Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1242–1246, February 1995
Neurobiology

Two closely linked but separable promoters for human neuronal nitric oxide synthase gene transcription

(neuronal gene expression/CpG islands/transient expression/exon mapping)

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Communicated by Martha Vaughan, National Institutes of Health, Bethesda, MD, October 21, 1994

ABSTRACT **In this report we demonstrate that the human cerebellum contains neuronal nitric oxide synthase (nNOS) mRNAs with two distinct 5′-untranslated regions that are encoded through use of closely linked but separate promoters. nNOS cDNA clones were shown to contain different 5′ terminal exons spliced to a common exon 2. Genomic cloning and sequence analysis demonstrate that the unique exons are positioned within 300 bp of each other but separated from exon 2 by an intron that is at least 20 kb in length. A CpG island engulfs the downstream 5′-terminal exon. In contrast, most of the upstream exon resides outside of this CpG island. Interestingly, the upstream exon includes a GT dinucleotide repeat. A fusion gene with a 414-bp nNOS genomic fragment that includes a portion of the upstream 5′-terminal exon and its immediate 5′-flanking DNA is expressed in transfected HeLa cells. Also expressed is a fusion gene that contains the luciferase reporter under transcriptional control by a 308-bp genomic fragment that includes the region separating both 5′-terminal exons. These results indicate that expression of these exons is subject to transcriptional control by separate promoters. However, the proximity of these promoters raise the possibility that complex interactions may be involved in regulating nNOS gene expression at these sites.**

The free radical gas nitric oxide (NO) is a signaling molecule that is involved in a large number of physiological processes (1). In the central nervous system (CNS), NO has been postulated to exert both neurotoxic and neuroprotective effects (2), and it may play physiologic roles as a mediator of both long-term potentiation (3) and synaptic plasticity and remodeling (4).

Based on sequence analysis of cDNA clones, at least three distinct isoforms of nitric oxide synthase (NOS), the enzyme responsible for NO biosynthesis, are expressed in different cells and tissues. Distinct calcium/calmodulin-dependent enzymes are expressed in endothelial cells (5) and in granule and basket cells of the cerebellum as well as 1–2% of the neurons throughout the CNS (6, 7). These NOS isoforms are sometimes referred to as constitutive NOS, or cNOS, to distinguish them from inducible NOS, or iNOS, an enzyme that does not require calcium/calmodulin for activity but appears to be subject to transcriptional regulation by cytokines (8).

Despite its moniker, transcription of the gene encoding the NOS isoform expressed in neurons is likely to be tightly regulated. For example, expression of NOS mRNA in the developing tectum of the chicken correlates with the onset of innervation by axons from retinal ganglion cells (9). In primary cultures of cerebellar granule cell neurons, expression of NOS appears to be diminished by electrical activity (10). Neuronal NOS (nNOS) is also dramatically induced in spinal motor neurons (11) as well as in dorsal root ganglia (12) in response to avulsion and proximal transection, respectively. Because of the diverse and profound effects of NO, control of NOS gene expression in neurons is of clear relevance to CNS function. To acquire a more general understanding of transcriptional control of the human nNOS gene,§ we have applied molecular biologic procedures to characterize nNOS transcripts present in human cerebellum. Our data reveal a previously unappreciated complexity.

MATERIALS AND METHODS

DNA Cloning and Sequencing. All procedures were performed using standard molecular biologic protocols (13). A human cerebellar cDNA library was purchased from Clontech. Genomic libraries were constructed in the vector λEMBL3 using human placental DNA after partial digestion with *Mbo* I or complete digestion with *Bam*HI and isolation of the approximately 20-kb or 9-kb fractions, respectively, from low-melting-point agarose gels. Libraries were screened by plaque hybridization. Nucleotide sequences were deduced by the chain-termination method (14) from single-stranded phage or denatured double-stranded plasmid templates.

