) Within the bm/vm progenitor domain, *Phox2b* in combination with *Nkx2.2* drives *Mash1* expression, which in turn promotes generic neuronal differentiation. Repression of *Hes* and *Id* gene expression by *Phox2b* may be necessary to overcome a negative feedback on neuronal differentiation triggered by Mash1. In the progenitor domain, *Nkx2.2* and *Phox2b* repress *Pax6* and *Olig2*, which can be supposed to inhibit bm/vm specification. *Phox2b* is postulated to boost expression of the *Nkx6* genes that are required for proper development of bm/vm neurons. The inhibitory interaction between *Pax6* and *Phox2b* is reciprocal. (B) Upon misexpression in the dorsolateral spinal cord and caudal hindbrain, *Phox2b* is postulated to cooperate with an unknown HD factor (X) to upregulate *Ngn2*, which in turn drives pan-neuronal differentiation. By boosting expression of *Hes5* and *Id2*, *Ngn2* triggers a negative feedback loop that is relieved by *Phox2b*. In addition, *Phox2b* directs a bm/vm fate, in part by repressing *Pax6* and *Olig2*, which are not permissive for this fate, and by upregulating expression of the *Nkx6* genes, which are compatible with it. In A,B, the positive autoregulation of *Phox2b* has been omitted for clarity.
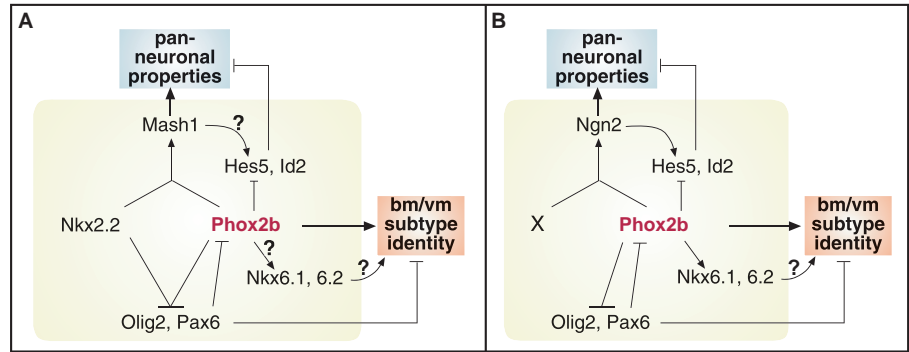
part by proneural bHLH factors. *Phox2b* misexpression expanded the expression domain of *Ngn2*, which has been shown to promote generic neuronal differentiation in a wide variety of settings, including the chick neural tube (Mizuguchi et al., 2001; Novitch et al., 2001). Moreover, in combination with *Nkx2.2* it induced *Cash1*, which is normally co-expressed with *Phox2b* in bm/vm progenitors, is downregulated in the absence of *Phox2b* (Pattyn et al., 2000) and (like *Ngn2*) capable of driving neuronal differentiation in the chick neural tube. Another result supporting the view that up-regulation of proneural genes is required for premature neurogenesis in response to *Phox2b* is that co-expression of *Id2*, a natural inhibitor of bHLH factor activity, greatly attenuates the effect of *Phox2b* on generic neuronal differentiation. There is now ample evidence suggesting that *Mash1* and *Ngn2*, in addition to their proneural function, participate in neuronal subtype specification (Fode et al., 2000; Scardigli et al., 2001; Lo et al., 2002). The fact that *Phox2b* directs bm/vm differentiation, whether in combination with *Mash1* in its endogenous expression domain or with *Ngn2* after forced expression in the spinal cord, suggests that all that Ngn2 and Mash1 provide in this context is their proneural function.

Phox2b not only upregulates expression of the *Ngn2* or *Mash1* proneural genes, but also represses inhibitors of neurogenesis such as Hes or Id family members (Ishibashi et al., 1995; Lyden et al., 1999; Ohtsuka et al., 1999; Cai et al., 2000; Cau et al., 2000). As overexpressing *Ngn2* increased rather than decreased chick *Hes5b* and *Id2* expression, downregulation of these genes cannot be a consequence of *Ngn2* induction and probably occurs by a separate pathway. Several lines of evidence support the idea that downregulation of chick *Hes5b* and *Id2* plays a causal role in the neurogenesis-promoting function of *Phox2b*. Forced expression of *Phox2b* results in rapid repression of chick *Hesb5* and *Id2*, independently of cell cycle stage, suggesting that their downregulation precedes the last S phase. Furthermore, constitutive expression of either gene counteracts the premature production of neurons caused by *Phox2b* misexpression.

