Tissue- and Development-specific Expression of Multiple Alternatively Spliced Transcripts of Rat Neuronal Nitric Oxide Synthase

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Abstract

Nitric oxide (NO) functions as an intercellular messenger and mediates numerous biological functions. Among the three isoforms of NO synthase that produce NO, the ubiquitously expressed neuronal NO synthase (nNOS) is responsible for a large part of NO production, yet its regulation is poorly understood. Recent reports of two alternative splice-forms of nNOS in the mouse and in man have raised the possibility of spatial and temporal modulation of expression. This study demonstrates the existence of at least three transcripts of the rat nNOS gene designated nNOSa, nNOSb, and nNOSc, respectively, with distinct 5′ untranslated first exons that arise from alternative splicing to a common second exon. Expression of the alternative transcripts occurs with a high degree of tissue and developmental specificity, as demonstrated by RNase protection assays on multiple tissues from both fetal and adult rats. Furthermore, terminal differentiation of rat pheochromocytoma-derived PC12 cells into neurons is associated with induction of nNOSa, suggesting, likewise, development- and tissue-specific transcriptional control of nNOS isoform expression. Physical mapping using a rat yeast artificial chromosome clone shows that the alternatively spliced first exons 1a, 1b, and 1c are separated by at least 15–60 kb from the downstream coding sequence, with exons 1b and 1c being positioned within 200 bp of each other.

These findings provide evidence that the biological activity of nNOS is tightly and specifically regulated by a complex pattern of alternative splicing, indicating that the notion of constitutive expression of this isoform needs to be revised. (J. Clin. Invest. 1997. 100:1507–1512.) Key words: nitric oxide • nitric oxide synthase • alternative splicing • gene expression • transcription

Introduction

Nitric oxide (NO)1 is synthesized by nitric oxide synthase (NOS) during the NADPH-dependent conversion of L-arginine to L-citrulline. Three isoforms of NOS encoded by distinct genes have been identified. The neuronal and endothelial NO synthases (nNOS, eNOS) are calcium/calmodulin dependent; their expression has in the past been viewed as constitutive (1–4). In contrast, the inducible isoform (iNOS) isolated from macrophages is calcium/calmodulin-independent, and expression is induced by cytokine stimulation (5, 6). NO is a multifunctional intercellular messenger affecting diverse aspects of mammalian physiology including regulation of vascular tone, macrophage-mediated cytotoxicity, neuronal differentiation, and synaptic plasticity (7). While the transinteraction of cytokine-responsive elements within the promoter region is thought to affect the transcriptional regulation of the iNOS gene (8), the mechanisms by which the ubiquitous expression of the nNOS gene is regulated are unknown.

The highest expression of NOS in the rat has been demonstrated in the brain, in particular in the cerebellum. The gene is also expressed in skeletal muscle, in the myenteric plexus in the gut, in the macula densa of the kidney, and in the heart (9, 10). Based on its widespread expression, nNOS is generally considered to be responsible for the largest proportion of tissue NO synthase activity. Although traditionally labeled constitutive, the expression of nNOS may in fact be tightly regulated, as suggested by a number of recent observations. nNOS expression was found to be subject to regulation by estrogen (11), and was induced after axotomy, in parallel with an increased expression of the transcription factors jun and krox (12, 13). Furthermore, transient changes in levels of expression of nNOS have been observed in the developing rat nervous system and lung (14, 15). Likewise, the onset of NOS expression in the developing tectum in chicken coincides with the onset of innervation by axons from retinal ganglion cells (16).

Recently, two alternatively spliced nNOS transcripts arising from two alternative promoters and differing in their 5′-untranslated regions (5′ UTR) have been described in human and murine nNOS genes (17, 18). The use of multiple alternative promoters in conjunction with alternative splicing may represent a mechanism by which spatial as well as temporal regulation of nNOS gene transcription is achieved.

