ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(4): 247–254 **CN** 31-1940/Q

Identification of Nuclear Factor-κB Responsive Element within the Neuronal Nitric Oxide Synthase Exon 1f-specific Promoter

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Abstract Transcriptional regulation of the neuronal nitric oxide synthase gene (*nNOS*) is particularly complex as 12 distinct transcripts derived from different first exons are expressed in a tissue- and cell-specific manner. The exon 1f mRNA is relatively highly expressed in nervous system and relies upon exon 1f-specific promoter activity. Using conventional and real-time reverse transcription-polymerase chain reaction, we found exon 1f mRNA was the major transcript of the *nNOS* gene in human neuroblastoma SK-N-SH cells. We analyzed a 1090 bp fragment of 1f promoter by TRANSFAC-TESS and Match softwares and luciferase assay, and found an important positive transcriptional regulation region that contained a putative nuclear factor (NF)-κB binding site. Subsequently, using electrophoresis mobility shift and chromatin immunoprecipitation assays, we identified this site to be the NF-κB responsive element, a crucial positive regulator in the activation of the *nNOS* 1f promoter. Taken together, our study identified an NF-κB responsive element within *nNOS* 1f promoter and showed that it plays an important role in the transactivation of *nNOS* 1f mRNA, the major transcript of *nNOS* in SK-N-SH cells.

Key words *nNOS*; NF-κB; transactivation; nervous system

Nitric oxide (NO) plays highly diversified biological roles in synaptic plasticity, development, differentiation, regeneration and other physiological processes of nervous tissue. It is also involved in neurodegenerative disorders and stroke as a mediator of neurotoxicity. NO is synthesized from *L*-arginine by three isoforms of NO synthase (NOS): neuronal (nNOS); endothelial (eNOS); and inducible (iNOS). nNOS is prominent in the nervous system [1–4].

NO biosynthesis derived from nNOS is regulated on many levels. Acute control of nNOS activity is mediated by allosteric enzyme regulation, post-translational modification and subcellular targeting of the enzyme. But it is more important to note that nNOS levels are dynamically regulated by transcriptional activity, which affords long-lasting changes in tissue NO levels. Some observations suggest *nNOS* mRNA is tightly regulated in response to different physiological and pathophysiological stimuli. Therefore, considerable efforts have been directed toward identifying the mechanisms that activate and limit *nNOS* expression. *nNOS* transcriptional regulation is rather complicated due to 12 distinct transcripts, derived from different first exons (named exon 1a–1l) of the *nNOS* gene, leading to different 5′-untranslated regions, translational efficiencies, and biological activity in a cell-, tissue-, and site-specific manner. The expression of different *nNOS* transcripts is subject to the specific promoter strength and specificity [5].

In order to assess the regulation mechanism of *nNOS* exon 1f transcript abundant in brain [6], we detected the *nNOS* 1f mRNA level as well as the 1f promoter activity in human neuroblastoma SK-N-SH cells. Furthermore, we identified a nuclear factor (NF)-κB responsive element in the *nNOS* 1f promoter and determined its crucial role in *nNOS* transactivation.

Materials and Methods

Received: November 16, 2006 Accepted: January 30, 2007
This work was supported by the grants from the National Natural Science Foundations of China (No. 30400463 and 30370785)
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DOI: 10.1111/j.1745-7270.2007.00280.x

Cell culture

The human neuroblastoma cell line SK-N-SH was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in minimum essential medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2.

Antibodies, plasmids and reagents

Rabbit polyclonal antibodies against p65 (sc-109 and sc-109X) and p50 (sc-114 and sc-114X) and normal rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit anti-β-actin polyclonal antibody was obtained from Cell Signaling Technology (Beverly, USA). pMD18-T vector was obtained from TaKaRa (Otsu, Japan) and pGL3-Basic and pRL-TK luciferase reporter vectors were from Promega (Madison, USA). Trizol reagent was purchased from Gibco (Los Angeles, USA). The reverse transcription system and the Dual Luciferase reporter assay system were obtained from Promega. AxyPre plasmid miniprep kits were purchased from Axygen (Union City, USA). Lipofectamine 2000 transfection reagents were obtained from Invitrogen (Carlsbad, USA). Protein A/G-agarose beads were purchased from Santa Cruz Biotechnology.

