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Involvement of the GC-rich sequence and specific proteins (Sp1/Sp3) in the basal transcription activity of neurogranin gene

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Abstract

Neurogranin (Ng), a neuronal protein implicated in learning and memory, contains a TATA-less promoter. Analysis of 5′-deletion mutations and site-directed mutations of the mouse Ng promoter revealed that a 258 bp 5′-flanking sequence (+3 to +260) conferred the basal transcription activity, and that the GC-rich sequence (+22 to +33) served as an important determinant of the promoter activity. Transient transfection of the Sp1 expression plasmid transactivated the reporter activity in neuroblastoma N2A cells while knocking down of endogenous Sp1 expression resulted in a 2.5-fold reduction of the reporter activity in HEK 293 cells. Exogenous expression of Sp3 in HEK 293 cells, however, repressed the reporter activity by 50%. Nevertheless, by gel shift assays, Sp1 and Sp3 were not found to be responsible for the protein-DNA complexes formed by the GC-rich sequence. Moreover, a nuclear factor from the mouse brain tissues was discovered to bind to multiple AT-rich regions in Ng promoter.

Keywords: Neurogranin; GC-rich sequence; Sp1; Sp3; Transcription factors; Basal transcription activity; EMSAs; AT-rich motif; Promoter binding proteins

Neurogranin/RC3 (Ng) is a postsynaptic protein that binds to calmodulin (CaM) in the absence of or in low levels of Ca^{2+} [1,2]. It regulates the availability of Ca^{2+}/CaM complex and modulates the homeostasis of intracellular calcium in neurons [3–5]. The Ng level in the central nervous system (CNS) is regulated by thyroid hormone, retinoic acid, and vitamin A [6–10], whose insufficient levels are concomitantly linked with an age-related decrease in Ng level [11–14]. Ng gene transcription could be induced by triiodothyronine (T3) in hypothalamic GT1-7 cells [15]. Ng gene is composed of four exons and three introns and the first two exons encode the Ng protein. A thyroid hormone responsive element was identified in the first intron of the human Ng gene homolog [16,17]. Ng promoter lacks the canonical TATA, GC, and CCAAT boxes in the proximal upstream region of the start sites and the basal promoter activity could be up-regulated by phorbol ester and phorbol ester-binding PKCs in HEK 293 cells [18]. Sequence analysis on rat Ng promoter showed that a GC-rich sequence (+11 to +40) is probably an overlapping binding site for transcriptional factors and may be important for basal transcription activity of Ng [18]. Several possible cis-regulatory elements including GR, GCF, SP-1, AP-1, AP-2, and PEA3-binding sites were predicted to reside in the 5′-flanking region of Ng gene [18,19]. A 20 kDa chromosomal non-histone high-mobility-group protein (HMG) that binds to AT-rich regions of Ng promoter was revealed to be a PKC substrate whose binding affinity to Ng promoter was dramatically reduced upon phosphorylation [18,20].

Currently, only very sparse information is available for the roles of *cis*-regulatory elements and *trans*-factors in the transcription regulation of Ng. The goal of the

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present study was to characterize the possible roles of Sp-family transcription factors and the aforementioned GC-rich sequence in conferring the basal transcription activity of Ng. In addition, we were interested in identifying novel DNA-binding factors for Ng promoter. We herein provide evidence that Sp1 and Sp3 transcription factors as well as the GC-rich sequence (+22 to +33) are important determinants in the basal transcription activity of Ng. In addition, an unknown nuclear factor from the mouse brain tissues was discovered to have binding affinity to AT-rich motif at multiple sites of the 5′-flanking sequence of Ng gene.

Materials and methods

Cell culture. Human embryonic kidney 293 (HEK 293), neuroblastoma Neuro-2a (N2A) (ATCC, Rockville, MD), and hypothalamic GT1-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 25 mM Hepes, 3.7 g/L NaH_2CO_3, 100 U/ml penicillin, and 100 μg/ml streptomycin, with a final pH of 7.3–7.4. Cells were cultured in a humidified incubator with 5% CO_2 at 37 °C and frequently checked for *mycoplasma* contamination.

Cloning of Ng promoter and construction of the luciferase reporter plasmid. For constructing the luciferase reporter plasmid with the 5′-flanking sequence of the mouse Ng gene, the mouse (Swiss albino) genomic DNA was extracted by NecleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany). By using the genomic DNA as a template and pNg_F_(full)/pNg_R_(full) as PCR primers (the sequence of primers used is listed in Table 1), a 2.2 kb PCR product encompassing the 5′-flanking sequence of the mouse Ng gene was amplified by a long range PCR using a DNA Polymerase kit (Finzyme, Espoo, Finland). A series of 5′-deletion mutations of Ng promoter were generated by PCR using various forward primers corresponding to different 5′-flanking regions (see Table 1 for the sequence of these primers), number in the bracket indicates the 5′-end position relative to transcriptional start site (TSS). For generating Ng promoter containing m1_GC mutation, m2_GC mutation or del_GC mutation (see Fig. 1B for the mutated bases), the plasmid pGL3-pNg (+3) was used as a template and primed with the forward primer pNg_(m1A_GC), pNg_(m2A_GC), and pNg_(delA_GC), respectively. The PCR product amplified by the forward primer SacI_pNg_(m1A) was used as a template to prime with the primer SacI_pNg_(m1B) in the second PCR. Likewise, PCR product amplified by the forward primer pNg_(m2A_GC) and pNg (delA_GC) was paired with SacI_pNg_(m2B_GC) and SacI_pNg (delB_GC), respectively, in the second PCR. XhoI_pNg was used as the reverse primer in all PCRs. All final PCR products were recovered from agarose gels by a gel extraction kit (Qiagen, Hilden, Germany) and cloned into pGL3 basic vector (Promega, Madison, WI) through *Sac*I and *Xho*I enzyme sites. The sequences of all primers are listed in Table 1.