In the *Phox2b* knockout mice, *Hes5* expression is downregulated rather than stimulated (Dubreuil et al., 2000)

and there is no change in the expression of Id family members (data not shown). Downregulation of *Mash1* and *Delta* and the ensuing reduced activity of the Notch pathway most probably accounts for reduced *Hes5* expression. In the light of our evidence that *Ngn2* stimulates *Id2* expression, reduced proneural activity may also explain why *Id* expression is unchanged in the absence of Phox2b. Hence, a sustained lack of *Phox2b* activity as occurs in knock out mice may induce feedback loops that prevent upregulation of Hes or Id genes and this may explain why some *Phox2b*$^{-/-}$ progenitors are still able to exit the cell cycle.

Finally, the question should be asked why forced expression of *Ngn2* or *Mash1* are able to promote neuronal differentiation in the presence of endogenous chick Hes5b and Id2. First, Hes5, which downregulates expression of proneural genes in addition to counteracting their activity (de la Pompa et al., 1997; Kondo and Raff, 2000; Ohtsuka et al., 1999), cannot repress expression of the transfected genes driven by artificial promoters. Second, high-level expression of the transgenes as achieved by transfection may titrate out the repressor molecules. Hence, forced expression of the proneural bHLH factors may be sufficient to overcome the influence of these negative regulators of neurogenesis, while counteracting them by transcription factors such as Phox2b may be required in a physiological setting.

Phox2b changes the pattern of transcription factor expression in the progenitor domain

In the ventral hindbrain, *Phox2b* is expressed both by cycling bm/vm progenitors and by their postmitotic descendants. Our data argue that during its neuroepithelial phase of expression, *Phox2b* acts not only to promote cell cycle exit but also as a patterning gene, controlling the identity and fate of dividing progenitors. *Pax6* and *Olig2* expression defines the dorsal limit of the *Nkx2.2*$^{+}$, *Phox2b*$^{+}$ bm/vm progenitor domain (Ericson et al., 1997; Briscoe et al., 1999; Mizuguchi et al., 2001). In the absence of Pax6, spinal interneurons and motoneurons do not properly develop, and in the caudal hindbrain, sm neurons appear to switch to a vm fate (Ericson et al., 1997). Olig2 directs a spinal motoneuronal fate and elicits expression of downstream factors appropriate for this fate (Mizuguchi et al.,

2001; Novitch et al., 2001). According to the derepression model of spinal cord neurogenesis (Muhr et al., 2001; Lee and Pfaff, 2001), both factors should thus suppress a bm/vm fate. The pronounced downregulation of the two factors by *Phox2b* misexpression may thus be a necessary step in the chain of events that result in the ectopic induction of bm/vm markers. *Pax6* and *Phox2b* appear to maintain cross-inhibitory interactions because, in the absence of Pax6, the *Phox2b* expression domain in the ventral hindbrain expands dorsally (Mizuguchi et al., 2001). One consequence of *Phox2b* overexpression is premature neuronal differentiation, which alone might explain the decrease in progenitor factor expression. We argue against this explanation, as *Phox2b* does not repress *Ngn2* and synergizes with *Nkx2.2* to activate *Cash1*, two genes that in the spinal cord are restricted to progenitor domains. Because *Olig2* expression depends on *Pax6* (Mizuguchi et al., 2001; Novitch et al., 2001), its downregulation may be a consequence of decreased *Pax6* expression, but may also occur by a separate pathway.

Nkx6.1 and *Nkx6.2* are co-expressed with *Phox2b* in the bm/vm progenitors (Qiu et al., 1998) and are required for their proper development (J. Ericson and M. Sander, personal communication). In line with this, *Phox2b* misexpression results in dorsal expansion of their expression domains. These HD factors may thus cooperate with Phox2b in the implementation of a bm/vm phenotype both in the ventral hindbrain and after misexpression in the spinal cord. However, *Nkx6.1* is expressed throughout the ventral third of the neural tube and, when ectopically expressed, directs sm and V2 neuronal fates (Briscoe et al., 2000). This shows that by itself, *Nkx6.1* does not induce a bm/vm fate and that Phox2b activity is required to achieve this.