Analysis by Reverse Transcription (RT)–PCR. Total RNA (2 μg) from human cerebellum, skeletal muscle, or leukocyte-depleted platelets was reverse transcribed in the presence or absence of random hexamers using SuperScript RNase H⁻ reverse transcriptase (BRL/GIBCO) according to the manufacturer's instructions except that actinomycin D (50 μg/ml) was included in the 15-μl reaction mixture. One-tenth of the reaction mixture was denatured for 5 min at 95° followed by 35 cycles of PCR (denaturation at 95°C for 20 sec, annealing at 57°C for 30 sec, and extension at 72°C for 90 sec) using an antisense strand oligonucleotide specific for exon 2 [5′-CGGATCAGATCTGAGGCATC-3′; nt 17–36 of pNOS5 (7)] and sense-strand oligonucleotides specific for either exon 5′1 (5′-CGGAGGATTCAGAACCCGGAG-3′; nt 776–796 of Fig. 3) or exon 5′2 (5′-GGGTGAGGAGCTACTTAGCG-3′; nt 443–462 of Fig. 3).

Plasmid Constructions. p2.3NOS-L and p2.3SON-L were constructed as follows: a 3-kb *Hin*dIII–*Bam*HI fragment derived from λNOS16 (see Fig. 3) was subcloned into pBluescript SK+ (Stratagene), resulting in pNOS16HB. The genomic fragment contains three *Sma* I sites. One is within the 5′1 untranslated region (UTR; see Fig. 3), one is several hundred base pairs downstream of the 5′1 UTR, and one is juxtaposed to the *Bam*HI site. Digestion of pNOS16HB with *Sma* I followed by religation yielded pNOS16HBΔS, a plasmid with

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Abbreviations: NOS, nitric oxide synthase; RT, reverse transcription; UTR, untranslated region; CNS, central nervous system; nNOS, neuronal NOS.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U15666).

the *Sma* I site within the 5′1 UTR now juxtaposed to the *Bam*HI site. Subcloning the *Hin*dIII–*Bam*HI fragment of pNOS16HBΔS into pXP2 and pXP1 (15), which were digested with *Hin*dIII and either *Bgl* II or *Bam*HI, yielded p2.3NOS-L and p2.3SON-L, respectively. After subcloning the 5-kb *Xho* I–*Bam*HI fragment derived from λNOS16 (see Fig. 3) into pBluescript SK+, p4.3NOS-L was constructed based on a strategy similar to that used to construct p2.3NOS-L. p5′1ANOS-L was constructed by subcloning an *Eag* I fragment containing a portion of exon 5′1 and its 5′ flanking DNA (nt 500–807 of Fig. 3) into pBluescript SK+, followed by insertion of a *Hin*dIII–*Sst* I fragment into pXP2. p5′1BNOS-L, p5′2ANOS-L, and p5′2BNOS-L were constructed by using PCR to generate fragments containing nt 579–798, 50–462, or 234–462 (Fig. 3), respectively, as well as *Kpn* I and *Bgl* II restriction sites at the 5′ and 3′ ends, respectively, followed by subcloning into pXP2. Structures of all fusion genes were confirmed by DNA sequencing.

Analysis of Transient Gene Expression. Calcium phosphate-mediated gene transfer of HeLa cells and rat L6 myoblasts (16) and assay of extracts for the amount of total protein (17), luciferase activity (18), and β-galactosidase activity (19) were performed by published procedures. For each experiment, the data are expressed as mean values ± SD obtained after assay of the extracts from three separate transfections. Each experiment was performed on several occasions with similar results.

Analysis by Primer Extension. Poly(A)$^+$ RNA from human cerebellum (2 μg) was reverse transcribed with end-labeled antisense oligonucleotides designed to hybridize to exon 5′1- or exon 5′2-containing mRNAs (5′-CGCTCCGGGTTCTGAATCC3′ or 5′-CTGGGCTCCAAAGCATA-3′; nt 780–798 or 235–251 of Fig. 3, respectively) followed by autoradiographic analysis of the products after polyacrylamide gel electrophoresis.