Construction of Sp1 expression plasmids and purification of the recombinant human Sp1 protein. The eukaryotic expression plasmid pcDNA3.1-Sp1-778C was constructed by PCR-mediated method. The plasmid pSp1-778C was used as a PCR template; NheI_Sp1_F/ XhoI_Sp1_R were used as PCR primers. The PCR product was cloned into the pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) by *Nhe*I and *Xho*I enzyme sites. To construct the proeukaryotic expression plasmid pQE30-Sp1-774C, KpnI_Sp1_F/ SalI_Sp1_R were used as PCR primers and the plasmid pSp1-778C was used as a PCR template. For efficiently expressing His-tag at 5′-end of Sp1 protein, the DNA sequence encoding for the first four amino acids (including two ATGs) of Sp1 protein was omitted in the forward primer, KpnI_Sp1_F. The PCR product was digested and cloned into the pQE30 expression vector (Qiagen) through *Kpn*I and *Sal*I enzyme sites. cDNA inserts were confirmed by DNA sequencing. As for the expression and purification of recombinant human Sp1 protein, the plasmid pQE30-Sp1-774C was transformed to competent M15 (pREP4) cells and a positive clone was cultured at 37 °C in Luria–Bertani (LB) medium containing 100 μg/ml ampicillin and 30 μg/ml kanamycin. The induction of expression was started at $OD_{600} = 0.8$, by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture at a final concentration of 0.5 mM. M15 cells were harvested after 4 h induction and the protein was purified by Ni–NTA column (Qiagen) according to the manufacturer's protocol. The purified Sp1 protein in eluate was subjected to size-exclusion FPLC with Superose

Table 1
The sequence of primers and oligonucleotides used for PCRs and EMSAs

Primer	Sequence
pNg_F_(full)	5′ GCTTGGCTGTTTGAGGTCC 3′
pNg_R_(full)	5′ GTGTTGAGGGTCCTTGGCT 3
SacI_pNg_(−1942)	5′ CTGGAGCTCGCTTGGCTGTTTGAGGTC 3
SacI_pNg_(−1628)	5′ CAGGAGCTCACCCCAGATGTTCCTTTC 3′
SacI_pNg_(−1150)	5′ CAGGAGCTCAGGCTCAGGAAACAT 3′
SacI_pNg_(−643)	5′ CAGGAGCTCACGTAGAATCAACGTGTG 3′
SacI_pNg_(+3)	5′ TAGGAGCTCGGTCCTCGCTCCAGTTCT 3′
SacI_pNg_(+34)	5′ TAGGAGCTCTGCAGAAAGTGTCTTCTG 3′
SacI_pNg_(+43)	5′ TAGGAGCTCGTGTCTTCTGATTGGCTTC 3′
pNg_(delA_GC)	5′ TCGCTCCAGTTCTTGCAGAAAGTGTCTTCTGAT 3′
SacI_pNg_(delB_GC)	5′ TAGGAGCTCGGTCCTCGCTCCAGTTCTTGCAGAA 3′
pNg_(m1A_GC)	5′ CAGTTCTCCCTTCCCACCCTGCAGAAAGTGTC 3′
SacI_pNg_(m1B_GC)	5′ TAGGAGCTCGGTCCTCGCTCCAGTTCTCCCTTCCCAC 3′
pNg_(m2A_GC)	5′ AGTTCTAATTATTATTTATGCAGAAAGTGTCTTCTGA 3′
SacI_pNg_(m2B_GC)	5′ TAGGAGCTCGGTCCTCGCTCCAGTTCTAATTATTATTTATG 3′
XhoI_pNg	5′ TGTTCTCGAGTGCCGGTGTTGAGGGTC 3′
NheI_Sp1_F	5′ ATTGCTAGCATGGATGAAATGACAGCTGT 3′
XhoI_Sp1_R	5′ TCGCTCGAGTCAGAAGCCATTGCCACT 3′
KpnI_Sp1_F	5′ CCTGGTACCACAGCTGTGGTGAAAATTG 3′
SalI_Sp1_R	5′ CTAGTCGACTCAGAAGCCATTGCCACTGAT 3′
GC-rich sequence (+12 to +43)	5′ CTCCAGTTCTCCCCGCCCACCCTGCAGAAAGT 3′
AT-rich motif	5′ AGAGGACACATAGATAAAAATACATGCA 3′