Phox2b acts as an activator in inducing Ngn2 and cranial motoneuronal markers

The available evidence suggests that Phox2b functions as a transcriptional activator rather than as a repressor, in line with the fact that it lacks an EH1 domain that confers repressor activity to other HD proteins (Muhr et al., 2001). First, Phox2b binds to and transactivates the promoters of the dopamine β-hydroxylase and *Phox2a* genes (Yang et al., 1998; Yokoyama et al., 1999; Adachi et al., 2000; Hong et al., 2001; Flora et al., 2001). Second, the *PHDEnR* construct, which should act as a repressor, prevents induction of tyrosine hydroxylase and dopamine β-hydroxylase by BMP2 in neural crest cells (Lo et al., 1999). We show that *PHDVP16* but not *PHDEnR* mimics the ectopic induction of *Ngn2* and Islet1 in the chick spinal cord, suggesting that Phox2b functions as an activator.

Phox2b thus appears to provide an activator function, which is necessary and sufficient for the initiation of bm/vm differentiation. According to the derepression model of neuronal cell type specification in the spinal cord (Muhr et al., 2001; Vallstedt et al., 2001), the factors that activate expression of downstream determinants of neuronal identity are thought to be expressed in the neural tube in a topologically unrestricted manner. Our results suggest that such transcriptional activators can also be deployed in spatially restricted domains.

Functional redundancy among neural HD genes

When misexpressed in spinal regions, *Phox2b* affects the expression of *Pax6, Olig2*, *Nkx6.1* and *Nkx6.2*. Nevertheless, expression of these four genes is not detectably affected in the hindbrain of *Phox2b* mutant mice (data not shown), suggesting that other genes are redundant with *Phox2b* in this capacity. In the case of *Pax6* and *Olig2*, *Nkx2.2*, the expression of which does not depend on *Phox2b* and which in GOF experiments represses both genes (Muhr et al., 2001; Novitch et al., 2001), is probably sufficient to exclude *Pax6* and *Olig2* from bm/vm progenitors in the absence of Phox2b. Conversely, despite the fact that *Nkx2.2* has the capacity to repress *Pax6*, lack of Nkx2.2 does not lead to ventral expansion of the *Pax6* domain (Briscoe et al., 1999). In the hindbrain, this may be due to functional redundancy with *Nkx2.9*, but on the basis of our results this may also be due to the presence of Phox2b. Likewise, bm/vm neurons appear to develop normally in the absence of Nkx2.2 (Briscoe et al., 1999), which again may be attributed to the persistent expression of either *Nkx2.9* or *Phox2b*.

It has been argued that redundant roles have been selected as backup against developmental error, whereby one of the genes fails to be expressed adequately at the appropriate site, thus helping to maintain the spatiotemporal precision of embryonic patterning (Cooke et al., 1997). Not surprisingly, the complex process of neuronal patterning appears to provide striking examples of this. The alternative possibility is that the redundant roles we observe are selected because they are non-redundant at other expression sites.

Synchronizing neuronal fate determination with timing and extent of neurogenesis

Neurogenesis involves the parallel activation of a program that controls commitment to cell cycle exit and generic neuronal differentiation and of a program that specifies the identity of the neurons to be generated. Recent studies suggest that one way these two programs are coordinated is by the type-specification properties of bHLH genes themselves. Examples are the bHLH transcription factors Math1 and Ngn1, the ectopic expression of which in the neural tube both drives the cells to move to the ML and fosters production of distinct classes of interneurons (Gowan et al., 2001), and Mash1, the misexpression of which in the dorsal forebrain and ventral spinal cord promotes the appearance of ventral cell types (Fode et al., 2000) and V2 interneurons (Parras et al., 2002), respectively. Another example is provided by *Olig2* expressed in spinal motoneuron progenitors (Mizuguchi et al., 2001; Novitch et al., 2001). When misexpressed in the spinal cord or hindbrain, it drives cells to exit the cell cycle and to migrate to the ML and, in addition, directs expression of determinants of sm or V2 neuronal fates. Furthermore, *Olig2* misexpression expands the domain of *Ngn2* expression suggesting that Olig2, like Phox2b, promotes generic neurogenesis by boosting the expression of downstream proneural genes. Hence, Olig2 and Phox2b may have comparable roles in sm and bm/vm progenitors, respectively. However, one important mechanistic difference between Phox2b and Olig2 is that Phox2b acts as an activator in inducing *Ngn2* and motoneuronal markers, whereas Olig2 functions as a transcriptional repressor. (Whether Olig2 also represses negative regulators of neuronal differentiation has not been examined.) It will be interesting to learn which transcription factors play this role in other neuronal lineages and how they work.

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