BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 145–151 (1996)
ARTICLE NO. 1324

# Cloning of a Novel Neuronal Nitric Oxide Synthase Expressed in Penis and Lower Urinary Tract[1]

Thomas Magee, Angela M. Fuentes, Hermes Garban, Tripathi Rajavashisth, Diana Marquez, Jose Antonio Rodriguez, Jacob Rajfer, and Nestor F. Gonzalez-Cadavid[2]

*Division of Urology, Harbor-UCLA Medical Center, Torrance, California 90509*

Received July 29, 1996

The neuronal nitric oxide synthase (nNOS) is the main NOS isoform in the urogenital tract catalyzing the synthesis of nitric oxide, the mediator of penile erection and presumably an important factor in the control of urinary voiding. We have cloned from the rat penile corpora cavernosa a cDNA coding for a novel nNOS differing from the cerebellar nNOS by the presence of a 102 nucleotides stretch and other features. This new species is the only nNOS mRNA expressed in the rat penis, urethra, prostate, and skeletal muscle, coexists with the cerebellar nNOS in the pelvic plexus and bladder, and is detectable in the cerebellum. The novel insert is present in human penile RNA and is transcribed from intron 16. The features and distribution of the penile nNOS suggest that it is may be regulated differentially from the cerebellar nNOS. 

Nitric oxide (NO) plays a fundamental role in the maintenance of the smooth muscle tone of the lower urogenital tract (1). In the penis, it is the mediator of penile erection (1,2) and it is synthesized predominantly in the non-adrenergic non-cholinergic nerve terminals by the neuronal nitric oxide synthase (nNOS). NO elicits the relaxation of the corpora cavernosal smooth muscle through the activation of guanyl cyclase and subsequent Ca2+ mobilization. In the rat, erectile dysfunction is accompanied by a decrease in penile NOS activity, and in some cases by a reduction in nNOS content (3-7). NO synthesized by nNOS appears to be an important factor in the relaxation of the urethral, bladder, and prostatic smooth muscle (1,8,9) involved in micturition and seminal fluid ejaculation.

nNOS is found in nerves along the urogenital system stemming from the pelvic plexus (1) and determined in the soluble fraction of tissue homogenates (3-7). In these respects, it is indistinguishable from the nNOS cloned originally from the cerebellum of the rat (10,11), mouse (12), and human (13,14), and a single gene has been identified in these species. However, due to the particular organization of its promoter (15.16), alternative start sites and nNOS proteins of different sizes are possible. A differential tissue splicing has been described for the mouse nNOSmRNA (12).

The differential roles played by NO in the central and peripheral nervous system (17-19) would argue in favor of the existence of tissue-specific nNOS differing in amino acid regions that may confer functional responsiveness to local regulatory factors. In addition, most physiological functions dependent on NO-related neurotransmission, including penile erection, remain unaffected in cerebellar nNOS knockout mice (20). In this report we describe the cloning of

[1] This work was funded by grants from the Tobacco Related Disease Research Program of the University of California, and NIREC Inc.

[2] Corresponding author. Harbor-UCLA Medical Center, Division of Urology, Building F-6, 1000 West Carson Street, Torrance, CA 90509. Fax: (310) 222-1914.

Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; RPnNOS, rap penile nitric oxide synthase; PnNOS, penile nitric oxide synthase.



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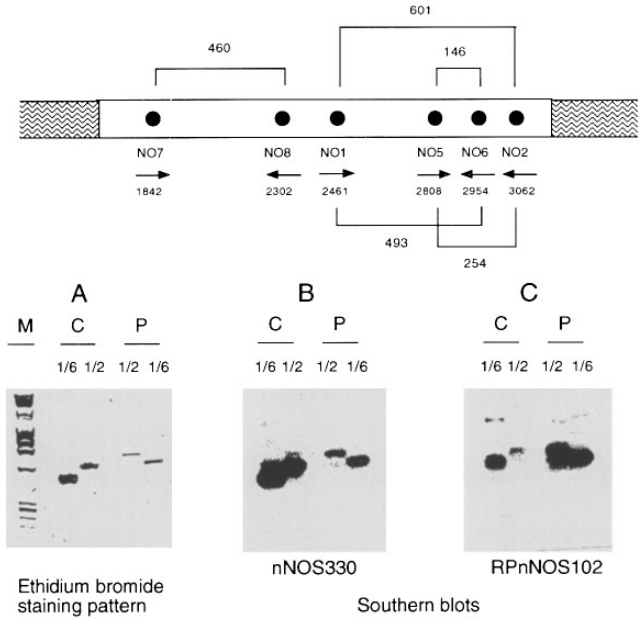