RESULTS

Expression of Multiple nNOS Transcripts Distinguished by Their 5′ UTRs. cDNA clones that encode human nNOS have been reported (7). The clones span 4780 nt and include a 430-nt 5′ UTR, a 4299-nt open reading frame that encodes a protein of 1433 amino acids, and 51 nt of the 3′ UTR. We screened a human cerebellar λgt10 cDNA library and isolated two clones that hybridized to the 5′-terminal *Eco*RI–*Bam*HI fragment of pNOS5 (7). The cDNA inserts were subcloned into pBluescript SK+ and designated pNOS5′1 and pNOS5′2, respectively.

The 3′-terminal sequences of the inserts within pNOS5′2 and pNOS5′1 (2.7 kb and 2.9 kb, respectively) align precisely with the published sequence of nNOS cDNA (7). The 5′ terminus of pNOS5′2 extends 62 nt beyond pNOS5 but is otherwise identical. The 5′-terminal 161 nt of pNOS5′1 are not contained within pNOS5′2 or pNOS5 (see Fig. 3). Moreover, alignment of nt +162 of pNOS5′1 initiates at nt +74 and +12 of pNOS5′2 and pNOS5, respectively. Hence, pNOS5 and pNOS5′2 on one hand and pNOS5′1 on the other hand appear to be derived from nNOS mRNAs with distinct 5′ UTRs. Fig. 1 illustrates the relationship among these cDNAs.

Analysis by RT–PCR confirms that RNAs corresponding to the structures predicted by pNOS5′1 and pNOS5′2 are expressed. An antisense strand primer derived from sequence common to pNOS5′1 and pNOS5′2 and downstream of the splice junction discussed in the next section was coupled with sense-strand primers representing sequence upstream of the splice junction and unique to either pNOS5′1 or pNOS5′2. As shown in Fig. 2, after RT of human cerebellar RNA, the expected PCR products are produced. In each case, appearance of the PCR product is RNA dependent. A low level of RNA transcripts corresponding to pNOS5′1 and pNOS5′2 is also found in skeletal muscle (Fig. 2). An RNA preparation

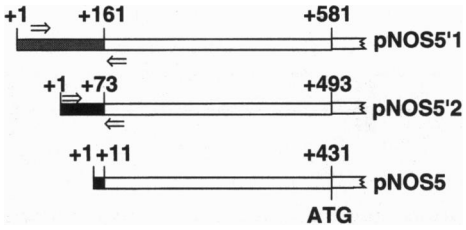

FIG. 1. Comparison of the 5′-UTRs of nNOS cDNAs. Sequence common to pNOS5′1, pNOS5′2, and pNOS5 (7) are indicated as open bars. Sequences unique to either pNOS5′1 (nt +1 to +161) or pNOS5′2 (nt +1 to +73) and pNOS5 (nt +1 to +11) are indicated as shaded and black bars, respectively. Also shown are the positioning of primers used for analysis by RT–PCR (⇐ and ⇒) and the positions of the common initiation codon (ATG) located at position +581, +493, and +431 of pNOS5′1, pNOS5′2, and pNOS5, respectively.

derived from leukocyte-depleted platelets did not contain either transcript (data not shown).

Two models of nNOS gene organization and expression are compatible with these data. First, alternative splicing of a single primary transcript could give rise to mRNAs with a common first exon adjacent to one of two different internal exons. In this model, the 5′ terminal sequences of pNOS5′1 and pNOS5′2 would represent alternatively spliced internal exons that are both positioned downstream from a single nNOS promoter. Second, distinct promoters could give rise to two primary transcripts that are spliced to yield mRNAs with different first exons spliced to a common second exon. This latter model, in which the 5′-terminal sequences of pNOS5′1 and pNOS5′2 represent different first exons under transcriptional control by separate promoters, is supported by our analysis of nNOS gene structure and function.

