Characterization and promoter analysis of the mouse nestin gene ☆

Leping Cheng[1,2], Zhigang Jin[1], Li Liu, Ye Yan, Tingting Li, Xueliang Zhu, Naihe Jing*

Laboratory of Molecular Cell Biology, Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, PR China

Received 20 January 2004; revised 29 March 2004; accepted 29 March 2004

First published online 15 April 2004

Edited by Ned Mantei

Abstract The intermediate filament protein nestin is expressed in the neural stem cells of the developing central nervous system (CNS). Promoter analysis revealed that the minimal promoter of the mouse nestin gene resides in the region −11 to +183 of the 5′-non-coding and upstream flanking region, and that two adjacent Sp1-binding sites are necessary for promoter activity. Electrophoretic mobility-shift assays (EMSA) and supershift assays showed that Sp1 and Sp3 proteins selectively bind to the upstream Sp1 site. These results demonstrate an important functionality of Sp1 and Sp3 in regulating the expression of the mouse nestin gene.

Keywords: Mouse nestin gene; Promoter; Sp1; Sp3; P19 cell

1. Introduction

Nestin, an intermediate filament protein, is expressed in the neural stem cells of the developing central nervous system (CNS) [1–4]. In situ hybridization demonstrated the presence of nestin mRNA in the neural tube neuroepithelial and radial glia cells of mouse embryos at embryonic day 10.5 (E10.5) [5]. The nestin protein was found in the rat neural tube prior to but not post neurogenesis [6]. Consistent with this observation, nestin expression exhibits a sharp decline in the motor neurons of spinal cord and marginal layer neurons of the telencephalon accompanied by the cessation of mitosis of these cells [5]. From E15.5 to postnatal day 0, nestin mRNA is expressed predominantly in the cerebellum, the ventricular and subventricular telencephalon [5], as well as some regions of the adult brain [7]. Nestin expression is not restricted to the neural stem cells, as it has also been detected in myogenic precursor cells, tooth buds, limb buds, the heart, testis, and eyes [8–13].

Human, rat and mouse nestin share considerable similarity in their gene structures [3,14,15]. Studies in transgenic mice demonstrated that two tissue-specific enhancers in the first and second introns of the rat nestin gene are required and sufficient to drive reporter gene expression in developing muscle and neural precursors, respectively [14]. The neural stem cell-specific enhancer resides in the 3′-region of the second intron [16,17], and a *cis*-element for the POU transcription factors is responsible for nestin expression in the CNS [17]. It has been shown that nestin is a target gene of thyroid transcription factor-1 (TTF-1, also known as Nkx2.1), which is involved in the organogenesis of the forebrain [18]. However, the structure and function of the nestin gene promoter remained unclear.

Our previous study described the cloning of mouse nestin gene and the functional expression of its cDNA in COS7 cells [15,19]. The mouse nestin gene was found to be expressed in the developing eye and lens [13], and also in the growth cones of P19 neurons and cerebellar granule cells [20]. In order to understand the molecular mechanisms of regulation of the nestin gene expression, we have characterized its promoter. We found that the proximal promoter is localized to the region between −11 and +183 of the 5′-non-coding and upstream flanking region, and that two adjacent Sp1-binding sites are required for the promoter activity. Our results further indicate that Sp1 and Sp3 proteins selectively bind to the upstream Sp1 site.

2. Materials and methods

2.1. RNA purification

Poly(A)$^+$ RNA were isolated from embryonic day 15.5 (E15.5) mouse embryo brain tissues and P19 cells by using the Oligotex® mRNA kit according to the manufacturer's protocol (Qiagen, Hilden, Germany).

2.2. Primer extension

An oligonucleotide corresponding to positions +178 to +159 (primer PE, 5′-GCACTAGAGAAGGGAGTGGC-3′) of exon 1 was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (TaKaRa, Dalian, China). 2 μg poly(A)$^+$ RNA or 10 μg yeast tRNA was incubated with 2.5 pmol of the labelled oligonucleotide for 15 min at 75 °C, then cooled on ice for 5 min, and mixed with Superscript II reverse transcriptase (Gibco, Rockville, MD, USA). Elongation was performed for 1 h at 42 °C. The primer-extended fragment was analyzed on a 6% polyacrylamide/7 M urea sequencing gel along with a dideoxy chain termination sequencing reaction with a genomic clone as template and the PE primer as the sequencing primer.

2.3. Cell culture and co-transfection assays

Cell lines including mouse embryonic carcinoma P19 cells, NIH3T3, CHO, F9, C6, and SY5Y cells were maintained in

☆ The nucleotide sequence reported has been submitted to the DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AY331185.

* Corresponding author. Fax: +86-21-54921011.
E-mail address: njing@sibs.ac.cn (N. Jing).

[1] These authors contribute equally to this work.
[2] Present address: Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA.

Abbreviations: AP, activator protein; CNS, central nervous system; EMSA, electrophoretic mobility-shift assay; LUC, luciferase; NE, nuclear extracts; Sp, specificity protein; SV40, simian virus 40

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doi:10.1016/j.febslet.2004.03.097

DMEM/F12 1:1 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37 °C in 5% CO_2. Cells were seeded into 24-well plates at a density of $2–4 \times 10^4$ cells/well. Twenty four hours later, 0.4 μg of firefly luciferase (LUC) reporter and 0.1 μg of pRL-TK plasmids (Promega, Madison, WI, USA) containing the *Renilla* LUC gene downstream of the herpes simplex virus thymidine kinase (TK) promoter were co-transfected into cells using LipofectAMINE reagent (Gibco) according to the manufacturer's protocol. After 40 h, cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Ten microlitres of the supernatant was analyzed for LUC reporter activity on a luminometer by the dual LUC reporter system (Promega). The transfection efficacy was normalized by *Renilla* LUC. A minimum of triplicates of all experiments was carried out and similar results were obtained.

2.4. Generation of reporter gene constructs

A series of reporter gene constructs was prepared in the pGL3-Basic vector (Promega) to analyze promoter activity of the 5′-upstream region of the mouse nestin gene. Two sets of primers (P85F/P85R and P83F/P85R, Table 1) and the pNX7 plasmid were used to amplify an 887-bp and a 2660-bp fragment both at the 5′-non-coding and upstream flanking region of the mouse nestin gene. The fragments were cloned into the pGEM-T-easy vector (Promega) and the DNA sequences were verified. These fragments were then subcloned into pGL3-Basic vector to make the reporter gene constructs, pNESP−704/+183 and pNESP−2477/+183. A 3.2-kb fragment from the *Sac*I and *Aat*II digestion of plasmid pNX7 was inserted into pNESP-704/+183 to generate the plasmid pNESP−3809/+183. A *Kpn*I–*Sac*I polylinker from pBluescript II SK (−) was introduced into pNESP−3809/+183 to create pNESP(SK)−3809/+183. Then, three 5′-deletion constructs were generated through different restriction enzyme digestion and self-ligation of pNESP(SK)−3809/+183: pNESP−1672/+183 (PstI), pNESP−161/+183 (ApaI) and pNESP−3007/+183 (*Eco*RV and *Pvu*II). Similarly, two 3′-deletion constructs were obtained from pNESP−3809/+183: pNESP−3809/−2155 was created by *Nco*I digestion and self-ligation; pNESP−3809/−598 was made by digesting with *Aat*II and *Xho*I, blunting the ends with mung bean nuclease (Gibco), and then self-ligating.