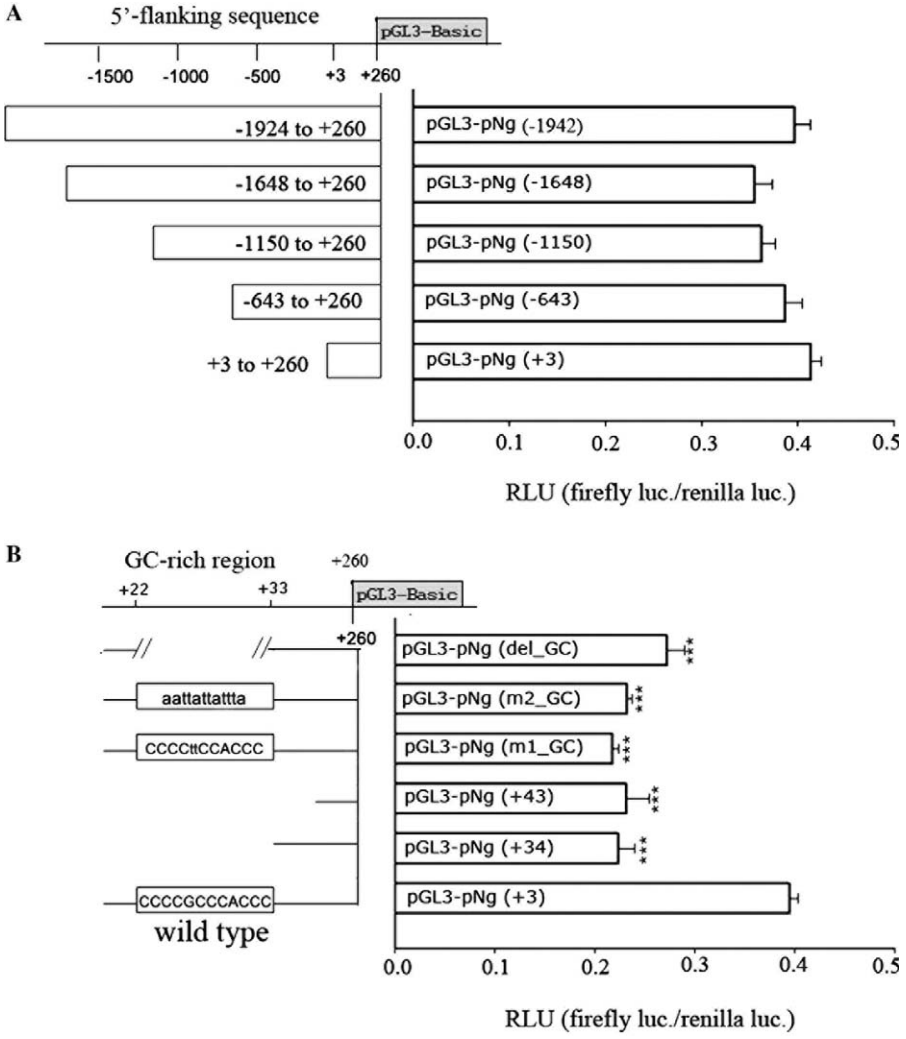

Fig. 1. Characterization of the mouse Ng promoter and the role of the GC-rich sequence. Schematic diagram shows the structure of reporter construct containing either deletion mutation of Ng promoter or site-directed mutation of the GC-rich sequence. The reporter construct was transfected to HEK 293 cells and the reporter activity was measured. Data are means ± SE ($n = 3$). Difference was compared by one-way ANOVA. ***$p < 0.001$ [as compared with pGL3-pNg (+3)]. (A) Analysis of the deletion mutations of Ng promoter. The starting 5′-end position of Ng promoter was indicated in the bracket of the name for the construct. (B) Analysis of Ng promoter containing mutated or deleted GC-rich sequence, or further deletion mutations. Mutated bases are indicated as lower case letters.

12 prep-grade column. The protein fractionated in the major peak was pooled and concentrated by a centriprep YM 3 centrifugal filter (Millipore, Bedford, MA). The final purified human recombinant Sp1 protein was confirmed by Western blotting.

Transient transfection of mammalian cells and the luciferase activity measurement. Plasmids subjected to transfection were extracted and purified by the plasmid midiprep system (Promega). Transfections were conducted in 6-well culture plates (Nunc, Roskilde, Denmark) by the TranIT transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. One hundred and fifty nanograms of the *Renilla* luciferase expression plasmid pRLSV40 was co-transfected for normalization of the transfection efficiency. In some experiments, 2 μg Sp1 expression plasmid pcDNA3.1-Sp1-778C or Sp3 expression plasmid pN3-Sp3FL-New was co-transfected to cells. The human Sp1 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and knock-down of endogenous Sp1 expression in HEK 293 cells was conducted following the manufacturer's protocol. The siRNA-A (Santa Cruz) was used as a negative control. Cells were harvested at 48 h post-transfection and the luciferase activity was measured by a Turner TD 20/20 luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Western blotting. Nuclear protein or purified recombinant human Sp1 protein was quantified by Bradford assay. After denaturation, 30 μg of the protein sample was separated by a 12% polyacrylamide gel. Electrophoresis was conducted by a SE 250 vertical electrophoresis apparatus (Hoefer, San Francisco, CA) with a constant current of 20 mA. Proteins were electro-blotted to the Hybond-C nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blot was blocked in 1× TBST with 5% nonfat dried milk overnight at 4 °C on a rocking platform. After short rinse of the membrane, the rabbit polyclonal anti-Sp1 antibody (Bethyl, Montgomery, Texas) or the rabbit anti-Sp3 antibody (Upstate, Charlottesville, VA) was added. Incubation was performed overnight in 1× TBST with 5% nonfat dried milk. After washing, the membrane was then incubated with the horseradish peroxidase-conjugated anti-rabbit IgG (Chemicon, Temecula, CA) for 2 h at room temperature with the same blocking buffer. After stringent washing in TBST, the membrane was then incubated with the SuperSignal West Pico chemiluminescent substrate solution (Pierce, Rockford, IL), followed by exposure to the CL-X Posure Film (Pierce).