Exons 5′1 and 5′2 Are Closely Linked. Genomic cloning and sequencing studies establish that the 5′-terminal exons of pNOS5′1 and pNOS5′2 are positioned within 300 bp of each other but separated from exon 2 by an intron that is at least 20 kb in length. We isolated two genomic clones, λNOS15 and λNOS16, based on hybridization to probes derived from 5′-UTR sequences of pNOS5 and pNOS5′1, respectively. Restriction maps of the genomic clones are shown (Fig. 3 *Upper*). Sequence data establish the intron–exon structure that is shown. The 5′-terminal 161 nt of pNOS5′1 and the 5′-terminal 73 nt of pNOS5′2 are each encoded by single exons (exons 5′1 and 5′2, respectively) that precede canonical 5′ splice junctions (Fig. 3 *Lower*). Moreover, these two exons are closely linked. The 3′ boundary of exon 5′2 (nt +73 of pNOS5′2) is located 223 nt upstream of the genomic sequence that specifies nt +1 of pNOS5′1 (Fig. 3 *Lower*). At least 20 kb downstream, a

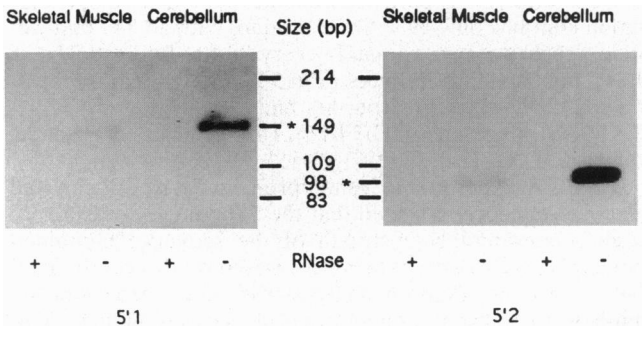

FIG. 2. RT–PCR of the 5′-UTR of pNOS5′1 and pNOS5′2. RT was performed using RNAs derived from human cerebellum or skeletal muscle with or without prior RNAse treatment (+ and −, respectively) followed by PCR using an antisense primer directed against exon 2 and sense primers specific for either exon 5′1 (*Left*) or exon 5′2 (*Right*). DNA size markers and the expected size of the PCR products are indicated.

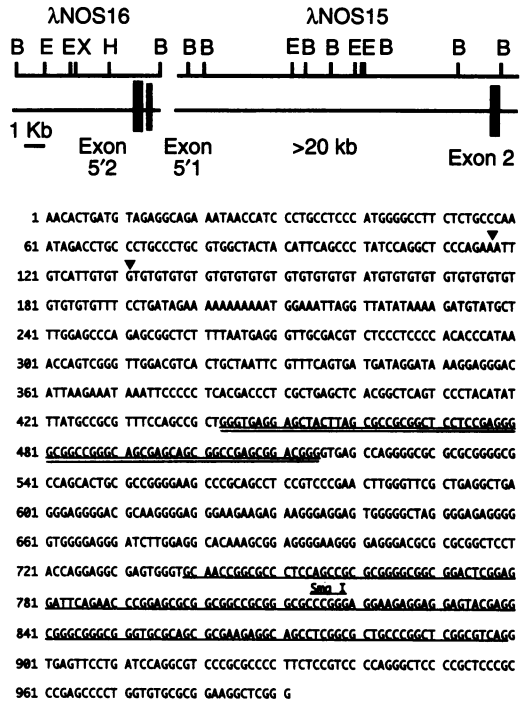

FIG. 3. Genomic structure encoding the 5′-UTRs of pNOS5′1 and pNOS5′2. (*Upper*) The nonoverlapping genomic clones λhNOS15 and λhNOS16 were mapped with respect to the restriction enzymes *Bam*HI (B) and *Eco*RI (E). λhNOS16 was also mapped with respect to *Hin*dIII (H) and *Xho* I (X). The position of unique exons 5′1 and 5′2 as well as the common exon 2, based on DNA sequence data, are indicated by solid vertical bars. (*Lower*) Nucleotide sequence of the genomic DNA that includes exons 5′2 and 5′1. Sequences corresponding to nt 1–161 of pNOS5′1 and nt 1–73 of pNOS5′2 are indicated with single and double underlines, respectively. Exon 5′2 transcription start sites are indicated (▼).

canonical 3′ splice junction (5′-CTCCTGTTCTTCCTCT-TCAG-3′) precedes the common exon 2 initiated at nt +162, +74, and +12 of pNOS5′1, pNOS5′2, and pNOS5, respectively (data not shown).

