Mol. Cells, Vol. 10, No. 5, pp. 566–574

Molecules and Cells

© Springer-Verlag 2000

5′-Flanking Sequence and Promoter Activity of the Rabbit Neuronal Nitric Oxide Synthase (nNOS) Gene

Yongsu Jeong†, Jaejoon Won, Changsoo Kim, and Jeongbin Yim*

National Creative Research Initiative Center for Genetic Reprogramming, Institute for Molecular Biology and Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea.

(Received on June 14, 2000)

We have isolated a rabbit neuronal nitric oxide synthase (nNOS) cDNA encoding a protein of 1,435 amino acids. Using the cDNA clones as probes, the 5′-flanking region of the nNOS gene was isolated from a rabbit genomic DNA library. 5′RACE and primer extension analysis of rabbit brain total RNA mapped multiple transcription initiation sites localized 474–487 bp upstream from the translation start codon. Analysis of 5,197 bp of the 5′-flanking sequence revealed that the rabbit nNOS gene promoter lacks canonical TATA or CCAAT boxes and, instead, contains a GC-rich region and multiple Sp1 sites. Farther from the +1 start, various putative *cis*-elements including AP-1, AP-4, NF-κB, STAT, CREB, C/EBP and c-Myc were observed. The functional promoter activity of the 5′-flanking region was demonstrated by its ability to drive the expression of a β-galactosidase reporter gene in several cell types. Serial deletion analysis of the promoter region revealed that the -291 to -172 region, which contains two Sp1 sites, is essential for basal transcriptional activity. These results suggest that the rabbit nNOS promoter contains characteristics of inducible genes.

Keywords: Gene Regulation; Neuronal Nitric Oxide Promoter; Rabbit; Synthase.

Introduction

Nitric oxide (NO) has been identified as an important mediator with a wide range of physiological activities. In the brain, normal physiology of NO has been implicated in synaptic plasticity. Recent studies with cultured hippocampal neurons (Arancio *et al*., 1996) or NOS-knockout mice (Son *et al*., 1996) demonstrated that NO acts as a retrograde messenger during long term potentiation (LTP) of excitatory neurotransmission in the hippocampus. In the peripheral nervous system, NO induces relaxation of smooth muscle important in the adaptation to a bolus of food and peristalsis. In the gut, smooth muscle relaxation depends on nonadrenergic-noncholinergic neurons that mediate their effects via NO (Huang *et al*., 1993). In addition to these beneficial effects, NO may also be detrimental in certain pathophysiological conditions. For example, neuronal damage following focal cerebral ischemia has been reported to be attributed to NO (Quast *et al*., 1995). NO is also involved in cellular damage in immunological diseases such as rheumatoid arthritis and septic shock. Because of its dual nature, tight regulation of NO biosynthesis is necessary. Indeed, NOS activity is regulated at multiple levels (Nathan and Xie, 1994). Although nNOS activity is strictly controlled by Ca^{2+}/calmodulin, additional mechanisms such as phosphorylation (Brüne and Lapetina, 1991; Nakane *et al*., 1991) and NO feedback regulation (Griscavage *et al*., 1994) may contribute to nNOS regulation. Signalling actions of NO can also be modulated by association of nNOS with several proteins. The postsynaptic density protein PSD-95 or α1-syntrophin mediate subcellular localizations of nNOS (Brenman *et al*., 1995; 1996). The protein inhibitor of nNOS (PIN) interacts physically with nNOS and destabilizes its dimeric form (Jaffrey and Snyder, 1996).

In spite of the constitutive expression of nNOS, several studies have shown that transcription of the nNOS gene can also be tightly regulated. In trkA-

† Present address: Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia 19104-6145, USA.

* To whom correspondence should be addressed.
Tel: 82-2-880-6702; Fax: 82-2-871-4315
E-mail: jyim@plaza.snu.ac.kr

Abbreviations: NGF, nerve growth factor; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; RT-PCR, reverse transcription-polymerase chain reaction; 5′RACE, 5′ rapid amplification of cDNA ends.

expressing cholinergic neurons and PC12 cells, nerve growth factor (NGF) induced the expression of nNOS, resulting in elevation of its mRNA levels (Holtzman *et al.*, 1996; Sheehy *et al.*, 1997). Also, pregnancy and estrogen treatment increased the amount of mRNA for eNOS and nNOS in the skeletal muscle (Weiner *et al.*, 1994). Several findings have suggested that the nNOS content may be modulated during certain pathophysiological conditions. Nerve injury such as peripheral axotomy caused up-regulation of nNOS mRNA (Verge *et al.*, 1992). Guo *et al.* (1997) showed that prolonged *in vivo* hypoxia caused a time-dependent increase in nNOS mRNA and protein expression in rat. These observations indicate that the biological activity of nNOS is specifically regulated at transcriptional or post-transcriptional levels, and that therefore the notion of the constitutive expression of this isoform needs to be reconsidered. Contrary to inducible NOS, however, little is known about transcriptional control processes of the nNOS gene since the accumulation of evidence is only just beginning.

Indeed to date, only the organization of the human nNOS gene has been reported (Hall *et al.*, 1994), and there are few studies demonstrating the effects of external signals on nNOS mRNA levels. In the present study, we have isolated the rabbit nNOS cDNA and characterized its 5′ flanking region as a prerequisite step in understanding the regulatory processes of nNOS gene expression.

Materials and Methods

Cloning of rabbit nNOS cDNA Rabbit brain Total RNA was reverse transcribed with 100 pmole of oligo(dT) or random primer and 40 units of AMV reverse transcriptase (Promega). The first-strand of cDNA was amplified with 2 units of *Taq* DNA polymerase (Poscochem) and 50 pmole of degenerate primers corresponding to amino acid sequences shared by rat and human nNOS. The three PCR products were subcloned into a PCR cloning vector, pGEM-T Easy (Promega), and then sequenced. The resulting plasmids, pTA23, pTA89, and pTA1011, were linearized, and utilized as templates to generate radioactive RNA run-off transcripts with 10 μM [α-^{32}P]UTP (800 Ci/mmol; Amersham) and 20 units of T7 RNA polymerase (Poscochem) or SP6 RNA polymerase (Poscochem). These riboprobes were used to screen a rabbit brain cDNA library (Clontech) as described before (Jeong *et al.*, 1998). The inserts of positive clones were subcloned into the *Eco*RI site of pGEM7zf (Promega) and sequenced.

Screening of rabbit genomic library cDNA subclones were linearized and utilized as templates to synthesize radiolabeled RNA run-off transcripts. This riboprobe was used to screen an EMBL SP6/T7 rabbit genomic library (Clontech). Phage hybridizations were carried out on Hybond-N membranes (Amersham). Nylon membranes were hybridized at 43–45°C for 16 h in 50% formamide, 2× Denhardt's solution, 150 mM sodium phosphate, pH 6.8, 250 mM sodium chloride, 1 mM EDTA, 1% SDS, 10% polyethylene glycol and the ^{32}P-labelled riboprobe. The blots were washed at 55–60°C with 0.1× SSC, 1% SDS and 10 mM sodium phosphate, pH 6.8, and exposed overnight at −80°C. Several positive clones were isolated, and phage DNAs were purified for restriction-site mapping. The inserts were subcloned and used as templates to generate riboprobes for further screening.

5′ Rapid amplification of cDNA ends Twenty to fifty μg of rabbit brain total RNA were dissolved in 50 mM Tris-HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, and 1 mM dithiothreitol, and mixed with a NOS gene-specific primer, NR4 (5′-AAGAGACGGACCGAGATGAC-3′), corresponding to nucleotides +43 to +62 relative to the ATG translation initiation codon. After heating at 70°C for 10 min and cooling down to 43°C, the RNA-primer mixture was then incubated with 20 units of AMV reverse transcriptase for 1 h at 43°C. The 3′-end of the cDNA was tailed at 37°C for 20 min with 10 units of terminal transferase in 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 200 μM dATP. The tailed cDNA pool was then amplified by PCR using an oligo dT-anchor primer (5′-GACCACGC GTATCGATGTCG-$ACT_{(16)}$V–3′) and a nested NOS gene-specific primer, NR5 (5′-GCCAAACACGTGCTCCTCCAT-3′), corresponding to nucleotides +1 to +21 relative to the ATG translation start codon. The products were ligated into pGEM-T Easy, and independent clones were sequenced. According to the sequencing result, the following primers were synthesized and used to perform a second 5′RACE: NR10 for RT, 5′-GAGTAGACAACATCTGCCACA-3′ (+332 to +352); NR11 for the first-round of PCR, 5′-GATCGGCTGT-GCACCTCAC-3′ (+377 to +395); NR-13 for the second-round of PCR, 5′-TCTCAGCGGCGGCGGCAG-3′ (+410 to +427).

