PHOX2B Regulates Its Own Expression by a Transcriptional Auto-regulatory Mechanism*

Revised for publication, July 29, 2005, and in revised form, September 2, 2005. Published, JBC Papers in Press, September 6, 2005, DOI 10.1074/jbc.M508368200

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The specification of neuronal identity is a result of interactions between the following two distinct classes of determinants: extrinsic factors that include secreted or cell membrane-associated signals in the local environment, and intrinsic factors that generally consist of ordered cascades of transcription factors. Little is known about the molecular mechanisms underlying the interplay between these extrinsic and intrinsic factors and the transcriptional processes that establish and maintain a given neuronal phenotype. Phox2B is a vertebrate homeodomain transcription factor and a well-established intrinsic factor in developing autonomic ganglia, where its expression is triggered by the bone morphogenetic proteins secreted by the dorsal aorta. In this study we characterized its proximal 5’-regulatory region and found that it contained five putative DNA sites that potentially bind homeodomain proteins, including PHOX2B itself. Chromatin immunoprecipitation assays showed that PHOX2B could bind its own promoter in vivo, and electromobility gel shift assays confirmed that four of the five sites could be involved in PHOX2B binding. Functional experiments demonstrated that 65% of the transcriptional activity of the PHOX2B promoter in neuroblastoma cells depends on this auto-regulatory mechanism and that all four sites were required for full self-transactivation. Our data provide a possible molecular explanation for the maintenance of PHOX2B expression in developing ganglia, in which initially its expression is triggered by bone morphogenetic proteins, but may become independent of external stimuli when it reaches a certain nuclear concentration and sustains its own transcription.

In general, intrinsic factors eventually cause cells to become independent of extrinsic signals and autonomously control the differentiation processes by directing the genetic program. This progression from extrinsic to intrinsic signals is well known in the development of the nervous system, but it is still poorly characterized at the molecular level (1).

The specification of sympathetic autonomic neurons has been widely investigated in studies that have produced an invaluable body of information for understanding the relationships between extrinsic and intrinsic factors (2–4). In particular, bone morphogenetic proteins (BMP3-2, -4, and -7) have been identified as essential extrinsic signals that are necessary to trigger the sympathetic neuronal differentiation of mammalian and avian neural crest cells (5). They are members of the transforming growth factor-β superfamily of growth factors expressed in bone, brain, skin, and kidney, in which they regulate cell growth, differentiation, and embryogenesis (6, 7). In the differentiation of sympathetic neurons, they control the expression of a set of transcription factors (Mash1, PHOX2A, PHOX2B, and dHand) representing a network of control genes that have hierarchically distinct roles but also partially overlapping functions (2, 3). Mash1 and PHOX2B are thought to be two independent intrinsic factors whose expression is absolutely required for the formation of sympathetic and parasympathetic ganglia (8), which do not develop in mouse embryos lacking one of the two genes (9, 10). The actions of these transcription factors converge on PHOX2A and dHand in order to regulate their expression. Although many of the phenomenological and functional aspects of the transcriptional cascade that takes place in the developing ganglia have been clarified, little is known about the molecular mechanisms underlying such processes. We have demonstrated previously that PHOX2B regulates the transcription of the PHOX2A gene by directly binding and transactivating its promoter (11). In this study, we characterized the human PHOX2B promoter and demonstrated by means of biochemical and functional criteria that most of its transcriptional activity is sustained and maintained by an auto-regulatory mechanism in which PHOX2B binds and transactivates its own promoter, thus providing a possible molecular explanation for the progression from extrinsic to intrinsic signals in the developing ganglia.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—The IMR32 human neuroblastoma cell line and the DAOY medulloblastoma cell lines were grown in RPMI 1640, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. The HeLa cell line was grown in Dulbecco’s

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3 The abbreviations used are: BMP, bone morphogenetic proteins; EMSA, electromobility gel shift assay; ChIP, chromatin immunoprecipitation; CCHS, congenital central hypoventilation syndrome; UTR, untranslated region; DAPI, 4,6-diamidino-2-phenylindole; h, human; CCHS, congenital central hypoventilation syndrome.
modified Eagle’s medium, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

**RNA Preparation and Northern Blot Analyses**—The RNAs were prepared, and the Northern blot analysis was performed as described by Flora et al. (12). The RNAs were hybridized with the human cDNA probes for PHOX2A and PHOX2B. The human PHOX2A probe was obtained by digesting the Sacl-NcoI construct (11) with Eagl and NcoI and corresponds to the 5′-UTR region (nucleotides +26/+219).

The PHOX2B probe was obtained by means of PCR amplification of human genomic DNA using primers designed on the region specifying the 5′-UTR of the mRNA (nucleotides −299/+90 with respect to the ATG; GenBankTM accession number NM_003924). The upper primer was 5′-GGGCCAGCCCATAGCAGG-3′ (nucleotides −195/−174 in Fig. 1B), and the lower primer was 5′-CTCAACGCTGCTTCCCAAACTG-3′ (nucleotides −7/+15 in Fig. 1B).

**Molecular Cloning of the Human PHOX2B 5′-Flanking Region**—The human PHOX2B probe described above was used to screen a commercial human genomic library (Clontech) from normal peripheral blood leukocytes, cloned in the λ vector EMBL3 SP6/T7. After three rounds of screening, we isolated four different clones, one of which was analyzed in detail by means of restriction and Southern blotting analyses. The phage contained a 4.5-kb fragment mainly belonging to the PHOX2B 5′-regulatory sequence, as confirmed by partial sequencing. The 4.5-kb fragment was subcloned in the XhoI and Spel sites of pBluescript II KS(+).

**Plasmid Construction**—Total RNA from SYSY, IMR32 cells, and HeLa cells was used to perform primer extension experiments using a 32p-labeled oligonucleotide (5′-ATCCCAAGCCCATACAGGCTGTAG-3′) complementary to the +142/+165 human PHOX2B sequence (underlined in Fig. 1B). The experiment was carried out as described in Flora et al. (11).