Different sequence classes flank the 5′1 and 5′2 exons. The genomic sequence that surrounds exon 5′1 (nt 460–991 of Fig. 3) is very G+C-rich (76%) with a CpG dinucleotide appearing approximately every 8 bp. This composition suggests that exon 5′1 is embedded within a CpG island, an ≈1 kb genomic structure that is distinguished by an abundance of nonmethylated CpG dinucleotides (20). In sharp contrast, a portion of exon 5′2, as well as all of its associated 5′ flanking DNA, lie outside of this G+C-enriched domain (nt 1–459; Fig. 3). This region contains only 49% G+C residues with a CpG dinucleotide appearing approximately every 38 bp. Interestingly, as shown below, transcription of exon 5′2 initiates near a repeating stretch of alternating purines and pyrimidines, 5′-$(TG)_{17}$-$(TA)_1(TG)_{13}$-3′ (nt 126–187; Fig. 3). This sequence has potential to form Z-DNA (21).

RT–PCR experiments using primers derived from the genomic sequence indicated that the 5′ boundary of exon 5′2 extends considerably upstream of the sequence contained within pNOS5′2 (data not shown). Based on this result, analysis by primer extension analysis was performed using an end-labeled antisense oligonucleotide corresponding to nt 235–251 of Fig. 3. Primer extension data shown in Fig. 4*A* indicate that transcription of exon 5′2 initiates at two sites corresponding to nt 117 and 131 of Fig. 3. A sequence resembling a transcription initiator element (22) is flanked by these two sites (5′-GTCATTGT-3′; nt 121–128 of Fig. 3).

Primer extension was also performed using an end-labeled antisense oligonucleotide designed to hybridize to exon 5′1-

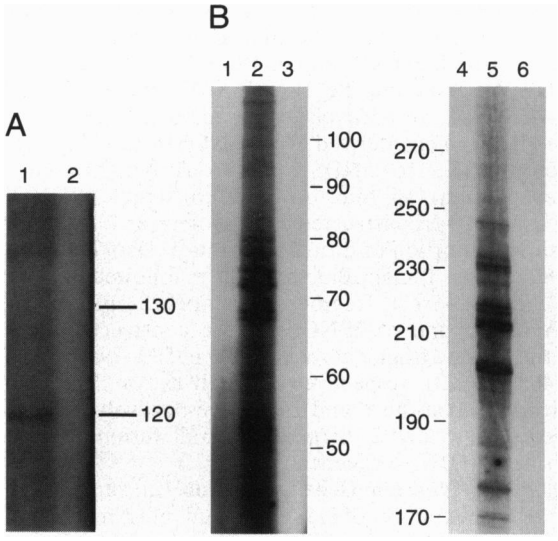

FIG. 4. Primer extension analysis of transcripts that include exon 5′1 or exon 5′2. (*A*) Reverse transcription was performed in the presence (lane 1) or absence (lane 2) of human cerebellar RNA using an end-labeled antisense primer specific for exon 5′2 (nt 235–251; Fig. 3) followed by polyacrylamide gel electrophoresis. (*B*) Reverse transcription was performed in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of human cerebellar RNA using an end-labeled antisense primer specific for exon 5′1 (nt 780–798; Fig. 3), or the primer and RNA were incubated without reverse transcriptase (lanes 1 and 4) followed by PAGE (first loading, lanes 4–6; second loading, lanes 1–3) and autoradiography. In both *A* and *B*, the sizes (in nucleotides) derived from sequencing reactions are indicated.

containing mRNA (nt 780–798 of Fig. 3). Data shown in Fig. 4*B* reveal a large number of primer-extended products that span an ≈200-nt range. These data suggest that exon 5′1 transcription initiates at a multitude of sites in the region corresponding to nt 550–750 of Fig. 3. However, because mRNA secondary structure may cause abortive RT, corroborative studies are required to definitely establish whether exon 5′1 transcription initiates at multiple sites.

