Biochemical and Biophysical Research Communications 375 (2008) 11–15

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Identification and characterization of mouse plexin B3 promoter

Liivi Tiismus [a,b], Piret Laht [a,b], Maarja Otsus [b], Andres Veske [a,b,*]

[a] Tallinn University of Technology, Department of Gene Technology, Tallinn, Akadeemia tee 15, 12618 Tallinn, Estonia
[b] Competence Center for Cancer Research, Tallinn, Akadeemia tee 15, Tallinn 12618, Estonia

ARTICLE INFO

*Article history:*
Received 14 July 2008
Available online 3 August 2008

*Keywords:*
Plexin B3
Promoter
Neurogenin 3
Myeloid zinc finger protein 1 (MZF-1)

ABSTRACT

Plexin B3 is a neurally expressed transmembrane receptor protein participating in growth cone remodelling and synaptic plasticity. Despite their biological importance no plexin promoter has been characterized so far. Regulation of mouse plexin B3 transcription was investigated using in silico analysis of promoter and inititation area, 5′RACE, reporter-gene assays, gelshift and co-expression experiments. As a result we have described a novel 5′ exon and show that a 234 basepair region upstream of it exhibits promoter activity. Further analysis indicated that this region contains predicted binding sites for myeloid zinc finger protein 1 (MZF-1) and neurogenin 3 (Ngn3). Oligonucleotides corresponding to the recognition sequences of these factors produced a specific mobility shift in EMSA. Expression of the reporter gene attached to the 234 region was increased 2-fold by Ngn3 and reduced twice in response to MZF-1 overexpression. These results help to better comprehend mechanisms used for plexin B3 transcriptional regulation.



Plexins emerged in evolution with the appearance of multicellular animals. The family of plexins comprises two genes in *Caenorhabditis elegans* and *Drosophila* and nine genes (A1-4, B1-3, C1, and D1) in mammals [1,2]. Plexins are receptors for semaphorins—secreted or membrane associated proteins that play an important role in axon and dendrite guidance. In addition, the plexin family controls adhesion and migration in a variety of non-neural cell types. It is therefore implicated in regulation of organogenesis and cancer formation [3,4].

Plexin B3, a receptor for semaphorin 5A, is a bifunctional molecule that can, induce growth cone collapse or promote cellular migration and growth on the context-dependent manner. Sema5A can activate Met signalling by plexin B3 leading to the invasive growth [5]. Plexin B3 can also stimulate neurite outgrowth through homophilic interaction *in trans*. This highly specific interaction is mediated by the extracellular Sema domain [6].

Mouse plexin B3 gene is located on chromosome X and spans ~17 kbp. It is predicted to encode a 1902 amino acid protein (Accession No. NP062533). The expression of B3 is scarcely detectable before birth. Postnatally it is predominantly expressed in central nervous system, peaking at P9 and remaining relatively high to adulthood [6–8]. Plexin B3 is expressed also in several normal tissues of the epithelial origin and in many tumor tissues [9]. Modern human specific haplotype of plexin B3 has been associated with brain white matter volume and verbal performance [10] in accordance with its neurite growth promoting effect, indicating that plexin B3 is involved in brain development and functioning.

In the present study, we characterized the genomic organization of the mouse plexin B3 promoter, mapped the transcriptional start sites, determined the minimal region required for transcriptional activity, and analyzed its regulation by transcription factors MZF-1 and Ngn3. This is the first promoter to be described for the plexin gene family.

## Materials and methods

*5′RACE.* FirstChoice® RLM-RACE Kit (Ambion) was used along with rat (Ref636622) and mouse (Ref636601) total brain RNA (BD BioSciences) following the manufacturer's instructions. Nested gene-specific primers used for amplification are listed in Supplementary Materials. PCR products were cloned into pCR2.1®- TOPO® (Invitrogen) and recombinant plasmids were sequenced.

*Database analysis.* Nucleotide sequences lying 4 kbp upstream of the annotated translation initiation sites of mouse, rat and human plexin B3 genes were aligned with ClustalW [11]. Transcription factor binding sites were predicted using the MatInspector software [12] and TFSEARCH [13]. Also, the CpG Island Searcher program (http://cpgislands.usc.edu/) was used.

