The *TLX2* homeobox gene is a transcriptional target of PHOX2B in neural-crest-derived cells

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The *TLX2* (*HOX11L1*, *Ncx*, *Enx*) and *PHOX2B* genes encode transcription factors crucial in the development of neural-crest-derived cells, leading to ANS (autonomic nervous system) specific neuronal lineages. Moreover, they share a similar expression pattern and are both involved in downstream steps of BMP (bone morphogenetic protein) signalling. In an attempt to reconstruct the gene network sustaining the correct development of the ANS, we have undertaken an *in vitro* experimental strategy to identify direct upstream regulators of the *TLX2* gene. After characterizing a sequence displaying enhancer property in its 5′ flanking region, we confirmed the functional link between the human *PHOX2B* and *TLX2* genes. Transient transfections and electrophoretic-mobility-shift assays suggested that PHOX2B is able to bind the cell-specific element in the 5′ regulatory region of the *TLX2* gene, determining its transactivation in neuroblastoma cells. Such interaction was also confirmed *in vivo* by means of chromatin immunoprecipitation assay and, in addition, up-regulation of endogenous *TLX2* mRNA level was demonstrated following PHOX2B over-expression, by quantitative real-time PCR. Finally, PHOX2B proteins carrying mutations responsible for CCHS (congenital central hypoventilation syndrome) development showed a severe impairment in activating *TLX2* expression, both *in vitro* and *in vivo*. Taken together, these results support the PHOX2B–*TLX2* promoter interaction, suggesting a physiological role in the transcription-factor cascade underlying the differentiation of neuronal lineages of the ANS during human embryogenesis.

Key words: autonomic nervous system (ANS), congenital central hypoventilation syndrome (CCHS) mutations, neural-crest development, PHOX2B, *TLX2* promoter.

INTRODUCTION

TLX2, also known as *Ncx* [1,2], *Enx* [3] or *HOX11L1* [4], belongs to a family of orphan homeobox genes, including two other members, *TLX1* and *TLX3,* and encodes a protein of 285 amino acids which is specifically expressed in several tissues derived from neural-crest cells at the end of their migration. In particular, *Tlx2* mRNA has been detected in dorsal-root ganglia, cranial (V, VII, IX and X) and enteric-nerve ganglia, parasympathetic ganglia of the heart and adrenal glands in mouse embryos between E9.5 (embryonic day 9.5) and E13.5, persisting in adrenal glands, intestine and heart in adult mice [2,3]. Induction of the *Tlx2* gene expression by BMP (bone morphogenetic protein) or retinoic acid treatment has been reported and a sequence putatively responsible for tissue specificity identified; however, no specific molecular mechanisms underlying such regulatory processes have been proposed so far [5,6]. More recently, *cis*-acting regulatory elements in the 3.3 kb genomic region located 5′ to the *Tlx2* transcription start site were confirmed to be sufficient to guarantee site-specific expression of this gene in mice [7]. Following the observation of *Tlx2*-null mice showing embryonal lethality at the gastrulation stage, this homeobox gene has been proposed as a mediator of BMP signalling during primitive streak and mesoderm development [5]. However, two other animal models have been described, both exhibiting small-intestinal hypoganglionosis, megacaecum, and hyperplasia of myenteric and submucosal neurons [2,3]. This prompted the authors to refer to these mice as models for a human congenital disorder termed INDB {intestinal neuronal dysplasia type B [OMIM (Online Mendelian Inheritance in Man) registry number 601223]}, an orphan pathological phenotype with controversial clinical significance [8]. An additional, the *Tlx2*-null mouse has been reported to exhibit signs of pseudo-obstruction and intestinal hyperganglionosis [7]. Nevertheless, no mutation affecting the coding region and 2 kb of 5′-flanking region has been found in a set of patients selected on the basis of clinical diagnosis of IND or chronic idiopathic intestinal pseudoobstruction [9,10].

The *PHOX2B* gene plays a pivotal role in ANS (autonomic nervous system) development, encoding a homoeoprotein which is expressed by both sympathetic and parasympathetic neurons [11]. The *PHOX2B* transcript has been detected in the developing hindbrain and peripheral nervous system, as well as in all noradrenergic centres and in specific neuronal groups. Accordingly, *Phox2b*$^{-/-}$ mice show a lack of intestinal innervation and all central and peripheral neurons that express noradrenergic traits [12]. Finally, *PHOX2B* in-frame duplications within the 20-alanine stretch of exon 3, leading to expansions from +5 to +13 alanine residues, and *PHOX2B* frameshift mutations, leading to aberrant C-terminal regions, have been detected in patients affected with CCHS (congenital central hypoventilation syndrome), a disorder characterized by failure of autonomic respiratory control due to abnormal ventilatory response to hypoxia and