To further analyze the promoter of the mouse nestin gene, additional deletion constructs were prepared. Plasmid pNESP−704/+183 was digested with *Mlu*I and *Sma*I, end-blunted with Klenow and self-ligated to produce pNESP−11/+183. Plasmid pNESP−161/+183 was digested with *Xho*I and *Sma*I, and further processed similarly to make pNESP−161/−12. Other deletion plasmids were constructed by amplifying pNX7 with different sets of primers and inserting these PCR fragments into the pGL3-Basic vector. All these constructs were confirmed by DNA sequencing. The constructs and corresponding primers are: pNESP+64/+183, P95F/P95R; pNESP−11/+73, P114F/P114R; pNESP+28/+131, P116F/P116R; pNESP−11/+131, P114F/P116R; pNESP−11/+112, P114F/P119R; pNESP+28/+138, P116F/P95R; pNESP−161/+27, P117AF/P118BR and rpNESP−161/+183, P120F/P120R. The sequences of the primers are listed in Table 1.

2.5. Mutagenesis

Site-directed mutagenesis of *cis*-elements in the nestin promoter region was performed as described previously [21]. All constructs were made by using pNX7 as the PCR template and pGL3-Basic digested by *Mlu*I–*Xho*I as the vector. The AP2 site-mutant pNESP AP2* was constructed by subcloning a *Mlu*I–*Eco*RI fragment amplified by the primer set P117AF/P117AR and an *Eco*RI–*Xho*I fragment amplified by the primer set P117BF/P117BR. Similarly, the TATA-like-mutant pNESP TATA-like*, the Sp1(U) site-mutant pNESP Sp1(U)* and the Sp1(D) site-mutant pNESP Sp1(D)* were constructed with the primer sets P117AF/P130AR + P130BF/P117BR, P117AF/P131AR + P131BF/P117BR, and P117AF/P132AR + P132BF/P117BR, respectively. The two Sp1 sites-mutant pNESP Sp1(U,D)* was constructed by subcloning an *Mlu*I–*Eco*RI fragment amplified by the primer set P117AF/P133AR using pNESP Sp1(D)* as the template and a *Eco*RI–*Xho*I fragment amplified by the primer set of P132BF/P117BR using pNESP−161/+183 as the template. All these constructs were verified by DNA sequencing. The sequences of the primers are listed in Table 1.

Table 1
Sequences of primers used for construction of plasmids

Primers	Sequences	Positions
P83F	5′-GAGAACGCGTGGCAGGTGGCTCACAACTATCT-3′	−2477/−2456
P85F	5′-GAGAACGCGTAGAGCCGCGTAACTTCTTCACT-3′	−704/−683
P85R	5′-GAGACTCGAGGTGGAGCACTAGAGAAGGGAGT-3′	+162/+183
P95F	5′-GAGAACGCGTATGAATACCCTCGCTTCAGCTC-3′	+64/+85
P95R	5′-GAGACTCGAGGTGGAGCACTAGAGAAGGGAGT-3′	+162/+183
P114F	5′-GAGAACGCGTGGGCTGTGTGTTGCACT-3′	−11/+6
P114R	5′-GAGACTCGAGGGGTATTCATACTCCCG-3′	+57/+73
P116F	5′-GAGAACGCGTTAGGGACCGCCCCTTTT-3′	+28/+44
P116R	5′-GAGACTCGAGGAGTAGCGGCGACAGTG-3′	+115/+131
P118BR	5′-GAGACTCGAGGGCCTTAACCCTTTAGAG-3′	+10/+27
P119R	5′-GAGACTCGAGCCAGCGGAAGCGGAGGA-3′	+96/+112
P120F	5′-GAGACTCGAGGGGCCCAGTTCTGTGCA-3′	−161/−145
P120R	5′-GAGAACGCGTTGGAGCACTAGAGAAGG-3′	+166/+182
P117AF	5′-GAGAACGCGTTGGGGCCCAGTTCTGT-3′	−163/−148
P117AR	5′-TCATACTgaattCGGCCGGGCGGAAAAG-3′	+40/+67
P117BF	5′-CCGGCCGaattcAGTATGAATACCCTCGC-3′	+49/+77
P117BR	5′-GAGACTCGAGGGAGCACTAGAGAAGGG-3′	+165/+181
P130AR	5′-AGGGTAagatctCTCCCGCCGGCCGGGC-3′	+47/+74
P130BF	5′-CGGGAGggatccTACCCTCGCTTCAGCTC-3′	+57/+85
P131AR	5′-GAAAAGaGatctTCCCTAGGCCTTAACC-3′	+18/+45
P131BF	5′-TAGGGAggatCCCTTTTCCGCCCGGCCGG-3′	+28/+56
P132AR	5′-GCCGGCgaattcGAAAAGGGGCGGTCCC-3′	+30/+57
P132BF	5′-CTTTTCgaattcGCCGGCGGGAGTATGAA-3′	+40/+68
P133AR	5′-GCCGGCgaattcGAAAAGGGatccTCCC-3′	+30/+57

Primers P83F, P85F, P85R, P95F, P95R, P114F, P114R, P116F, P116R, P118BR, P119R, P120F, P120R, P117AF and P117BR introduced a restriction enzyme recognition site for *Mlu*I or *Xho*I for convenient digestion of the PCR product. Primers P117AR and P117BF were used for pNESP AP2* and contain *Eco*RI sites. Primers P130AR and P130BF were used for pNESP TATA-like* and contain *Bgl*II and *Bam*HI sites, respectively. Primers P131AR and P131BF were used for pNESP Sp1(U)* and contain *Bgl*II and *Bam*HI sites, respectively. Primers P132AR and P132BF were used for pNESP Sp1(D)* and contain *Eco*RI sites. Primer P133AR was used for pNESP Sp1(U,D)* and contains *Eco*RI and *Bam*HI sites. The lowercase letters denote mutated nucleotides.