**Construction of the Human PHOX2B Promoter Reporter Plasmids**—The 4.5-kb XhoI-Spel plasmid was used as a template to amplify the 5′-UTR of PHOX2B with the aim of mutagenizing the nucleotides surrounding the translation start codon in order to generate an Ncol site. This allowed the direct fusion of the PHOX2B 5′-UTR and the Renilla luciferase coding sequence. The 181-bp XbaI-Ncol PCR product and the 745-bp HindIII-Xbal fragment were cloned by means of three-way ligation into the HindIII and NcoI sites of phRG-b (Promega), thus generating the HindIII/NcoI [mut2] construct. The HindIII/NcoI [mut2] was then used as a template to produce the HindIII/Ncol [mut2,3] construct, using HindIII/NcoI [mut2] as a template and introducing point mutations in the ATTA3 site as described above. HindIII/Ncol [mut2,3] was then used as a template to produce the HindIII/Ncol [mut2,3,4] construct, and finally, HindIII/Ncol [mut2,3,4,5] was used as a template to generate HindIII/Ncol [mut5].

**Transient Transfections and Luciferase Assays**—The cells were transiently transfected by means of lipofection using 8 × 10⁴ IMR32 or 4 × 10⁴ HeLa cells. The experiments were carried out as described by Flora et al. (11). The RSV-luc plasmid expresses the firefly luciferase reporter gene under the control of the Rous sarcoma virus promoter and was co-transfected in each sample in order to normalize transfection efficiency. In the co-transfection experiments with PHOX2B, 150 fmol of the PHOX2B expression construct was added to the reporter and pRSV-luc plasmid and incubated with 2 μl of FuGENE 6. Firefly and Renilla luciferase activities were detected using the dual luciferase reporter assay system (Promega) according to the supplier’s instructions. All of the transfections were performed in duplicate, and each construct was tested in at least three independent experiments using different plasmid preparations batches. All of the plasmids were purified using Qiagen (Hilden, Germany) columns. The transient transfections data were analyzed as described previously (14).

**Synthetic Peptide Preparation and Antibody Production**—Two peptides corresponding to the sequences of human PHOX2A (CKPG-PALKTNLF) and PHOX2B (CGAKAALVKSSMF), both of which are located in the carboxyl terminus of the proteins, were synthesized by means of a solid-phase method in an automated Applied Biosystem model 433A peptide synthesizer using Fmoc-(9-fluorenylmethoxycarbonyl) chemistry (15). The peptides were cleaved from the resin and side chain deprotected by reagent K, a scavenger-containing solution of trifluoroacetic acid (16).

The immunogens were produced by conjugating the peptides with mariculture keyhole limpet hemocyanin (Pierce) by using the heterobifunctional reagent sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane 1-carboxylic acid (Pierce) according to the procedure proposed by Liu et al. (17). The affinity-purified polyclonal antibodies from chicken 4 D. Fornasari, unpublished results.
egg yolk were produced by Davids Biotechnologie (Ragensburg, Germany).

Preparation of Nuclear Extracts—The nuclear extracts were obtained as described by Terzano et al. (18).

In Vitro Protein Expression—The in vitro expression of PHOX2A and PHOX2B was obtained by means of a commercial rabbit reticulocyte lysate system (TNT Quick-coupled Transcription/Translation System, Promega, Madison, WI). Briefly, PHOX2B/pCDNA3 or PHOX2A/pCDNA3 constructs were linearized with XhoI and in vitro transcribed by the T7 RNA polymerase. The purified plasmid was added together with methionine (1 mM final concentration) in the reaction tube provided by the manufacturer, which contained all of the reagents needed for transcription and translation in a final volume of 50 μl. The reaction was carried out at 30 °C for 90 min. The negative controls were unprogrammed lysates in which the reactions were carried out without plasmids. [35S]Methionine was sometimes added to the reaction in order to be able to detect the protein product by autoradiography and confirm the correctness of its size.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSAs were performed as described by Terzano et al. (18) and Flora et al. (11).

When the nuclear extracts were replaced by in vitro expressed proteins, 2 μl of reticulocyte lysates programmed with PHOX2B/pCDNA or PHOX2A/pCDNA were added. Unprogrammed lysates were also used as negative controls.

The oligonucleotides used in the EMSA experiments are indicated. EMSAs (1, 2), bearing the ATTA1 and ATTA2 sites, 5′-AAGTAGTGAGTTAAATTG ACTTTCAAAATGGAGATGAGTCA-3′; EMSA[1mut,2mut], bearing mutated ATTA1 and mutated ATTA2, 5′-GATGCA-3′; ATTA3 and ATTA4 sites, 5′-GGATGCA-3′; ATTA5 site, 5′-GGATGCA-3′; EMSA (1), bearing the ATTA1 site, 5′-GGATGCA-3′; EMSA[3mut,4], bearing the mutated ATTA3 and ATTA4 sites, 5′-GATGCA-3′; EMSA (2), bearing the ATTA2 site, 5′-GGATGCA-3′; EMSA (3, 4), bearing the ATTA3 and ATTA4 sites, 5′-GATGCA-3′; EMSA (5), bearing the ATTA5 site, 5′-GATGCA-3′. All of the oligonucleotides were purchased from Invitrogen.

The homeodomain-binding site oligonucleotide has been described by Flora et al. (11) and corresponds to the PHOX2A promoter region that contains the DNA element for PHOX2B.

Western Blotting—Fifteen micrograms of nuclear extract or 2 μl of in vitro expressed proteins were separated by means of 11% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The expressed proteins were separated by means of 11% SDS-PAGE in vitro and transferred to nitrocellulose membranes (Schleicher & Schuell). The negative controls were unprogrammed lysates in which the reactions were carried out without plasmids. [35S]Methionine was sometimes added to the reaction in order to be able to detect the protein product by autoradiography and confirm the correctness of its size.