Electrophoresis mobility shift assays (EMSAs). The Swiss Albino mice of 4 weeks were sacrificed and their brain tissues were taken out and quickly frozen in liquid nitrogen and homogenized. The nuclear extract from HEK 293 cells and the mouse brain tissues were prepared by the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's protocol. Oligonucleotides were synthesized by Research Biolabs (Singapore). The sequences of synthesized oligonucleotides corresponding to the GC-rich sequence (+12 to +43) and a computer-based predicted AT-rich motif are indicated in Table 1 without showing their complementary strands. Each DNA oligo (1 μM) was labeled with biotin by a 3′-end DNA labeling kit (Pierce). Complementary DNA oligos were annealed with each other by heating at 95 °C for 3 min separately followed by one hour incubation of the two DNA oligos together at room temperature. EMSAs were performed by using a LightShift chemiluminescent EMSA kit (Pierce). One hundred and fifty nanograms of purified recombinant human Sp1 protein or 10 μg nuclear extract was used in each assay. For the competition assay, the nuclear extract was pre-incubated with unlabeled probe for 15 min before labeled DNA was added. For supershift assays, anti-Sp1 or anti-Sp3 antibody was pre-incubated with the nuclear extract for 30 min with gentle shaking at room temperature before respective labeled probe was added. All incubations were loaded on 6% non-denaturing polyacrylamide gel prepared in 0.5× TBE. Electrophoresis was performed at 300 V in 0.5× TBE and stopped when bromophenol blue just ran out of the gel. The biotin labeled DNA was electro-blotted onto Hybond-N nylon membrane (Amersham) and the membrane was cross-linked by a 312 nm UV transilluminator for 1 min. The signal was developed with a chemiluminescent nucleic acid detection module (Pierce) following the manufacturer's protocol.

Computer-based analysis. Putative DNA motifs in the 5′-flanking sequence of the mouse Ng gene were predicted by Multiple Em for Motif Elicitation (MEME, http://meme.sdsc.edu/meme/meme.html) [21] using a 2.2 kb 5′-flanking sequence of the mouse Ng gene (GenBank Accession No. AF230869) as an input. The transcription factor binding sites were analyzed by Transcription Element Search System (TESS, http://www.cbil.upenn.edu/tess) with Ng promoter sequence from position +3 to +260 as an input.

Results and discussion

The GC-rich sequence (+22 to +33) in the 5′-flanking sequence is an important determinant in the basal transcription activity of the mouse Ng gene

To characterize the mouse Ng promoter, a 2.2 kb sequence corresponding to the 5′-flanking sequence of the mouse Ng gene was amplified by PCR. Luciferase reporter plasmids containing a series of 5′-deletion mutations of Ng promoter were constructed and transfected to HEK 293 cells. Promoter analysis showed that a 258 bp 5′-flanking sequence of the mouse Ng gene that encompasses a region from +3 to +260 conferred the full promoter activity. This construct showed similar reporter activity as that was showed by the construct containing full-length promoter (−1942 to +260) or other constructs containing progressive deleted 5′-flanking sequence of the mouse Ng gene (−1648 to +260, −1150 to +260, and −643 to +260) (Fig. 1A). Further deletion of the 5′-flanking sequence of Ng gene (+33 to +260 and +43 to +260) resulted in a 2-fold reduction of the reporter activity (Fig. 1B) indicating that important determinants reside in the 5′-flanking sequence between +3 and +33 of the mouse Ng gene. These findings agree with previous study on the rat Ng promoter [18] and the sequence alignment has revealed that the mouse and the rat share high sequence similarity in proximal 5′-flanking sequence of the Ng gene. Our data further revealed that disruption or deletion of a GC-rich sequence (+22 to +33) resulted in a decrease in promoter activity by 50% in HEK 293 cells. Our results from site-directed mutation showed that only two base pairs' replacement in the GC-rich sequence (pNg_m1_GC) was enough to reduce the reporter activity to a level similar to those of all GCs in this region were replaced by ATs (pNg_m2_GC) or deletion of the whole GC-rich sequence (pNg_del_GC) (Fig. 1B). This was the first experimental evidence to show that the GC-rich sequence in the 5′-flanking sequence of Ng gene is an important determinant in conferring the basal transcription activity of Ng.

DNA-protein complexes formed by the GC-rich sequence are not involved with Sp1 and Sp3 transcription factors

The GC-rich sequence (+22 to +33) in the 5′-flanking region of Ng gene, structurally, is very similar to the consensus Sp1-binding sequence: G/T-GGGCGG-G/A-G/A-CT [22]. Before we examined the likelihood of Sp1 and Sp3 transcription factors binding to the GC-rich sequence of Ng promoter, the expression pattern of the two transcription factors in three cell lines (HEK 293, N2A, and GT1-7) was examined by Western blotting. Surprisingly, though Sp1 and Sp3 proteins were reported as the major isoforms of Sp-family transcriptional factors that are ubiquitously expressed in most tissues [23–25], the Sp1 transcription factor was only found in the nuclear extract of HEK 293 cells (Fig. 2D). The Sp3 transcription factor was not expressed in any of the three cell lines. This was not due to the fault of the Sp3 antibody since the immunoactivity of the Sp3 protein could be seen with three molecular forms in the nuclear protein sample from HEK 293 cells transfected with the Sp3 expression plasmid, pN3-Sp3FL-new (Fig. 2D, lane 1).