Abbreviations used: ANS, autonomic nervous system; BMP, bone morphogenetic protein; CCHS, congenital central hypoventilation syndrome; ChIP, chromatin immunoprecipitation assay; E9.5 (etc.), embryonic day 9.5 (etc.); EMSA, electrophoretic-mobility-shift assay; HBS, homeodomain-binding sequence; HSCR, Hirschsprung disease; IND, intestinal neuronal dysplasia; NE, nuclear extract; RARE, retinoic acid response element; RT-PCR, reverse-transcription–PCR; SV40, simian virus 40.

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hypercapnia, often associated with defects of the enteric innervation, leading to either HSCR (Hirschsprung disease) or severe constipation [13,14].

In an attempt to clarify the transcription-factor cascade underlying the differentiation of neuronal lineages arising from neural-crest cells, we report in the present paper a detailed analysis of the regulatory elements in the 5′-flanking region of the human *TLX2* gene. In particular, we have identified a cell-specific region whose activity relies on the presence of 'ATTA' repeats, known to be recognized by homeodomain transcription factors. On the basis of similarities between *TLX2* and *PHOX2B* with respect to expression pattern and involvement in differentiation of neural-crest derivatives, we have tested the hypothesis of a possible PHOX2B–*TLX2* promoter interaction, thus demonstrating that PHOX2B acts as *TLX2* transcriptional activator by binding a distal cell-specific sequence with an enhancer property.

EXPERIMENTAL

Reporter-gene constructs

Cloning of 1849 bp upstream of the ATG (+1) of *TLX2* isoform 1, between nucleotides −1885 and −37, has already been reported [10]. Progressive deletions of the promoter sequence were obtained by the use of both proper restriction enzymes and exonuclease III digestion (Erase-a-Base System; Promega).

Mutated constructs were generated by PCR, starting from the 1849-bp wild-type construct, as previously described [15]. Oligonucleotides used for site-directed mutagenesis were: 5′-GGGGAAGGTA**CATGC**ATTCCGGCCC-3′ (−1472/−1497) for the HBSs (homeodomain-binding sequences); 5′-GCCACCACG**GA**TCAGAGGTA-3′ (−1647/−1621) for the E-box. Mutated nucleotides are shown in bold.

Assay for the promoter activity

Each cell line was transfected by means of the polyethylenimine method as already described [10]. *Renilla* luciferase reporter plasmid pRL-CMV was used as a transfection efficiency control, while the promoterless pGL3-basic vector and the pGL3-control vector, containing an SV40 (simian virus 40) promoter and enhancer, were used as negative and positive controls respectively. Luciferase activities in cell lysates were determined using Dual Luciferase Reporter Assay System (Promega). Values reported in each Figure represent mean results for duplicate experiments involving at least three independent transfections, and the error bars indicate the S.D. values. The expression plasmid containing the murine *Phox2B* cDNA was obtained from Dr Christos Goridis (Developpement et Evolution du Systeme Nerveux, UMR 8542 CNRS, Ecole Normale Superieure, Paris, France). Human and murine PHOX2B protein sequences are identical. Human wild-type and mutated *PHOX2B* cDNAs were obtained as already reported [16].

EMSA (electrophoretic-mobility-shift assay)

NE (nuclear extract) was prepared as previously described [17] from IMR32 cells. Double-stranded oligonucleotides were end-labelled with [γ-^{32}P]ATP and purified on a Sephadex G-25 column (Roche).

A 6 μg portion of NE was incubated with the radiolabelled probe for 20 min at room temperature in binding buffer [20 mM Hepes, pH 7.9, 20 % (v/v) glycerol, 1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol and 0.5 mM PMSF] with 2 μg of poly(dI-dC).

For competition binding assays, unlabelled double-strand oligonucleotides were added to the reaction mixture in molar excess before the probe. For supershift assays, antibodies were incubated with the NE mix for 30 min on ice before adding probe HBS (−1495/−1471) 5′-GGGGAAGGTAATGTAATTCCGGCCC-3′, whose mutated version is reported above. A polyclonal antibody against a peptide corresponding to sequence in the C-terminus of PHOX2B was produced in chicken egg yolk. Its specificity has been tested by *in vitro PHOX2B* transcription/translation [18].

ChIP (chromatin immunoprecipitation assay)

ChIP was performed as recently described using IMR32 cells [18].

A genomic sequence of interest, from −1724 to −1283 of Figure 1, was amplified by PCR using primer 5′-CGGGAACC-AGCAGGATGGAG-3′ with primer 5′-GAGAAGGGAGGTGG-GGAAAGAC-3′.

