Integration of anteroposterior and dorsoventral regulation of *Phox2b* transcription in cranial motoneuron progenitors by homeodomain proteins

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Summary

Little is known about the molecular mechanisms that integrate anteroposterior (AP) and dorsoventral (DV) positional information in neural progenitors that specify distinct neuronal types within the vertebrate neural tube. We have previously shown that in ventral rhombomere (r)4 of *Hoxb1* and *Hoxb2* mutant mouse embryos, *Phox2b* expression is not properly maintained in the visceral motoneuron progenitor domain (pMNv), resulting in a switch to serotonergic fate. Here, we show that *Phox2b* is a direct target of Hoxb1 and Hoxb2. We found a highly conserved *Phox2b* proximal enhancer that mediates rhombomere-restricted expression and contains separate Pbx-Hox (PH) and Prep/Meis (P/M) binding sites. We further show that both the PH and P/M sites are essential for Hox-Pbx-Prep ternary complex formation and regulation of the *Phox2b* enhancer activity in ventral r4. Moreover, the DV factor Nkx2.2 enhances Hox-mediated transactivation via a derepression mechanism. Finally, we show that induction of ectopic *Phox2b*-expressing visceral motoneurons in the chick hindbrain requires the combined activities of Hox and Nkx2 homeodomain proteins. This study takes an important first step to understand how activators and repressors, induced along the AP and DV axes in response to signaling pathways, interact to regulate specific target gene promoters, leading to neuronal fate specification in the appropriate developmental context.

Key words: Hoxb1, Hoxb2, Nkx2.2, Pbx1a, Prep1, Motoneuron, Hindbrain, Transcriptional activation, Derepression, Ternary complex, PH and P/M binding sites, AP and DV integration

Introduction

How specific neuronal types are generated at defined locations within the developing neural tube is still poorly understood. During early development of the central nervous system (CNS), neural progenitors acquire regionally restricted positional addresses by responding to graded inductive signals intersecting along the AP and DV axes (Jessell, 2000; Lumsden and Krumlauf, 1996). A grid-like set of positional cues is established within an initially equivalent group of cells that, together with the activation of a neurogenesis program (Bertrand et al., 2002), is translated into specific neuronal fates. Moreover, distinct neuronal types are generated in specific temporal order, often from common pools of neural progenitors (Jessell, 2000; Pattyn et al., 2003a).

In vertebrates, distinct sets of spatially restricted homeodomain (HD) transcription factors provide transcriptional readouts of AP and DV positional addresses in neural progenitors. Along the AP axis, distinct progenitor domains are generated by the nested expression patterns of the Hox HD-containing genes (Lumsden and Krumlauf, 1996). In the ventral neural tube, DV positional addresses are instead conferred by Nkx-, Dbx-, Pax- and Irx-class HD proteins (Briscoe et al., 2000). Along both axes, auto- and crossregulatory activities among HD factors are required to refine and/or maintain progenitor domains (Briscoe et al., 2000; Dasen et al., 2003; Maconochie et al., 1997; Popperl et al., 1995). The combined activity of HD genes is thought to activate other sets of transcription factors that, in turn, regulate the expression of unique neuronal phenotypes (Lee and Pfaff, 2001). Neuronal identity is therefore the result of a complex regulatory network of transcription factors acting sequentially. Despite the increasing knowledge about genetic cascades and epistatic relationships among HD factors, little is known about their direct downstream targets and how AP and DV molecular

inputs are integrated for precise spatiotemporal transcriptional regulation.

Hox genes are involved in the specification of motoneuron (MN) subtype identities along the AP axis, both at spinal cord and hindbrain levels (Barrow and Capecchi, 1996; Cooper et al., 2003; Dasen et al., 2003; Davenne et al., 1999; Gaufo et al., 2000; Gaufo et al., 2003; Gavalas et al., 1997; Goddard et al., 1996; Guidato et al., 2003; Jungbluth et al., 1999; Pattyn et al., 2003a; Studer et al., 1996; Tiret et al., 1998). In ventral r4, for example, the development of facial branchiomotor (BM) neurons depends on *Hoxb1* and *Hoxb2* functions, raising the question of their direct molecular target(s). Transcriptional specificity of Hox factors is achieved upon heterodimerization with Pbx HD factors, murine homologs of *Drosophila* extradenticle (*exd*), and binding of bipartite PH sites (Chan et al., 1994; Maconochie et al., 1997; Mann and Affolter, 1998; Mann and Chan, 1996; Popperl et al., 1995). Pbx-Hox binding and transcriptional activity are further enhanced by Prep or Meis proteins, murine homologs of *Drosophila* homothorax (*hth*), and additional members of the TALE (three-amino acid-loop-extension) class of HD factors (Burglin, 1997). By binding of distinct P/M sites in the vicinity of PH sites and direct interaction with Pbx, Prep/Meis/Hth proteins facilitate the formation of transcriptionally active ternary complexes (Berthelsen et al., 1998a; Berthelsen et al., 1998b; Ferretti et al., 2000; Gebelein et al., 2002; Jacobs et al., 1999; Ryoo et al., 1999). For example, Hox-Pbx-Prep complexes are involved in the maintenance of *Hoxb1* and *Hoxb2* transcription in r4 (Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997; Popperl et al., 1995). Despite these insights into the regulation of Hox-mediated transcription, and Hox gene involvement in MN development, so far no direct Hox target gene has been identified that is required for MN specification and/or differentiation.

In the ventral neural tube, specification of neuronal progenitors requires HD factors that function as repressors of other repressors, that is by transcriptional derepression of downstream targets (Briscoe et al., 2000; Muhr et al., 2001). Similar derepression strategies have been conserved in various tissues or animal systems, indicating that they are efficient ways of keeping control of target gene expression (Barolo and Posakony, 2002). Nonetheless, a derepression strategy involves the existence of transcriptional activators driving neuronal specification in suitably derepressed environments. Recently, retinoic acid signaling was identified as one of such activator pathways, involved in somatic MN (sMN) specification in the spinal cord (Novitch et al., 2003). While providing a rationale for how activators and repressors may interact to drive cell fate in a specific progenitor domain, the molecular mechanisms that allow switching from transcriptional repression to activation of target genes remain poorly understood. It is also important to investigate whether neuronal specification in other regions of the vertebrate CNS may rely on similar mechanisms. In the hindbrain, for example, it is unknown how the repressor activities of Nkx HD proteins may integrate with Hox factors to achieve spatially restricted regulation of MN programs and downstream targets.

Here, we have investigated the transcriptional regulation of the paired HD transcription factor *Phox2b*. In the mouse hindbrain, *Phox2b* is expressed in longitudinal columns, spanning several rhombomeres, that identify distinct populations of neural progenitors and postmitotic neurons (Pattyn et al., 1997). *Phox2b* is an obligatory determinant of cranial BM and visceral motor (VM) neuron specification (Dubreuil et al., 2002; Pattyn et al., 2000). BM and VM neurons – collectively referred to as vMN – innervate the muscles of the branchial arches and the parasympathetic ganglia, respectively. All vMNs are generated from a common ventral progenitor domain, pMNv, which is equivalent to the spinal p3 domain generating V3 interneurons (Briscoe et al., 2000; Pattyn et al., 2003a; Pattyn et al., 2003b). Throughout the hindbrain, the pMNv domain expresses *Nkx2.2* and *Nkx2.9*, as well as *Nkx6.1* and *Nkx6.2* (Briscoe et al., 2000; Pattyn et al., 2003b). However, the distribution of vMN subtypes is rhombomere specific. Rhombomere 1 does not generate vMNs, r2-4 generate only BM neurons, whereas r5-7 generate both BM and VM neurons. In *Phox2b* knockout mice, vMN progenitors either do not exit the cell cycle or switch to a serotonergic fate (Dubreuil et al., 2000; Pattyn et al., 2000; Pattyn et al., 2003a). Thus, *Phox2b* acts as a binary switch in the selection of vMN or serotonergic fate. In *Hoxb1* and *Hoxb2* knockout mice, maintenance of *Phox2b* expression is impaired and this results in facial BM to serotonergic fate switch in ventral r4 (Pattyn et al., 2003a).

We show that *Phox2b* is a direct target of Hoxb1 and Hoxb2. We identify a conserved *Phox2b* enhancer containing separate PH and P/M sites, both of which are essential for ventral r4 regulation. We further show that transcriptional cooperation among Hox, Pbx, Prep factors and Nkx2.2, via a derepression mechanism, is an important component of *Phox2b* enhancer activity. In addition, cooperation between Hox paralogs 1 or 2 and Nkx2 factors is required in vivo to generate ectopic *Phox2b*-expressing vMNs. These findings provide a molecular rationale to explain how AP and DV inputs are integrated on the *Phox2b* promoter to drive restricted expression in the r4 pMNv domain and facial MN fate.