In the present study, the recombinant human Sp1 protein was expressed in *Escherichia coli*. and purified by a His-tag affinity column (Fig. 2A) and gel filtration (Fig. 2B). The purity of recombinant human Sp1 protein was examined by Western blotting (Fig. 2C). Based on the knowledge we obtained on the endogenous expression of Sp1 and Sp3 in the three cell lines investigated, EMSAs were performed in such a way that the nuclear extract from either HEK 293 cells or HEK 293 cells transfected with the Sp3 expression plasmid, or purified recombinant Sp1 protein was incubated with the biotin-labeled GC-rich sequence (corresponding to the 5′-flanking sequence from +12 to +43 of Ng gene). As shown in Fig. 2E, when incubated with the nuclear extract of HEK 293 cells, the GC-rich sequence formed three major DNA-protein complexes (C1, C2, and C3). The binding specificity of these complexes was determined by the competition assay with excess unlabeled GC-rich sequence. Data revealed that only the complexes C2 and C3 could be competed off by 100× excess

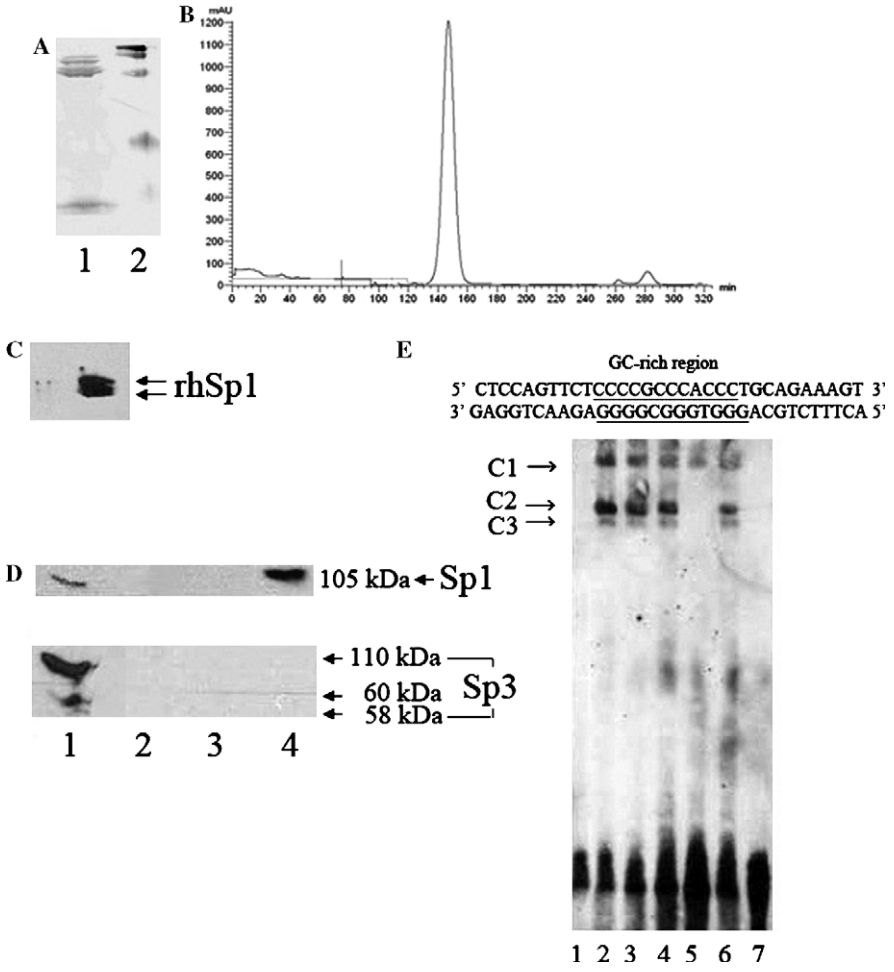

Fig. 2. Analysis of the binding affinity of Sp1 and Sp3 transcription factors to the GC-rich sequence of the mouse Ng promoter (A) Comassie blue-staining of 12% polyacrylamide gel. Lane 1, His-tagged human Sp1 purified by Ni-NTA column; lane 2, kaleidoscope prestained protein standards (Bio-Rad). (B) Purification of recombinant human Sp1 by FPLC. (C) The Western blot for the purified recombinant Sp1 protein. rhSP1, recombinant human Sp1. (D) The Western blot for the endogenous expression of Sp1 and Sp3 in three cell lines. The molecular weights of Sp1 and Sp3 are indicated. Protein samples are from: lane 1, HEK 293 cells transfected with the Sp3 expression plasmid pN3-Sp3FL-New; lane 2, N2A cells; lane 3, GT1-7 cells; lane 4, HEK 293 cells. (E) EMSAs of the nuclear extract of HEK 293 cells and purified recombinant human Sp1 protein with the GC-rich sequence. The sequence used for EMSAs is indicated on the top with the GC-rich sequence underlined. Protein-DNA complexes are indicated as C1, C2, and C3. Lane 1, without the nuclear extract; lane 2, with the nuclear extract of HEK 293 cells; lane 3, with the nuclear extract of HEK 293 cells and the Sp1 antibody; lane 4, with the nuclear extract of HEK 293 cells and the Sp3 antibody; lane 5, with the nuclear extract of HKE 293 cells and 100-fold excess molar unlabeled GC-rich sequence; lane 6, with the nuclear extract of HEK 293 cells transfected with pN3-Sp3FL-new plasmid; lane 7, with the recombinant human Sp1 protein.