**FIG. 1.** Novel nNOS mRNA (RPnNOS) in the rat penile corpora cavernosa. (Top) NO1-8 start at indicated nucleotides (even numbers: antisense; odd numbers: sense). Primers have 24 bases of the RCnNOS sequence plus a 9-base restriction site at the 5′end, except NO5 (20-mer). Restriction sites are CCGGAATTC (EcoRI; NO1,7) or GCCGGATTCC (BamHI; NO2,6,8). Sizes of fragments encompassed by each set of primers are shown. (Bottom, A) Staining of RT/PCR bands. C: rat cerebellum; P: penile corpora cavernosa. M: size markers. 1/6, 1/2: NO primer combinations. (Bottom, B,C) Southerns with cDNA probes as indicated. nNOS1/2: region of nNOS encompassed by primers NO1/NO2; nNOS330: 5′end region of nNOS1/2, generated by Ban1 restriction digestion; RPnNOS102: 102 bp insert present in RPnNOS and absent in RCnNOS.

a novel nNOS cDNA from the rat penis, and its expression in other organs of the rat, mainly in the urogenital tract.

## MATERIALS AND METHODS

*Reverse transcription (RT) and PCR.* RNA was isolated from rat penises (skin-denuded bulb and shaft) and cerebellum, and polyA+ RNA was selected as described (21). After DNAse treatment RNAs (0.5 $\mu$g) were incubated with MMLV reverse transcriptase (100 U) and 0.5 $\mu$M primers NO2 or NO6 (Fig. 1, top). Primers were synthesized based on the sequence (10) of the rat cerebellar nNOS (RCnNOS) on the calmodulin/FMN region Primers NO1 and NO2 encompass a 601 bp DNA sequence. Primers NO5 and NO6 encompass an internal 146 bp fragment. The PCR-amplified cDNAs are 18 bp larger because the primers have non-NOS sequences as 5′ tails (restriction sites). 1/5 of the RT mix was heated at 65 C and submitted to PCR with NO1/NO2 or NO1/NO6 primers (0.25 $\mu$M) for 36 cycles at 94 C (45 sec), 55 C (30 sec), and 72 C (2 min). An aliquot was used for a second PCR round at 62 C annealing temperature. Products (1/8) were run and stained with ethidium bromide on 1.5% Nusieve gels. Fragments were eluted, purified and cloned in plasmid PCRII (Invitrogen, La Jolla, CA). Southern blots were hybridized (21) against the [$^{32}$P]-labeled probes.

*cDNA library cloning.* Penile polyA+ RNA (10 $\mu$g) was reverse transcribed with oligodT primers (XhoI site at end), followed by ligation with EcoRI adaptors and cleavage of the XhoI site. The cDNA inserts were cloned into the XhoI/EcoRI sites of lamdaZap vector arms (Stratagene, La Jolla, CA). Initial screening was performed with a probe (RPnNOS5/6) generated by RT/PCR as above with NO5/NO6 primers. Selected clones were submitted to secondary and tertiary screening with RPnNOS5/6, and RPnNOS102 (probe for the novel 102 bp fragment; see Results). Two clones (#1 and #2) were used for pBS SK− phagemid excision and DNA isolation. EcoRI and XhoI digestion was followed by Southern blotting and sequential hybridization with the previous two probes. A 3.1 kb insert was found in both clones but only clone #1 was sequenced (5′end: nt1684).