Materials and methods

Expression vectors and DNA constructs

Expression vectors were as described: mouse Hoxa2 (Pasqualetti et al., 2000), mouse Hoxb1, human HOXB2, and Pbx1a (Di Rocco et al., 1997), Prep1 (Berthelsen et al., 1998a), and chick Nkx2.2, Nkx2.2HD-VP16, Nkx2.2HD-EnR and Nkx2.2ΔTN (Muhr et al., 2001). To yield P2b_10/*lacZ*, a *Sal*I-*Nde*I 10 kb fragment, including the *Phox2b* endogenous promoter and 5′ sequences, was cloned in front of a NLS-*lacZ* reporter cassette. P2b_2.8/*lacZ* was obtained by cloning the *Xba*I-*Xba*I 2.8 kb proximal fragment (Fig. 1) into the BGZ40 reporter (Studer et al., 1996). The P2b_0.38 fragment was PCR-amplified using the following primers: 5′GTTGACTAGTG-GACGAAGAAGGGGGGAAACA3′ (sense) and 5′ACTTACTAG-TAGTATATAGTCCTCATAATAAACTTG3′ (antisense). P2b_0.38 was cloned into the *Spe*I site of BGZ40 (P2b_0.38/*lacZ*) or into p-βglob-*Luc* (P2b_0.38/*Luc*), containing a human β-globin minimal promoter driving *Luciferase* expression. P2b_0.38mPH/*Luc*, P2b_0.38mPH/*lacZ*, P2b_0.38ΔPM/*lacZ* and P2b_0.38mPM/*lacZ* were generated with the Stratagene QuickChange™ Site-directed Mutagenesis Kit. To introduce nucleotide changes in the PH site the following primers were used: 5′TTCCAAGTAGCGTCGTTCGAT-TAAAGGGCAGGAGCTGGTTAGAAG3′ (sense) and 5′CTTCT-AACCAGCTCCTGCCCTTTAATCGAACGACGCTACTTGGAA3′ (antisense). To introduce mutations in the P/M site the following primers were used: 5′GCCCAATAGACGGATGAGTTAGTAAA-AAGCGCCAGCAATAAG3′ (sense) and 5′CTTATTGCTGGCG-

CTTTTTACTAACTCATCCGTCTATTGGGC3′ (antisense) for P2b_0.38ΔPM/*lacZ*; and 5′GCCCAATAGACGGATGAGTTATCTG-TATGTAAAAAGGCGCAGC3′ (sense) and 5′GCTGGCGCTTTT-TACATACAGATAACTCATCCGTCTATTGGGC3′ (antisense) for P2b_0.38mPM/*lacZ*. To construct P2b_10ΔPH/*lacZ*, a 1.3 kb *Nhe*I-*Xma*I fragment containing the P2b_0.38 enhancer was subcloned from the P2b_10/*lacZ* into pBluescript KS (Stratagene). Deletion of 11 bp, including the PH site was obtained by using the following primers: 5′TCATTATTTCCAAGTAGCGTAGGGCAGGAGCTGGTTAGAA3′ (sense) and 5′TTCTAACCAGCTCCTGCCCTACGCTACTTGGA-AATAATG3′ (antisense). The wild-type 1.3 kb fragment in P2b_10/*lacZ* was replaced by the fragment including the PH deletion to obtain P2b_10ΔPH/*lacZ*. The p3xPH/*Luc* and p3xPH/*lacZ* reporter plasmids were obtained by cloning into the *Nhe*I site of p-βglob-*Luc* or the *Xba*I site of BGZ40, respectively, the following double stranded fragment containing three copies of the PH site and adjacent sequences (17 bp): 5′ACTTACTAGTCGTGATTGAATTAAAGGC-GTGATTGAATTAAAGGCGTGATTGAATTAAAGGACTAGTACT-T3′ with its complementary antiparallel oligonucleotide. The reporter plasmid p3xmPH/*Luc* was constructed as p3xPH/*Luc*, except that the PH site was mutated as in P2b_0.38mPH/*Luc* and P2b_0.38mPH/*lacZ*.

In ovo electroporation

Chick eggs were incubated in a humidified chamber, and embryos were staged according to (Hamburger and Hamilton, 1951). DNA constructs were injected into neural tubes of stage HH10-12 chick embryos. Electroporation was performed as described previously (Itasaki et al., 1999) using a square wave electroporator. Construct concentrations were: 1.5 mg/ml reporter construct, 1 mg/ml expression vector and 0.5 mg/ml co-injected pCMV/EGFP as a tracer of electroporated cells. Embryos were harvested 24-48 hours after electroporation and processed for immunohistochemistry, in situ hybridization or β-galactosidase staining.

Transient transfection assays

P19 embryonic carcinoma cells were cultured in Dulbecco minimal essential media supplemented by 5% fetal calf serum and 5% delipidated fetal calf serum. Cells were seeded, incubated for 36 hours and transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In a typical experiment, 500 ng of reporter plasmid, 250-500 ng of each expression construct and 100 ng of pCMV-β-gal (as a control of transfection efficiency) were used per well in six-well plates. Cells were lysed 40-45 hours after transfection then assayed for luciferase activity. Values were normalized by β-gal activity. Data represent means of duplicate values from representative experiments. All transfections were independently repeated at least three times.

Transient transgenic analysis in mouse embryos

Generation of mouse transgenic embryos was performed as described (Popperl et al., 1995). Embryos were harvested at E10.5. β-Gal was detected either by whole-mount in situ hybridization with a *lacZ* probe (a kind gift of M. Kmita) or by X-Gal staining.

Electrophoretic mobility shift assays (EMSAs)

Proteins were in vitro translated using the coupled TNT transcription/translation system (Promega) in the presence of ^{35}S methionine. Proteins were visualized by SDS-PAGE, followed by autoradiography. EMSA was performed according to (Ferretti et al., 2000). Antibodies used are polyclonal anti-Hoxb1 (Babco), polyclonal anti-Pbx1 and anti-Prep1 (Santa Cruz Biotechnology).

Immunostaining and in situ hybridization

Monoclonal anti-Isl1/2 (Developmental Studies Hybridoma Bank) was used for immunohistochemistry, as described (Tsuchida et al., 1994). Chick *Phox2b* (Ernsberger et al., 1995), *Isl2* (Tsuchida et al., 1994) and *Hb9* (Lee and Pfaff, 2001) probes were used for in situ hybridization as described previously (Gavalas et al., 1997).

Results

Identification of *Phox2b* regulatory regions driving restricted expression in the mouse hindbrain

In transient mouse transgenic assay, a 10 kb genomic construct driving the *lacZ* reporter gene, including the *Phox2b* promoter and its upstream sequences (P2b_10/*lacZ*, Fig. 1A) recapitulated most of the endogenous *Phox2b* expression pattern at E10.5 (Fig. 1B,D; data not shown). In the hindbrain, the r2-r6 dorsal columns of *Phox2b* expression were readily identified, as well as the two ventral columns that included the developing cranial vMNs (Fig. 1B) (Pattyn et al., 1997). To further identify fragments driving expression in *Phox2b* subset domains, we deleted the 5′-most 7 kb from the 10 kb genomic fragment. The resulting 2.8 kb region was placed upstream of an heterologous promoter driving the *lacZ* reporter (P2b_2.8/*lacZ*). Three out of eight P2b_2.8/*lacZ* transgenic embryos displayed detectable reporter levels at E10.5. Interestingly, *lacZ* expression was restricted to the ventral neural tube (Fig. 1C; data not shown). In particular, two out of three embryos displayed expression in two ventral columns running throughout the hindbrain and spinal cord (data not shown). In the third embryo, reporter expression was restricted to the ventral region of r4 (Fig. 1C), matching the *Phox2b* endogenous domain. Thus, the 2.8 kb fragment contains cis-regulatory sequences integrating both AP and DV positional information to drive spatially restricted *Phox2b* expression in ventral r4.

Characterization of a conserved proximal enhancer and analysis of its regulatory potential in the chick neural tube

To identify conserved cis-regulatory elements within the 2.8 kb fragment potentially involved in r4 restricted regulation, we compared its sequence with *Phox2b* genomic sequences from other organisms. A stretch of 376 base pairs (bp) in the mouse proximal promoter region (GenBank Accession Number AY 640178) was highly conserved in human and rat (97% at the nucleotide level), as well as pufferfish (Fugu) and zebrafish (Danio) *Phox2b* genomic regions (Fig. 2A,B). To test for regulatory potential of this conserved region, we used in ovo electroporation in the chick hindbrain. This is a suitable system to study conservation of transcriptional regulatory mechanisms (e.g. Itasaki et al., 1999; Manzanares et al., 2001). We electroporated a *lacZ* reporter construct carrying the mouse 376 bp *Phox2b* conserved enhancer (P2b_0.38/*lacZ*) into neuroepithelial cells along one side of the neural tube of stage HH10-12 chick embryos. High reporter expression levels were restricted to r4 and, to a lesser extent, to r2, whereas only weak activation was found in other hindbrain regions or rostral spinal cord (Fig. 3C). In r4, expression always extended more ventrally than in r2 (arrow, Fig. 3C). Electroporation of a control construct without the enhancer did not result in any activation (data not shown).

In summary, the conserved P2b_0.38 enhancer responded in a rhombomere-restricted fashion to the activity of endogenous factors in the chick hindbrain. Such response reminded that of

Hox-regulated enhancers (Maconochie et al., 1997; Jacobs et al., 1999; Ferretti et al., 2000; Popperl et al., 1995), suggesting that Hox-responsive sequences are present in the P2b_0.38 enhancer.