*Plasmid construction. PCR-amplified mouse plxnB3* promoter fragments were cloned into pCR2.1®-TOPO® vector (Invitrogen). Primers used are listed in Supplementary Materials. Fragments were subcloned into Acc65I and XhoI sites of pSEAP2 Basic (Clontech) vector. Short fragments of 234 and 437 bp were generated by

* Corresponding author. Address: Tallinn University of Technology, Department of Gene Technology, Tallinn, Akadeemia tee 15, 12618 Tallinn, Estonia. Fax: +372 620 4401.
*E-mail address:* andres.veske@ttu.ee (A. Veske).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.07.122

restriction endonuclease treatment of pSEAP/772 construct (using Acc65I with Mph1103I or Bsp1407I, respectively).

Mouse MZF-1 and Ngn3 coding regions were PCR amplified from mouse brain cDNA and cloned into the EcoRI and Acc65I sites of pFLAG-CMV-4 (Sigma).

*Cell culture.* NIH3T3 (mouse embryonic fibroblast), Neuro2A (mouse neuroblastoma) and B10F10 (mouse melanoma) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml).

*Secreted alkaline phosphatase assay.* Experiments were carried out in 24-well plates. SEAP reporter plasmid (0.8–1 μg) and β-galactosidase internal control plasmid (0.25 μg) were transiently transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. For cotransfection experiments 0.8 μg of pSEAP/234, 0.8 μg of transcription factor expression constructs in pFLAG-CMV-4 and 0.4 μg of β-galactosidase internal control plasmid were used. Each transfection was performed in triplicate and repeated at least five times with every cell line. Cells were harvested 48 h after transfection and analyzed with Great EscAPE system (BD Biosciences Clontech) and β-galactosidase Enzyme Assay System (Promega) according to manufacturers' protocols. The relative alkaline phosphatase activity was normalized to β-galactosidase activity and was calculated as a ratio of construct's activity to pSEAP2 Basic activity.

*Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)*

Nuclear extracts were prepared and EMSA was carried out using the online protocol suggested by the molecular cloning laboratory manual (www.molecularcloning.com). Protein concentration of the nuclear extracts from cell lines was measured with Bradford reagent (Sigma). 2000 cpm of labeled double stranded DNA oligonucleotide (listed in Supplementary Materials) was used per reaction and mixed with 0.75 μg of nuclear protein mix and 1 μg of non-specific competitor poly-dCdI (Serva). The final mix contained also 45 mM NaCl, 6 mM Hepes–KOH pH 7.9; 60 mM KCl; 0.15 mM DTT and 10% glycerol. Unlabeled oligos were used as specific competitors in 250-fold excess. Reactions were incubated at room temperature for 15 min.

## Results

*Characterization of mouse plxnB3 transcription start sites*

To determine the transcription start sites (TSSs) of mouse *plxnB3*, RLM-RACE was performed using mouse total brain RNA. A total of 26 clones revealed five different 5′ ends in three different genomic regions (Fig. 1B): (1) near the beginning of the first coding exon 35 nucleotides upstream of ATG (Accession No. EU529858); (2) at positions −1432, −1449, and −1474 (EU529860, EU529859, EU529857) in the second exon clustering together with previously described TSSs available from GenBank entry for mouse *plxnB3* (Fig. 1C); and (3) 1969 basepairs upstream of the start codon (EU529857). The latter marks the beginning of a yet undescribed 5′ exon ranging from −1969 to −1684. The following intron (−1683 to −1467) is flanked by consensus sites used by the major spliceosome. One alternative splicing event was observed whereby second exon was joined with exon three at position +54 instead of the conventional −5 (EU529857). This leads to the elimination of ATG at +1 and translation could potentially be initiated at position +217.

5′ RACE analysis was also carried out for rat plexin B3 gene. In case of the first exon in rat, the start site was 23 bp downstream from mouse *plxnB3* TSS, at −1946 (EU643794). Major TSS in the second exon was at position −1432 (EU529862), with alternative sites at −1474 (EU529864) and −1419 (EU529863). No clones starting from coding (third) exon were detected. The splice sites were identical to the mouse gene. Alignment of mouse, rat and human plexin B3 promoter areas revealed that human *plxnB3* does not share the first exon with rodents but has a completely different one further upstream according to GenBank ESTs.

A panel of cDNA-s derived from mouse tissues (mainly from various brain regions) was used to analyze the usage of first two exons by semiquantitative PCR. As expected, *plxnB3* was rather difficult to detect in embryonic mouse tissue, but the mRNA level rose fast postnatally. Both exons were equally expressed all over the brain, but were not detected in other tissues (Supplementary Fig. 1).