RNA isolation and RT-PCR

Total RNA was prepared from SK-N-SH cells using Trizol reagent. cDNA was synthesized with the reverse transcription (RT) system following the protocol recommended by the manufacturer. In detecting the expression of total *nNOS* mRNA, polymerase chain reaction (PCR) was carried out with an intron-spanning pair of exon 10- and exon 11-specific primers present in all known human *nNOS* transcripts to generate a 164 bp fragment: forward, 5′-CCCTTCAGTGGCTGGTACAT-3′; and reverse, 5′- ACCGCGATATTGATCTCCAC-3′. The exon 1f-specific transcript was amplified using intron-spanning primers complementary to exon 1f and the common exon 2 to generate a 152 bp fragment: forward, 5′-ACATATTTATGCCGCGTTTC-3'; and reverse, 5′- TATCTTCATTGCCAGCCTGT-3′. For *β-actin* mRNA as an internal control, the primers were: forward, 5′-CTTCTACAATGAGCTGCGTG-3′; and reverse, 5′-TCATGAGGTAGTCAGTCAGG-3′. These primers were used to generate a 308 bp fragment.

To assess the absolute copy number of total and 1f-specific *nNOS* mRNA, quantitative real-time PCR was carried out using the primers above as well as *Taq*man probes 5′-FAM-CGACTACTGTGACAACTCCCGCTACAAT-TAMARA-3′ for total *nNOS* mRNA and 5′-FAM-AGCTACTTAGCGCCGCGGCT-TAMARA-3′ for 1f-specific *nNOS* mRNA. The PCR products of total and 1f-specific *nNOS* were cloned into the pMD18-T vectors and confirmed by sequencing. The plasmid DNA was quantified by an ultraviolet spectrophotometer to get absolute copy numbers and then serially diluted to create the standard curves of total or 1f-specific *nNOS* mRNA. The sample was amplified in triplicate to obtain average copy numbers. Reactions without cDNA templates were used as the negative control.

Analysis of *cis*-acting elements in *nNOS* 1f promoter and construction of plasmids

According to the sequence provided by Marsden's group [7], a 1090 bp (–1063/+27) sequence of exon 1f promoter was analyzed using TRANSFAC-TESS and Match (http://www.cbil.upenn.edu/cgi-bin/tess/tess and http://www.gene-regulation.com) searches. A series of truncated *nNOS* 1f promoter fragments was amplified by PCR, spanning –1063/+27, –897/+27, –631/+27, –464/+27 and –260/+27, using different forward primers and the same reverse primer. Then fragments were cloned into pMD18-T vectors, subcloned into the unique *Sac*I/*Hin*dIII sites of pGL3-Basic vector, and named pGL1063w, pGL897w, pGL631w, pGL464w and pGL260w, respectively. Constructs with a mutated or deleted NF-κB site at position –893/–884 (named pGL897m and pGL897d) were generated with the forward primer (the mutated nucleotides are underlined) 5′-TGGTGGGTTT-ACTCTTTGCCTCT-3′ or 5′-TTCTCTGCGCAACTCG-AAGC-3′, respectively, and the same reverse primer as pGL897w. All constructs were confirmed by sequencing, and coding frame shift of the luciferase gene was excluded.

Transient transfection and luciferase assay

SK-N-SH cells were seeded in 96-well plates, grown to 80%–90% confluence, and transiently transfected using Lipofectamine 2000 transfection reagents. pRL-TK plasmid with a cDNA encoding Renilla luciferase was used as an internal control in each experiment. Five hundred nanograms of one version of the pGL constructs and 0.05 μg pRL-TK were co-transfected into cultured cells. Using the Dual Luciferase reporter assay system, the activities of firefly and Renilla luciferases were determined sequentially from a single sample using a Lumat LB 9507 luminometer (Bethold Technologies, Bad Wildbad, Germany).