Transactivation of the *Phox2b* enhancer by Hox proteins and Pbx and Prep co-factors

To investigate transactivation by Hox transcription factors, we transfected murine embryonal carcinoma P19 cells, a suitable system for the analysis of the transcriptional activity of Hox proteins (Di Rocco et al., 2001; Saleh et al., 2000). A *Luciferase* reporter construct driven by the conserved *Phox2b* enhancer in front of a minimal promoter (P2b_0.38/*Luc*) was co-transfected along with Hoxb1, HOXB2 or Hoxa2 expression vectors. A three- to fourfold increase of P2b_0.38/*Luc* basal transcriptional activity was observed with either one of the Hox vectors (Fig. 3A; not shown). To extend these findings in vivo, we co-electroporated Hox vectors with P2b_0.38/*lacZ* (Fig. 3E,G,I) in the chick neural tube. We observed a marked upregulation of P2b_0.38/*lacZ* activity in the hindbrain and rostral spinal cord of embryos co-electroporated with either Hoxb1 (n=21/32), HOXB2 (n=10/12) or Hoxa2 (n=12/18), when compared with P2b_0.38/*lacZ* alone (Fig. 3C). Unlike the modest transactivation observed in P19 cells (Fig. 3A), this robust effect indicated that the in vivo activity of electroporated Hox proteins may be enhanced by the presence of endogenous co-factors. We therefore tested whether Hox transcriptional activity on P2b_0.38/*Luc* was improved by co-transfections with Pbx1a and Prep1 expression vectors (Fig. 3A). Pbx1a or Prep1 alone were unable to stimulate the reporter activity more than two- to threefold. By contrast, co-transfection of either Hoxb1, HOXB2 or Hoxa2 with Pbx1a and Prep1 co-factors resulted in a significant 13-14-fold enhancement of transcription (Fig. 3A; and not shown).

In summary, the *Phox2b* conserved enhancer can be transactivated by Hoxb1, HOXB2 or Hoxa2 both in cultured cells and chick neural tube. Furthermore, the observed Hox-mediated transcriptional activity is enhanced by the co-factors Pbx1a and Prep1. These results strongly suggest that DNA binding site(s) for Hox proteins and their co-factors are present in the P2b_0.38 enhancer.

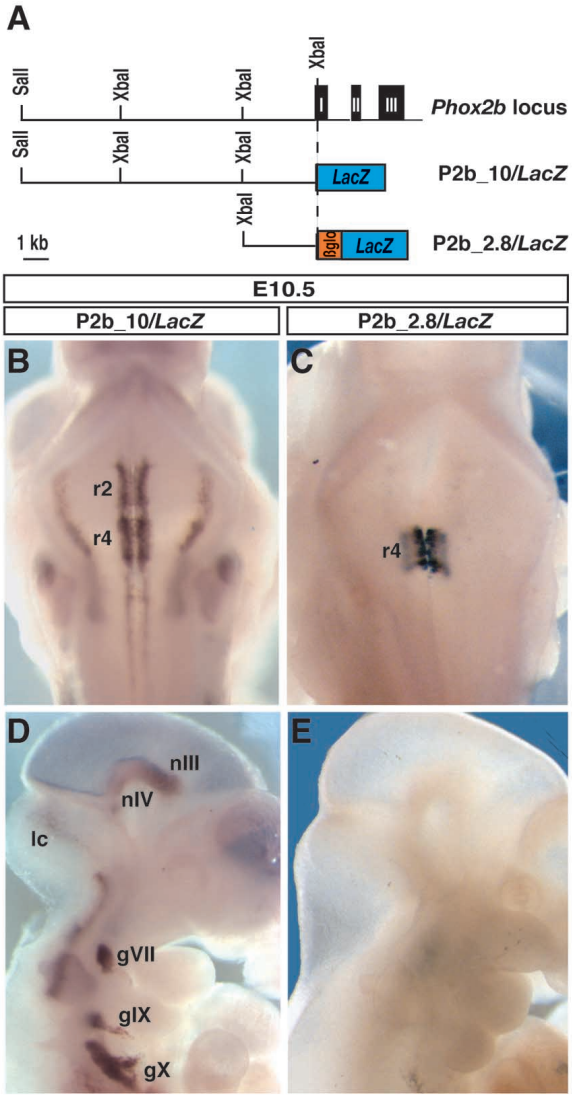

Fig. 1. Analysis of *Phox2b* regulatory regions in transgenic mice. (A) The *Phox2b* locus and *lacZ* reporter constructs used in transgenic mouse assay. Black boxes represent *Phox2b* exons I, II, and III; the red box represents the β-globin (β-glo) minimum promoter. (B,D) Dorsal (B) and lateral (D) views of E10.5 embryos carrying the P2b_10/*lacZ* construct. *lacZ* expression is detected by whole-mount in situ hybridization. (C,E) Dorsal (C) and lateral (E) views of E10.5 embryos carrying the P2b_2.8/*lacZ* construct, stained for β-gal activity. Expression of P2b_10/*lacZ* recapitulates most of *Phox2b* expression pattern in hindbrain and sensory ganglia (Pattyn et al., 1997) (B,D), whereas that of the P2b_2.8/*lacZ* is selectively restricted to ventral r4 (C) and not expressed in the ganglia (E). r, rhombomere; nIII, oculomotor nucleus; nIV, trochlear nucleus; lc, locus cœruleus; gVII, gIX and gX, geniculate, petrose and nodose ganglia of cranial nerves, respectively.

The *Phox2b* enhancer contains conserved Pbx-Hox and Prep/Meis binding sites

Indeed, sequencing of the *Phox2b* enhancer revealed the presence of a putative bipartite PH-binding site (TGATTGAA) (Fig. 2B). Notably, its nucleotide sequence was identical to that of the low-affinity PH binding site of repeat 2 (R2) of the *Hoxb1* autoregulatory (b1-ARE) r4 enhancer (Popperl et al., 1995) (Fig. 2C). Moreover, it shared fairly high conservation with the PH site present in the *Hoxb2* r4 enhancer, also regulated by *Hoxb1* (Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997) (Fig. 2C). Similar to the *Hoxb1* and *Hoxb2* r4 enhancers, we also found a conserved P/M site (TTGTCATG), downstream of the PH site (Fig. 2B,C). The *Phox2b* P/M site and its flanking nucleotides exactly matched the sequence found in the *Hoxb1* r4 enhancer and shared six out of eight nucleotides with that in the *Hoxb2* r4 enhancer (Ferretti et al., 2000; Jacobs et al., 1999). Interestingly, unlike the previously identified PH and P/M sites lying in relative proximity to each other, the *Phox2b* P/M site was 147 nucleotides distant from the PH site (Fig. 2B).

Hox, Pbx, and Prep proteins form a ternary complex on the *Phox2b* enhancer

To test for direct binding, we run EMSA assays using Hox,

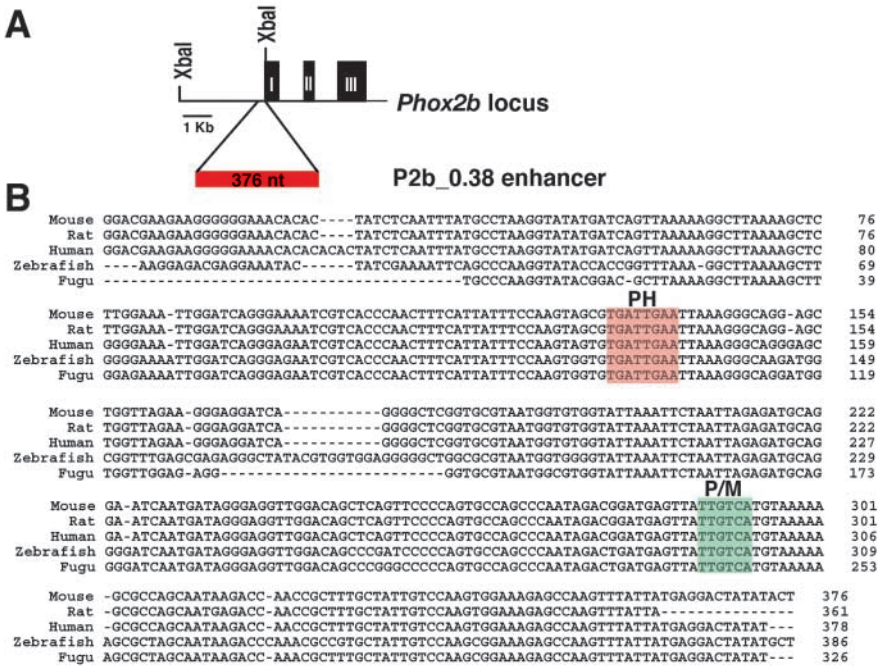

Fig. 2. A *Phox2b* enhancer that contains conserved Pbx-Hox and Prep/Meis sites. (A) The mouse *Phox2b* locus highlighting (red box) a 376 nucleotide (nt) proximal enhancer (P2b_0.38). (B) Sequence alignment between mouse, rat, human, zebrafish and fugu, showing high conservation. PH and P/M binding elements are boxed in red and green, respectively. (C) Comparison between PH and P/M sequences in *Phox2b*, *Hoxb1* (ARE repeat 2) (Popperl et al., 1995) and *Hoxb2* (Maconochie et al., 1997) r4 enhancers. The *Phox2b* and *Hoxb1* PH and P/M core sequences are identical.

Pbx1a and/or Prep1 in vitro translated proteins on a 30 bp oligonucleotide probe containing the *Phox2b* PH site and flanking sequences, or on a PCR-amplified radiolabeled fragment (233 bp) including both PH and P/M sites in their native context (Fig. 4A,B). As for the *Hoxb1* and *Hoxb2* r4 enhancers (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Popperl et al., 1995), the PH-containing probe (Fig. 4A) was readily bound by Pbx-Prep heterodimers, whereas no binding was observed with Pbx or Prep on their own (Fig. 4A, lane 4 arrow; not shown). Moreover, nucleotide changes of the PH site (same mutation as in Fig. 3B,D and 6B,D; see below) abrogated binding of the Pbx-Prep heterodimer (not shown). By contrast, we did not detect Hoxb1-Pbx1a heterodimers nor Hoxb1-Pbx1a-Prep1 heterotrimers, indicating low in vitro binding affinity of the PH site for Hox-containing multimeric complexes (Fig. 4A, lanes 3,5). Such binding behavior was consistent with that of the PH site in the R2 of the b1-ARE r4 enhancer (Popperl et al., 1995), which did not show cooperative binding of Pbx-Hox complexes in vitro, while contributing to *Hoxb1* r4 expression in vivo (Popperl et al., 1995) (see Discussion).