*Functional analysis of the promoter activity of the plxnB3 gene 5′ flanking sequences*

To identify *cis*-acting sequences that regulate plexin B3 transcription, a series of secreted alkaline phosphatase reporter constructs containing fragments of the mouse *plxnB3* gene 5′ flanking region were generated, ranging in length from 234 bp to 1.9 kbp and covering possible activating regions in front of three first exons, (see Fig. 2). The constructs were transiently cotransfected with the β-galactosidase reporter (to control for differences in transfection efficiency) into Neuro2A and NIH3T3 cell lines (which are *plxnB3* positive and negative, respectively, Fig. 3B). Mouse melanoma cell line (B16F10) was also used and showed similar results with Neuro2A (data not shown).

The longest fragment (1904 bp) did not exhibit any transcription activating abilities (Fig. 2). The same was observed for shorter fragments in front of the second exon (1301 and 376 bp). The region just in front of the third exon (−269 to +78) had little promoting ability in both cell lines. High levels of alkaline phosphatase activity were observed in case of pSEAP/437 (−2364 to −1927) and its truncated version pSEAP/234 (−2161 to −1927). They showed 7- to 14-fold increase in reporter-gene expression in Neuro2A but only 2- to 3-fold in NIH3T3, thus being specific for plexin B3 positive cells. Approximately 4-fold increase of activity was detected in case of pSEAP/772 (−2699 to −1927), which is a longer variant of the former two. Such results suggest the presence of repressor elements between positions −2699 and −2365.

*Identifying nuclear factor binding sites within plxnB3 proximal promoter*

Analysis of promoter core elements showed the presence of initiator (Inr), but no TATA-box, TFIIB recognition or downstream core promoter elements (BRE or DPE). The sequence up to 10 kbp upstream from mouse *plxnB3* translation start site was also screened for CpG islands, but none were found during that process. MatInspector and TFSEARCH software detected several potential regulatory elements that may have functional importance for *plxnB3*. The highest core similarity and matrix similarity scores (both 1.00) were achieved with two factors: myeloid zinc finger protein 1 (MZF-1) and TG-interacting factor belonging to TALE class of homeodomain factors (TGIF). The binding sites of these factors were present in the proximal promoter in several copies (Fig. 1C, Supplementary Table 2). One TGIF site located immediately in front of TSS, thus potentially playing an important role in transcription initiation, overlapped with a neurogenin 1/3 (Ngn1/3) binding site.

To obtain experimental evidence for the efficiency and specificity of transcription factor binding to *plxnB3* promoter (from