Exons 5′1 and 5′2 Are Transcribed from Separable Promoters. Assays of transient gene expression were performed to test whether functional promoters resided in the 5′-flanking regions of either exon 5′1 or 5′2. Initially, a genomic restriction fragment with one boundary located within exon 5′1 (nt 819 of Fig. 3) and the other boundary located ≈2.3 kb upstream was inserted into the promoterless expression vectors pXP1 and pXP2 (15). The pXP2- and pXP1-based constructs contain the putative nNOS promoter cloned immediately upstream of cDNA encoding firefly luciferase and positioned in the forward (p2.3NOS-L) and reverse (p2.3SON-L) orientations, respectively. Also constructed was p4.3NOS-L, a fusion gene with the same 3′ boundary as pNOS2.3-L but with a 5′ boundary that is an additional 2 kb upstream. To test for promoter activity, HeLa cells were transfected with pRSV-βgal (23) and either p2.3NOS-L or p2.3SON-L followed by assay for β-galactosidase and luciferase activities. Luciferase activities were normalized to β-galactosidase activities so that errors due to differences in transfection efficiency were minimized. As positive and negative controls, transfections were performed with either pRSV-L (18) or pXP2, respectively. Data shown in Fig. 5*A* demonstrate that the luciferase activity obtained after transfection with p2.3NOS-L is much greater than the activity obtained after transfection with either pXP2 or p2.3SON-L and is similar to the activity obtained after transfection with pRSV-L. Similar results were observed after transfection of L6 myoblasts (data not shown). In addition, the luciferase specific activity obtained after transfection with p2.3NOS-L was ≈2-fold higher than that obtained after transfection with

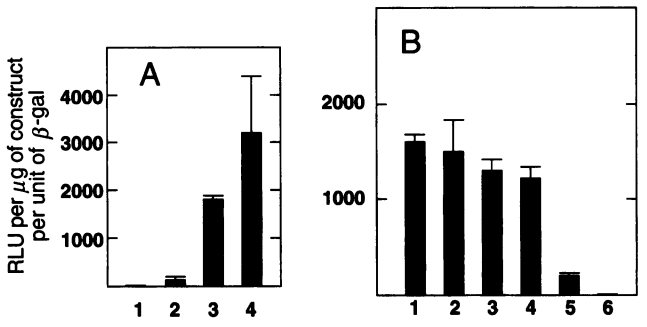

FIG. 5. Expression of nNOS–luciferase fusion plasmids in transfected HeLa cells. Cells were transfected by calcium phosphate-mediated gene transfer with 10 μg of pRSV-βgal and 25 μg of test plasmid followed by assay for transient gene expression after 48 hr. Data are expressed as a ratio of luciferase activity per microgram of test plasmid divided by β-galactosidase activity [relative light units (RLU) per μg of construct per unit of β-galactosidase activity (β-gal)] to normalize variations in transfection efficiency. (*A* and *B*) Separate experiments using the following test plasmids. (*A*) Lane 1, pXP2; lane 2, p2.3SON-L; lane 3, p2.3NOS-L; lane 4, pRSV-L. (*B*) Lane 1, p2.3NOS-L; lane 2, p5′1ANOS-L; lane 3, p5′1BNOS-L; lane 4, p5′2ANOS-L; lane 5, p5′2BNOS-L; lane 6, pXP2.

p4.3NOS-L (data not shown). These data demonstrate that the genomic nNOS fragment has promoter activity when positioned upstream of the luciferase reporter in the proper orientation.