amount of unlabeled GC-rich sequence (Fig. 2E, lane 5), indicating that the complex C1 was a non-specific DNA-protein complex. The purified Sp1 could not form any complex with the GC-rich sequence (Fig. 2E, lane 7). Western blotting revealed that our purified human recombinant Sp1 protein was composed of two major molecular forms around 100 kDa (Fig. 2C). It might be a result from the multiple transcription of Sp1 gene, nevertheless the variable molecular form of Sp1 was unlikely to affect the binding affinity to its DNA targets since the DNA-binding structure and the 3′-end Zn(II) fingers of Sp1 protein were least likely to be altered in the *E. coli* expression system [26,27]. It is apparent that the complexes C2 and C3 were not formed by Sp3 protein because HEK 293 cells are Sp3-free cells as shown in Fig. 2D. EMSAs showed that the nuclear extract of HEK 293 cells transfected with the Sp3 expression plasmid did not form any new complex other than the complexes C2 and C3 (Fig. 2E, lane 6). These results indicated that neither Sp1 nor Sp3 was able to bind to the GC-rich sequence of Ng promoter.

To consolidate our findings, supershift assays were conducted using Sp1 and Sp3 antibodies. Results showed that addition of Sp1 and Sp3 antibodies did not supershift the complex C2 and/or C3 (Fig. 2E, lanes 3 and 4). It is the cogent evidence that the C2 and C3 complexes were not formed by Sp1 or Sp3 protein. It has been suggested that the Sp1 transcription factor binds to its target sequence in assembled nucleosomes [28]. In this regard it is possible that the Sp1 protein needs to recruit co-activators, for example CRSP (cofactor required for Sp1) [29], before it would be able to bind to the GC-rich sequence of Ng promoter.

The transcription factor Sp1 transactivates Ng transcription while Sp3 represses the transcription

TESS analysis of the 5′-flanking sequence of Ng gene revealed a tandem of Sp1 sites at the proximal promoter region (Fig. 3). In the present study, to determine if the Sp1 transcription factor could transactivate Ng promoter activity, the Sp1 expression plasmid pcDNA 3.1-Sp1_778C was co-transfected to Sp1-free N2A cells with the minimal Ng promoter construct pGL3-pNg (+3). As shown by the Western blotting, Sp1 expression was detectable in the nuclear extract but not the cytoplasmic extract of N2A cells (Fig. 4B), indicating the Sp1 transcription factor successfully translocated to the nucle-

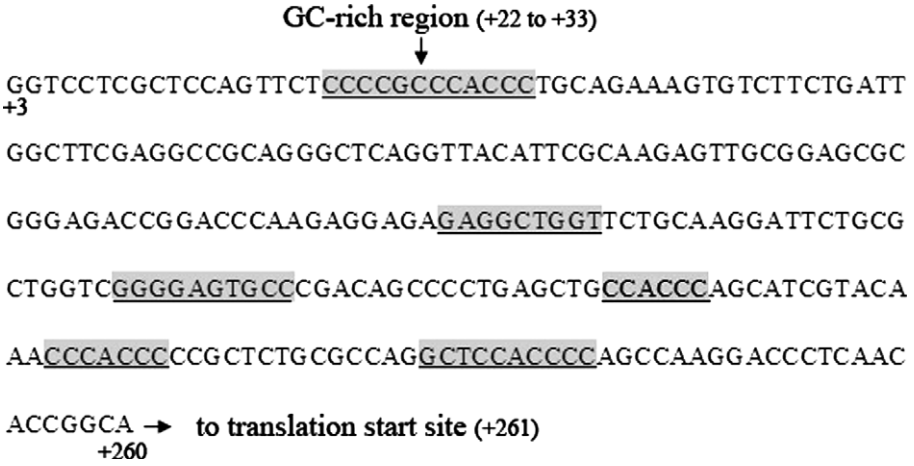

Fig. 3. Computer-based prediction of Sp1/Sp3-binding sites in the 5′-flanking sequence of Ng gene. The 5′-flanking sequence of the mouse Ng gene from +3 to +260 was analyzed by TESS. Possible Sp1/Sp3-binding sites are shaded and underlined. The GC-rich sequence and the translation start site are indicated.