Immunofluorescence—DAOY cells plated on 1.7 × 1.7-cm² glass coverslips were grown to 50% confluency, transfected with pcDNA3/PFOX2A or pcDNA3/PFOX2B, or mock-transfected with the empty vector. After 24 h, they were fixed with 4% paraformaldehyde in 0.120 M sodium phosphate buffer, pH 7.4, for 30 min at 37 °C. The monolayers were permeabilized and processed for immunofluorescence as described previously (19). The primary antibody was used at a dilution of 1:400 and revealed by means of an anti-chicken Texas Red conjugated secondary antibody (Jackson Immunoresearch). Competition assays were performed by incubating the related primary antibody with the specific peptide in a mass ratio of 10:1. Before mounting, the cells were stained with DAPI. The preparations were observed through a Zeiss Axiosplan microscope equipped for epifluorescence, and the images were acquired using a Zeiss AxioCam HRm.

Chromatin Immunoprecipitation (ChiP)—Chromatin immunoprecipitation was carried out following the protocols of Ballas et al. (20) and Battaglioli et al. (21) with some modifications.

IMR32 or HeLa cells from a 100-mm dish (~70% confluent) were cross-linked by means of the addition of formaldehyde to a 1% final concentration for 10 min at room temperature; the reaction was stopped by adding glycerine to a final concentration of 0.125 M. The cells were harvested and washed in PBS, and the pellets were resuspended in cell lysis buffer (5 mM HEPES, pH 8, 85 mM KCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The isolated nuclei were resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, 1 mM phenylmethylsulfonyl fluoride) and sonicated to obtain DNA fragments with a length between 200 and 1000 bp. Sonicated chromatin was precleaned with monoclonal anti-chicken IgY-agarose beads (Davids Biotechnologie, Ragensburg, Germany), 0.01 μg/μl chicken pre-immune IgY (Davids Biotechnologie, Ragensburg, Germany), 20 μg/μl tRNA, and 10 μg/μl salmon sperm DNA at 4 °C for 1 h.

The precleared chromatin was incubated overnight at 4 °C with 5 μg of each antibody (anti-hPHOX2A and anti-hPHOX2B antibodies (Davids Biotechnologie, Ragensburg, Germany); anti-Sp1 and anti-ac.H4 (Upstate); and chicken pre-immune IgY (Davids Biotechnologie, Ragensburg, Germany)), and the immunocomplexes were collected on monoclonal anti-chicken IgY-agarose beads pre-adsorbed with 20 μg/μl tRNA and 10 μg/μl salmon sperm DNA. Following washes and elution, cross-linking was reversed by heating to 65 °C overnight, and the samples were purified on columns (High Pure PCR product purification kit, Roche Applied Science).

For the PCR detection of immunoprecipitated chromatin, 5% of the purified DNA was used as a template with the following primers: ChIP[2bprom]UP, 5′-CAAGCTTATTTTCTAAATGGAGATGAGTCA-3′; ChIP[2bprom]LOW, 5′-GCCTCTCTATGAGATGCCTT-GTCTGA-3′, to amplify the PHOX2B promoter; ChIP[2bCDS]UP, 5′-TATGAGATGCAGGAATCAATGATAGGG-3′; ChIP[2bCDS]LOW, 5′-GATATGGAGAAGGTGGCTGGAGTG-3′, to amplify the PHOX2B coding sequence; ChIP[5]UP, 5′-ATATCCTTCACTGGT-CAGCCTTGGAG-3′, and ChIP[5]LOW, 5′-CAGCAGCGGATTACCATCTTTGTC-3′, to amplify the DBH promoter; ChIP[2aprom]UP, 5′-CTCCGTCCGTCGCTCCATCAG-3′, and ChIP[2aprom]LOW, 5′-GATATGGAGAAGGTGGCTGGAGTG-3′, to amplify the PHOX2A promoter; ChIP[α5prom]UP, 5′-CTCCGTCCGTCGCTCCATCAG-3′, and ChIP[α5prom]LOW, 5′-GATATGGAGAAGGTGGCTGGAGTG-3′, to amplify the α5 promoter.

The DNA samples were heated to 95 °C for 2 min, followed by 47 cycles of heating to 95 °C for 30 s, and combined annealing/extension at 55 °C for 45 s.
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FIGURE 1. Structural features of the 5′-flanking region of the human PHOX2B gene. A, schematic representation of the HindIII-Ncol region. The striped box indicates the DNA sequence specifying the 5′-UTR of the mRNA; the black region corresponds to the first part of the coding sequence. The arrows indicate the principal transcription start sites, B, nucleotide sequence of the HindIII-Ncol region. The positions are numbered from the major transcription start site (indicated by the thick arrow), as defined by primer extension analysis. The underlined sequence corresponds to the primer used in the primer extension experiments. The nucleotides in boldface correspond to the putative TATA-box and the start codon. The putative DNA-binding sites for homeodomain transcription factors are boxed and numbered. C, primer extension mapping of the transcription start sites of the human PHOX2B gene. A 32P-labeled oligonucleotide, complementary to the DNA region specifying the 5′ end of the mRNA, was annealed to total RNA purified from the indicated cell lines (lanes 1–3) and then extended. Yeast RNA (lane 4) was used as a negative control. The arrows indicate the transcription start sites. D, Northern blot analysis of the expression of the human PHOX2A and PHOX2B genes in different human cell lines. The labeled PHOX2A and PHOX2B probes were hybridized to 20 μg of total RNA purified from the indicated cell lines. E, functional characterization of the 5′-regulatory region of the human PHOX2B gene. IMR32 and HeLa cells were transiently transfected with the HindIII-Ncol construct (in which the renilla luciferase reporter gene is under the control of the −825/+105 5′-regulatory region of PHOX2B) or the promoterless pHRG-B vector. The luciferase assays were performed 48 h later. The bars represent the relative luciferase activity of the constructs given as a percentage of the activity of the pHRG-B vector. The data are the mean values ± S.E. (error bars, here undetectable) of at least three independent experiments performed in duplicate.

RESULTS

Structural and Functional Characterization of the Human PHOX2B 5′-Regulatory Region—In order to isolate the 5′-flanking region of the PHOX2B gene, we screened a human genomic library with a probe corresponding to the DNA region specifying the 5′-UTR of PHOX2B mRNA. We identified four different clones, one of which (containing 930 bp immediately upstream of the translation start codon) was further characterized (Fig. 1, A and B).