Primer extension analysis An oligonucleotide primer (5′-TCCCCCGTCCGTGAGGTGCACAGCCGATCT-3′) complementary to the 5′-end of the rabbit nNOS mRNA was end-labeled with [γ-^{32}P]ATP (800 Ci/mmol; Amersham) and T4 polynucleotide kinase (Poscochem). Approximately 1×10^5 cpm of this primer was hybridized to 50 μg of rabbit brain total RNA at 30°C for 16 h in 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl and 80% formamide. The primer was extended at 37°C for 2 h with 100 units of Moloney murine leukemia virus reverse transcriptase in 50 mM Tris, pH 8.3, 7 mM $MgCl_2$, 40 mM KCl, 1 mM dNTPs, 10 mM dithiothreitol, and 40 units of RNasin. Primer extension products were digested with RNase A (Sigma), extracted with phenol/chloroform, precipitated with ethanol, and then analyzed on an 8% denaturing polyacrylamide gel in parallel with dideoxynucleotide chain termination sequencing ladders.

Construction of promoter-β-galactosidase plasmids A 6 kb-*Xho*I fragment of λnosJ was cloned into pBluescriptIIKS to generate subclone pJ 6.0, and then a 4 kb-*Sfo*I fragment of pJ 6.0 was ligated into the *Sma*I site of pβgal-Basic (Clontech) to obtain a reporter-promoter construct, pGalS4. To make serial deletion constructs, -3,319, -2,643, -1,999, -1,105, -728, -291, and -171, pGalS4 was partially cut with *Eco*RI, *Ear*I, *Bam*HI, *Apa*I, *Afl*I, *Nae*I, and *Sac*I in combination with *Xma*I

respectively, and appropriate restriction fragments isolated from the gel were self-ligated.

Cell culture and transfection LLC-RK1 (rabbit kidney) cells were maintained in medium 199 supplemented with 10% heat-inactivated horse serum (Gibco-BRL) under 5% CO_2. HIG-82 (rabbit synoviocyte) cells were cultured in Ham's F12 medium with 10% fetal bovine serum (Gibco-BRL). PC12 cells were maintained in Ham's F12K medium supplemented with 15% horse serum and 2.5% fetal bovine serum. For PC12 cell transfection, plastic dishes were treated overnight with 15 μg/ml poly-D-lysine (> 300,000 Da, Sigma), rinsed with distilled water, and then plated with PC12 cells. About 50% confluent cells in 35-mm dishes were transfected with 1 μg of various promoter-reporer constructs, 1 μg of pEGFP-N1 (Clontech) and 12 μg Lipofectamin (Gibco-BRL) in 1 ml of Opti-MEM (Gibco-BRL), and incubated for 8 h at 37°C under 5% CO_2. After 2 d of culture in the respective serum-containing medium, the β-galactosidase activity was assayed using the Luminescent β-gal Detection kit II (Clontech) and luminometer (EG & G Berthold). The fluorescence of the EGFP protein was also measured to normalize differences in transfection efficiency or the preparation of cell extracts.

Results

Isolation of rabbit nNOS cDNA Fully degenerate oligonucleotide primers based on the amino acid sequences of human and rat nNOS were synthesized for RT-PCR with the aim to generate probes useful for screening a rabbit brain cDNA library. After reverse transcription, the primers of each set were added to the reaction, and cDNA sequences were amplified by PCR. A rabbit brain λgt10 cDNA library was screened with NOS-specific PCR fragments, and six positive clones were isolated. Each cDNA insert of the clones was ligated into the *Eco*RI site of pGEM7zf, and the nucleotide sequences determined. Sequence analysis indicates that the 4,920-bp cDNA sequence of rabbit neuronal nitric oxide synthase contains a 4,305-bp open reading frame encoding a polypeptide of 1,435 amino acids with a calculated molecular mass of 161 kDa (Fig. 1). The deduced amino acid sequence of nNOS reveals several consensus sites. The amino acid sequence of rabbit nNOS displays an overall 90% identity to other neuronal enzymes from human, rat, and mouse. Like other nNOSs, the rabbit protein possesses recognition sites for calmodulin, FMN, FAD, and NADPH in the carboxyl terminal half of the polypeptide (Fig. 1).

Isolation of the 5′ flanking genomic region The rabbit genomic DNA library was screened with ^{32}P-labeled riboprobes derived from linearized cDNA subclones as templates. Three positively hybridizing phage clones, λnosA, λnosB, and λnosC, were isolated and the inserts were subcloned (Fig. 2). Since partial sequence analysis showed that these clones contained exons 3 to 10, repeated screening was performed with the same probe. λnosP, λnosQ, λnosS, and λnosL were isolated and partially sequenced. Compared with the nNOS cDNA sequence, these clones contained only exon 2. Using radioactive riboprobes derived from λnosL subclones

```
MEEHVFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGG    50
AAEQSGLIQAGDIILAVNGRPLVDLSYDSALEVLRGVASETHVVLILRGP   100
EGFTTNLETTFTGDGTPKTIRVTQPLGAPTKAVDLSHQPPSAGKEQPRPV   150
DGAAGPGSWPQPTQGHGQEAGSPSRANGLAPRTSSQDPAKKSGWAGLQGS   200
GDKNELLKEIEPVLTLLAGGSKAVDGGGPAKAETRDTGVQVDRDFDAKSH   250
KPLPLGVENDRVFSDLWGKGSAPVVLNNPYSEKEQPPASGKQSPTKNGSP   300
SKCPRFLKVKNWETDVVLTDTLHLKSTLETGCTEHICMGSIMFPSQHTRR   350
PEDIRTKEQLFPLAKEFIDQYYSSIKRFGSKAHMERLEEVNKEIESTSTY   400
QLKDTELIYGAKHAWRNASRCVGRIQWSKLQVFDARDCTTAHGMFNYICN   450
HIKYATNKGNLRSAITIFPQRTDGKHDFRVWNSQLIRYAGYKQPDGSTLG   500
DPANVQFTEICIQQGWKPPRSRFDVLPLLLQANGNDPELFQIPPELVLEV   550
PIRHPKFEWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWYMGTEIG   600
VRDYCDNSRYNILEEVAKKMNLDMRKTSSLWKDQALVEINIAVLYSFQSD   650
KVTIVDHHSATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQE   700
MLNYRLTPCFEYQPDPWNTHVWKGTNGTPTKRRAIGFKKLAEAVKFSAKL   750
                                  CaM
MGQAMAKRVKATILYATETGKSQAYAKTLCEIFKHAFDAKVMSMEEYDIV   800
HLEHETLVLVVTSTFGNGDPPENGEKFRCALMEMRHPNSLQEERKSYKVR   850
FNSVSSYSDSRKSSGDGPDVRDHFESAGPLANVRFSVFGLGSRAYPHFCA   900
                                      FMN
FGHAVDTLLEELGGERILKMREGDELCGQEEAFRTWAKKVFKAACDVFCV   950
GDDVNIEKANNSLISNDRSWKRNKFRLTYVAEAPGLTQGLSSVHKKRVSA  1000
ARLLSRQNLQSPKSSRSTIFVRLHTNGSQELQYQPGDHLGVFPGNHEDLV  1050
                                    FAD-PPi
NALIERLEDAPPANQMVKVELLEERNTALGVISNWKDEPRLPPCTVFQAF  1100
KYYLDITTPPTPLQLQQFASLASNEKEKQRLLVLSKGLQEYEEWKWGKNP  1150
TIVEVLEEFPSIQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLTVAI  1200
                              FAD-ISO
VSYHTRDGEGPIHHGVCSSWLNRIPADEVVPCFVRGAPSFRLPRNPQVPC  1250
ILVGPGTAFAPFRSFWQQRQFDIQHKGMSPCPMVLVFGCRQSKIDHIYRE  1300
  NADPH-ribose
EALQAKNKGVFRELYTAYSREPDKPKKYVQDILQEQLAEQVYRALKEQGG  1350
HIYVCGDVTMAADVLKAVQRIMAQQGKLSAEDAGVFISRLRDDNRYHEDI  1400
  NADPH-adenine
FGVTLRTYEVTNRLRSESIAFIEESKKDTDEVFSS                 1435
```