*Isolation of the cDNA 5′ region.* It was generated (nt 248 to 2956 by extra long PCR using 20-mer primers: a)

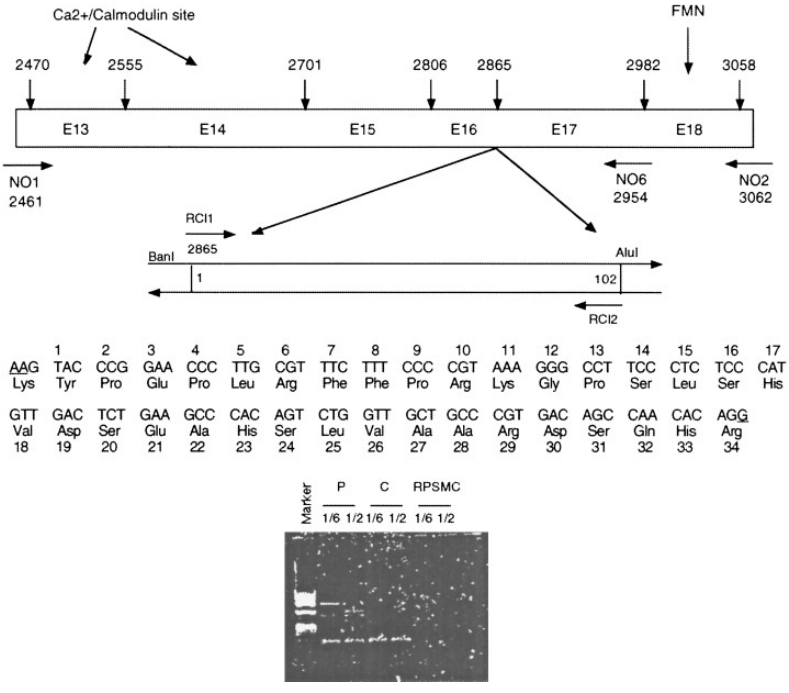



**FIG. 2.** Position and sequence of a 102 bp insert (RPnNOS102). (Top) Representation of RCnNOS in region encompassed by primers NO1/2, showing exon boundaries (E) 13-18. The insert was found in the 722 bp penile cDNA isolated from lane 1/2 on Fig. 1, in position 2865. (Middle) Nucleotide and amino acid sequences. (Bottom) Staining of the 102 bp fragment generated by PCR from both the penile and cerebellar fragments 1/6 and 1/2 in Fig. 1.

RCnNOSATG (sense, from the rat nNOS (10), 5′ end: nt 248), and RC14 (antisense, from the novel 102 bp insert, TGTCACGGGCAGCAACCAGA). 1 $\mu$g of total rat penis RNA was incubated with AMV reverse transcriptase (Promega, Madison, WI), and amplified with the GeneAmp XL PCR kit (Perkin-Elmer, Branchburg, NJ), using optimized conditions in conjunction with rTH DNA polymerase. The PCR fragment was cloned by blunt end ligation into EcoRV cut pZero2.1 (Invitrogen, San Diego, CA) and designated pZRCnNOSATG-RCI4.

*DNA sequencing.* PCR fragments and cloned cDNAs were submitted to dideoxy sequencing in both directions using an automatic sequenator (Applied Biosystems, Foster City, CA). Differences with the nNOS sequence (13) were confirmed by repeated sequencing.

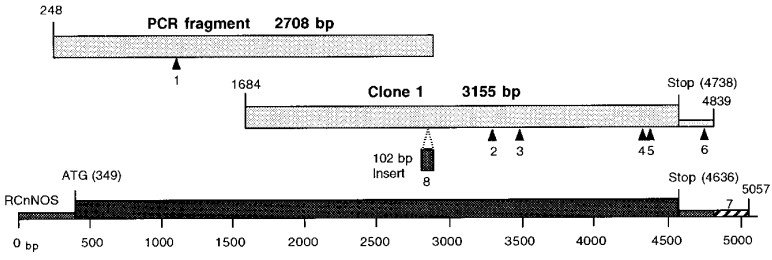
*Determination of novel nNOS expression.* RNA was obtained from rat organs as described above, and incubated (2 $\mu$g) with MMLV reverse transcriptase (100 U) using NO2, NO6, or oligoDT as primer for first strand DNA synthesis. 1/5 of each reaction was used for PCR with primers NO1/NO6 or NO5/NO6. Samples were run, blotted, and hybridized as above.

*PCR of genomic DNA.* Human lung and rat liver DNA were isolated (Easy DNA, Invitrogen, La Jolla), and 1 $\mu$g was subjected to extra long PCR with primers for exons 16 and 17 on the nNOS sequence (10) and on the novel 102 bp fragment. They were: a) primers RCI4 on the 3′end of RPnNOS102 (antisense), and NO-E16F on the middle of exon 16 (nt 2826; sense); b) primers NO-17E17 on the 3′end of exon 17 (nt 2960; antisense) with either NO-16F on the middle of exon 16 (nt 2865; sense) or with RCI3 on the 5′end of RPnNOS102 (GTACCCGGAACCCTTGCGTT; sense). PCR fragments were isolated from 0.8% agarose gels, purified and sequenced.