Here we present evidence for the presence of three differentially spliced nNOS variants in the rat as well as a comprehensive analysis of spatial and temporal expression patterns that further supports the notion that nNOS expression is modulated by specific and complex regulatory mechanisms on the transcriptional level.

1. Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; NGF, nerve growth factor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; UTR, untranslated regions; YAC, yeast artificial chromosome.
Methods

Rapid amplification of cDNA ends (RACE). Total RNA was extracted from frozen rat tissues by the guanidium thiocyanate–cesium chloride gradient method. Genomic DNA was removed by incubation with RNase-free DNase (Promega Corp., Madison, WI) followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. RACE was carried out as previously described (19). In brief, first-strand cDNA was synthesized from 3 μg total RNA of various tissues with primer N1 (5′-ggtgctcaagtctgcgattttct-3′) located at position 211 of the published sequence for the rat nNOS gene (3), using Superscript II RNase H–Reverse Transcriptase (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD). The cDNA was column-purified, tailed with terminal transferase (Life Technologies, Inc.) in the presence of dCTP (Promega Corp., Madison, WI). The labeled oligonucleotides for nNOSa (A1 5′-gaccttcaacagcctgcagcc-3′), nNOSb (By1 5′-ctgtggaatcggcgtcgtgctg-3′), and nNOSc (By2 5′-ggaactctgaaagccgctct-3′) were combined with the 5′ end extension at 72°C for 10 min. Amplicons were fractionated on a 20% agarose gel, purified, and cloned into the TA Cloning Kit (Invitrogen Corp., San Diego, CA). Clones were sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit using primer N3 on an ABI373A automatic sequencer (Applied Biosystems Inc., Foster City, CA). All newly determined sequences have been deposited with the GenBank database (accession numbers: AF008911, AF008912, AF008913).

RNase protection assay. cDNA clones obtained by RACE–PCR were linearized at appropriate restriction sites at the 5′ end of the insert. Complementary RNAs were transcribed from 1 μg plasmid in the presence of [α-32P]UTP (DuPont-NEN, Boston, MA) with SP6 RNA polymerase using the MAXiScript kit (Ambion Inc., Austin, TX). Labeled probes were purified over Chromaspin-100 columns (Clontech, Palo Alto, CA). RNase protection assay was carried out using the RPAII kit (Ambion Inc.). In brief, 100,000 cpm of labeled probe was hybridized against 100 μg total RNA or tRNA overnight at 45°C. Samples were digested with RNase A and T1, precipitated, and run on a 5.5% denaturing polyacrylamide gel.

Cell culture and reverse transcription PCR (RT-PCR). Differentiation of rat pheochromocytoma–derived PC12 cells into neurons was performed with the internally located oligonucleotide N3 labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA).

Screening of a rat yeast artificial chromosome library. PCR amplification of screening pools of a rat YAC library (22) was carried out using oligonucleotide sets specific for the alternative first exons of nNOSa (Ay1 5′-ggtcgctgctgctaccgttcttct-3′; Ay2 5′-ctgctgctgctgctaccgtctttc-3′), and nNOSb (By1 5′-ctgtggaatcggcgtcgtgctgctg-3′; By2 5′-ggaactctgaaagccgctct-3′) and nNOSc (C1 5′-ctgcagcctcttgagctagct-3′), respectively, were combined with the common antisense primer N1. A cDNA pool derived from brain, embryo, and kidney was included as positive control. To monitor expression of nNOSa, nNOSb, and nNOSc, RNase protection assay was carried out using probes generated by PCR using the same primers as for the library screening. Probes were labeled with [α-32P]dCTP (DuPont NEN) in the presence of random hexamers using T7 DNA polymerase (Pharmacia Diagnostics AB).