Fig. 1. Deduced amino acid sequence of the rabbit neuronal NOS. Consensus sites for calmodulin (CaM), FMN, FAD pyrophosphate (FAD-PPi), FAD isoalloxazine (FAD-ISO), NADPH ribose, NADPH adenine are boxed. The nucleotide sequence has been submitted to GenBank with the accession number U91584.

```
GGCTGGGCGGGCCTGCAAGGCAGTGGCGATAAGAACGAGCTCCTGAAGGAGATTGAGCCCGTGCTGACCCTCCTCGCCAGTGGGAGCAAGGCAGTCGACGGAGGGGGCCCTGCCAAGGCG
GlyTrpAlaGlyLeuGlnGlySerGlyAspLysAsnGluLeuLeuLysGluIleGluProValLeuThrLeuLeuAlaGlySerLysAlaValAspGLyGlyGlyProAlaLysAla  232
GAGACGAGAGACATGGGAGTCCAGGTGGACAGgtgagatgcccaggtgtgtgtgtgcgtgtgtgtgtgcgtgtctgtgtgcacgcgggagtctcagcctcacagcccagagctgctcaga
GluThrArgAspThrGlyValGlnValAspAr                                                                                          243
tcatgcgcatgcatttggccacccttgaccttgcccccaa . . . . . Intron 2 . . . . tggattgaggcagtgatctggccgttccttgtgtgtctcctgcctcacca
ccccgccttgcttgcagGGATTTCGATGCCAAGTCGCACAAGCCTCTGCCTCTTGGCGTGGAGAACGACCGCGTCTTCAGTGACCTGTGGGGGAAGGGCAGCGCGCCTGTGGTCCTCAAC      Exon 3
                gAspPheAspAlaLysSerHisLysProLeuProLeuGlyValGluAsnAspArgValPheSerAspLeuTrpGlyLysGlySerAlaProValValLeuAsn  277
AACCCCTATTCAGAGAAGGAGCAGgtaagtgtgcgaggcgagcctggcgcccacactcccacaccgccctgaaccctcccggggagaagccgagggg
AsnProTyrSerGluLysGluGln                                                                                                  285
```

Fig. 2. Genomic map of the rabbit nNOS gene. Overlapping phage clones are shown as horizontal lines. The first ten exons are indicated by vertical lines or an empty box. Exon 2 contains the ATG translational start site. E, *Eco*RI; B, *Bam*HI.

and the 5′RACE result, we have isolated two positive clones, λnosK and λnosJ, and determined their restriction-site maps. Comparison of genomic and complementary DNA sequences revealed that the λnosJ clone contained exon 1 and a 5′ flanking region for the nNOS gene. A 6 kb-*Xho*I fragment of λnosJ was subcloned into pBluescriptIIKS and the nucleotide sequence was determined (Fig. 3).

Determination of the transcriptional initiation site 5′RACE analysis was performed to identify the transcription initiation site of nNOS and orient its promoter relative to the coding sequence. When NR4 was used as the first-strand cDNA synthesis primer, and NR5 as the nested PCR primer, a -470 bp product was amplified, and subcloned into pGEM-T Easy. Sequence analysis of twenty individual clones mapped the transcriptional start sites at positions 474, 481, 487 bp upstream with respect to the first nucleotide of the translational initiator codon. These were consistent with the result from a second 5′RACE using primers near the 5′ end. Shown in Fig. 4 is a primer extension analysis that was also used to confirm the start site of transcription. Three distinct extended products were observed in lane 1. Adjacent lanes (GATC) display a dideoxy nucleotide sequencing ladder used to measure the lengths of the extension products. In agreement with the result of 5′RACE, alignment of the nNOS primer extension products with the sequencing ladder revealed that the rabbit nNOS mRNA began at multiple sites, which are -487 bp, -481 bp or -474 bp upstream of the initiator methionine codon. Sequence inspection of the proximal upstream region of the start sites did not identify a consensus TATA motif. The occurrence of multiple transcriptional initiation sites is not uncommon in genes whose promoter lacks a TATA box.

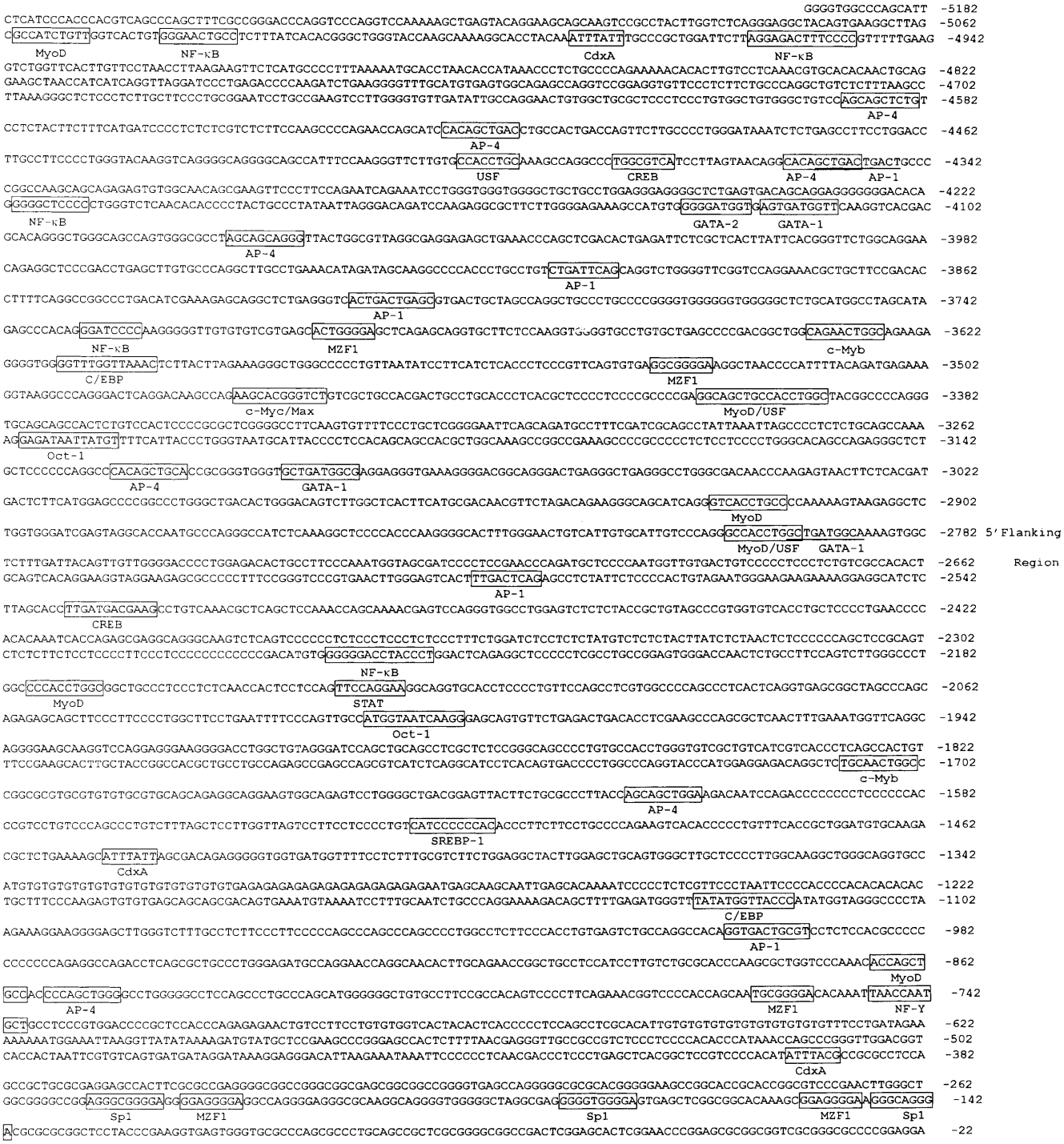
DNA sequence analysis of the rabbit nNOS 5′ flanking region Analysis of the 5,197 bp promoter region of the rabbit nNOS gene revealed that it has an overall GC content of 60%. In particular, the proximal region of the nNOS gene promoter has a high GC content of 78% between -399 bp and -98 bp. The sequence immediately upstream of the transcriptional start site does not have an identifiable TATA or CCAAT box. Instead, computational analysis of the promoter region shows that there are various consensus sequences for binding potential transcription regulatory factors (Fig. 3). Multiple Sp1 binding sites were evident at nt -246, -185, and -145. The Sp1 site is commonly required for the transcription of numerous TATA-less genes by RNA polymerase II. Five myeloid zinc finger 1 (MZF1) elements are found in the 5′flanking region, of which two are located at positions -235 and -55 contiguous to the Sp1 sites. Three consensus sequences for CdxA were observed at nt -4,985, -1,445 and -396. In addition, there are five AP-1 sites at nt -4,351, -3,906, -3,811, -2,596 and -1,002 as well as seven AP-4 sites at nt -4,588, -4,520, -4,356, -4,068, -3,123, -1,616 and -852. In the distal promoter region, five putative binding sites for NF-κB were detected at nt -5,037, -4,958, -4,216, -3,729 and -2,253. The 5′-flanking region of the rabbit nNOS gene contains two cyclic AMP-responsive elements (CRE) at positions -4,379 and -2,528 of the transcription start. Located at nt -5,056, -3,407, -3,400, -2,925, -2,803, -2,174 and -864 are potential MyoD elements, of which two overlap with the upstream stimulating factor (USF) at nt -3,400 and -2,803. Sequences for binding possible transcription factors such as c-Myc, SREBP 1 (sterol regulatory element-binding protein 1), STAT (signal transducers and activators of transcription), and NF-Y can also be found at nt -3,466, -2,134, -1,523, and -744, respectively (Fig. 3). Other putative *cis*-acting elements observed in the promoter of the nNOS gene are sites for C/EBP at nt -3,608 and -1,125, GATA-1 at nt -4,119, -3,101 and -2,795, GATA-2 at nt -4,129, Oct-1 at nt -3,253 and -2,008, and c-Myb at nt -3,633 and -1,708.