*PCR of human cDNA library.* PolyA+ RNA was isolated from human corpora cavernosa and a cDNA library was constructed as above. DNA was isolated using the Wizard Lamda Preps DNA kit (Promega, Madison, WI) and PCR was performed with primers RCI-3 and NO6.

## RESULTS AND DISCUSSION

*Detection of novel nNOS mRNA in the rat penile corpora cavernosa.* A comparative RT/PCR on polyA+ RNA isolated from rat corpora cavernosa and cerebellum was performed in




| Differences | Amino acid position | RPnNOS codon | RPnNOS AA | RCnNOS* codon | RCnNOS* AA |
|---|---|---|---|---|---|
| 1 | 269 | GTC | Val | ATC | Ile |
| 2 | 953 | GCG | Ala | CCG | Pro |
| 3 | 1008 | TCC | Ser | TTC | Phe |
| 4 | 1299 | GCC | Ala | GCT | Ala |
| 5 | 1311 | GTC | Val | GCC | Ala |

| | Nucleotide position** | Differences other than base substitutions |
|---|---|---|
| 6 | 4772 | Two bp GG insert in RPnNOS clone compared to RCnNOS |
| 7 | 4839 | RCnNOS is 319 nts longer than RPnNOS at the 3' end |
| 8 | 2865 | 102 bp insert in RPnNOS clone compared to RCnNOS |

* Reference: Bredt et al., 1991, Nature 351, 714-718. ** Numbering takes into account the 102 bp insert.

**FIG. 3.** Cloning of RPnNOS coding region and sequence comparison with RCnNOS. (Top) 3′ clone from cDNA library, and 5′ cDNA fragment generated by PCR from the library. Arrows indicate positions of amino acid substitutions on RPnNOS as compared to RCnNOS. The site of the 102 bp in frame insert is indicated below clone 1. (Bottom) RCnNOS coding region. Chart: nucleotide changes resulting in amino acid substitutions as compared to RCnNOS (RCnNOS numbering conserved).

order to determine whether different nNOS cDNAs were generated from both organs. Fig. 1, bottom, A, shows that in the case of the rat cerebellum (C), primers NO1/2 (lane 3) and 1/6 (lane 2) gave the expected 619 bp and 511 bp bands, respectively, on the staining pattern (lane 1). However, the bands generated from penile RNA are larger: 720 bp (lane 4) and 615 bp (lane 5), respectively. No bands were seen in the negative controls (no RNA or no reverse transcriptase; not shown)). Similar bands were generated by just one PCR round amplification at the highest stringency. Sequencing of the NO1/2 and NO1/6 fragments showed a complete homology between cerebellar nNOS and penile nNOS RNA (RPnNOS), with the exception of a 102 bp insert, in RPnNOS, designated RPnNOS102. This insert indicates that the penis contains a novel nNOS isoform RNA. No RCnNOS fragments were visible in the penis.

Two primers (RCI-1 and RCI-2) for both extremes of RPnNOS 102 (see below) were used to generate by PCR the 102 bp probe that was cloned into vector PCR-I. A probe (nNOS330) common to the 5′end of both RPnNOS1/2 and RCnNOS1/2 (outside RPnNOS102) was prepared by digestion of RPnNOS1/2 with BanI. Southern blot of the gel with nNOS330 (Fig. 1, bottom panel B) confirmed the staining pattern and showed that although the known nNOS species is predominant in the cerebellum, it is accompanied by a minor expression of the novel nNOS RNA. Further proof was obtained by hybridization with RPnNOS102 (Fig. 1, bottom panel C), showing that in the penis no regular RCnNOS is expressed and the only penile nNOS species is the novel RPnNOS, whereas in the cerebellum RCnNOS coexists with traces of RPnNOS. Sequencing of the NO1/2 and NO1/6 PCR fragments originated from penile RNA and cloned RPnNOS102 gave a common sequence for the 102 bp insert (Fig. 2, middle). This coresponds to a 34 amino acid stretch which lacks significant homology at the nucleotide

