# Functional characterization of a minimal sequence essential for the expression of human *TLX2* gene

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**TLX2 is an orphan homeodomain transcription factor whose expression is mainly associated with tissues derived from neural crest cells. Recently, we have demonstrated that PHOX2A and PHOX2B are able to enhance the neural cell-type specific expression of human TLX2 by binding distally the 5′-flanking region. In the present work, to deepen into the TLX2 transcription regulation, we have focused on the proximal 5′-flanking region of the gene, mapping the transcription start site and identifying a minimal promoter necessary and sufficient for the basal transcription in cell lines from different origin. Site-directed mutagenesis has allowed to demonstrate that the integrity of this sequence is crucial for gene expression, while electrophoretic mobility shift assays and chromatin immunoprecipitation experiments have revealed that such an activity is dependent on the binding of a PBX factor. Consistent with these findings, such a basal promoter activity has resulted to be enhanced by the previously reported PHOX2-responding sequence.[BMB reports 2009; 42(12): 788-793]**

## INTRODUCTION

*TLX2*, also known as *Hox11l1* (1), *Ncx* (2) or *Enx* (3), is an orphan homeobox gene which belongs to a family including two other members, *TLX1*, also known as *HOX11* (4) and *TLX3*, also known as *HOX11L2* (1) or *Rnx* (5). These *TLX* family members are characterized by a divergent homeodomain with threonine replacing isoleucine or valine at a critical site (residue 47), a substitution which, altering the DNA recognition motif, suggests a unique set of target genes for this family of transcription factors (4).

The transcriptional regulation of *TLX1* has widely been investigated, identifying specific proximal and long range elements upstream of the ATG start codon (6) and putative regulatory sequences in the 3′-untranslated region (7). On the

other hand, our previous studies of the 5′-flanking regions of the other two family members could disclose a minimal promoter sequence without cell-specificity for *TLX3* (8) and a neural-specific PHOX2B/PHOX2A binding site with enhancer property for *TLX2* (9, 10). This latter transcriptional pathway accounts for the observation that *TLX2* is mainly expressed in tissues derived from neural crest cells at the end of their migration. Indeed, the gene has been detected in dorsal root ganglia, cranial (V, VII, IX, X), vesical and enteric nerve ganglia, parasympathetic ganglia of the heart and adrenal glands in mouse embryos between E9.5 and E13.5, persisting in adrenal glands, intestine and heart in adult mice (2, 3). Consistently, the generation of several mice models has shown the crucial role of *TLX2* in the development of the Enteric Nervous System (ENS). Two reports described *TLX2* null mice both exhibiting small intestinal hypoganglionosis, megacaecum, hyperplasia of myenteric and submucosal neurons (11, 12). In contrast, another research group observed embryonal lethality at the gastrulation stage (11). More recently an additional *TLX2* null mouse has been reported to exhibit signs of pseudo-obstruction and intestinal hyperganglionosis (13). In the current study, we have further focused on the *TLX2* expression regulation thus identifying a short sequence, immediately flanking the transcription start site, necessary and sufficient for the basal transcription in cell lines from different origin, and demonstrating that such an activity is dependent on the binding of a PBX factor.

## RESULTS

### Mapping of the transcription start site

To determine the transcription initiation site of the *TLX2* gene, we set up a 5′-RACE using an uncloned library of adaptor-ligated double-strand cDNA derived from a pool of total human fetuses. In particular, we performed a nested PCR, with the forward primers specific for the adaptor and the two reverse primers lying in the first exon of the gene and, after purification from agarose gel, we cloned the amplification products in TA-vector for sequencing. We analyzed a total of nine clones derived from two independent experiments, identifying five clones all exactly mapping 338 bp upstream of the first ATG codon, very close to the initiation start point

already known in the mouse sequence. Conversely, the other four clones, 25-30 bp shorter than the above five ones, resulted all different from each other and were therefore discarded. The mRNA sequence actually deposited in Gene-Bank (NM_016170) lacks 15 nucleotides at the 5′ end.