To test whether separable promoters drive nNOS transcription, additional fusion plasmids were constructed and tested for transient expression in HeLa cells. The plasmids, designated p5′2A-NOS-L and p5′2BNOS-L, contain either nt 50–462 or nt 234–462 (Fig. 3), respectively, upstream of the luciferase reporter. Data shown in Fig. 5*B* indicate that transient expression of p5′2ANOS-L is similar to that of p2.3NOS-L. Expression of p5′2BNOS-L, although well above background levels obtained with pXP2, is dramatically diminished. Thus, a fusion gene that contains a portion of exon 5′2, its transcription start sites, and its immediate 5′ flanking DNA drives high level expression of the luciferase reporter. Deletion of the 5′ flanking DNA and exon 5′2 transcription start sites markedly attenuates expression.

Independent promoter activity is encoded within exon 5′1. The plasmids p5′1ANOS-L and p5′1BNOS-L contain nt 500–807 or nt 579–798 (Fig. 3), respectively, upstream of the luciferase reporter. Moreover, as shown in Fig. 5*B*, each of these fusion plasmids express well in HeLa cells. These results establish that separable promoters reside in the vicinity of both exons 5′1 and 5′2. However, as discussed below, the intriguing structural relationship that exists between these promoters and the fact that they function in transient assays of HeLa cells raise interesting questions with respect to transcriptional control of nNOS gene expression.

DISCUSSION

These data establish that human nNOS mRNAs with distinct 5′-UTRs are expressed in cerebellum and, to a lesser extent, skeletal muscle. Alternative splicing of internal exons present within a single primary transcript or alternative promoter usage to yield primary transcripts with different 5′ terminal exons are both consistent with this structural variation. Our data demonstrate that two closely linked but separate promoters function in transient assays, suggesting that multiple promoters are responsible for nNOS transcription *in vivo*.

The structural relationship that exists between the two nNOS promoters identified in this report is an interesting one. Exon 5′1 is embedded within a CpG island, a structure thought to be associated with the 5′ ends of roughly one-half of all genes (24). The nNOS promoter associated with this CpG island lacks a readily identifiable TATA box or initiator element (22), and transcription is initiated at multiple sites in this region. The CpG island terminates within exon 5′2. Moreover, an initiator element (22) appears to specify transcriptional initiation of this upstream exon at limited sites. Thus, transcription of the nNOS mRNAs represented by pNOS5′1 and pNOS5′2 appears to be driven by two juxtaposed but separable elements representing both major classes of eukaryotic promoters.

Why should the nNOS gene be subject to transcriptional control by more than one promoter? First, the multiple promoters obviously produce multiple species of nNOS mRNA. Although identical proteins are likely to be encoded by these mRNAs, differences in 5′-UTR structure could affect mRNA processing, localization, stability, or translation efficiency. Hence, posttranscriptional regulation of nNOS gene expression might exploit the differences in nNOS mRNA 5′-UTR structure reflected by pNOS5′1 and pNOS5′2. Second, multiple promoters offer an opportunity to extensively regulate transcription of a single gene in different tissues and in response to cellular changes. Although our data indicate coordinated expression of exon 5′1 and exon 5′2 in cerebellum and in skeletal muscle, this is not necessarily the case in all tissues or situations. In this context, it is important to note that other genes are also encoded by multiple promoters (25). However, the spatial proximity of the nNOS promoters described in this report is unusual, and it is tempting to speculate that the structure has a specific role in transcriptional control. For example, the clustering of promoters may serve to coordinate expression of exon 5′1- and exon 5′2-containing nNOS mRNAs via communal use of cis-acting elements. Promoter-specific modulatory effects might then superimposed upon such a coordinated pattern of expression.