 Primer extension experiments were performed to determine the initiation of transcription of the PHOX2B gene. A major transcription start site was identified in both neuronal cell lines (Fig. 1C, lanes 2 and 3), and as expected, no signal was detectable in HeLa cells and yeast RNAs (Fig. 1C, lanes 1 and 4). Upstream of this site, computer-assisted analysis revealed the presence of a TATA-box consensus sequence in the canonical position of −34 bp (Fig. 1B, in boldface), which is in line with the hypothesis that the region corresponded to the PHOX2B promoter. Two minor transcription start sites were also detected in the SYSY cells at positions −112 and −202 (Fig. 1C, lane 3), whose origin and significance remain to be investigated.

 In order to establish whether the examined genomic PHOX2B region contained relevant regulatory elements capable of driving transcription on the basis of functional criteria, we generated the HindIII-Ncol construct for transfection purposes. To this end, we used Northern blotting to evaluate the expression of PHOX2B mRNA in human neuroblastoma cell lines and found that SYSY and IMR32 cells expressed appreciable amount of the transcript (Fig. 1D, lower panel, lanes 1 and 2). Most surprisingly, no PHOX2B mRNA was detectable in SK-N-BE or SK-N-BE (2C) cells (Fig. 1D, lower panel, lanes 3 and 4). All four neuroblastoma cell lines expressed the PHOX2A transcript (Fig. 1D, upper panel, lanes 1–4), and as expected, the HeLa cells did not show any hybridization signal (Fig. 1D, upper and lower panels, lane 5).

On the basis of these results, the HindIII-Ncol construct was transfected into IMR32 and HeLa cells, and its transcriptional activity was evaluated by means of luciferase assays and compared with that of the promoterless pHRG-B plasmid, which contained the Renilla luciferase reporter gene but no upstream regulatory region. The HindIII-Ncol construct showed as much as 8.5 times the activity of pHRG-B in IMR32 cells, whereas the activity of the two plasmids was similar in HeLa cells (Fig. 1E). These data thus suggested that the −825/+105 PHOX2B region was transcriptionally competent, with an activity profile restricted to neuronal cells.

Characterization of a Polyclonal Antibody Directed against hPHOX2B—In order to follow the expression of hPHOX2B protein, we produced an anti-peptide polyclonal antibody in chicken eggs and verified its specificity by means of various criteria and techniques. We first made a Western blot analysis using nuclear extracts prepared from IMR32 or HeLa cells in parallel with the mouse Phox2b protein obtained by means of in vitro transcription/translation. It is worth noting that the two Phox2b orthologues are virtually identical in their primary sequences, with a unique conservative amino acid substitution outside

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![Graphical representation of PHOX2B Transactivates Its Own Promoter](image)

**FIGURE 2. Anti-human PHOX2B antibody characterization.** A. Western blot analysis. Two microliters of *in vitro* expressed human PHOX2B protein or unprogrammed lysate (lanes 1 and 3) and 15 µg of nuclear extracts (lanes 2 and 4) were separated by means of SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), and probed with the anti-anti-PHOX2B antibody. The arrow indicates the expected and identified major band of ~32 kDa. In lanes 5 and 6, the IMR32 nuclear extract or the *in vitro* expressed human PHOX2A protein was also probed with the anti-PHOX2B antibody preincubated with an excess of the Phox2b peptide used for chicken immunization. B. EMSA. Gel shift assays were carried out using the oligonucleotide homeodomain-binding site, which corresponds to the PHOX2A promoter sequence-binding PHOX2B (11). The arrow on the left indicates a retarded complex that formed after incubation of the labeled probe with the IMR32 nuclear extract (lane 2) or the *in vitro* expressed Phox2b protein (lane 3). The asterisk on the right indicates the supershifted complex obtained by incubating the IMR32 nuclear extract or the *in vitro* expressed Phox2b protein with the anti-peptide anti-PHOX2B antibody (Ab) under examination (lanes 4 and 7, respectively). The triangle on the right indicates a nonspecific complex that forms in the presence of the reticulocyte lysate (lanes 5–8). C. Immunofluorescence. DAOY cells were transfected with PHOX2B/pcDNA3, a vector bearing the human PHOX2B cDNA (panels c–f), or with the empty vector pcDNA3 (panels a and b). The nuclei were visualized by DAPI (panels a, c, and f) and the anti-hPHOX2B antibody (panels b, d, and e). In panel f the antibody was preincubated with an excess of the Phox2b peptide used for chicken immunization.

It has been shown that PHOX2B regulates the expression of PHOX2A by binding to a homeoprotein-binding site in its 5′-regulatory region (11, 22). We used the homeodomain-binding site oligonucleotide corresponding to the PHOX2A promoter sequence that binds PHOX2B (11) in EMSAs in order to verify whether the examined antibody was capable of supershifting complexes containing PHOX2B. Fig. 2B shows that a prominent retarded band formed in the presence of the IMR32 nuclear extract and that its formation was prevented by an excess of cold oligonucleotide (lanes 2 and 3). When the anti-hPHOX2B antibody was added to the reaction, a supershifted complex was detected (Fig. 2B, lane 4). To confirm these data, we also carried out EMSAs using the *in vitro* expressed Phox2b protein; a specific retarded band was observed, which was partially competed by an excess of cold oligonucleotide and supershifted by the anti-hPHOX2B antibody (Fig. 2B, lanes 5–7). The sizes of the retarded and ultra-retarded bands obtained using the nuclear extracts or the *in vitro* expressed protein were similar, but their intensities were quite different, suggesting that additional events (such as post-translational modifications) not occurring in the lysate are required in order to stabilize the PHOX2B interactions with DNA. As expected, no specific band was detectable in the unprogrammed lysate (Fig. 2B, lane 8).