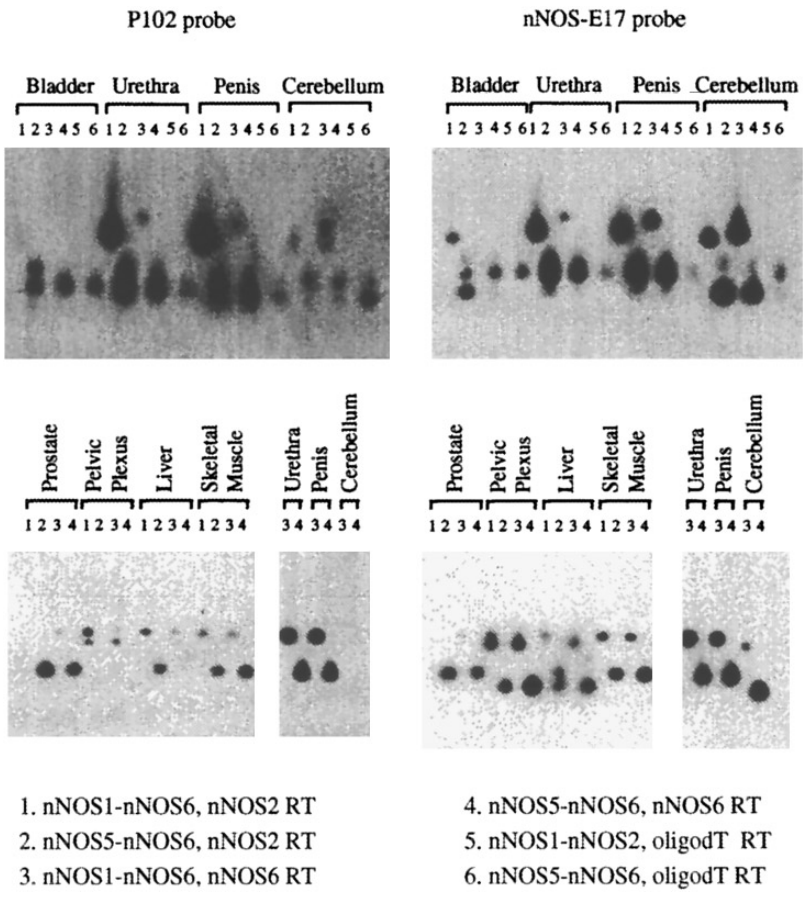



**FIG. 4.** Tissue expression of RPnNOS and RCnNOS. RNA was reversed and the cDNA amplified with the indicated primer sets. Fragments were separated on 1.5% Nusieve, and the gel was submitted to Southern blotting). Sizes expected for RCnNOS are 511 bp (lanes 1,3), 155 bp (lanes 2,4,6), and 619 bp (lane 5). Each fragment is 102 bp longer for RPnNOS.

or amino acid level with any sequence in the DNA data bank. The insert occurs at nucleotide position 2865 (Fig. 2, top), breaking the triplet for lysine 839 without changing this amino acid or the reading frame. This corresponds to the boundary between exons 16 and 17 in the nNOS gene (15,16).

Confirmation of the expression of RPnNOS102 in the cerebellum by staining was obtained when the RT was carried out with either NO2 or NO6, followed by a first round of PCR with NO1/NO2 or NO1/NO6, and a second round with RCI-1/RCI-2 (Fig. 2 bottom). No 102 bp band was visible in the RNA from RPSMC, thus indicating the absence of RPnNOS in the penile smooth muscle that is the target for NO.

*Cloning and sequencing of RPnNOS cDNA.* Sequencing of clone 1 revealed, in addition to the 102 bp insert, four nucleotide and amino acid changes (Fig. 3) with RCnNOS. The 2.7 kb fragment generated by ''extended'' PCR using a primer at the 3′end of RPnNOS102 (RCI-4) showed another amino acid change. The non-coding 3′ tail is shorter by 319 bp in RPnNOS.