Western blot analysis

Nuclear extract was prepared from SK-N-SH cells as

previously described [8]. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane at 4 ºC. Membranes were subsequently incubated with rabbit polyclonal IgG against p65, p50 or β-actin (1:500) as the primary antibody, followed by incubation with goat anti-rabbit IgG (1:2000) as the secondary antibody. Signals were then detected.

Electrophoresis mobility shift assay

Electrophoresis mobility shift assay (EMSA) was carried out as previously described [8]. Briefly, nuclear extracts were prepared from SK-N-SH cells. Oligonucleotides containing the wild-type and mutant putative NF-κB element at position –893/–884 upstream of the exon 1f transcription start site of the *nNOS* gene were synthesized and annealed. Probes were radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Binding reactions were carried out in a 15 μl volume containing approximately 5000 cpm of the labeled probe, 5 μg of nuclear extract, 0.5 μg/μl poly(dI-dC), and 1×binding buffer (10 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol). For the competition assay, 10-fold or 100-fold molar excess of cold competitor was added into the binding reaction mixture. Reactions were also carried out by incubating nuclear extract with anti-p50 or anti-p65 antibodies for 30 min at 4 ºC before adding the probe. After incubation on ice for 1 h, reaction mixtures were loaded onto an 8% polyacrylamide gel and run at 150 V in 0.375×Tris-Borate-EDTA (TBE) for 4 h. The gel was then dried under vacuum and autoradiographed.

Chromatin immunoprecipitation

Formaldehyde (1%) was added to the cultured SK-N-SH cells and incubated for 20 min at 37 ºC. Cells were harvested, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), incubated at 4 ºC for 10 min, then sonicated to generate DNA fragments ranging in size from 100 to 300 bp. One quarter of the lysate was used as the DNA input control. The remainder was diluted 10-fold with chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, 150 mM NaCl) followed by incubation with specific anti-p50 and anti-p65 antibodies, or with nonimmune rabbit IgG as the negative control at 4 ºC overnight. Immunoprecipitated complexes were collected by protein A/G-agarose beads. Precipitates were extensively washed and incubated at room temperature for 20 min. Cross-linking of protein-DNA complexes was reversed at 65 ºC for 5 h, and then DNA was extracted with phenol/chloroform. PCR was carried out using *nNOS* promoter-specific primers (5′-ATGTGGAAGACAGCATAGACC-3′ and 5′-TGGTGGTTTTCCTCTTTGCCTCT-3′) to generate a 189 bp *nNOS* 1f promoter fragment. PCRs using *WNK4* (with no lysine k kinase 4) gene promoter-specific primers (5′-CACTGACCTCTCCGTTCGGC-3′ and 5′-GGAGCA-TCCTCCCGCACTAA-3′) to generate a 190 bp fragment without p65/p50 binding were used as a negative control.

Results

Expression of total and exon 1f-specific *nNOS* mRNA in SK-N-SH cells

The *nNOS* gene is scattered over 237 kb of genomic DNA on chromosome 12q24.2–24.3 and its structure is rather complex due to 12 distinct first exons. As shown in **Fig. 1(A)**, the 5′-half of the gene contains the different first exons, and the 3′-half the common exons 2 to 29, the coding sequence for nNOS protein [7,9].

In this study, RT-PCR and quantitative real-time RT-PCR were carried out with intron-spanning primers and *Taq*man probes complementary to common exon 10 and exon 11 or exon 1f and common exon 2 [**Fig. 1(A)**] to test the expression level of total and exon 1f-specific *nNOS* mRNA. The RT-PCR results suggested exon 1f mRNA expressed highly and was an important transcript of the *nNOS* gene in SK-N-SH cells [**Fig. 1(B)**]. To further quantify the expression level of 1f mRNA, real-time RT-PCR was carried out to obtain absolute copy numbers and showed *nNOS* exon 1f mRNA accounted for 53% of total *nNOS* mRNA [**Fig. 1(C)**].