### Functional analysis of the human *TLX2* minimal promoter

Transient transfections of constructs containing fragments of various lenghts of the 5′-flanking region of *TLX2* gene fused to firefly *luciferase* gene had already shown that a proximal sequence, demonstrated to span 522 nt upstream of the ATG start codon, was sufficient to guarantee a residual level of promoter activity in cell lines from different origin (9). In the light of the presently identified transcription initiation site (nt +1), this sequence starts at position -186. On the other hand, a further deletion of 97 nucleotides, downstream the start site, did not show induction of any luciferase activity (Fig. 1). Therefore, we propose that the 186 nucleotides sequence upstream of the transcription start point represents a minimal promoter, sufficient for a residual activity in all the analized cell lines, namely SK-N-BE and IMR32 neuroblastoma, U2-OS osteosarcoma and HEK 293 embryonic kidney. In the light of previous reports demonstrating PBX proteins as activators of *TLX1* transcriptional activity (14), we focused on an element located at -22/-12 with respect to the transcription start point containing the motif 5′-NTCANTCA-3′ described as the PBX consensus sequence (15). In particular, a site-specific mutation of the above site (Fig. 2A) was introduced into the promoter-reporter construct containing 1,649 bp upstream of the ATG translation start codon and thus including also the distal cell-specific enhancer element already described (9). The transfection of this plasmid displayed a strongly reduced gene reporter induction both in SK-N-BE and in HEK 293 (Fig. 2B),
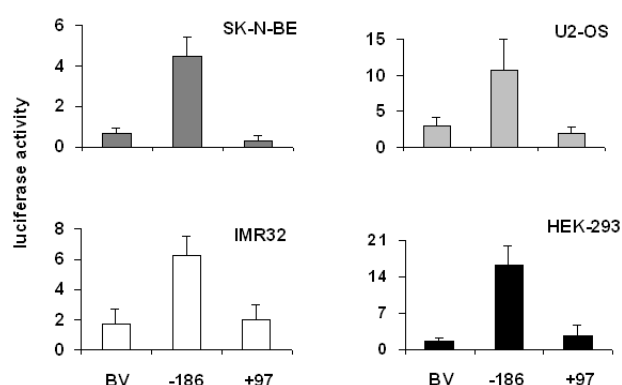
confirming the pivotal role of this binding site for the minimal sequence activity.

### *In vitro* analysis of PBX binding

To investigate whether a PBX factor was able to bind the minimal promoter sequence, EMSA was performed using NE from SKNBE and IMR32 neuroblastoma cells, two lines known to display similar *TLX2* promoter activity (9). Incubation with a radiolabeled probe containing the putative PBX site induced the formation of two major specific complexes (Fig. 3A and 3B, lanes 1) which were competed by a molar excess of the cold oligonucleotide (Fig. 3A, lane 2, and Fig. 3B, lanes 2 and 3). On the contrary, the oligonucleotide bearing the mutation tested in transfection experiments, and an oligonucleotide representing the consensus binding site for AP1, used as negative controls, failed to compete the above complexes (Fig. 3A, lanes 3 and 4, and Fig. 3B, lanes 4 and 5). Using an α-PBX 1, 2, 3 antibody (Santa Cruz Biotechnology), we observed the appearance of a supershifted band, which definitely demonstrated that this sequence is a PBX binding site (Fig. 3A, lane 5 and Fig. 3B, lane 6). An α-CEBPβ antibody, used as non specific control, did not induce any change (data not shown).

### *In vivo* analysis of PBX binding

To confirm that the interaction between a PBX factor and the



**A.**

Wt  5'-GCCGATCCGTCAGTCACTGCCCCA-3'
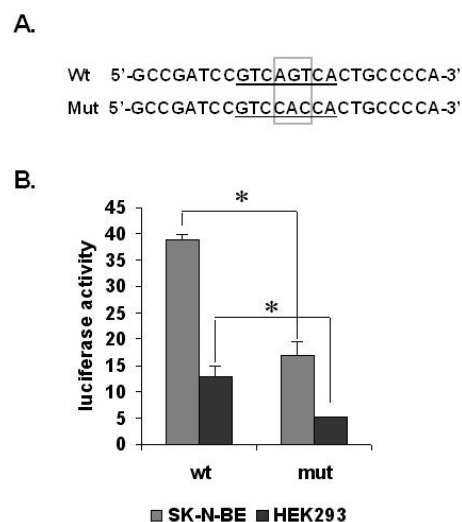
Mut 5'-GCCGATCCGTCCACCACTGCCCCA-3'