Next, we tested whether the binding of a Hoxb1-Pbx1a-Prep1 ternary complex would be stimulated by the simultaneous presence of both PH and P/M motifs, as for the *Hoxb1* and *Hoxb2* r4-regulated enhancers (Ferretti et al., 2000; Jacobs et al., 1999). Indeed, a slower migrating band was detected upon incubation of Hoxb1, Pbx1a and Prep1 with a probe containing both the PH and P/M sites in their native context, even though spaced by 147 nucleotides (arrow in Fig. 4B, lane 5). This band did not form with only Hoxb1-Pbx1a (lane 3) or Pbx1a-Prep1 (lane 4) pairs, nor with single proteins (data not shown). The presence of all three HD proteins in the complex was further confirmed, as the addition of either anti-Hoxb1, anti-Pbx or anti-Prep antibodies selectively inhibited its formation (Fig. 4B, lanes 6-8). Importantly, deletion of eleven nucleotides encompassing the PH site (same mutation tested in mouse transgenic analysis; Fig. 5) in the context of the entire probe containing the wild-type P/M site prevented all complex formation, demonstrating the requirement of the PH site for efficient multimeric complex association (Fig. 4B, lane 11). Moreover, a point mutant of the P/M site (same mutation tested in chick hindbrain; Fig. 3) in the presence of a wild-type PH site was also unable to bind a ternary complex (Fig. 4B, lane 16), indicating that both the PH and P/M sites are essential for binding of a Hoxb1-Pbx1a-Prep1 complex.

In summary, the combination of low-affinity PH and distant P/M motifs on the *Phox2b* enhancer can support the assembly of a Hoxb1-Pbx1a-Prep1 ternary complex in vitro. Moreover, both the PH and P/M sites are essential for ternary complex binding. These results support the transcriptional cooperation of the three HD proteins observed in cell culture (Fig. 3A), and

raise the question of the specific contribution of the identified sites to the transcriptional regulation of the *Phox2b* enhancer.

The PH and P/M sites are both essential for *Phox2b* enhancer activity in ventral r4

To test the involvement of the PH site in the activity and spatial regulation of the *Phox2b* enhancer, we introduced nucleotide mutations changing TGATTGAA to TcgTTcgA, while leaving the rest of the enhancer unaltered. Similar nucleotide changes were previously shown to impair PH site activity in the *Hoxb1* and *Hoxb2* enhancers (Ferretti et al., 2000).

A *Luc* construct carrying the mutated enhancer (P2b_0.38mPH/*Luc*) was co-transfected in P19 cells along with Hoxb1, HOXB2, Hoxa2, Pbx1a and/or Prep1 vectors. The transcriptional activity of P2b_0.38mPH/*Luc* induced by each protein alone remained just above the basal level (compare Fig. 3A with 3B; and not shown). Notably, the enhanced transcriptional response induced by Hox/Pbx or Hox/Pbx/Prep combinations on the wild-type enhancer (Fig. 3A) was abrogated on the P2b_0.38mPH/*Luc* construct (Fig. 3B).

To investigate the PH site requirement for Hox-mediated spatial regulation, a *lacZ* construct carrying the mutated *Phox2b* enhancer (P2b_0.38mPH/*lacZ*) was electroporated in the neural tube of stage HH10-12 chick embryos. Twenty-four hours later, two main expression differences were observed with the mutant P2b_0.38mPH/*lacZ* construct, when compared with the wild-type P2b_0.38/*lacZ* (compare Fig. 3C,D). First, a general decrease of β-gal expression levels and/or number of expressing cells throughout the expression domain. Second, the ventral domain of r4 expression was invariably lost or severely impaired (*n*=34/34; arrow, Fig. 3D). Mutation of the PH site also severely reduced Hox-induced upregulation

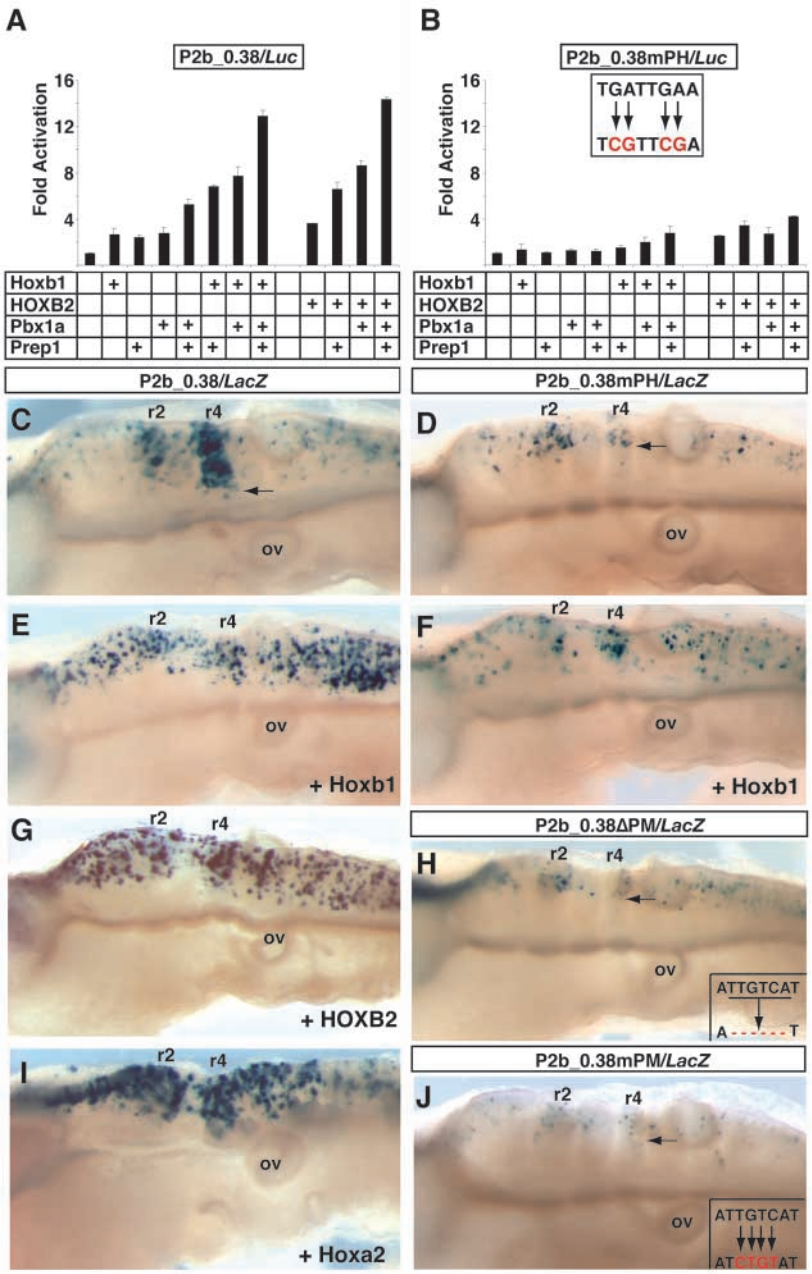

Fig. 3. The PH and P/M sites are both essential for *Phox2b* enhancer regulation in cell culture and ventral r4. (A,B) Fold activation of luciferase activity assayed from P19 cells transiently co-transfected with combinations of Hoxb1, HOXB2, Pbx1a or Prep1 vectors along with P2b_0.38/*Luc* (A) or mutant P2b_0.38mPH/*Luc* (B) reporter constructs. The box in B shows the nucleotide changes in the PH site of P2b_0.38mPH/*Luc*. Note that Hox, Pbx and Prep synergistic activity depends on an intact PH site. (C-J) Dorsolateral views (anterior towards the left) of stage 17-18 chick embryo hindbrains electroporated with P2b_0.38/*lacZ* (C), P2b_0.38mPH/*lacZ* (D), P2b_0.38ΔPM/*lacZ* (H) or P2b_0.38mPM/*lacZ* (J) constructs. P2b_0.38mPH/*lacZ* carries the same mutation as P2b_0.38mPH/*Luc*. P2b_0.38ΔPM/*lacZ* and P2b_0.38mPM/*lacZ* carry P/M site mutations shown in H and J, respectively. (C) High reporter expression is restricted to r4 and, to a lesser extent, to r2. (D,H,J) Overall β-gal levels decrease and ventral r4 expression is lost (arrows). Thus, both PH and P/M sites are required for ventral r4 expression. Co-electroporation of Hoxb1 (E), HOXB2 (G) or Hoxa2 (I) vectors significantly enhances expression from wild type P2b_0.38/*lacZ* but not mutated P2b_0.38mPH/*lacZ* (F). ov, otic vesicle.