Transient expression data shown in Fig. 5 clearly identify functional promoters in the 5′-flanking DNA of both exon 5′1 and exon 5′2. However, because nNOS expression is limited to selected cells *in vivo*, the results obtained after HeLa cell transfections are enigmatic. Several potential mechanisms are consistent with these data. First, the constructs tested may all lack critical cis-acting elements that serve to limit transcription to certain cells. Accordingly, ectopic expression in HeLa cells may reflect the absence of an important level of transcriptional control on the functional promoters. Second, although the luciferase activities in HeLa cells are orders of magnitude above background, it is possible that even higher activities are achievable in selected neuronal populations. However, because NOS promoter fragments are similar to the promiscuous Rous sarcoma virus long terminal repeat in their ability to drive luciferase expression in HeLa cells, we do not favor this possibility. Third, critical regulatory influences imposed by chromatin structure might be lost in these transient assays (26). Finally, exons 5′1 and 5′2 are closely linked to each other but separated from a common exon 2 by an intron that is at least 20 kb in length. As noted by others (25, 27, 28), transcription and processing of genes with large introns present special problems to the cell, raising the fourth possibility that complex and tissue-specific posttranscriptional mechanisms might be involved in formation of nNOS mRNA.

Elucidating the molecular mechanisms that control nNOS gene expression should prove to be both interesting and useful. Our description of closely linked but separable nNOS promoters provides an important foundation for this subsequent research.

We thank Dr. Hawley K. Linke for the gift of human placental tissue and Dr. Michael Seiff for gifts of cerebellar and muscle tissue. This research was supported by National Institutes of Health Grant EY05063 (A.P.Y.).

1. Bredt, D. S. & Snyder, S. H. (1992) *Neuron* **8,** 3–11.
2. Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S. V., Sucher, N. K., Loscaizo, J. & Stamler, J. S. (1993) *Endothelium* **1,** Suppl., 7.
3. Schuman, E. M. & Madison, D. V. (1991) *Science* **254,** 1503–1506.
4. Hess, D. T., Patterson, S. I., Smith, D. S. & Skene, J. H. P. (1993) *Nature (London)* **366,** 562–565.
5. Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B. & Bloch, K. D. (1992) *J. Biol. Chem.* **267,** 14519–14522.
6. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351,** 714–718.
7. Nakame, M., Schmidt, H. H. H. W., Pollock, J. S., Forstermann, U. & Murad, F. (1993) *FEBS Lett.* **316,** 175–180.
8. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. (1992) *Science* **256,** 225–228.
9. Williams, C. V., Nordquist, D. & McLoon, S. C. (1994) *J. Neurosci.* **14,** 1746–1755.
10. Schilling, K., Schmidt, H. H. H. W. & Baader, S. L. (1994) *Neuroscience* **59,** 893–903.
11. Wu, W. (1993) *Exp. Neurol.* **120,** 153–159.
12. Verge, V. M., Xu, Z., Xu, X. J., Wiesenfeld-Hallin, Z. & Hokfelt, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11617–11621.
13. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
15. Nordeen, S. K. (1988) *BioTechniques* **6,** 454–457.
16. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
17. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–256.
18. DeWet, J. R., Wood, K. W., DeLuca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.
19. Norton, P. A. & Coffin, J. M. (1985) *Mol. Cell. Biol.* **5,** 281–290.
20. Cross, S. H., Charlton, J. A., Nan, X. & Bird, A. P. (1994) *Nat. Genet.* **6,** 236–244.
21. Rich, A., Nordheim, A. & Wang, A. H. J. (1984) *Annu. Rev. Biochem.* **53,** 791–846.
22. Smale, S. T. & Baltimore, D. (1989) *Cell* **57,** 103–113.
23. MacGregor, G. R., Mogg, A. E., Burke, J. F. & Caskey, C. T. (1987) *Somatic Cell Mol. Genet.* **13,** 253–265.
24. Antequera, F. & Bird, A. (1993) in *DNA Methylation: Molecular Biology and Biological Significance*, eds. Jost, J. P. & Salutz, H. P. (Birkhaeuser, Basel), pp. 169–185.
25. Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M. & Persson, H. (1993) *Neuron* **10,** 475–489.
26. Wolfe, A. P. (1994) *Cell* **77,** 13–16.
27. Fraser, P., Cummings, P. & Curtis, P. (1989) *Mol. Cell. Biol.* **9,** 3308–3313.
28. Bernards, A., Ruben, C. M., Westbrook, C. A., Paskind, M. & Baltimore, D. (1987) *Mol. Cell. Biol.* **7,** 3231–3236.