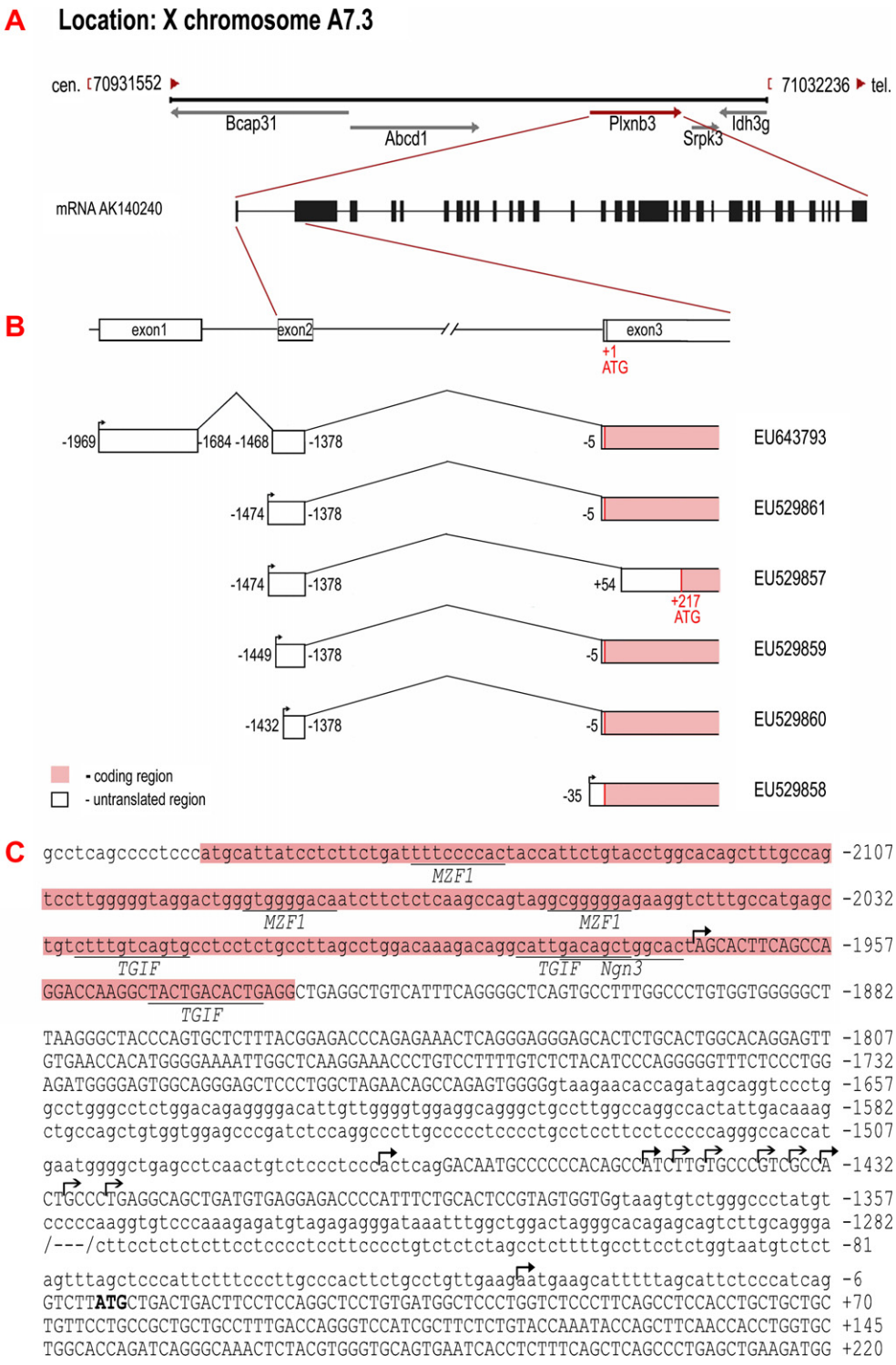


**Fig. 1.** Genomic organization of mouse *plxnB3* promoter. (A) Location of plexin B3 gene on X-chromosome and exon–intron structure of representative mRNA AK140240. (B) TSSs of *plxnB3* identified by 5′RACE. Boxes indicate exons, numbering is relative to the translation start site. Corresponding GenBank Accession Nos. are at the end of each line. (C) Nucleotide sequence of *plxnB3* promoter area. ATG at +1 is in bold, exons are in uppercase and introns in lowercase. The minimal region showing promoter activity is shadowed and potential transcription factor binding sites are underlined. Closed arrows mark the TSSs identified during this study, open arrows mark the TSSs of previous GenBank ESTs.

−2161 to −1927), the protein binding was surveyed by EMSA using Neuro2A, B16F10 and NIH3T3 nuclear extracts. $^{32}$P-labeled double-stranded oligonucleotide probes containing the binding sites of MZF-1, TGIF, and Ngn3 were generated. Also, oligonucleotides with mutated core sequences for these factors were used to confirm the specificity of the binding event (Fig. 3A). In case of TGIF, no specific shift was observed. A specific DNA-protein complex appeared with MZF-1 and Ngn3 probes. This shift was successfully out-competed with a 250-fold excess of non-labeled oligonucleotides. Also, mutating the core sequence (Ngn3