Analysis of endogenous *TLX2* mRNA

Expression of *PHOX2B* and *TLX2* genes in the selected cell lines was analysed by RT-PCR (reverse-transcription–PCR) after RNA isolation performed using TRYZOL. Specific primers for *PHOX2B* (5′-GGCTGAGCCATCCAGAACCT-3′ with 5′-GTC-CGTGAAGAGTTTGTAAG-3′) and for *TLX2* (5′-GGTTCT-CCTCGGCCCAGA-3′ with 5′-GCCGATCGGACGGGCGT-3′) were used.

The PHOX2B-induced endogenous levels of *TLX2* mRNA were assessed in SK-N-BE neuroblastoma cells (700 000 cells/60 mm-diameter Petri dish) transfected by means of FuGENE6™ (Roche) with 4 μg of wild-type or mutated *PHOX2B* cDNA expression constructs. After 48 h, total RNA was extracted using both Tryzol (Gibco–BRL) and RNeasy Mini Kit (Qiagen) and following the manufacturer's instructions. DNase (Qiagen) treatment was also performed. A 500 ng portion of spectrophotometer-quantified RNA from each sample was retro-transcribed using Advantage RT-for-PCR kit (BD-Clontech). Real-time quantitative PCR was carried out using inventoried Assays-on-Demand™ provided by Applied Biosystems (Hs.00818124_g1 for *TLX2* and Hs.99999907_m1 for for the gene encoding β_2-microglobulin, used as endogenous reference). PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). After ascertaining that *TLX2* and the gene encoding β_2-microglobulin have approximately equal amplification efficiencies, changes in mRNA amount of *TLX2* were quantified by using the comparative CT Method (Sequence Detection System Chemistry Guide, Applied Biosystems). Real-time PCR amplification was performed in triplicate and repeated three times.

Statistical analysis

Statistical association between the length of polyalanine-expanded tracts in *PHOX2B* mutant constructs and the target *TLX2* promoter activity (independent variable) was determined by the Spearman rank test for correlation.

A permutation–randomization test was used to assay differences between the means between the wild-type and each frameshift mutation under analysis (http://www.bioss.ac.uk/smart).

RESULTS

Characterization of the 5′-flanking region of the human *TLX2* gene

Starting from the indication of two regions highly conserved between human and mouse (−1517/−1187 and −801/−638 in human) [6], we used a construct, containing 1849 bp of the

Figure 1 Sequence of the 1849 bp construct cloned from the 5′-flanking region of the human *TLX2* gene

Some of the main putative binding sites for transcription factors are boxed. The four sequences included in the deletion constructs (see also Figure 2) can be deduced here from the marked (del) corresponding distal starting points (see arrows). The proximal limit of the four deletions is in each case at nucleotide −37. Positions are numbered from the ATG (+1), corresponding to the first codon of the gene. The start site deposited in GeneBank® (NM_016170) is indicated. Regions already reported as highly conserved between human and mouse [6] are boxed.

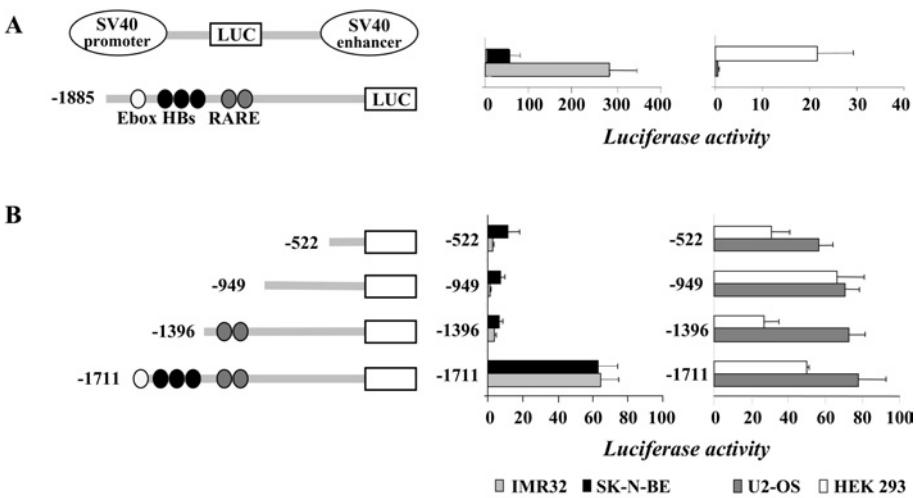

Figure 2 Functional characterization of the 5′-flanking region of the human *TLX2* gene

(**A**) Luciferase activity encoded by the 1849-bp-long construct is reported as percentage of that of the pGL3-control vector (100 %). Both constructs are shown on the left. Scale on the left graph: 0–400 %; scale on the right graph: 0–40 %. (**B**) Luciferase activity displayed by the deletion constructs, reported as percentage of the activity of the 1849-bp-long sequence (100 %). Each construct is schematically represented at the left of the corresponding graph lanes. In each case, reported activity was significantly higher than that of pGL3 promoterless basic vector, transfected as a negative control. Reported values represent means for duplicate experiments from at least three independent transfections. Error bars indicate the S.D. values.