Table 2
Sequences of synthetic oligonucleotides used in EMSA

Oligonucleotides	Mutations	Sequences
Probe Sp1	Wild type	5′-GCCTAGGGACCGCCCCTTTTCCGCCCGG-3′
Probe Sp1(U)*	Sp1(U)	5′-GCCTAGGGAagatCCCTTTTCCGCCCGG-3′
Probe Sp1(D)*	Sp1(D)	5′-GCCTAGGGACCGCCCCTTTTCgaattcG-3′
Probe Sp1(U,D)*	Sp1(U), Sp1(D)	5′-GCCTAGGGAggatCCCTTTTCgaattcG-3′
Probe AP2, TATA-like	Wild type	5′-GGCCGGCGGGAGTATGAATACCC-3′
Probe SIE	Wild type	5′-GATCCAGTTCCCGTCAATCGATC-3′

For double-stranded oligonucleotides used in EMSA, only the sense strands are shown. Putative binding sites are underlined. The lowercase letters denote mutated nucleotides.

2.6. Electrophoretic mobility-shift assay (EMSA) and supershift analysis

Nuclear extracts (NE) were prepared from P19 cells and from mouse postnatal day 5 (P5) cerebella as described previously [22,23]. Nuclear proteins (3–6 μg) were preincubated with 2 μg poly(dI-dC) · poly(dI-dC) (Amersham/Pharmacia, Uppsala, Sweden) on ice for 30 min in 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl and 50 mM Tris–HCl (pH 7.5). ^{32}P-labelled DNA probe was added to the binding reaction mixture and incubated at 25 °C for 30 min. Competition experiment was performed by preincubation of a 100-fold molar excess of unlabelled probe together with nuclear protein. In the case of the supershift assay, the antibodies (1 μg) against Sp1 (Sp1(PEP2), Santa Cruz Biotechnology, Santa Cruz, CA, USA), Sp3 (Sp3(D-20), Santa Cruz) and β-tubulin (Sigma, St. Louis, MO, USA) were added to the reaction mixture before the addition of labelled probe. Protein–DNA complexes were fractioned on a prerun non-denaturing, 6% polyacrylamide gel and subjected to electrophoresis in 0.5 × TBE buffer. Gels were dried and exposed to X-ray film with an intensifying screen at −80 °C. Oligonucleotide sequences of probes and competitors are reported in Table 2.

3. Results

3.1. Determination of transcription initiation sites

To map the 5′ end(s) of the mouse nestin gene, primer extension was performed with an anti-sense oligonucleotide (primer PE) located in exon 1. Three end-points were obtained in poly(A)$^+$ RNA from E15.5 mouse embryo brain and from P19 cells, but not in tRNA from yeast (Fig. 1A), indicating three transcription initiation sites of the gene. These three end-points were aligned with a dideoxy nucleotide sequencing ladder prepared using the same primer (Fig. 1B), indicating that these sites corresponded to positions of 216, 126 and 110 bp upstream of the translation start codon (ATG). The most 5′-upstream transcription initiation site was defined as +1, followed by initiation sites at positions +91 and +107. These results suggest that mouse nestin mRNA is transcribed from at least three different initiation sites in mouse fetal brain and in P19 cells.

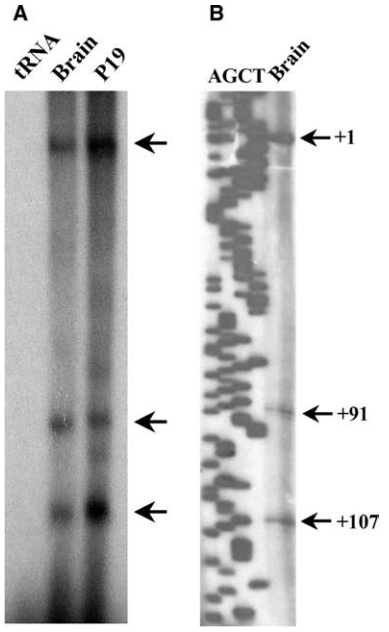

Fig. 1. Determination of transcription initiation sites of the mouse nestin gene. A, B: Primer extension assays were performed with 2 μg of poly(A)$^+$ RNA from E15.5 mouse brain (lane "Brain") or from P19 cells (lane "P19"), or with 10 μg of yeast tRNA (lane "tRNA") as negative control by using a ^{32}P-labelled oligonucleotide within exon 1 as described in Section 2. The transcription initiation sites are indicated by arrows and the numbers on the right denote their corresponding positions relative to the first transcription initiation site.

3.2. Promoter analysis of the mouse nestin gene

Mouse embryonic carcinoma P19 cells can be induced by retinoic acid (RA) to differentiate into neural cells including neurons and glial cells [24]. Nestin mRNA and protein could be detected in non-induced P19 cells. Analogous to its expression in the neural stem cells in vivo, nestin was up-regulated upon RA induction, and declined along with neural cell maturation during RA-induced P19 cell neural differentiation ([20] and Tang, K. and Jing, N. unpublished observation). Therefore, P19 cells were used as an in vitro system for promoter analysis of the mouse nestin gene.

To identify potential regulatory elements in the 5′-flanking region of the mouse nestin gene, we generated a series of promoter-LUC 5′-deletion constructs. The longest construct pNESP−3809/+183 displayed a higher promoter activity in P19 cells than that of the control plasmid, pGL3-Basic (Fig. 2A). Deletion constructs pNESP−3007/+183 and pNESP−2477/+183, however, reduced the promoter activity by 31% and 42%, respectively. Further deletions to positions −1672 (pNESP−1672/+183), −704 (pNESP−704/+183), and −161 (pNESP−161/+183) resulted in a gradual increase in the promoter activity. The highest promoter activity was observed in the construct of pNESP−161/+183, suggesting that there are inhibitory elements in the nestin promoter within the region −3809 to −161. The 3′-truncation deletions from position +183 to −598, pNESP−3809/−598 and to −2155, pNESP−3809/−2155 completely abolished the promoter activity. All these results suggest that the isolated 4 kb genomic fragment of the mouse nestin gene contains a functional promoter and that the core sequence of the basal promoter is localized in the region −161 to +183.