Promoter activity of the rabbit nNOS 5′-flanking region We have subcloned the 5′-flanking region into the pβgal-Basic plasmid vector in front of the reporter β-galactosidase gene to test its ability to function as a transcriptional promoter. This plasmid was used to transfect some cultured cell lines, while the promoterless pβgal-Basic served as a negative control. The transfection efficiency was normalized by co-transfection with green fluorescent protein DNA driven by the CMV promoter. As shown in Fig. 5, the two rabbit cell lines transfected with pGalS4 showed significantly more β-galactosidase activity than cells transfected with the control plasmid that lacked the promoter. Similar activity was shown with PC12 (rat Pheochromocytoma) cells. These results demonstrate that the 5′-flanking region of the rabbit nNOS gene is capable of functioning as a transcriptional promoter.

Deletion analysis of the rabbit nNOS promoter To determine the *cis* elements essential for the transcriptional activity of the nNOS gene, we performed serial deletion analysis of the rabbit nNOS promoter (Fig. 6). Deletion of the -1,105 to -729 region led to a 30% reduction in transcriptional activity in PC12 cells. A further deletion from -728 to -292 led to only a slight reduction in transcriptional activity. The loss of more than 70% promoter activity in construct -171 compared to construct -291 suggests that the region from -291 to -172 is essential for the basal promoter activity.

Discussion

Identification of the nNOS potential *cis*-regulatory DNA sequences We have characterized the structure of the promoter by DNA sequence analysis and examined its