Immunofluorescence was used to confirm that the examined antibody recognized PHOX2B in its native state. DAOY cells, which do not endogenously express the antigen, were transfected with PHOX2B/pcDNA3 and probed with the antibody. Fig. 2C shows the nuclei of transfected DAOY cells stained with DAPI (panel c) or revealed by the anti-hPHOX2B antibody (panel d). The antibody immunostained the nuclei of a few cells, as may be expected in a transient transfection experiment, but did not produce any signal when mock-transfected cells were probed (Fig. 2C, panels a and b). Similarly, no signal was detectable in PHOX2B/pcDNA3-transfected cells if the antibody was previously incubated with an excess of the PHOX2B peptide (Fig. 2C, panels e and f). The anti-hPHOX2B antibody was also tested against the *in vitro* expressed PHOX2A protein by means of Western blotting, which proved that it did not cross-react (data not shown), as was expected on the basis of the high specificity of the Phox2b peptide sequence used to produce antibodies. The *in vitro* expressed Phox2b protein was recognized by the antibody as a prominent band of ~32 kDa, and no signal was detectable by using the unprogrammed lysate (Fig. 2A, lanes 1 and 3). Remarkably, a band of the same size was detected in the IMR32 nuclear extract, and as expected, no band was observed in HeLa cells (Fig. 2A, lanes 2 and 4). In order to corroborate these data, we carried out competition experiments in which an excess of the Phox2b peptide used for immunization was added. As shown in Fig. 2A, the specific PHOX2B band disappeared from both lanes containing the *in vitro* expressed Phox2b protein or the IMR32 nuclear extracts (lanes 5 and 6). A minor band was also observed with the *in vitro* expressed Phox2b protein, which may have been due to the use of one of the potential downstream translational start codons (Fig. 1B) or simply represent a degradation product.

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5 A. Flora, unpublished results.
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Detection of the in Vivo Binding of PHOX2B to Its Own Promoter by Means of ChIP Assay—Sequence analysis of the human PHOX2B promoter revealed a number of putative sites for homeodomain proteins (boxed in Fig. 1B), which also include PHOX2B itself.

As the establishment of auto-regulatory loops is a recognized mechanism by which the expression of certain transcription factors may become independent from external stimuli during development, we wondered whether PHOX2B was capable of regulating the transcription of its own gene. We started to investigate this by ChIP assays. The previously characterized anti-hPHOX2B antibodies were used to immunoprecipitate chromatin from IMR32 cells, and the associated DNA fragments were amplified using primers flanking the five “ATTA” sites contained in the PHOX2B promoter. An expected band of 301 bp was observed, thus suggesting that PHOX2B is associated with its putative DNA sites in chromatin (Fig. 3A, upper panel, lane 2). Bands of the same size were also observed with antibodies directed against the acetylated form of histone H4 (Ac.H4) (Fig. 3A, upper panel, lane 6), a well known marker of transcriptionally active promoters, and Sp1 (Fig. 3A, upper panel, lane 7), a ubiquitous transcription factor involved in PHOX2B expression.4 Remarkably, the antibodies directed against PHOX2A were also capable of immunoprecipitating the same band (Fig. 3A, upper panel, lane 3), thus suggesting that this transcription factor also participates in the transcriptional complex assembled on the PHOX2B promoter; the functional significance of this is currently under investigation. No amplification products were observed using pre-immune IgY immunoglobulins (Fig. 3A, lane 4) or when a 298-bp fragment of the PHOX2B coding sequence was amplified instead of the promoter region (Fig. 3A, lower panel, lanes 2–4 and 6); as expected, the 298-bp fragment only appeared when the chromatin was precipitated with the antibody against the Ac.H4. We also tested the specificity of the obtained bands by using the anti-hPHOX2B, -PHOX2A, and -Sp1 antibodies with chromatin prepared from HeLa cells, which did not express PHOX2B. No signals were detected with these antibodies or the anti-AcH4 antibody (Fig. 3A, upper and middle panels, lanes 2–6).

In order to have a positive control to prove the quality of the HeLa chromatin, we amplified a fragment of the promoter controlling the expression of the α5 nicotinic receptor subunit gene, which is expressed in HeLa cells and has its transcription under the control of Sp1 (11, 23). As expected, a 370-bp band appeared with the antibodies directed against Ac.H4 and Sp1 (Fig. 3B, lower panel, lanes 5 and 6) but not with the pre-immune antibodies (Fig. 3B, lower panel, lane 4).

In Vitro Analysis of the Molecular Interactions between PHOX2B and the ATTA Sites from Its Own Promoter—In order to evaluate the contribution of each putative homeodomain protein DNA site to the binding of PHOX2B to its own promoter, we carried out EMSAs using the nuclear extracts obtained from the IMR32 neuroblastoma cell line or the in vitro expressed Phox2b protein as sources of hPHOX2B. When a labeled oligonucleotide containing ATTA sites 1 and 2 (Fig. 1B) was incubated with the IMR32 nuclear extract, a retarded band was detectable (Fig. 4A, lane 2), which could be competed by a molar excess of cold oligonucleotide and supershifted in the presence of the anti-hPHOX2B antibody (Fig. 4A, lanes 3 and 4). Similarly, the in vitro expressed Phox2b protein was also capable of causing the appearance of a retarded band that was competed by the cold oligonucleotide and supershifted by the antibody (Fig. 4A, lanes 5–7). In order to confirm that the binding of PHOX2B to the oligonucleotide occurred through the ATTA sites as expected, we generated an oligonucleotide bearing two point mutations in each site, which competition experiments showed to be unable to compete (Fig. 4B, compare the lanes 3 and 4). When the mutated oligonucleotide was labeled and incubated with the in vitro expressed Phox2b protein, no band was detectable (Fig. 4C, compare lanes 1–4 with lanes 5–8). In order to establish whether the two sites were equally relevant for the binding of PHOX2B, we generated two oligonucleotides centered around one or the other ATTA site, labeled them, and carried out EMSAs. The oligonucleotide bearing ATTA site 2 was capable of binding PHOX2B as a nuclear extract (Fig. 4E, lanes 1–4) or in vitro expressed protein (Fig. 4E, lanes 5–8), but the oligonucleotide bearing site 1 was completely unable to bind it (Fig. 4D, lanes 1–8).