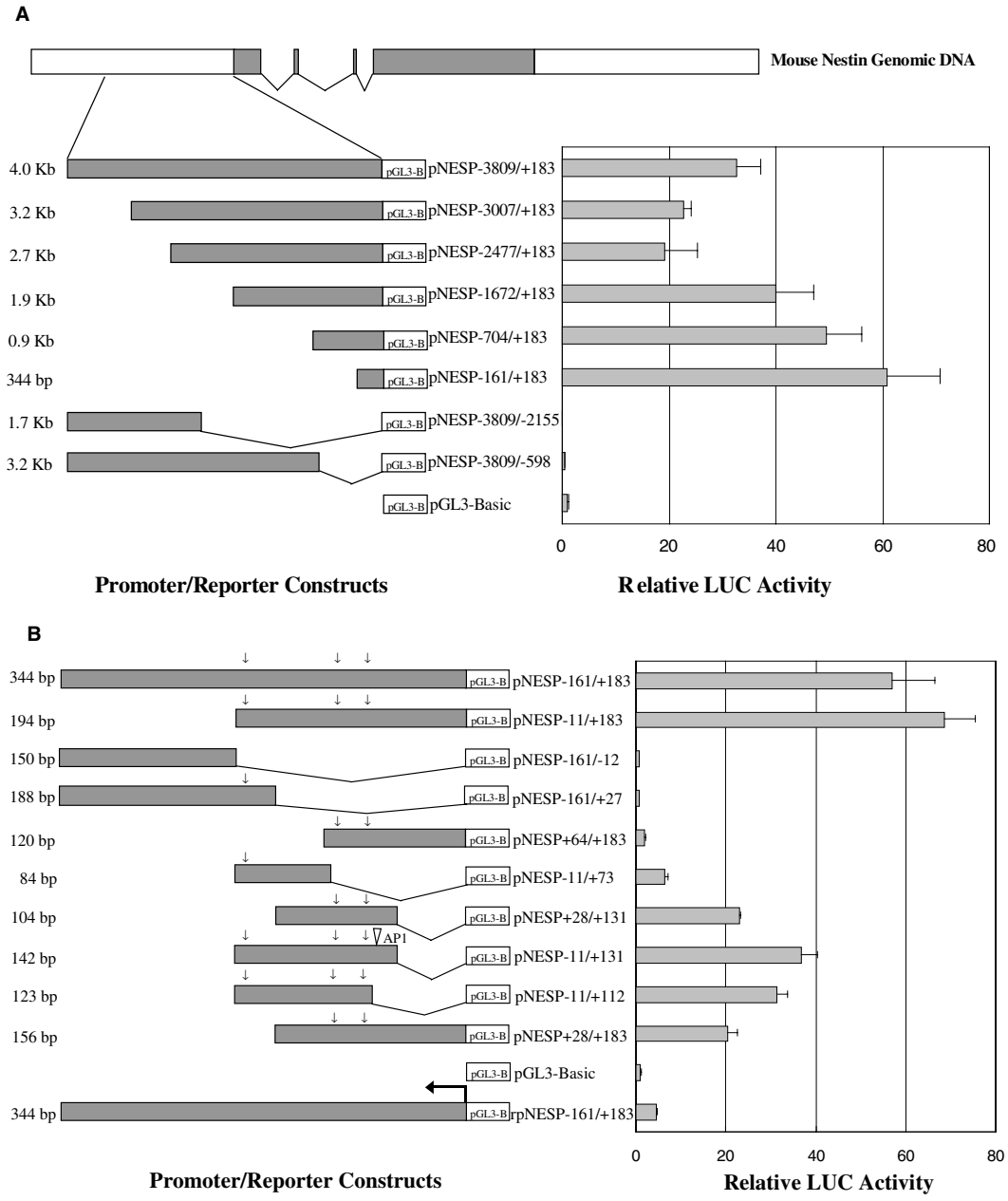

Fig. 2. Functional analysis of the mouse nestin gene promoter. (A) The promoter activity of the mouse nestin gene resides in the region from −161 to +183. Left panels: schematic representation of the fragments used for generating the different deletion constructs tested in transfection experiments. Right panels: LUC activities obtained after transfection in P19 cells. (B) Refining the functional region in the nestin gene promoter. Left panels: schematic representation of promoter deletion constructs used for transient transfection. Transcription initiation sites are marked by filled arrowheads and a putative AP1 element is indicated by an open arrowhead. Right panels: LUC activities obtained after transfection in P19 cells. Transfection and LUC assays were performed as described in Section 2. The LUC activity of each construct was graphed as the fold increases over that of pGL3-Basic vector. The results are presented as means ± S.D. Each experiment was repeated at least three times and similar results were obtained.

3.3. Refining the functional region in the nestin gene promoter

To further characterize the core promoter region, additional deletion and truncation constructs were generated and transfected into P19 cells (Fig. 2B). The −161/+183 region was first divided into a 3′-proximal 194-bp fragment (pNESP−11/+183) and a 5′-proximal 150-bp fragment (pNESP−161/−12). We found that the downstream 194-bp fragment possessed full promoter activity, while the upstream 150-bp region had no promoter activity. When we further divided the −11/+183 region into two overlapping fragments, a 3′-proximal 120-bp fragment (pNESP+64/+183) and a 5′-proximal 84-bp fragment (pNESP−11/+73), the promoter activities of both constructs decreased to very low levels. Therefore, the region from −11 to +183 probably possesses the minimal promoter activity. To test whether any possible element resides in the 5′-upstream region, we extended (pNESP−161/−12) another 38-bp towards the 3′-terminus, from position −12 to position +27 (pNESP−161/+27), which is beyond the first initiation site.

However, we still could not detect any activity. These results suggest that the nestin promoter localizes in the 5′-non-coding region and a few basepairs upstream of the mouse nestin gene.

As the next step, we constructed internal deletions within the established minimal promoter region (Fig. 2B). An 104-bp fragment from position +28 to +131 (pNESP+28/+131) showed a significant increase in promoter activity as compared to that of pNESP+64/+183 and pNESP−11/+73, implying the importance of potential *cis*-elements and two downstream transcription initiation sites (+91, +107) within this region. As we further extended another 38-bp upstream from position +28 to give pNESP−11/+131, promoter activity increased by approximately 60% as compared to that of pNESP+28/+131. This 38-bp region contains the first transcription initiation site. Additional transcripts starting at this site might account for the increased promoter activity. However, deletion of 20 bp at the 3′-end to position +112 (pNESP−11/+112), thereby removing an AP1-binding site, only caused a slight decrease of the promoter activity, suggesting that the AP1-binding site is not critical for the nestin promoter. The reporter gene expression was very low if the fragment −161/+183 was in the reversed orientation (rpNESP−161/+183). As a summary, these results indicate that the proximal promoter is primarily localized in the region from −11 to +183, and the AP1-binding site may not be required for the promoter activity of the mouse nestin gene.