5′-flanking region of the human *TLX2* gene (−1885/−36) upstream of the reporter firefly-luciferase gene [10], to study the *TLX2* promoter activity by cellular transient transfection assays. Four deleted constructs were obtained by removing DNA fragments of increasing lengths at the distal end of the region under analysis (Figures 1 and 2). In particular, the first deletion (−1711) did not remove either of the conserved regions, the second deletion (−1396) interrupted the distal conserved region, maintaining the RARE (retinoic acid response element) previously described in the murine promoter [6], the third deletion (−949) completely eliminated the distal conserved region and the fourth deletion (−522) erased the proximal conserved region, leaving only 200 bp upstream of the transcription initiation site.

For subsequent *in vitro* experiments, we selected two neuroblastoma cell lines, SK-N-BE and IMR32, which are derived from the neural crest and which express the *TLX2* gene, and two non-neural cell lines, HEK-293 and U-2OS. As shown in Figure 2(A), after transfecting the largest (−1885) reporter construct, the

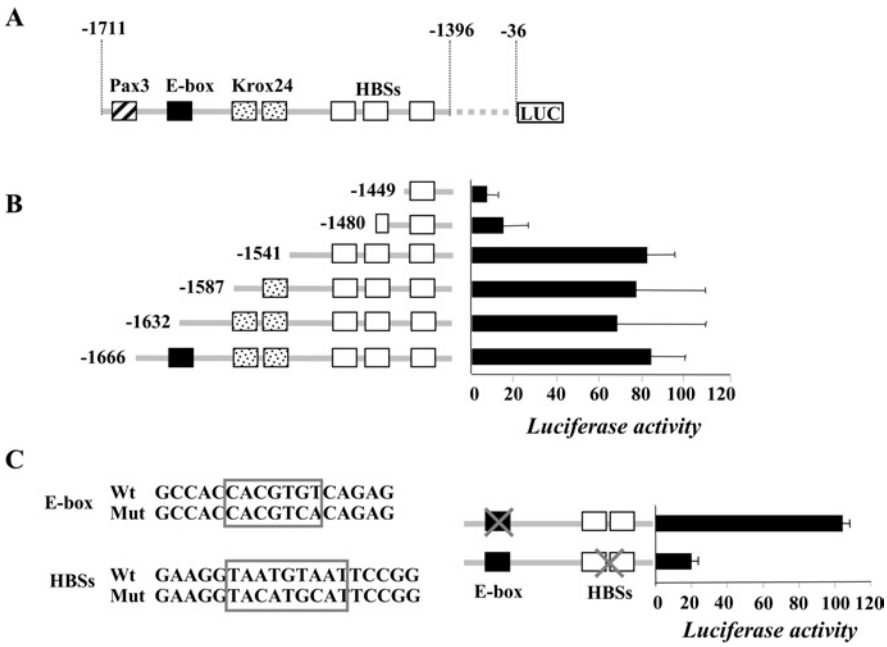

Figure 3 Functional characterization of the cell-specific sequence (−1711/−1396)

(**A**) The reporter construct containing the region of interest (−1711/−1396) is schematically shown, together with the candidate and relevant functional elements. (**B**) Deletion constructs under analysis are schematically represented on the left of the corresponding histogram bars. In each case the proximal limit is at −36 nucleotide. (**C**) The nucleotide substitutions, obtained by site-directed mutagenesis performed in the 1849-bp-long construct and specifically impairing the candidate functional elements, are reported on the left. Values of luciferase activity after transfection in SK-N-BE cells are reported in the (**B**) and (**C**) right-hand histograms as percentage of the activity of that (100%) of the 1849-bp-long sequence and represent the means for duplicate experiments in at least three independent assays. Error bars indicate S.D. values.

former cells displayed significantly higher promoter activities, namely 293 ± 65% in IMR32 and 54 ± 26% in SK-N-BE relative to the activity of a construct containing the SV40 promoter and enhancer. Under the same conditions, the HEK-293 cell line, not expressing *TLX2*, revealed easily-detectable levels of the 1849-bp-long promoter activity (21 ± 7%), whereas the osteosarcoma cell line U-2OS, though expressing the gene, did show a very low activity of the same promoter (0.6 ± 0.2%). In each case, endogenous expression of the gene was tested by RT-PCR (results not shown).