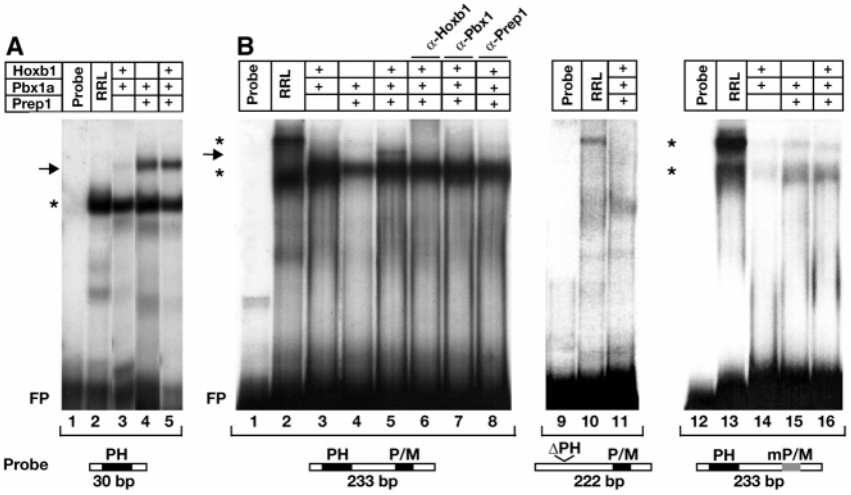

Fig. 4. Binding of a Hox-Pbx-Prep ternary complex requires intact PH and P/M sites. (A,B) In vitro synthesized proteins were subjected to EMSA with different radiolabeled probes (drawn below the gels) containing the PH and/or P/M sites. (A) Pbx-Prep (arrow in lanes 4,5), but not Pbx-Hox, heterodimers are formed on the 30 bp probe. (B) A retarded band (arrow in lane 5) forms only in the presence of Hoxb1, Pbx1a and Prep1 proteins on a 233 bp probe containing PH and P/M sites in their native context (left panel), but not on a probe carrying a PH site deletion (middle panel) or a P/M site mutation (same as that in Fig. 3J) (right panel). The ternary complex is selectively inhibited by adding specific antibodies (lanes 6-8). Asterisks indicate unspecific bindings. RRL, unprogrammed rabbit reticulocyte lysate; FP, free probe.

of the enhancer, as assessed by co-electroporation of P2b_0.38mPH/*lacZ* and Hoxb1 or Hox paralog group 2 vectors (compare Fig. 3E,F; data not shown). Thus, the PH site is an essential component of Hox-mediated regulation in ventral r4.

As the PH site contributes to ventral r4 expression in the context of the *Phox2b* enhancer, we next asked whether it was also sufficient to direct spatially restricted expression in the chick hindbrain. However, unlike the full enhancer (Fig. 3C), the p3xPH/*lacZ* construct, driven by three copies of a 17 bp oligonucleotide containing the *Phox2b* PH site and flanking sequences, was weakly active and did not display spatially restricted reporter expression in electroporated embryos (*n*=32/32, Fig. 6E; see Discussion). Thus, interactions with additional sequences within the *Phox2b* enhancer are required for the PH site to fully function in vivo. To determine if the P/M motif also contributes to enhancer activity, we generated a six-nucleotide deletion (Fig. 3H) or a four-nucleotide exchange (Fig. 3J; same mutation that abolishes ternary complex binding in vitro in Fig. 4) of the P/M site in the *Phox2b* enhancer and tested their effects on reporter activity in chick electroporation. Notably, both P/M mutations phenocopied the PH mutation and abrogated ventral reporter expression in r4 (*n*=30/30, Fig. 3H; *n*=12/12, Fig. 3J).

Thus, the PH and P/M sites are both essential for Hox-mediated transcriptional regulation of the *Phox2b* enhancer activity in ventral r4, strongly supporting the involvement of a Hox-Pbx-Prep transcriptionally active ternary complex in vivo.

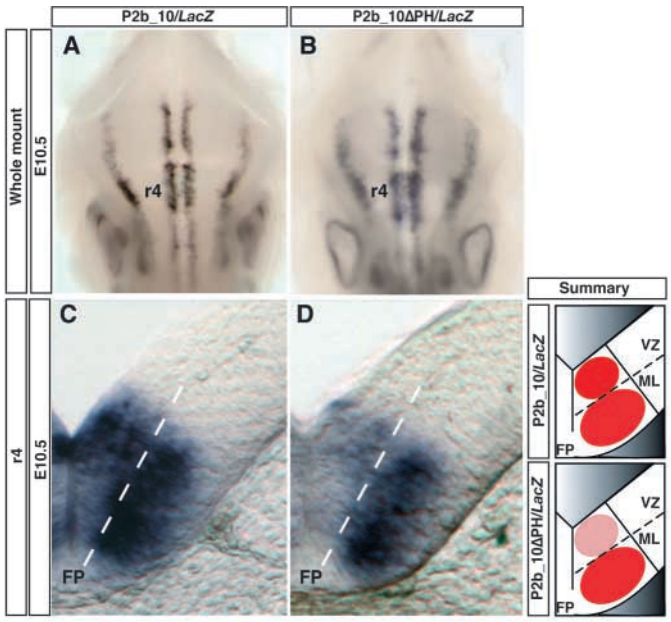

Fig. 5. The PH site contributes to mouse *Phox2b* regulation in ventral r4 progenitors. (A,B) Dorsal views of whole-mount E10.5 mouse embryos carrying (A) P2b_10/*lacZ* or (B) P2b_10ΔPH/*lacZ*, containing an 11 bp deletion of the PH site (same as in Fig. 4B). No overt differences are apparent between mutated and wild-type constructs. (C,D) Cross-sections through ventral r4 of transgenic embryos carrying P2b_10/*lacZ* (C) or P2b_10ΔPH/*lacZ* (D). (D) Reporter expression is severely reduced in the ventricular zone (VZ) (delimited by the broken line) of the pMNv domain but not in the mantle layer (ML). *lacZ* expression is detected by in situ hybridization. The summary represents the expression pattern differences at E10.5 in ventral r4 of wild type P2b_10/*lacZ* and mutant P2b_10ΔPH/*lacZ* transgenic embryos. Red circles, strong expression; pink circle, weak residual expression. FP, floorplate.

An intact PH site is essential for *Phox2b* regulation in the mouse ventral r4 progenitor domain

To investigate the relevance of the PH motif to Hox-mediated regulation of mouse *Phox2b*, we generated an 11 bp deletion of the PH site (same mutation that abolishes ternary complex formation in vitro; Fig. 4) in the context of the 10 kb *Phox2b* genomic construct (P2b_10ΔPH/*lacZ*), and tested it in mouse transgenic embryos (Fig. 5). Analysis of whole-mount hindbrains of E10.5 transgenic embryos did not reveal overt differences in reporter expression between mutated and wild-type constructs (compare Fig. 5A,B; Fig. 1B; data not shown), indicating that the establishment of the major domains of *Phox2b* expression in the hindbrain does not depend on an intact PH site. However, a selective, spatially restricted, difference was detected in ventral r4 upon sectioning of embryos carrying the mutant construct (Fig. 5D). B-gal expression in the pMNv progenitor domain was eliminated (six out of 11 embryos) or severely reduced (the five remaining embryos; Fig. 5D), whereas mantle layer (ML) expression was not significantly affected (Fig. 5B,D; data not shown).

The effect of the PH site deletion precisely mirrored the loss or reduction of *Phox2b* expression in the r4 pMNv progenitor domain of E10.5 *Hoxb1*$^{-/-}$ or *Hoxb2*$^{-/-}$ mutant embryos, respectively (Davenne et al., 1999; Gaufo et al., 2000) (see Pattyn et al., 2003a). In these mutants, ventral r4 expression of *Phox2b* at E10.5 is not properly maintained, leading to a facial MN to serotonergic switch of progenitor fate. Our results therefore strongly suggest that maintenance of *Phox2b* expression by Hoxb1 and/or Hoxb2 in ventral r4 progenitors is directly regulated through the PH site. Moreover, the PH site appears to integrate both AP and DV regulatory inputs as its mutation affects ventral regulation in r4.

Transcriptional cooperation of Hox and Nkx2 proteins on the *Phox2b* enhancer

Nkx2 proteins are good candidates for providing DV regulatory inputs that restrict *Phox2b* expression to the pMNv domain. In fact, *Nkx2.2/2.9* and *Phox2b* expression patterns are co-extensive in the pMNv domain (Pattyn et al., 2003a). Moreover, gain-of-function studies have involved Nkx2 proteins in ectopic *Phox2b* activation (Pattyn et al., 2003b). Thus, Nkx2 patterning factors may interact with Hox and their co-factors to allow high *Phox2b* expression levels specifically in the ventral r4 progenitor domain. To test whether Hox and Nkx2 factors transcriptionally cooperate to regulate the *Phox2b* enhancer, we first examined the transcriptional activity of Nkx2 factors on P2b_0.38/*Luc* in P19 cells. Co-transfection of Nkx2.2 or Nkx2.9 vectors alone did not stimulate reporter activity more than two- to threefold, comparable with the modest activity induced by Hoxb1 alone (Fig. 6A; data not shown). Co-transfection of Nkx2.2 with Pbx and Prep, in the absence of Hoxb1, did not stimulate reporter activity more than fourfold (Fig. 6A). Notably, when Hoxb1 was co-expressed with Nkx2.2, reporter activity was cooperatively stimulated up to tenfold. Further addition of Pbx and Prep co-factors resulted in a synergistic enhancement of transcription up to 20-fold, significantly exceeding the transcriptional enhancement observed with only Hox, Pbx and Prep (Fig. 6A). Importantly, mutation of the PH site within the context of the full enhancer abolished the transcriptional cooperation between Hox, its HD co-factors and Nkx2.2 (P2b_0.38mPH/*Luc*; Fig. 6B).