3.4. Two Sp1-binding sites indispensable for promoter activity

Sequence analysis of the core promoter region −161/+183 revealed several putative *cis*-elements such as AP1 and AP2-binding sites, a TATA-like sequence, and two Sp1-binding sites (Fig. 3A). In order to evaluate the biological significance of these regulatory elements, reporter constructs carrying mutated sites were generated and transfected into P19 cells. As shown in Fig. 3B, mutations of the AP2-binding site (pNESP AP2*) and the TATA-like sequence (pNESP TATA-like*) exhibited a slight decrease of promoter activity as compared with that of the wild-type construct (pNESP−161/+183). In contrast, mutation of the downstream Sp1-binding site (pNESP Sp1(D)*) resulted in a moderate decrease of promoter activity to 64%, mutation of the upstream Sp1 site (pNESP Sp1(U)*) caused a sharp reduction to 27% and mutation of both Sp1 sites (pNESP Sp1(U,D)*) led to a minimum activity of 15% as compared to the wild-type. Consistently, the deletion of these two adjacent Sp1 sites from +28 to +64 in exon 1 decreased the reporter activity more than 10-fold (Fig. 2B,

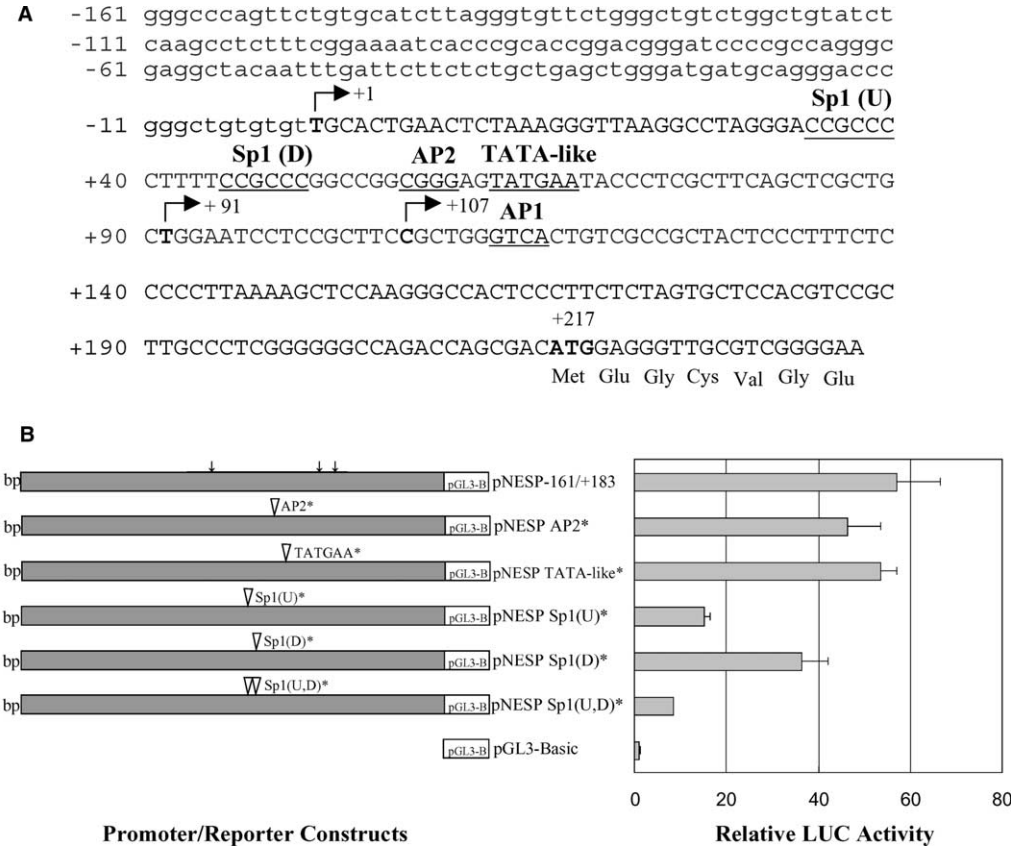

Fig. 3. Identification of the potential *cis*-elements in the −161/+183 region. (A) Positions of potential regulatory elements in the 5′ flanking and non-coding region −161 to +183 of the mouse nestin gene. The position of the first transcription initiation site is defined as +1. The core sequences of the putative *cis*-elements are underlined. The three transcription initiation sites are indicated by bent arrows. (B) Two adjacent Sp1-binding sites are essential to the nestin gene promoter activity. Left panel: schematic representation of site mutation reporter gene constructs used for transfection experiments. The transcription initiation sites are marked by filled arrowheads and the putative *cis*-elements are indicated by open arrowheads. The asterisks indicate that these sites are mutated. Right panels: LUC activities obtained after transfection in P19 cells. Transfection and LUC assays were performed as described in Section 2. The LUC activity of each construct was graphed as the fold increases over that of pGL3-Basic vector. The results are presented as means ± S.D. Each experiment was repeated at least three times and similar results were obtained.

pNESP+28/+183 vs. pNESP+64/+183). These results suggest that these two Sp1-binding sites contribute to the transcriptional activity of the mouse nestin promoter.

3.5. *Sp1 and Sp3 binding to the minimal promoter of mouse nestin gene*

To determine the protein-binding activity of the putative *cis*-elements in the minimal promoter region, we prepared NE from P19 cells and performed EMSA experiments. Two shifted bands were observed with radiolabelled oligonucleotides containing two Sp1 sites (Probe Sp1, position +25/+52, Table 2) (Fig. 4A, lane 1). The broad band of lower mobility in Fig. 4 was actually composed of two closely spaced Sp1 and Sp3 complexes (see below), and this was resolved with a lower exposure of the autoradiograph (data not shown). All shifted bands were successfully competed away with the non-radiolabelled probe (Fig. 4A, lane 2), but not with an irrelevant competitor such as Probe SIE (Fig. 4A, lane 3). To further confirm the protein-binding specificity of Sp1-binding sites, three Sp1-mutated oligonucleotides (Probe Sp1(U)*, Probe Sp1(D)* and Probe Sp1(U,D)*) were used as competitors. The oligonucleotide containing a mutation in the downstream Sp1 site (Probe Sp1(D)*) effectively competed with the radiolabelled probe (Fig. 4A, lane 5), while the oligonucleotides containing mutations in the upstream (Probe Sp1(U)*) or in both Sp1 sites (Probe Sp1(U,D)*) failed to compete (Fig. 4A,