We next characterized ATTA sites 3 and 4 by using the same approach. A labeled oligonucleotide bearing the two sites was incubated with the IMR32 nuclear extract or the in vitro expressed Phox2b protein and, in both cases, detected an identical retarded band (Fig. 5A, lanes 2 and 6) that was competed by an excess of wild-type cold oligonucleotide (Fig. 5A, lanes 3 and 7) and supershifted if the anti-hPHOX2B antibody was added (Fig. 5A, lanes 4 and 8). In order to confirm that the binding of PHOX2B to the oligonucleotide occurred through the ATTA sites, we carried out EMSAs using an oligonucleotide bearing two point mutations in each of the two sites. Competition experiments showed that this oligonucleotide was unable to compete with its labeled wild-type counterpart (Fig. 5B, compare the lanes 3 and 4). When the mutated oligonucleotide was labeled and incubated with the in vitro expressed Phox2b protein, no band was detectable (Fig. 5C, compare the lanes 1–4 with lanes 5–8). In order to establish whether the two sites were equally relevant for the binding of PHOX2B, we generated two oligonucleotides that were alternatively mutated in sites 3 or 4; the oligonucleotide mutated in site 4 still bound PHOX2B contained in the nuclear extract (Fig. 5D, lanes 1–4) but not as in vitro expressed protein (Fig. 5D, lanes 5–8), whereas the oligonucleotide mutated in site 3 was
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Molecular characterization of the ATTA1 and ATTA2 sites by EMSA. EMSAs were performed using probes containing both (A–C) or only one of the ATTA1 and ATTA2 sites (ATTA1 in D and ATTA2 in E). The arrows indicate the specific retarded complexes that formed after the labeled probes were incubated with the IMR32 nuclear extract or the in vitro expressed PHOX2B protein; the asterisks indicate the supershifted complexes obtained in the presence of the anti-PHOX2B antibody, and the triangles indicate the nonspecific complexes. The competition experiments were performed in the presence of a 1000- to excess of the unlabeled oligonucleotides or their mutated version in which the two ATTA sites were mutagenized. A, the labeled oligonucleotide was incubated alone (lane 1), with the IMR32 nuclear extract (lanes 2–4), or in the vitro expressed Phox2b protein (lanes 5–7). The anti-PHOX2B antibody was added to the samples run in lanes 4 and 7. The competition experiments were carried out using an excess of the wild-type cold oligonucleotide (lanes 3 and 6). B, the labeled oligonucleotide was incubated alone (lane 1), with the IMR32 nuclear extract (lanes 2–5), and in the presence of the anti-PHOX2B antibody (lane 5). The competition experiments were carried out with the wild-type or the mutated oligonucleotide in which both the two ATTA sites had been mutagenized (lanes 3 and 4). C, the wild-type (lanes 1–4) or mutated oligonucleotides (lanes 5–8) were labeled and incubated alone (lanes 1 and 5) or with the in vitro expressed Phox2b protein. The anti-PHOX2B antibody was added to the samples run in lanes 4 and 8. The competition experiments were carried out with the wild-type (lane 3) or mutagenized oligonucleotide (lane 7). D, the labeled oligonucleotide containing only the ATTA1 site was incubated alone (lane 1), with the IMR32 nuclear extract (lanes 2–4), the in vitro expressed Phox2b protein (lanes 5–7), or the unprogrammed lysate (lane 8). Competition experiments (lanes 3 and 6) were carried out with an excess of unlabeled oligonucleotide. The anti-PHOX2B antibody was added to the samples run in lanes 4, 7, and 8.

still capable of binding PHOX2B regardless of its sources, although the in vitro expressed protein seemed to bind with less affinity (Fig. 5E, lanes 1–8). Finally, when a labeled oligonucleotide bearing ATTA site 5 was incubated with the IMR32 nuclear extract, a retarded complex was observed that could be competed by a molar excess of unlabeled oligonucleotide, and supershifted in the presence of the anti-hPHOX2B antibody (Fig. 6A, lanes 1–4); however, once again, the in vitro expressed Phox2b protein did not produce any retarded band (Fig. 6, lanes 5–8). In order to confirm that the binding of PHOX2B to the oligonucleotide nevertheless required the presence of the ATTA, we carried out competition experiments using a mutated oligonucleotide, which was unable to compete with the labeled wild-type counterpart for the formation of the retarded band (Fig. 6B, compare lanes 3 and 4). In brief, in vitro DNA-protein interaction experiments confirmed that the ATTA sites localized in the 5’- regulatory region of the PHOX2B promoter are involved in the binding of PHOX2B to its own promoter, with some exceptions and restrictions; in particular, ATTA site 1 did not bind PHOX2B at all, and sites 3 and 5 were unable to bind the in vitro expressed protein.

PHOX2B Transactivates Its Own Promoter—In order to demonstrate the functional significance of the binding of PHOX2B to its own promoter, we carried out co-transfection experiments using HeLa cells into which the PHOX2B/pcDNA3 effector plasmid or the empty pcDNA3 vector was co-transfected with the wild-type PHOX2B promoter reporter plasmid or its ATTA site-mutated variants (Fig. 7B). The wild-type Phox2b promoter HindIII/NcoI was strongly transactivated by PHOX2B at all, whereas a PHOX2B reporter plasmid in which all ATTA sites were mutagenized only marginally responded to the expression of PHOX2B (Fig. 7B).