GGGGTGGCCCAGCATT -5182
CTCATCCCACCCACGTCAGCCCAGCTTTCGCCGGGACCCAGGTCCCAGGTCCAAAAAGCTGAGTACAGGAAGCAGCAAGTCCGCCTACTTGGTCTCAGGGAGGCTACAGTGAAGGCTTAG -5062
CGCCATCTGTTGGTCACTGTGGGAACTGCCTCTTTATCACACGGGCTGGGTACCAAGCAAAAGGCACCTACAAATTTATTTGCCCGCTGGATTCTTAGGAGACTTTCCCCGTTTTTGAAG -4942
MyoD NF-κB CdxA NF-κB
GTCTGGTTCACTTGTTCCTAACCTTAAGAAGTTCTCATGCCCCTTTAAAAATGCACCTAACACCATAAACCCTCTGCCCCAGAAAAACACACTTGTCCTCAAACGTGCACACAACTGCAG -4822
GAAGCTAACCATCATCAGGTTAGGATCCCTGAGACCCCAAGATCTGAAGGGGTTTGCATGTGAGTGGCAGAGCCAGGTCCGGAGGTGTTCCCTCTTCTGCCCAGGCTGTCTCTTTAAGCC -4702
TTAAAGGGCTCTCCCTCTTGCTTCCCTGCGGAATCCTGCCGAAGTCCTTGGGGTGTTGATATTGCCAGGAACTGTGGCTGCGCTCCCTCCCTGTGGCTGTGGGCTGTCCAGCAGCTCTGT -4582
AP-4
CCTCTACTTCTTTCATGATCCCCTCTCTCGTCTCTTCCAAGCCCCAGAACCAGCATCCACAGCTGACCTGCCACTGACCAGTTCTTGCCCCTGGGATAAATCTCTGAGCCTTCCTGGACC -4462
AP-4
TTGCCTTCCCCTGGGTACAAGGTCAGGGGCAGGGGCAGCCATTTCCAAGGGTTCTTGTGCCACCTGCAAAGCCAGGCCCTGGCGTCATCCTTAGTAACAGGCACAGCTGACTGACTGCCC -4342
USF CREB AP-4 AP-1
CGGCCAAGCAGCAGAGAGTGTGGCAACAGCGAAGTTCCCTTCCAGAATCAGAAATCCTGGGTGGGTGGGGCTGCTGCCTGGAGGGAGGGGCTCTGAGTGACAGCAGGAGGGGGGACACA -4222
GGGGGCTCCCCCTGGGTCTCAACACACCCCTACTGCCCTATAATTAGGGACAGATCCAAGAGGCGCTTCTTGGGGAGAAAGCCATGTGGGGGATGGTGAGTGATGGTTCAAGGTCACGAC -4102
NF-κB GATA-2 GATA-1
GCACAGGGCTGGGCAGCCAGTGGGCGCCTAGCAGCAGGGTTACTGGCGTTAGGCGAGGAGAGCTGAAACCCAGCTCGACACTGAGATTCTCGCTCACTTATTCACGGGTTCTGGCAGGAA -3982
AP-4
CAGAGGCTCCCGACCTGAGCTTGTGCCCAGGCTTGCCTGAAACATAGATAGCAAGGCCCCACCCTGCCTGTCTGATTCAGCAGGTCTGGGGTTCGGTCCAGGAAACGCTGCTTCCGACAC -3862
AP-1
CTTTTCAGGCCGGCCCTGACATCGAAAGAGCAGGCTCTGAGGGTCACTGACTGAGCGTGACTGCTAGCCAGGCTGCCCTGCCCCGGGGTGGGGGGTGGGGGCTCTGCATGGCCTAGCATA -3742
AP-1
GAGCCCACAGGGATCCCCAAGGGGGTTGTGTGTCGTGAGCACTGGGGAGCTCAGAGCAGGTGCTTCTCCAAGGTGGGTGCCTGTGCTGAGCCCCGACGGCTGGCAGAACTGGCAGAAGA -3622
NF-κB MZF1 c-Myb
GGGGTGGGGTTTGGTTAAACTCTTACTTAGAAAGGGCTGGGCCCCCTGTTAATATCCTTCATCTCACCCTCCCGTTCAGTGTGAGGCGGGGAAGGCTAACCCCATTTTACAGATGAGAAA -3502
C/EBP MZF1
GGTAAGGCCCAGGGACTCAGGACAAGCCAGAAGCACGGGTCTGTCGCTGCCACGACTGCCTGCACCCTCACGCTCCCCTCCCCGCCCCGAGGCAGCTGCCACCTGGCTACGGCCCCAGGG -3382
c-Myc/Max MyoD/USF
TGCAGCAGCCACTCTGTCCACTCCCCGCGCTCGGGGCCTTCAAGTGTTTTCCCTGCTCGGGGAATTCAGCAGATGCCTTTCGATCGCAGCCTATTAAATTAGCCCCTCTCTGCAGCCAAA -3262
AGGAGATAATTATGTTTTCATTACCCTGGGTAATGCATTACCCCTCCACAGCAGCCACGCTGGCAAAGCCGGCCGAAAGCCCCGCCCCCTCTCCTCCCCTGGGCACAGCCAGAGGGCTCT -3142
Oct-1
GCTCCCCCCAGGCCCACAGCTGCACCGCGGGTGGGTGCTGATGGCGAGGAGGGTGAAAGGGGACGGCAGGGACTGAGGGCTGAGGGCCTGGGCGACAACCCAAGAGTAACTTCTCACGAT -3022
AP-4 GATA-1
GACTCTTCATGGAGCCCCGGCCCTGGGCTGACACTGGGACAGTCTTGGCTCACTTCATGCGACAACGTTCTAGACAGAAGGGCAGCATCAGGGTCACCTGCCMyoD
TGGTGGGATCGAGTAGGCACCAATGCCCAGGGCCATCTCAAAGGCTCCCCACCCAAGGGGCACTTTGGGAACTGTCATTGTGCATTGTCCCAGGGCCACCTGGCTGATGGCAAAAGTGGC -2782 5′Flanking
MyoD/USF GATA-1
TCTTTGATTACAGTTGTTGGGGACCCCTGGAGACACTGCCTTCCCAAATGGTAGCGATCCCCTCCGAACCCAGATGCTCCCCAATGGTTGTGACTGTCCCCCTCCCTCTGTCGCCACACT -2662 Region
GCAGTCACAGGAAGGTAGGAAGAGCGCCCCCTTTCCGGGTCCCGTGAACTTGGGAGTCACTTTGACTCAGAGCCTCTATTCTCCCCACTGTAGAATGGGAAGAAGAAAAGGAGGCATCTC -2542
AP-1
TTAGCACCTTGATGACGAAGCCTGTCAAACGCTCAGCTCCAAACCAGCAAAACGAGTCCAGGGTGGCCTGGAGTCTCTCTACCGCTGTAGCCCGTGGTGTCACCTGCTCCCCTGAACCCC -2422
CREB
ACACAAATCACCAGAGCGAGGCAGGGCAAGTCTCAGTCCCCCCTCTCCCTCCCTCTCCCTTTCTGGATCTCCTCTCTATGTCTCTCTACTTATCTCTAACTCTCCCCCCAGCTCCGCAGT -2302
CTCTCTTCTCCTCCCCTTCCCTCCCCCCCCCCCCGACATGTGGGGGACCTACCCTGGACTCAGAGGCTCCCCCTCGCCTGCCGGAGTGGGACCAACTCTGCCTTCCAGTCTTGGGCCCT -2182
NF-κB
GGCCCCACCTGGCGGCTGCCCTCCCTCTCAACCACTCCTCCAGTTCCAGGAAGGCAGGTGCACCTCCCCTGTTCCAGCCTCGTGGCCCCAGCCCTCACTCAGGTGAGCGGCTAGCCCAGC -2062
MyoD STAT
AGAGAGCAGCTTCCCTTCCCCTGGCTTCCTGAATTTTCCCAGTTGCCATGGTAATCAAGGGAGCAGTGTTCTGAGACTGACACCTCGAAGCCCAGCGCTCAACTTTGAAATGGTTCAGGC -1942
Oct-1
AGGGGAAGCAAGGTCCAGGAGGGAAGGGGACCTGGCTGTAGGGATCCAGCTGCAGCCTCGCTCTCCGGGCAGCCCCTGTGCCACCTGGGTGTCGCTGTCATCGTCACCCTCAGCCACTGT -1822
TTCCGAAGCACTTGCTACCGGCCACGCTGCCTGCCAGAGCCGAGCCAGCGTCATCTCAGGCATCCTCACAGTGACCCCTGGCCCAGGTACCCATGGAGGAGACAGGCTCTGCAACTGGCC -1702
c-Myb
CGGCGCGTGCGTGTGTGCGTGCAGCAGAGGCAGGAAGTGGCAGAGTCCTGGGGCTGACGGAGTTACTTCTGCGCCCTTACCAGCAGCTGGAAGACAATCCAGACCCCCCCCTCCCCCCAC -1582
AP-4
CCGTCCTGTCCCAGCCCTGTCTTTAGCTCCTTGGTTAGTCCTTCCTCCCCTGTCATCCCCCCACACCCTTCTTCCTGCCCCAGAAGTCACACCCCCTGTTTCACCGCTGGATGTGCAAGA -1462
SREBP-1
CGCTCTGAAAAGCATTTATTAGCGACAGAGGGGGTGGTGATGGTTTTCCTCTTTGCGTCTTCTGGAGGCTACTTGGAGCTGCAGTGGGCTTGCTCCCCTTGGCAAGGCTGGGCAGGTGCC -1342
CdxA
ATGTGTGTGTGTGTGTGTGTGTGTGTGTGAGAGAGAGAGAGAGAGAGAGAGAGAATGAGCAAGCAATTGAGCACAAAATCCCCCTCTCGTTCCCTAATTCCCCACCCCACACACACAC -1222
TGCTTTCCCAAGAGTGTGTGAGCAGCAGCGACAGTGAAATGTAAAATCCTTTGCAATCTGCCCAGGAAAAGACAGCTTTTGAGATGGGTTTATATGGTTACCCATATGGTAGGGCCCCTA -1102
C/EBP
AGAAAGGAAGGGGAGCTTGGGTCTTTGCCTCTTCCCTTCCCCCAGCCCAGCCCAGCCCCTGGCCTCTTCCCACCTGTGAGTCTGCCAGGCCACAGGTGACTGCGTCCTCTCCACGCCCCC -982
AP-1
CCCCCCCAGAGGCCAGACCTCAGCGCTGCCCTGGGAGATGCCAGGAACCAGGCAACACTTGCAGAACCGGCTGCCTCCATCCTTGTCTGCGCACCCAAGCGCTGGTCCCAAACCACCAGCT -862
MyoD
GCCACCCCAGCTGGGGCCTGGGGGCCTCCAGCCCTGCCCAGCATGGGGGGCTGTGCCTTCCGCCACAGTCCCCTTCAGAAACGGTCCCCACCAGCAATGCGGGGACACAAATTAACCAAT -742
AP-4 MZF1 NF-Y
GCTGCCTCCCGTGGACCCCGCTCCACCCAGAGAGAACTGTCCTTCCTGTGTGGTCACTACACTCACCCCCTCCAGCCTCGCACATTGTGTGTGTGTGTGTTTCCTGATAGAA -622
AAAAAATGGAAATTAAGGTTATATAAAAGATGTATGCTCCGAAGCCCGGGAGCCACTCTTTTAACGAGGGTTGCCGCCGTCTCCCTCCCCACACCCATAAACCAGCCCGGGTTGGACGGT -502
CACCACTAATTCGTGTCAGTGATGATAGGATAAAGGAGGGACATTAAGAAATAAATTCCCCCCTCAACGACCCTCCCTGAGCTCACGGCTCCGTCCCCACATATTTACGCCGCGCCTCCA -382
CdxA
GCCGCTGCGCGAGGAGCCACTTCGCGCCGAGGGGCGGCCGGGCGGCGAGCGGCGGCCGGGGTGAGCCAGGGGGCGCGCACGGGGGAAGCCGGCACCGCACCGGCGTCCCGAACTTGGGCT -262
GGCGGGGCCGGAGGGCGGGGAGGGGAGGGCGAGGGGAGGGCGCAAGGCAGGGGTGGGGGCTAGGCGAGGGGTGGGGATGTGAGCTCGGCGGCACAAAGCGGAGGGGAAGGGCAGGG -142
Sp1 MZF1 Sp1 MZF1 Sp1
ACGCGCGCGGCTCCTACCCGAAGGTGAGTGGGTGCGCCCAGCGCCCTGCAGCCGCTCGCGGGGCGGCCGACTCGGAGCACTCGGAACCCGGAGCGCGGCGGTCGCGGGCGCCCCGGAGGA -22
AGCGGCGGCGGGCGGCGGGCGCGCGGAGCAGGAGCGAGGAGCGAGAAGGCAGCCCTAGCGCTGCCCGTCTCAGCGGCGGCGGCAGgtgagttctaggcgcaggctgcccgcgccctctt Exon 1
cctgtccccaggqctcgcctctcccgcgcgcaaggtttgcgccgtgtcccaacctcgttcgggcagcctcgttgtcctccatgcaggcggtcccgggcagaggggctccggtgccctgg
gggtcgctgtgcccgcctggctccaacttgcagggaccc . . . . . Intron 1 . . . . . gaggttggtggttgcaggtggggcctgtctgctggctacc
gtttccgtcttagctttgagagtggtacgtgtactccacgtcagtgtcttgtttttcttcttcttttttacgccctccctcttctccttttcctcagCTCCGGGAGCCCCAGATCGGCTGTG
CACCTCACGGACGGGGGACCACGATGCCCAGGGAGTAGACAACATCTGCCACATGTCCCCTTGACCCCTCCGCCAGACGCTTCTCCCCCCTCAGCCTGTTCTGACCCCGTTGATCAGAAC
GTCCCCCCGCCACCTCTCCCGACGTCTGCCTGGTCAGCCATTGCTTCCTTGGAGAACGAGGACGGCGGCAGACGCATGAAATCATGCCACCGGCAGCCGGGCGGCCGTGACTGGGTGACG
CCGGGTTGACGTCAGAGCCGGAGAAGACTGAGAGAGCTCGGCGGCCCCAGCTCCCGGCCCCTTCCCTCGGCGAACCTGGGCGCCCAGCACCTCCTGGTAAAGTCTTCCCGGCTTGATAGG
TGTTGAGGTCTCCCGCCGGGCACCATGGAGGAGCACGTGTTTGGCGTTCAGCAAATCCAGCCCAATGTCATCTCGGTCCGTCTCTTCAAGCGCAAGGTGGGGGGCCTGGGGTTCCTGGTG
MetGluGluHisValPheGlyValGlnGlnIleGlnProAsnValIleSerValArgLeuPheLysArgLysValGlyGlyLeuGlyPheLeuVal 32
AAGGAGCGCGTCAGCAAGCCCCCCGTGATCATCTCCGACCTGATTCGAGGCGGGGCCGCCGAGCAGAGCGGCCTTATCCAGGCCGGCGACATCATTCTCGCCGTCAACGGCAGGCCCTTG
LysgluArgValSerLysProProValIleIleSerAspLeuIleArgGlyGlyAlaAlaGluGlnSerGlyLeuIleGlnAlaGlyAspIleIleLeuAlaValAsnGlyArgProLeu 72
GTGGACCTGAGCTACGACAGCGCCCTGGAAGTGCTCCGGGGTGTTGCCTCGGAGACCCATGTCGTCCTCATCCTGAGGGGCCCCGAGGGCTTCACCACGCACCTGGAGACCACCTTCACT Exon 2
ValAspLeuSerTyrAspSerAlaLeuGluValLeuArgGlyValAlaSerGluThrHisValValLeuIleLeuArgGlyProGluGlyPheThrThrAsnLeuGluThrThrPheThr 112
GGAGACGGCACCCCCAAGACCATCCGGGTAACCCAGCCCCTGGGTGCCCCCACCAAAGCCGTCGATCTATCCCACCAGCCGCCGTCGGCCGGCAAAGAGCAGCCACGGCCAGTGGACGGG
GlyAspGlyThrProLysThrIleArgValThrGlnProLeuGlyAlaProThrLysAlaValAspLeuSerHisGlnProProSerAlaGlyLysGluGlnProArgProValAspGly 152
GCCGCGGGGCCTGGCAGCTGGCCTCAGCCCGCCCAAGGTCACGGGCAGGAAGCCGGCTCACCCTCCCGCGCCAATGGCCTGGCCCCCAGAACGTCAAGCCAGGACCCTGTCAAGAAAAGT
AlaAlaGlyProGlySerTrpProGlnProThrGlnGlyHisGlyGlnGluAlaGlySerProSerArgALaAsnGlyLeuAlaProArgThrSerSerGlnAspProAlaLysLysSer 192