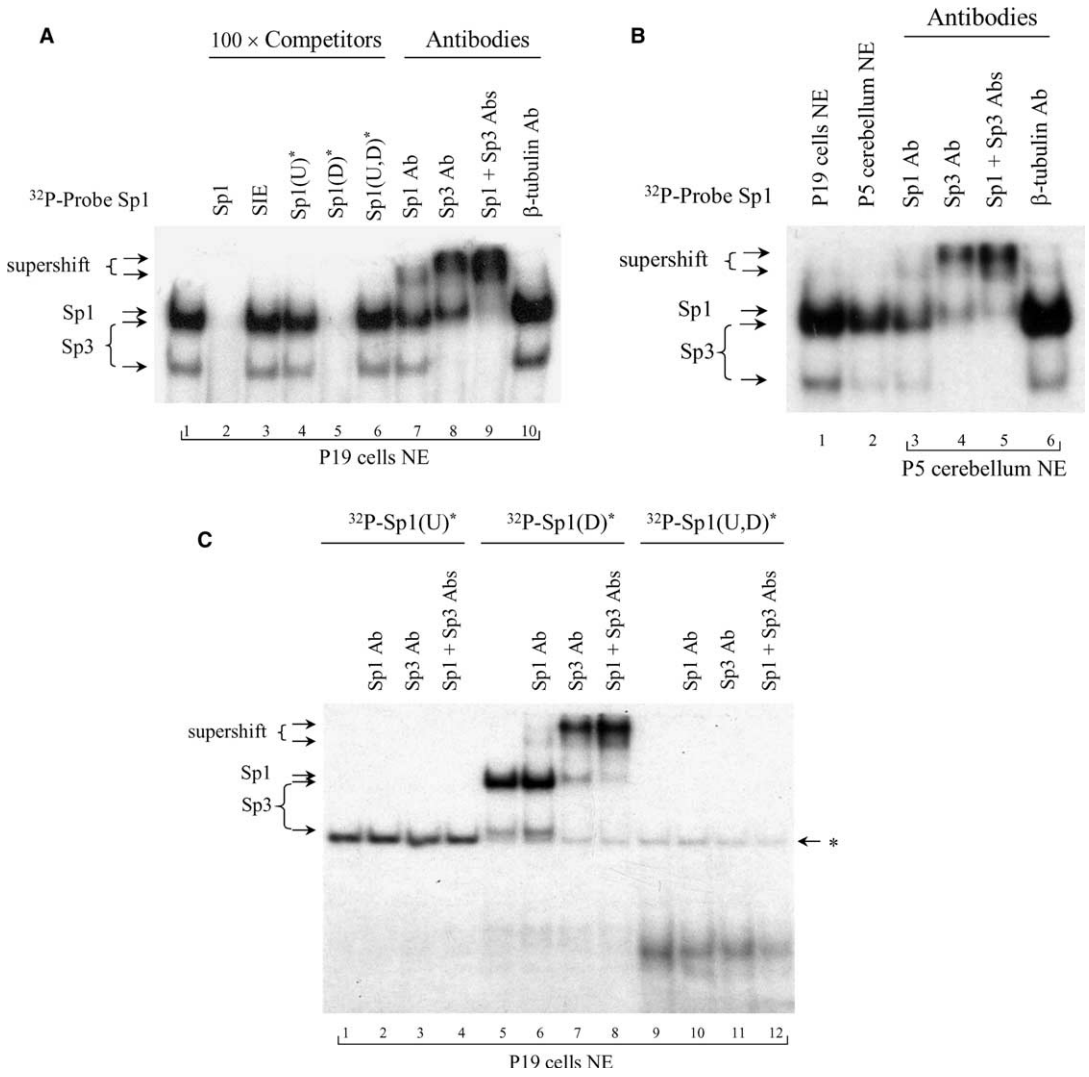

Fig. 4. EMSA of the +25 to +52 region of the mouse nestin gene promoter. (A) ^{32}P-labelled Sp1 probe (+25/+52) was incubated with NE from P19 cells (P19 cells NE) (lanes 1–10). The competition experiments were performed with 100-fold excess of wild-type Sp1 oligonucleotide (Sp1) (lane 2), non-relevant oligonucleotide (SIE) (lane 3), and mutated Sp1 sites oligonucleotides (Sp1(U)*, Sp1(D)*, Sp1(U,D)*) (lanes 4–6). The supershift assays were performed with antibodies against Sp1 protein (Sp1 Ab) (lane 7), Sp3 protein (Sp3 Ab) (lane 8), Sp1 and Sp3 proteins (Sp1+Sp3 Abs) (lane 9) and non-relevant β-tubulin protein (β-tubulin Ab) (lane 10). Note that there are the supershifted bands in lanes 7–9. (B) ^{32}P-labelled Sp1 probe (+25/+52) was incubated with NE from P19 cells (lane 1) and from mouse P5 cerebellum (P5 cerebellum NE) (lanes 2–6). The supershift assays were performed with antibodies against Sp1 protein (Sp1 Ab) (lane 3), Sp3 protein (Sp3 Ab) (lane 4), Sp1 and Sp3 proteins (Sp1+Sp3 Abs) (lane 5) and non-relevant β-tubulin protein (β-tubulin Ab) (lane 6). Note that there are the supershifted bands in lanes 3–5. (C) EMSA with mutated Sp1 probes. The NE from P19 cells (P19 cells NE) were incubated with a ^{32}P-labelled oligonucleotide carrying a mutation in the upstream Sp1 site (^{32}P-Sp1(U)*) (lanes 1–4), the downstream Sp1 site (^{32}P-Sp1(D)*) (lanes 5–8), or both Sp1 sites (^{32}P-Sp1(U,D)*) (lanes 9–12). The supershift assays were performed with antibodies against Sp1 protein (Sp1 Ab) (lanes 2, 6, 10), Sp3 protein (Sp3 Ab) (lanes 3, 7, 11), Sp1 and Sp3 proteins (Sp1+Sp3 Abs) (lanes 4, 8, 12). Note that there are gel-shifted bands in lane 5 and supershifted bands in lanes 6–8.

lanes 4, 6). The radiolabelled Probe AP2, TATA-like (position +51/+73) containing the AP2-binding site and TATA-like motif did not form any shifted bands when assayed with nuclear proteins from P19 cells (data not shown). These results suggest that the Sp1 family of transcription factors participate in binding to the minimal promoter and that the 5′-upstream Sp1-binding site is more important for the binding of nuclear proteins.

To identify the proteins binding to these Sp1 sites, supershift assays were performed with antibodies specific for Sp1 and Sp3 proteins (Fig. 4A, lanes 7–10). The slowest migrating protein–DNA complex was supershifted by an antibody against Sp1 (Fig. 4A, lane 7), while the two fast migrating bands were supershifted by the Sp3-specific antibody (Fig. 4A, lane 8). All three bands were supershifted when both antibodies were present (Fig. 4A, lane 9). No supershifted band was observed, however, when the non-relevant antibody against β-tubulin was used (Fig. 4A, lane 10). These results indicated that Sp1 and Sp3 proteins bound specifically to the +25/+52 region containing two Sp1 sites.