*Expression of nNOS species in different tissues.* The RT/PCR was repeated on RNA from rat urogenital organs and other tissues. Fig. 4, top left shows the presence of intense signals for the 102 bp insert in the penis and urethra, and visible bands in the cerebellum and bladder,

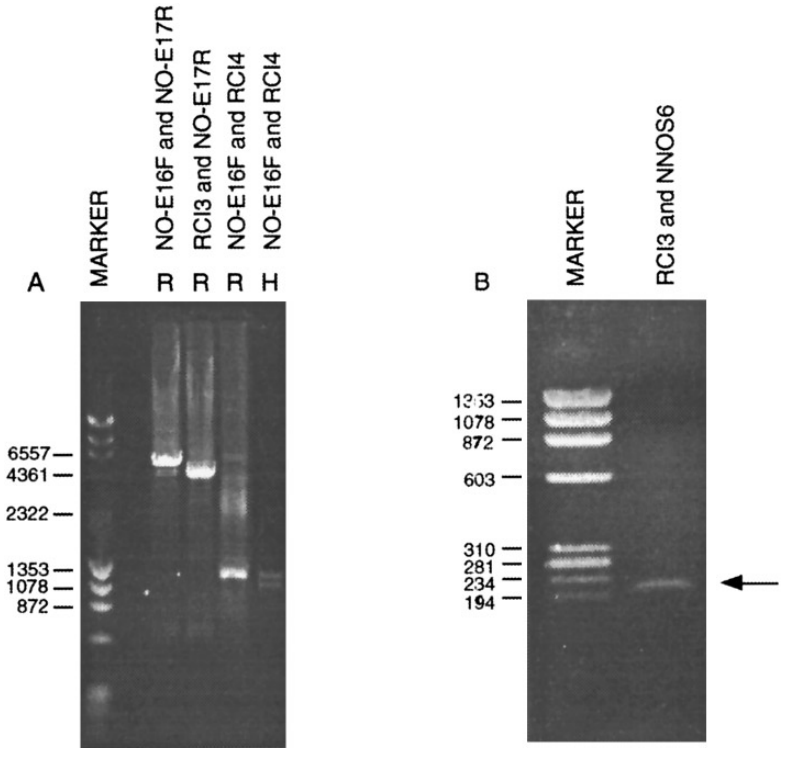



**FIG. 5.** Detection of the 102 bp insert on rat and human intron 16 and in human penile RNA. (A) Rat (R) and human (H) DNAs were amplified with the indicated set of primers, and fragments were separated on 1% agarose and stained. (B) DNA from a human penile cDNA library was submitted to PCR with the indicated primers (expected size: 207 bp), and fragments were separated on 1.5% Nusieve and stained.

in all PCRs that worked (combinations 1-4). The nNOS-E17 probe common for both RPnNOS and RCnNOS (top right) replicated the staining pattern (not shown), and gave single larger bands for penis and urethra, as expected. The bladder showed larger fragments accompanying the shorter ones, and the cerebellum had traces of the larger band.

When the experiment was duplicated with primer combinations 1-4 and additional tissues, the prostate and skeletal muscle gave signals with the P102 probe (bottom left), of the same size as in penis and urethra and nearly as intense (prostate). The pelvic plexus and liver had little RPnNOS102. The staining pattern was similar to this hybridization (not shown). The common nNOS-E17 probe (bottom right) confirmed that RPnNOS is unique in penis, urethra, prostate, and skeletal muscle, but much less abundant than RCnNOS in pelvic plexus and liver.

*Intron location of the novel nNOS sequence and expression in human penile tissue.* The location of RPnNOS102 within intron 16 was established by extra-long PCR using primers spanning different regions of this intron. Fig. 5, left shows that it is 1.2 kb from exon 16 (R, 3rd lane) and 4.3 kb from exon 17 (R, 2nd lane), within a 5.4-5.6 kb intron (R, 1st lane). The insert is present in human DNA (H) at the same location as in the rat DNA and also in a smaller bottom band, as confirmed by partial sequencing around the 102 bp insert. The consensus sequence for the initial 96 nucleotides in this 102 bp region in human DNA was virtually identical to RPnNOS102, with only two nucleotides changes (AGC to AAA, on amino acid #31 in insert; serine changed to lysine). The insert is present in the RNA

from human corpora cavernosa, as indicated by PCR of a human penile cDNA library with primers RCI-3/NO6 giving the expected 207 bp fragment, and the sequencing confirming the 102 region (not shown).