Structure analysis and activity determination of *nNOS* 1f promoter

To characterize the *nNOS* exon 1f-specific promoter, we initially analyzed a 1090 bp 5′- flanking sequence (GenBank accession number: U11422) of *nNOS* 1f transcript for transcription factor binding sites with TRANSFAC-TESS and Match searches. The results showed a typical TATA-box motif at position –28/–20 and several potential transcription factor binding sites including CCAAT/enhancer-binding protein (C/EBP), glucocorticoid receptor (GR) and NF-κB (**Fig. 2**). Subsequently, a series of luciferase reporter vectors containing truncated *nNOS* 1f promoter was constructed, spanning –1063/+27, –897/+27, –631/+27, –464/+27 and –260/+27. Results of luciferase assay revealed that two positive regulatory regions were located at position –897/–631 and –631/

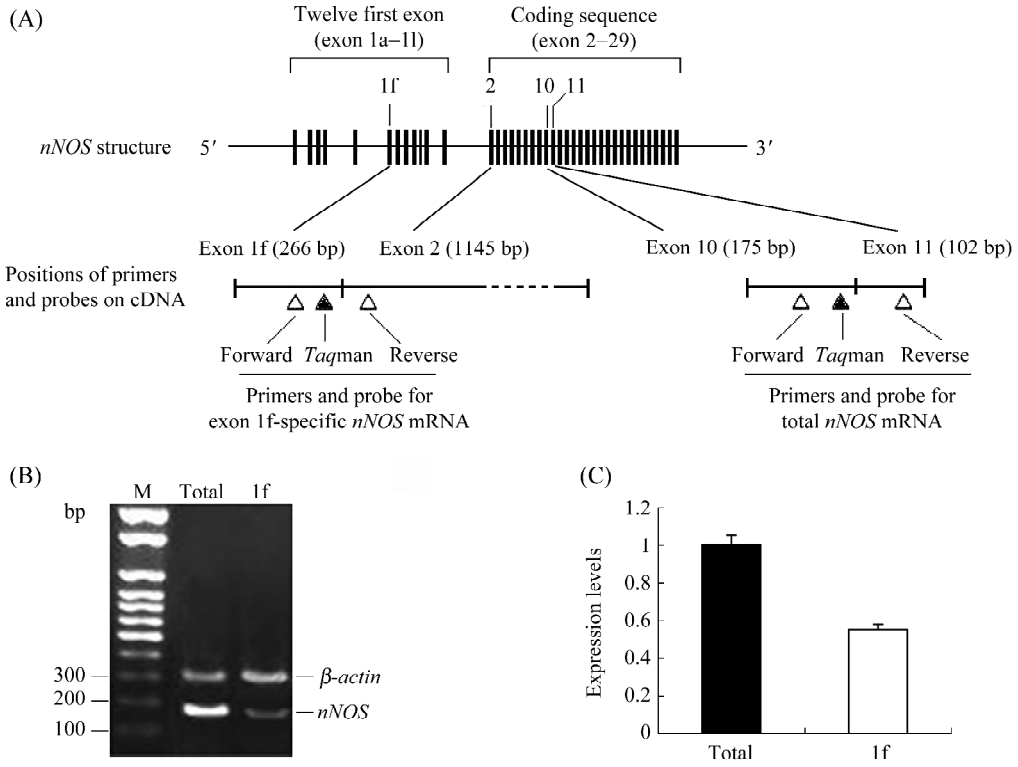

Fig. 1 Total and exon 1f-specific neuronal nitric oxide synthase (*nNOS*) mRNA expression in human neuroblastoma SK-N-SH cells

(A) Structure of the *nNOS* gene and the positions of primers and probes used in this study. (B) Total and exon 1f-specific *nNOS* mRNA expression with β-*actin* as an internal control by RT-PCR. M, 100 bp DNA ladder. (C) Real-time RT-PCR results of total and 1f *nNOS* mRNA levels.