Since the nestin gene is expressed primarily in the neural stem cells, it is interesting to see whether these Sp1-binding sites are also involved in the development of CNS. We thus prepared NE from the mouse cerebellum of postnatal day 5 (P5) when nestin was expressed [5] and performed EMSA with radiolabelled Probe Sp1 (Fig. 4B). Three retarded protein–DNA complexes were also observed in NE of mouse cerebellum at P5 (Fig. 4B, lanes 1, 2). These complexes were supershifted by adding the antibodies against Sp1 and Sp3 (Fig. 4B, lanes 3–5), but not with the antibody against β-tubulin (Fig. 4B, lane 6). Therefore, we conclude that the Sp1 sites in the minimal promoter of the mouse nestin gene might also be occupied in vivo by the transcription factors Sp1 and Sp3.

3.6. Selective binding of Sp1 and Sp3 proteins to the 5′-upstream Sp1 site

To further distinguish the different binding activities between the two adjacent Sp1-binding sites, radiolabelled oligonucleotides with the single or double mutated Sp1 site(s) (Probe Sp1(U)*, Probe Sp1(D)*, Probe Sp1(U,D)*) were used to perform supershift assays with NE from P19 cells (Fig. 4C). Only one complex was observed with probes Sp1(U)* or Sp1(U,D)* (Fig. 4C, lanes 1–4 and 9–12). In contrast, four complexes were obtained with probe Sp1(D)*. Only the three complexes unique to Sp1(D)* could be supershifted by the Sp1- and Sp3-specific antibodies (Fig. 4C, lanes 5–8). The complex common to all probes is presumably non-specific, or at least does not contain Sp1 or Sp3, since it could not be supershifted. Overall, these results show that Sp1 and Sp3 can bind selectively to the more upstream Sp1 binding site.

3.7. The functions of the mouse nestin gene promoter in different cell lines

To examine whether the promoter of the mouse nestin gene possesses regulatory elements in mediating cell-type specific expression, tests were carried out with cell lines of various origins, such as mouse embryonic carcinoma cell F9, rat glioma C6, Chinese hamster ovary (CHO), mouse fibroblast NIH3T3, and human neuroblastoma SH-SY5Y (SY5Y). The reporter gene constructs pNESP−3809/+183 and pNESP−161/+183 were transfected into these cells, and the simian virus 40 (SV40) promoter driven reporter gene (pGL3-Promoter) was used as the positive control (Fig. 5). Both pNESP−161/+183 and pNESP−3809/+183 displayed a significant level of promoter activity compared with that of the SV40 promoter in these cell lines. The fact that comparable promoter activities were observed in different cell line suggests that there are no cell-type specific elements in the promoter region of the mouse nestin gene up to position −3809.

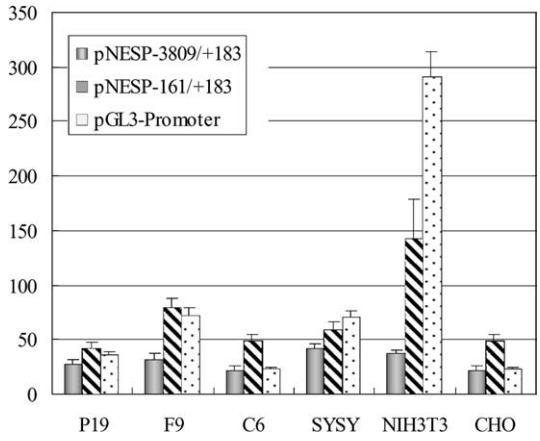

Fig. 5. Promoter activity of the mouse nestin gene in different mammalian cell lines. The reporter gene constructs, pNESP-3809/+183, pNESP−161/+183 and pGL3-Promoter, were transfected into P19, F9, C6, SY5Y, NIH3T3 and CHO cells. The LUC activities were assayed as described in Section 2, and were graphed as the fold increases over that of pGL3-Basic vector. The results are presented as means ± S.D. Each experiment was repeated at least three times, and similar results were obtained.

4. Discussion

In this study, we determined the transcription initiation sites of the mouse nestin gene. Analysis of its 5′-flanking and non-coding region revealed that the minimal promoter is localized in the region −11/+183. Two closely spaced Sp1-binding sites are critical for the expression of the nestin gene. EMSA and supershift assays showed that the 5′-upstream Sp1 site was selectively occupied by Sp1 and Sp3 proteins from P19 cells and the P5 mouse cerebellum.

In order to identify regulatory *cis*-elements of the mouse nestin gene, a series of deletion constructs from 5′-flanking region up to position −3809 were analyzed. We found that a construct containing mainly 5′-non-coding region of exon 1 (−11/+183) demonstrated a full promoter activity, and therefore we believe that potential binding sites for Sp1 and other putative transcription factors in this region are important for the expression of the nestin gene. Indeed, a significant decrease in reporter gene expression was observed when the sequences of the two adjacent Sp1 sites were either deleted or mutated (Figs. 2B and 3). Thus, it is quite likely that these Sp1 consensus sequences contribute greatly to the expression of the mouse nestin gene.

Gel retardation assays were used to examine the binding activity of proteins to these Sp1 consensus sequences. Sp1 and Sp3 proteins from NE of P19 cells and of P5 mouse cerebellum were found to bind to an oligonucleotide containing these Sp1 sites (Figs. 4A and B). The Sp4 protein, another Sp family member, could also recognize the GC-box motif (GGGCGG)

with similar affinity as the Sp1 and Sp3 proteins [25]. The antibodies specific to Sp1 and Sp3, however, could supershift all the protein–DNA complexes formed by P19 cell NE. This result indicates that it is indeed Sp1 and Sp3 proteins that bind to these Sp1 sites. The competition assays suggested that Sp1 and Sp3 proteins selectively bind to the upstream Sp1 site, since an oligonucleotide carrying a mutation in the upstream Sp1 could not interfere with the formation of the shifted bands (Fig. 4A). This hypothesis was proven by utilizing the Sp1 site-mutated oligonucleotides as the radiolabelled probes in the supershift assays (Fig. 4C).

This study showed that the nestin promoter lacks a functional TATA box, that it has three transcription initiation sites, and that the two Sp1 sites are important for basal transcription activity. The presence of multiple transcription start sites is characteristic of TATA-less GC-rich promoters, as in the promoters of the mouse sulfonylurea receptor 1 gene (SUR1) [26], the human death receptor 5 gene (DR5) [27] and the endoplasmic reticulum protein, *ERp29*, gene [28]. Moreover, it has been indicated that selective binding to several putative Sp1 sites might be a common mechanism for this type of promoters, by which transcription factors Sp1 and/or Sp3 mediate the transactivation, as in the cases of the human c-kit gene [29], the transforming growth factor β type I receptor gene (TGF-βRI) [30], and the nicotinamide adenine dinucleotide phosphate cytochrome P-450 oxidoreductase gene [31].