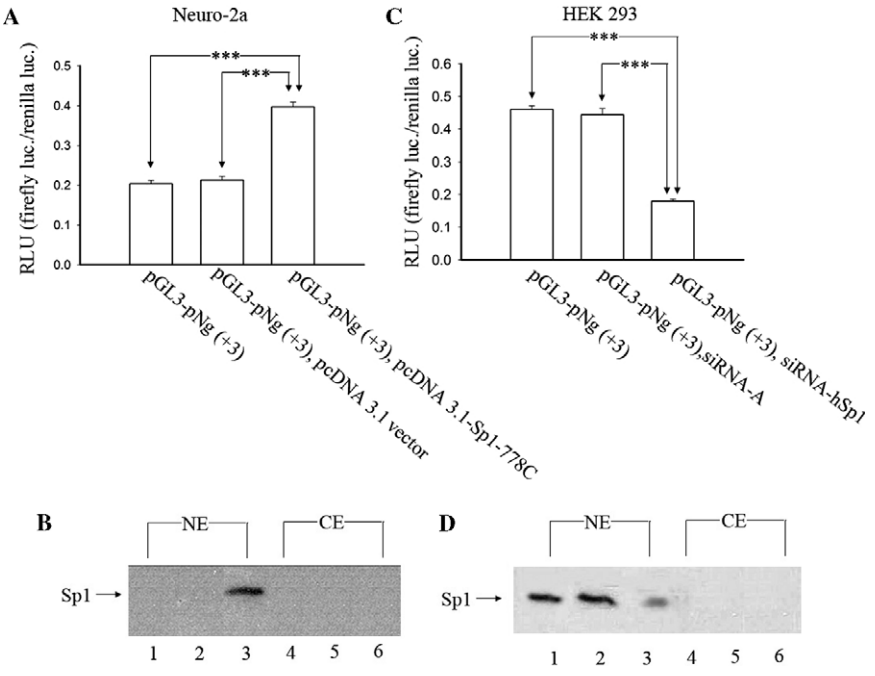

Fig. 4. The Sp1 transcription factor transactivates the transcription activity of Ng. Data for (A) and (C) are means ± SE ($n = 3$). Difference was compared by one-way ANOVA. ***$p < 0.001$. In (B) and (D), NE, nuclear extract; CE, cytoplasmic extract. (A) The reporter activity of pGL3-pNg (+3) with or without transfection of the Sp1 expression plasmid pcDNA 3.1-Sp1-778C in N2A cells. (B) The Western blotting for expression of Sp1 in N2A cells. Lanes 1 and 4, the N2A cells transfected with pGL3-pNg (+3); lanes 2 and 5, cells transfected with pGL3-pNg (+3) together with pcDNA 3.1 vector; lanes 3 and 6, cells transfected with pGL3-pNg (+3) together with pcDNA 3.1-Sp1-778C. (C) The reporter activity of pGL3-pNg (+3) in the presence or absence of siRNA-hSp1 in HEK 293 cells. siRNA-A in the middle bar is an unspecific siRNA used as a control. (D) The expression of Sp1 in HEK 293 cells. Lanes 1 and 4, HEK 293 cells transfected with pGL3-pNg (+3); lanes 2 and 5, cells transfected with pGL3-pNg (+3) together with siRNA-A; lanes 3 and 6, cells transfected with pGL3-pNg (+3) together with siRNA-hSp1.

us of cells. Promoter analysis revealed that exogenous expression of Sp1 in N2A cells induced a 2-fold increase in the reporter activity, compared with the control cells in which the pcDNA 3.1 vector or the reporter construct pGL3-pNg (+3) alone was transfected (Fig. 4A). The Ng promoter activity decreased by 2.5-fold (Fig. 4C) when endogenous expression of Sp1 protein in HEK 293 cells was inhibited by the short interfering RNA targeting for human Sp1 (Fig. 4D, lane 3). Densitometric analysis of the signals showed that the expression level of Sp1 was suppressed by 60–70% (Fig. 4D, lane 3) of its original level (Fig. 4D, lanes 1 and 2). Based on these findings, it is obvious that Sp1 plays an important role in Ng basal transcription activity. Various lines of evidence have shown that multiple Sp1 sites could efficiently drive transcription of TATA-less promoter [30,31]. Now that the Sp1 transcription factor could not interact directly with the GC-rich region (+22 to +33) according to our EMSA results, it is very likely that Sp1 transactivates Ng transcription by binding to the tandem Sp1 sites which are more adjacent to the translation start site of Ng gene (Fig. 3).

To determine the role of Sp3 transcription factor in regulating Ng basal transcription activity, the Sp3 expression plasmid pN3-Sp3FL-New was transiently transfected to HEK 293 cells. The expression and nucleus translocation of the Sp3 transcription factor were examined by Western blotting (Fig. 5B). Analysis of the reporter activity of pGL3-pNg (+3) revealed that ectopic expression of Sp3 in HEK 293 cells resulted in a 2-fold reduction of the basal transcription activity of Ng, compared with the control (pN3 vector or reporter construct alone was transfected) (Fig. 5A). This result indicated that Sp3 protein could be a negative regulatory transcription factor for Ng gene, at least in the context of HEK 293 cells. The Sp3 transcription factor belongs to the Sp-family and recognizes GC-box virtually identical to the sequence of Sp1-binding site due to the structural similarity among different members of Sp-family [23]. However, the Sp3 transcription factor has been shown in many instances to inhibit the Sp1-induced activation of gene transcription [32–34]. In the present study, the Sp3 transcription factor was shown for the first time to down-regulate the basal transcription of Ng.