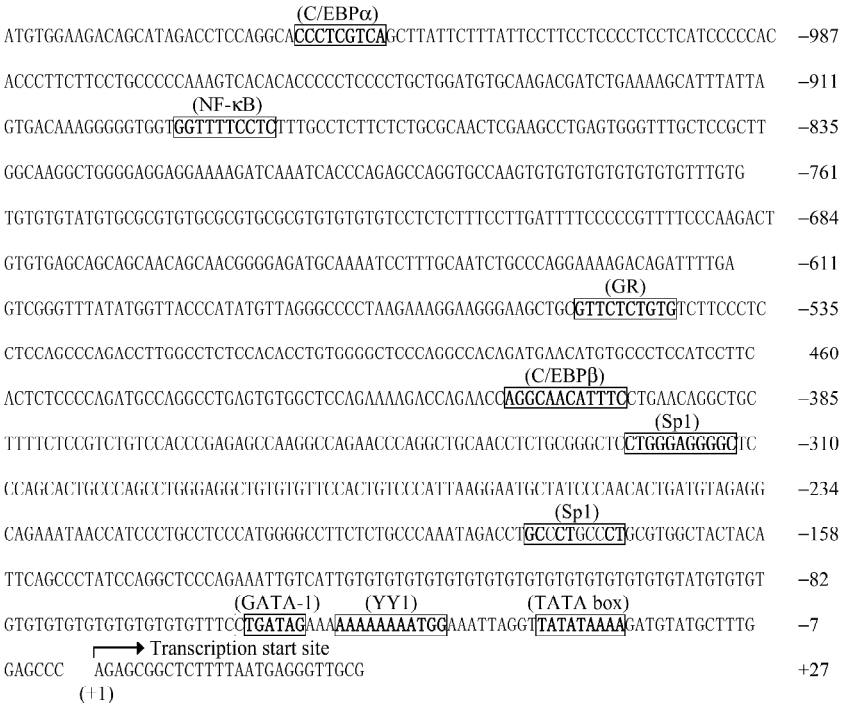

Fig. 2 Putative *cis*-acting elements within neuronal nitric oxide synthase (*nNOS*) 1f promoter by TRANSFAC-TESS and Match searches

A typical TATA-box motif is shown to be located between −28 and −20, and several potential transcription factor-binding sites were revealed. The nuclear factor (NF)-κB element is the one studied in this work. C/EBP, CCAAT/enhancer-binding protein; GR, glucocorticoid receptor; Sp1, stimulatory protein 1; YY1, Yin Yang 1.