Transient transfections of constructs containing fragments of various lengths of the promoter fused to the firefly-luciferase gene showed a remarkable activity reduction associated with the removal of the region between −1711 and −1396 in neuroblastoma cells (Figure 2B). By contrast, non-neural cells did not display significant changes in promoter activity, irrespective of the length of the deletion. The −1711/−1396 region is therefore a candidate to contain a cell-type-specific sequence able to enhance the transcriptional activity. Since this segment does not include the above-mentioned RARE, an active role of this latter element in determining the cell-specific expression of *TLX2* can be excluded.

Functional analysis of the cell-specific sequence

A MatInspector analysis [19] showed that the cell-specific region identified between −1711 and −1396 presents putative binding sites for different transcription factors such as PAX3 and KROX24, a canonical E-box and three tandem 'TAAT/ATTA' repeats, this latter being known to bind HBSs (Figure 3A). To identify the functional elements, we performed serial deletions of the −1711/−1396 segment in the −1711 construct using the Erase-a-Base system, and the constructs thus obtained were transfected in the SK-N-BE cell line. A strong reduction in the luciferase activity was determined by the deletion of the region located between −1541 and −1480, which includes two tandem HBSs (Figure 3B). As shown in Figure 3(C), site-directed mutation of HBSs (−1489/−1480) severely affected the activity of the 5′-flanking region. On the other hand, as expected, the E-box mutation did not affect the promoter activity.

Transactivation of the human *TLX2* gene by PHOX2B

To test whether the homeodomain protein PHOX2B could be the HBS binding cell-specific factor able to effectively transactivate *TLX2*, we performed co-transfection assays in SK-N-BE cells, a line selected on the basis of its neural origin and lack of *PHOX2B* expression (Figure 4A), thus being suitable to detect even slight effects of PHOX2B activity. Co-transfection of a *PHOX2B* construct increased reporter gene expression driven by the 1849-bp-long *TLX2* promoter construct by approx. 3-fold, in comparison with the empty pcDNA3.1 expression vector (Student's *t* test; $P < 0.05$) (Figure 4B). Accordingly, specific mutations at the HBSs prevented such a PHOX2B-mediated transactivation (Figure 4B). In addition, when we co-transfected the *PHOX2B* expression construct together with the −1541 and −1480 deletion constructs, bearing and lacking the two distal HBSs respectively, transactivation was observed only in the former case (results not shown). The relative PHOX2B-mediated induction of the reporter gene transcription observed after co-transfection was confirmed also in HEK-293 cells, though PHOX2B transactivation levels were much less than those observed in neuroblastoma cells (results not shown).

To address the question of whether PHOX2B activates luciferase-gene transcription by directly interacting with the HBSs identified at −1489/−1480, we investigated DNA–protein interactions at the putative binding site by EMSA. When a labelled