**B.**

**Fig. 2.** Site-directed mutagenesis analysis. (A) The nucleotide substitutions introduced in the longest 5′-flanking sequence (1,849 bp) construct and specifically impairing the candidate functional element, are reported. (B) Values of *Luciferase* activity, after transfection in SK-N-BE and HEK 293 cells, are reported on the graph as fold induction with respect to the aspecific activity induced by the empty vector, and represent the means of duplicate experiments from at least three independent assays. Error bars indicate standard deviations. wt = 1,849 bp-long sequence carrying wild type PBX binding site (-22/-12); mut = 1,849 bp-long sequence carrying mutant PBX site; *Indicate *t*-test with P ≤ 0.05.



**Fig. 1.** Relative *luciferase* activity displayed by two deletion constructs in four different cell recipients. Numbers on the x-axis define the extention of such deletions, referring to the transcription start site reported in the first paragraph of Results section. BV = pGL3 promoterless basic vector, transfected as negative control. Reported values represent averages of duplicated experiments from at least three independent transfections. Error bars indicate standard deviations.
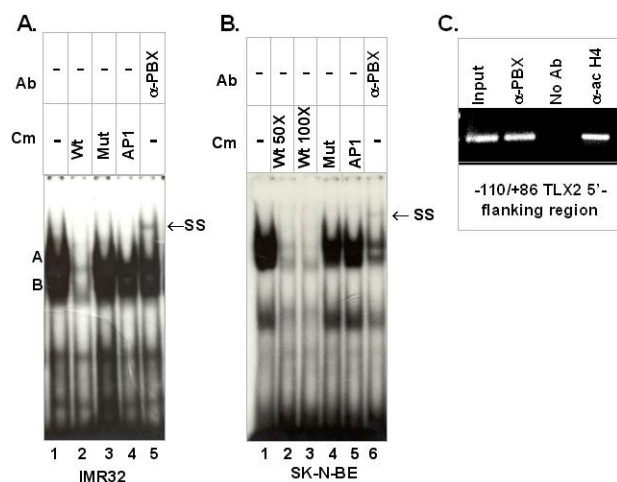
**Fig. 3.** *In vitro* binding analysis. EMSA experiments were performed using nuclear extracts from IMR32 (A) or SK-N-BE (B) cells and a $\gamma$ $^{32}$P end-labeled probe containing the PBX putative binding site. For competition binding assays, non-labeled double strand oligonucleotides were added in the reaction mix in specific molar excess (100x, if not indicated) before the radiolabeled probe. Wt = wilde-type cold oligonucleotide; Mut = mutated cold oligonucleotide (for sequence see Fig. 2); AP1 = cold oligonucleotide containing the AP1 consensus binding sequence; SS=supershift. (C) *In vivo* binding analysis. ChIP was performed on IMR32 cells. Amplification product corresponds to the *TLX2* promoter region from −110 to +86 with respect to the transcriptional start site indicated by +1. "NoAb" is the control immunoprecipitation performed without antibody. "Input" represents 1% of the total extracted chromatin.

minimal promoter occurs *in vivo*, we performed chromatin immunoprecipitation (ChIP) using formaldehyde cross-linked and sonicated chromatin from IMR32 cells together with the antibody used in the EMSA experiments. PCR results, represented in Fig. 3C, showed that the antibody against PBX 1, 2, 3 effectively immunoprecipitated the *TLX2* regulatory sequence under analysis while the control assay performed without antibody did not present any amplification product. Moreover, since the same result was achieved using the antibody against acetil-Histone H4, we can conclude that the region under analysis resulted transcriptionally active.