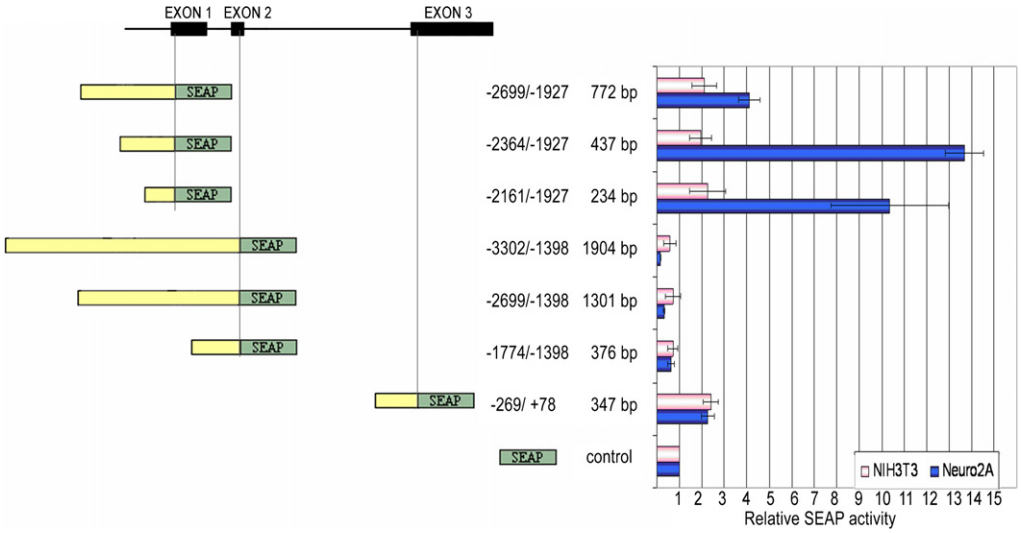


**Fig. 2.** Functional analysis of mouse *plxnB3* promoter by SEAP reporter assay. Neuro2A and NIH3T3 cell lines were transfected with different reporter-gene constructs indicated on the left. Numbering is the same as in Fig. 1. SEAP activity was measured and normalized to β-galactosidase activity and is presented as fold increases over the pSEAP2 Basic activity. Results shown are the averages (means ± standard deviations) of three independent experiments performed in triplicate.