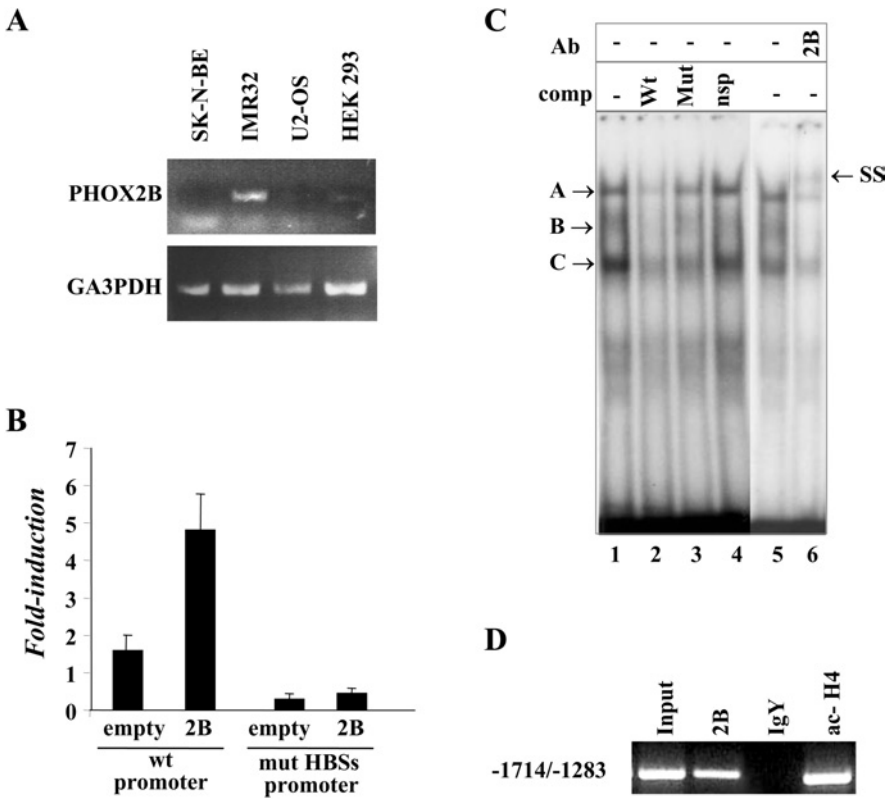

Figure 4 Transactivation of the human *TLX2* gene by PHOX2B

(**A**) Expression of *PHOX2B* in the cell lines selected for the study, as obtained by RT-PCR. (**B**) Luciferase activity displayed by either wild-type or HBS mutant 1849-bp-long reporter construct measured on lysates from SK-N-BE cells co-transfected with either the empty pcDNA 3.1 and pcDNA 3.1-PHOX2B expression constructs. Values represent fold induction versus activity derived from transfection of wild-type 1849-bp-long construct alone and were obtained in three indipendent experiments performed in duplicate. (**C**) EMSA was performed using NE from IMR32 cells and a probe containing the HBSs (−1489/−1480). Competition tests were performed in presence of 100-fold molar excess of unlabelled oligonucleotides. Wt, wild-type competitor; Mut, mutated competitor; nsp, non-specific competitor; 2B, α-PHOX2B; SS, supershift. (**D**) Formaldehyde-cross-linked and sonicated chromatin from IMR32 cells was incubated with α-PHOX2B (lane 2), normal chicken IgY (lane 3) or α-acetyl-H4 (lane 4). Immunoprecipitated DNA was analysed by PCR of the region −1714/−1283 of the *TLX2* promoter. 'Input' represents 1 % of the total chromatin extract.

oligonucleotide corresponding to the two HBSs was incubated with PHOX2B-expressing IMR32 NE (Figure 4A), three retarded bands labelled as 'A', 'B' and 'C' in Figure 4(C) were observed (lanes 1 and 5). These bands were competed for by a molar excess of unlabelled oligonucleotide (lane 2), whereas a mutant unlabelled oligonucleotide and a non-specific competitor were unable to release the proteic complex from our labelled DNA probe (lanes 3 and 4). The pre-incubation of IMR32 NE with specific polyclonal antibody α-PHOX2B induced the supershifted band 'SS', thus demonstrating direct binding of PHOX2B at the probe (lane 6). Finally, this interaction was attested *in vivo* through ChIP. As shown in Figure 4(D), a genomic sequence containing the HBSs under analysis was selectively amplified by PCR from chromatin precipitated by anti-PHOX2B antisera, but not from chromatin treated with normal chicken IgY. The same sequence was also amplified from samples precipitated by α-acetyl-histone H4, thus confirming that this region is transcriptionally active in neuroblastoma cells.

Effect of CCHS-associated *PHOX2B* mutations on *TLX2* transactivation

To determine whether the PHOX2B interaction is physiologically relevant for *TLX2* expression in humans, we have taken advantage of a recently developed collection of mutated versions of *PHOX2B* carrying CCHS-associated defects and already demonstrated to impair activation of two other downstream promoter targets of PHOX2B, namely *DBH* and *PHOX2A* [16]. Constructs carrying expanded polyalanine regions decreased the *TLX2* promoter activity by 20 % on average, with respect to the wild-type *PHOX2B* expression plasmid (100 %). No correlation ($r = 0.542$; $P = 0.209$) between reduction of luciferase activity and increasing length of the co-transfected polyalanine tracts could be demonstrated, as shown in Figures 5(A) and 5(B). On the other hand, frameshift mutations of *PHOX2B* (c.614-618delC, c.930insG, c.862-866insG and c.721-758del38nt) induced a marked decrease in *TLX2* promoter activity, namely 40–60 % with respect to the wild-type PHOX2B (Figure 5C), an effect similar to that already demonstrated on the *DBH* promoter, another PHOX2B target gene [16].

Moreover, overexpression of wild-type PHOX2B gave a three-fold increase in the endogenous *TLX2* mRNA level, as quantified by real-time PCR, in comparison with the basal level in mock-transfected SK-N-BE cells. On the other hand, and under the same conditions, each of the four PHOX2B mutant versions failed to induce a significant change in endogenous *TLX2* expression (Figure 6).