### Confirmation of the minimal promoter activity by PHOX2 enhancer element

In a previous report, we described a distal sequence at the *TLX2* locus displaying enhancer property due to the binding of PHOX2A and PHOX2B, as summarized in Fig. 4A (9). To confirm the role of the *TLX2* minimal promoter currently identified, we cloned such PHOX2-responding sequence, downstream the *luciferase* gene, this latter under the control of the minimal promoter of either *TLX2* or SV40. *In vitro* transfection assays were carried out in the SK-N-BE line, a suitable cell recipient to study such a sequence activity (9). The results reported in Fig. 4B clearly show that the enhancer element

strongly increased the activity of both the promoters. In particular, the construct bearing the *TLX2* minimal promoter showed a luciferase induction of more than 23-fold, confirming that this sequence is necessary for the gene transcription.

## DISCUSSION

While a previous report on the murine *TLX2* 5′-flanking region suggested the presence of a basal promoter in the proximal 856 bp, without indicating any possible regulatory factor (16), the present work has reduced the minimal region to 186 bp upstream of the transcriptional initiation site, demonstrating that its activity mainly relies on sequence recognized by a PBX factor. The *PBX* family belongs to the superfamily of highly conserved homeodomain-containing proteins named TALE (Three Amino Loop Extension) which normally act as HOX co-factors by forming complexes binding DNA and activating various target genes to regulate developmental gene expression (17, 18). The identification of further PBX partners has suggested additional roles of these factors in several biological pathways (19). In Mammals, four separate genes encode Pbx family members, which share remarkable sequence identity within and flanking their DNA-binding homeodomains. *Pbx1*, *Pbx2*, and *Pbx3* are widely expressed, whereas *Pbx4* mRNA has been identified only in the testis (20). Moreover, some of these genes give rise to several isoforms by alternative splicing (20). Recently the differential contribution of each single Pbx protein to mammalian development has been established (21). Despite a tissue-specific regulation mediated by different *PBX* species has been suggested, probably due to an *in vivo* differential recruitment of transcriptional co-factors, the DNA-binding properties of Pbx proteins appear similar *in vitro* (22). In fact, after demonstrating the binding of PBX2, but not PBX1 and PBX3, to *TLX1* promoter in leukaemia cell lines (14), the analysis of the spleen organogenesis in mouse embryos has displayed the capability of *TLX1* to act synergistically with *PBX1* to bind its own promoter, thus auto-inducing its expression (23). This confirms that different PBX family members can act in the transactivation of the same target gene depending on the specific tissue or developmental stage and strengthens the hypothesis of overlapping functions of Pbx family members, at least in specific organ systems and in tissues showing similar expression patterns (19).

In the present report, the dramatic impairment of the gene reporter activity driven by the mutated *TLX2* 5′-flanking sequence demonstrates that the integrity of the minimal promoter, and in particular of the PBX binding site, is essential for the gene expression. In the light of the above considerations, we have used an antibody not discriminating between PBX1, PBX2 and PBX3 to perform supershift experiments and ChIP assays. We have therefore confirmed the specificity of binding of a PBX protein. The activity is requested in cells from different origin, irrespectively of whether they normally express *TLX2* or not. This is not surprising, particularly in the
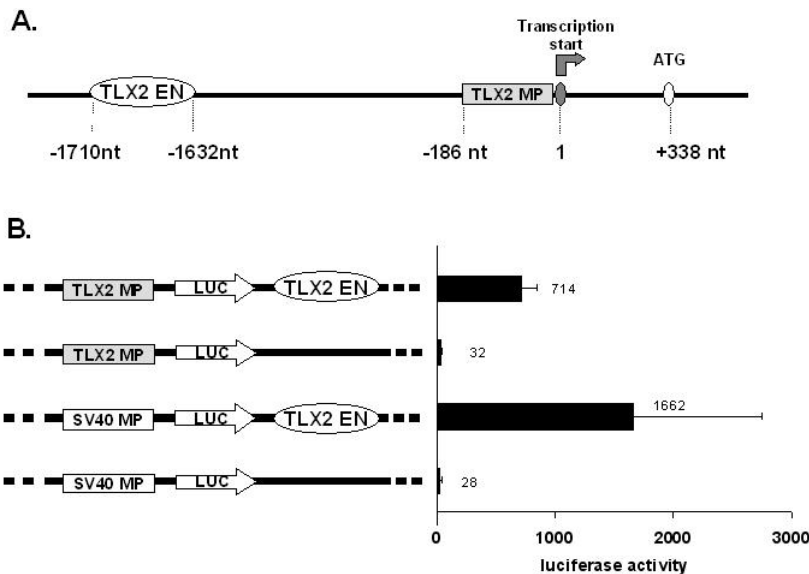
**A.**