◀

Fig. 3. Nucleotide sequences of the 5′-flanking region and the first three exons of the rabbit neuronal NOS gene. Exons are numbered and the amino acid sequences are shown below the nucleotide sequence. Intron sequences are in lower-case. The transcription start point was determined by 5′RACE and denoted by an underline. In the 5′-flanking region, putative *cis*-regulatory elements are identified by computer-based sequence analysis and indicated by open boxes. The nucleotide sequence has submitted to the GenBank with the accession number AF115281, AF126538-9.

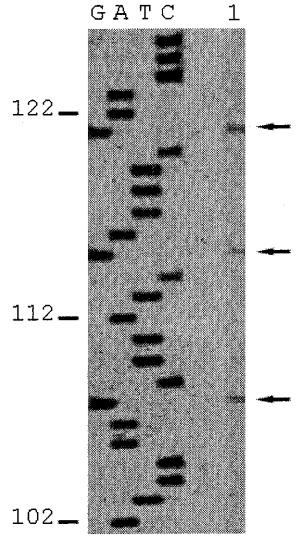


Fig. 4. Primer extension analysis. Lane 1 contained the extended products. A related sequence ladder (lane GATC) was used to measure the length of the extension products.

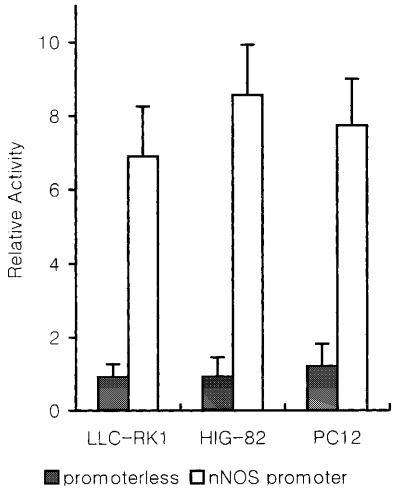


Fig. 5. Promoter activity of the rabbit nNOS 5′-flanking sequence. Transfected cells were cultured for 2 d and harvested. β-galactosidase activity and GFP fluorescence in cell lysates were measured. Open bars, promoter transfectants; solid bars, promoterless transfectants.