DISCUSSION

Several different mouse models, and the specific pattern of expression during embryogenesis, have suggested the *TLX2* gene

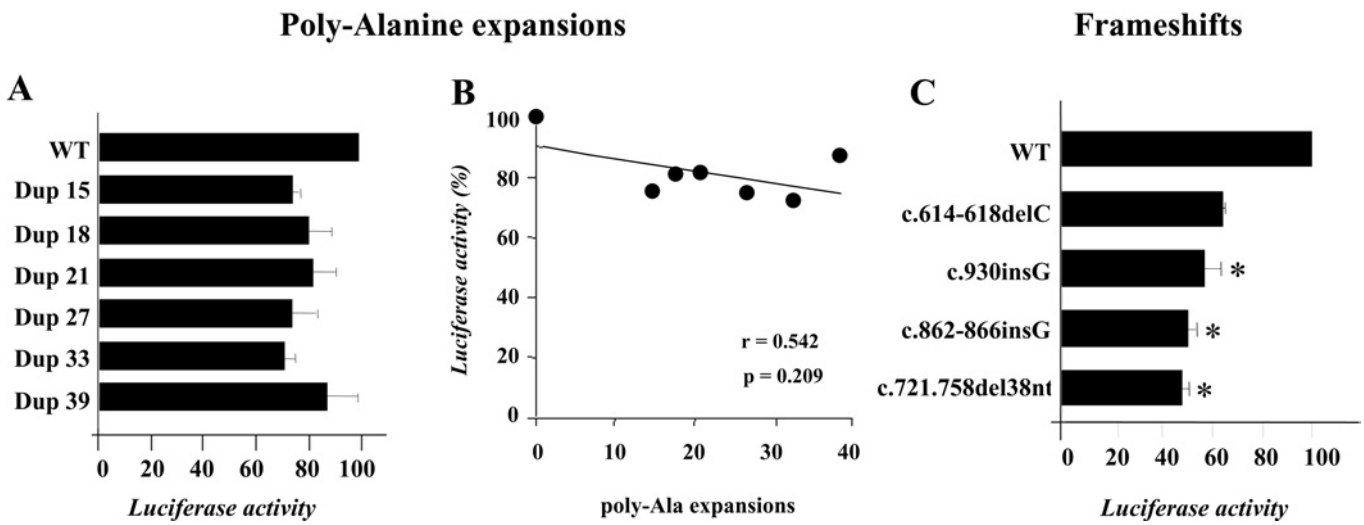

Figure 5 Co-transfection of mutant *PHOX2B* constructs with the 1849-bp-long sequence from the *TLX2* 5′-flanking region

Luciferase activity obtained by co-transfection of the *TLX2* promoter construct with *PHOX2B* expression constructs carrying polyalanine-expanded tracts (indicated as 'Dup' followed by the number of nucleotides involved in the duplication) (**A**) and frameshift mutations (**C**). Mean values of luciferase activation induced by each *PHOX2B* mutation [16] under analysis, and obtained from two independent experiments performed in duplicate, are reported as percentage of that induced by the wild-type (WT) construct. (**B**) In the case of polyalanine expansions, a regression line has been traced and the significance of the correlation thus obtained tested. Significant differences ($P < 0.05$) in the means between the wild-type and specific frameshift mutations, tested by permutation–randomization analysis, are reported with asterisks in (**C**).

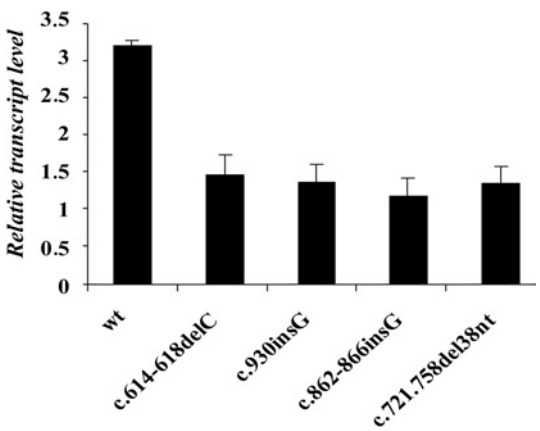

Figure 6 Amount of *TLX2* mRNA following over-expression of wild-type or mutant PHOX2B [16] in SK-N-BE cells

The amount of *TLX2* mRNA is calculated in comparison with the level of β_2-microglubulin mRNA, used as standard reference, by means of the comparative CT Method. Bars represent the mean values obtained in three independent real-time quantitative PCR experiments, each performed in triplicate. Error bars indicate S.D. values.

as a key player in the differentiation of neural-crest progenitors to autonomic neurons [1,3,5,7]. Such a developmental step is likely to involve a number of different pathways and factors which are presently either unknown or known, like TLX2, but not yet recognized as interconnected. Clarification of the underlying gene network(s) will allow us to understand correct ANS development, single gene contributions and their involvement in human pathology. Since the transcriptional cascade in which TLX2 is involved has still to be disclosed, and none of its direct regulators and target genes have been identified so far, we have commenced a study focused on the regulation of *TLX2* expression.