Transcription start

ATG

TLX2 EN      TLX2 MP

-1710nt    -1632nt        -186 nt    1    +338 nt

**B.**

TLX2 MP — LUC — TLX2 EN      714

TLX2 MP — LUC      32

SV40 MP — LUC — TLX2 EN      1662

SV40 MP — LUC      28

0    1000    2000    3000
**luciferase activity**

**Fig. 4.** TLX2 minimal promoter and the PHOX2-binding enhancer element. (A) Schematic representation of the TLX2 locus. The experimentally determinated transcriptional start site (see first paragraph of the results) is indicated as nucleotide +1. (B) Values of *luciferase* activity obtained from tranfected SK-N-BE lysates are reported on the graph as fold induction with respect to the aspecific activity induced by the empty vector, and represent the means of duplicate experiments from at least three independent assays. Error bars indicate standard deviations. On the left, a schematic representation of the pGL3 constructs. TLX2-EN = PHOX2-responding enhancer; TLX2-MP = minimal promoter; LUC = luciferase reporter gene.

light of a report demonstrating that PBX and cofactors constitutively bind Myo-D dependent myogenin promoter prior to initiation of muscle differentiation and in absence of Myo-D (24). Therefore PBX might act by penetrating repressive chromatin and marking specific genes for activation by tissue-specific regulators. Indeed, in the progenitor cells for autonomic neurons PBX could bind *TLX2* promoter thus allowing the anchoring of tissue-specific activators, such as the PHOX2 factors (9, 10) at the correct developmental stage. This hypothesis has been strenghtened by the results obtained using suitable constructs bearing both the minimal promoter and the enhancer sequence. Moreover, the presence of a nearly ubiquitous factor bound to the minimal promoter in cells from different origin has already been described for *TLX3* (8).

Finally, since *TLX1* is transactivated by PBX1 and PBX2 (14), our results suggest a common mechanism driving the transcriptional regulation for the whole *TLX* gene family.

## MATERIALS AND METHODS

### Cell cultures
SK-N-BE and IMR32 neuroblastoma cells were grown in RPMI medium (Gibco-BRL, Gaithersburg, Maryland), U2-OS osteosarcoma cells in DMEM (Gibco-BRL) and the HEK293 cells in MEM (Gibco-BRL). All media were supplemented with 2 mM L-glutamine (Euroclone, Wetherby, West York UK), 10 % fetal bovine serum (New Zeland, Gibco-BRL), 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. 2 mM non-essential amino acids and 1 mM sodium pyruvate were also added to IMR32 and HEK293 media. Cells were grown at 37°C in a humified atmosphere containing 5 % $CO_2$.

### Constructs and transfections
Cloning of *TLX2* 5′-regulatory region has already been reported (25). The mutated construct was generated by PCR starting from the corresponding wt construct, as previously described (26). Oligonucleotides used for site-directed mutagenesis were 5′-CGATCCGTC**CAC**CACTGCC-3′ and 5′-GGCA GTG**GTG**GACGGATCG-3′, where mutated nucleotides are represented in bold. Each construct was controlled by direct sequencing.

*TLX2* enhancer sequence has been digested with PmlI and BamHI restriction enzymes (NEB). The fragment thus obtained has been cloned at 3′-end of the *luciferase* gene in both the construct carrying the *TLX2* minimal promoter and pGL3- promoter vector (Promega), this latter bearing the SV40 minimal promoter. Vectors have been digested in succession with SalI, Klenow fragment and BamHI. Linearized plasmids and insert have been purified on agarose gel and by columns (Millipore). Ligation has been performed by LigaFast (Promega).