Sp1 and Sp3 proteins are known to be ubiquitous transcription factors and are involved in the transcriptional regulation of various ''house-keeping'' genes. It has been well documented that binding of Sp proteins to GC-box motif can trigger basal gene expression [32]. Results in this study illustrated the importance of of the Sp1-binding sites and Sp family proteins for the basal promoter activity of the mouse nestin gene. Consistent with this, the nestin gene promoter showed no cell-type specificity, and it could function in different mammalian cell lines (Fig. 5). Questions remain, however, as to how the nestin gene establishes its tissue specific expression in neural stem cells in vivo. A reasonable speculation is that the specific CNS expression may be mediated by the tissue-specific enhancer in the second intron of the nestin gene [14,16–18]. Similar to the nestin gene promoter, many intermediate filament gene promoters are also regulated by Sp1 and Sp3 proteins. Activation of the lamin A proximal promoter is mediated by Sp1 and Sp3 transcription factors, and the function of Sp3 is more pronounced than that of Sp1 [33]. Sp1 also controls the expression of a type-III intermediate filament protein, peripherin [34]. Moreover, several GC-rich sequences are located in the promoter of the human vimentin gene, while the one close to the transcription initiation site is required for promoter function and gene expression [35].

In general, Sp1 can activate gene expression, whereas Sp3 can both repress and activate transcription. For the activation of gene expression, Sp family members may form either homodimer or heterodimer complexes with other nuclear proteins [32]. Further studies will be needed to elucidate the possible interaction(s) between Sp proteins and other transcription factors during the transactivation of the mouse nestin gene promoter.

Acknowledgements: We thank Drs. K. Yokoyama, H. Kondoh and S. Kulish for valuable advices and their critical reading of this manuscript, and Dr. M. Berlyn (*E. coli* Genetic Stock Center) for providing host strain GBE180. This work was supported in part by grants from the National Key Basic Research and Development Program (G1999054000, 2002CB713802), National Natural Science Foundation of China (39870283, 39930090, 90208011).

References

[1] Frederiksen, K. and McKay, R.D. (1988) J. Neurosci. 8, 1144–1151.
[2] Lendahl, U., Zimmerman, L.B. and McKay, R.D. (1990) Cell 60, 585–595.
[3] Dahlstrand, J., Zimmerman, L.B., McKay, R.D. and Lendahl, U. (1992) J. Cell Sci. 103, 589–597.
[4] Reynolds, B.A. and Weiss, S. (1992) Science 255, 1707–1710.
[5] Dahlstrand, J., Lardelli, M. and Lendahl, U. (1995) Brain Res. Dev. Brain Res. 84, 109–129.
[6] Hockfield, S. and McKay, R.D. (1985) J. Neurosci. 5, 3310–3328.
[7] Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U. and Frisen, J. (1999) Cell 96, 25–34.
[8] Sejersen, T. and Lendahl, U. (1993) J. Cell Sci. 106, 1291–1300.
[9] About, I., Laurent-Maquin, D., Lendahl, U. and Mitsiadis, T.A. (2000) Am. J. Pathol. 157, 287–295.
[10] Wroblewski, J., Engstrom, M., Edwall-Arvidsson, C., Sjoberg, G., Sejersen, T. and Lendahl, U. (1997) Differentiation 61, 151–159.
[11] Kachinsky, A.M., Dominov, J.A. and Miller, J.B. (1995) J. Histochem. Cytochem. 43, 843–847.
[12] Frojdman, K., Pelliniemi, L.J., Lendahl, U., Virtanen, I. and Eriksson, J.E. (1997) Differentiation 61, 243–249.
[13] Yang, J., Bian, W., Gao, X., Chen, L. and Jing, N. (2000) Mech. Dev. 94, 287–291.
[14] Zimmerman, L., Parr, B., Lendahl, U., Cunningham, M., McKay, R., Gavin, B., Mann, J., Vassileva, G. and McMahon, A. (1994) Neuron 12, 11–24.
[15] Cheng, L., Yang, J. and Jing, N. (2000) Acta Biochim. Biophys. Sin. 32, 649–652.
[16] Lothian, C. and Lendahl, U. (1997) Eur. J. Neurosci. 9, 452–462.
[17] Josephson, R., Muller, T., Pickel, J., Okabe, S., Reynolds, K., Turner, P.A., Zimmer, A. and McKay, R.D. (1998) Development 125, 3087–3100.
[18] Lonigro, R., Donnini, D., Zappia, E., Damante, G., Bianchi, M.E. and Guazzi, S. (2001) J. Biol. Chem. 276, 47807–47813.
[19] Yang, J., Cheng, L., Yan, Y., Bian, W., Tomooka, Y., Shiurba, R. and Jing, N. (2001) Biochim. Biophys. Acta 1520, 251–254.
[20] Yan, Y., Yang, J., Bian, W. and Jing, N. (2001) Neurosci. Lett. 302, 89–92.
[21] Takashima, S., Yoshida, Y., Kanematsu, T., Kojima, N. and Tsuji, S. (1998) J. Biol. Chem. 273, 7675–7683.
[22] Andrews, N.C. and Faller, D.V. (1991) Nucleic Acids Res. 19, 2499.
[23] Planas, A.M., Berruezo, M., Justicia, C., Barron, S. and Ferrer, I. (1997) J. Neurochem. 68, 1345–1351.
[24] Bain, G., Ray, W.J., Yao, M. and Gottlieb, D.I. (1994) Bioessays 16, 343–348.
[25] Castoldi, M. and Chu, M.L. (2002) Biochem. J. 362, 41–50.
[26] Hernandez-Sanchez, C., Ito, Y., Ferrer, J., Reitman, M. and LeRoith, D. (1999) J. Biol. Chem. 274, 18261–18270.
[27] Yoshida, T., Maeda, A., Tani, N. and Sakai, T. (2001) FEBS Lett. 507, 381–385.
[28] Sargsyan, E., Baryshev, M., Backlund, M., Sharipo, A. and Mkrtchian, S. (2002) Gene 285, 127–139.
[29] Park, G.H., Plummer III, H.K. and Krystal, G.W. (1998) Blood 92, 4138–4149.
[30] Ji, C., Casinghino, S., McCarthy, T.L. and Centrella, M. (1997) J. Biol. Chem. 272, 21260–21267.
[31] O'Leary, K.A., McQuiddy, P. and Kasper, C.B. (1996) Arch. Biochem. Biophys. 330, 271–280.
[32] Suske, G. (1999) Gene 238, 291–300.
[33] Muralikrishna, B. and Parnaik, V.K. (2001) Eur. J. Biochem. 268, 3736–3743.
[34] Ferrari, N., Desmarais, D. and Royal, A. (1995) Gene 159, 159–165.
[35] Izmailova, E.S., Wieczorek, E., Perkins, E.B. and Zehner, Z.E. (1999) Gene 235, 69–75.