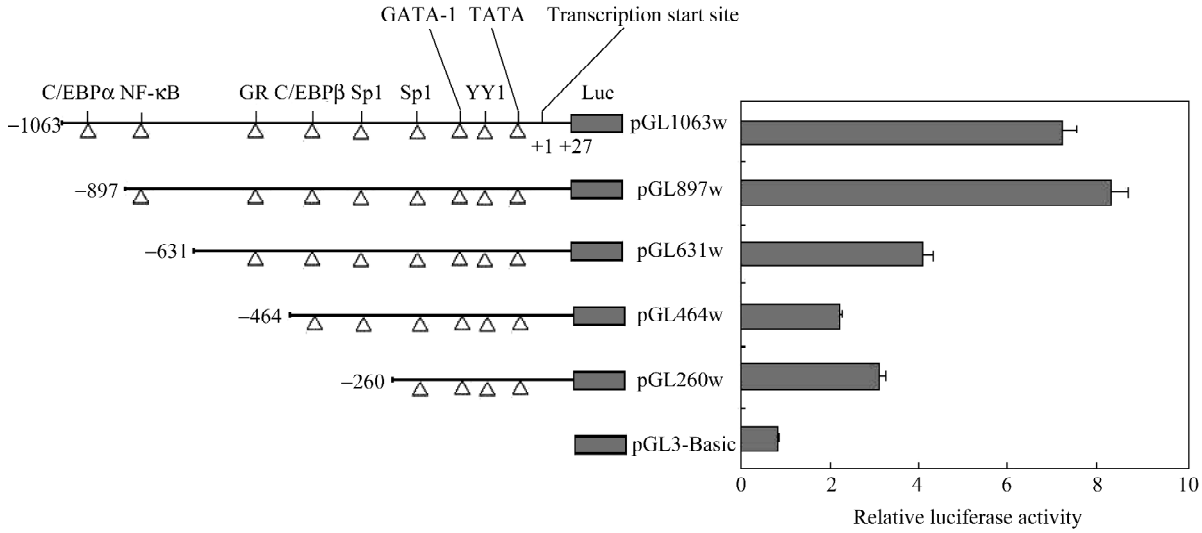

Fig. 3 Results of luciferase (Luc) assay of truncated neuronal nitric oxide synthase (*nNOS*) 1f promoter

Two positive transcriptional regulation regions (positions −897/−631 and −631/−464) and two negative regulation regions (positions −1063/−897 and −464/−260) were suggested.

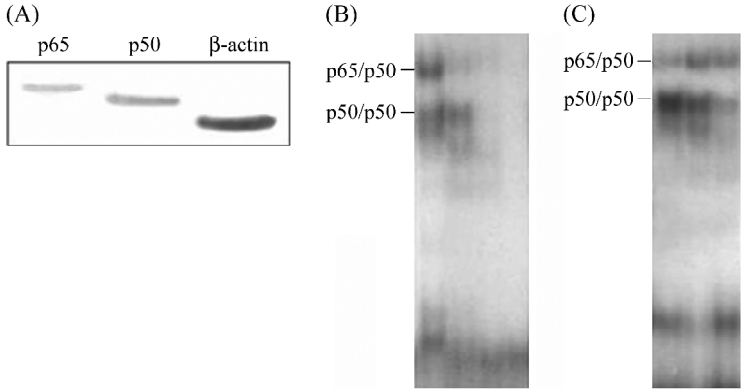

Fig. 4 Identification of nuclear factor (NF)-κB responsive element located at position −893/−884 of neuronal nitric oxide synthase (*nNOS*) 1f promoter by electrophoresis mobility shift assay

(A) NF-κB p65 and p50 subunits were detected in the nucleus of human neuroblastoma SK-N-SH cells by Western blotting. (B) Nuclear extract from SK-N-SH cells formed two complexes (p65/p50 heterodimer and p50/p50 homodimer) with a wild-type probe that was displaced by the unlabeled cold competitor. The mutant probe did not form complexes. (C) p65-specific and p50-specific antibodies (Ab) both blocked the formation of the complexes.

−464, and two negative regulatory regions were located at position −1063/−897 and −464/−260 (**Fig. 3**).

Identification of NF-κB responsive element in *nNOS* 1f promoter

To probe the mechanism of the high expression level of *nNOS* exon 1f mRNA, we paid attention to the region −897/−631 of the *nNOS* 1f promoter as the deletion of pGL897w construct to −631 significantly reduced the promoter activity. We noted a putative NF-κB responsive element (located at −893/−884) was suggested in this region. To identify the existence of NF-κB in the nucleus of SK-N-SH cells, Western blot analysis was carried out and both NF-κB p65 and p50 subunits were detected [**Fig. 4(A)**].

EMSA was carried out and the labeled wild-type and mutant oligonucleotides were incubated with nuclear extracts from SK-N-SH cells. As shown in **Fig. 4(B,C)**, wild-type probe formed two complexes (p65/p50 heterodimer and p50/p50 homodimer) that were both displaced by the unlabeled cold competitor. When site-directed mutagenesis was carried out on the NF-κB element to change two corresponding T and C residues to G and A