SK-N-BE neuroblastoma and HEK293 cells ($10^5$) were transfected using polyethylenimine (PEI) method, as already reported (9) or FuGene 6 (Roche). The promoterless pGL3 vector was transfected as negative control and used as reference for graphical data presentation. Luciferase activity in cell lysates was determined using Promega protocol (Dual Luciferase Reporter Assay System) and luminometer (Turner designs, Sunnyvale, CA). Each construct was transfected in duplicate and each test was repeated at least three times.

### Determination of the transcriptional initiation site
The transcriptional initiation site of the human *TLX2* was

determined by Rapid Amplification of 5'-cDNA Ends (5'-RACE) analysis using an uncloned library of adaptor-ligated double strand cDNAs (Marathon-ready, Clontech, Palo Alto, CA) derived from human fetus. In brief, 3 μl of library were mixed to 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA), 5% glycerol, 1.5 mM MgCl$_2$, 200 μM dNTPs containing 7-deaza-dGTP, the anchor primer provided by the manufacturer and the specific primer (5'-CAAGTGAGCCGGC GGGACCGTAG-3'), lying in the first exon, in 1 : 5 concentrations respectively. After an initial denaturation at 95$^o$C for 10 minutes, PCR was run as follows: 5 cycles at 94$^o$C for 5 sec and 72$^o$C for 4 min, 5 cycles at 94$^o$C for 5 sec and 70$^o$C for 4 min, 25 cycles at 94$^o$C for 5 sec, 65$^o$C for 30 sec and 68$^o$C for 4 min. Nested PCR was performed under the same conditions after mixing 5 μl of the first PCR product diluted 1 : 50 in Trycine-EDTA buffer to 1 unit of Taq polymerase (Applied Biosystems), 5% glycerol, 1.5 mM MgCl$_2$, 200 μM dNTPs containing 7-deaza-dGTP, the nested anchor primer provided by the manufacturer and the specific primer (5'-CGTGGTGTGGG AGGTTGTGTGGACC-3'). Amplification products thus obtained were separated on a 1% agarose gel and, after band purification (Qiagen, Hilden, Germany), cloned in TA-vector (Invitrogen) for sequencing. We sequenced a total of four clones derived from a pool of clones displaying the same sequence length.

### *In vitro* and *in vivo* PBX binding analysis

Nuclear extracts (NE) were prepared as previously described (27) from SK-N-BE and IMR32 cells. EMSA assays were performed using 6 μg of NE and the following radiolabeled probe 5'-GCC GATCCGTCAGTCACTGCCCCA-3', lying between -30 and -7 nucleotides. Corresponding mutated double strand oligonucleotides are reported above. Sequence for AP1 consensus probe used as competitor is the following: 5'-CGCTTGATGAC TCAGCCGGAA-3'. For supershift assays, PBX 1, 2, 3 antibody (Santa Cruz) was incubated with the NE mix for 30 min on ice before adding the radiolabeled probe.

To study PBX-minimal promoter interaction *in vivo*, ChIP was performed on IMR32 cells following the protocol provided by the manufacturer (Upstate Biotechnology). After formaldeyde crosslinking, lysis, sonication and preclearing, a volume of the supernatant corresponding to 1% of the total chromatin was kept as "input" material, whereas the remaining cleared chromatin was incubated overnight in different tubes: with or without anti-PBX 1, 2, 3 (Santa Cruz) or anti-acetil-Histone H4 (Upstate). Immune-complexes were recovered by salmon sperm DNA-protein A agarose beads (Upstate biotechnology). Chromatin was isolated by reversing crosslinking at 65$^o$C for four hours, proteinase K treatment and, finally, phenol-chloroform purification.

Genomic sequence of the TLX2 promoter region under investigation was amplified by PCR using the primers 5'-TT GGCGCAAACAGGCGTC-3' (-111 nt/-93 nt) and 5'-GAGCT TCCTTGCTGGCTCTTA-3' (+64 nt/+85 nt).

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