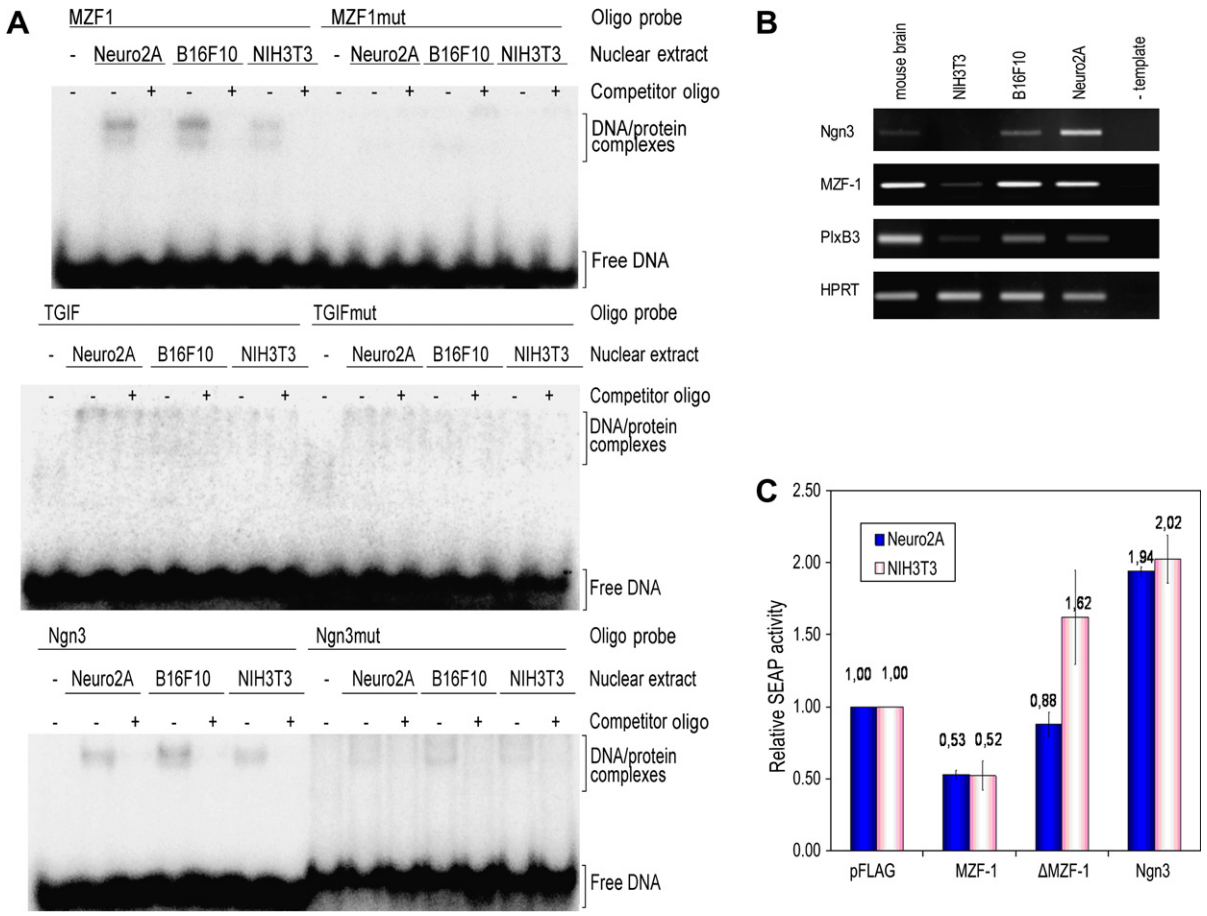


**Fig. 3.** *PlxnB3* promoter is regulated by MZF-1 and Ngn3. (A) EMSA: Neuro2A, B16F10, and NIH3T3 nuclear extracts were incubated with $^{32}$P-labeled oligonucleotides corresponding to predicted binding sites of MZF-1, TGIF, and Ngn3 in *plxnB3* promoter. Same oligonucleotides with mutated core sequences were used as negative controls. Unlabeled oligonucleotides were used in 250-fold excess for competition. (B) Semi-quantitative analysis of Ngn3, MZF-1, and plexin B3, and control HPRT mRNA expression by RT-PCR in mouse cell lines. Mouse brain mRNA was used as positive control. (C) The influence of Ngn3 and MZF-1 over-expression on *plxnB3* promoter construct pSEAP/234 activity. Empty pFLAG was used as negative control and results were calculated relative to it.

CTGG → ATGG, MZF-1 GGGG → GAGG) prevented formation of that complex. We therefore concluded that MZF-1 and Ngn3 are able to physically interact with *plxnB3* promoter and this may have a biological significance.

*Over-expression of MZF-1 and Ngn3 affects the activity of plxnB3 promoter*

Next we examined whether MZF-1 and Ngn3 could transactivate the pSEAP/234 construct in Neuro2A and NIH3T3 cells. As MZF-1 is expressed with or without corepressor-binding N-terminal SCAN-domain, we used both full-length and the truncated isoform. MZF-1 repressed transcription—an effect that disappeared in case of ΔMZF-1 indicating the requirement of SCAN-domain for this process. Ngn3, on the other hand, clearly possessed activating properties. The results of three independent experiments are summarized in Fig. 3C.

## Discussion

Plexin B3 is an important receptor in neural network development and subsequent functioning of the brain. The aim of this study was to describe the promoter of mouse plexin B3 as the regulation of plexins on transcriptional level has not been studied. Intriguingly, the region predicted to contain *plxnB3* promoter according to database ESTs did not show any transactivation although several TSSs mapped there. Instead we report a novel 5′UTR exon of this gene and point out a 234 bp sequence with transcription promoting properties in front it. Sequence analysis indicated that this promoter is TATA-less with Inr elements. EMSA analysis revealed that consensus binding sites for MZF-1 and Ngn3, but not for TGIF, are involved in its regulation. When overexpressed in cell culture, Ngn3 increased and MZF-1 decreased the activity of *plxnB3* minimal promoter. Our results prove for the first time experimentally that the mouse *plxnB3* gene expression is regulated by MZF-1 and Ngn3 factors.

Neurogenin 3 is a proneuronal bHLH protein that participates in many signalling and metabolic pathways. Among other functions Ngn3 regulates receptor-mediated axon growth repulsion, dendritic and synaptic plasticity, and apoptosis [14–16]. Expression patterns of Ngn3 and its close relative Ngn1 overlap in many aspects with plexin B3 in CNS, and are likely to regulate its transcription.

MZF-1 is involved in the apoptosis, proliferation and differentiation of myeloid progenitors [17], and also plays a role in supression of tumorigenesis and has been shown to repress transcription in non-hematopoietic cells [18]. Plexin B3, functioning in the signaling pathway in the control of cell migration and invasive growth, might well be regulated via MZF-1, and it may be important for down-regulation of plexin B3 expression in non-neuronal tissues.

In summary, we successfully identified and described the mouse plexin B3 promoter. The unique features of found promoter provide help in gaining a greater insight into the complicated network of transcriptional regulation of plexin B3.

## Acknowledgments

We thank Dr. M. Speek and Dr. R. Tamme for the comments and suggestions. This work was supported by the Competence Centre for Cancer Research and ESF Grant No. 5915.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.122.