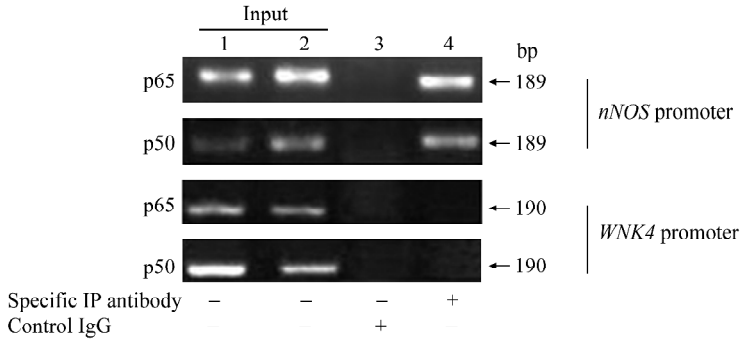

Fig. 5 Identification of nuclear factor (NF)-κB binding site using chromatin immunoprecipitation assay

A 189 bp fragment of neuronal nitric oxide synthase (*nNOS*) 1f promoter was detected when nuclear extract from human neuroblastoma SK-N-SH cells was immunoprecipitated with p65 or p50 antibody, but no detectable signal was found when immunoprecipitated with normal rabbit IgG. A 190 bp fragment of *WNK4* gene promoter was used as a negative control. Lane 1 was the input control of lane 3, and lane 2 was the input control for lane 4. IP, immunoprecipitation.

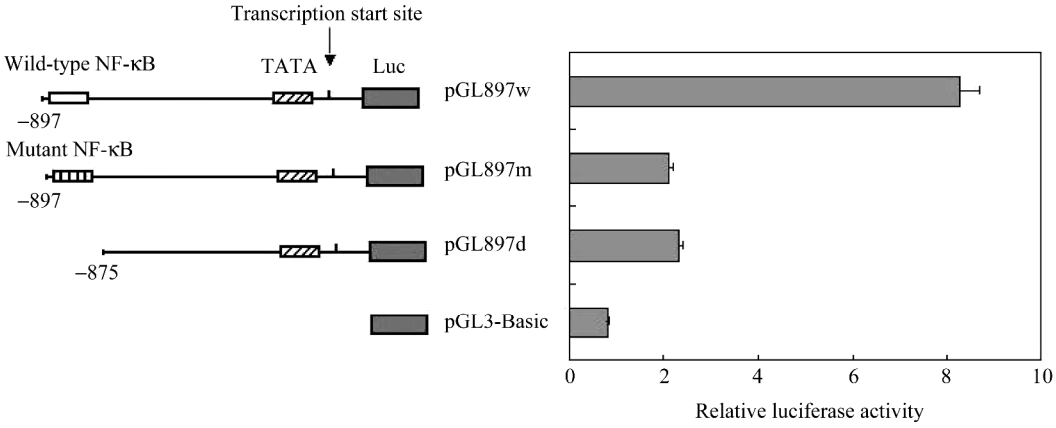

Fig. 6 Activity of promoter with wild-type, mutated or deleted nuclear factor (NF)-κB element

High promoter activity was detected with wild-type NF-κB element (pGL897w), but the activity was greatly decreased with mutation or deletion of the element (pGL897m or pGL897d). Luc, luciferase.

(5′-GGGTTTACTC-3′), the binding disappeared with the mutant probe. Specific anti-p65 and anti-p50 antibodies both blocked the binding of NF-κB to the element.

ChIP assay was used to identify the binding under *in vivo* conditions. As **Fig. 5** shows, binding of both p65 and p50 was specific as immunoprecipitation with normal rabbit IgG did not show a detectable *nNOS* 1f promoter fragment and PCRs using *WNK4* promoter primers did not form fragments when nuclear extracts were immunoprecipitated with p65 or p50 antibody. These results showed that the region −893/−884 of the *nNOS* 1f promoter was an NF-κB responsive element.

Role of NF-κB responsive element in activation of *nNOS* 1f promoter

To further assess the function of the identified NF-κB site at position −893/−884 of the *nNOS* 1f promoter in *nNOS* transactivation, we constructed luciferase reporter vectors containing mutation or deletion of this site (named pGL897m and pGL897d, respectively). Luciferase assay showed high promoter activity with wild-type NF-κB, but the activity was predominantly decreased (reaching about one-quarter of that of pGL897w) with mutation or deletion of the element (**Fig. 6**). This result indicated a crucial role of the NF-κB element in *nNOS* 1f promoter activation.