Transcriptional activation by Hox factors is enhanced by Nkx2.2-mediated derepression

In principle, the transcriptional enhancement mediated by Nkx2.2 in the presence of Hoxb1 and its co-factors may require DNA binding on the conserved *Phox2b* enhancer. However, in EMSA assays Nkx2.2 did not bind any potential Nkx consensus binding site or core HD-binding sequence from the *Phox2b* enhancer (data not shown). Most compellingly, co-

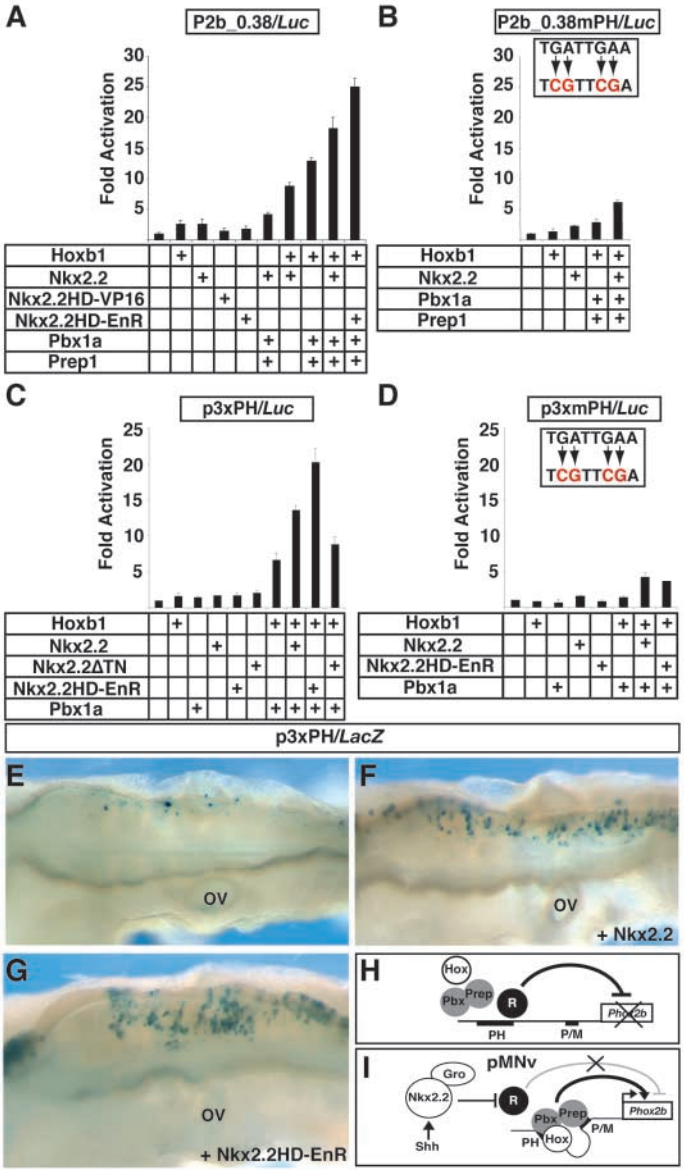

Fig. 6. *Nkx2*-mediated derepression of the *Phox2b* enhancer enhances Hox-dependent regulation at the PH site. (A-D) Fold activation of luciferase activity assayed from P19 cells co-transfected with different vector combinations (as indicated below the graphs) along with P2b_0.38/*Luc* (A) or p3xPH/*Luc* (C) carrying three copies of the PH site, or their mutated versions P2b_0.38mPH/*Luc* (B) and p3xmPH/*Luc* (D). The nucleotide changes in the PH site are shown in the boxes in B and D. Cooperative activation by Hoxb1, Pbx1a and Prep1 is further enhanced by Nkx2.2 or Nkx2.2HD-EnR repressor proteins (A,C), and it requires an intact PH site (B,D). Co-transfection of P2b_0.38/*Luc* with Nkx2.2HD-VP16 does not activate reporter expression (A), suggesting that Nkx2.2 HD does not bind to the enhancer. Co-transfection of Nkx2.2ΔTN, carrying a deletion of the Groucho-interacting domain (Muhr et al., 2001), almost abolishes Nkx2-dependent transcriptional cooperation (C). (E-G) Dorsolateral views (rostral to the left) of stage 17-18 chick embryo hindbrains electroporated with p3xPH/*lacZ* carrying three copies of the PH site (E), and with Nkx2.2 (F), or Nkx2.2HD-EnR (G) vectors. p3xPH/*lacZ* is weakly active and does not display spatially restricted reporter expression (E). (F,G) p3xPH/*lacZ* expression is enhanced throughout the hindbrain by Nkx2.2 (F) or Nkx2.2HD-EnR (G) repressors. (H,I) Model for integration of Hox-dependent activation and Nkx2-mediated derepression on the *Phox2b* enhancer. (H) In the ventral neural tube, dorsal to the pMNv domain (i.e. in the absence of Nkx2 factors) *Phox2b* is repressed, despite the presence of Hox activators and their Pbx and Prep co-factors, through the binding of a putative repressor (R) at, or in the vicinity of, the PH site. (I) In the pMNv domain, the repressor activity of Nkx2.2/Groucho (Gro) inhibits R, either directly or transcriptionally. After derepression, a Hox-Pbx-Prep ternary complex can bind to the PH and P/M sites and stimulate high levels of *Phox2b* transcription. Shh, sonic hedgehog; ov, otic vesicle.

transfection of a Nkx2.2HD-VP16 chimeric construct, consisting of the HD of Nkx2.2 (Nkx2.2HD) coupled to the VP16 activator domain (Muhr et al., 2001), did not stimulate transcription of P2b_0.38/*Luc* (Fig. 6A), in keeping with the idea that Nkx2.2 does not bind the enhancer.

In the ventral neural tube, Nkx transcription factors work as transcriptional repressors (Muhr et al., 2001). However, on the *Phox2b* enhancer Nkx2.2 stimulates transcriptional activation, even though only in the presence of Hoxb1 and its co-factors (Fig. 6A). In principle, this positive effect could also be the result of Nkx2.2 acting as a repressor, by relieving an inhibition on the transcriptional activation stimulated by Hox and co-factors.

We therefore examined the activity of a hybrid construct consisting of Nkx2.2HD coupled to the Engrailed repressor domain (Nkx2.2HD-EnR). This construct functions as a repressor in transfection assays, and mimics the repressive ability of full-length Nkx2.2 in the chick neural tube (Muhr et al., 2001). Strikingly, co-transfection of Nkx2.2HD-EnR in the presence of Hoxb1, Pbx1a and Prep1 led to a 25-fold stimulation of the P2b_0.38/*Luc* reporter transcription, while co-transfection of Nkx2.2HD-EnR alone had no effect (Fig. 6A). Thus, the observed Nkx2.2-mediated enhancement of Hox, Pbx and Prep-induced transcription is accounted for by its repressor activity, as it can be mimicked by the EnR domain.

Next, we asked whether the PH site is sufficient to mediate Hox and Nkx2.2 cooperation. Co-transfection in P19 cells of the p3xPH/*Luc* construct, which contains three copies of a 17 bp oligonucleotide including the PH site and its flanking sequences, along with Hoxb1, Pbx1a, Nkx2.2 or Nkx2.2HD-EnR vectors alone did not stimulate reporter activity more than twofold (Fig. 6C). Co-expression of Hoxb1 and Pbx1a enhanced p3xPH/*Luc* activation by about sevenfold. Importantly, co-transfection of Hoxb1 and Pbx1a with Nkx2.2 or Nkx2.2HD-EnR resulted in a robust synergistic stimulation of reporter activity by 14-fold and 20-fold, respectively (Fig. 6C), reproducing the effect observed with the full enhancer. This effect was abolished upon mutation of the PH site (p3xmPH/*Luc*; Fig. 6D). Notably, co-expression of Hoxb1 and Pbx1a with a truncated version of Nkx2.2 lacking the N-terminal TN domain (Nkx2.2ΔTN), which mediates interaction with co-repressors of the Groucho (Gro)/TLE family (Muhr et al., 2001), almost abolished transcriptional synergy (Fig. 6C).

Finally, the modest activity observed with Nkx2.2 (or Nkx2.2HD-EnR) when co-transfected alone with p3xPH/*Luc* (Fig. 6C), suggested that Nkx2.2 is not sufficient on its own to stimulate transcription at the PH site in the absence of Hox and Pbx co-factors, the endogenous levels of which are low in P19 cells (Saleh et al., 2000). We therefore tested whether overexpressing Nkx2.2 stimulated transcriptional activity at the PH site in the chick neural tube, a context in which Hox and its co-factors are endogenously available. Interestingly, although weakly active alone (Fig. 6E; see above), the p3xPH/*lacZ* reporter expression was significantly stimulated by co-electroporation of either Nkx2.2 or Nkx2.2HD-EnR vectors (n=14/16, Fig. 6F; n=10/12, Fig. 6G).

Altogether, these data indicate that Nkx2.2-mediated derepression, partially regulated through interaction with Gro corepressor(s), alleviates a repressive activity at, or in the vicinity of, the PH site, allowing transcriptional activation by Hox and Pbx factors (Fig. 6H,I; see Discussion). These data provide a molecular framework for understanding how AP and DV molecular inputs are integrated on the *Phox2b* enhancer and have relevance for the mechanism of generation of vMNs at specific hindbrain locations.