Results

Isolation of multiple nNOS transcripts with unique 5′UTRs. Cloning and sequencing of cDNA by RACE from brain, kidney, heart, intestine, and embryo led to the identification of three different nNOS mRNA species that are designated as nNOSa, nNOSb, and nNOSc. nNOSa was overall the most abundant isoform, found especially in the brain, while nNOSb was almost exclusively expressed in embryonic tissue and nNOSc in the kidney. A common 3′ terminal sequence consisting of 30 bp was found to be shared by all isoforms extending 5′ from position +2 of the published sequence of the rat nNOS cDNA (3). Based on PCR analysis of genomic DNA, this 30-bp common sequence is contiguous with the published 5′ end of the gene, and therefore represents the actual 5′ end of the second exon (previously perceived as the first exon) of the rat nNOS gene. The unique 3′ terminal sequences represent differential exon 1 sequences of nNOSa, nNOSb and nNOSc that are spliced to this newly identified 30-bp domain of the second exon (Fig. 1). The newly identified unique first exons of nNOSa, nNOSb, and nNOSc are 442, 134, and 151 bp in length, respectively. RT-PCR with isoform-specific sense and antisense primers located at various positions of the cDNA as well as direct sequencing of PCR products yielded no evidence for additional nNOS mRNA species or further variations within the coding sequence, except for a previously described 102-bp insert between exon 16 and 17 (23) that was detectable in testicular muscle and embryo (data not shown). Comparative databank searches using the BLAST module showed that nNOSa shares a high degree of homology with one of the two alternative first exons identified in the mouse, while neither nNOSb nor nNOSc showed homology to any of the other alternative first exons described in mouse or human (17, 18).

Tissue- and development-specific expression of nNOS mRNA isoforms. The expression pattern of the different transcription variants was determined by RNase protection assay using cDNA clones obtained by RACE as probes that included part of the published sequence corresponding to position +2
Because all three nNOS mRNA isoforms shared the additional common, newly recognized stretch of 30 bp in exon 2, a 160-bp fragment was expected to be protected, and was indeed found to be present in all tissues expressing any of the nNOS variants. In addition, specific transcript signals corresponding to the 5' terminus of each nNOSa, nNOSb, and nNOSc, were found depending on tissue-specific isoform expression. Consistent with its high prevalence among RACE clones, nNOSa was the most abundant isoform. It showed highest expression in brain, followed by kidney, intestine, E18 embryo, adrenal, heart, and skeletal muscle. In contrast, nNOSb was exclusively expressed in E18 embryo, while nNOSc expression was found in kidney, skeletal muscle, and embryo (Fig. 2). Absolute expression levels of nNOSc are low compared to the other isoforms, and signals were detectable only after prolonged exposure to autoradiographic material. The only tissue that consistently tested negative for all three isoforms of nNOS was the testis.

**Induction of nNOSa in terminally differentiated PC12 cells.** Using sense primers A1, B1, and C1 specific for the alternative first exons in combination with the antisense primer N1 specific for exon 2 of the rat nNOS gene, RT-PCR followed by Southern blotting was carried out on cDNA obtained from rat pheochromocytoma-derived PC12 cells before and after induction of neuronal differentiation by NGF. While nNOS expression was almost undetectable under basal conditions, strong induction of nNOSa mRNA was seen in differentiating cells (Fig. 3).

**Figure 1.** Schematic presentation of alternative first exons in rat, mouse, and human nNOS gene. (A) The light grey bar denotes the published nNOS cDNA sequence of the rat containing the translation initiation site (ATG). A 30-bp extension from position 2 (+2) of the published sequence common to all alternative 5'UTRs is indicated by a dark grey bar. Unique sequences of the alternative 5'UTRs are indicated as hatched bar (nNOSa) or open bars (nNOSb, nNOSc). A1, B1, and C1: sense primer used for RT-PCR; N1, N2, and N3: antisense primer used for RACE and RT-PCR. Clones used in RNase protection assays included a 160-bp stretch common to all nNOS isoforms. Alternative 5' sequences of the mouse nNOS gene (nNOSb and nNOSγ) and of the human nNOS gene (nNOS5'1 and nNOS5'2; C). Homologous first exons (nNOSa of the rat and nNOSb of the mouse) are indicated by hatched bars.
least 15 kb, but of not more than 20 kb upstream of exon 2. Exons 1b and 1c were placed approximately an additional 40 kb upstream of exon 1a (Fig. 4).