Discussion

It has been reported that 12 distinct first exons (1a to 1l) of the *nNOS* gene are selectively used in a tissue-specific manner and the expression of exon 1f mRNA is relatively

high in brain. In human neuroblastoma cell line SK-N-MC, exon 1f transcript accounts for 51% of total *nNOS* mRNA [5]. Therefore, we studied the expression of 1f transcript in human neuroblastoma SK-N-SH cells, a related cell line. RT-RCR and quantitative real-time RT-PCR results revealed exon 1f mRNA was the major transcript of the *nNOS* gene in SK-N-SH cells and accounted for 53% of total *nNOS* mRNA, a fraction closely resembling that obtained from SK-N-MC cells.

A number of studies suggest that promoter strength and specificity determine the *nNOS* expression profile and prove that transcription factors of stimulatory protein (Sp) and zinc finger (ZNF) families transactivate the *nNOS* exon 1c promoter [10], Ca^{2+} influx dynamically regulates *nNOS* expression through the binding of cAMP response element binding protein (CREB) to cAMP response elements (CREs) within *nNOS* promoter [11] and exon 1f expression is increased by cAMP-activating agents through the cAMP response element of the exon 1f promoter, while other mRNAs remain unchanged [6,9]. Therefore, the generation of different transcripts of the *nNOS* gene is facilitated by specific promoters. Recently published research showed that exon 1f-specific promoter activity is in good accordance with the exon 1f transcript profile in representative neuronal cells [5]. Hence, to probe the regulation mechanism of the high expression of *nNOS* exon 1f mRNA in SK-N-SH cells, we analyzed a 1090 bp fragment of the *nNOS* 1f promoter with TRANSFAC-TESS and Match searches. This analysis revealed potential binding sites of some important transcription factors, such as C/EBP, GR and NF-κB (**Fig. 2**). To our knowledge, these responsive elements have not been identified yet.

Luciferase assay of a series of truncated *nNOS* 1f promoter fragments revealed two positive regulatory regions (–897/–631 and –631/–464, respectively). The region –897/–631 plays an important role in transcriptional regulation, as pGL897w showed high transcription activity and deletion of this region greatly reduced the promoter activity. Interestingly, a putative NF-κB responsive element was shown in this region, and NF-κB is an important transcription factor present in neurons and glia. Recent research has detailed the role of NF-κB in the regulation of both physiological and pathological processes in the nervous system, such as memory formation, and Parkinson's and Alzheimer's diseases [12–14]. The study by Chan *et al*. examined the crucial role of NF-κB activation in *nNOS* expression during the development of hyperalgesia [15]. Therefore, DNA-binding experiments were carried out to identify this element. We showed that two complexes were bound to this site, competed by excess of cold competitor, and blocked by antibody against NF-κB p65 or p50 subunits. ChIP assay further showed the binding was specific as immunoprecipitates with normal control IgG did not show detectable *nNOS* 1f promoter fragment. Subsequent luciferase assay revealed this NF-κB responsive element was crucial for the high *nNOS* 1f promoter activity in SK-N-SH cells, as the mutated or deleted version of this site (pGL897m or pGL897d) sharply reduced the promoter transcription activity. It is worth noting that the activity of pGL897m or pGL897d (**Fig. 6**) was even lower than that of pGL631 (**Fig. 3**). A possible explanation is that the region –897/–631 contains some negative regulatory elements as well as the powerful positive NF-κB site.

In summary, our study showed that exon 1f mRNA is a major transcript of the *nNOS* gene in human neuroblastoma SK-N-SH cells. Furthermore, we identified an NF-κB responsive element within the *nNOS* 1f promoter and proved its crucial role in promoter activation. This might be a mechanism by which *nNOS* exon 1f mRNA is expressed highly in SK-N-SH cells.

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Edited by
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