In vivo cooperation of Hox and Nkx2 factors generates ectopic *Phox2b*-expressing branchiomotor neurons

The ectopic expression of *Hoxb1* or *Hoxa2* in r1, an area normally devoid of BM neurons, led to the generation of ectopic facial or trigeminal BM neurons, respectively (Jungbluth et al., 1999). However, ectopic BM neurons were only detected ventrally, despite widespread *Hox* expression throughout the dorsoventral extent of r1, suggesting the requirement for an additional ventral input for BM neuron specification (Jungbluth et al., 1999). Nkx2 proteins could provide this ventral regulatory input, since electroporation of *Nkx2.2* in the chick hindbrain is sufficient to induce ectopic *Phox2b* expression and generation of BM neurons at dorsal neural tube levels (Pattyn et al., 2003b).

To investigate in vivo cooperation of Hox and Nkx2 factors in ectopic BM neuron generation, we first evaluated the AP distribution of ectopic *Phox2b*-expressing cells induced by forced *Nkx2.2* expression. Stage HH10-12 embryos were electroporated and analyzed 48 hours later. Interestingly, ectopic *Phox2b*-expressing cells were detected at dorsal levels but only up to r2, i.e. within the *Hox*$^+$ domain, and never in r1 (Fig. 7C). Conversely, forced Hoxb1 or Hoxa2 expression throughout the hindbrain resulted in ectopic *Phox2b*-expressing cells in r1, but only ventrally, i.e. within the *Nkx2.2*$^+$ domain (Fig. 7A,B). No ectopic *Phox2b* expression was detected at dorsal levels (the dorsoventral extent of electroporation was assessed by GFP co-injection; data not shown). Interestingly, only the combination of either Hoxb1/Nkx2.2 or Hoxa2/Nkx2.2 vectors could stimulate the generation of ectopic *Phox2b*$^+$, *Isl1*$^+$, *Isl2*$^-$, *Hb9*$^-$ vMNs in dorsal r1 (Fig. 7D-I; data not shown).

Thus, the generation of ectopic *Phox2b*-expressing BM neurons requires in vivo cooperation of Hox paralogs 1 or 2 and Nkx2 HD factors.

Discussion

Phox2b is a direct target of *Hoxb1* and *Hoxb2* in ventral rhombomere 4

Hox transcription factors direct the patterning of a variety of structures in the developing embryo and are thought to regulate numerous genes, but to date only a few direct targets have been identified, mainly in *Drosophila* (Capovilla et al., 1994; Capovilla et al., 2001; Galant et al., 2002; Lohmann et al., 2002; Vachon et al., 1992). In the vertebrate hindbrain, Hox genes regulate rhombomere-specific neuronal patterning, but it is unclear how they may link early neural patterning to the establishment of neuronal fates, as their direct downstream effectors remain elusive. We provide the first direct link between Hox function in the hindbrain and the expression of a downstream effector, *Phox2b*, that is an obligatory determinant of cranial vMN specification (Brunet and Pattyn, 2002).

We have focused on the regulation of *Phox2b* expression in the pMNv domain of ventral r4. The r4 pMNv domain gives

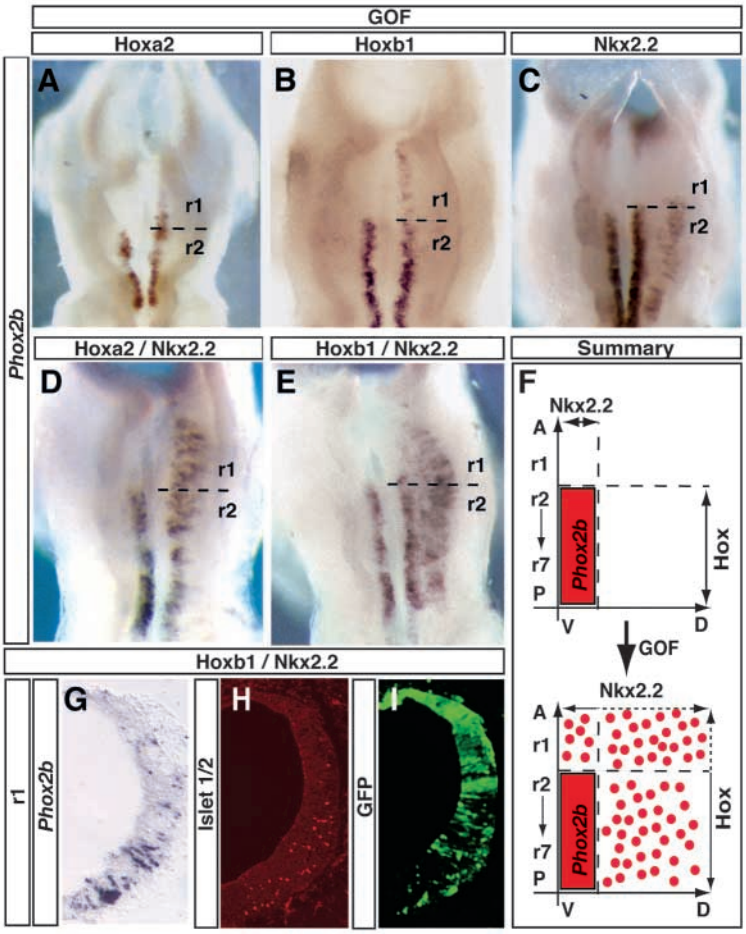

Fig. 7. Generation of ectopic *Phox2b*$^{+}$ motoneurons requires the combined activities of Hox and Nkx2 factors. Dorsal views of whole-mounts (A-E) or cross-sections through r1 (G-I) of stage 21-22 chick embryos electroporated, on the right-hand side, with the vectors indicated above each panel, and assayed for *Phox2b* in situ hybridization (A-E,G) or Isl1/2 immunohistochemistry (H). Hoxa2 (A) or Hoxb1 (B) misexpression induces ectopic *Phox2b* expression only in ventral r1. Conversely, Nkx2.2 misexpression (C) induces ectopic *Phox2b* expression at dorsal levels, though not in r1. Co-electroporation of Nkx2.2 with Hoxa2 (D) or Hoxb1 (E) additionally induces ectopic *Phox2b*$^{+}$ (G), *Isl1/2*$^{+}$ (H) motoneurons in dorsal r1. (I) GFP fluorescence, showing the dorsoventral distribution of electroporated cells. (F) Summary showing, on AP and DV coordinate axes, the requirement for the combined activities of Hox and Nkx2.2 proteins to induce ectopic *Phox2b* expression in the hindbrain. GOF, gain of function; r, rhombomere.

rise to the facial BM and inner ear efferent neurons of the VIIth cranial nerve (Bruce et al., 1997; Pattyn et al., 2003a; Simon and Lumsden, 1993; Tiveron et al., 2003), and analysis of knockout mice revealed that Hoxb1 and Hoxb2 are required for maintenance of the late phase of *Phox2b* expression in this progenitor domain (Pattyn et al., 2003a). We provide several lines of evidence supporting a direct regulation by Hoxb1 and/or Hoxb2, involving Pbx and Prep/Meis proteins as co-factors. First, ectopic expression of *Hoxb1* or *Hox* paralog group 2 in the chick neural tube can induce ectopic *Phox2b* expression (Fig. 7). Second, a conserved 376 bp enhancer, enclosed within a 2.8 kb *Phox2b* genomic fragment that drives ventrally restricted r4 expression in the mouse (Fig. 1), contains separate PH and P/M sites whose conserved sequences are hallmarks of Hox-mediated transcriptional regulation in r4 (Fig. 2) (Ferretti et al., 2000; Jacobs et al., 1999; Popperl et al., 1995). An intact PH site is required for Hoxb1-, HOXB2- or Hoxa2-mediated transactivation of the *Phox2b* enhancer, in both P19 cells and chick hindbrain (Fig. 3). Importantly, both the PH and P/M motifs are essential for binding in vitro of a Hox-Pbx-Prep ternary complex and for enhancer activity in ventral r4 of chick embryos. Finally, mutation of the PH site selectively impairs the regulation of a mouse 10 kb *Phox2b* transgenic construct, recapitulating endogenous *Phox2b* expression (Fig. 1), in the ventral r4 pMNv domain (Fig. 5). Moreover, the effect of the PH mutation faithfully mimics the endogenous *Phox2b* downregulation observed in *Hoxb1* and *Hoxb2* knockout mice (Pattyn et al., 2003a).

Our results further suggest that *Hoxa2* could also directly regulate the *Phox2b* enhancer. However, analysis in *Hoxa2* knockout mice did not reveal obvious *Phox2b* expression defects in ventral r4, indicating a major role for *Hoxb1* and *Hoxb2* at that level. By contrast, in *Hoxa2* mutants *Phox2b* expression is lost in the r2-r3 dorsal columns (Davenne et al., 1999). Sequences mediating regulation by Hoxa2 in dorsal columns may reside outside the 2.8 kb *Phox2b* genomic construct, as this fragment drives only ventral expression in transgenic mice (Fig. 1C).

Altogether, our data lead us to conclude that, in the ventral r4 pMNv domain, *Phox2b* is a direct target of Hoxb1 and Hoxb2.

Functional differences between PH-P/M modules in the *Phox2b* and other Hox-regulated r4 enhancers

Similar to the *Hoxb1* and *Hoxb2* r4 enhancers, we found separate PH and P/M sites embedded within the *Phox2b* enhancer. Nevertheless, the in vivo output of Hox regulation on these three enhancers is rather different, as the *Phox2b* PH or P/M sites mediate a transcriptional response restricted to ventral progenitors, despite widespread Hoxb1 and Hoxb2 distribution throughout r4. This is in keeping with the observation that endogenous *Phox2b* expression is upregulated in sharp columns of selected progenitor domains at distinct DV

levels. Comparing the nature and function of bipartite PH and P/M sites in the context of the *Hoxb1*, *Hoxb2* and *Phox2b* enhancers may therefore provide clues of how *Phox2b* regulation is spatially constrained.