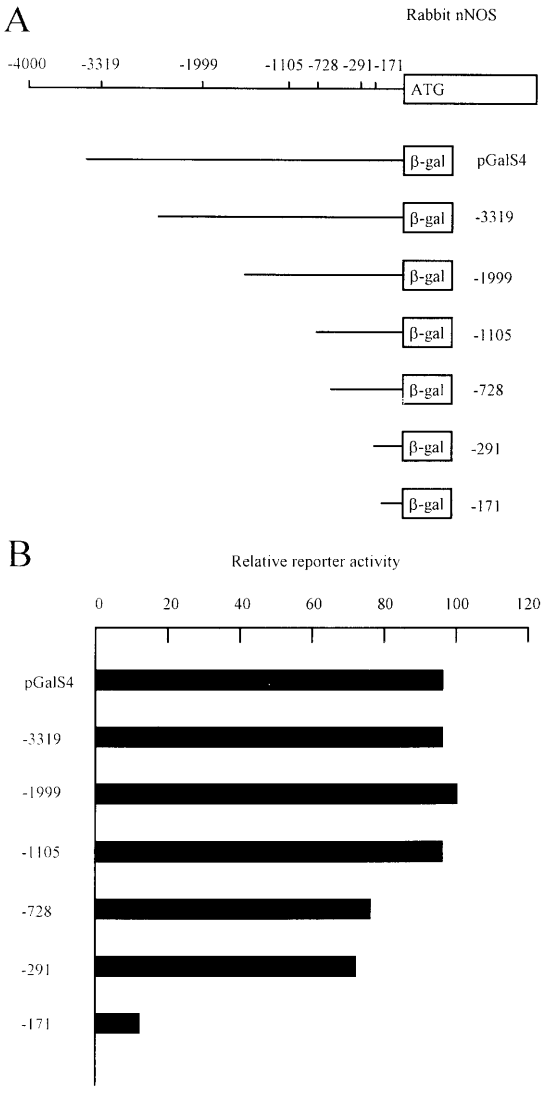


Fig. 6. Deletion analysis of the rabbit nNOS promoter. **A.** Schematic illustration of the β-galactosidase reporter plasmids with serial deletions of the rabbit nNOS promoter. **B.** β-galactosidase activity of the deletion constructs in PC12 cells. β-galactosidase activity was expressed as a percentage of the activity of the -2,643 plasmid

functional activity with transient transfection. The rabbit nNOS promoter has a high GC content of 60% overall and 78% within -399 to -98 bp. The canonical TATA and CCAAT boxes are not present within the proximal region upstream of the transcription start. Three distinct transcription start sites for exon 1 were detected by 5′RACE and primer extension analysis. These features have also been observed in the promoters of many other eukaryotic genes. The TATA motif controls the accuracy of transcription through site-specific initiation (McKnight and Kinsburg, 1982). The lack of a TATA box does not affect the rate of transcription, but causes promiscuous initiation by RNA polymerase II, resulting in multiple initiation

sites. TATA-less promoters have regions with GC-rich sequences recognized by Sp1 which has been documented to be commonly required for the transcription of many TATA-less gene promoters (Blake *et al*., 1990; Kadonaga *et al*., 1986).

From the serial deletion analysis of the rabbit nNOS promoter, it was found that the -291 to -172 region is essential for the basal promoter activity and the -1,105 to -729 region is necessary for full activity. The -291 to -172 region contains two Sp1 sites. Cooperative actions of transcription factors are well documented. Sp1-Sp1 self interaction can activate transcription synergistically (Courey *et al*., 1989). In addition, Sp1 cooperates with other transcription factors. One potential regulatory binding site for AP-1 is found in the -1,105 to -727 region. In myeloid cells, AP-1 acts synergistically with Sp1 to facilitate gene expression (Noti *et al*., 1996). In many promoter contexts, Sp1 cooperates with NF-Y (Inoue *et al*., 1999; Jun *et al*., 1997; Roder *et al*., 1999). So, NF-Y located in the -1,105 to -727 region (-744 bp) may cooperate with Sp1 in the -291 to -172 region for transcriptional activation of the nNOS gene. Although not necessary for basal transcriptional activity, some elements located in other regions may function in response to some external signals. GATA-1, which binds to the sequence SNNGATNNNN (-4,119, -3,101 and -2,795 bp), is implicated in the gene expression of erythroid, megakaryocyte and mast cells, and synergizes with Sp1 (Merika and Orkin, 1995). The GATA-2 element (NNNGATRNNN), which is found at nt -4,129 in the promoter of the nNOS gene, can potentiate the action of AP-1 (Kawana *et al*., 1995). Five binding elements for NF-κB were also observed in the distal region of the promoter. The NF-κB family has been shown to respond to a wide variety of signals and to regulate the expression of a large number of genes. NF-κB can interact cooperatively with Sp1 or C/EBP (Stein *et al*., 1995). Cooperation with Sp1 is also known to be required for sterol-regulated gene expression mediated by SREBP, which is located at nt -1,523 in the nNOS promoter (Sanchez *et al*., 1995). SREBP can also stimulate transcription through functional interaction with NF-Y (-744 bp) (Ericsson *et al*., 1996). Although the possible *cis*-elements responsible for the basal transcriptional activity of the rabbit nNOS promoter were identified by deletion analysis, further functional studies related to the signal-dependent regulation of the nNOS promoter activity are needed to fully understand the regulation of the nNOS gene.

Interspecies comparison of genes coding for nNOS The organization of the rabbit nNOS gene was compared with that of its human counterpart. So far, only the structure of the human nNOS gene has been described (Hall *et al*., 1994). The 1.6 kb-promoter region of the human nNOS gene was sequenced, and various putative *cis*-elements were described by Hall *et al*. In humans, a TATA box was evident 28 nt upstream from the single defined transcriptional start site. But the rabbit promoter is TATA-less and drives transcriptional initiation from multiple sites. On comparing the nucleotide composition, the rabbit sequences have a higher GC content (63% in the proximal region from -1,634 to -1 bp and 60% overall) than that of the human nNOS promoter (53% for the -1,634 to -1 bp). Besides, the unusually high GC content (78% composition), which provides three potential binding sites for Sp1 (-246, -185 and -145 bp), exists within the proximal region (-399 to -98 bp) from the transcriptional initiation site of the rabbit nNOS, but not in the human promoter which does not have any Sp1 sites. Comparing the farther upstream region, several putative sites for AP-2, Ets, NF-1, NRF-1 and TEF-1/MCBF are located in the human promoter, while computer analysis of the rabbit 5′-flanking region did not detect any of those elements. The NF-κB element, which was identified in the human iNOS gene, was also observed between these species. But, the locations and numbers of this consensus motif are different. While two NF- κB sites are present within the region from nt -501 to -308 of the human sequences, five are dispersed in the rabbit promoter ranging from nt -5,041 to -2,246. Although the amino acid sequence of the rabbit nNOS is highly homologous to that of human, many dissimilarities exist in the promoter region, and the nNOS genes may be subject to different transcriptional regulation in different species.

The rabbit nNOS promoter has inducible characteristics for its transcription Three distinct NOS isoforms responsible for NO biosynthesis are expressed in many different cells and tissues. The two enzymes eNOS and nNOS are often referred to as constitutive NOS (cNOS) to distinguish them from inducible NOS (iNOS) which is subject to transcriptional regulation by cytokines. Several studies, however, have shown that transcription of the nNOS gene can also be tightly regulated (described in **Introduction**). Characterization of the rabbit nNOS gene and analysis of the putative *cis*-elements in the promoter are likely to provide important information regarding its inducible expression. Intriguing is the presence within the 5′-flanking region of multiple regulatory elements which respond to certain external signals or abnormal conditions such as treatment of NGF or estrogen, hypoxia and nerve injury. Hypoxia is an important pathophysiological condition that occurs during injury, ischemia, reperfusion and stroke, and induces gene expression. Exposure of cells to hypoxia results in IkappaB degradation, increased NF-κB DNA binding activity, and transactivation of certain genes (Koong *et al*., 1994; Schmedtje *et al*., 1997). In rat dorsal root ganglia, Schwann cells and PC12 cells, NF-κB is selectively activated by NGF (Carter *et al*., 1996;

Wood, 1995). CRE (a cAMP-response element) has been shown to mediate the induction of VGF gene expression by NGF (Hawley *et al*., 1992). Besides, in rat brain and PC12 cells, hypoxia and estrogen treatment induces the phosphorylation of CREB, which is required for CRE-mediated transcriptional activation (Beitner-Johnson and Millhorn, 1998; Zhou *et al*., 1996). AP-1 and C/EBP are also neuronal transcriptional regulators activated by NGF in PC12 cells (Alheim *et al*., 1996; Sterneck and Johnson, 1998).