Cell-transfection assays have allowed us to characterize a sequence in the *TLX2* 5′-flanking region that displays a cell-specific property. The homeoprotein PHOX2B binds two tandem 'TAAT/ATTA' repeats in this sequence, determining a significant enhancement of the endogenous level of *TLX2* mRNA in neuroblastoma cells.

A previous study [6] had already defined a region, but no binding factors, upstream of the tandem TAAT/ATTA, as a regulatory sequence governing the tissue-specific expression of the murine *Tlx2* gene. The authors concluded that additional specific nuclear factors were required to control lineage-restricted expression of the *Tlx2* gene in neural-crest-derived cells. In the present work we have identified *PHOX2B* as one of these factors. Previous observations on PHOX2B and its paralogue, PHOX2A, binding identical HBSs [20] and increasing the dopamine β-hydroxylase promoter activity with comparable efficiency [21,22], can explain the detection in our present transfection assays of a cell-specific activity in both IMR32 cells, which express PHOX2B and PHOX2A, and in SK-N-BE cells, which express PHOX2A but not PHOX2B (Figure 2). Consequently, PHOX2A might represent an additional regulator transactivating the *TLX2* gene – a hypothesis deserving further investigation.

Following the observation that *Phox2b*$^{-/-}$ mice lack the entire ANS [12], this transcription factor has been considered an early gene in the differentiating nervous system, expressed after *SOX10* in the precursor cells derived from the neural crest and thus common to sympathetic (noradrenergic) and parasympathetic (cholinergic) innervations [23]. The observation of bowel dismotility in *Tlx2*$^{-/-}$ mice, accompained by increased cholinergic innervation [24], suggests the lack of noradrenergic neurons, thus supporting the hypothesis that *TLX2* expression is required for sympathetic development. Although BMPs represent the earliest signal inducing a transcription-factor cascade required for the differentiation of neural-crest cells to autonomic neurons [23], we propose that *TLX2* is one of the *PHOX2B* effectors in this BMP signalling, leading to final noradrenergic specification.

The functional significance of PHOX2B-induced *TLX2* expression is confirmed by the analysis of mutant versions of *PHOX2B* detected in patients affected with CCHS, a human

disorder characterized by severely impaired autonomic respiration, often associated with defects of enteric innervation, leading to either HSCR or severe constipation [13,14]. In contrast with what has already been reported for different target gene promoters, showing a severe polyalanine-length-dependent impairment of PHOX2B-induced reporter-gene activation [16], the weak effect of the polyalanine mutant PHOX2B on the *TLX2* promoter observed here suggests that the polyalanine domain is not crucial for *TLX2* activation. On the other hand, co-transfections of *PHOX2B* constructs carrying frameshift mutations with the *TLX2* regulatory regions resulted in a dramatic decrease in luciferase activity and in a lack of a significant enhancement of endogenous *TLX2* mRNA. The different frameshift mutations produced an altered C-terminal region of PHOX2B that could modify the folding of the protein, leading to either defective binding to the *TLX2* regulatory region identified by us or altered binding to factors co-operating in PHOX2B transcriptional activity. Nevertheless, the questions of how the PHOX2B domain(s) defective in CCHS normally works and which other protein(s) such domain(s) it makes contact with to regulate its targets, including *TLX2*, are still open.

In conclusion, although many aspects of the regulatory network specifying autonomic neuron fate remain to be clarified, the present study contributes to its reconstruction, linking for the first time the activity of two genes, unrecognized so far as closely related, namely *TLX2* and *PHOX2B*, and suggesting a further mechanism by which PHOX2B mutations lead to altered differentiation of the ANS.

We thank Dr Christo Goridis for kindly providing the murine *Phox2b* expression construct, and to Dr Francesca Puppo for her helpful assistance in setting up the real-time procedure. The financial support of the Compagnia di San Paolo and Italian Telethon (grant GGP04257 to IC) is gratefully acknowledged.

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Received 24 August 2005/3 January 2006; accepted 10 January 2006
Published as BJ Immediate Publication 10 January 2006, doi:10.1042/BJ20051386