In order to evaluate the functional contribution of each site to the transactivation of PHOX2B promoter, we carried out co-transfection experiments using a series of reporter plasmids bearing mutations in a single ATTA site (Fig. 7B) and, remarkably, obtained similar results with
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**FIGURE 5.** Molecular characterization of the ATTA3 and ATTA4 sites by EMSA. EMSAs were performed using probes containing both (A–C) or only one of the ATTA3 and ATTA4 sites (ATTA3 in D and ATTA4 in E). The arrows indicate the specific retarded complexes that formed after the labeled probes were incubated with the IMR32 nuclear extracts or the in vitro expressed Phox2b protein; the asterisks indicate the supershifted complexes obtained in the presence of the anti-PHOX2B antibody, and the triangles indicate the nonspecific complexes. The competition experiments were performed in the presence of a 1000× excess of the unlabeled oligonucleotides or their mutated version in which both ATTA sites were mutagenized. A, the labeled oligonucleotide was incubated alone (lanes 1 and 3), with the IMR32 nuclear extract (lanes 2–4), the in vitro expressed Phox2b protein (lanes 6–8), or the unprogrammed lyssate (lane 9). The anti-PHOX2B antibody was added to the samples run in lanes 4, 8, and 9. The competition experiments were carried out using an excess of the wild-type cold oligonucleotide (lanes 3 and 7, 8), the labeled oligonucleotide was incubated alone (lane 1), or with the IMR32 nuclear extract (lanes 2–5), and in the presence of the anti-PHOX2B antibody (lane 3). The competition experiments were carried out with the wild-type or the mutated oligonucleotide, in which the two ATTA sites had been mutagenized (lanes 3 and 4); C, the wild-type (lanes 1–4) or mutated oligonucleotides in which both the ATTA3 and 4 sites were mutagenized (lanes 5 and 6) or with the in vitro expressed Phox2b protein. The anti-PHOX2B antibody was added to the samples run in lanes 4, 8, and E, the labeled oligonucleotide containing only the ATTA3 site (the ATTA4 site had been mutagenized) was incubated alone (lane 1), or with the IMR32 nuclear extract (lanes 2–5), or the unprogrammed lyssate (lane 6). The competition experiments (lanes 3 and 6) were carried out using an excess of unlabeled wild-type oligonucleotide. The anti-PHOX2B antibody was added to the samples run in lanes 4, 7, and 8. The retarded bands were much less intense than those observed with the sites 2 and 4, although it is possible that the ultra-retarded bands appeared to be more intense than the retarded bands only in the case of sites 3 and 5 as all of the tested mutants; the PHOX2B promoter was transactivated, but the level of transactivation never reached that of the wild-type reporter plasmid.

In order to validate these results in neurons, we transfected IMR32 cells with the same reporter constructs. The plasmid bearing mutations in all of the ATTA sites showed a drastically decreased transcriptional activity (Fig. 7C). However, the constructs bearing mutations in single ATTA sites also showed similar reductions (Fig. 7C), thus confirming and extending our previous observation that the presence of all of the sites was required to ensure self-stimulation of the promoter by PHOX2B.

**DISCUSSION**

We studied the transcriptional mechanisms that govern the expression of the human PHOX2B gene, focusing particularly on the presence of five putative sites for homeodomain proteins that might bind PHOX2B itself, thus providing the molecular evidence that it is capable of auto-regulating its own expression. These sequences are perfectly conserved across species, such as humans, mice, rats, Zebrafish, and fugu (25), thus suggesting that they play an essential role that has been preserved during evolution. Chromatin immunoprecipitation assays showed that PHOX2B can bind to its own promoter in vivo, thus providing robust evidence for the hypothesis of an auto-regulatory mechanism at the transcriptional level, and EMSAs confirmed that the binding of PHOX2B to DNA was mediated by four of the five putative sites (with the exclusion of the ATTA1). EMSAs were carried out by using in parallel the IMR32 nuclear extracts or the in vitro expressed protein as sources of PHOX2B and revealed interesting differences in the binding ability of the four sites. ATTA sites 2 and 4 bound PHOX2B regardless of its source, although the retarded bands obtained using the in vitro expressed protein were less intense than those obtained with the nuclear extracts, despite the amount of PHOX2B protein was more abundant in the former. On the contrary, sites 3 and 5 bound PHOX2B only as a nuclear extract, and the retarded bands were much less intense than those observed with the sites 2 and 4, although it is possible that the in vitro expressed protein bound to these sites but with undetectable affinity. Most interestingly, the ultra-retarded bands appeared to be more intense than the retarded bands only in the case of sites 3 and 5 as...
Molecular characterization of the ATTA5 site by EMSA. EMSAs were performed using a probe containing the ATTA5 site (A and B). The arrows indicate the specific retarded complexes that formed after the labeled probe was incubated with the IMR32 nuclear extract or the in vitro expressed Phox2b protein. The asterisks indicate the supershifted complexes obtained in the presence of the anti-PHOX2B antibody. The competition experiments were performed in the presence of a 1000-fold excess of the unlabeled oligonucleotides or their mutated version in which the ATTA site was mutagenized. A, the labeled oligonucleotide was incubated alone (lanes 1 and 5), or with the IMR32 nuclear extract (lanes 2–4) or the in vitro expressed Phox2b protein (lanes 6–8). The anti-PHOX2B antibody was added to the samples run in lanes 4 and 8. The competition experiments were carried out using an excess of the wild-type cold oligonucleotide (lanes 3 and 7). B, the labeled oligonucleotide was incubated alone (lane 1), or with the IMR32 nuclear extract (lanes 2–5), and in the presence of the anti-PHOX2B antibody (lane 5). The competition experiments were carried out with the wild-type or the mutated oligonucleotide in which the ATTA site had been mutagenized (lanes 3 and 4).

if the anti-PHOX2B antibody stabilized the DNA-protein interactions. Taken together, these findings indicate that the in vitro expressed transcription factor seems to bind to DNA with less affinity than the PHOX2B contained in the nuclear extracts. It has been reported that PHOX2A, a paralogue of PHOX2B, can undergo post-translational modifications, such as phosphorylation, which modify its affinity for DNA (24), and it is possible that the binding of PHOX2B to DNA is also affected by post-translational modifications that cannot occur in in vitro expressed samples. An alternative explanation implies the presence of additional factors in the nuclear extract that may stabilize the binding of PHOX2B to DNA by means of protein-protein interactions.