*Physiological significance.* No evidence is available on whether the novel nNOS is an alternative splicing product (12), or it is encoded by another nNOS gene. Irrespective of this, the existence of a novel nNOS poorly represented in the cerebellum and having a long insert, strongly suggests that this sequence confers special properties to the penile nNOS protein that are particularly suited to its role in the mechanism of penile erection. The other amino acid differences may enhance the functional specificity of this nNOS, designated as PnNOS for both rat and human tissues. Since virtually no cerebellar nNOS is present in the penis, we postulate that PnNOS and not CnNOS is responsible for the synthesis of NO during penile erection.

The abundance of PnNOS in prostate, urethra, and to a lower extent in bladder, is an indicator that PnNOS may synthesize NO as a peripheral neurotransmitter in nerve terminals controlling endothelium-independent smooth muscle relaxation (17-19). This would occur through an isoform-specific modulation of NOS enzyme activity different from the one operating in the central nervous system. We hypothesize that the tone of the urethra and prostate, and partially the bladder may be controlled by PnNOS. The basal levels of PnNOS coexisting with CnNOS in the cerebellum and pelvic plexus suggest that PnNOS may be located in neurons and nerve fibers separate from the ones for CnNOS.

## REFERENCES


1. Burnett, A. L. (1995) *Urology* **45,** 1071–1083.
2. Lugg, J., Gonzalez-Cadavid, N. F., and Rajfer, J. (1995) *J. Androl.* **16,** 2–4.
3. Garban, H., Vernet, D., Freedman, A., Rajfer, J., and Gonzalez-Cadavid, N. F. (1995) *Am. J. Physiol.* **268,** H467–H475.
4. Lugg, J., Rajfer, J., and Gonzalez-Cadavid, N. F. (1995) *Endocrinology* **136,** 1495–1501.
5. Lugg, J., Ng, Ch., Rajfer, J., and Gonzalez-Cadavid, N. F. (1996) *Am. J. Physiol.*, in press.
6. Vernet, D., Cai, L., Garban, H., Babbitt, M. L., Murray, F., Rajfer, J., and Gonzalez-Cadavid, N. F. (1995) *Endocrinology* **136,** 5709–5717.
7. Xie, Y., Ng, Ch., Rajfer, J., and Gonzalez-Cadavid, N. F. (1996) *J. Urol.*, in press.
8. Bennet, B. C., Kruse, M. N., Roppolo, J. R., Flood, H. D., Fraser, M., and de Groat, W. C. (1995) *J. Urol.* **153,** 2004–2009.
9. Jen, P. Y. P., Dixon, J. S., Gearhart, J. P., and Gosling, J. A. (1996) *J. Urol.* **155,** 1117–1121.
10. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) *Nature* **351,** 714–718.
11. Charles, I. G., Chubb, A., Gill, R., Clare, J., Lowe, P. N., Holmes, L. S., Page, M., Keeling, J. G., Moncada, S., and Riveros-Moreno, V. (1993) *Biochem. Biophys. Res. Commun.* **196,** 1481–1489.
12. Ogura, T., Yokoyama, T., Fujisawa, H., Kurashima, Y., and Esumi, H. (1993) *Biochem. Biophys. Res. Commun.* **193,** 1014–1022.
13. Hartneneck, C., Klatt, P., Schmidt, K., and Mayer, B. (1994) *Biochem. J.* **304,** 683–686.
14. Fujisawa, H., Ogura, T., Kurashima, Y., Yokoyama, T., Yamashita, J., and Esumi, H. (1994) *J. Neurochem.* **63,** 140–145.
15. Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994) *J. Biol. Chem.* **269,** 33082–33090.
16. Xie, J., Roddy, P., Rife, T. K., Murad, F., and Young, A. P. (1995) *Proc. Natl. Acad. Sci. U.S.* **92,** 1242–1246.
17. Zhang, J., and Snyder, S. H. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 231–233.
18. Rand, M. J., and Li, C. G. (1995) *Annu. Rev. Physiol.* **57,** 659–682.
19. Garthwaite, J., and Boulton, C. L. (1995) *Annu. Rev. Physiol.* **57,** 683–706.
20. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993) *Cell* **75,** 1273–1286.
21. Hung, A., Vernet, D., Rajavashisth, T., Rodriguez, J. A., Rajfer, J., and Gonzalez-Cadavid, N. F. (1995) *J. Androl.* **16,** 469–481.