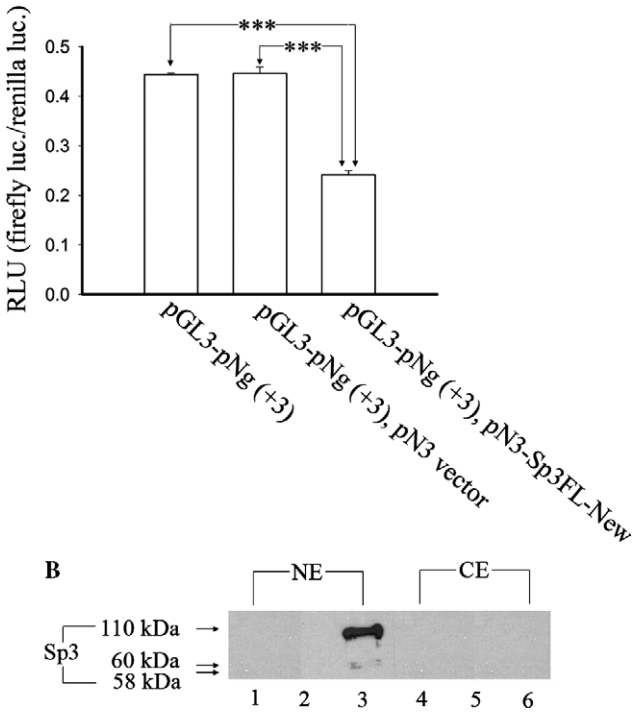

Fig. 5. The Sp3 transcription factor represses the transcription activity of Ng. Data for (A) are means ± SE ($n = 3$). Difference was compared by one-way ANOVA. ***$p < 0.001$. In (B), NE, nuclear extract; CE, cytoplasmic extract. (A) The reporter activity of pGL3-pNg (+3) with or without transfection of the Sp3 expression plasmid pN3-Sp3FL-New. (B) The Western blot for expression of Sp3 in HEK 293 cells. Lanes 1 and 4, the cells transfected with pGL3-pNg (+3) alone; lanes 2 and 5, cells transfected with pGL3-pNg (+3) together with pN3 vector; lanes 3 and 6, cells transfected with pGL3-pNg (+3) with pN3-Sp3FL-New. Different molecular weights of Sp3 are indicated.

A nuclear factor from the mouse brain tissues binds to the AT-rich motif at multiple sites in the 5′-flanking sequence of the mouse Ng gene

Though the cellular and physiological functions of Ng have been extensively studied, little is known about the promoter-binding proteins. In the present study, we analyzed the 5′-flanking sequence of the mouse Ng gene by MEME, a powerful DNA motif discovery tool, and found four possible DNA motifs. Gel shift assays showed that three of them failed to bind to any nuclear protein extracted from the mouse brain tissues (data are not shown). The result from EMSAs showed that an AT-rich sequence displayed the binding affinity to the nuclear extract of the mouse brain tissues and formed two major complexes (Fig. 6, C1a and C2a). The specificity of the complexes was examined by competition assays. The protein-DNA complex of C2a was partially competed off by pre-incubation with nuclear extract with 100-fold excess molar unlabeled AT-rich sequence (cool probe) (Fig. 6, lane 4). When 200-fold excess molar unlabeled probe was used, the complex C2a was totally diminished (Fig. 6, lane 6). As shown in lanes 2, 3, and 5, the complex C2a was gradually intensified with the increasing amount of nuclear proteins (6, 8, and 10 μg, respectively). However, the intensity of C1a did not change in either case, suggesting that C1a was a non-specific complex.

A 20 kDa protein was previously reported to bind to an AT-rich sequence of Ng and PKCγ gene [18,20]. The sequence alignment revealed that the AT-rich motif found in our study is different from the one previously reported. MEME prediction showed that the AT-rich motif in our study is repeatedly distributed in the whole 5′-flanking sequence of the mouse Ng gene. Analysis of 5′-deletion mutations indicated that tapering the number of the AT-rich motif in the 5′-flanking sequence of Ng gene did not result in significant changes of the reporter activity

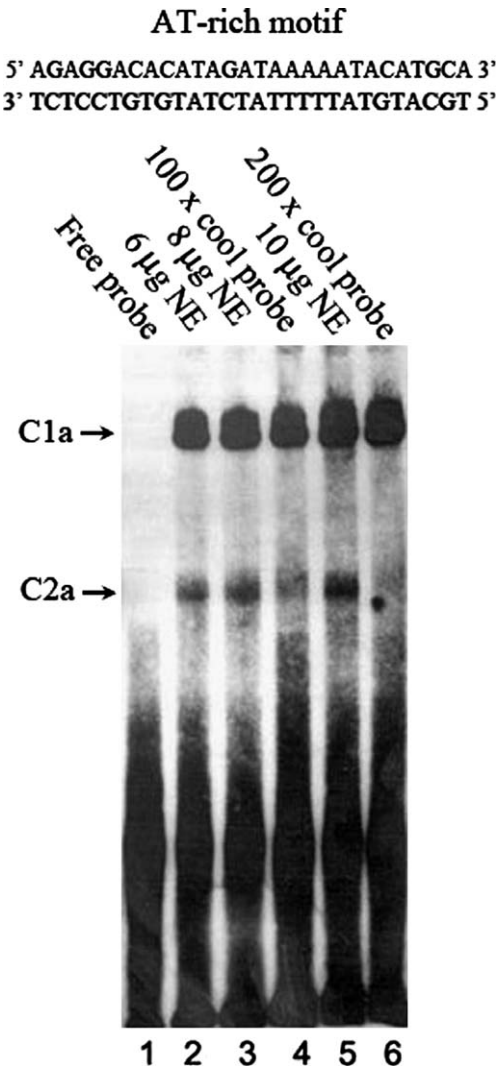

Fig. 6. EMSAs of the nuclear extract of the mouse brain tissues with AT-rich sequence. The sequence of AT-rich motif is shown on the top. Protein-DNA complexes are shown as C1a and C2a. Lane 1, without the nuclear extract; lanes 2, 3 and 5, with the indicated amount of nuclear extract; lanes 4 and 6, with the indicated amount of unlabeled probe (cool probe).

(refer to Fig. 1A). It implied that this newly discovered nuclear factor might be a structural scaffold protein. However, before the nuclear protein was isolated and characterized, it is premature to define its role. Data based on EMSAs suggested that the binding affinity and specificity of the unknown nuclear factor to the AT-rich motif were strong. This would make it possible to isolate this protein by a sequence-specific DNA-affinity column [35] for future research.

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