One general question raised by the EMSA results concerns the functional significance of these sites: are they redundant in such a way that the occupancy of one or two is sufficient to produce the full auto-transactivation of the PHOX2B promoter or do they cooperate and have additive or synergistic effects? Our co-transfection experiments in HeLa cells clearly showed that the mutation of any one site prevents the full transactivation of the promoter, thus suggesting that all of the sites are required. Transfection of the mutated variants of the PHOX2B promoter into the more physiological context of IMR32 cells confirmed and extended these observations: ~65% of the transcriptional activity of the PHOX2B promoter in neuroblastoma cells depends on self-transactivation (as indicated by the construct mutated in all four ATTA sites), whereas the remaining 35% may be attributable to other transcription factors. The mutation of just one of the ATTA sites substantially prevents the self-transactivation of the PHOX2B promoter, thus suggesting that the sites work synergistically and may establish a sort of threshold mechanism by which self-transactivation can occur only when all four sites are occupied. It is well known that one mechanism by which transcription factors can remain expressed is through a positive feedback loop that relies on transcriptional auto-regulation (1). This process is extremely important during development because it allows progenitors and precursors to acquire independence from extrinsic signals, whose production may be extinguished or become ineffective once the cells have migrated far enough away from their sources. PHOX2B is a well-established intrinsic factor in developing autonomic ganglia where its expression is triggered by the BMPs secreted by the dorsal aorta (for review see Ref. 4). To place our findings in a physiological perspective, we hypothesize that the PHOX2B protein accumulates during the development of sympathetic neurons in response to BMPs, and it reaches a defined nuclear concentration that allows it to occupy all of its cognate sites on its own promoter and autonomously sustains its own expression. One of the possible consequences is that this mechanism would make PHOX2B, whose expression persists into adulthood in

FIGURE 6. Molecular characterization of the ATTA5 site by EMSA. EMSAs were performed using a probe containing the ATTA5 site (A and B). The arrows indicate the specific retarded complexes that formed after the labeled probe was incubated with the IMR32 nuclear extract or the in vitro expressed Phox2b protein. The asterisks indicate the supershifted complexes obtained in the presence of the anti-PHOX2B antibody. The competition experiments were performed in the presence of a 1000-fold excess of the unlabeled oligonucleotides or their mutated version in which the ATTA site had been mutagenized. A, the labeled oligonucleotide was incubated alone (lanes 1 and 5), or with the IMR32 nuclear extract (lanes 2–4) or the in vitro expressed Phox2b protein (lanes 6–8). The anti-PHOX2B antibody was added to the samples run in lanes 4 and 8. The competition experiments were carried out using an excess of the wild-type cold oligonucleotide (lanes 3 and 7). B, the labeled oligonucleotide was incubated alone (lane 1), or with the IMR32 nuclear extract (lanes 2–5), and in the presence of the anti-PHOX2B antibody (lane 5). The competition experiments were carried out with the wild-type or the mutated oligonucleotide in which the ATTA site had been mutagenized (lanes 3 and 4).

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FIGURE 7. PHOX2B transactivates its own promoter. A, schematic representation of the reporter constructs used in the transfection experiments. The arrow indicates the major transcription start site. The gray oval represents the Renilla luciferase reporter gene; the dots indicate the mutated ATTA sites. B, luciferase assays were performed to measure the activity of the PHOX2B promoter reporter constructs after their cotransfection in HeLa cells with the expression vector PHOX2B/pcDNA3 that harbors PHOX2B cDNA (black bars), or with the empty vector pcDNA3 (gray bars). The bars represent the transcriptional activity of the constructs given as a percentage of the activity of the constructs when cotransfected with the empty pcDNA3. The data are the mean values ± S.E. (error bars) of at least three independent experiments performed in duplicate. C, luciferase assays. IMR32 cells were transiently transfected with the indicated constructs. The bars represent the transcriptional activity of the constructs given as a percentage of the activity of the wild-type construct HindIII/NcoI. The data are the mean values ± S.E. (error bars) of at least three independent experiments performed in duplicate.
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human autonomic ganglia, independent of BMPs, whose expression in adult dorsal aorta is likely to be extinguished.

A molecular characterization of the human PHOX2B promoter has been published by Hong et al. (26), who found that PHOX2B modestly transactivates its own promoter and that their P2/H3 site (corresponding to our ATTA 2 site) actually binds PHOX2B. However, they did not provide any mutation analysis of this or other sites, and so no information is available concerning the role of PHOX2B in the activity of its own promoter in the neural model used by Hong et al. (26).

PHOX2B is also precociously expressed in a few areas of the developing central nervous system, such as the ventral hindbrain in which it is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is a direct target of Hoxb1 and Hoxb2, involving Pbx and Prep/Meis proteins as co-factors (25). The 376-bp proximal PHOX2B regulatory sequence actually contains separate Pbx-Hox (PH) and Prep/Meis (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1, Pb1a, and Prep-1 and that, in cooperation with Nkx (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1 and Hoxb2, involving Pbx and Prep/Meis proteins as co-factors (25). The 376-bp proximal PHOX2B regulatory sequence actually contains separate Pbx-Hox (PH) and Prep/Meis (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1, Pb1a, and Prep-1 and that, in cooperation with Nkx (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1 and Hoxb2, involving Pbx and Prep/Meis proteins as co-factors (25). The 376-bp proximal PHOX2B regulatory sequence actually contains separate Pbx-Hox (PH) and Prep/Meis (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1, Pb1a, and Prep-1 and that, in cooperation with Nkx (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1 and Hoxb2, involving Pbx and Prep/Meis proteins as co-factors (25). The 376-bp proximal PHOX2B regulatory sequence actually contains separate Pbx-Hox (PH) and Prep/Meis (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1, Pb1a, and Prep-1 and that, in cooperation with Nkx (P/M)-binding sites that promote the formation of a ternary complex containing Hoxb1 and Hoxb2, involving Pbx and Prep/Meis proteins as co-factors (25).

Mutations in the PHOX2B gene have been implicated in various human diseases related to the development of the neural crest, including neuroblastomas, Hirschsprung disease, and congenital central hypoventilation syndrome (CCHS). CCHS or “Ondine’s curse” is an autosomal dominant disease that is characterized by a lack of adequate autonomic breathing control especially during sleep and decreased sensitivity to hypoxia and hypercapnia (for review see Ref. 29). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28). It has been shown recently that, in this neural district, PHOX2B is already present in the dividing progenitors of the neuroepithelium and controls the generation of branchial and visceral motoneurons (27, 28).

In conclusion, we have provided molecular evidence of an auto-regulatory mechanism that allows PHOX2B to control and sustain its own expression. We believe that these findings open up new scenarios concerning the epistatic cascades governing the specification of the different neural structures in which PHOX2B is involved and that they may provide new insights into the pathogenetic mechanisms of human PHOX2B-related diseases.