Discussion

Our results demonstrate that nNOS in the rat occurs in at least three isoforms characterized by the presence of different first exons, and that the previously published information regarding the 5' end of what we now recognize as exon 2 of the rat nNOS cDNA was incomplete. In addition, we report that the three isoforms of the gene show markedly different expression patterns that are spatially and developmentally distinct. While the relative abundance of nNOS mRNA in the tissues analyzed is consistent with previous studies, our findings demonstrate that overall mRNA levels in some tissues are composed of different amounts of the mRNA variants of nNOS. Further studies using in situ hybridization are necessary to elucidate the spatial and temporal expression of nNOS-mRNA on a tissue and cellular level. These observations suggest a more complex and intricate pattern of modulation of expression for this enzyme than previously appreciated, and challenge the heretofore held perception that nNOS is a constitutively expressed gene.

The newly identified first exons 1a, 1b, and 1c of the rat nNOS gene are located upstream of the coding sequence, and therefore, do not result in a modification of the encoded protein. Recently, two nNOS mRNAs with alternative 5'UTRs were identified in the mouse as well as in humans (17, 18). While rat nNOSa shows close homology to one of the mouse mRNA isoforms (nNOSb), however, no similarity was detectable between nNOSb and nNOSc and the second 5'UTR (nNOSγ) in the murine nNOS gene. Likewise, no homology was found between any of the three first exons in the rat and the two reported alternative exons in humans (nNOS 5' and nNOS 5'). This observation may simply reflect species-specific differences, or point to the presence of further yet to be identified alternative 5' ends in the rat that are homologous with the alternative first exon in mouse and the two human isoforms, and that may have escaped our present 5'RACE analysis due to low abundance. Although the heterogeneity of the 5' ends of nNOS mRNA could be explained by alternative splicing events, it is likely that activation of differential promoters is involved in their generation. In support of this, the two human nNOS mRNA species are thought to be transcribed from two closely linked, but separable promoters (17).
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doing of developmentally specific expression patterns may shed exclusive expression during development (14, 15). Our observation of developmentally specific expression patterns may shed some light on recent observations obtained in mice carrying a deletion of exon 2 of the nNOS gene (25). We speculate that the relative absence of developmental pathology in this model may be explained by predominance of the still functional transcript nNOSb from the first alternative exon, that retains 85% activity during critical phases of development (18, 25). The presence of multiple promoters would allow extensive regulation of transcription of a single gene both spatially and temporally, as suggested by the tissue- and development-specific expression pattern of nNOS mRNA species presented here.

Rat nNOS Transcriptional Variants

Furthermore, the alternative use of multiple first exons flanked by tissue-specific promoters has been demonstrated for a number of other genes that are ubiquitously expressed, such as for the gene encoding aromatase (which, like nNOS, is a member of the cyotochrome P-450 superfamily) (24) and the gene encoding the glucocorticoid receptor (24). The hypothesis that expression of nNOSb may be driven by a developmentally regulated promoter is consistent with its transient and exclusive expression during development (14, 15). Our observation of developmentally specific expression patterns may shed some light on recent observations obtained in mice carrying a deletion of exon 2 of the nNOS gene (25). We speculate that the relative absence of developmental pathology in this model may be explained by predominance of the still functional transcript nNOSb from the first alternative exon, that retains 85% activity during critical phases of development (18, 25). The presence of multiple promoters would allow extensive regulation of transcription of a single gene both spatially and temporally, as suggested by the tissue- and development-specific expression pattern of nNOS mRNA species presented here.

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