In the *Hoxb2* enhancer, only one PH site is present that shows cooperative binding of Hoxb1 and Pbx/Exd proteins in vitro and is required for r4 expression in vivo (Maconochie et al., 1997). By contrast, the b1-ARE enhancer contains three PH motifs (R1-R3). Mutational analysis in the mouse indicated that all three PH sites are cooperatively required for high levels of r4 expression (Popperl et al., 1995), although with distinct individual contributions. Among the three *Hoxb1* PH sites, the R2 sequence precisely matches that of the *Phox2b* PH octamer core (Fig. 2C). Like the *Phox2b* PH site, the R2 repeat did not bind Hoxb1/Exd heterodimers in vitro, nor Hoxb1 or Exd alone, although it is necessary for optimal r4 activity (Popperl et al., 1995). Thus, the *Hoxb1* R2 repeat requires cooperative interactions with adjacent sequences in the b1-ARE to fully function in vivo. Similarly, a trimerized *Phox2b* PH site was not sufficient on its own to direct r4 restricted expression in the chick hindbrain (Fig. 6E), unlike the sufficiency for r4 expression of multimerized *Hoxb1* R3 or *Hoxb2* high-affinity PH sites (Maconochie et al., 1997; Popperl et al., 1995). Nonetheless, the PH motif was necessary, in the context of the *Phox2b* enhancer, for mediating the transcriptional cooperation of Hox, Pbx and Prep/Meis co-factors and for in vivo regulation in ventral r4 both in chick and mouse hindbrain (Figs 3, 5). Thus, the *Phox2b* low-affinity PH site, while representing a necessary site of integration of r4 activity, operates in vivo mainly through cooperative interactions with its surrounding regulatory environment, even in the presence of high endogenous levels of binding factors.

Cooperative interactions of PH sites with nearby sequences are important for in vivo specificity of Hox-Pbx complexes in both vertebrate and invertebrate Hox-regulated enhancers (Jacobs et al., 1999; Ferretti et al., 2000; Manzanares et al., 2001; Di Rocco et al., 2001; Mann and Affolter, 1998). We show that a distant P/M site makes an essential contribution to the binding specificity of the PH element, allowing formation of a Hox-Pbx-Prep complex in vitro, as ternary complexes were not observed on DNA probes containing mutations of either PH or P/M sites (Fig. 4). Moreover, regulation of the *Phox2b* enhancer in ventral r4 requires the integrity of both PH and P/M sites (Fig. 3), indicating the formation of transcriptionally active Hox-Pbx-Prep complexes in vivo. Although this functional behavior is reminiscent of that of the P/M element in the *Hoxb2* r4 enhancer, it differs from that of the *Hoxb1* P/M motif, functionally redundant with the R1-R3 elements (Jacobs et al., 1999; Ferretti et al., 2000). In addition, it should be noted that the *Hoxb1*, *Hoxb2* and *Phox2b* enhancers differ in the spacing and relative orientations of their P/M and PH motifs. Although the *Hoxb2* and *Hoxb1* (R2) P/M sites are located close to the 5′ and 3′ ends of their PH sites, respectively, the mouse *Phox2b* P/M element is located 147 nucleotides 3′ to the PH motif (Fig. 2). Different configurations and spacing might correlate with distinct spatial and/or levels of activity of Hox-regulated r4 enhancers (Jacobs et al., 1999). In this respect, the organization of PH-P/M modules in the *Hoxb1* and *Hoxb2* enhancers vary among vertebrate species that show fine regulatory differences in r4 and its derivatives (Popperl et al., 1995; Scemama et al., 2002). The permissivity of the different PH-P/M spacing could be explained by looping-out or bending of the intervening DNA (Fig. 6I), to allow formation of a trimeric complex. In the *Phox2b* enhancer, the unusual spacing of the P/M site might introduce further constraint on the ability of the low-affinity PH site to be activated in vivo, despite high endogenous Hoxb1, Hoxb2 and co-factor levels.

In conclusion, unlike the *Hoxb1* or the *Hoxb2* r4 enhancers that contain multiple and/or high-affinity PH sites readily activated by threshold levels of endogenous Hox proteins and their co-factors, the low-affinity *Phox2b* PH motif must integrate additional inputs in order to be fully functional in vivo. It is tempting to speculate that similar low-affinity PH sites are present in the enhancers of Hox target genes, the expression of which is tightly regulated in sharp columns in the hindbrain and the activation of which outside their normal domains would have deleterious consequences for neuronal patterning.

Maintenance of Hox target gene expression in r4 through PH-P/M modules

Our data strongly suggest that the *Phox2b* PH-P/M module is involved in the maintenance of high *Phox2b* expression levels in ventral r4. First, inactivation of the *Phox2b* PH site mirrors the effect of *Hoxb1* or *Hoxb2* loss-of-function in mice, i.e. the lack of maintenance of *Phox2b* expression in the r4 pMNv domain (Pattyn et al., 2003a). Second, other conserved PH-P/M cis-regulatory modules in *Hoxb1*, *Hoxb2*, *Hoxa3* and *Hoxb4* enhancers are all involved in Hox-dependent maintenance of rhombomere-restricted expression (Popperl et al., 1995; Jacobs et al., 1999; Ferretti et al., 2000; Maconochie et al., 1997; Manzanares et al., 2001; Gould et al., 1997). Clearly, other elements must then be required for the initiation of *Phox2b* expression in the pMNv domain. In this respect, forced expression of Hox and Nkx2 factors is sufficient to induce ectopic *Phox2b* expression and generates ectopic BM neurons in the chick hindbrain (Fig. 7), indicating that these factors could also mediate *Phox2b* activation through additional sequences other than the identified PH or P/M sites.

In conclusion, three key r4 targets of *Hox* paralog 1 and 2 genes, i.e. *Phox2b*, *Hoxb1* and *Hoxb2* bear conserved PH-P/M modules, arguing for a cis-regulatory signature that could be shared by a more ample collection of Hox direct targets requiring temporal maintenance in r4.

Integration of AP and DV transcriptional inputs via Nkx2-mediated derepression at the *Phox2b* PH site

We discussed how formation of a Hox-Pbx-Prep ternary complex results in transcriptional cooperation and contributes to overcome insufficient activation at the low-affinity PH site (Fig. 3A). However, as Hox, Pbx and Prep factors are present throughout r4 (Ferretti et al., 1999; Popperl et al., 1995; Schnabel et al., 2001), this model cannot solely explain how *Phox2b* expression is sharply restricted in ventral r4 to the pMNv progenitor domain. Our results indicate that cooperation with Nkx2.2 is an additional component of the regulation of the *Phox2b* enhancer.

How does Nkx2.2 contribute to the *Phox2b* enhancer regulation? First, Nkx2.2 binding to the *Phox2b* enhancer is not required (Fig. 6A; data not shown). Second, transcriptional activation by Hox and co-factors is further enhanced by the

activity of Nkx2.2 as a repressor (Fig. 6A). Third, an intact PH site is an essential component of the Hox and Nkx2.2 cooperation on the *Phox2b* enhancer (Fig. 6B). Moreover, a trimerized PH site is sufficient to respond to Nkx2.2 activity in the presence of Hox factors, both in P19 cells and chick hindbrain (Fig. 6C,F,G). Nkx2.2 activity is mediated in part through association with the Gro/TLE class of co-repressors, as deletion of the TN interacting domain impairs Nkx2.2 activity on the *Phox2b* enhancer (Fig. 6C). One possibility is that Nkx2.2/Gro could transcriptionally repress, or sequester, a putative repressor (R) normally bound at, or in the vicinity of, the PH site (Fig. 6H). In the absence of Nkx2 proteins, i.e. dorsal to the pMNv domain, R could prevent the formation of a Hox-Pbx-Prep ternary complex and consequently the activation of high *Phox2b* expression levels. Within the pMNv progenitor domain, the presence of Nkx2.2 would relieve repression by blocking the activity or the expression of R acting on the *Phox2b* enhancer (Fig. 6I). After recruitment of Hoxb1 or Hoxb2 by Pbx and binding of Prep1 to the P/M site (Ferretti et al., 2000; Jacobs et al., 1999), a ternary complex would then form and stimulate high levels of transcription (Fig. 6I).

Nonetheless, in the chick hindbrain reporter expression driven by the *Phox2b* enhancer was not restricted in a columnar pattern (Fig. 3C), unlike endogenous *Phox2b*. Thus, although the PH and P/M sites embedded within the 376 bp enhancer are required for ventral r4 regulation (Figs 3, 5), additional inhibitory inputs from regulatory regions outside the enhancer are also needed to achieve columnar regulation. In this respect, ventral restriction of reporter expression is obtained with the 2.8 kb construct (Fig. 1C). Therefore, the proposed repressor (Fig. 6H), although required, may not be sufficient to restrain the *Phox2b* enhancer activity outside the pMNv domain, when the enhancer is tested in isolation from its genomic context. Although an important site of integration of AP and DV regulatory inputs, the *Phox2b* enhancer may require interaction with distant regulatory elements for precise columnar regulation.

In conclusion, our results take a first significant step in understanding how the transcriptional activity of repressors and activators converges on a specific target gene promoter to direct expression in a specific progenitor domain in the mammalian central nervous system.

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