Taken together, the rabbit nNOS gene promoter contains many inducible characteristics, which may be responsible for its regulated transcription.

Acknowledgments This work was supported by the National Creative Research Initiative Program. We thank Dr. Yutaka Akiyama (Kyoto University) for help with the transcription factor data base search. Jaejoon Won is a recipient of the Brain Korea 21 Fund from the Korean Ministry of Education.

References

Alheim, K., Mcdowell, T. L., Symons, J. A., Duff, G. W., and Bartfai, T. (1996) An AP-1 site is involved in the NGF induction of IL-1 alpha in PC12 cells. *Neurochem. Int*. **29**, 487–496.

Arancio, O., Kiebler, M., Lee, C. J., Lev-Ram, V., Tsien, R. Y., Kandel, E. R., and Hawkins, R. D. (1996) Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. *Cell* **87**, 1025–1035.

Beitner-Johnson, D. and Millhorn, D. E. (1998) Hypoxia induces phosphorylation of the CREB by a novel signalling mechanism. *J. Biol. Chem*. **273**, 19834–19839.

Blake, M. C., Jambou, R. C., Swick, A. G., Kahn, J. W., Clifford, J., and Clifford Azizkhan, J. (1990) Transcriptional initiation is controlled by upstream GC-box interactions in a TATA-less promoter. *Mol. Cell. Biol*. **10**, 6632–6641.

Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995) Nitric oxide synthase is complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* **82**, 743–752.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* **84**, 757–767.

Brüne, B. and Lapetina, E. Q. (1991) Phosphorylation of nitric oxide synthase by protein kinase A. *Biochem. Biophys. Res. Commun*. **181**, 921–926.

Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaie, R., Baeuerle, P. A., and Barde, Y. A. (1996) Selective activation of NF-κB by NGF through the neurotrophin receptor p75. *Science* **272**, 542–545.

Courey, A. J., Holtzman, D. A., Jackson, S. P., and Tjian, R. (1989) Synergistic activation by the glutamate-rich domains of human transcription factor Sp1. *Cell* **59**, 827–836.

Ericsson, J., Jackson, S. M., and Edwards, P. A. (1996) Synergistic binding of SREBP and NF-Y to the farnesyl diphosphate synthase promoter is critical for sterol-regulated expression of the gene. *J. Biol. Chem*. **271**, 24359–24364.

Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994) Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. *J. Biol. Chem*. **269**, 21644–21649.

Guo, Y., Ward, M. E., Beasjours, S., Mori, M., and Hussain, S. N. (1997) Regulation of cerebellar nitric oxide production in response to prolonged *in vivo* hypoxia. *J. Neurosci. Res*. **49**, 89–97.

Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C., and Marsden, P. A. (1994) Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J. Biol. Chem*. **269**, 33082–33090.

Hawley, R. J., Scheibe, R. J., and Wagner, J. A. (1992) NGF induces the expression of the VGF gene through a cAMP response element. *J. Neurosci*. **12**, 2573–2581.

Holtzman, D. M., Lee, S., Li, Y., Chua-Couzens, J., Xia, H., Bredt, D. S., and Mobley, W. C. (1996) Expression of neuronal NOS in developing basal forebrain cholinergic neurons: regulation by NGF. *Neurochem. Res*. **21**, 861–869.

Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **75**, 1273–1286.

Inoue, T., Kamiyama, J., and Sakai, T. (1999) Sp1 and NF-Y synergistically mediate the effect of vitamin D(3) in the p27(Kip1) gene promoter that lacks vitamin D response elements. *J. Biol. Chem*. **274**, 32309–32317.

Jaffrey, S. R. and Snyder, S. H. (1996) PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* **274**, 774–777.

Jeong, Y., Won, J., and Yim, J. (1998) Cloning and structure of a rabbit protein inhibitor of neuronal nitric oxide synthase (PIN) gene and its pseudogene. *Gene* **214**, 67–75.

Jun, D. Y., Kim, M. K., Kim, I. G., and Kim, Y. H. (1997) Characterization of the murine cyclinD2 gene: exon/intron organization and promoter activity. *Mol. Cells* **7**, 537–543.

Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) Promoter specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci*. **11**, 20–23.

Kawana, M., Lee, M. E., Quertermous, E. E., and Quertermous, J. (1995) Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. *Mol. Cell. Biol*. **15**, 4225–4231.

Koong, A. C., Chen, E. Y., and Giaccia, A. J. (1994) Hypoxia causes the activation of NFκB through the phosphorylation of IκB alpha on tyrosine residues. *Cancer Res*. **54**, 1425–1430.

Mcknight, S. L. and Kinsburg, R. (1982) Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217**, 316–324.

Merika, M. and Orkin, S. H. (1995) Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol. Cell. Biol*. **15**, 2437–2447.

Mermod, N., Williams, T. J., and Tjian, R. (1988) Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription *in vitro*. *Nature* **332**, 557–561.

Nakane, M., Mitchell, J., Forstermann, U., and Murad, F. (1991) Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. *Biochem. Biophys. Res. Commun*. **180**, 1396–1402.

Nathan, C. and Xie, Q. (1994) Regulation of biosynthesis of nitric oxide. *J. Biol. Chem*. **269**, 13725–13728.

Noti, J. D., Reinemann, B. C., and Petrus, M. N. (1996) Sp1 binds two sites in the CD11c promoter *in vivo* specifically in myeloid cells and cooperates with AP-1 to activate transcription. *Mol. Cell. Biol.* **16**, 2940–2950.

Quast, M. J., Wei, J., and Huang, N. C. (1995) Nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester decreases ischemic damage in reversible focal cerebral ischemia in hyperglycemic rats. *Brain Res.* **677**, 204–212.

Roder, K., Wolf, S. S., Larkin, K. J., and Schweizer, M. (1999) Interaction between the two ubiquitously expressed transcriptional factors NF-γ and Sp1. *Gene*. **234**, 61–69.

Sanchez, H. B., Yieh, L., and Osborne, T. F. (1995) Cooperation by sterol regulatory element-binding protein and Sp1 in sterol regulation of LDL receptor gene. *J. Biol. Chem.* **270**, 1161–1169.

Schmedtje, J. F. Jr., Ji, Y. S., Liu, W. L., Dubois, R. N., and Runge, M. S. (1997) Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. *J. Biol. Chem.* **272**, 601–608.

Sheehy, A. M., Phung, Y. T., Riemer, R. K., and Black, S. M. (1997) Growth factor induction of nitric oxide synthase in rat PC12 cells. *Brain Res. Mol. Brain Res.* **52**, 71–77.

Son, H., Hawkins, R. D., Martin, K., Kiebler, M., Huang, P. L., Fishman, M. C., and Kandel, E. R. (1996) Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* **87**, 1015–1023.

Stein, B., Cogswell, P. C., and Baldwin, A. S. Jr. (1995) Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. *Mol. Cell. Biol.* **13**, 3964–3974.

Sterneck, E. and Johnson, P. F. (1998) CCAAT/enhancer binding protein beta is a neuronal transcriptional regulator activated by nerve growth factor receptor signaling. *J. Neurochem.* **70**, 2424–2433.

Verge, V. M. K., Xu, Z., Xu, X-Z., Wiesenfeld-Hallin, Z., and Hokfelt, T. (1992) Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. *Proc. Natl. Acad. Sci. USA* **89**, 11617–11621.

Weiner, C. P., Lizasoanin, I., Gaylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994) Induction of calcium-dependent nitric oxide synthase by sex hormones. *Proc. Natl. Acad. Sci. USA* **91**, 5212–5216.

Wood, J. N. (1995) Regulation of NF-κB activity in rat dorsal root ganglia and PC12 cells by tumor necrosis factor and nerve growth factor. *Neurosci. Lett.* **192**, 41–44.

Zhou, Y., Watters, J. J., and Dorsa, D. M. (1996) Estrogen rapidly induces the phosphorylation of the cAMP-response element binding protein in rat brain. *Endocrinology* **137**, 2163–2166.