## References

[1] L. Tamagnone, S. Artigiani, H. Chen, Z. He, G. Ming, H. Song, A. Chedotal, M.L. Winberg, C.S. Goodman, M. Poo, M. Tessier-Lavigne, P. Comoglio, Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates, Cell 99 (1) (1999) 71–80.
[2] R. Fiore, A.W. Püschel, The function of semaphorins during nervous system development, Front. Biosci. 8 (2003) 484–499.
[3] M. Negishi, I. Oinuma, H. Katoh, Plexins: axon guidance and signal transduction, Cell. Mol. Life Sci. 62 (2005) 1363–1371.
[4] T.S. Tran, A.L. Kolodkin, R. Bharadwaj, Semaphorin regulation of cellular morphology, Annu. Rev. Cell Dev Biol. 23 (2007) 263–292.
[5] S. Artigiani, P. Conrotto, P. Fazzari, G.F. Gilestro, D. Barberis, S. Giordano, Plexin-B3 is a functional receptor for semaphorin 5A, EMBO Rep. 5 (2004) 710–714.
[6] C. Hartwig, A. Veske, S. Krejcova, G. Rosenberger, U. Finckh, Plexin B3 promotes neurite outgrowth, interacts homophilically, and interacts with Rin, BMC Neurosci. 6 (2005) 53.
[7] T. Worzfeld, A.W. Püschel, S. Offermans, R. Kuner, Plexin-B family members demonstrate non-redundant expression patterns in the developing mouse nervous system: an anatomical basis for morphogenetic effects of Sema4D during development, Eur. J. Neurosci. 19 (2004) 2622–2632.
[8] N.M. Perälä, T. Immonen, H. Sariola, The expression of plexins during mouse embryogenesis, Gene Exp. Pat. 5 (2005) 355–362.
[9] Š. Krejčová, Genomics, expression and developmental analysis of murine and human Plexin B3, Ph.D. Thesis, University of Hamburg 2005.
[10] D. Rujescu, E.M. Meisenzahl, S. Krejcova, I. Giegling, T. Zetzsche, M. Reiser, C.M. Born, H.J. Moller, A. Veske, A. Gal, U. Finckh, Plexin B3 is genetically associated with verbal performance and white matter volume in human brain, Mol. Psych. 12 (2) (2007) 190–194.
[11] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, ClustalW and ClustalX version 2, Bioinformatics 23 (21) (2007) 2947–2948.
[12] K. Quandt, K. Frech, H. Karas, E. Wingender, T. Werner, MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data, Nucleic Acids Res. 23 (23) (1995) 4878–4884.
[13] T. Heinemeyer, E. Wingender, I. Reuter, H. Hermjakob, A.E. Kel, O.V. Kel, E.V. Ignatieva, E.A. Ananko, O.A. Podkolodnaya, F.A. Kolpakov, N.L. Podkolodny, N.A. Kolchanov, Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL, Nucleic Acids Res. 26 (1998) 364–370.
[14] N.R. Treff, R.K. Vincent, M.L. Budde, V.L. Browning, J.F. Magliocca, V. Kapur, J.S. Odorico, Differentiation of embryonic stem cells conditionally expressing neurogenin 3, Stem Cells 24 (11) (2006) 2529–2537.
[15] P. Salama-Cohen, M.A. Arévalo, R. Grantyn, A. Rodríguez-Tébar, Notch and NGF/p75NTR control dendrite morphology and the balance of excitatory/inhibitory synaptic input to hippocampal neurones through Neurogenin 3, J. Neurochem. 97 (5) (2006) 1269–1278.
[16] V. Dror, V. Nguyen, P. Walia, T.B. Kalynyak, J.A. Hill, J.D. Johnson, Notch signalling suppresses apoptosis in adult human and mouse pancreatic islet cells, Diabetologia 50 (12) (2007) 2504–2515.
[17] L. Bavisotto, K. Kaushansky, N. Lin, R. Hromas, Antisense oligonucleotides from the stage-specific myeloid zinc finger gene MZF-1 inhibit granulopoiesis in vitro, J. Exp. Med. 174 (5) (1991) 1097–1101.
[18] M. Gaboli, P.A. Kotsi, C. Gurrieri, G. Cattoretti, S. Ronchetti, C. Cordon-Cardo, H.E. Broxmeyer, R. Hromas, P.P. Pandolfi, Mzf1 controls cell proliferation and tumorigenesis, Genes Dev. 15 (13) (2001) 1625–1630.